Evolution and Morphological Shape Ontogeny of the Brown ticks (Acari: Ixodida: Ixodidae: *Rhipicephalus*)

Deon Kahlil Bakkes

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Department of Botany & Zoology

Stellenbosch University

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

Rhipicephalus constitute a species-diverse genus of mammal ectoparasites mainly distributed in the Afrotropics that are characterised by generally inornate, uniform brown body colour, short hypostome and palps, basis capituli approximately hexagonal, eyes present and male adanal plates present. They transmit microparasites such as *Rickettsia* spp., *Theileria* spp. and *Babesia* spp. to livestock and humans alike, and some inject neurotoxins during feeding that lead to tick paralysis in livestock. This work infers the phylogeny of *Rhipicephalus* from molecular lines of evidence (12S, 16S, COI and 28S-D2) and uses this as a basis to infer aspects of their evolutionary history, ecology and evolutionary-development based on geographic distribution data and basis capitulum shape data. Analyses included estimation of divergence times, ancestral area optimisations, ancestral host-use optimisations in immatures and adults, as well as estimations of ancestral climate niches. Basis capitulum morphology is quantified to determine evolutionary-developmental modifications, which are linked to similar patterns in overall body size. Major outcomes of studying *Rhipicephalus* evolution are 1) radiations coincide with mammal evolution and dispersal, 2) host-use at immature stages partially explain extent of geographic ranges as well as basis capitulum morphology for boring into thick host skin, 3) evolutionary host switches were facilitated by off-host periods and nested connections in predator-prey food webs, 4) speciation partially resulted from niche partitioning along temperature variation gradients, which was reinforced by interspecific competition, and 5) evolutionary-developmental modification (basis capitulum and overall body size) resulted from responses to distinct sets of selection pressure in on- and off-host environments taking into account one-, two- and three-host life cycles. The persistent taxonomic problem of *R. turanicus* between Palearctic and Afrotropical regions was investigated using integrated lines of evidence to test the species boundary in an iterative framework. This revealed two distinct species in these regions, and the Afrotropical species is described under the name *R. afranicus*. Another aim of this work was to test the hypothesis of phylogenetic recapitulation in post-embryonic stages of *Rhipicephalus* basis capituli, where early developmental stages resemble ancestral adults. However, findings indicate no signal for phylogenetic recapitulation is present, most likely due to the action of selection that shape basis capituli over evolutionary timescales. Selection supersedes any possible background action of condensing selection through development that would produce phylogenetic recapitulation. As such, this work serves as a first step for investigating phylogenetic recapitulation using shape data, and suggests future investigation should consider embryonic life stages, alternative features under less selection, or wider phylogenetic comparisons.

Preface

This dissertation comprises six chapters that investigate various aspects of *Rhipicephalus* evolution. The first chapter provides a discussion of the general methodology and philosophical aspects of the work, as well as an introduction of the organisms under study. This chapter includes key questions and hypotheses that direct the investigation. The second, third and fourth chapters comprise 'data chapters' that deal with various aspects of *Rhipicephalus* evolution. The fifth chapter provides a general discussion of the work, and includes answers to key questions set out in the first chapter. The sixth chapter, is an addendum data chapter that validates the geometric morphometric method used to quantify morphological shape in chapters three and four. All data chapters have been compiled as separate manuscripts, ready for publication – this resulted in the unavoidable repetition of certain introductory elements. Each chapter contains its own set of references and supporting information. Publication of taxonomic names in this work are disclaimed under article 8.2 of the International Code of Zoological Nomenclature.

The chapters published in peer-reviewed journals are listed below:

Chapter 4

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Without deviation from the norm, progress is not possible – Frank Zappa

1 Evolution and Historical Biogeography as a Science, Overview of *Rhipicephalus*, General Methodology, and Philosophy of Approach

Organismal variation across time and space is a phenomenon produced by the natural world that captivates curiosity and inspires attempts to describe and understand it. To do this, patterns of biodiversity are described using species as principal units, which can be ordered into nested hierarchies representing shared ancestry and descent with modification (De Queiroz and Gauthier, 1992). If these classification systems accord with the processes that formed them, significant predictive power is afforded to any given species name or nested class such that similar traits are present among closely related constituents. To study the processes that produce divergence, similarity and relationships between organisms with shared ancestry, comparative information is drawn from several levels of biological variation and is used to answer downstream questions, as well as solve real world problems involving biological elements (Carroll et al., 2014; Mayr, 1997).

Evolution is the process of biological change over time, and acts on individual organisms to produce effects at all levels of biology, consequently uniting diverse aspects of biodiversity in a nested hierarchy proportional to time. Changes at the molecular level form the basis for changes at higher levels (Dobzhansky, 1937). This process is highly complex, but generally comprised of two main components; neutral changes (Duret, 2008; Kimura, 1991, 1983, 1968; Ohta, 2002) and selective adaptation (Darwin, 1859; Fisher, 1958; Kocher, 2004). Neutral changes generally result from synonymous substitutions at the DNA level that are not selected for, but may indicate shared ancestry along stochastic patterns of drift in allele frequencies (Dobzhansky, 1937). Alternatively, non-synonymous changes generally confer a benefit or hindrance to an organism, which may become fixed or disappear in the population due to selective adaptation. However, one example of the complexity of molecular changes is noted in certain synonymous mutations which may be associated with splicing error, resulting in disease and becoming selected against (Chamary et al., 2006). Irrespective, it is generally understood that selective changes occur under adaptation to natural selection (including sexual selection) for traits that provide advantages to fitness, and these changes tend to stabilise trait frequencies and limit variation over time (Mayr, 1942). Following this, neutral evolution was subsequently adapted to the near neutral theory of evolution (Ohta, 1973), which serves as a null hypothesis for molecular evolution (Duret, 2008).

Evolution is understood in terms of phylogeny based on shared ancestry, which delimits separately evolving clades and species, as well as their genealogical relationships to one another (De Queiroz 2005; De Queiroz 2007). Clades are monophyletic (=holophyletic), where all individuals of a clade share a single most recent common ancestor. Evidence for their common ancestry is found in ‘special similarities’ of their natural variation (Hennig, 1965). These special similarities refer to synapomorphies and symplesiomorphies (shared derived

and shared ancestral features). Synapomorphies and symplesiomorphies are produced by shared genealogical histories that undergo descent with modification by drift and selective adaptation. Over large timescales, these processes generate variability in organismal form and function. The resulting lineages that link these clades are traceable and diagnosable by evaluating these features across a group of organisms with consideration of shared similarities (De Queiroz, 2007).

Evolutionary History: Phylogenetics and Ancestral State Estimation

Similarity among lineages has traditionally been measured in discrete, qualitative characters that are observable by eye (morphology and anatomy). These characters form the basis of proxies for phylogeny reconstruction in a comparative framework (Kitching, 1998; Lipscomb, 1998). However, being proxies, these characters miss some level of information. For example, when coding continuous characters into states for a data matrix, some level of variation is almost always misrepresented and summarised non-optimally; because state delimitation is arbitrary (Poe and Wiens, 2000). Furthermore, determining evolutionary polarity between states is often done arbitrarily (Poe and Wiens, 2000). To remedy this, contemporary biology includes molecular sequence data for nucleotides in DNA and RNA strands to characterise organisms more robustly (Dickinson, 1995; Patterson, 1987; Petersen and Seberg, 1998). An additional benefit of molecular data is the propensity for nucleotide substitutions to be inherited in an approximately clock-like manner (Bromham and Penny, 2003; Drummond et al., 2006; Ho and Larson, 2006). From this process, divergence time estimates between clades can be made, which offer additional evidence to answer research questions pertaining to evolutionary history. As such, nucleotide sequence data for DNA and RNA are well suited for addressing evolutionary history, and to facilitate more conclusive inferences of phylogeny.

Another contemporary data source which can augment molecular data is quantitative shape data derived from geometric morphometrics. This method stemmed from traditional morphometrics, which uses linear distances of morphological features. In geometric morphometrics however, linear distances are discarded in favour of two-dimensional co-ordinates that indicate landmark positions on a feature. Landmarks are placed on homologous features or along feature outlines, to capture multidimensional variability and measure shape. This affords high resolution in a robust statistical framework based on multivariate ‘shape space’ for capturing features that often have complex shapes (Adams et al., 2013, 2004; Mitteroecker and Gunz, 2009; Richtsmeier et al., 2002; Slice, 2007). A unique advantage is that these methods naturally lend themselves to graphical representation of the data in an easy-to-interpret form which resembles the studied feature. Pertinent to the focus of the

present work, this method has been successfully used to characterise shape differences in many Arthropoda groups (Dujardin et al., 2014; Dupraz et al., 2016; Horton and Lewis, 2005; Karanovic et al., 2016; Mutanen and Pretorius, 2007; Pretorius and Clarke, 2000). However, phylogenetic inference from such data is problematic as many levels of information are present in morphology, including shape changes that are non-evolutionary and add homoplasy which does not reflect phylogeny (Rohlf, 2002, 1998). Nevertheless, a molecular phylogeny can be used in combination with shape data for reconstructing the evolutionary history of shape changes, and thereby inferring aspects of the evolutionary history of a given study group (Klingenberg and Gidaszewski, 2010). One prominent shortcoming of geometric morphometrics is that non-shape variation and measurement error may be present, and these should be reported and dealt with adequately (Arnqvist and Martensson, 1998; Cardini et al., 2015; Fruciano, 2016; Rohlf, 2003).

Integrating molecular and morphological data facilitates better confidence in phylogenies, and may be done by iterating relevant research questions on both datasets (Yeates et al., 2011). However, the characteristics of molecular data are completely different compared to qualitative or quantitative morphology. Molecular data not only provide a large amount of characters useful for phylogeny estimation, but also manifest at much finer scales with greatly reduced complexity (A, G, C, or T). This means that different laws govern its evolutionary processes, and the reduced complexity of molecular data allows variation to be readily quantified and generalised. In this context, concatenated molecular data derived from unlinked genes provide independent data points which can be used to compare with findings from morphology. Neutral evolutionary changes in nucleotide sequences that vary due to drift are an effective way to approximate shared ancestry. This enables a framework of hypothesis testing which allows claims to be repeatable and falsifiable (Popper, 1959), where molecular data is tested against morphological data. Generally, correlation between molecular and morphological data is expected, but exceptions do exist. Changes in parts of genes and in morphology that arise due to selective pressure, disrupt phylogenetic signal and may induce incongruence between molecular and morphological phylogenies (Wiens et al., 2005). This remains a complex issue for which a solution is unclear in both morphological and molecular data. In some cases, neutral evolution of particular genes may also induce incongruence by factors such as homoplasy or incomplete lineage sorting, when alleles of a gene evolve either faster or slower than species divergence (Maddison, 1997). Methods are currently available to model sequence evolution (Gascuel and Guindon, 2007) and to estimate species trees in the context of incongruent genes trees based on coalescent theory (Tonini et al., 2015). Additionally, species tree methods perform better in analysis of recent timescales than concatenation (Gatesy and Springer, 2014), but concatenation methods remain reliable in

many cases (Tonini et al., 2015). Overall, factors that induce incongruence must be taken into account when studying phylogeny, and integrative (iterative) approaches are best to obtain confidence in results.

A phylogeny may be used for making further inferences about evolutionary processes in a set of organisms. Inference of geographical structuring through time across a clade of organisms can be made because genetic distances often approximate geographic distances (Novembre et al., 2008). This means molecular data has a lot to offer for studying historical biogeography and geographic movements in the evolutionary history of a clade. Historical biogeography can be defined as the series of geographical movements between constituents of a clade (Morrone and Crisci, 1995; Posadas et al., 2006; Wiens and Donoghue, 2004). A phylogeny informs patterns of relatedness between species, which can then be used as a null hypothesis with extant distribution patterns to test biogeographic area relationships in history. Contemporary methods such as S-DIVA (Yu et al., 2010) and BayArea (Landis et al., 2013) take into account the entire dataset of phylogenetic trees from a pseudo-replicate bootstrap analysis to account for phylogenetic and optimization uncertainty. Biogeographic data of the tree terminals are coded, and used to estimate probabilities for ancestral area occupancy at each node (Yu et al., 2015). DIVA optimises ancestral area probability based on a cost matrix (Ronquist, 1997; Yu et al., 2010), while BayArea samples posterior probabilities in a Bayesian framework (Landis et al., 2013). These analyses are useful to characterise geographic components of historical biogeography across a clade of organisms, which facilitates an understanding of the geographic placement of speciation events in evolutionary history. This information can be compared with climatic niches, host relationships among parasite species, and other factors that contribute to evolution to elucidate the complex set of factors present in the evolution of group of organisms.

Ancestral traits that are continuous rather than discrete, may also be inferred from a phylogeny and trait dataset. One such trait is climatic niche, and in an evolutionary context we may ask whether 1) multiple divergent climate niches are present across a clade, and 2) to what degree these niches are conserved (Losos, 2008; Münkemüller et al., 2015; Peterson, 2011; Wiens, 2004; Wiens and Graham, 2005). Answers to these questions aid in placing climatic niches across a clade into evolutionary context to provide a better understanding of dynamics that, in turn, may offer insight for downstream questions. However, due to considerable assumptions that must be made, any inference of ancestral climatic niche must be made with caution. The procedure typically incorporates climate data that are derived from point localities, but these conditions may not reflect climate tolerance of a species adequately (especially when samples are few). Moreover, species occurrences and associated climate conditions may be principally driven by biogeographic history rather than by climate tolerance

(Estrada-Peña et al., 2012; Vieites et al., 2009). Consequently, the first crucial assumption is to validate whether the climate data do accurately, or approximately, model the physical distribution to obtain good coverage of the climatic niche (Vieites et al., 2009). This may be done by comparing the fit between model and known distributions. Models based on climate data alone will seldom match a known distribution perfectly due to biotic factors and processes of historical biogeography that are unaccounted for (Pearson and Dawson, 2003; Peterson, 2001). Nevertheless, a good approximation between model and known distributions, indicates that climate data approximate the climatic niche of a given species, such that ancestral climatic niche estimation may proceed. Nevertheless, the use of physiological tolerance data is encouraged when available (Kearney and Porter, 2009; Vieites et al., 2009). The second assumption that must be met for confidence in results is that trait evolution should proceed in a manner similar to Brownian motion (Vieites et al., 2009) – a concept borrowed from physics. When Brownian motion is applied as a model of evolutionary change it represents a stochastic force that makes evolutionary changes proportional to branch length, and thus provides a vantage point for testing correlations between trait evolution and phylogeny (Diaz-Uriarte and Garland, 1996; Edwards and Cavalli-Sforza, 1964; Felsenstein, 1985a, 1988). Thus, Brownian motion in the context of trait evolution predicts that traits evolve in a manner that is proportional to the corresponding branch length in a phylogeny (Felsenstein, 1988; Schluter et al., 1997; Vieites et al., 2009). A close fit between inferred trait evolution and Brownian motion indicates that evolution proceeds in a manner similar to genetic drift, and excludes trait evolution by selection. Climatic niche evolution should generally proceed in such a manner, but deviations may present themselves in cases where evolution is driven by environmental variables or competitive exclusion such that selection conserves a particular set of traits. For example, salamanders need specific levels of moisture in their habitat for physiological function. This conserves their ecological niche, and consequently disrupts Brownian motion in their evolution, as well as obfuscates phylogenetic signal. In such a case, ancestral niche estimation cannot proceed with confidence and results should be treated with explicit caution (Vieites et al., 2009).

Phylogenetic Recapitulation and Evolutionary-Developmental Modification

Another kind of similarity among species that is incompletely understood is the relationship between ontogeny (development) and phylogeny (Alberch and Blanco, 1996). Ernst Haeckel (1866) postulated that ontogeny recapitulates phylogeny, whereby distantly related species that descended from a common ancestor pass through embryological stages that resemble the adults of their common ancestor. This claims that development for any given organism summarises the phylogenetic changes of its deep phylogenetic history during

embryonic development. However, studies have shown that there is no explicit recapitulation of ancestral adult stages during ontogeny (Ehrlich et al., 1963; Gould, 1977; Kalinka and Tomancak, 2012). Instead, an approximate parallelism exists where similarity of some features appear to recapitulate phylogeny (Alberch and Blanco, 1996; Gould, 1977; Kalinka and Tomancak, 2012; Ziermann and Diogo, 2013). For example, human embryos possess gill slits that are homologous with fish and bird embryos, but which are lost later in development. Moreover, older functional genes that presumably arose in ancestors, tend to be predominantly expressed at early life stages in *Drosophila* and zebrafish (Domazet-Lošo and Tautz, 2010). This indicates phylogenetic parallelism during development may result from a kind of conservation of features. However, the distinction must be made between embryonic and post-embryonic stages. The former occur in protected environments with minimal selection pressure, that are instead dominated by developmental constraints and gene regulation (Domazet-Lošo and Tautz, 2010). Moreover, the role of post-embryonic selection and its complex interactions with developmental processes must be included. As such, the discussion must widen to include evolutionary-developmental modifications that would slow down or accelerate development (Klingenberg, 1998), as well as factors that conserve the sequence of developmental features due to functional and developmental constraints (Mayr, 1994). Complex interactions between these factors may conserve or erode parallelism between phylogeny and development.

An example of developmental parallelism with phylogeny is in the sequence and timing of head segmentation in early development of pterygote hexapods (Orthoptera, Hemiptera, Coleoptera and Diptera) and the expression patterns of *Engrailed* (*En*) genes required for segment border formation (Rogers and Kaufman, 1997). Expression patterns are divided in the intercalary area of the procephalon in most insects (Insecta: Pterygota). Yet, these remain undivided (continuous) in basal Thysanura (Insecta: Apterygota) (Rogers and Kaufman, 1997). This suggests developmental variation that parallels phylogeny where the ancestral state is present in basal groups as a symplesiomorphy, and not by reversal. In contrast, deviations by reversal are also present and one example is flightlessness that is synapomorphic in some insects. Wings and ovaries develop simultaneously in female insects (Johnson, 1969), and short-winged individuals are generally more fecund than their long-winged counterparts because more energy is available for developing ovaries (Roff, 1986). As such, wing development can become selected against in favour of offspring abundance, and remain in a juvenile state. This promotes the tendency for delayed development (neoteny), which produces paedomorphic adults in terms of wing development. Female Mutillidae (Insecta: Pterygota: Hymenoptera) are an example of such a process. Such reversals can be mistaken for the basal symplesiomorphy of winglessness, where directional selection pressure

destabilises signal for phylogenetic recapitulation by inducing evolutionary-developmental modifications.

It is possible to envision a two-component mechanism for phylogenetic parallelism that would produce developmental phenotypes apparently consistent with recapitulation, based on arguments of selection borne intrinsically during development. The first component considers developmental constraints where developmental features that deviate too greatly destabilise subsequent functions and processes in development (Mayr, 1994). Developmental destabilisation can render a deleterious effect on survival, and initiate a selective advantage for individuals that retain ancestral developmental pathways. This results in developmental conservation (Fig. 1.1A) and forces significant changes to occur primarily towards the end of development to maintain viability of developmental processes (McKinney and McNamara, 1991). Evolutionary-developmental changes that occur toward the end of a life cycle have been termed 'terminal modification' by Alberch & Blanco (1996), and are important in cases of accelerated development (peramorphosis) where developmental time is still available for changes to take place after morphogenesis is complete, but before growth ends or the organism dies (Fig. 1.1A). The second component is complex and involves greater timescales (Fig. 1.1B). Developmental features that are beneficial and arise early will confer a selective advantage to increase fitness, such as sexual maturity (Ekstig, 1994; Müller, 1869). Ancestral features have more time and opportunities for selection to move them toward an earlier onset, and this will produce a tendency for beneficial ancestral features to become expressed earlier in development (Clune et al., 2012). Moreover, due to ancestral features accumulating over evolutionary time, but finite developmental time in which to express them, features that are non-essential for subsequent processes will be selected against. Phenotypes able to better optimise developmental energy and reach sexual maturation quickly will be favoured. This will fragment development such that only features which are essential to subsequent development will remain, and their expression will become truncated to the lower limit of their duration of utility. This process has been termed 'condensation' (Gould, 1977) and 'Müller's force' (Clune et al., 2012; Fig. 1.1B), and suggests there always exists a tendency for selection to accelerate development (Ekstig, 1994). Phylogenetic parallelism that approximates recapitulation will result as a special case of accelerated development where key features of development interact with developmental pathway constraints and become conserved as symplesiomorphic developmental characters. In this case, selection arises primarily from intrinsic sources to optimise resource availability and hasten sexual maturity, as opposed to arising from environmental and ecological sources with different sets of selection pressure to survive. As such, condensing selection to retain key ancestral features and remove others, would emerge over great timescales in the absence of environmental selection (Fig. 1.1B). This makes the

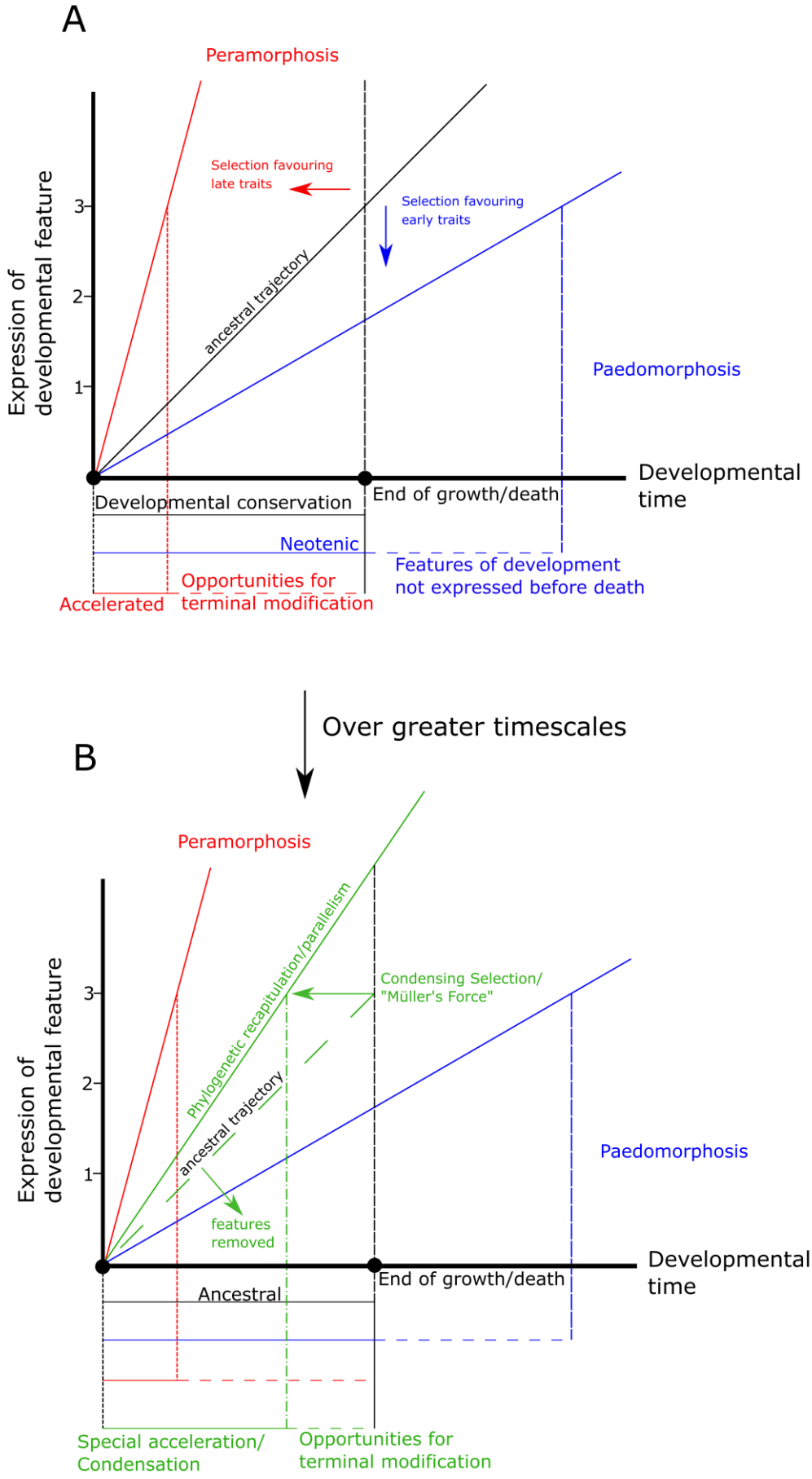

Figure 1.1. Diagram of evolutionary-developmental modification (A) and phylogenetic recapitulation (B). Paedomorphosis (blue) and peramorphosis are useful in testing for phylogenetic recapitulation, where paedomorphosis excludes phylogenetic recapitulation, and peramorphosis is a prerequisite. However, it must be noted that peramorphosis is not exclusive to phylogenetic recapitulation. Colours represent distinct processes of evolutionary-developmental modification (Black: Developmental Conservation; Blue: Neoteny/Post-displacement; Red: Acceleration/Pre-displacement; Green: Condensation/Phylogenetic Recapitulation).

phylotypic embryo stage something of a 'developmental basket' where ancestral features important for subsequent development are conserved up to the lower limit of their developmental utility. Moreover, condensing selection simultaneously facilitates opportunities for morphological novelty to emerge by developmental system drift (Haag and True, 2018) in subsequent developmental stages. This is similar to terminal modification, but is relative to condensed stages rather than to ancestral adult stages (*sensu* Alberch and Blanco, 1996). This makes complex processes and constraints in development crucial to the origin of novelty in morphological evolution (Ostachuk, 2016). Taken together, the interaction between complex developmental mechanisms and environmental selection pressures facilitate morphological adaptation that enable organisms to enter novel niches. In this way, deep phylogenetic signal can be retained, albeit in a summarised, fragmented and truncate form.

A simulation study in digital Avida organisms demonstrated the tendency for ontogeny to recapitulate phylogeny, and recovered the two factors described above (Clune et al., 2012). A third factor was noted where increasing complexity throughout development generated a tendency for recapitulation to occur (Clune et al., 2012). This was termed 'complexity correlation', and is expected to arise as organismal complexity increases through evolution. Increased complexity bolsters developmental stability through interaction effects between changes in developmental pathways where a prerequisite constraint exists to maintain prior functions of ancestral development along iterative steps into novel evolutionary-developmental modifications (developmental conservation). This simulation indicates that factors of selection, conservation and interaction effects should exist in development that produce a tendency for phylogenetic parallelism to arise that resembles recapitulation.

In principle, this process should run continuously as a baseline for evolutionary-developmental modification given constant selection to optimise developmental resources and accelerate sexual maturity to maximise fitness (Ekstig, 1994). In phenotypes that interact with environments however, recapitulation is likely obscured by different sets of directional selection pressure that select for solving different problems at different life stages (Grandjean, 1957; Klompen et al., 1989). As such, recapitulation is most clearly observable in embryos sheltered from the environment, and probably rare in life stages interacting with the environment. The disruptive mechanisms of neoteny and non-special acceleration are termed 'heterochronies' where changes evolve in both rate and timing of developmental features in descendant species compared with common ancestors (Gould, 1977). These result in adult morphology which resembles that of juveniles in the case of paedomorphosis, or overdevelopment in the case of peramorphosis. These states indicate development proceeds under neoteny, post-displacement or progenesis (paedomorphosis) versus acceleration, pre-displacement or hypermorphosis (peramorphosis) (Gould, 1977; Reilly et al., 1997). Following

this, environmental selection that favours early developmental features will prolong expression and slow development to override any subsequent ancestral features, and override signal for previous recapitulation (Fig. 1.1A). For example, adults of some axolotl species retain gills from juvenile stages (paedomorphosis) due to neotenic selection for living in aquatic environments, and this induces loss of parallel phylogenetic signal between trees drawn from morphological and molecular data (Wiens et al., 2005). Similarly, selection pressure which favours later developmental features (such as feeding apparatus or sexual maturity) induces earlier onset or accelerated development of those features (peramorphosis), and will obscure phylogenetic signal. In the case of peramorphosis however, signal for recapitulation can be present or absent depending on whether the features are old (deeply ancestral), and important for subsequent development. If so, they will be retained throughout evolution and their development will be moved earlier or accelerated by condensing selection. If not, novel phenotypes will replace or displace them and signal for recapitulation will be lost. Taken together, peramorphosis can arise by recapitulation (special acceleration) or non-special acceleration, while paedomorphosis can only arise by neoteny and similar process, excluding the possibility of recapitulation. This means testing for recapitulation may proceed by first detecting signal for peramorphosis, where ancestral adult traits resemble contemporary juveniles, and then by comparing the order in which features arise in development and phylogeny. Heterochrony and selection can cause deviations from developmental conservation that would otherwise result in phylogenetic recapitulation by condensing selection over great timescales. As such, the risk of conflating special-case acceleration (condensation and recapitulation) with non-special acceleration is always present and must be taken into account.

The historical focus on temporal components of development such as heterochrony has also hindered our understanding of recapitulation (Alberch and Blanco, 1996). This is especially true given heterochrony represents deviations from recapitulation – which may be a baseline evolutionary-developmental process. The spatial component of evolutionary changes in development hold additional evidence to test for phylogenetic signal along developmental trajectories (Zelditch et al., 2000; Zelditch and Fink, 1996). This was originally framed in terms of heterotopy by Haeckel (1866), which refers to changes in organismal form through development of descendant species. Form is best measured as geometric shape (Bookstein, 1991), and when applying this to development, form is measured as shape change along developmental trajectories. Changes in developmental shapes between species comprise both temporal and spatial elements that are mostly correlated (Klingenberg, 1998; Zelditch and Fink, 1996). If spatial elements are not similar, inference of temporal changes may be misrepresented, which may result as inaccurate inference of parallelism or deviation

from developmental conservation (Zelditch and Fink, 1996). This is an important assumption and highlights the value of conducting such a study in a comparative framework on a group of closely related, monophyletic and morphologically similar organisms where developmental time is standardised. Shape changes in this context, refer to emergence of developmental features (y-axis in Fig. 1.1). Geometric morphometric analyses should be employed to rigorously capture multivariate shape changes along developmental trajectories of features that are inherently multidimensional in shape (Zelditch et al., 2004). Moreover, dimensionality bias is a problem when using linear measurements, but is bypassed when using a geometric morphometric approach (Webster and Zelditch, 2005).

Rhipicephalus in Context

Ticks of the genus *Rhipicephalus* Koch, 1844 are obligate bloodfeeding parasites of vertebrates that are important for agriculture and human health (Keirans and Durden, 2005). They transmit microparasites such as *Rickettsia* spp., *Theileria* spp. and *Babesia* spp. to livestock and humans alike (Walker et al., 2000). Additionally, they may inject neurotoxins during feeding that can lead to tick paralysis. Control measures are limited to traditional acaricides, which may be harmful to ecology, especially when abused by farmers, or even rendered ineffective due to resistance (Graf et al., 2004; Willadsen, 2006). Interspecific distinctiveness based on morphology is low, making species difficult to distinguish, but at the same time intraspecific variability can be high, complicating taxonomy, systematics and identification (Beati and Keirans, 2001). Nevertheless, alpha taxonomy of *Rhipicephalus* is relatively well resolved, with many species delimited and described by Walker *et al.* (2000) in an exhaustive taxonomic revision. *Rhipicephalus* comprises ~75 species worldwide (depending on species definitions), and of these ~64 are Afrotropical endemics (Barker and Murrell, 2004; Camicas et al., 1998; Guglielmone et al., 2014; Horak and Camicas, 2003). They are one of the largest genera of Ixodidae Koch, 1844 and are morphologically distinguished by short hypostome and palps, basis capituli approximately hexagonal, eyes present, male adanal plates present and generally inornate, uniform brown body colour with exception of four ornate species (Keirans, 1992). The first described species was *R. sanguineus* (Latreille, 1806) and was placed in *Ixodes* Latreille, 1795. Later, Koch (1844) recognised differences between this species and others he described, and established the genus *Rhipicephalus* named for their brown cephalothorax that is fused with the abdomen, imparting a head-like appearance to the body. Evolutionary relationships between species and factors that drive *Rhipicephalus* evolution have received attention from a few workers, but are not fully understood (Barker and Murrell, 2004, 2002; Beati and Keirans, 2001; Burger et al., 2014; Labruna et al., 2009; Mangold et al., 1998; Murrell et al., 2001, 2000). Concomitantly,

nucleotide sequence data for Afrotropical tick species is greatly insufficient (Estrada-Peña et al., 2012; Mans et al., 2016). Most attention has focused on economically important species such as *R. microplus* (Canestrini, 1888), and consequently a comprehensive baseline understanding of tick biology, rooted in tick evolution and biodiversity is lacking for many taxa. Such knowledge would facilitate better understanding of tick distributions and disease relationships, as well as lead to development of more sophisticated control measures.

Diversification in ticks has been considered from a variety of perspectives. Host specificity first dominated hypotheses, with the suggestion that mouthparts and coxae are specially adapted for particular hosts, indicating co-evolution (Hoogstraal and Kim, 1985). Detracting from this however, are the generalist host-use patterns displayed by many ticks (Klompen et al., 1997). Another possibility is that host size may play a role given adults feed on larger hosts and immatures on smaller hosts, and these hosts are often phylogenetically distant (Murrell et al., 2001). Moreover, host mobility also plays some role given the observed effects of host-use patterns on tick geneflow (Matthee, 2020; Sands et al., 2017a). Abiotic factors of climate have recently come under investigation for their role in tick diversification and limiting geographic ranges. Distribution patterns in Afrotropical ticks are not similar to host distribution patterns, and are probably limited by some combination of environmental variables instead (Cumming, 1999). Climate niche models approximate geographic distributions, and indicate abiotic factors such as temperature and precipitation have a primary role in limiting tick distribution (Cumming, 2002, 2000). This imposes an alternative hypothesis for tick diversification, where adaptation is to habitats and not hosts, and arises as a consequence of ecology and biogeography driving diversification. Anecdotal support for this is noted in ticks that parasitise phylogenetically distant hosts, but with similar nesting and behavioural habits (Klompen et al., 1997). Moreover, a combination of host use patterns, competition for hosts and abiotic environmental factors may lead to complex interactions that drive tick diversification and radiations (Cangi et al., 2013; Cumming, 2002; Klompen et al., 1997; Sands et al., 2017a). Adversely, a first inquiry into the dynamics of climate niches in Afrotropical ticks found that principal component variation for monthly temperature and rainfall is not correlated with genus-level taxonomy (*i.e.* a proxy for evolutionary relationships) (Estrada-Peña *et al.* 2012). Instead, tick genera present overlapping climatic niches, indicating that shared biogeographic history is a main factor limiting distributions at genus level. However, this may not be true at lower taxonomic levels, such as between species or among clades, because closely related species exhibit some specialisation and tend to occupy differentiated niches (Estrada-Peña et al., 2012). In general, these findings indicate that tick diversification is influenced by a complex of factors that require careful and comprehensive investigation.

Placing tick climate niches in phylogenetic context and determining the ancestral niche will aid in better understanding the processes of diversification in ticks due to the abiotic environment.

Ticks pass through one inactive and three active life stages that comprise egg, larva, nymph and adult; the last being the sexually mature stage (Fig. 1.2; Apanaskevich and Oliver, 2014). In *Rhipicephalus*, larvae are more simplified in morphology, having only six legs and basis capitulum cornua generally absent (Anderson and Magnarelli, 2008). Nymphae become more complex, having cornua either present or absent (depending on species) as well as having lateral extensions of their basis capituli generally present either posteriorly, anteriorly or transverse centrally. Additionally, nymphae have an extra pair of posterior legs that bring the final count of eight legs observed in Arachnida. Adults have more robust basis capituli where cornua are generally present and lateral extensions may persist or become reduced (Fig. 1.2). Adult females develop porous areas on their basis capituli, and their basis capitulum shapes differ from that of males (Fig. 1.2). Adult ticks also develop genital apertures which are absent in larval and nymphal stages (Durden and Beati, 2014). These life stages are punctuated by moults which follow from single bloodmeals (Apanaskevich and Oliver, 2014). Throughout its life, an individual may utilise one, two or three hosts depending on the habits of the species. Most *Rhipicephalus* ticks utilise three hosts where the larvae, nymphae and adults each drop off the host after feeding in order to moult. Exceptions include two-host ticks that will utilise the same host as larvae and nymphs but then drop off and feed on a different host as adults (Apanaskevich and Oliver, 2014). These ticks include the evertsi group comprising *R. bursa* Canestrini & Fanzago, 1878; *R. evertsi* and *R. glabroscutatum* Du Toit, 1941 (Walker et al., 2000). Furthermore, one-host species will utilise a single host for the entire life cycle and only drop off to lay eggs. These life cycles are exclusive to the subgenus *Boophilus*. The evertsi group (subgenus *Digineus*) has been demonstrated as phylogenetically close to subgenus *Boophilus*, suggesting the possibility that host truncation to two-host life cycles evolved once in *Rhipicephalus* that was followed by transition to one-host life cycles (Barker and Murrell, 2002). The study of morphological shape development is well suited to *Rhipicephalus* ticks because they exhibit three active life stages that are distinctive in morphology. Moreover, moulting allows for simple and effective co-ordination of age (developmental time) amongst samples. Age is vitally important to such a study, however chronological time is not the same as developmental time (Alberch and Blanco, 1996; Godfrey and Sutherland, 1995; Reiss, 1989). Consequently, chronological time does not serve as a good proxy for age, despite many studies having used chronological time. Size is a better measure of development, but must be coupled with appearance of a feature to approximate developmental time effectively (Alberch and Blanco, 1996; Klingenberg, 1998). In ticks, morphology changes significantly with moults, enabling comparison between developmental

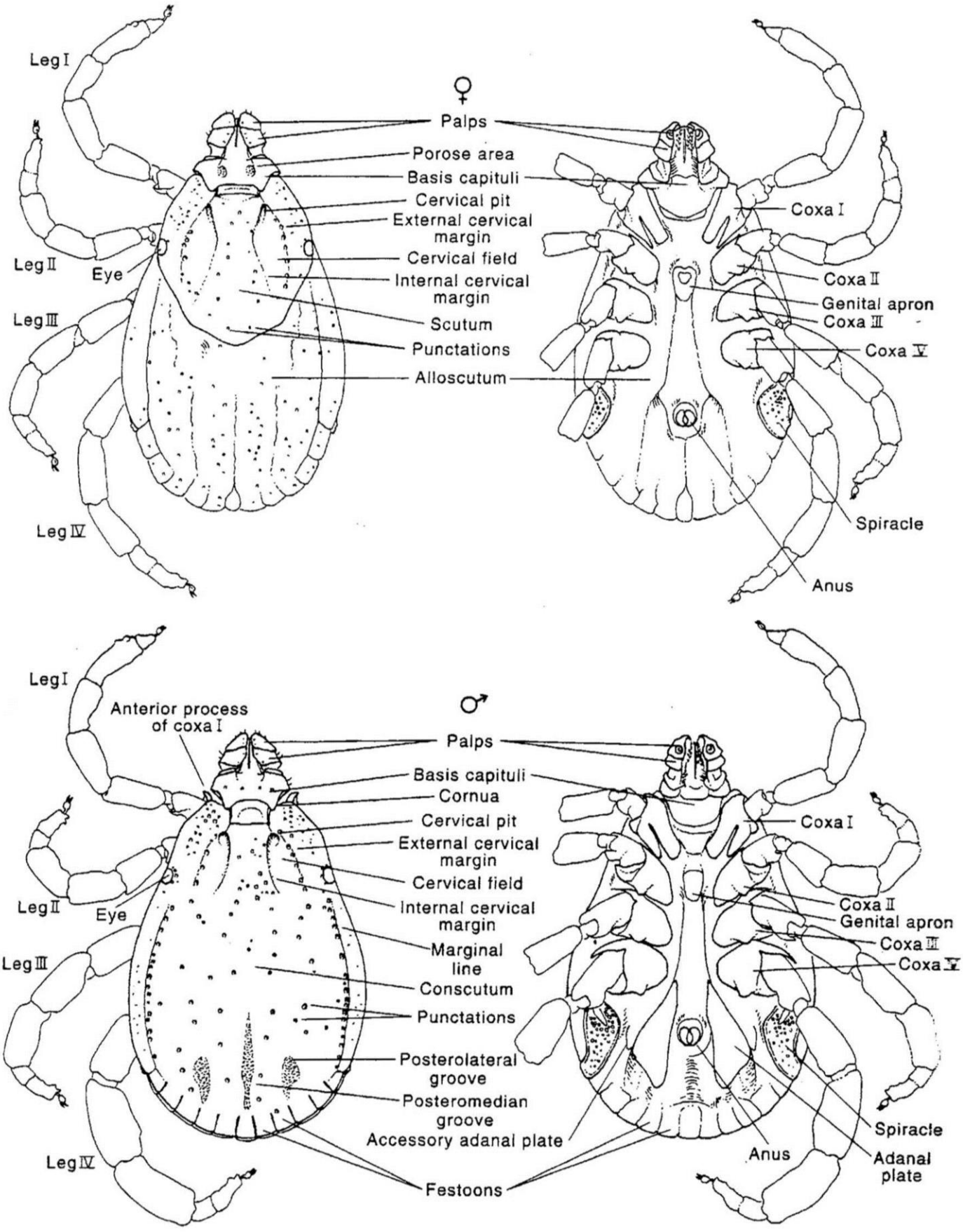

Figure 2.2. Diagram of generalised *Rhipicephalus* morphology for adult females (top) and adult males (bottom).

trajectories in terms of shape and size, where size approximates age. In this work, development is measured as shape change that is correlated with stage-linked size increase (allometry) along the y-axis (vertical), and stage along the x-axis (horizontal).

Comprehensive phylogenies often initiate taxonomic changes and elucidate new species due to the breadth of taxon sampling that enables effective comparison between taxonomic units (Charles and Godfray, 2002; Dayrat, 2005; Padial et al., 2010; Schlick-Steiner et al., 2014; Wiens, 2007; Yeates et al., 2011). Several *Rhipicephalus* species already represent unsolved taxonomic problems for which qualitative morphology is inconclusive, and one such example is *Rhipicephalus turanicus* Pomerantsev, 1940 (Pegram et al., 1987a; Walker et al., 2000). This species is a member of the *R. sanguineus* group and closely resembles *R. sanguineus* in morphology. Their geographic distribution covers almost all of Africa and Eurasia, raising the possibility that the taxon may contain more than one species (Pegram *et al.* 1987a; Walker *et al.*, 2000; Dantas-Torres *et al.*, 2013; Hekimoğlu *et al.*, 2016; Zemtsova *et al.*, 2016). Geographically distant members of *R. turanicus* have divergent morphology between African and Cypriot (Greece) strains (Pegram et al., 1987a). However, these strains readily interbreed and produce viable progeny with hybrid vigour, presumably by heterosis (Pegram et al., 1987a). Of all crosses, 90% produced offspring, however greater amounts were produced by crosses between strains (*circa* 5000 in strain-hybrids vs *c.* 4000 in same strain crosses). Preliminary sequence data from Genbank (COI, 12S, 16S, ITS2) suggest there may be two lineages of *R. turanicus* in the Palearctic which could indicate two different species; one in Uzbekhistan and surrounds (type locality), and one in western Europe and the middle east (Hekimoğlu et al., 2016; Latrofa et al., 2013; Moraes-Filho et al., 2011; Nava et al., 2012). Furthermore, limited sequence data from Genbank also suggest that the Afrotropical strain is highly divergent in COI, 12S & 16S genes of both putative Palearctic lineages (Dantas-Torres et al., 2013; Hekimoğlu et al., 2016).

Research Aims

This study aims to estimate phylogenetic relationships for *Rhipicephalus*, to serve as a basis for further investigations in an evolutionary and comparative context. Of particular interest, is to investigate mechanisms that gave rise to the diversity of the genus that may also be applied to other tick genera. The first data chapter (chapter 2) investigates the evolutionary history of *Rhipicephalus* with respect to species genealogical relationships, divergence times, historical biogeography as well as ancestral climate niches and host-use patterns. These inferences will provide evidence to better understand factors that played a role species diversification, range expansions and contractions. Additionally, these findings can aid to establish subgenera for *Rhipicephalus* founded on evolutionary grounds that maximise

predictive power associated with taxonomic names. Predictive power is valuable for the scientific community and the public to underpin downstream research and applications ranging from tick proteomics to distribution modelling, especially in the light of impending climate change and associated epidemiological risk.

One avenue of downstream work that will be based on phylogeny, is testing phylogenetic recapitulation and evolutionary-developmental modification in basis capitulum shapes. These structures were selected for study due to variable morphology during development and between species. Variation is necessary to provide adequate signal for shape changes between species and throughout development. When measured in a geometric morphometric framework, shape variation can be cross referenced with phylogeny and ecological factors to determine whether shape change is stochastic or directional (under selection). Determining causes and mechanisms underlying developmental evolution in a substructure of tick mouthparts will contribute to the forefront of evolutionary biology and the emerging field of evo-devo from a perspective above the level of gene complexity, by using a comprehensive developmental shape dataset which has not been done before.

Finally, a species that presents a challenge to tick taxonomy, *R. turanicus* between Afrotropical and Palearctic regions, will be investigated for validity of species status by including molecular and morphological shape data to test a species boundary hypothesis. Morphological features chosen for study include male adanal plates as well as female spiracles, because they are known as useful species diagnostic features for *Rhipicephalus* taxonomy. Findings pertaining to the presence or absence of this species boundary will aid in better understanding the mechanisms involved in tick speciation and may establish a new species to be considered for vector competence. Moreover, this will demonstrate modern tools that are useful to delimit closely related species.

Key Research Questions and Hypotheses

Chapter Two – *Rhipicephalus* Phylogeny and Historical Biogeography:

1) What are the phylogenetic relationships between *Rhipicephalus* species and groups?

Hypothesis: Congruent with Murrell *et al.* (2001). *R. sanguineus* and related species are basal, with sequential phylogenetic branching of *R. appendiculatus* group and *R. evertsi* group, with *R.* (*Boophilus*) as most derived.

2) Are the nine sub-generic species groups of Walker *et al.* (2000) monophyletic?

Hypothesis: The nine species groups of Walker *et al.* (2000) are monophyletic.

3) Do ancestral area and divergence time estimates indicate an African origin for *Rhipicephalus*?
Hypothesis: Divergence time estimates post-date the breakup of Gondwanaland (Beati and Klompen, 2019; Mans et al., 2019), supporting the hypothesis for African origin to explain the abundance of basal species in the Afrotropics.

4) Does (i) host-use, (ii) climate or (iii) geography correlate best with *Rhipicephalus* phylogeny?
Hypothesis: Climate is the primary factor that correlates with *Rhipicephalus* phylogeny (Cumming, 2002).

5) To what degree are climate niches conserved in *Rhipicephalus*?
Hypothesis: Two non-overlapping niches are present (wet and dry) such that clades are adapted exclusively to either one.

Chapter Three – Evolutionary-Developmental Modification in *Rhipicephalus*:

1) Does selection affect post-embryonic basis capitulum evolutionary-development?
Hypothesis: Selection is not the primary factor driving shape change in *Rhipicephalus*.

2) Is there signal for paedomorphosis (neoteny), peramorphosis (recapitulation) or no evolutionary-developmental modification between life stages among the 24 species of *Rhipicephalus* studied?
Hypothesis: Phylogenetically ancestral adults and contemporary immatures resemble one another in terms of shape variables, indicating peramorphosis and suggesting recapitulation may be present.

Chapter Four – *Rhipicephalus turanicus* species delimitation:

1) Do molecular markers support a monophyletic *R. turanicus*?
Hypothesis: Molecular markers support distinct clades of *R. turanicus* in the Afrotropical and in the Palearctic regions (Dantas-Torres et al., 2013; Hekimoğlu et al., 2016).

2) Do female spiracle and male adanal plate shapes support a monophyletic *R. turanicus*?
Hypothesis: Shape variation in female spiracles and male adanal plates support two distinct clades of *R. turanicus*, one in the Afrotropical region and another in the Palearctic region.

2 Adaptive Radiation and Speciation in *Rhipicephalus* ticks: A Medley of Novel Hosts, Nested Predator-Prey Food Webs, Off-host Periods and Dispersal Along Temperature Variation Gradients

Abstract

Rhipicephalus are a species-diverse genus of ticks, mainly distributed in the Afrotropics with some species in the Palearctic, Oriental and Australasian regions. Current taxonomic consensus comprise nine informal species groups/lineages based primarily on immature morphology. This work explores their phylogeny and historical biogeography by integrating biogeographic and molecular lines of evidence. Phylogenetic analysis based on four genes (12S, 16S, 28S-D2 and COI) recovered five distinct clades with nine descendant clades that are generally congruent with current taxonomy based mainly on morphology, but with some exceptions. Historical biogeography is reconstructed from estimates of molecular divergence times, ancestral areas, ancestral host-use and ancestral climate niches based on four bioclimatic variables of annual, seasonal and diurnal temperature variation that correlate with phylogeny. Estimates indicate *Rhipicephalus* originated in central Africa during the Paleocene-Eocene (52-72 Mya), with their origin linked to either an adult stage host shift from predators to early Ungulata that entered Africa and provided novel niches for ticks (if *Rhipicentor* is sister), or to radiation of early Ungulata and Rodentia (if *Hyalomma* is sister). Within Africa, two ancestral lineages dispersed southward during the Oligocene (20-35 Mya) in a period of faunal turnover associated with grassland and savannah expansion. From these southern lineages, at least three descendant lineages dispersed back to central Africa during the Miocene (10-20 Mya) in the period of Ruminantia diversification that followed their dispersal into Africa from Asia via the Gomphotherium Land Bridge (Arabian Peninsula). Once Ruminantia dispersed and diversified in southern Africa, *Rhipicephalus* ticks utilised these novel hosts and dispersed into established host populations northward. Dispersals out of Africa occurred at least twice, first along a northwest Africa–west Mediterranean route (18-31 Mya), then along the Arabian Peninsula (1-17 Mya). Notably, sanguineus group ticks dispersed back into Africa during the late Miocene (9 Mya). Ancestral climate niche estimates corroborate these dispersal events by indicating hypothetical *Rhipicephalus* ancestors moved into environments with different annual, seasonal and diurnal temperature variation along latitudinal gradients. Novel hosts may have been accessible to ticks by nested predator-prey food web connections combined with off-host periods that characterise tick life cycles. Host-use at different life stages was important for dispersal and diversification of *Rhipicephalus*. Tick species that utilise large and mobile hosts (Ungulata and Carnivora) early in development could disperse further. Conversely, species that utilise small and relatively immobile hosts (Rodentia, Lagomorpha and Afroinsectivora) early in development, are more dependent on the mobility of hosts for adult stages during dispersal. Taken together, these indicate

Rhipicephalus radiations were initiated by host switches in response to novel niches provided by host dispersals, and that off-host periods are important for providing host-use flexibility. Speciation is partially driven by climate niche partitioning along annual, seasonal and diurnal temperature variation gradients in latitudinal shifts that differentially approximate ancestral niches in novel environments along dispersal routes. Another driver of speciation may be reduced geneflow that results from utilising small hosts with limited dispersal ability early in development. Moreover, competitive interactions can reinforce these processes and also drive speciation. Off-host periods facilitate both processes of speciation (climate niche partitioning) and adaptive radiation by facilitating host switches along nested predator-prey connections in food webs, but at the cost of environmental exposure that partition niches among dispersing progenitors. As such, evolution and niches of the Brown ticks are characterised by trade-offs between on- and off-host periods, and these trade-offs interact with nested predator-prey connections in food webs, host-use of different life stages and associate host size, dispersal of hosts, as well as latitudinal annual, seasonal and diurnal temperature variation gradients to drive adaptive radiation and speciation.

Introduction

Organisms undergo evolutionary radiations when they enter and adapt to a niche that is open and novel relative to the native niche of the organism (Gavrilets and Losos, 2009). Adaptive radiations are often associated with a 'key innovation' in the phenotype that can facilitate better competence for using the novel niche (Gavrilets and Vose, 2005). However, these processes are often linked to ecological opportunity rather than innovation alone (Gavrilets and Vose, 2005), and are characterised by speciation events (Schluter, 1996). In the case of pollinators or parasites, radiations may be linked to coevolution where biological interactions drive innovation (Ehrlich and Raven, 1964). However, radiations of parasites have often been misinterpreted as coevolution with their hosts due to the appearance of close association between organisms, but which lack a history of interrelated evolutionary responses (Janzen, 1980).

Phylogenies provide the framework that can be used to make inference about evolutionary processes in a group of organisms, such as indicating speciation associated with radiations, or inferring ancestral states for a given trait (Pagel et al., 2004; Schluter et al., 1997). A set of phylogenetic relationships offer comparative data for testing patterns of trait distribution among species given non-independence due to shared ancestry (Adams and Collyer, 2015). Phylogenetic signal can be measured from these tests where a high correlation between trait distribution and phylogenetic distances indicate traits are phylogenetically structured by drift (based on genes where changes are generally neutral and fixed by drift),

and low correlation indicates traits may be under selection or play a role in driving radiations (Blomberg et al., 2003; Münkemüller et al., 2012; Pagel, 1999). Geographic occurrences confined to specific regions for a prolonged period of time is one example of discrete traits (Ronquist, 1997), where large genetic distances fixed by drift, will approximate large geographic distances provided recent human-mediated movement is absent (Novembre et al., 2008). Ancestral states for continuous traits may also be inferred, and one example is parameters that form climate niches (Budic and Dormann, 2015; García-Navas and Rodríguez-Rey, 2019; Pie et al., 2017; Vieites et al., 2009). In an evolutionary context, this determines whether multiple divergent niches are present in a clade, and to what degree these niches are conserved within clades (Münkemüller et al., 2015; Peterson, 2011; Wiens and Donoghue, 2004). This places climate niches into phylogenetic context to facilitate better understanding of trait dynamics for downstream questions.

Estimates of ancestral climate niches, however, must be made cautiously with two critical assumptions tested. The procedure incorporates climate data derived from a range of point localities to model and predict geographic distributions, but these data may misrepresent climate tolerances, because they represent realised niches determined by biogeographic history or biotic interactions in addition to climate tolerance (Estrada-Peña et al., 2012; Peterson, 2001; Vieites et al., 2009). Evaluating the fit between predicted and known geographic distributions can test whether climate data accurately represent climate niches, and whether estimates are trustworthy (Vieites et al., 2009). The second assumption to be met for accurately estimating ancestral climates niches (using maximum likelihood for example - Schluter et al., 1997) is that trait evolution should proceed in a manner similar to Brownian motion (Vieites et al., 2009). Brownian motion (borrowed from physics) accounts for all components in a system dispersing randomly to gradually maximise all distances. In evolution, this represents a stochastic factor which approximates genetic drift and makes change proportional to phylogenetic distances, providing a vantage point to test trait correlation across a phylogeny (Felsenstein, 1988; Schluter et al., 1997; Vieites et al., 2009). A close fit to Brownian motion indicates trait evolution proceeds in a manner approximating genetic drift, and excludes trait evolution by stabilising selection (Blomberg et al., 2003; O'Meara et al., 2006; Pagel, 1999). Deviation from Brownian motion occurs when stabilising selection from environmental variables have significant limiting effect on a species. For example, salamanders need specific moisture conditions for physiological functions. This conserves their ecological niche, disrupts stochastic Brownian motion-like processes of neutral evolution, and obfuscates phylogenetic signal (Vieites et al., 2009). In such a case, ancestral niche estimates should be treated with explicit caution.

Ticks of the genus *Rhipicephalus* Koch, 1844 (Brown ticks) are obligate bloodfeeding ectoparasites of vertebrates that are of medical and veterinary importance (Keirans and Durden, 2005; Sonenshine, 1991). They transmit blood parasites such as *Rickettsia* spp., *Theileria* spp. and *Babesia* spp. to livestock as well as humans. Some species can also inject neurotoxins during bloodfeeding that cause tick paralysis (Walker et al., 2000). Interspecific distinctiveness between some species is problematic due to the lack of clear morphological synapomorphies, and intraspecific variability can also be large, complicating taxonomy and systematics (Beati and Keirans, 2001). Nevertheless, alpha taxonomy of *Rhipicephalus* is relatively well resolved, with many species described and groups delimited by Walker *et al.* (2000) and Horak et al. (2018) in exhaustive taxonomic revisions.

Rhipicephalus comprise ~75 species worldwide (depending on species definition), and ~64 are Afrotropical endemics (Camicas et al., 1998; Guglielmone et al., 2014; Horak et al., 2018; Horak and Camicas, 2003). They are hard ticks and represent one of the largest genera in Ixodidae Koch, 1844. Members of this genus are morphologically distinguished by short hypostome and palps, basis capitulum approximately hexagonal, eyes present, male adanal plates present, and uniform brown body colour with exception of four ornate species (Keirans, 1992). Species have traditionally been classified in four subgenera (Camicas et al., 1998), comprising *Digineus* Pomerantsev, 1936; *Hyperaspidion* Pomerantsev, 1936; *Pterygodes* Neumann, 1913 and *Rhipicephalus* Koch, 1844 (*sensu* Zumpt, 1950). However, Walker *et al.* (2000) questioned the validity of these taxa because they were based on adult stages only. She defined loose groupings based on shapes of basis capituli and palps throughout immature life stages, and delimited the following nine informal groups/lineages: evertsi (=*Digineus*), appendiculatus (containing *R. armatus* Pocock, 1900 from *Hyperaspidion*), Afrotropical sanguineus, Non-Afrotropical sanguineus, simus, follis, capensis, pravus, and haemaphysaloides (Oriental and Indo-malaysian species), leaving four species unplaced (*R. fulvus*, *R. cuspidatus*, *R. longicoxatus*, and *R. theileri*). Immature life stages for fifteen species are unknown and not designated to any group due to lack of data for comparative morphology.

Subgenus *Boophilus* (Curtice, 1891) was previously considered a separate genus, but is currently considered a derived lineage of *Rhipicephalus* most closely related to *R. pravus* Dönitz, 1910, *R. punctatus* Warburton, 1912 and *R. evertsi* Neumann, 1897. This was based on data from mitochondrial DNA for COI, 12S rDNA, 16S rDNA, and nuclear ITS2 sequence data in combination with revised morphology (Barker and Murrell, 2002; Beati and Keirans, 2001; Mangold et al., 1998; Murrell et al., 2001, 2000; Murrell and Barker, 2003). Neumann (1904) first proposed that *Boophilus* are derived *Rhipicephalus* stating their generic characters as insufficient for warranting generic status. This was corroborated much later by Camicas & Morel (1977) and cladistic analysis of morphology (Klompen *et al.* 1997). Overall, several

subgenera may be valid within *Rhipicephalus*, and it is important to test the validity of these groups in order to maximise predictive power that taxonomic names can offer to other fields.

Evolutionary relationships between *Rhipicephalus* species and factors driving their evolution have received some attention, but due to limited taxonomic sampling, remain unclear (Barker and Murrell, 2004, 2002; Beati and Keirans, 2001; Beati and Klompen, 2019; Burger et al., 2014; Coimbra-Dores et al., 2018; Klompen et al., 1996; Labruna et al., 2009; Mangold et al., 1998; Murrell et al., 2000, 2001). The same applies to their historical biogeography which aims to reconstruct geographic movements between constituent groups in a clade (Morrone and Crisci, 1995; Posadas et al., 2006; Wiens and Donoghue, 2004). Monophyly of *Rhipicephalus*, *Hyalomma* and *Rhipicentor* within the Rhipicephalinae has been widely supported by higher order taxonomic studies based on molecular data (Barker and Murrell, 2002; Beati and Keirans, 2001; Beati and Klompen, 2019; Mans et al., 2019), and these studies place *Rhipicentor* as basal among these taxa, with *Hyalomma* sister to *Rhipicephalus*. However, morphology suggest *Rhipicentor* is sister to *Rhipicephalus* instead (Klompen et al., 1997). Basal *Rhipicephalus* lineages occur only in the Afrotropics, suggesting an Afrotropical origin for the genus (Barker and Murrell, 2004). Divergence time estimates indicate *Rhipicephalus* origin between 20 Mya (18S rDNA - Beati and Klompen, 2019), and 100 Mya (protein alignment of mitochondrial genomes - Mans et al., 2019). These estimates are wide, however both confirm an African origin for *Rhipicephalus* that postdates the breakup of Gondwanaland at about 120 Mya (Seton et al., 2012). Africa was mostly isolated during this time, which explains why most *Rhipicephalus* are limited to the Afrotropics, with the few groups present in Palearctic and Australasian regions likely associated with post-Gondwanaland dispersal– excluding human-mediated dispersal of ticks to Nearctic and Neotropical regions (Dantas-Torres et al., 2018; Gray et al., 2013; Laatamna et al., 2020). Dispersal to the Palearctic and Australasia must have occurred after landmass and faunal connections were established at about 20 Mya onwards (Barker and Murrell, 2002; Cox and Moore, 1993; Sen, 2013; Seton et al., 2012). Most tick clades, including *Rhipicephalus*, are estimated to have radiated after the Cretaceous-Tertiary extinction (about 50-60 Mya), leading to the hypothesis that mammalian radiations played a significant role in tick dispersal and evolution (Beati and Klompen, 2019; Hoogstraal and Kim, 1985; Sands et al., 2017b).

Two main hypotheses have been considered for ecological drivers of tick evolution; parasite-host associations (Hoogstraal and Aeschlimann, 1982; Hoogstraal and Kim, 1985; Sands et al., 2017a) and climate niche (Cumming, 2002, 1999; Estrada-Peña et al., 2012; Zemtsova et al., 2016). Parasite-host associations were initially thought of as the sole factor in limiting geographic distribution among tick species due to coevolution, but this hypothesis was shown as incomplete because abiotic off-host conditions also play an important role

(Balashov, 2004; Klompen et al., 1996). This was supported by occurrence data that revealed tick species distributions did not match host distributions, with approximately 50% of known species having smaller geographic ranges than their hosts (Cumming, 1999; Olwoch et al., 2003). Moreover, variables related to minimum and maximum temperatures, as well as precipitation have been recovered as good predictors of tick species distribution (Cumming, 2002). Taken together, this indicates factors apart from host associations are involved in driving tick distribution and evolution, with climate proposed as a strong possibility for limiting distributions. That said, the role of hosts cannot be entirely ignored as they act as dispersal agents, and provide a buffer from off-host environmental conditions during part of the tick life cycle (Estrada-Peña and De La Fuente, 2014; McCoy et al., 2003; Norte et al., 2020; Olwoch et al., 2003; Sands et al., 2017a; Sonenshine and Mather, 1994). Moreover, off-host periods are an important factor because the availability of hosts for immatures plays a role in structuring populations (Cangi et al. 2013; Matthee, 2020; Sands et al., 2017a). Other factors such as microclimate and phenotypic plasticity of the ticks themselves, as well as interspecific competition for hosts likely also play a role (Bournez et al., 2015; Cangi et al., 2013; Estrada-Peña and De La Fuente, 2014). As such, the proposed scenario of coevolution between ticks and their hosts, is likely a misidentification of evolutionary radiations (Janzen, 1980) that are driven by a multitude of complex factors (Gavrilets and Losos, 2009; Schluter, 1996).

The aim of the present study is to estimate phylogenetic relationships for *Rhipicephalus* ticks using molecular data from four genes to test the validity of proposed subgenera and species groups based on a suite of morphological characters. Secondly, historical biogeography is investigated by estimating divergence times and ancestral areas to test the hypothesis of an African origin for *Rhipicephalus*, as well as whether or not the phylogeny correlates with host radiation and intercontinental dispersals. Moreover, ancestral climate niches and host-use patterns are estimated to test correlations of climate versus host-use on *Rhipicephalus* evolution. Lastly, ancestral climate niche estimates are used to determine the degree to which climate niches are conserved in *Rhipicephalus*. The present study adds valuable nucleotide sequence data for Afrotropical species that are underrepresented in public nucleotide sequence databases. Nucleotide sequence data for many Afrotropical tick species are insufficient, greatly reducing the integrity and depth of conclusions than can be made (Estrada-Peña et al., 2012; Mans et al., 2016). Most effort has focused on economically important species such as *R. microplus* (Canestrini, 1888), and consequently a comprehensive baseline understanding of *Rhipicephalus* biology rooted in evolution and biodiversity is lacking. Such knowledge will facilitate better understanding of *Rhipicephalus* evolution, distribution and disease relationships, as well as contribute to the development of better control measures.

Methods

Sampling, DNA extraction, sequencing and alignment

Tick samples were collected directly from hosts or by dragging in a range of localities (Table 2.1). Collected individuals were placed in absolute ethanol and subsequently stored at -20ºC until DNA extraction. Specimens were provided and identified *a priori* by Ivan Horak and Dmitry Apanaskevich according to established taxonomic characters (Horak et al., 2018; Walker et al., 2000). Voucher specimens are housed at the Gertrud Theiler Tick Museum (GTTM), Agricultural Research Council - Onderstepoort Veterinary Research, South Africa and the United States National Tick Collection (USNTC), Georgia Southern University, USA. Up to four legs were removed from individual samples for DNA extraction using prepGEM (ZyGEM, Hamilton, New Zealand) and QIAamp DNA Mini (Qiagen, Venlo, Netherlands) extraction kits, and further procedures followed manufacturer instructions.

Four genes were targeted for sequencing to represent a range of mutation rates and selection pressures appropriate for species and genus-level phylogenetic signal. These included two mitochondrial ribosomal genes (12S and 16S rDNA), one protein-encoding mitochondrial gene (cytochrome oxidase I - COI), and one nuclear ribosomal gene (28S-D2). Fragments were amplified with polymerase chain reactions (PCR) using primers designed in previous studies (12S: Norris et al., 1999 | 16S: Black and Piesman, 1994 | 28S-D2: Sonnenberg et al., 2007 | COI: Chitimia et al., 2010; Folmer et al., 1994), and reactions were made up to a final volume of 50 µL containing 25 µL Emerald Amp® PCR Mastermix (TAKARA BIO INC., Otsu, Shiga, Japan), 10pmol of each primer in the presence of 2-20 ng of extracted genomic DNA template. Cycling parameters comprised initial denaturation: 94 °C (2 min); 40 cycles of 94 °C (30 s), 58±3 °C (30 s), 72 °C (2 min); final elongation: 72 °C (2 min). Successfully amplified products were purified and sequenced at the Central Analytical Sequencing Facility at Stellenbosch University, South Africa, following the Sanger method. All sequences were viewed, assembled and edited in CLC Genomics Workbench v9.5.2 (http://www.clcbio.com). New sequences were submitted to Genbank (accessions: 12S – MW080169-MW080209; 16S – MW080135-MW080168; COI – MW079312-MW079338; 28S-D2 – MW080093-MW080134), and additional sequences to maximise *Rhipicephalus* species representation, test sequence authenticity, and contribute data for appropriate outgroups were retrieved from Genbank (Table 2.1).

Sequences were aligned using MAFFT (Q-INS-i, 200PAM / k=2, Gap opening penalty: 1.53) (Katoh et al., 2002). Optimal nucleotide substitution models for each gene fragment were selected using Bayesian Information Criterion calculations in W-IQ-TREE (Trifinopoulos et al., 2016). Optimal models and associated parameters were applied to all subsequent analyses

Table 2.1: *Rhipicephalus* specimens sampled and analysed in the present study. Country of sampling origin is presented where known and sequences obtained from Genbank are indicated. Origin of sequences indicated by superscript numbers in each row where appropriate. Terminal names correspond to Fig. 2.1. '--' indicates missing data. Outgroup taxa used in the study are also listed in the same form separately.

Terminal name (Sample voucher name | Species | Locality)	12S	16S	28S-D2	COI
INGROUP TAXA				
R. afranicus SOUTH AFRICA[1]/ZIMBABWE[2]/ZAMBIA[3] (Genbank)	AF150017[1]	GU553080[2]	--	DQ859260[3]
RD13 *R. afranicus* SOUTH AFRICA (Genbank)	MN945326	--	--	--
RD12 *R. afranicus* SOUTH AFRICA (Genbank)	MW080209	MK158990	--	--
Ri1 *R. annulatus* CAMEROON	--	MW080159	MW080122	MW079335
R. annulatus GUINEA-BISSAU[1]/EGYPT[2]/INDIA[3] (Genbank)	KU568498[1]	MF946466[2]	--	MH612832[3]
R. appendiculatus SOUTH AFRICA[1]/ZAMBIA[2] (Genbank)	MH751457[1]	L34301[1]	KY457501[1]	DQ901363[2]
Ri9 *R. appendiculatus* SOUTH AFRICA	MW080196	--	MW080120	MW079333
R. australis AUSTRALIA[1]/NEW CALEDONIA ISL.[2] (Genbank)	KC503255[1]	KC503255[1]	--	KY678121[2]
Ri13 *R. bequaerti* ETHIOPIA	MW080192	MW080157	MW080115	--
Ri14 *R. bergeoni* ETHIOPIA	MW080199	MK941248	MW080124	--
R. bursa SPAIN[1]/IRAN[2] (Genbank)	AF150053[1]	AJ002956[1]	--	KT313103[2]
Ri16 *R. bursa* GREECE	--	--	MW080134	MW079338
R. camicasi ETHIOPIA (Genbank)	FJ536556	MN944881	--	--
Ri18 *R. capensis* SOUTH AFRICA	MW080185	MW080150	MW080109	MW079328
IV11 *R. carnivoralis* KENYA	MW080186	MW080151	MW080110	MW079329
Ri21 *R. compositus* ZAMBIA	MW080183	MW080148	MW080107	MW079326
R. compositus ZIMBABWE (Genbank)	AF031860	--	--	AF132834
R. decoloratus MALI[1]/KENYA[2]/SOUTH AFRICA[3] (Genbank)	KF569940[1]	MN266916[2]	KY457485[3]	KY67813[3]
Ri24 *R. distinctus* SOUTH AFRICA	MW080188	MW080153	MW080112	--
R. duttoni ANGOLA (Genbank)	MF425966	MF425974	--	MF425991
Ri25 *R. duttoni* ANGOLA	MW080203	MW080164	MW080129	--
Ri27 *R. e. evertsi* SOUTH AFRICA	MW080175	MW080141	MW080099	MW079318
R. e. evertsi SOUTH AFRICA[1]/LESOTHO[2] (Genbank)	MH751456[1]	KJ613642[1]	KY457503[1]	MN453671[2]
R. e. mimeticus NAMIBIA[1]/ANGOLA[2] (Genbank)	MF425965[1]	MF425975[2]	--	AF132836[1]
Ri28 *R. e. mimeticus* SOUTH AFRICA	MW080206	MW080165	--	--
Ri29 *R. exophthalmos* ORIGIN UNKNOWN	MW080179	MW080145	MW080103	MW079322
Ri30 *R. follis* SOUTH AFRICA	MW080194	--	MW080117	MW079331
Ri3 *R. geigyi* UPPER GUINEA	MW080197	--	MW080121	MW079334
R. geigyi GUINEA-BISSAU[1]/EGYPT[2]/INDIA[3] (Genbank)	MF425937[1]	KF569942[2]	--	KU568512[3]
Ri32 *R. gertrudae* SOUTH AFRICA	MW080173	MW080139	MW080097	MW079316
Ri33 *R. glabroscutatus* SOUTH AFRICA	MW080172	MW080138	MW080096	MW079315
R. guilhoni NIGERIA (Genbank)	KC243814	KC243854	--	KC243900
Ri35 *R. haemaphysaloides* NEPAL	MW080207	--	MW080132	--
Ri40 *R. kochi* ORIGIN UNKNOWN	MW080177	MW080143	MW080101	MW079320
R. leporis IRAQ (Genbank)	FJ536557	--	--	KM235720
Ri42 *R. longiceps* NAMIBIA	MW080182	MW080147	MW080106	MW079325
Ri44 *R. longus* ETHIOPIA	MW080202	MW080163	MW080128	--
R. maculatus SOUTH AFRICA[1]/KENYA[2] (Genbank)	AF150026[1]	KP858499[2]	KY457505[1]	KP862678[2]
R. microplus SOUTH AFRICA (Genbank)	EU921764	EU918182	KY457506	KY678117
Ri5 *R. microplus* MOZAMBIQUE	--	--	MW080130	MW079337
Ri50 *R. muehlensi* ORIGIN UNKNOWN	MW080169	MW080135	MW080093	MW079312
DN80 *R. muhsamae* NIGERIA	MW080187	MW080152	MW080111	--
Ri51 *R. muhsamae* ORIGIN UNKNOWN	MW080204	MK941210	--	--
R. muhsamae IVORY COAST[1]/NIGERIA[2] (Genbank)	--	KY111471[1]	--	KC243926[2]
Ri53 *R. nitens* SOUTH AFRICA	MW080198	MW080160	MW080123	--
Ri59 *R.* nr. *pravus* TANZANIA	--	MN994299	MW080118	MW079332
Ri63 *R.* nr. *punctatus* ZIMBABWE	MW080170	MW080136	MW080094	MW079313
Ri56 *R. pilans* INDONESIA	MW080205	--	MW080131	--

Ri58 *R. praetextatus* TANZANIA	MW080201	MK941207	MW080127	--
IV12 *R. praetextatus* KENYA	MW080208	MW080166	--	--
IV3 *R. pravus* KENYA	MW080174	MW080140	MW080098	MW079317
K8 *R. pravus* KENYA	--	MW080162	MW080126	MW079336
R. pravus KENYA (Genbank)	MF361837	--	--	KT307494
Ri61 *R. pulchellus* TANZANIA	MW080184	MW080149	MW080108	MW079327
R. pulchellus TANZANIA[1]/EGYPT[2]/ORIGIN UNKNOWN[3] (Genbank)	AF150024[1]	MK774738[2]	--	AY008682[3]
R. pumilio RUSSIA[1]/ORIGIN UNKNOWN[2] (Genbank)	AF150023[1]	--	--	AY008684[2]
R. pusillus SPAIN[1]/PORTUGAL[2] (Genbank)	MF425936[1]	MN944865[1]	--	MF425999[2]
R. rossicus RUSSIA[1]/ROMANIA[2] (Genbank)	AF150021[1]	KY111472[2]	--	JX394215[2]
R. sanguineus s.l. "southeastern lineage" EGYPT (Genbank)	KY413802	KY413783	--	--
LCD348 *R. sanguineus* s.l. "southeastern lineage" ROMANIA (Genbank)	MN945316	MN944863	--	--
R. sanguineus s.s. "temperate lineage" FRANCE[1]/PORTUGAL[2] (Genbank)	MN996254[1]	MH630342[1]	--	MF426012[2]
LCD340 *R. sanguineus* s.s. "temperate lineage" GREECE (Genbank)	MN945319	MN944856	--	--
Ri67b *R. sanguineus* s.l. "tropical lineage" SOUTH AFRICA	MW080190	MW080155	MW080113	--
R. sanguineus s.l. "tropical lineage" ANGOLA[1]/IVORY COAST[2]/CHINA[3] (Genbank)	MF425968[1]	MG651947[2]	--	KX757914[3]
Ri70 *R. sculptus* ORIGIN UNKNOWN	MW080181	MW080146	MW080105	MW079324
R. senegalensis GUINEA-BISSAU (Genbank)	MF425972	MF425985	--	MF426005
Ri71 *R. senegalensis* IVORY COAST	--	MW080167	MW080133	--
Ri73 *R. simpsoni* GHANA	MW080180	MK941214	MW080104	MW079323
Ri74 *R. simus* SOUTH AFRICA	MW080171	MW080137	MW080095	MW079314
R. simus SOUTH AFRICA (Genbank)	MF425938	KJ613641	KY457509	AF132840
R. sulcatus ZIMBABWE (Genbank)	MN945335	MN944868	--	--
R. turanicus UZBEKISTAN[1]/KYRGYZSTAN[2]/KAZAKHSTAN[3] (Genbank)	FJ536579[1]	KT382459[2]	--	MN853166[3]
DN63 *R. turanicus* ISRAEL (Genbank)	MW080193	MN944864	MW080116	--
RD10 *R. turanicus* GREECE (Genbank)	MK158987	MK158992	--	--
RD11 *R. turanicus* GREECE (Genbank)	MK158986	MK158991	--	--
Ri80 *R. warburtoni* SOUTH AFRICA	MW080178	MW080144	MW080102	MW079321
R. warburtoni SOUTH AFRICA (Genbank)*	AF031865	--	--	AF132838
Ri81 *R. zambeziensis* SOUTH AFRICA	MW080176	MW080142	MW080100	MW079319
R. zambeziensis SOUTH AFRICA[1]/ZIMBABWE[2] (Genbank)	DQ849237[1]	KY457544[1]	KY457509[1]	AY008683[2]
Ri83 *R. zumpti* SOUTH AFRICA	MW080191	MW080156	MW080114	--
OUTGROUP TAXA				
Amblyomma dissimile HONDURAS/BRAZIL/PANAMA (Genbank)	AY342249	KJ569692	--	KF200170
Amblyomma rotundatum BRAZIL[1]/PERU[2] (Genbank)	MH236879[1]	KU720248[2]	--	KU720278[2]
Dermacentor andersoni CANADA[1]/U.S.A.[2] (Genbank)	U95868[1]	FM955611[1]	--	KX360398[2]
Dermacentor rhinocerinus SOUTH AFRICA (Genbank)	KY457526	KY457526	--	KY457526
Dermacentor silvarum CHINA (Genbank)	KP258209	KP258209	--	KP258209
Nosomma monstrosumi ORIGIN UNKNOWN[1]/SRI LANKA[2] (Genbank)	AF031858[1]	KU130405[2]	--	AF132832[2]
Hyalomma kumari PAKISTAN (Genbank)	--	KU130443	--	KU130608
Hyalomma hussaini ORIGIN UNKNOWN[1]/PAKISTAN[2] (Genbank)	AY008689[1]	KU130433[2]	--	MN72899[2]
Hyalomma rufipes SOUTH AFRICA[1]/ZIMBABWE[2]/EGYPT[3]/ISRAEL[4] (Genbank)	U95875[1]	MK737650[2]	KY457486[3]	AF132823[4]
Hyalomma truncatum SOUTH AFRICA[1]/ZIMBABWE[2]/NAMIBIA[3] (Genbank)	AF150031[2]	KU130479[3]	KY457496[1]	AF132824[1]
Ri101 *Haemaphysalis elliptica* SOUTH AFRICA	MW080195	MW080158	MW080119	--
Haemaphysalis elliptica SOUTH AFRICA (Genbank)	HM068955	HM068961	--	--
Haemaphysalis flava CHINA (Genbank)	KJ747360	KC844859	KX450284	KY021819
Haemaphysalis inermis SLOVAKIA[1]/IRAN[2] (Genbank)	U95871[1]	U95872[1]	--	MH532295[2]
Ixodes cookei CANADA[1]/U.S.A.[2] (Genbank)	U95882[1]	U95883[1]	--	KP836459[2]
Ixodes ovatus JAPAN (Genbank)	U95899	AB819244	--	AB231670
Rh1 *Rhipicentor bicornis* NAMIBIA	MW080189	MW080154	--	MW079330
Rhipicentor bicornis D.R.C. (Genbank)	U95917	L34304	--	--
Rhipicentor nuttalli SOUTH AFRICA (Genbank)	MF818020	MF818020	MF818019	MF818020
RD3 *Rhipicentor nuttalli* SOUTH AFRICA	MW080200	MW080161	MW080125	--

* mis-identified as *R. punctatus* on Genbank - historical *R. punctatus* in South Africa have been shown to be *R. warburtoni* (Horak et al., 2018)

where appropriate. To determine phylogenetic congruence (Campbell et al., 2011), pairwise p-distance matrices were calculated in MEGA v10.1.7 (Kumar et al., 2018) for 55 samples with data for all mtDNA genes, and were used in a CADM test with 1000 permutations in the ape package (Paradis and Schliep, 2019) in R v3.6.1 (R Core Team, 2020) and RStudio v1.2.5001 (RStudio Team, 2020). Due to the potential reticulate nature of some closely related species within *Rhipicephalus* and prevalence of misidentification, phylogenetic networks for each gene were estimated in SplitsTree v4.14.3 (Huson and Bryant, 2006) using neighbour network analysis with 1000 bootstrap replicates (Fig. S2.1-4: supplementary information). Alignments were concatenated into a single dataset (including 28S-D2) using SequenceMatrix v1.8 (Vaidya et al., 2011), and comprised samples with sequences for at least two mtDNA genes. The final dataset comprised 1620 nucleotide characters across 100 samples, comprising 53 *Rhipicephalus* species and 17 outgroup species, with 16.67% missing data for mitochondrial genes, and 44.68% missing data for nuclear 28S-D2.

Phylogenetic analyses

Parsimony analysis in PAUP* v4.0a168 (Swofford, 2003) employed a heuristic tree search protocol with the following parameters: all characters equally weighted and unordered, uninformative characters excluded; starting tree obtained with tree-bisection-reconnection (TBR) branch-swapping algorithm using 10 replicates of random stepwise addition of sequences; initial 'maxtrees' set to 200 with automatic increase by 100; bootstrap support values (BP) (Felsenstein, 1985b) were calculated from a heuristic search using TBR branch-swapping replicated in 1000 resampled datasets with simple addition of 10 sequences. Maximum likelihood analysis in RAxML-HPC v8.1.20 (Stamatakis, 2014) used separate nucleotide substitution models for each gene partition with empirical base frequencies estimated. Bootstrap support was based on 1000 replicates and was followed by a thorough maximum likelihood search. Bayesian inference in MrBayes v3.2 (Ronquist and Huelsenbeck, 2003) was performed using a variable rate prior, flat Dirichlet priors and unlinked parameters for each partition to obtain separate estimates for each gene. The analysis was run in parallel using four Monte-Carlo-Markov-Chains (MC^2) respectively for 10 million iterations sampling every 100^{th} iteration. The first 25% of trees sampled from each MC^2 run were discarded, and both runs were combined to produce a majority-rule consensus tree with posterior probabilities (PP) for each node. Tracer v1.6 (Drummond and Rambaut, 2007) was used to assess the convergence and sampling efficiency between runs, and all ESS values were greater than 200 indicating effective sampling.

Divergence time estimates

Phylogenetic divergence times were estimated in BEAST v2.6.2 (Bouckaert et al., 2014) using a relaxed clock under an uncorrelated lognormal model with a Yule speciation process using data from all genes. Separate substitution models were applied to each data partition with model and clock parameters unlinked across all partitions. Clock rates were estimated from uniform priors. The analysis was set to 300 million iterations, with every 1000th iteration sampled, and was run four times starting from different random seeds. Log and tree outputs were combined using LogCombiner v2.6.2 (Drummond and Rambaut, 2007) with the first 25% of trees discarded. Convergence and sampling efficiency between runs was assessed using Tracer v1.7.1 (Drummond and Rambaut, 2007), and all ESS values were greater than 200 indicating effective sampling. A maximum clade credibility tree was prepared using TreeAnnotator v2.6.2 (Drummond and Rambaut, 2007), and median node ages were calculated with 95% high posterior density confidence intervals. Information for fossil vertebrates was retrieved from The Paleobiology Database (www.paleobiodb.org).

Fossil dates for ticks published in the literature served as calibration points for four outgroup nodes. Calibrations were each modelled under lognormal prior distributions comprising hard minimum ages, and soft maximum ages, with an offset, mean and standard deviation that set 95% of the priors to fall within minimum and maximum ages of selected fossils. Fossils for outgroup taxa were used given the lack of fossils for *Rhipicephalus*. The fossil larva of *Amblyomma* spp. (30-40 Mya; Lane & Poinar, 1986) from Dominican amber closely resembles *A. dissimile* (Keirans et al., 2002) and was used to calibrate the most recent common ancestor (MRCA) of *A. dissimile* and *A. rotundatum* (closely related to *A. dissimile* - Keirans and Durden, 1998; Lampo et al., 1997). The fossil of *A. birmitum* (99 Mya; Chitimia-Dobler et al., 2017a) is the oldest *Amblyomma* and was used to calibrate the stem node for divergence of all *Amblyomma*. The fossil of *I. succineus* (44-49 Mya; Weidner, 1964) from Baltic amber was recently demonstrated as closely related to *I. ovatus* based on morphology under microtomographic investigation (Dunlop *et al.*, 2016). A close extant species to *I. ovatus*, is *I. cookei* based on 16S (Chitimia-Dobler et al., 2016) and 12S similarity (BLAST searches on Genbank), and the node for this MRCA was calibrated using the fossil dates for *I. succineus*. The fossil of *Haemaphysalis. cretacea* (100-110 Mya: Chitimia-Dobler et al., 2018) is placed among subgenus *Alloceraea*. Within this group, sequence data for *Ha. inermis* is available, but data for other subgenus *Alloceraea* species are unavailable. However, somewhat distantly related subgenus *Rhipostoma* species, *Ha. elliptica* (Koch, 1844) and *Ha. flava* Neumann, 1897, have more complete molecular datasets available. Following this, the MRCA for *Ha. inermis* and *Ha. elliptica* + *Ha. flava* is taken to approximate basal subgenus *Alloceraea*, making this node close to *Ha. cretacea*. Divergence time estimation tends to

overestimate shallow node ages when calibrating deep nodes (Duchêne et al., 2014; Rodríguez-Trelles et al., 2002), as such nodes for replicates of 25 extant species were calibrated under lognormal prior distributions (hard minimum/soft maximum) that set 95% of the priors within 0.33-3 Mya with median at 1 Mya. These bounds were chosen because intraspecific variation in mitochondrial mutations in 12S, 16S and COI data had a median of 2.04±1.72 (95% confidence interval) mutations between conspecific replicates. This makes the median lineage divergence time within species around 0.74 Mya, based on the rate of 2.69 mutations per million years for Tenebrionidae (Papadopoulou et al., 2010) with similar reproductive rates and r-selected reproductive strategies (De Los Santos et al., 1988). Species with more than two replicates were only calibrated at the node uniting all replicates (deepest node for that species) to maximise representation of intraspecific haplotype diversity.

Ancestral area estimates

Ancestral area probabilities were estimated in RASP v4.2 (Yu et al., 2020) using a dataset of 3 000 trees obtained from BEAST. These trees were pruned to exclude species duplicates and distantly related outgroup taxa so that unequal taxon representation was minimized. However, *Rhipicentor* species were retained to provide deep resolution for visualisation. Note, *Rhipicentor* were treated as sister to *Rhipicephalus* due to similarity in morphology (Klompen et al., 1997), despite inconclusive findings from molecular data (also see discussion for *Rhipicephalus* origin below). Biogeographic regions generally following Linder et al. (2012) were divided into 15 areas that approximated distinct *Rhipicephalus* species distributions in the Afrotropics (Horak et al., 2018; Walker et al., 2003; Walker et al., 2000). Areas outside of the Afrotropics followed more expansive regional landmass divisions, but also approximated known *Rhipicephalus* distributions (Horak et al., 2018; Walker et al., 2003; Walker et al., 2000). These areas are as follows: A - Cape, B - Natal-Lowveld, C - Namib-Karoo, D - Kalahari-Highveld, E - Zambezian, F - Guinea-Congo, G - Somali-Masai, H - West Sahel-Savanna, I - East Sahel-Savanna, J - Mediterranean-NW Africa, K - SE Europe-NE Africa, L - Middle East, M - India-SE Asia mainland, N - Indo-Malaysia, O - Australasia. Species were assigned to an area, or set of areas, according to known distribution ranges (Horak et al., 2018; Walker et al., 2003; Walker et al., 2000). The decision was made to pursue better data 'granularity' by subdividing broad regions which allows for deeper inference given generally well-known geographic limits for most *Rhipicephalus* species. The dataset was analysed under three models of ancestral area estimation: S-DIVA (Statistical Dispersal-Vicariance Analysis: Yu et al., 2010), S-DEC (Statistical Dispersal-Extinction-Cladogenesis: Beaulieu et al., 2013), and BBM (Bayesian Binary MCMC: Ronquist and Huelsenbeck, 2003). S-DIVA and S-DEC analyses were done using 3 maximum areas for each node with no range

constraints. BBM analysis was done using an F81+G model with 5 maximum areas per node, and was run for 10 million iterations across 10 chains, sampling every 1000th iteration, discarding the first 25%.

Ancestral host-use estimates

Ancestral host-use probabilities for immature and adult stages were estimated in RASP using the same BEAST trees dataset from ancestral area estimation. Host-use categories were divided into five groups representing mammal taxa that form primary hosts for each species based on compiled host-use data (Horak et al., 2018; Walker et al., 2003; Walker et al., 2000). Host-use categories are as follows: A - Artiodactyla, B - Perissodactyla, C - Carnivora, D - Hyracoidea, E - Rodentia + Lagomorpha + Afroinsectivora (small mammals). Two models of ancestral state estimation were applied. S-DIVA ran with four maximum hosts per node and no constraints. BBM applied an F81+G model with 4 maximum hosts per node, and ran for 10 million iterations across 10 chains, sampling every 1000th iteration, discarding the first 25%. Tests for correlation between host-use and phylogeny were performed using 1000 permutation in the phyloint package (Eklöf and Stouffer, 2016) in R.

Ancestral climate niche estimates

Locality data for 53 *Rhipicephalus* species and two *Rhipicentor* species were compiled from the Gertrud Theiler Tick Museum (Agricultural Research Council-Onderstepoort Veterinary Research, South Africa) and Cumming (1999). Datasets for seven species had less than ten locality records (2: *R. haemaphysaloides*, *R. pilans*, *R. pumilio*, *R. pusillus*, *R. rossicus*; 6: *R. australis*, *R. zumpti*), the remaining 48 had more than ten records ranging from 13-3319 (median: 76.5). Coordinates were used to extract climate data for nineteen bioclim variables from the worldclim 2-5m dataset (www.worldclim.org) in DIVA-GIS v7.5 (Hijmans et al., 2004). Means for each variable were calculated across geographic ranges for each species and used as terminal values in ancestral state estimation based on the majority-rule consensus tree from Bayesian inference. Tests of phylogenetic signal for each bioclim variable based on Blomberg's K and Pagel's λ were done using 1000 permutations in the phytools package (Revell, 2012) in R and RStudio. Bioclim variables having significant phylogenetic signal for both K and λ ($p < 0.05$) were used for ancestral climate estimation. These variables were similar enough to Brownian motion to warrant ancestral state estimation by maximum likelihood (Schluter et al., 1997; Vieites et al., 2009) as implemented in phytools in R. However, these variables had some deviation from Brownian motion (K and $\lambda \neq 1$), indicting weak selection is present. These variables were also used to model species distributions and evaluate model performance in DIVA-GIS. Duplicates from same grid cell were removed, and

extent was set to include Afrotropical, southern Palearctic and Australasian regions. Species distribution models were evaluated using Receiver Operating Characteristic (ROC) curves to calculate Area Under Curve (AUC) values. Seven species datasets with six or less records were excluded from model evaluation.

Results

Phylogenetic analyses

Optimal nucleotide substitution models were determined as 12S/COI – TIM+F+I+G4 (Posada, 2003), 16S – TIM3+F+G4 (Posada, 2003), and 28S-D2 – K3Pu+F+I (Kimura, 1981). Conserved sites for 12S, 16S, 28S-D2 and COI were 37.68%, 44.76%, 93.51%, and 51.44% respectively. The CADM test indicated all p-distance matrices were congruent ($p = 0.001$), confirming gene tree topologies were similar and nucleotide data could be concatenated. Phylogenetic networks confirmed samples from different localities represented closely related lineages and thus can be assumed to represent the same species (Fig. S2.1-4). For Bayesian inference, average standard deviation of split frequencies was 0.0022 indicating convergence between both chains. The majority-rule consensus tree with posterior probabilities from Bayesian inference is presented in Fig. 2.1, along with bootstrap support values for parsimony and maximum likelihood analyses. Nodes with strong support are taken as bootstrap values ≥ 70, and significant posterior probability values ≥ 0.95 (Erixon et al., 2003; Hillis and Bull, 1993; Rannala and Yang, 1996). Nodes below 0.80 posterior probability were collapsed in TreeGraph v2.15 (Stöver and Müller, 2010), however weakly supported nodes above this threshold were kept as bifurcating to maximise information of possible associations. Tree topologies from all analyses were mostly congruent with a few exceptions at terminal nodes. Parsimony analysis of 604 parsimony-informative nucleotide base pair characters yielded 72 most parsimonious tree (length = 4147 steps, consistency index (CI) = 0.263, retention index (RI) = 0.605).

Rhipicephalus as a whole formed a well-supported monophyletic group in comparison to the outgroup taxa (Fig. 2.1). Five deep clades were recovered with strong support that separate *Rhipicephalus* into (clade I) *R.* (*Boophilus*) + *R.* (*Digineus*) + *R. duttoni*/*R. nitens* (appendiculatus group ii) + pravus group, (clade II) pulchellus group, (clade III) appendiculatus group i, (clade IV) sanguineus group, and (clade V) simus group + capensis groups i/ii + follis group + haemaphysaloides group. However, a basal polytomy was recovered indicating uncertainty with regards to which clade is basal in *Rhipicephalus*. Moreover, *R. senegalensis* was recovered as part of a polytomy with sanguineus group (clade IV), and simus group + capensis groups i/ii + follis group + haemaphysaloides group (clade V).

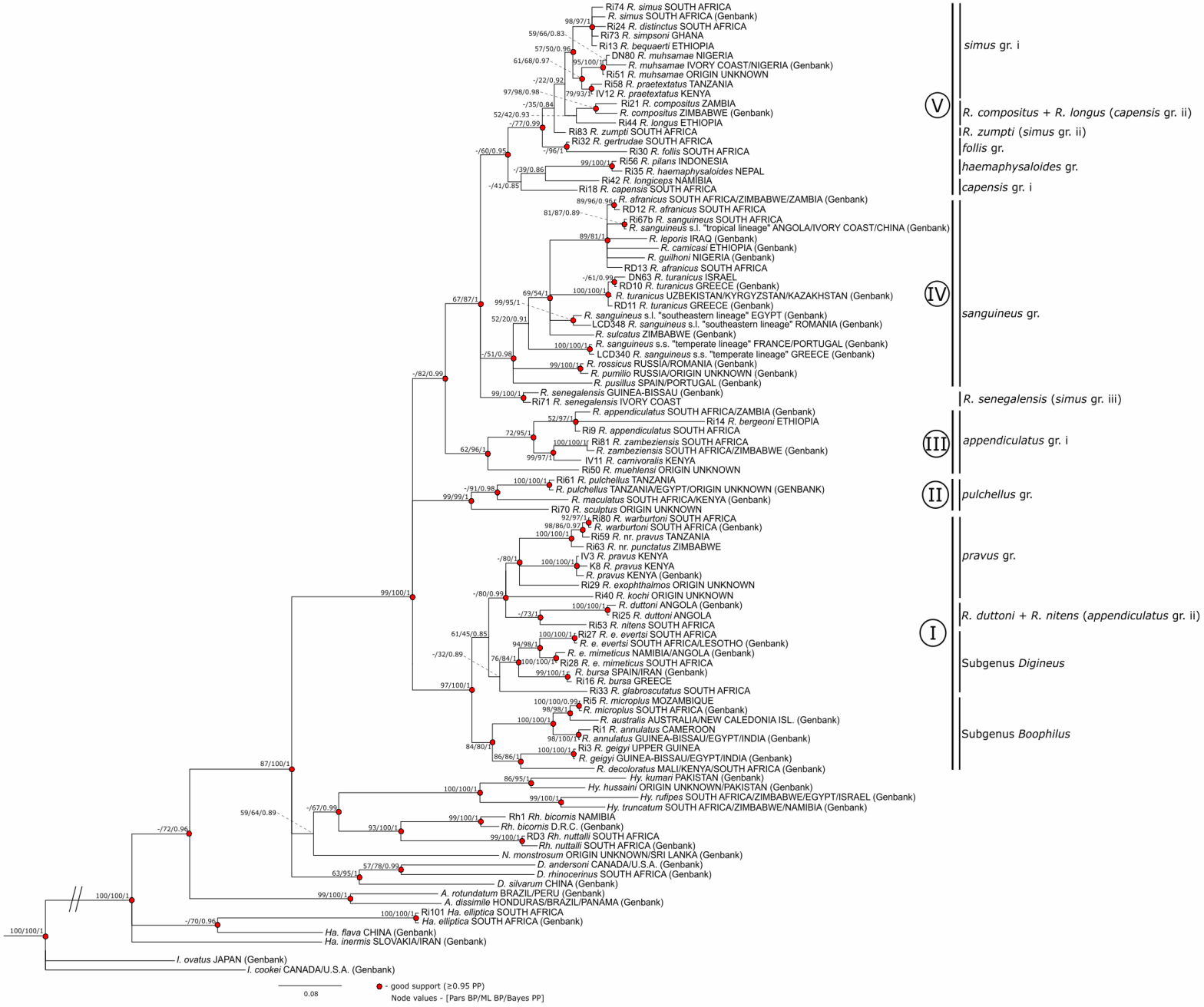

Figure 2.1. Consensus tree recovered from Bayesian inference. Nodal support values represent parsimony bootstraps (left), maximum likelihood bootstraps (middle), and Bayesian posterior probabilities (right). Nodes with significant posterior probabilities are indicated by a red circle. Labels are provided for sample codes, Genbank accession numbers, species names, localities, species groups and clades, as represented in Table 2.1. Uppercase Roman numerals indicate five major radiations (clades), while lowercase Roman numerals indicate subclades/subgenera/species groups.

Currently accepted subgeneric and species group divisions (Walker et al., 2000) were mostly recovered as monophyletic with notable exceptions including the traditional pulchellus group (clade II) as separate from appendiculatus group i (clade III), as well as polyphyly among appendiculatus, simus and capensis groups. Polyphyletic relationships among simus groups i and ii (clade V), as well as capensis groups i and ii (clade V), are contentious as these clades had weak support and limited taxon sampling. However, polyphyly between appendiculatus groups i and ii (clades III and I) which place *R. duttoni* + *R. nitens* closer to the pravus group, is robust because support was strong, and these clades were adequately sampled.

Divergence time estimates

The maximum clade credibility tree with divergence time estimates (95% HPD) was congruent with the majority-rule consensus tree from Bayesian inference (Fig. 2.2). Stem *Rhipicephalus* is estimated to have begun diversification late Cretaceous, 72.53 Mya (95% HPD interval = 59.74-84.33 Mya). Subsequently, crown *Rhipicephalus* is estimated to have begun diversification in the early Eocene, 52.27 Mya (95% HPD interval = 41.74-63.18 Mya). Clade I, containing *R.* (*Boophilus*) + *R.* (*Digineus*) + *R. duttoni*/*R. nitens* + pravus group, last shared a common ancestor in the late Eocene, 35.20 Mya (27.37-44.45 Mya). Clade II, containing pulchellus group, last shared a common ancestor in the late Oligocene, 28.15 Mya (17.84-39.82 Mya). Clade III, containing appendiculatus group i, last shared a common ancestor in the late Oligocene, 29.58 Mya (20.96-39.22 Mya). Clade IV, containing sanguineus group, last shared a common ancestor in the late Oligocene, 25.04 Mya (19.08-32.18 Mya). Clade V, containing simus group + capensis groups i/ii + follis group + haemaphysaloides group, last shared a common ancestor in the late Oligocene, 25.59 Mya (19.29-32.37 Mya). Radiations of descendant clades of clade I include subgenus *Boophilus* in the early Oligocene, 28.26 Mya (19.97-36.58 Mya), subgenus *Digineus* in the late Oligocene, 24.64 Mya (16.69-32.04 Mya), and appendiculatus group ii + pravus group in the early Miocene, 24.38 Mya (17.71-31.14 Mya). The Afrotropical sanguineus group (clade IV) last shared a common ancestor in the late Miocene, 9.08 Mya (5.55-13.51 Mya). Radiations of descendant clades of clade V include simus group i in the middle Miocene, 9.26 Mya (5.97-13.08 Mya), follis group in the Pliocene/Pleistocene, 4.46 Mya (2.00-7.62 Mya), and capensis group i + haemaphysaloides group in the early Miocene, 20.88 Mya (13.81-27.99 Mya), with the haemaphysaloides group diversifying in the Pliocene/Pleistocene, 2.17 Mya (0.12-7.31 Mya).

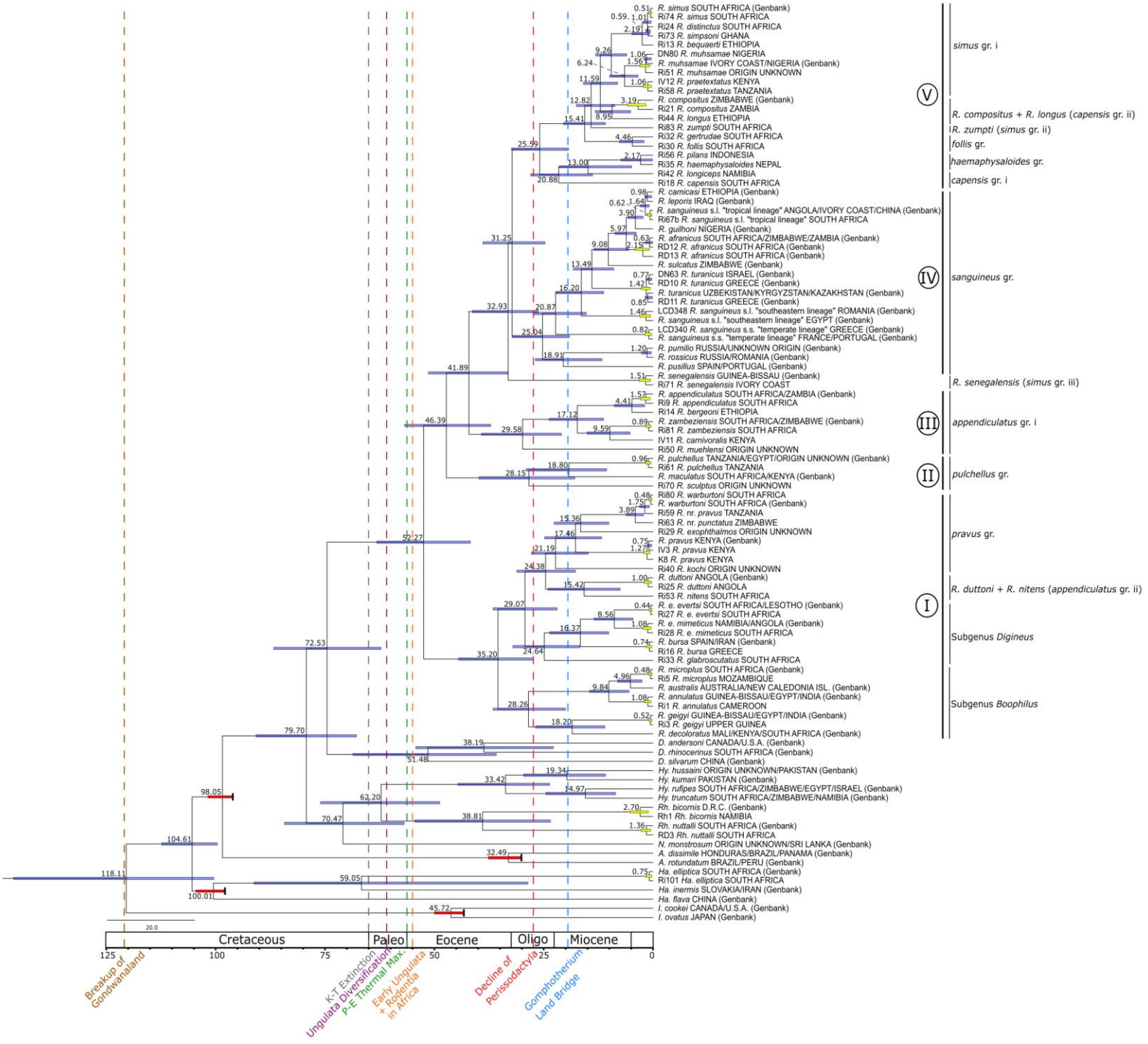

Figure 2.2. Divergence time estimates tree from BEAST analysis. Node values indicate median divergence time estimates, and purple bars indicate 95% HPD intervals. Red bars indicate fossil calibrations, while yellow bar represent species replicate calibrations. Labels are provided for sample codes, Genbank accession numbers, species names, localities, species groups and clades, as represented in Table 2.1. Uppercase Roman numerals indicate five major radiations (clades), while lowercase Roman numerals indicate subclades/subgenera/species groups. Dotted lines indicate events significant for *Rhipicephalus* evolutionary history. Timescale is in millions of years ago (Mya).

Ancestral area estimates

Ancestral area estimates under three models (S-DIVA, S-DEC and BBM) were generally congruent in terms of most likely ancestral areas, with results from BBM generally most resolved (Fig. 2.3). The ancestral area for each node was taken as the majority outcome (at least 2/3) of the most likely state ('pie slice') from each model, and interpreted as individual areas where areas were either combined by the analysis or individually represented. The origin of stem-*Rhipicephalus* is largely unresolved and contentious given the lack of a distinct sister genus (Fig. 1), however the majority of estimates that were resolved indicate sub-Saharan Africa in general (congruent majority: CG | S-DIVA: CFG [0.059]-unresolved: 0.823; S-DEC: CEG [0.011]-unresolved: 0.989; BBM: C [0.377]-unresolved: 0.210). Similarly, the origin of crown-*Rhipicephalus* is largely unresolved, but with majority of resolved estimates indicating central Africa (FG+E+G | FG [0.044]-0.956; E [0.081]-0.860; G [0.282]-0.345). Crown progenitors of subgenus *Boophilus* (clade I) (F | F [0.496]-0.008; J [0.097]-0.567; FH [0.459]-0.258), pulchellus group (clade II) (G | G [0.947]-0.053; G [0.567]-0.038; G [0.748]-0.038), appendiculatus group i (clade III) (E | E [0.393]-0; E [0.396]-0.037; BEG [0.470]-0.057), and simus group + sanguineus group (clade V) + *R. senegalensis* (HI | FHI [0.151]-0.095; HI [0.126]-0.316; H [0.140]-0.453) remained in central Africa. Some descendant species from these lineages, *R. appendiculatus* (terminal: BE) and *R. decoloratus* (terminal: ABDEFHI), later expanded their ranges southwards into southern Africa, in the middle-late Miocene (4-12 Mya). Palearctic descendants of Subgenus *Boophilus* (clade I) dispersed out of Africa into the eastern Palearctic (FL | FLN [0.056]-0; O [0.917]-0.083; FHIL [0.137-0.601) and then to Indo-Malaysia and Australasia (NO | NO [1]-0; O [0.808]-0.001; LNO [0.313]-0.320) in the middle-late Miocene (4-10 Mya). Crown progenitors of the sanguineus group (clade IV) dispersed out of Africa into the western Palearctic (J | J [0.700]-0.105; J [0.451]-0.224; J [0.782]-0.162) in the Oligocene (25-30 Mya), and expanded eastwards to southeastern Europe and the Middle East (JK | IJK [0.232]-0.096; K [0.298]-0.107; JK [0.402]-0.118) in the Early Oligocene (about 15-24 Mya). Subsequently, crown progenitors of the Afrotropical sanguineus group (clade IV) dispersed back into Africa via the Middle East and East Sahel-Savanna (I | I [1]-0; O [0.452]-0.46; DEHI [0.243]-0.438) in the Miocene (about 13-9 Mya). After this, the *R. leporis* + *R. camicasi* lineage expanded eastwards (GL | GL [1]-0; GL [0.828]-0; G [0.356]-0.644), with *R. leporis* later diverging and dispersing into the Middle East (terminal: L) in the Pliocene/Pleistocene (about 1 Mya). A southward dispersal from central to southern Africa was estimated among crown progenitors of subgenus *Digineus* + *R. duttoni*/*R. nitens* + pravus group (clade I) (A | AKL [0.089]-0.017; E [0.182]-0.234; A [0.787]-0.213), as well as simus groups i/ii + capensis groups i/ii + follis group + haemaphysaloides group (clade V) (A | A [0.403]-0.033; E [0.215]-0.272; A [0.487]-0.118) in the Oligocene (20-35 Mya).

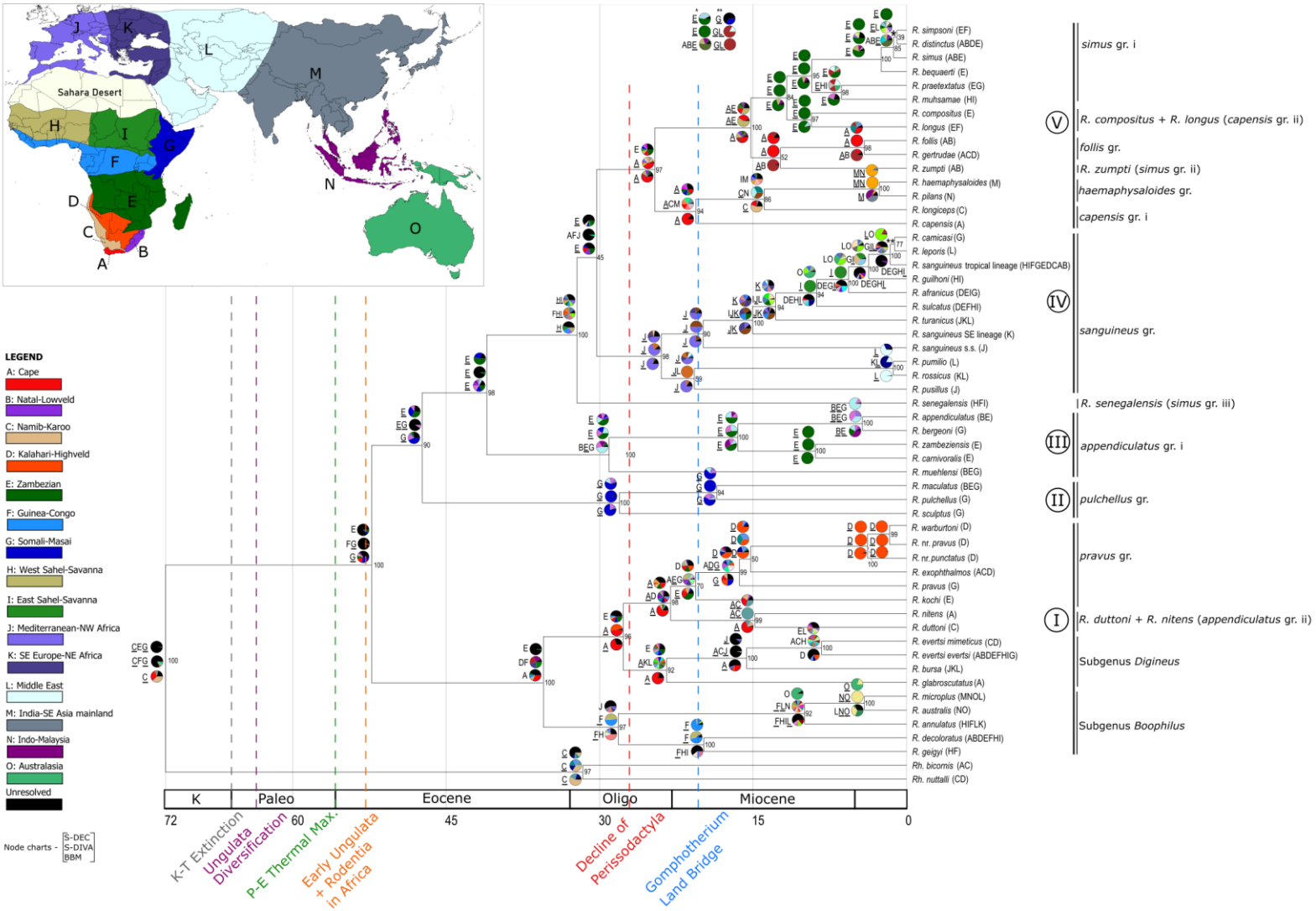

Figure 2.3. Ancestral area estimates tree from RASP analyses. Nodal pie charts indicate ancestral area probabilities for S-DEC (top), S-DIVA (middle) and BBM (bottom) with areas of highest probability indicated by capital letters (left) represented in the legend. Note that colours for combinations of ancestral areas represented by two-four letters, are not indicated in the legend due to space constraints. Underlined letters for each model at each node represent congruent majority area taken as ancestral area result for that node. Node values (right) indicate node frequency as percentage. Labels are provided for sample codes, Genbank accession numbers, species names, localities, species groups and clades, as represented in Table 2.1. Uppercase Roman numerals indicate five major radiations (clades), while lowercase Roman numerals indicate subclades/subgenera/species groups. Dotted lines indicate events significant for *Rhipicephalus* evolutionary history. Timescale is in millions of years ago (Mya).

Their descendant lineages *R. kochi* (E | AEG [0.173]-0.036; D [0.186]-0.246; E [0.371]-0.130), *R. pravus* (G | ADG [0.216]-0; D [0.391]-0.250; G [0.258]-0.257), *R. bursa* (AJ | ACJ [0.027]-0.973; J [0.054]-0.893; A [0.264]-0.547) (clade I), and simus group i + capensis group ii (clade V) (E | E [0.990]-0.010; E [0.977]-0.023; E [0.704]-0.113) dispersed back northwards into central Africa or the Mediterranean-NW Africa (*R. bursa*) in the Miocene (10-20 Mya). Finally, pravus group descendants (*R. exophthalmos* to *R. warburtoni*; clade I) dispersed back southward to the Kalahari-Highveld (D | D [0.333]-0.001; D [0.740]-0.064; D [0.505]-0.0774) in the early Miocene (17 Mya). Estimates for the haemaphysaloides group + capensis group i (clade V) indicate origin in southwest Africa (A | ACM [0.164]-0.036; A [0.154]-0.270; A [0.863]-0.137) in the early Miocene (20 Mya), with subsequent dispersal to Oriental and Indo-Malaysian regions in the Miocene-Pliocene/Pleistocene (2-12 Mya) (haemaphysaloides group) (MN | MN [1]-0; MN [0.900]-0.017; M [0.407]-0.124). However, taxa from this clade are under sampled and nodes are weakly supported (Fig. 2.1), making this result somewhat contentious.

Ancestral host-use estimates

Ancestral host-use probabilities recovered were generally congruent between models and well resolved for each node with the combined consensus for both models taken as the ancestral host range for each node (Fig. 2.4). Permutation tests for phylogeny and host-use correlation indicate hosts for both immatures (p=0.003) and adults (p=0.001) correlate significantly with phylogeny. Ancestral hosts for stem and crown *Rhipicephalus* immatures were estimated as Artiodactyla, Perissodactyla and small mammals (Rodentia + Lagomorpha + Afroinsectivora) (combined consensus: ABE | S-DIVA: ABE [0.3367]-unresolved: 0.0199; BBM: AE [0.8484]-unresolved: 0.0551). Among groups with host-truncate life cycles (clade I excl. pravus group + appendiculatus group ii) Artiodactyla hosts were ancestral (A | A [1]-0; A [0.9631]-0.0369), with exception of basal *Digineus* that also parasitise rodents at early life stages (A | A [1]-0; AE [0.9829]-0.0171). In the pravus group (three-host life cycles), small mammals were ancestral (E | E [1]-0; E [0.9455]-0.0021) with exception of basal species *R. kochi* that also parasitise Artiodactyla at early life stages (AE | AE/E [0.5]-0; AE [0.9145]-0.0053), while in appendiculatus group ii (three-host life cycles), Artiodactyla hosts were ancestral (A | A [1]-0; A [0.9540]-0.0460). In the pulchellus group (clade II), Perissodactyla and Artiodactyla hosts were ancestral (AB | A/B [0.5]-0; AB [0.5837]-0.0528), and in appendiculatus group i (clade III), Artiodactyla and small mammal hosts were ancestral (AE | E [0.9867]-0.0133; AE [0.9722]-0.0278). In the sanguineus group (clade IV) small mammal hosts were ancestral at all nodes (E | E [1]-0; E [0.9064]-0.0022), with Carnivora hosts also

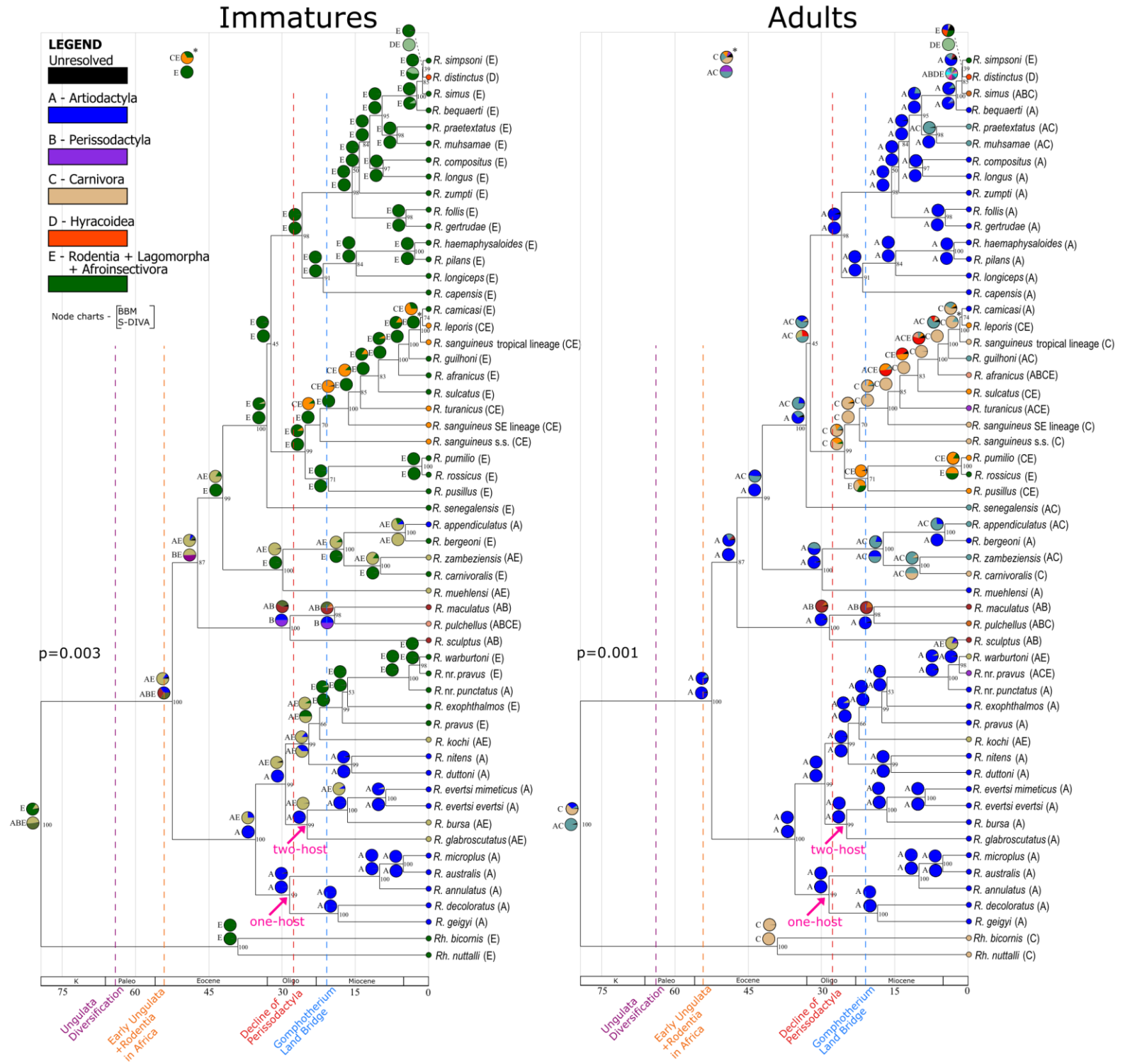

Figure 2.4. Ancestral host-use estimates for immatures and adults from RASP analyses. Nodal pie charts indicate ancestral host-use probabilities for BBM (top) and S-DIVA (bottom) with highest probability indicated by capital letters (left) represented in the legend. Note that colours for combinations of ancestral hosts represented by two-three letters, are not indicated in the legend due to space constraints. Node values (right) indicate node frequency as percentage. Labels are provided for species names, present host-use, and host-truncate clades. P-values indicate phylogenetic signal from 1000 permutations. Dotted lines indicate events significant for *Rhipicephalus* evolutionary history. Timescale is in millions of years ago (Mya).

ancestral at five nodes in BBM analysis. In all clade V groups, small mammals were ancestral (E | E [1]-0; E [09952]-0.0048), with notable specialisation to Hyracoidea in *R. distinctus*.

Ancestral hosts for stem *Rhipicephalus* adults were estimated as Artiodactyla and Carnivora (AC | A [0.9600]-0.0400; C [0.6242]-0.0155). However, this may be due to the assumption of *Rhipicentor* as sister instead of *Hyalomma*, and as such is contentious. Ancestral hosts for crown *Rhipicephalus* were Artiodactyla (A | A [0.9600]-0.0400; A [0.8732]-0.0192). In subgenus *Boophilus* + subgenus *Digineus* + appendiculatus group ii + pravus group (clade I), Artiodactyla hosts were ancestral for adults (A | A [1]-0; A [0.9929]-0.0071) with exception of some descendant pravus group species in BBM analysis. These exceptions include ancestral *R. warburtoni* + *R.* nr. *pravus* on Artiodactyla and small mammals (BBM: AE [0.8177]-0.0106), as well as extant *R. kochi* that parasitise rodents in adult life stages (terminal: AE), and *R.* nr. *pravus* that parasitise carnivores in adult life stages (terminal: ACE). In the pulchellus group (clade II), Perissodactyla and Artiodactyla hosts were ancestral (AB | A [0.9600]-0.0400; AB [0.8647]-0.0356), and in appendiculatus group i (clade III), Artiodactyla hosts were ancestral (A | A [1]-0; A [0.5332]-0.0045). Notably, descendant species were estimated to parasitise Artiodactyla and Carnivora as adults (AC | AC/A [0.5]-0; AC [0.7933]-0.0080). In the sanguineus group (clade IV), Carnivora hosts were ancestral (C | C [0.5943]-0.0690; C [0.7086]-0.0333) with Artiodactyla and small mammal hosts also ancestral at four nodes. Moreover, ancestral hosts in the basal clade (*R. pusillus* + *R. rossicus* + *R. pumilio*) were estimated as small mammals in addition to Carnivora (CE | CE/C/E [0.3333]-0.0001; CE [0.9533]-0.0467). In all clade V groups, Artiodactyla hosts were ancestral (A | A [1]-0; A [0.9537]-0.0463) with derived *R. muhsamae* + *R. praetextatus* also having Carnivora as ancestral hosts in BBM analysis (AC [0.9715]-0.0285). Moreover, derived *R. distinctus* + *R. simpsoni* were estimated to have Hyracoidea and other small mammals as adult ancestral hosts (DE | DE [1]-0; E [0.2946]-0.1837).

Ancestral climate niche estimates

Phylogenetic signal was limited, although significant, in variables related to temperature variability (Fig. 2.5): Annual Mean Diurnal Range (Bioclim2), Isothermality (Bioclim3), Temperature Seasonality (Bioclim4) and Temperature Annual Range (Bioclim7). Values for K and λ were all between 0.27-0.36 and 0.79-0.85 respectively, indicating some deviation from Brownian motion was present, such that close relatives tend to resemble one another, but excludes directional selection. This means selection was weak and ancestral state estimates are reliable for these climate variables. Moreover, species distribution models generated using these four variables had a mean AUC of 0.74±0.16, indicating the four climate variables generally approximated climate niches. Ancestral state estimates revealed a general

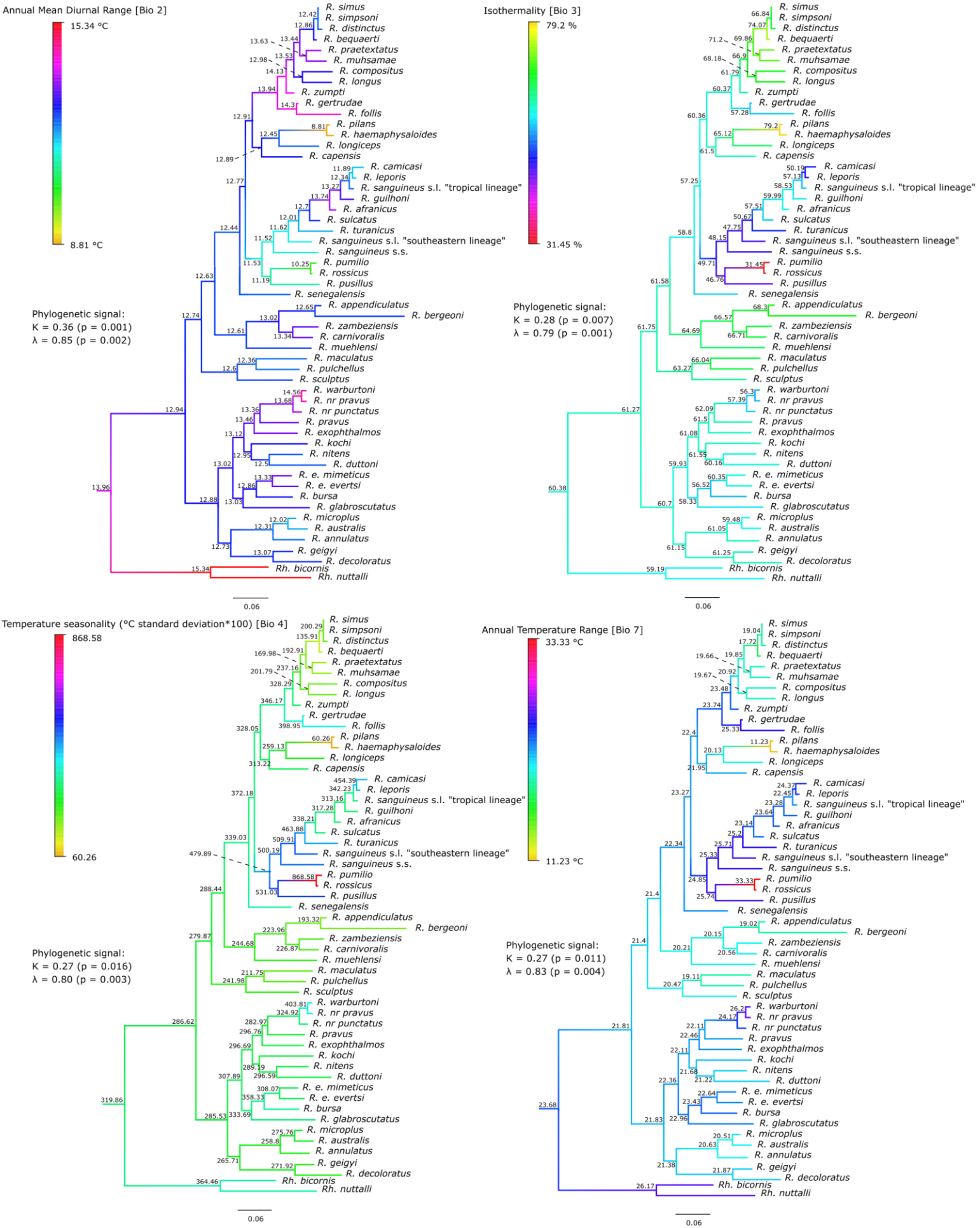

Figure 2.5. Ancestral climate niche estimates from phytools analyses for four Bioclim temperature variation variables. Nodal values indicate ancestral estimates form climate niche means. Branch colour legends indicate high to low temperature variation from red (variable climates) to yellow (stable climates). Labels are provided for species names.

pattern of clades being weakly structured by differential tolerance to variability in temperatures. Groups in the most temperature stable environments (mean temperature seasonality < 275.76), included subgenus *Boophilus* (clade I), appendiculatus group i (clade II), pulchellus group (clade III), haemaphysaloides group (clade V), and simus group i + capensis group ii (clade V). Groups in the most temperature variable environments (mean temperature seasonality > 282.97) included subgenus *Digineus* + pravus group + appendiculatus group ii (clade I), sanguineus group (clade IV), capensis group i (clade V), and follis group (clade V).

Discussion

Systematics and Phylogeny

Phylogenetic relationships among *Rhipicephalus* ticks were generally well supported, but with some polytomies present (Fig. 2.1). Relationships were generally congruent with previous phylogenetic studies based on molecular data (Barker and Murrell, 2002; Beati and Keirans, 2001; Beati and Klompen, 2019; Coimbra-Dores et al., 2018; Klompen et al., 1996; Mans et al., 2019), and with species groups based on adult and immature morphology (Camicas et al., 1998*;* Walker et al., 2000). However, five instances of incongruence regarding placement of taxa into species groups were recovered, with weak or strong support. In capensis and simus groups (clade V), incongruence split each group into two or three clades respectively (Fig. 2.1), but these were recovered with weak support and probably reflect data limitations in this clade due to the effect of missing data (17.01% - excluding conserved 28S-D2) and incomplete taxon sampling (~28%) (Hillis, 1998; Wiens, 2006; Zwickl and Hillis, 2002). Similarly, phylogenetic placement of *R. senegalensis* could not be confidently resolved (Fig. 2.1), however morphological evidence place them among simus group species (Walker et al., 2000). Incomplete taxon sampling of species in the clade (V) likely contributes to this uncertainty.

On the other hand, separate appendiculatus groups and distinct pulchellus group were recovered with strong support and likely reflect biological reality (Fig. 2.1). Polyphyletic appendiculatus group ii species *R. duttoni* and *R. nitens*, were recovered as sister to the pravus group with good support (Fig. 2.1). These two species parallel some disease transmission traits of pravus group species, where *R. duttoni* and *R. pravus* are able to transmit *Theileria parva* (*R. pravus* under laboratory conditions), and where salivary proteins in *R. nitens* and *R. warburtoni* can cause tick toxicosis and paralysis (Horak et al., 2018; Walker et al., 2000). Traditional pulchellus group species, *R. pulchellus* and *R. maculatus*, were recovered with *R. sculptus* (appendiculatus group *sensu* Camicas et al., 1998 and Walker et al., 2000), and formed a monophyletic group distinct from appendiculatus group i

(Fig. 2.1). This corroborates Coimbra-Dores et al. (2018), and suggests these species should be placed in the traditional pulchellus group (*sensu* Camicas et al., 1998), but including *R. sculptus* from appendiculatus group. Moreover, pulchellus group represent the most undifferentiated species among *Rhipicephalus* given their short branch length to extant species (Fig. 2.1), and similar morphology compared with *Rhipicentor*, *Cosmiomma*, and one *Dermacentor* species that have ornate or sculpted scutae, as well as patches of alloscutal setae in females (*D. rhinocerinus* and *R. pulchellus* only) (Horak et al., 2018; Walker et al., 2000). As such, it appears pulchellus group most likely represent the most basal group among *Rhipicephalus* despite the unresolved placement from molecular data (Fig. 2.1).

Rhipicephalus bergeoni was recovered among appendiculatus group i (clade III) (Fig. 2.1), but is traditionally among sanguineus group species (Morel, 1980), with some contention (Pegram et al., 1987b). Current species group designations exclude *R. bergeoni* because immature stages are unknown (Walker et al., 2000). However, similarity to *R. appendiculatus* in adult scutae/conscutae, male adanal plates and female genital aprons support it's placement in the appendiculatus group (Pegram et al., 1987b; Walker et al., 2000). Uncertainty remains however, because branch-length for *R. bergeoni* is abnormally long (Fig. 2.1) due to 12S rDNA data which place it in the sanguineus group (Fig. S2.1). This may indicate incomplete lineage sorting or historical introgression, similar to that suggested for *R. afranicus* (Bakkes et al., 2020; Chapter 4). Finally, *Rhipicephalus bequaerti* was recovered among simus group i (Fig. 2.1), resolving uncertainty due to unknown immature stages (Walker et al., 2000).

Origin of Rhipicephalus

The earliest diversification of *Rhipicephalus* is characterised by a basal polytomy in the crown group and long stem-branch (Fig. 2.1). A deeper ancestral polytomy was recovered among genera sister to *Rhipicephalus* that group *Hyalomma* + *Nosomma* + *Rhipicentor* separately from *Dermacentor*, albeit with weak support. This leaves the sister relationship of *Rhipicephalus* unresolved. Morphological data however, place *Rhipicentor* as sister to *Rhipicephalus* (Klompen et al., 1997), but previous molecular data place the *Hyalomma-Nosomma* group as sister instead (Barker and Murrell, 2004; Mans et al. 2019). Moreover, the pulchellus group (clade II), including *R. sculptus* from appendiculatus group (*sensu* Camicas et al., 1998 and Walker et al., 2000) are the closest to basal among *Rhipicephalus* based on shortest branch length to extant species (Fig. 2.1), and have morphology similar to *Cosmiomma*, *Dermacentor* and *Rhipicentor*. The polytomies result from fragmented and sometimes conflicting signal at the origin of stem- and crown-*Rhipicephalus*, which makes resolving phylogenetic relationships between genera difficult based on these data alone. The long branch of stem-*Rhipicephalus* indicates extinction may have occurred in *Rhipicephalus*

progenitors which obscures evolutionary history by incomplete taxon sampling (Crisp and Cook, 2009; Purvis et al., 2000). Alternatively, the long branch length and basal polytomy may indicate a lack of diversification due to limited diversity of mammal hosts at the time (52-72 Mya; Fig. 2.2), followed by diversification as a result of diversification in mammal hosts (Bininda-Emonds et al., 2007). However, the distinctive morphology of basal pulchellus group species that resemble distantly related genera, but have no gradual analogues leading to typical *Rhipicephalus* morphology (inornate with no alloscutal setal patches), suggest a 'gap' in the phylogeny that indicates the long stem-branch is more likely due to extinction than slow diversification. Under the assumption that molecular data represent the true phylogeny, *Rhipicephalus* must have originated on early Ungulata and Rodentia. However, no Rodentia fossils are known from the Paleocene in Africa, but at least one early Ungulata fossil is known from the Paleocene in north Africa (Gingerich, 1990). Under the assumption that morphological data represent the true phylogeny a different scenario is possible. It is likely that *Rhipicentor* were more diverse in the past given their divergence time estimates between 50-130 Mya (Fig. 2.1; Mans et al., 2019), but limited extant species diversity (Barker and Murrell, 2004). As such, a hypothesis of *Rhipicephalus* origin can be postulated whereby a diverse set of *Rhipicentor*-like progenitors lead to multiple lineages, of which only two survived. One of these surviving lineages would have given rise to extant *Rhipicentor* which remained in Africa. This lineage would have also given rise to ancestral *Hyalomma-Nosomma* that spread to Asia and diverged due to continental vicariance between Africa and Arabia (Sands et al., 2017b), or possibly Indian drift given similar biogeographic patterns, divergence time estimates and distinct morphological change as observed in other organisms (Bakkes et al., 2018a; Bossuyt et al., 2006; Sklenarova et al., 2013). The second surviving lineage would have given rise to stem-*Rhipicephalus* that went extinct after the diversification of crown-*Rhipicephalus* (Fig. 2.6). This hypothesis suggests *Rhipicephalus* originated from a now-extinct ancestor ('ghost lineage' *sensu* Norell, 1992) that increases stem-*Rhipicephalus* branch length due to the loss of evolutionary information (Fig. 2.6), and makes resolving phylogenetic relationships difficult (Crisp and Cook, 2009; Purvis et al., 2000). In this case, morphology provides an independent source of data that may retain ancestral synapomorphies for longer than molecular data, because morphological divergence tends to proceed slower than divergence in mitochondrial nucleotide sequences (De Queiroz, 2007, 2005). Indeed, phylogenetic analyses that include fossil morphology which represent ancestral character combinations tend to drastically alter relationships derived from molecular data (Koch and Parry, 2020). Moreover, extinctions tend to result in phylogenies with tipward-biased signal for divergence (Pybus and Harvey, 2000). This suggests the seven characters of capitulum, gland and setal morphology in larvae and females (Klompen et al., 1997) as well

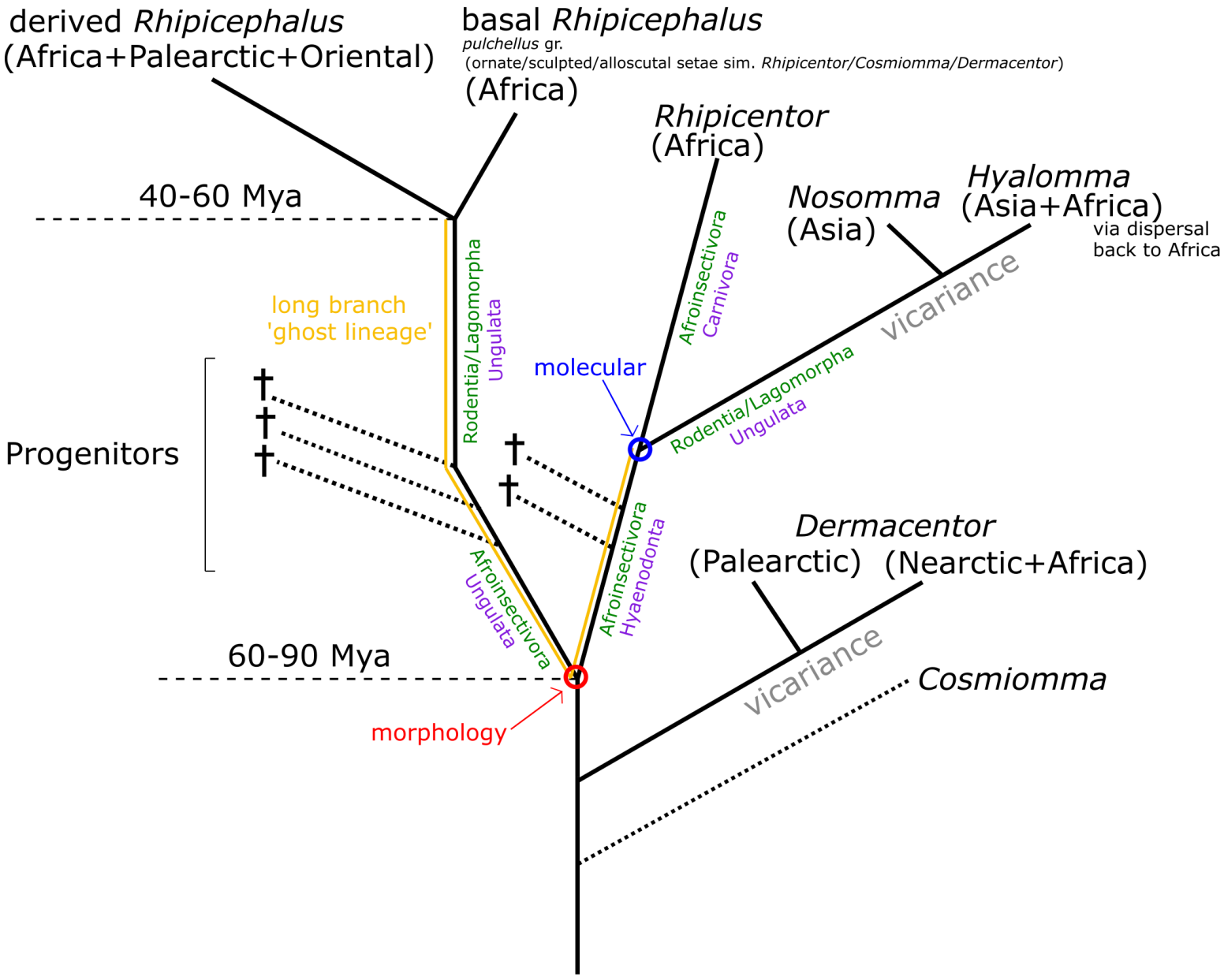

Figure 2.6. Diagram of putative extinction among *Rhipicephalus* and *Rhipicentor* progenitors that lead to conflicting phylogenetic signal between morphological and molecular data. Text along branches indicate putative host-use in immatures (green) and adults (purple). Yellow branch indicates putative 'ghost lineage' that makes *Rhipicentor* more distinct from *Rhipicephalus* due to extinction, and results from conflicting signal between morphological (red) and molecular data (blue). Placement of *Cosmiomma* is taken from Klompen et al., 1997).

as overall morphological appearance (Horak et al., 2018) which all support *Rhipicentor* as sister to *Rhipicephalus*, may do so because they detect signal for a deep node of the true phylogeny (Fig. 2.6). In contrast, faster evolving mitochondrial nucleotide sequence data detect more recent divergence, and lose signal for deeper divergences when deeper branches go extinct (Fig. 2.6). The alternative possibility of morphological convergent evolution cannot be excluded, however it seems unlikely given the limited functional or survival significance of these characters (palps compressed or not, cuticular glands unpaired or absent, one or two setal grooves in larvae, three or four larval capsule sensillae). Nevertheless, the origin of *Rhipicephalus* remains contentious due to the lack of data, and the nature of slow diversification or extinction that can produce uncertainty in phylogenies. This highlights the need to investigate *Rhipicephalus* phylogeny further by studying genes that mutate at a rate between highly conserved nuclear genes (28S, 18S) and rapidly evolving mitochondrial genes (12S, 16S, COI, ND1, ND2, ND4, CYTB), or by studying large genomic datasets (~1000 genes) and possibly SNPs to resolve deeper relationships.

Based on the evidence provided by morphology for a *Rhipicentor* + *Rhipicephalus* sister relationship (Klompen et al., 1997), it is of interest to consider the possible effects of host-use on the origin and diversification of the Brown ticks. *Rhipicentor* are specialist ectoparasites of Carnivora as adults, and prefer Macroscelidea (Afroinsectivora) as immatures (Fourie et al., 2005; Horak et al., 2018). Given the possibility of a sister relationship between stem-*Rhipicephalus* and stem-*Rhipicentor* (Fig. 2.6), it is possible to speculate that *Rhipicephalus* may have diverged from early *Rhipicentor*-like progenitors between 60-90 Mya (Fig. 2.2 – combined estimates due to polytomy in Fig. 2.1). However, this is long before Carnivora or Macroscelidea evolved (Polly et al., 2006; Seiffert, 2007). Nevertheless, extant *Rhipicentor* host preference for small mammals as immatures and carnivores as adults, suggests stem-*Rhipicentor* would have used early ecological analogues of these mammals as hosts. Before the Eocene, Africa was populated by Hyaenodonta predators both small (*Tinerhodon* and *Boualitomus*) and large (*Lahimia*), as well as early Ungulata and members of Afroinsectivora (Gheerbrant et al., 2006; Gingerich, 1990; Solé et al., 2009). As such, stem-*Rhipicephalus* may have originated from host switches in immature/adult stages from Afroinsectivora/Hyaenodonta to Afroinsectivora/early Ungulata (Fig. 2.4) along nested predator-prey connections in food webs (Britton, 2013; Graham et al., 2009; Lafferty et al., 2008). Small Hyaenodonta would prey on Afroinsectivora that would also become prey to large Hyaenadonta hypercarnivores that similarly preyed on large Ungulata. These connections would allow tick immatures to move onto novel hosts either directly from kills, or by progressively advancing into their geographic ranges on intermediate hosts, dropping off, moulting, and questing for a new host. Most descendant groups of the putative stem-

Rhipicentor have three-host life cycles (most *Rhipicephalus*, extant *Rhipicentor, Nosomma*, *Dermacentor*), and proceed through three off-host periods that offer ample opportunities for host switches between life stages. As such, off-host periods between moults enabled stem-*Rhipicephalus* ticks to respond to ecological opportunities when novel hosts appeared. Ticks that attach to novel hosts would gain a selective advantage to initiate adaptive radiation, because ectoparasite niches would be open. Indeed, the first Hyaenadonta and Condylarthra (early Ungulata) fossils are from the Paleocene (Gheerbrant et al., 2006; Gingerich, 1990; Solé et al., 2009), concurrent with the initial divergence of *Rhipicephalus* 60-90 Mya, indicating that novel predator-prey interactions could have played a role in adult host switches of *Rhipicephalus* progenitors from Hyaenodonta to early Ungulata in Africa. Stem-*Rhipicephalus* were most probably transitionary forms diverging from stem-*Rhipicentor* (Fig. 2.6 – based on morphology: Klompen et al., 1997) while adapting to novel hosts and food webs. Following from this, extinction of putative Hyaenodonta hosts, may explain the limited diversity of extant *Rhipicentor* as the only surviving lineage of Hyaenodonta ectoparasites that could adapt to modern predator-prey food webs. Importantly, the lineage leading to extant *Rhipicentor* did not switch to Ungulata hosts along with stem-*Rhipicephalus* (Fig. 2.6). Host switching, while possible for large population sizes, would have been limited by the inherent complexity of host switching between trophic levels along nested predator-prey connections in food webs given the low abundances and species diversity observed among *Rhipicentor* on hosts (Horak et a. 2018). Indeed, predator-prey host switches are limited within *Rhipicephalus* (Fig. 2.4), and generally arose in clades that have occur in high abundances (Horak et al., 2018; Walker et al., 2000). Moreover, host switches would have been limited even further due to competitive exclusion with stem-*Rhipicephalus*, *Dermacentor* and *Hyalomma-Nosomma* that already began to occupy niches on Ungulata. Alternatively, it remains possible that *Rhipicentor* may represent a unique genus of carnivore ectoparasites among rhipicephaline ectoparasites, which would invalidate the hypothesis for *Rhipicephalus* origin linked to Hyaenodonta as hosts. Ultimately, the lack of definitive evidence for the sister taxon to *Rhipicephalus* represents a significant challenge to understanding the origin of *Rhipicephalus*.

Account of Rhipicephalus Historical Biogeography

The origin of crown-group *Rhipicephalus* around 52 Mya (Eocene) (Fig. 2.2) is between estimates from other studies (20 Mya: Beati and Klompen, 2019, 100 Mya: Mans et al., 2019). This confirms an Afrotropical origin for *Rhipicephalus* after Africa had separated from all other remnants of Gondwanaland and remained relatively isolated until the Miocene (Barker and Murrell, 2004). This limited initial *Rhipicephalus* distribution to the Afrotropics, making host-linked dispersal out of Africa the only possible mode of subsequent global radiation. Timing

estimates of the earliest three divergences marginally postdate paleogeographic changes linked to the Cretaceous-Tertiary extinction, 65 Mya (Alvarez et al., 1980) and Paleocene-Eocene Thermal Maximum, 56 Mya (Wing et al., 2005). These events set conditions for the delayed radiation of many mammal groups (Bininda-Emonds et al., 2007), suggesting an influence of mammals on *Rhipicephalus* evolution (Beati and Klompen, 2019). Early Ungulata dispersed from Laurasia to Africa along a number of intermittent connections by at least the Eocene Ypresian (48-56 Mya) based on Condylarthra fossils (Adaci et al., 2007; Gheerbrant and Rage, 2006; Sudre et al., 1993). These hosts may have provided novel niches for the progenitors of *Rhipicephalus* to utilise and may have played a significant role in subsequent tick dispersal and radiation due to high mobility and large home ranges of these hosts (Bowman et al., 2002). This is especially pronounced in species that utilise descendants of these hosts at early life stages, many of which evolved host-truncate life cycles (Fig. 2.4). Moreover, intermittent dispersal routes from Laurasia allowed limited dispersal of early Ungulata which concurs with limited *Rhipicephalus* speciation (three events) around this time (Fig. 2.2). Central Africa was the most likely region for *Rhipicephalus* progenitors (Fig. 2.3), and this region was mostly dry woodland at the time (Jacobs, 2004; Jacobs and Herendeen, 2004) which favoured early Ungulata as primary herbivores with masticatory teeth (Hunter and Jernvall, 1995). Simultaneously, Rodentia began to radiate at about 60 Mya (Bininda-Emonds et al., 2007) and dispersed into Africa by 56 Mya (Adaci et al., 2007; Court and Hartenberger, 1992). These small mammals may have provided novel niches for tick immatures that presumably used Afroinsectivora as hosts before this time. Subsequently, host switches to Lagomorpha must have occurred much later given Lagomorpha only entered Africa by at most 14 Mya (Matthee et al., 2004). Three-host species with multiple off-host periods have increased risk of dehydration (Leal et al., 2020; Yoder et al., 2006), making them especially dependant on hosts. Higher rates of metabolism and activity in small mammals (Clarke et al., 2010), as well as greater abundances (Cotgreave, 1993), increase opportunities for tick attachment (Brunner and Ostfeld, 2008; Kowalski et al., 2015). As such, immatures that attached to small mammal hosts would have been selected for. This probably maintained the ancestral condition of three-host life cycles that utilise small mammals at early stages, as observed in *Rhipicentor* (Fourie et al., 2005; Horak et al., 2018). However, small mammals tend to have small home ranges, making them less important for dispersal events (Bowman et al., 2002). Instead, adult host-use is more important for dispersal of species that currently parasitise Rodentia, Lagomorpha and Afroinsectivora at early life stages, such as those in simus, capensis, follis and haemaphysaloides groups (Figs. 2.3-2.4).

Progenitors of two lineages (I excl. *Boophilus* and V) dispersed southwards between 20-35 Mya (Oligocene) after initial speciation events in central Africa, and left remaining

progenitors (subgenus *Boophilus*, pulchellus group, appendiculatus group i and sanguineus group) in central Africa (Figs 2.3, 2.7). During this time, grasslands and savannahs started expanding while equatorial forests and woodland receded (Keeley and Rundel, 2005; Lunt et al., 2007; Mannetje, 2007). This facilitated southward radiation of early Ungulata that easily adapted to grassland environments due to their masticatory teeth (Hunter and Jernvall, 1995). Speciation in their tick parasites followed due to climate niche partitioning associated with high annual, seasonal and diurnal temperature variation (Fig. 2.5 – temperature seasonality: ancestral 286.62 to 328.05 in V, and 307.89 in I excl. *Boophilus*). Species diversity increased following initial dispersal, but many of these species have small geographic ranges (Fig. 2.3 – range codes at terminals). This is possibly due to the small home ranges and reduced mobility of hosts for immature life stages of these species (Fig. 2.4), that reduce tick geneflow (Matthee, 2020; Sands et al., 2017a). Additionally, dispersal events impose a shift in environmental conditions that initiate niche partitioning when progenitors establish populations in areas with different approximations of their ancestral climate niche (Kozak and Wiens, 2010; Pyron et al., 2015; Warren et al., 2008). In these progenitors dispersing to southern Africa, speciation corresponds with a dispersal route along gradients in annual, seasonal and diurnal temperature variation (Fig. 2.5). Hypothetically, adults dispersed southward into novel environments on mobile hosts, where allopatric and peripatric divergence was driven by differential approximations of the ancestral climate niche and limited geneflow due to immature host-use. Subgenus *Digineus* is excluded from the latter process however, as their immatures parasitise large and mobile hosts (Fig. 2.4), which facilitates geneflow while expanding geographic ranges, consequently limiting speciation (Figs 2.1, 2.3). Later, descendants of these tick radiations (I: *R. kochi* + *R. pravus*, *R. bursa*, and V: simus group i) dispersed northward again between 10-20 Mya (Miocene) (Figs 2.3, 2.7). This time however, possibly as adults on novel Ruminantia and Suidae hosts (Fig. 2.4) following their dispersals into Africa from Asia along the Gomphotherium Land Bridge (Arabian Peninsula) that formed in the Miocene, 19 Mya (Cox, 2000; Frantz et al., 2016; Rögl, 1997; Sen, 2013). Once these animals radiated into southern Africa, they may have provided novel niches for southern African ectoparasites that extended back northward into established Ruminantia and Suidae populations. This would make historical tick dispersal advance in the opposite direction to that of host dispersal (Figs 2.3, 2.7). Notably, progenitors of *R. bursa* + *R. glabroscutatus* underwent a distinctly large dispersal extending into the Palearctic linked to the Miocene radiation of immature hosts that were highly mobile (Fig. 2.4), such as Caprinae and sister groups (Mead and Taylor, 2005; Ropiquet and Hassanin, 2005). This may have been aided by their two-host life cycle which limits exposure to harsh off-host conditions to only twice during life cycles (Barker and Murrell, 2004; Klompen et al., 1996).

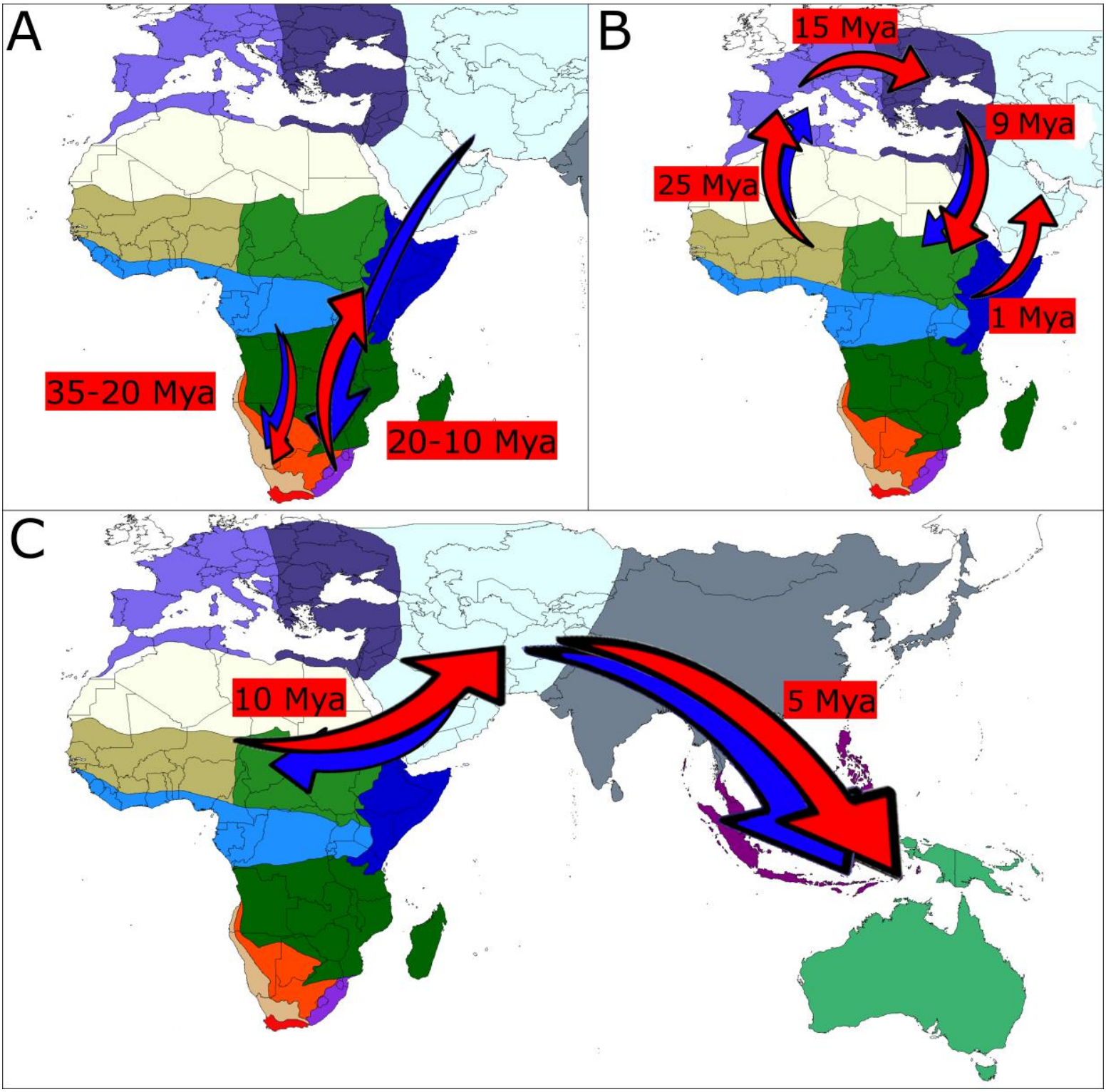

Figure 2.7. Hypotheses for *Rhipicephalus* dispersal events among subgenus *Digineus*, pravus group, simus group (A); sanguineus group (B) and subgenus *Boophilus* (C). Red arrows indicate direction of tick movement, while blue arrows indicate direction of host movement. Time stamps are in millions of years ago (Mya).

Progenitors of the pulchellus group likely remained in central Africa after initial *Rhipicephalus* divergences, and probably adapted to ancient Perissodactyla in Africa given their present-day host preference for Equidae and Rhinocerotidae (Fig. 2.4) (Horak et al., 2018; Walker et al., 2000). The earliest fossil of surviving African Perissodactyla (Equidae) is from the early Oligocene, 33 Mya (Dartevelle, 1935), and coincides with stem estimates of pulchellus group, 28-46 Mya (Fig. 2.2). Many *Rhipicephalus* ticks quest on short grasses near ground level to parasitise grazers (Browning, 1976; Speybroeck et al., 2003). Other Rhipicephalinae ticks, such as *Cosmiomma hippopotamensis* and *Dermacentor rhinocerinus*, quest on tree branches or tall grasses about 100cm above ground level, and specifically along rhinoceros paths, to parasitise browsers such as black rhinoceros (Apanaskevich et al., 2013; Horak et al., 2018; Walker, 1991). Hypothetically, ectoparasite niches on ancient Perrisodactyla were inaccessible to grazer-adapted ticks given many ancient Perissodactyla were browsers (Janis, 2008; Semprebon et al., 2011). These niches may have instead been filled by ticks such as *Cosmiomma* and *Dermacentor* with questing behaviour more suitable for parasitising browser hosts. As such, the rise of grazing Perissodactyla (Equidae and some Rhinocerotidae) would have provided novel niches for grazer-adapted *Rhipicephalus* to radiate into. Following from this, the decline of global Perissodactyla that started in the middle Oligocene, around 27 Mya (Cifelli, 1981; Langer, 1987), may have contributed to a loss of parasite diversity, and may explain the limited present-day diversity among *Cosmiomma* and Afrotropical *Dermacentori,* as well as putative extinction of stem-*Rhipicephalus* (post host switch to early Ungulata) discussed above. If true, this would make these ticks relict Perissodactyla ectoparasites that were more diverse in the Eocene, that were unable to switch to grazing Ruminantia and Suidae hosts that dispersed into Africa in the Miocene at 19 Mya (Cox, 2000; Frantz et al., 2016; Rögl, 1997; Sen, 2013). Moreover, ectoparasite niches on novel Ruminantia and Suidae grazers would have been filled by *Rhipicephalus* already adapted to grazing hosts, leaving browser adapted ticks too specialised and unable to compete for niches to switch hosts.

Estimates for the sanguineus group indicate their progenitors most likley dispersed out of Africa into the Mediterranean via a western dispersal route about 25 Mya (Oligocene-Miocene) (Figs 2.3, 2.7). This is based on the extant populations of basal *R. sanguineus* s.s. and *R. pusillus* in western Europe and northwest Africa (Fig. 2.3, Walker et al., 2000). Timing estimates generally predate the formation of the Gomphotherium Land Bridge (Arabian Peninsula) at 19 Mya (minimum 95% HPD = 19.08 Mya), and ancestral area estimates suggest a western, rather than eastern, dispersal event (Figs 2.3, 2.7). The west Mediterranean was scattered with island land bridges by the Oligocene Aquitanian, 21 Mya, with the opening of the Gulf of Lyon and Valencia trough (Schettino and Turco, 2006; Séranne,

1999; Seton et al., 2012). This allowed limited faunal interchange and some organisms with similar historical biogeographic patterns include trapdoors spiders (Opatova et al., 2016) and ground beetles (Faille et al., 2018). A host switch in *Rhipicephalus* adults from Ungulata to predators, also occurred during this divergence (Fig. 2.4) given the majority of sanguineus group species specialise on Canidae and Felidae hosts, while most other *Rhipicephalus* do not – with exception of *Rhipicephalus carnivoralis* (Horak et al., 2018; Walker et al., 2000). However, divergence time estimates predate the origins of Canidae and Felidae at around 10 Mya (Johnson et al., 2006; Wang et al., 2004). Following from the hypothesis for *Rhipicephalus* origin on Hyaenadonta, ectoparasite niches on Hyaenodonta predators may have been filled by progenitors of stem-*Rhipicentor* and stem-*Rhipicephalus* at the time, while crown-*Rhipicephalus* utilised Ungulatea (Fig. 2.4). However, the radiation of *Hyainailouros* by the late Oligocene (Kappelman et al., 2003; Leakey et al., 1995) coincides with initial divergence and adult host switch of the sanguineus group about 28 Mya (Fig. 2.2). As such, these predators may have provided novel niches for *Rhipicephalus* that initiated a reversal in adult host preference back to predators, as in adult *Rhipicentor* (Fig. 2.4). More specifically, African *Hyainailouros sulzeri* are a clade-unique instance of dispersal into Eurasia at exactly this time (Borths et al., 2016; Borths and Stevens, 2017). From this, we can hypothesise that sanguineus group ticks may have originated from a host-linked dispersal into the Mediterranean that shortly followed a host switch in adult stages to *Hyainailouros* predators. Many *Rhipicephalus* species, including the sanguineus group, have immatures with host preferences for small Lagomorpha and Rodentia that are prey to small predators (Fig. 2.4). Most Oligocene-Miocene Hyaenadonta hypercarnivores preyed on large animals, but some, such as smaller African Viverridae (Rasmussen and Gutierrez, 2009), would have preyed on small mammals such as Rodentia that were present at the time (Stevens et al., 2009). In turn, small carnivores would also become prey to large Hyaenadonta such as *Hyainailouros sulzeri*. Following this, food webs with nested predator-prey connections may have facilitated an adult host switch from Ungulata to *Hyainailouros sulzeri*, via tick immatures moving onto predators directly from kills, or by advancing into predator geographic ranges on small predators and prey, then dropping off to moult before questing and attaching to a new host. All sanguineus group ticks move through three off-host periods, providing ample opportunity for host switches to novel hosts (Balashov, 2004; Nava and Guglielmone, 2013). Ticks able to attach to novel and radiating *Hyainailouros* predators likely gained a selective advantage due to easily available niches. As such, nested predator-prey connections in food webs (Britton, 2013; Graham et al., 2009; Lafferty et al., 2008) may have again played a role in facilitating tick response to novel ecological opportunity to drive opportunistic tick radiation (Balashov, 2004). Moreover, the same process could have facilitated additional ectoparasite exchanges

between *Hyainailouros sulzeri* and Palearctic predator species after initial sanguineus group dispersal out of Africa. This may have contributed to Palearctic sanguineus group species switching to other predators, and eventually to extant Carnivora hosts (Fig. 2.4). In particular, Amphicyonidae had populations that extended into the Palearctic during the Miocene (Aguirre et al., 1982; Gagnaison, 2013), and may have provided novel niches to facilitate sanguineus group radiation in the western Palearctic and further east (Figs 2.3, 2.7). Estimates of ancestral climate support poleward movement in the initial dispersal of sanguineus group progenitors based on increasing temperature seasonality and annual temperature range (Fig. 2.5). Species diversity increased markedly following initial dispersal, but the resulting species have small geographic ranges (Fig. 2.3 – range codes at terminals). Differential approximations to novel conditions probably drove speciation due to accelerated allopatric and peripatric population divergence processes. Speciation may have been also driven by immature host preference for small and relatively immobile Rodentia and Lagomorpha hosts (Fig. 2.4) that limit geneflow (Matthee, 2020; Sands et al., 2017a).

After some time, the sanguineus group is estimated to have radiated back into Africa, around 9 Mya (Figs 2.3, 2.7), toward climates with lower temperature seasonality closer to the equator (Fig. 2.5). Adult stages of non-Afrotropical sanguineus frequently parasitise wolf and fox-like Canidae that dispersed to Africa by the late Miocene, 5-10 Mya (De Bonis et al., 2007; Morales et al., 2005; Sotnikova and Rook, 2010; Wang et al., 2004). This dispersal is estimated to have occurred along the Gomphotherium Land Bridge (Arabian Peninsula), and was approximately concurrent with Felidae, Canidae and other groups dispersing from Eurasia (Bibi, 2011; Johnson et al., 2006; Toussaint et al., 2019). Canidae hosts may have carried adults of sanguineus group species back to Africa, and simultaneously introduced them to African Carnivora and co-dispersed Felidae through shared food webs (Fig. 2.4). Supporting this contention, are basal *R. sulcatus* that have preference for both Canidae and Felidae hosts (Horak et al., 2018; Walker et al., 2000). Moreover, Ruminantia entering Africa at the same time may have similarly provided co-dispersing novel niches for adult stages of some Afrotropical sanguineus group species, which eventually lead to an adult host switch reversal back to Artiodactyla in *R. camicasi* (Fig. 2.4). The wide range of adult host preference for large hosts among Carnivora and Ruminantia probably facilitates the wide geographic ranges of most Afrotropical sanguineus group species (Fig. 2.3 – range codes at terminals), in spite of limitations imposed by immature life stages utilising small, relatively immobile host species. Host switches along nested predator-prey connections in food webs, linked by different tick life stages, may have proved important for tick evolution once again, because Hyaenodonta, Amphicyonidae and other hypothetical ancient hosts went extinct during this time due to competition with more adaptable Canidae and Felidae (Viranta, 1996). The Sahara

desert formed after this, about 5-7 Mya (Schuster et al., 2006; Zhang et al., 2014), and probably limited further dispersal events between Afrotropical and Asian regions (Douady et al., 2003; Gonçalves et al., 2012). However, oscillating climate history in this area due to Milankovich cycles, would have formed temporarily habitable conditions that facilitated ephemeral faunal connections (Bennett, 1990; Gonçalves et al., 2018). This may have contributed to reticulate evolution that maintained hybridisation potential between *R. afranicus* and *R. turanicus* (Bakkes et al., 2020; Chapter 4). Subsequently, Afrotropical sanguineus group descendants radiated throughout Africa, with *R. leporis* dispersing into the Middle East during divergence from *R. camicasi* about 1 Mya (Pleistocene) (Figs 2.3, 2.7), most likely on *Vulpes* foxes as these are preferred hosts for *R. leporis* (Walker et al., 2000). This final dispersal is supported by ancestral climate estimates which increase due to more variable temperatures encountered by their descendants in arid environments (Fig. 2.5).

Another clade that remained in central Africa became appendiculatus group i that generally prefer Artiodactyla grazing hosts at all life stages (Fig. 2.4). This may explain their generally wide geographic ranges (Fig. 2.3 – range codes at terminals), as a result of immature dispersal facilitated by large and mobile host-use. Grazer-adapted questing behaviour, where ticks quest at low heights (Browning, 1976; Speybroeck et al., 2003), may have enabled them to adapt to Ruminantia hosts along with most other *Rhipicephalus* groups, following dispersal of Ruminantia into Africa in the early Miocene, 19 Mya (Cox, 2000; Leakey et al., 1996; Sen, 2013). However, one species from this clade, *R. carnivoralis*, exclusively prefer extant Felidae hosts as adults (Walker et al., 2000) and originated after Felidae dispersed into Africa in the late Miocene about 10 Mya (Johnson et al., 2006). This host shift may have been driven by nested predator-prey connections in food webs with Felidae preying on Artiodactyla. Interesting to note, this divergence and host shift is estimated as concurrent with Afrotropical sanguineus re-introduction to Africa. However, most sanguineus group ticks parasitise both Felidae and Canidae hosts, suggesting they may have adapted to both host groups as they dispersed into Africa. On the other hand, *R. carnivoralis* prefer Felidae hosts which suggest a more specific host switch or that Felidae may have arrived in Africa before Canidae. Supporting the latter hypothesis are older divergence time estimates for Felidae, as well as their origin in Asia rather than North America as in Canidae (Johnson et al., 2006). Interesting to note, some mitochondrial introgression seems possible among *R. bergeoni* (appendiculatus group i) and *R. sanguineus* s.l. “southeastern lineage” (sanguineus group) (Fig. S2.1: 12S rDNA). These two species occur in Ethiopia and Egypt respectively, which are separated by the Sahara desert (Walker et al., 2000). However, these areas were likely connected intermittently during Milankovitch cycles which may have facilitated interbreeding with little selection against hybrids in a similar manner as proposed for *R. afranicus* and *R. turanicus*

(Bakkes et al., 2020; Chapter 4). This would have to occur after 7.82 Mya (maximum 95% HPD; Fig. 2.2) when *R. bergeoni* diverged from *R. appendiculatus*, because the same introgression is not present in *R. appendiculatus*. Indeed, Milankovitch cycles proceeded well into the Quaternary (Bennett, 1990; Gonçalves et al., 2018). Alternatively, this may also be explained as a result of *R. appendiculatus* geographic localisation southwards, which bypassed any putative interbreeding during periods of connectivity. Nevertheless, these hypotheses remain to be tested with sequence data from more *R. bergeoni* individuals.

Subgenus *Boophilus* is estimated to have originated in central Africa about 28 Mya (early Oligocene), and dispersed into eastern Palearctic, Indo-Malaysian and Australasian regions much later between 5-10 Mya (late Miocene) (Figs 2.3, 2.7). Bovidae are the preferred hosts for all life stages of these ticks (Fig. 2.4), and the radiation of bovids into Africa along the Gomphotherium Land Bridge (Bibi, 2013; Cox, 2000; Leakey et al., 1996; Sen, 2013) likely initiated tick dispersal out of Africa, in the opposite direction, along established populations of hosts novel to these ticks. This excludes recent human-mediated dispersal of ticks globally (Bram et al., 2009). Similar divergence time estimates for *Hyalomma* dispersals into Africa from Asia, as well as similar host preferences suggest they underwent the same dispersal event, but in the direction of host movement into Africa (Sands et al., 2017b). Moreover, these animals dispersed across the Afrotropics and probably facilitated dispersal and initiated divergence of *R. decoloratus* from their central African progenitors, which resulted in *R. decoloratus* wide extant distribution in the Afrotropics (Figs 2.3, 2.7). Ancestral climate estimates for subgenus *Boophilus* are congruent with a central African origin in climates with low annual, seasonal and diurnal temperature variation, and remained generally unchanged throughout their radiation (Fig. 2.5). This may suggest they were pre-adapted for climate conditions they would encounter as they dispersed into Asia. Host truncation to one-host life cycles is ancestral in subgenus *Boophilus* (Fig. 2.4), and most likely contributed significantly as a 'key innovation' (Gavrilets and Vose, 2005) to drive *Boophilus* radiation and geographic expansion (Barker and Murrell, 2004; Klompen et al., 1996). One-host life cycles on large and mobile hosts enable ticks to attach for longer periods to move further, and limits exposure to harsh off-host conditions to reduce abiotic selection pressure linked to dehydration (Klompen et al., 1996). This allows peripheral populations to disperse further with host movement, and makes establishment of tick range expansions more stable due to reduced exposure to harsh off-host conditions. Hypothetically, the combined effects of reduced niche partitioning due to dispersal into temperature stable environments similar to ancestral conditions, as well as one-host life cycles where immatures utilise large, mobile hosts may explain the wide geographic ranges among most species in subgenus *Boophilus* (Fig. 2.3 – range codes at terminals). Moreover, these combined factors may also limit speciation by increasing geneflow between

populations separated by large distances, and connecting populations in environments with similar annual, seasonal and diurnal temperature variation. Indeed, subgenus *Boophilus* comprise only six species, which is unusual given their wide global distribution (Guglielmone et al., 2014).

Progenitors of haemaphysaloides group + capensis group i are estimated to have originated in southwest Africa around 20 Mya (early Miocene), with haemaphysaloides group descendants dispersing to Oriental and Indo-Malaysian regions between 2-12 Mya (Miocene-Pliocene-Pleistocene) (Figs 2.3, 2.7). Long-range dispersal is not reasonable as all such possibilities can be disproved because some *Rhipicephalus* will occasionally use birds as hosts, but these are incidental and don't occur in high abundances (Horak et al., 2018; Walker et al., 2000). Moreover timing estimates significantly post-date Indian drift dispersal (Bakkes et al., 2018a; Bossuyt et al., 2006; Sklenarova et al., 2013). This leaves Gomphotherium Land Bridge dispersal out of Africa as the only possible explanation, linked with Ruminantia and Suidae host dispersal proceeding in the opposite direction (Bibi, 2013; Cox, 2000; Frantz et al., 2016; Sen, 2013) – as proposed for subgenus *Boophilus* (Fig. 2.7). Unfortunately, sequence data is lacking for species considered closely related based on morphology, and which have extant distributions near this dispersal route (Walker et al., 2000). These include east African *R. lunulatus*, *R. interventus*, *R. jeanneli* and *R. hurti*, as well as Oriental *R. ramachandrai* and *R. scalpturatus*. Nevertheless, the best hypothesis remains host-linked dispersal along the Gomphotherium Land Bridge out of Africa between 2-12 Mya (Figs 2.3, 2.7).

Drivers of Rhipicephalus Evolution

Four bioclimatic variables representing annual, seasonal and diurnal temperature variation were recovered as significant correlates of *Rhipicephalus* phylogeny (Fig. 2.5). These variables were previously recovered as significant for geographic distributions of *R. sanguineus*, *R. appendiculatus* and *R. microplus* (Leta et al., 2013; Silatsa et al., 2019; Sungirai et al., 2018; Zemtsova et al., 2016). The present findings indicate these variables are phylogenetically significant and pertain to a broad spectrum of *Rhipicephalus* species. Distribution models generated from these four variables performed well with a mean of 74% (±16%) true positive rate. This is important, given limitations for generalising species distribution models when using only four variables, and is valuable for future distribution modelling effort. Clades that have geographically widespread species, but are less species-diverse (such as subgenus *Boophilus*, appendiculatus group i and simus group i: Fig. 2.3 – range codes at terminals), were found to have evolved in climates with lower annual, seasonal and diurnal temperature variation (Fig. 2.5: 5-8 species per clade). On the other hand, more

diverse clades that have smaller geographic ranges per species (such as sanguineus group and subgenus *Digineus* + pravus group + appendiculatus group ii: Fig. 2.3 – range codes at terminals), evolved in climates with greater annual, seasonal and diurnal temperature variation (Fig. 2.5: 11-12 species per clade) – with exception of *R. evertsi evertsi* and *R. evertsi mimetcus* that evolved in more temperature variable environments (Fig. 2.5: ancestral temperature seasonality 308.07). This finding suggests off-host environments play an important role in limiting tick ranges (Klompen et al., 1996) and also suggests an important driver of tick speciation is niche divergence following shifts between climate regimes during dispersal (Kozak and Wiens, 2010), such as those from lesser to greater temperature seasonality (Fig. 2.5). Greater temperature variation annually, diurnally and between seasons provides more diverse conditions among progenitors dispersing poleward, and facilitates allopatric or peripatric speciation when populations establish different approximations to the ancestral niche along a dispersal route (Pyron et al., 2015; Warren et al., 2008). This is most clear in the distinction between sanguineus group that are species-diverse, but with species that have generally localised geographic ranges, and subgenus *Boophilus* that are not species-diverse and have global geographic ranges (Fig. 2.3 – range codes at terminals) – these examples exclude the possibility of human-mediated dispersal of ticks (Bram et al., 2009; Dantas-Torres et al., 2018; Gray et al., 2013; Laatamna et al., 2020). The sanguineus group possibly underwent dispersal on highly mobile predator hosts into the Palearctic, a region with the highest temperature seasonality and annual temperature range for *Rhipicephalus* ticks (Fig. 2.5). In contrast, subgenus *Boophilus* possibly underwent dispersal on similarly mobile hosts, but into regions with the lowest temperature seasonality and annual temperature range (Fig. 2.5). This process is likely underway in *R. appendiculatus* that vary diapause behaviour during life cycles along similar latitudinal gradients, where no diapause is present at lower latitudes (Madder et al., 2002). This may result in reproductive isolation by allochronic divergence (Taylor and Friesen, 2017; Helm and Womack, 2018), ultimately due to different local approximations of the ancestral climate niche.

Departure from Brownian motion in phylogenetic signal for variables of annual, seasonal and diurnal temperature variation was generally weak, suggesting additional factors play a role. Ultimately, the complex role of host associations for dispersing ticks and buffering them from off-host environments during parts of their life cycle cannot be excluded. One factor that hypothetically initiates *Rhipicephalus* speciation is host-linked dispersal into novel areas (founder events) which drive population expansion into diverse environments that present divergent niches, mainly in terms of annual, seasonal and diurnal temperature variation (Figs 2.3-2.5). During dispersal into such environments, niche partitioning would result in reduced geneflow and increased genetic diversity between allopatric and peripheral populations

(Kozak and Wiens, 2010; Mayr, 1947). Factors that counteract this include hosts with greater mobility, larger ranges and dense populations, that will readily move ticks within their home ranges to increase tick geneflow and limit speciation (Matthee, 2020). On the other hand, these hosts can also carry ticks along unidirectional dispersal routes (such as the Gomphotherium route) and introduce them to novel environments, initiating niche partitioning and decreasing geneflow to enforce speciation. Moreover, hosts with high mobility and large ranges but sparse populations, such as predators, may similarly limit geneflow and drive differential niche approximation to increase speciation. In such host-sparse environments, generalist host-use strategies could counteract speciation by increasing the effective geographic range of hosts and also increasing tick geneflow (Matthee, 2020). Finally, generalists in host-dense environments may increase geneflow and reduce speciation even further, possibly making tick ranges large. Geographic barriers such as altitudinal gradients, can create movement bottlenecks in hosts with large geographic ranges that may also contribute to reduced geneflow and increased genetic diversity to drive tick speciation (Bakkes et al., 2018b; Sands et al., 2017a).

The trade-off between on- and off-host periods is another important factor to consider, and this trade-off may have led to the evolution of host-truncate life cycles in some clades. For example, the number of off-host periods differ between species-diverse, geographically localised sanguineus group (three periods) and species-limited, geographically widespread subgenus *Boophilus* (one period). Hosts offer protection from off-host conditions and reduce selection by abiotic factors, such as dehydration (Klompen et al., 1996; Leal et al., 2020; Yoder et al., 2006), and also move ticks between populations to increase geneflow (Matthee, 2020). These may counteract the effect of niche partitioning along gradients of annual, seasonal and diurnal temperature variation, and effectively limit speciation. As such, fewer off-host periods and tighter associations with hosts may limit speciation and facilitate geographic range expansion. Ticks that have close associations with their hosts, by way of host truncation as a 'key innovation' (subgenera *Boophilus* and *Digineus*), may be favoured by selection due to reduced exposure to off-host conditions and risk of death due to dehydration. During on-host periods, selection occurs mainly by grooming and predation, as well as by host ex-sanguination if too many ticks attach. Hypothetically, selection for host-truncate life cycles may favour individuals that parasitise large hosts because they afford better protection from predation and ex-sanguination due to their size that can hide ticks more effectively, given the extended periods of time these ticks are on their hosts. Following this, immatures that successfully parasitise and moult on large hosts will gain a selective advantage because they stand to benefit more than adults because they can access these benefits sooner in their life cycle. This would lead to higher abundances of host-truncate ticks that may in turn drive

reproductive fitness. As such, the key innovation of host-truncate life cycles may be driven by selection for immatures that are able to parasitise large hosts. However, immatures also benefit from using small hosts given higher activity and abundance of small mammals that increase opportunities for attachment in order to reduce dehydration risk (Brunner and Ostfeld, 2008; Clarke et al., 2010; Cotgreave, 1993; Kowalski et al., 2015). Hypothetically, an alternative niche centred around using small hosts at early life stages possibly maintained the ancestral condition of three-host life cycles in *Rhipicephalus* (Fig. 2.4).

Generalist host-use strategies are enabled by off-host periods which facilitate opportunistic host-use (Nava and Guglielmone, 2013). As such, off-host environments initiate a trade-off between subjecting ticks to harsh conditions, and enabling host-use flexibility that may facilitate host switches when ecological opportunities arise. This may prove important for enabling evolutionary responses to emergent ecological changes by not being too closely aligned with a particular host, but still benefiting from some alignment to on-host conditions (Balashov, 2004). Food webs may also play a role in introducing tick species to novel hosts along nested predator-prey connections (Britton, 2013; Graham et al., 2009; Lafferty et al., 2008), and off-host periods could provide ample opportunity for host-use flexibility to arise. Successful tick attachments to novel hosts along these connections may hypothetically facilitate host switches and adaptive radiation into novel niche-space. Additionally, availability and mobility of hosts for immatures is another important factor for limiting tick distributions and dispersal events (Matthee, 2020; Sands et al., 2017a). Hosts with greater dispersal ability, such as Ungulata, will disperse immatures further than hosts with limited dispersal ability, such as Rodentia and other small mammals. Hypothetically, this makes host-use at immature stages important for dispersal and geneflow in species that use large, mobile hosts as immatures. Conversely, adult hosts may be more important for dispersal in species that use small, relatively immobile hosts as immatures, as is the case in most three-host species (Fig. 2.4). As such, host size, which plays a role in determining host ranges (Ofstad et al., 2016), may also play an important role in determining tick distribution ranges and geneflow (McCoy et al., 2013). Consequently, tick evolution may be influenced by the trade-off between on- and off-host periods that interact with food webs, novel hosts, host mobility and climate factors during unidirectional host dispersals, in order to drive opportunistic radiation.

Competitive interactions have also been shown as important for limiting tick geographic ranges, and are important for driving speciation by way of selecting against one or more species in sympatric zones (Bournez et al., 2015; Cangi et al., 2013; Šimková et al., 2002; Spickett et al., 1991). These interactions may also reinforce allopatric and peripatric boundaries derived from niche partitioning along gradients of annual, seasonal and diurnal temperature variation, and accelerate speciation. For example, many host-truncate species

have wide geographic distributions, but the widest of these is often limited to one or two species per subgeneric group (Horak et al., 2018; Walker et al., 2004; Walker et al., 2000). Among two-host subgenus *Digineus* ticks, *R. e. evertsi* has the widest distribution that generally excludes its close relatives (Horak et al., 2018; Walker et al., 2000). Partitioning between predilection sites may also play a role, as all *Digineus* species readily parasitise goats, but utilise different predilection sites as immatures (Walker et al., 2000). These differences in geographic distributions and predilection sites, suggest selection for different ecological niches may have taken place. Furthermore, close relatives of *R. e. evertsi* occupy diverging niches with regards to annual, seasonal and diurnal temperature variation (Fig. 2.5: ancestral temperature seasonality 308.07-358.33), suggesting climate niche partitioning may have taken place. However, possibly as a correlated outcome of initial competitive pressures.

Conclusions

The present study recovered five deep clades among *Rhipicephalus* ticks with nine descendant clades that generally support current informal species groups (Walker et al., 2000). Notable exceptions include the distinct pulchellus group and two distinct appendiculatus groups. Investigation into historical biogeography support an African origin for *Rhipicephalus* based on divergence time estimates that post-date the breakup of Gondwanaland. Moreover, ancestral area estimates support central Africa as the most likely region for the origin of crown-*Rhipicephalus* based on geographic distributions of basal clades in central Africa. Patterns of host-use, geography and climate correlate with *Rhipicephalus* phylogeny to some extents. Climatic niches that are generally conserved at the subgeneric/species group level include four variables pertaining to annual, seasonal and diurnal temperature variation. As such, drivers of *Rhipicephalus* evolution and diversification probably include a complex set of interactions between all of these factors. Hypothetically, host-use patterns and off-host periods between immatures and adults interact with host mobility, geographic ranges and host activity to mediate geneflow, and also interact with host-linked dispersal events into novel environments. Along dispersal routes, gradients in annual, seasonal and diurnal temperature variation may play a role to initiate climate niche partitioning and subsequent speciation. In turn, these divergences may become reinforced, or initiated, by competitive interactions.

Timing estimate for the majority of *Rhipicephalus* radiations correlate with predicted host dispersal events among Ruminantia and Suidae hosts in the Miocene. The origin of *Rhipicephalus* may be a result of adult host switches from Hyaenodonta predators to early Ungulata that entered Africa in the Paleocene-Eocene, however this is contentious given the lack of a definitive sister taxon to *Rhipicephalus*. One radiation suggests a host switch from

early Ungulata to extinct *Hyainailouros* predators and eventually to extant Carnivora, concurrent with a host-linked dispersal to the western Mediterranean. Hypothetically, these host switches may have involved tick immatures, small mammals and predators in shared food webs mediated by off-host periods. These radiations highlight the importance of off-host periods for tick evolution, which may enable opportunistic shifts to novel hosts and initiate *Rhipicephalus* evolution into novel niches. Two radiations were associated with the phenotypic innovation of host-truncate life cycles, into niches more closely aligned with large hosts that reduce exposure to harsh off-host conditions and increase host-linked mobility and geneflow. Host-linked dispersal events are themselves linked to environmental changes in climate, ecology and faunal connectivity between habitable landmasses. A clear pattern was found that indicate novel tick-host interactions could have provided novel niches for ticks to disperse and radiate into, sometimes advancing in the opposite direction of host movement. These dispersals would initiate population establishment in novel areas, especially where climate regimes are suitable. Temperature stable environments are inhabited by less diverse clades that are also characterised by wider geographic ranges, and the opposite is true for environments with higher annual, seasonal and diurnal temperature variation (Figs 2.3, 2.5). This suggests that shifts in climate regime may partially drive tick speciation by way of differential niche partitioning where diverging species establish populations along different approximations of their ancestral niche. However, host associations probably also play a role in driving and limiting speciation by moving ticks such that geneflow is maintained or reduced. Host-use at immature stages may be important for dispersal, where large and mobile hosts can disperse tick species further if utilised early in development. In contrast, species that utilise small and relatively immobile hosts early in development may not dispersed as effectively. Instead, adult hosts that tend to be large and mobile are more important for dispersal in these species. Following this, species that utilise small, relatively immobile hosts early in development could also be more affected by niche partitioning along annual, seasonal and diurnal temperature variation gradients due to reduced geneflow. Finally, on-host environments reduce exposure to harsh off-host conditions, and may also reduce the range limiting effects of climate niche partitioning, especially for species with truncate host-use, effectively limiting speciation. Likewise, generalist host-use patterns may also play a role, and these host-use strategies emerge from having at least one off-host period which may enable opportunistic response to ecological changes, while also ensuring ticks remain partially aligned with a set of hosts in order to benefit from on-host environments.

This study contributes novel sequence data for many Afrotropical *Rhipicephalus* species. These sequence data will prove valuable for tick biology in a number of fields, particularly for verification of species identifications. Previous phylogenetic studies lack data

for many Afrotropical *Rhipicephalus* species, making the present study the most comprehensive to date. As such, findings presented here allow for deeper and more confident inference into the evolutionary history of *Rhipicephalus*. Additionally, phylogenetically significant climate variables will prove valuable in future species distribution modelling. Future research should also aim to sequence molecular data for species omitted here to further elucidate *Rhipicephalus* phylogeny and historical biogeography. Moreover, genomic approaches should be considered to better resolve the root of the *Rhipicephalus* phylogeny and determine relationships between sister genera. After this, subgenera should be formally established using integrated morphological and molecular lines of evidence to stabilise *Rhipicephalus* taxonomy. This will maximise the predictive power of taxonomic names and serve as an effective framework grounded in phylogeny, in which to place all *Rhipicephalus* related research.

SUPPLEMENTARY INFORMATION:

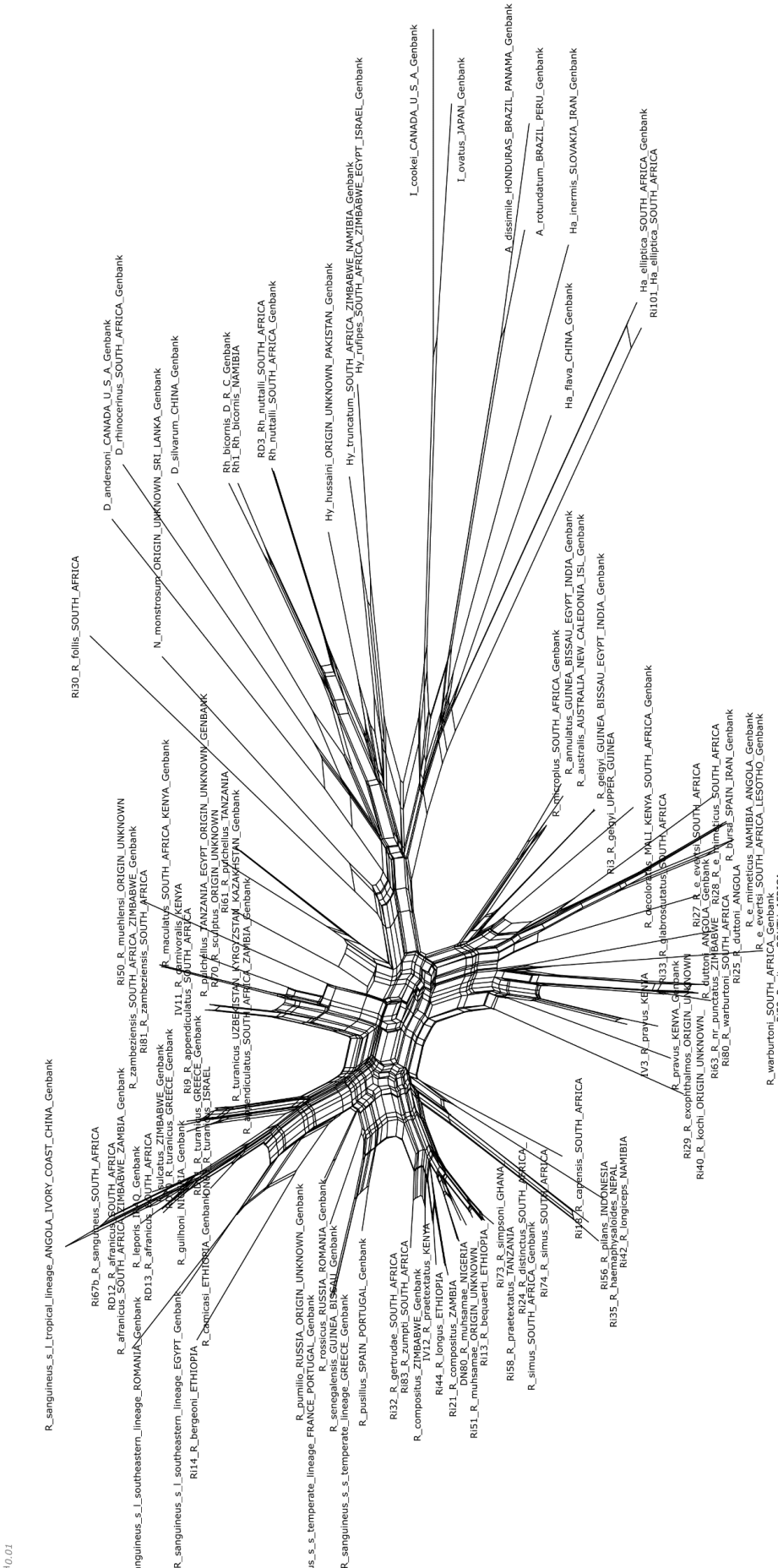

Figure S2.1. Splitstree for 12S gene partition.

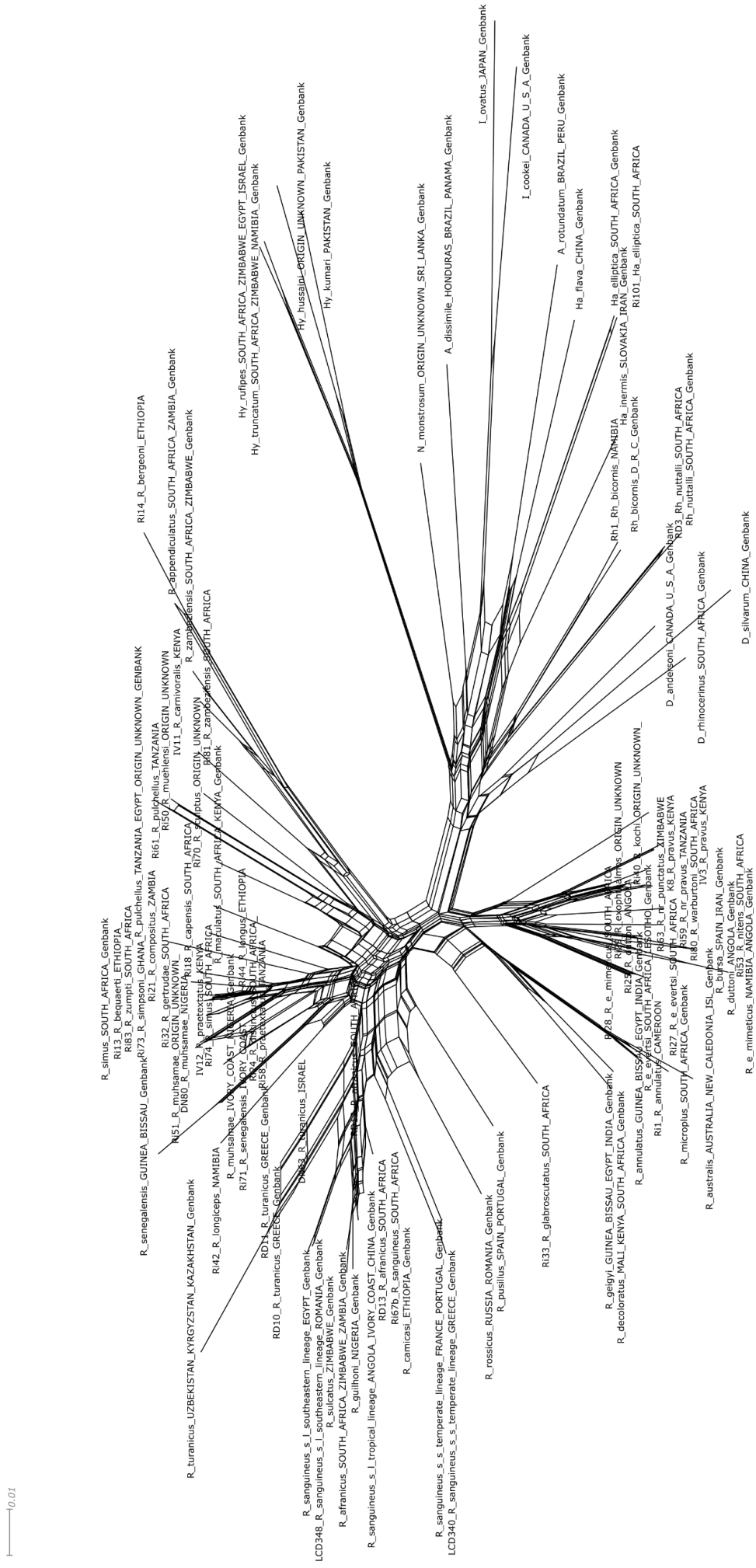

Figure S2.2. Splitstree for 16S gene partition.

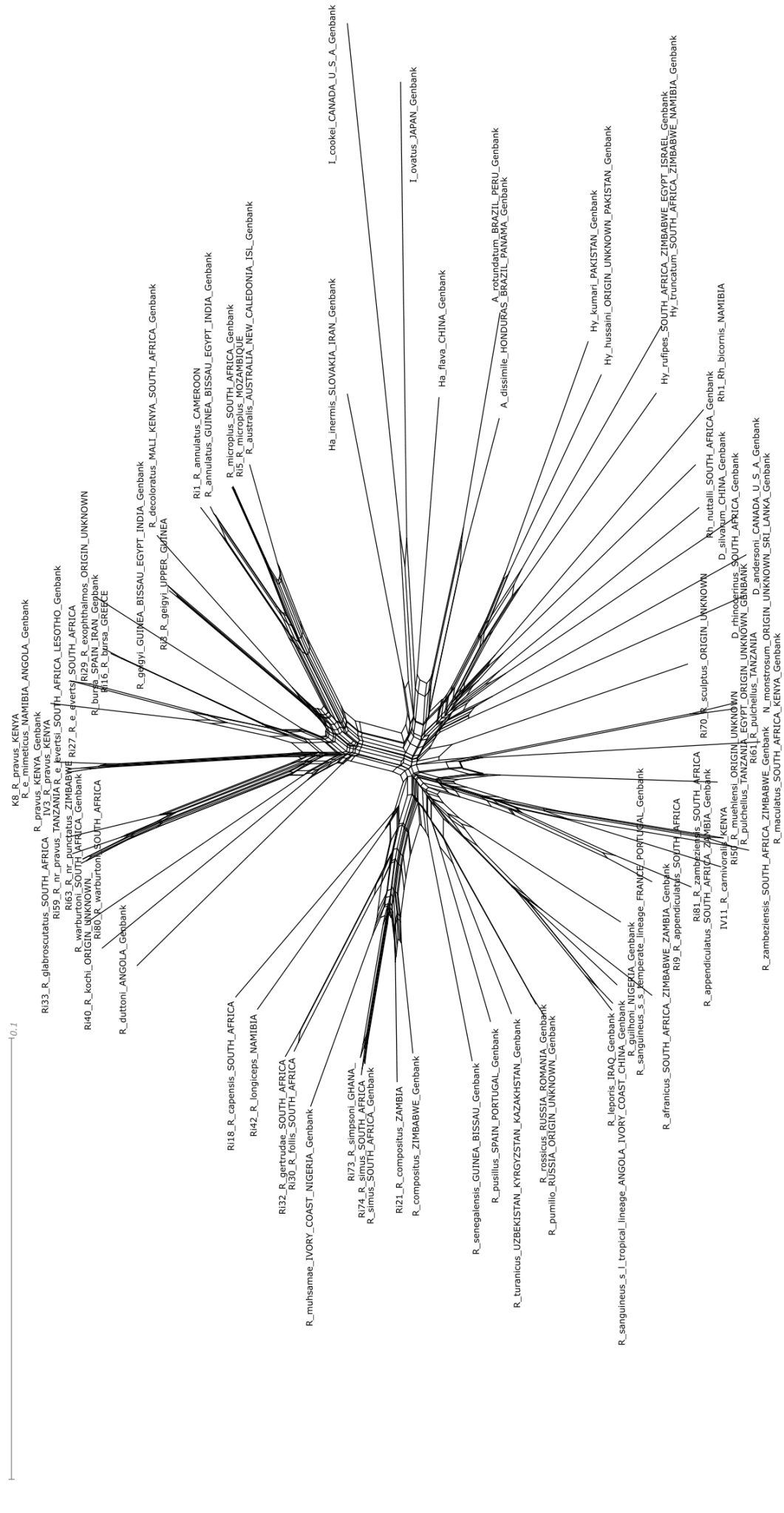

Figure S2.3. Splitstree for COI gene partition.

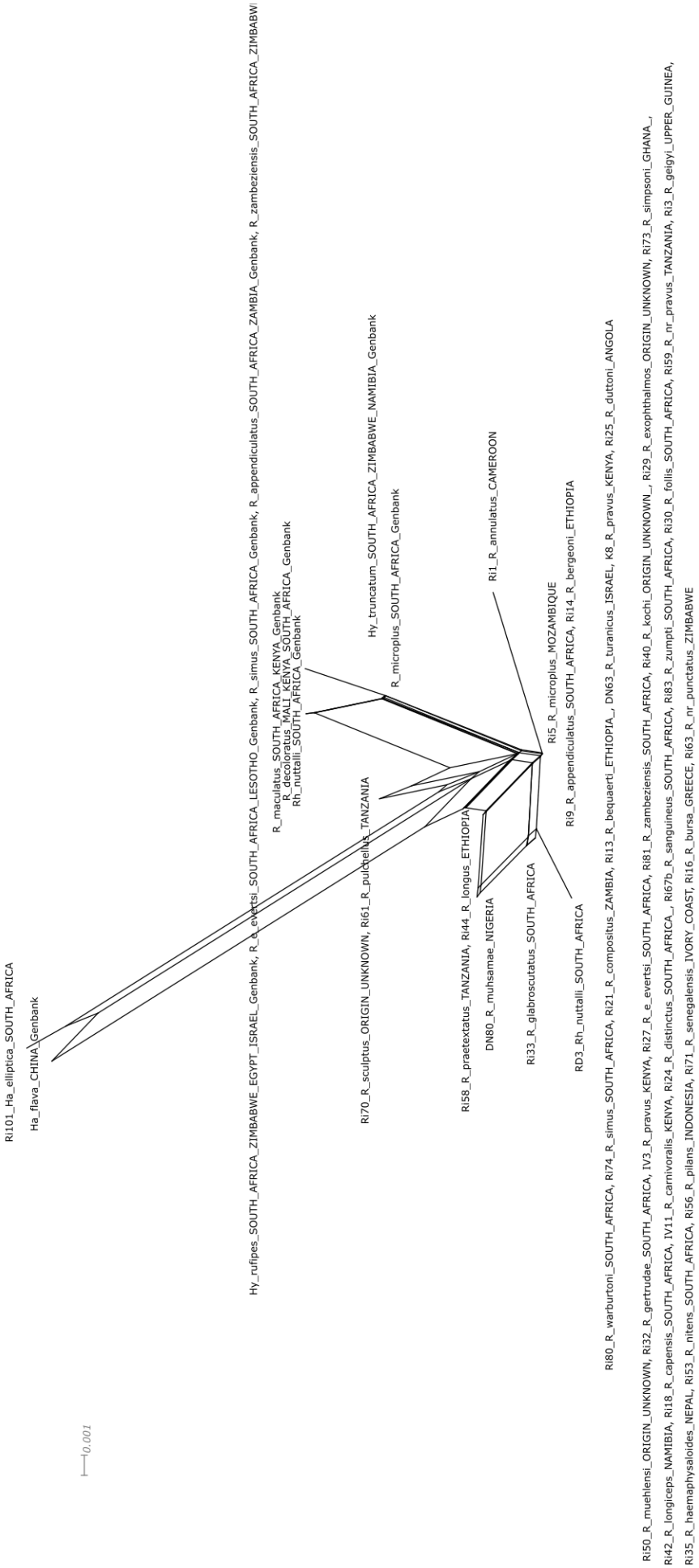

Figure S2.4. Splitstree for 28S-D2 gene partition.

3 Their Young Bite Better: Host-use Truncation in *Rhipicephalus* Ticks Driven by Neotenic Selection for Large Basis Capituli

Abstract

Developmental systems evolve by random mutations in genes and their regulatory networks that produce variable phenotypes for selection to act upon, or which may become fixed by drift if changes are selectively neutral. Life stages may be subject to different sets of selection pressure from the external environment that differentially influence phenotypic evolution of each stage. However, early life stages form the phenotypic basis for late life stages, making the evolution of developmental trajectories constrained towards a certain set of phenotypes near or along ancestral developmental trajectories based on internal selection pressure. Common evolutionary-developmental modifications include retention of immature features (paedomorphosis), or accelerated development (peramorphosis). This study investigates developmental shape variation in *Rhipicephalus* basis capituli with reference to phylogeny and host-use patterns, using geometric morphometric analysis based on 20 landmarks for 24 species. Basis capituli generate hydrostatic pressure in a cavity between the dorsal surface and hypostome which drive chelicerae protraction into host skin. Results indicate species that use large hosts at early developmental stages (host-truncate and some three-host species) have sagittally enlarged basis capituli with distinct anterior convexities, that correlate with host size (an approximation for skin stiffness). These features increase the volume of the hydrostatic cavity, facilitating additional force for chelicerae protraction into host skin. Large hosts provide advantages for ticks by reducing exposure to harsh off-host environments and host ex-sanguination. This suggests an adaptive response to selection for obtaining bloodmeals from large hosts with thick and stiff skin at early life stages. Small hosts however, provide more opportunities for attachment due to higher activity and abundance. This forms a separate viable niche that benefits immatures under dehydration risk. Moreover, using small hosts bypasses selection for attachment to thick and stiff skin. Developmental trajectories indicate one-host species undergo neotenic development, while two- and three-host species undergo distinct developmental phases in either early or late growth. Development is generally accelerated before the first off-host period for a given life cycle. This is due to selection for rapid development which produces larger individuals able to better maintain water balance while questing. However, three-host species that use small hosts at early have limited early development and nymphs remain small. These species bypass dehydration risk by attaching to highly active and abundant hosts in wetter microclimates close to the ground. Additionally, their small size may be selected for to facilitate higher on-host abundance given competition for attachment sites on small hosts. Three-host species that use large hosts at early life stages represent the ancestral condition, making three-host species that use small hosts at early

stages a derived condition that is developmentally post-displaced. Tests for mode of evolutionary-developmental modification indicate host-truncate species retain immature basis capitulum morphology through development, while three-host species do not. This is especially pronounced in subgenus *Boophilus* that also retain other immature features, such as small body size, pale colouration and circular spiracles. This indicates selection for large basis capituli with anterior convexities, combined with neotenic selection to retain these and other features during development, facilitated host truncation in *Rhipicephalus* ticks. Selection for small adults (neoteny) may be driven by limiting predation risk by reducing on-host visibility, as well as reduced developmental costs given lower dehydration risk in on-host environments. In conclusion, *Rhipicephalus* evolution is influenced by a complex set of trade-offs in tick body size in the context of on- and off-host environments while using small or large hosts that determine risks for dehydration, predation/grooming (visibility), competition and ex-sanguination. In turn, these trade-offs are affected by host-use patterns where basis capitulum morphology is constrained to be sagittally enlarged with distinct anterior convexities, in species that use large hosts with predilection sites with thick and stiff skin. Off-host periods, host-use and host truncation modulate these trade-offs by introducing or bypassing different sets of selection pressure. These findings suggest approaches to tick resistance by skin thickness and stiffness, may only be possible by enhancing skin thickness and stiffness beyond normal levels.

Introduction

Development of organisms (ontogeny) is the result of the genotype that becomes expressed in the phenotype over the lifespan of an organism. Developmental processes have a genetic and epigenetic basis that is heritable, and are regulated by developmental gene regulatory networks (Klingenberg, 2010; Young and Badyaev, 2007). Developmental variation has also been identified as an important source of evolutionary novelty (Love, 2006; Moczek, 2008; Ostachuk, 2016). Expressed features may vary through development due to a number of external and internal factors (Zelditch et al., 2003; Zelditch and Fink, 1996), and these variations provide the basic building blocks that selective pressure or drift can act upon. Under selection, organisms become optimised for survival in their respective environments through adaptive responses to selection pressure. Alternatively, a process termed ‘developmental system drift’ (True and Haag, 2001) or ‘phylogenetic drift’ (*sensu* Weiss and Fullerton, 2000) is partially responsible for developmental variation where chance mutations in developmental trajectories alter individual phenotypes and may be fixed by genetic drift. Moreover, modifications at early developmental stages can have impacts at later developmental stages, and if these are deleterious or disruptive to subsequent development, they will be selected

against (Mayr, 1994). As such, various traits of each life stage of an organism may be subjected to different selection pressures, which in turn can produce significant variation between species during development. Evaluating patterns that developmental systems produce across a set of related species can provide useful insight into the drivers and dynamics of evolution and development.

Differences in morphology across species are defined as disparity (*sensu* Sheets and Zelditch, 2013), and can be calculated using principal components of shape variation to compute Procrustes distance between species (Drake and Klingenberg, 2010). These differences can decrease or increase due to evolutionary changes in developmental phenotypes (Sheets and Zelditch, 2013). Some empirical studies have shown that shape disparities between closely related piranhas (Zelditch et al., 2003) and sea urchins (Mcnamara and Mckinney, 2005) tend to decrease through post-embryonic development, which make adult phenotypes more similar. Reduced disparity in adult phenotypes may result from internal factors such as ancestral developmental constraints or labile developmental systems that converge to produce similar adult phenotypes from disparate developmental trajectories (True and Haag, 2001; Zelditch et al., 2003). Ancestral developmental constraints will be more significant for later stages due to increased complexity of phenotypes (Clune et al., 2012), that make developmental systems more sensitive to small detrimental changes. In contrast, labile developmental systems placed under similar external selection pressure will tend to converge as a response to natural selection (Frankino et al., 2005). However, both processes of drift and selection operate simultaneously during developmental evolution where neutral variation among individuals increases the pool of possible phenotypic outcomes. These also serve as the ‘raw materials’ that selection acts upon to optimise phenotypes for certain environments (Gould, 1977; Mayr, 1994; McKinney and McNamara, 1991). As such, ancestral patterns of development set a ‘bias’ in morphospace that favours certain developmental patterns and features, which drive a proportion of evolutionary conservation in developmental systems. This occurs due to Brownian motion-like processes that change features in morphospace gradually, relative to previous iterations, in the absence of directional selection (Fusco et al., 2012). Taken together, the processes that shape the evolution of developmental systems are a complex of drift, selection and ancestral developmental constraints that occur at the individual level, where differentially successful phenotypes are taken up into species level variation (disparity) via genetic recombination in a population (Farine et al., 2015; Kingsolver and Pfennig, 2004; Nussey et al., 2007).

Variation in development is defined under two perspectives – heterochrony, which represent changes in timing of appearance of features, and heterotopy, which represent changes in spatial characteristics such as shape or position of features. These are not

mutually exclusive, and are likely correlated to affect one another during evolution of developmental processes (Klingenberg, 1998). Heterotopy is generally understudied, however the availability of modern geometric morphometric tools has reignited interest in this field due to the meticulous characterization of shape data afforded by these methods (Adams et al., 2013; Klingenberg, 2010; Mitteroecker and Gunz, 2009; Slice, 2007). Evolutionary-developmental modification of ancestral developmental pathways can arise by natural selection for early or later developmental traits. If early developmental traits provide a selective advantage that is relevant for later stages, phenotypic expression of those traits is prolonged (Gould, 1977; Reilly et al., 1997). This slows development and results in paedomorphosis (neoteny) in adults. Similarly, selection pressure that favours later developmental traits (such as sexual maturity) accelerate development (Stearns, 1992). This induces earlier onset of these traits and results in peramorphosis in juveniles. Moreover, accelerated development can initiate terminal addition where novel morphospace is reached towards the end of an organism's life cycle (Alberch and Blanco, 1996). Neoteny and acceleration form two broad classes of evolutionary-developmental modification that can be subdivided further based on changes in timing to onset and offset of features during development (Klingenberg, 1998). Alternatively, conservation of ancestral development results when no directional selection is present, or when features have already been optimised for their environment. Variation along conserved developmental pathways is termed 'ontogenetic scaling', where modifications in trait development are proportional to the expected ancestral trajectory (Klingenberg, 1998; Strelin et al., 2016).

Ticks of the genus *Rhipicephalus* Koch, 1844 are obligate bloodfeeding parasites of vertebrates that are important for biodiversity (Carlson et al., 2017), agriculture and human health (Keirans and Durden, 2005). They transmit microparasites such as *Rickettsia* spp., *Theileria* spp. and *Babesia* spp. to livestock and humans alike (Walker et al., 2000). Additionally, they may inject neurotoxins during feeding that can lead to tick paralysis (Horak et al., 2018; Walker et al., 2000). *Rhipicephalus* comprise ~75 species worldwide, and ~64 of these are Afrotropical endemics (Barker and Murrell, 2004; Camicas et al., 1998; Guglielmone et al., 2014; Horak and Camicas, 2003). Ticks pass through one inactive egg stage and three active life stages (larva, nymph and adult), with adults being the sexually mature stage that display high sexual dimorphism (Apanaskevich and Oliver, 2014). Larvae are more simplified in morphology (Anderson and Magnarelli, 2008) and have only six legs and no posterior protrusions (cornua) on their basis capituli (basal part of their mouthparts). Nymphs gain an extra pair of legs posteriorly, and a pair of spiracles behind these. Nymphal basis capituli are more complex with cornua either present or absent (depending on species), and lateral extensions that are generally present posteriorly, anteriorly or transverse centrally, imparting

a widened appearance. Adults also have eight legs, and have even more complex basis capituli with cornua generally present, and lateral extensions persisting or reduced. Important to note is that adult males and females are sexually dimorphic in basis capitulum shape, while larvae and nymphs are not. Moreover, adult females develop porous areas on their basis capituli that are linked to glands which secrete antioxidants that are suggested to protect lipids secreted by Gene's organ when females wax coat eggs during oviposition (Booth, 1989; Coons and Rothschild, 2008). Adult ticks also develop genital apertures which are absent in larval and nymphal stages (Sonenshine and Roe, 2014). Immature life stages are punctuated by moults which each follow from a single bloodmeal, and during their life cycle an individual may utilise one, two or three hosts depending on the species (Apanaskevich and Oliver, 2014; Hoogstraal and Aeschlimann, 1982). In three-host life cycles, each life stage requires a new host after moulting, and this comprises the majority of *Rhipicephalus* species. In ticks with two-host life cycles, such as *Rhipicephalus evertsi evertsi*, both larval and nymphal stages parasitize one host with the adult stage requiring a new host. In ticks with one-host life cycles, such as *Rhipicephalus decoloratus*, all life stages feed and moult on the same host, with adults dropping off the host for oviposition. Phylogenetic evidence suggests host-truncate life cycles evolved twice independently in *Rhipicephalus* and are phylogenetically conserved among subgenera *Boophilus* (one-host) and *Digineus* (two-host) (Barker and Murrell, 2002). Three-host life cycles are considered the ancestral state in *Rhipicephalus* ticks, and truncation of host-use in life cycles is considered evolutionarily derived (Barker and Murrell, 2002, chapter 2). Moreover, one-host life cycles in subgenus *Boophilus* may result from neotenic selection to retain small body size to avoid predation/grooming given that adults are generally smaller than most other *Rhipicephalus* adults (Arthur, 1960; Horak et al., 2018).

The aim of this study is to characterise species variation in *Rhipicephalus* basis capituli and determine whether evolutionary-developmental modification plays a role in evolution and morphogenesis. To do this, basis capituli shapes between life stages and species were studied in a geometric morphometric framework. Basis capituli form an integral part of the mouthparts in ticks, and house hypostomal musculature which originate under the scutum, pass through the ventral part of the basis capitulum, and connect underneath the base of the hypostome (Fig. 3.1). A cavity is present above the hypostome and musculature that extends posteriorly from the basis capitulum-hypostomal fusion (Balashov, 1972; Coons and Alberti, 1999). This cavity is used to generate hydrostatic pressure that drives protraction of chelicerae into host skin (Richter et al., 2013). Subsequently, muscles beneath the hypostome are used to retract chelicerae, driving the hypostome deeper into host skin in order to take a bloodmeal (Richter et al., 2013). The dorsal shape of the basis capitulum represents an approximate measure for the two-dimensional shape of the hydrostatic cavity (Fig. 3.1). As such, dorsal

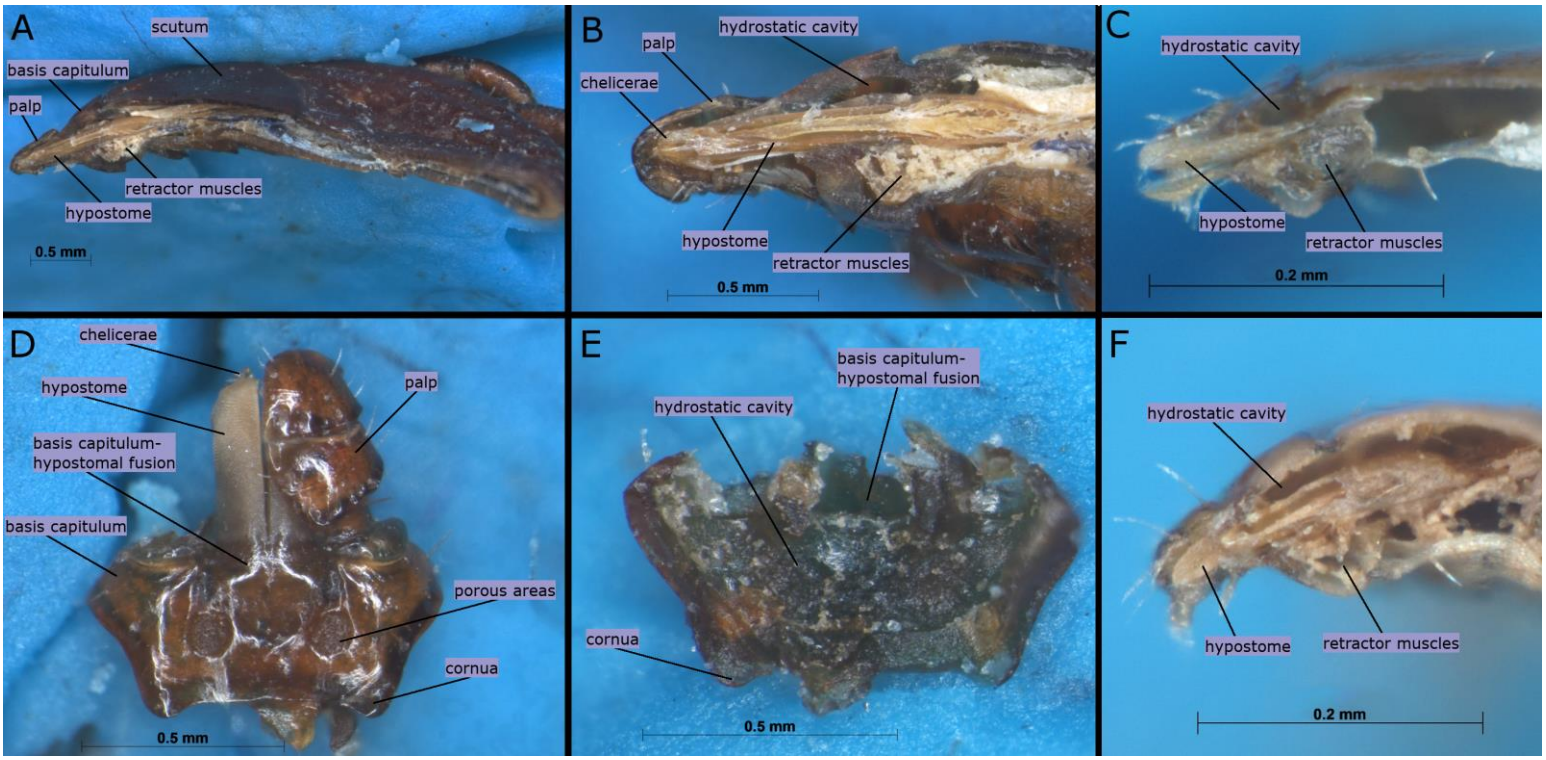

Figure 3.1. Photographs of *R. simus*: female body lateral cross-section (A), female capitulum cross-section (B), larval capitulum cross-section (C), female capitulum dorsal (D), female capitulum internal (E), and *R. microplus*: larval capitulum cross-section (F).

shape outlines of basis capituli were photographed and landmarked as a measure that approximates the shape of the hydrostatic cavities among *Rhipicephalus* species.

Morphological shape development is easily studied in ticks because they pass through three active life stages defined by moults where development between moults is limited (episodic growth), and each stage is distinct morphologically. As such, moulting facilitates clear boundaries of developmental time (age) among samples (Mcnamara and Mckinney, 2005). Developmental time is an important factor in studies of development, however chronological time does not always equal developmental time (Alberch and Blanco, 1996; Godfrey and Sutherland, 1995; Reiss, 1989). Size is a better measure of developmental time, but must be coupled with the appearance of a feature to approximate developmental time effectively (Alberch and Blanco, 1996; Klingenberg, 1998).

It is necessary to devise a tripartite hypothesis test to determine whether morphological evolution of *Rhipicephalus* basis capituli proceeded by neoteny (paedomorphosis), acceleration (peramorphosis), or no evolutionary-developmental modification where ancestral development is conserved (null hypothesis). These three categories can be defined and tested in terms of distances in shape between immatures and adults of contemporary species and ancestral estimates in various combinations. In the case of neoteny, signal for paedomorphosis can be detected when shape distances are smallest between ancestral early

stages and contemporary late stages for species in a given clade. For acceleration, signal for peramorphosis is present when distances are smallest between ancestral late stages and contemporary early stages. Finally, signal for developmental conservation (null hypothesis) can be detected when shape distances are smallest between same-stage comparisons involving contemporary and ancestral estimates. Immature-adult shape distances are based on estimates of ancestral shape and actual measurements of contemporary species. Estimates of ancestral shape are calculated by square-change parsimony at the root of a given clade, and are reliable provided there is no unidirectional selection pressure exerted on the clade that limits variation (Klingenberg and Gidaszewski, 2010). Null hypothesis comparisons that test for developmental conservation are expected to dominate signal for evolutionary-developmental modification by having the smallest set of shape distances. This is due to the effect of ancestral developmental constraints that maintain the viability of developmental pathways which limits developmental variation to approximate a set of ancestral pathways which produce similar phenotypes at similar life stages (Mayr, 1994). The tripartite test determines which of the two sets of shape distances for either paedo- or peramorphosis, is the significantly smaller set with reference to no evolutionary-developmental modification. The smallest set of distances is taken as the prevalent mode of change in evolutionary-developmental modification. The prevalent mode of change is expected to be comparable to developmental conservation, but cannot be smaller due to the effects of developmental constraints.

Methods

Samples, photographs and landmarking

Tick samples were compiled from collections housed at the Gertrud Theiler Tick Museum (GTTM), Agricultural Research Council - Onderstepoort Veterinary Research, South Africa (Table S3.1: supplementary information). Specimens in this museum have been identified over the last 100 years by Gerald Bedford, Gertrud Theiler, Jane Walker, Heloise Heyne and Ivan Horak. Three replicate photographs were taken of basis capituli for up to five individuals for each life stage and sex representing 24 *Rhipicephalus* species (total photographs = 1440 from 480 specimens). The majority of species have three-host life cycles, but three and four species have one-host and two-host life cycles respectively (*R. evertsi mimeticus* is treated as a distinct species here). Specimens were positioned on a rotational mount (Bakkes, 2017; Chapter 6), photographed using a Zeiss AxioCam MRc 5 camera, and stacked in Zeiss Axiovision v4.8. Each stacked image consisted of between 10 and 20 photographs. Outlines of basis capituli were digitized using COO v41 in the CLIC package by Jean-Pierre Dujardin (available at http://mome-clic.com/the-clic-package/) according to 20

landmarks (Fig. S3.1), and were scaled to a 0.2 mm scale bar. Operational definitions for each landmark are available in Fig. S3.1. Replicate photographs were digitized in batches by replicate to avoid digitization bias from operator memory. Landmarks were transformed in a Procrustes fit in MorphoJ v1.06d (Klingenberg, 2011) and a covariance matrix was generated for symmetric components previously shown as adequate to detect species disparity (Bakkes, 2017; Chapter 6). A Procrustes ANOVA was done to measure variability in individuals with replicate as the error effect, species as the main effect and stage/sex as the secondary effect. This was done to confirm whether species replicates could be averaged.

Data analysis

For the first set of analyses, observations were divided into separate datasets by stage/sex and averaged by individual. Regression scores for developmental trajectories were calculated using data averaged by a species-stage classifier, and then used in a regression of shape on size in MorphoJ. The regression score variable calculated in MorphoJ represents the shape variable most closely correlated with size (Drake and Klingenberg, 2010). This variable is adequate for modelling developmental trajectories in ticks because their size increases significantly with each moult, to accurately mark developmental time, and as such this variable represents growth in terms of change in shape/size. The resulting MorphoJ regression scores were grouped by life stage and used to estimate linear models of development for both male and female lines along overall and early/late component trajectories using Phylogenetic Generalised Least Squares regressions (PGLS) in the package caper (Orme, 2018) in R v3.6.1 (R Core Team, 2020) and RStudio v1.2.5001 (RStudio Team, 2020). Four models were estimated for different host-use strategies: one-host, two-host, three-host using small hosts at early life stages, and three-host using large hosts at early life stages. These models were compared using ANOVA for host-use effect. Phylogenetic relationships for the 24 species studied, were taken from Chapter 2, and used to plot phylomorphospaces for each stage/sex onto the first two principal components (PC) of shape, and centroid size (centre of gravity among landmarks which approximates feature size) using MorphoJ. These analyses included tests of phylogenetic signal based on 1000 random permutations of the data against the phylogeny. Phylogenetic Generalized Least Squares (PGLS) regressions were performed using centroid size and PC1 for each stage and sex, with host size as the predictor variable in the package caper in R and RStudio. This was done to determine the relationship between basis capituli morphology and host size. Data for host size as adult body mass (g) were obtained from the PanTHERIA dataset (Jones et al., 2009), and compiled per tick species (Table S3.2) taking the three most common wild/primary hosts for each tick species as adults and immatures (Horak et al., 2018; Walker, 2014; Walker et al.,

2000). Lastly, centroid sizes for each stage/sex were grouped by number of hosts used in life cycles and compared using ANOVA for host-use effect, Tukey HSD comparisons and boxplots in R and RStudio.

In the second set of analyses, data were subdivided into six datasetss to represent clades following Chapter 2 as well as the entire genus. Two of these represent clades that correspond to host-truncate life cycles. This was done to reduce noise from combining shape data for species nested in different clades during principal component analysis, so as to ensure principal components were relevant for each of the five subclades. Landmark data in each dataset were averaged by individual and a regression of shape on centroid size was performed, taking the regression residuals for further analysis to exclude correlates of size variation (size correction). Covariance matrices were calculated from regression residuals, and these were used in principal component analysis of shape for each of the five clade datasets. This was done to ensure principal components for separate stages/sex were comparable, after which PC scores were subdivided by stage/sex and averaged by a species-stage/sex classifier. The first two PC scores were used to plot phylomorphospace for each clade and infer estimates of the root ancestral shape using the package geomorph (Adams et al., 2020) in R and RStudio. Procrustes distances were calculated between all ancestral root shapes and contemporary shapes from phylomorphospace PC scores, and were grouped into the following hypotheses: paedomorphosis – ancestral root shape of early stages and contemporary shape of late stages; peramorphosis – ancestral root shape of late stages and contemporary shape of early stages; null hypothesis of evolutionary-developmental modification – ancestral root shape and contemporary shape of same-stages. These grouped distances were compared using ANOVA for evolutionary-developmental modification and Tukey HSD comparisons in R and RStudio.

Results

Procrustes ANOVA of shape indicate the effect of species was much larger than stage/sex ($df = 414$, $F = 34.15$, $p < 0.001$), which in turn was much larger than individual ($df = 1710$, $F = 56.52$, $p < 0.001$). Fluctuating asymmetry was marginally greater than replicate error ($df = 8802$, $F = 1.27$, $p < 0.001$), indicating replicates were approximately homogenous and could be confidently averaged by individual. The first two principal components of shape accounted for 64.70-87.01% of species disparity in each stage/sex (lowest to highest: males, females, nymphs, larvae). In larval shape, PC1 represented anterior convexity of basis capitulum-hypostomal fusion as well as sagittal length (anterior-posterior) of basis capitulum, while PC2 represented posterior development (Fig. 3.2). Similar features of sagittal length and anterior convexity were represented in PC1 and 2 in all other life stages, but these shape

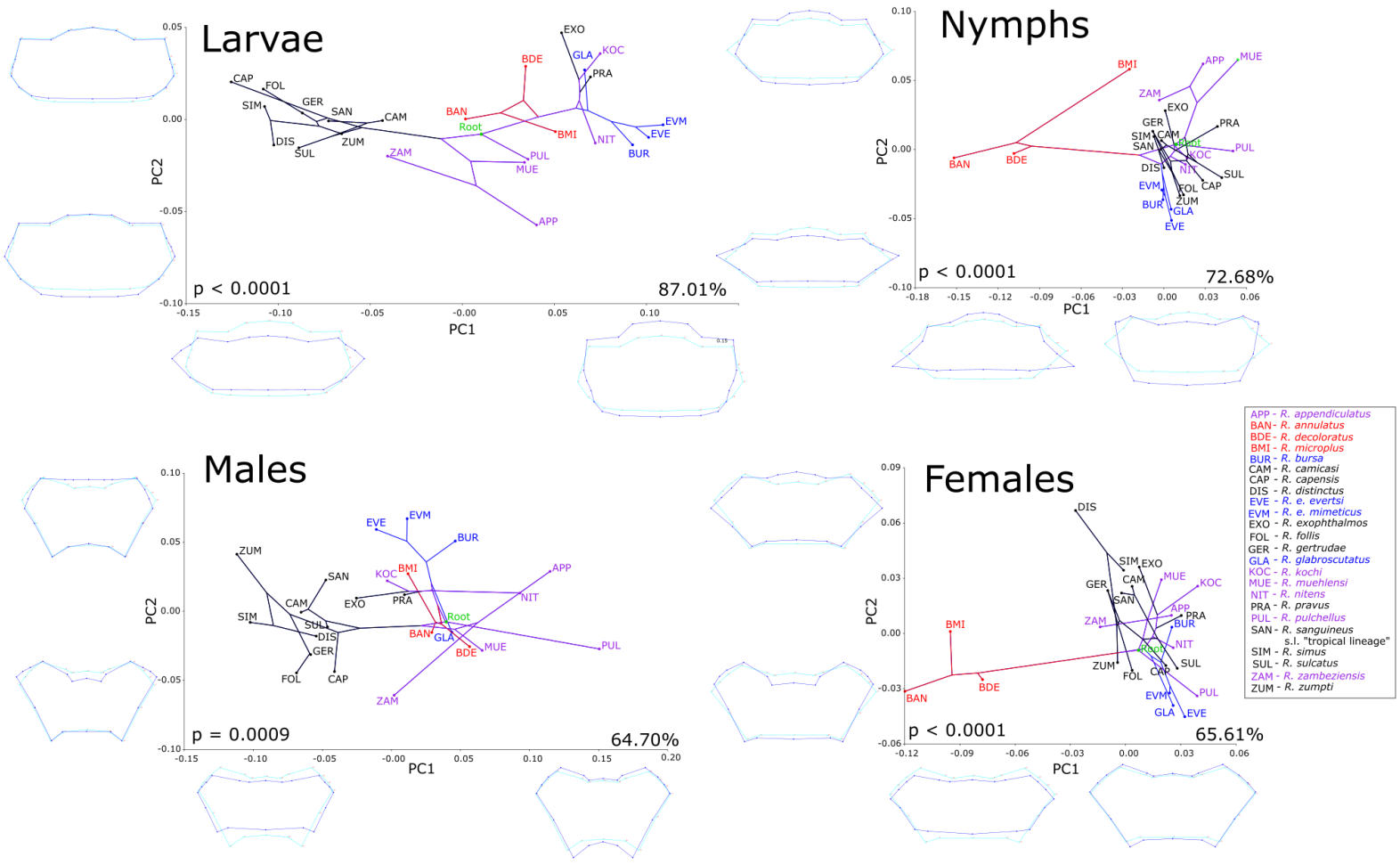

Figure 3.2. Principal component analysis of species averages mapped onto phylogeny (phylomorphospace) for each life stage/sex. Coloured taxa and branches represent host-use strategies (red: one-host, blue: two-host, black: three-host using small hosts at early stages, purple: three-host using large hosts at early life stages). Shape changes along PC (Principal Component) axes are to scale at minimum and maximum extents. Light blue traces represent the mean shape and dark blue traces represent deviation from the mean shape at the given extent. P-values indicate phylogenetic signal (1000 permutations) and percentages indicate PC1 and PC2 cumulative contribution to total variance.

changes were most pronounced in immature life stages (Fig. 3.2). Moreover, cornua are absent from adults of one-host species (Fig. 3.2). Host-use had a significant effect on overall and component developmental trajectories (at $\alpha = 0.05$), except in overall male development (Fig. 3.3, Table 3.1). For almost all PGLS linear models of development, adjusted R^2 values were between 0.5854–0.9904 with $p < 0.05$ (Table 3.1), indicating good fit. However, this is with exception of the component model for early development in three-host species that utilise small hosts at early life stages which had $R^2 = -0.0150$ and $p = 0.4162$, indicating high error associated with the linear model. Development of both male and female lines along overall trajectories indicate one-host species have the lowest slope (Fig. 3.3), followed by three-host species, and two-host species, with two-host species having the greatest overall slope (Fig. 3.3). Component trajectories indicate variation between early and late development (Fig. 3.3). Early developmental trajectories (larva-nymph) indicate three-host species with immatures that parasitise large hosts develop more rapidly compared to three-host species with immature

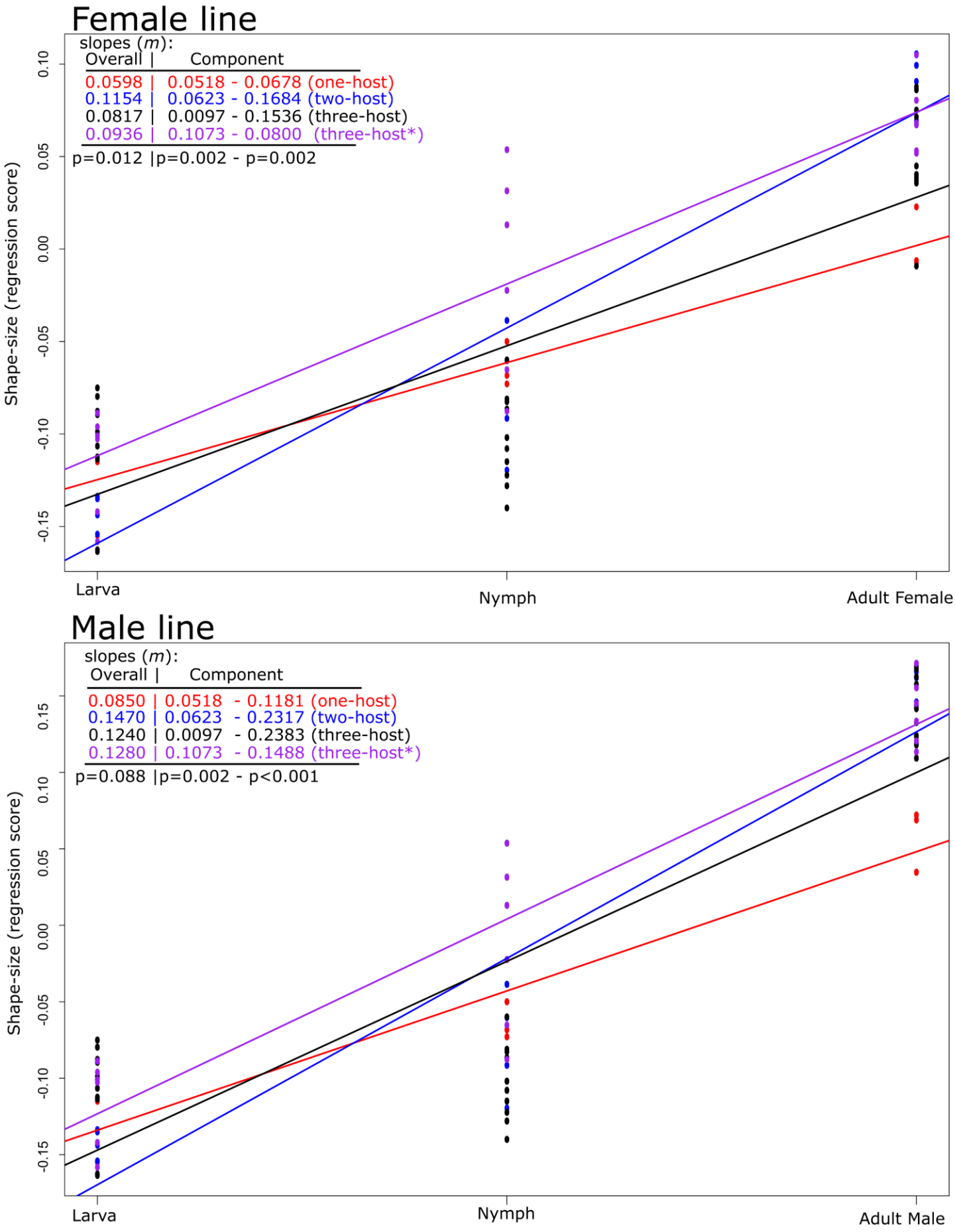

Figure 3.3. Developmental trajectories of *Rhipicephalus* species averages based on PGLS regressions of the shape variable most closely correlated with size (MorphoJ regression score), and life stage. Colours represent host-use strategies (red: one-host, blue: two-host, black: three-host using small hosts at early life stages, purple: three-host using large hosts at early life stages). Slopes of overall developmental trajectories are plotted. Slope values of overall as well as component early and late development are indicated. P-values indicate ANOVA for effect of host-use.

Table 3.1: Results from PGLS linear models of shape-size correlate (MorphoJ regression score) by life stage for male and female lines (Fig. 3.3). *denotes significance for PGLS regressions at α = 0.05. ANOVA values indicate effect of host-use on developmental trajectories.

	Overall (Female line)		Overall (Male line)		Larvae-Nymph		Nymph-Adult (Female line)		Nymph-Adult (Male line)	
	R^2	p-value	R^2	p-value	R^2	p-value	R^2	p-value	R^2	p-value
One-host	0.9093	< 0.0001*	0.8888	< 0.0001*	0.6632	0.0301*	0.9904	< 0.0001*	0.9780	0.0001*
Two-host	0.8836	< 0.0001*	0.8671	< 0.0001*	0.5854	0.0164*	0.9080	0.0002*	0.9540	< 0.0001*
Three-host	0.6971	< 0.0001*	0.7370	< 0.0001*	-0.0150	0.4162	0.9210	< 0.0001*	0.9651	< 0.0001*
Three-host*	0.8440	< 0.0001*	0.8923	< 0.0001*	0.6079	0.0017*	0.6009	0.0019*	0.8254	< 0.0001*
ANOVA (host-use)	F=3.9654, p=0.012		F=2.2737, p=0.088		F=5.8392, p=0.002		F=5.7933, p=0.002		F=10.462, p<0.001	

stages that parasitise small hosts (Fig. 3.3). Early development in one-host and two-host species are intermediate between these (Fig. 3.3). Two-host and three-host species that use small hosts at early life stages undergo accelerated development between later life stages, while one-host and three-host species that use large hosts at early life stages undergo reduced developmental changes. One-host species have similar rates of shape change over development Males deviate the most in late shape development due to sexual dimorphism, and this indicates female basis capituli shapes are generally nested within immature stage variation as a result of retention of distinct lateral extensions (Fig. 3.2).

Phylogenetic signal for shape (PC1, PC2) and centroid size was significant in all life stages/sexes, with notably strong support ($p < 0.012$) in centroid size for immature stages (Table 3.2, Fig. 3.4). PGLS regressions of species centroid size on host size for each stage/sex indicate a weak but significant correlation at $\alpha = 0.10$ in all life stages (Table 3.2). PGLS regressions of species PC1 (shape) on host size for each stage/sex indicate a weak but significant correlation in larval (Fig. 3.4) and nymphal stages, but no correlation in adult stages (Table 3.2). Host-use had a significant effect on centroid size for all life stages (all p-values < 0.001). Centroid sizes of one- and two-host immatures were significantly larger at $\alpha = 0.10$, and less variable, than three-host immatures (Fig. 3.5). One-host adults were significantly smaller, and less variable, than two- and three-host adults (Fig. 3.5). Tests for evolutionary-developmental modification indicate ancestral early stages and contemporary later stages significantly resemble one another in clades with host-truncate species, at $\alpha = 0.10$ (Figs. 3.6A, 3.7). In the entire genus dataset, distances to indicate signal for paedomorphosis was significantly smaller than those for both null and peramorphosis, with distances for peramorphosis the greatest (Fig. 3.7). In cladal datasets with three-host species, same-stage ancestors and contemporary adults (null hypothesis) most closely resemble one another and exclude both paedo- and peramorphosis, with exception of the sanguineus group where no significant differences between hypotheses were recovered (Fig. 3.7). Phylomorphospaces for each stage/sex in subgenus *Digineus* and simus+follis+capensis gr. are plotted along common principal component axes in Fig. 3.6 to display examples of paedomorphosis and developmental conservation (null hypothesis) in shape space.

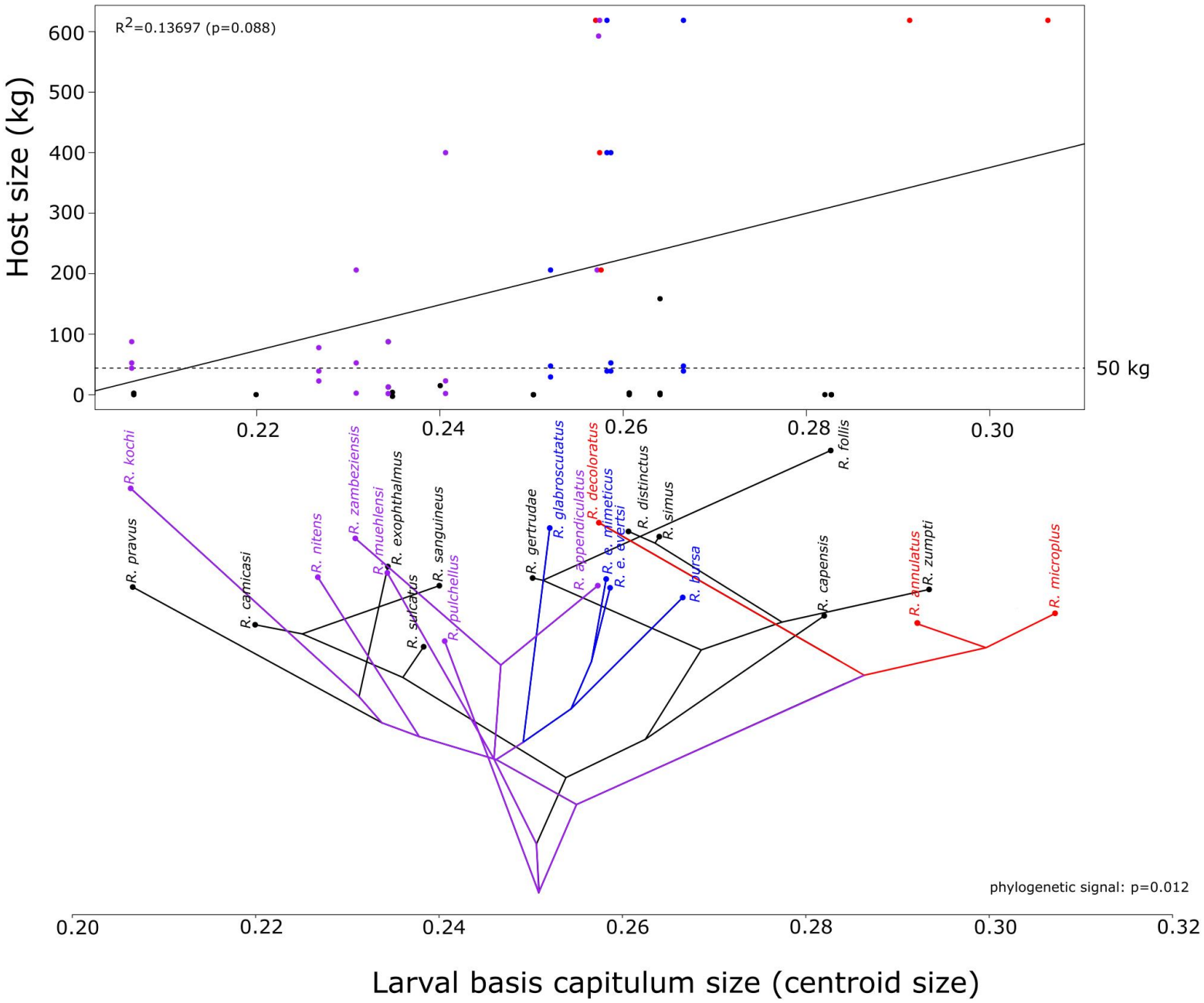

Figure 3.4. Centroid size for *Rhipicephalus* larval basis capituli (species averages) mapped onto phylogeny (below), and PGLS regression of larval centroid size (species averages) on host body mass (kg) for three primary hosts per species. Coloured taxa and branches represent host-use strategies (red: one-host, blue: two-host, black: three-host using small hosts at early stages, purple: three-host using large hosts at early life stages).

Table 3.2: Results from permutation tests for phylogenetic signal in size and shape (PC1-2) (left), as well as PGLS regressions of size and shape (PC1) on host size, for each life stage/sex (centre, right). *denotes PGLS regression significance at α = 0.10. Larval centroid size regression and phylogenetic signal represented in Fig. 3.4.

	Phylogenetic Signal (p-value)		Regression: Centroid size on host size		Regression: Shape (PC1) on host size	
	Centroid size	Shape (PC1-2)	R^2	p-value	R^2	p-value
Larvae	0.0120	< 0.0001	0.13697	0.088*	0.19260	0.035*
Nymphs	0.0081	< 0.0001	0.14424	0.081*	0.23333	0.018*
Females	0.0507	< 0.0001	0.23613	0.021*	0.01090	0.632
Males	0.0500	0.0009	0.25647	0.012*	0.02402	0.451

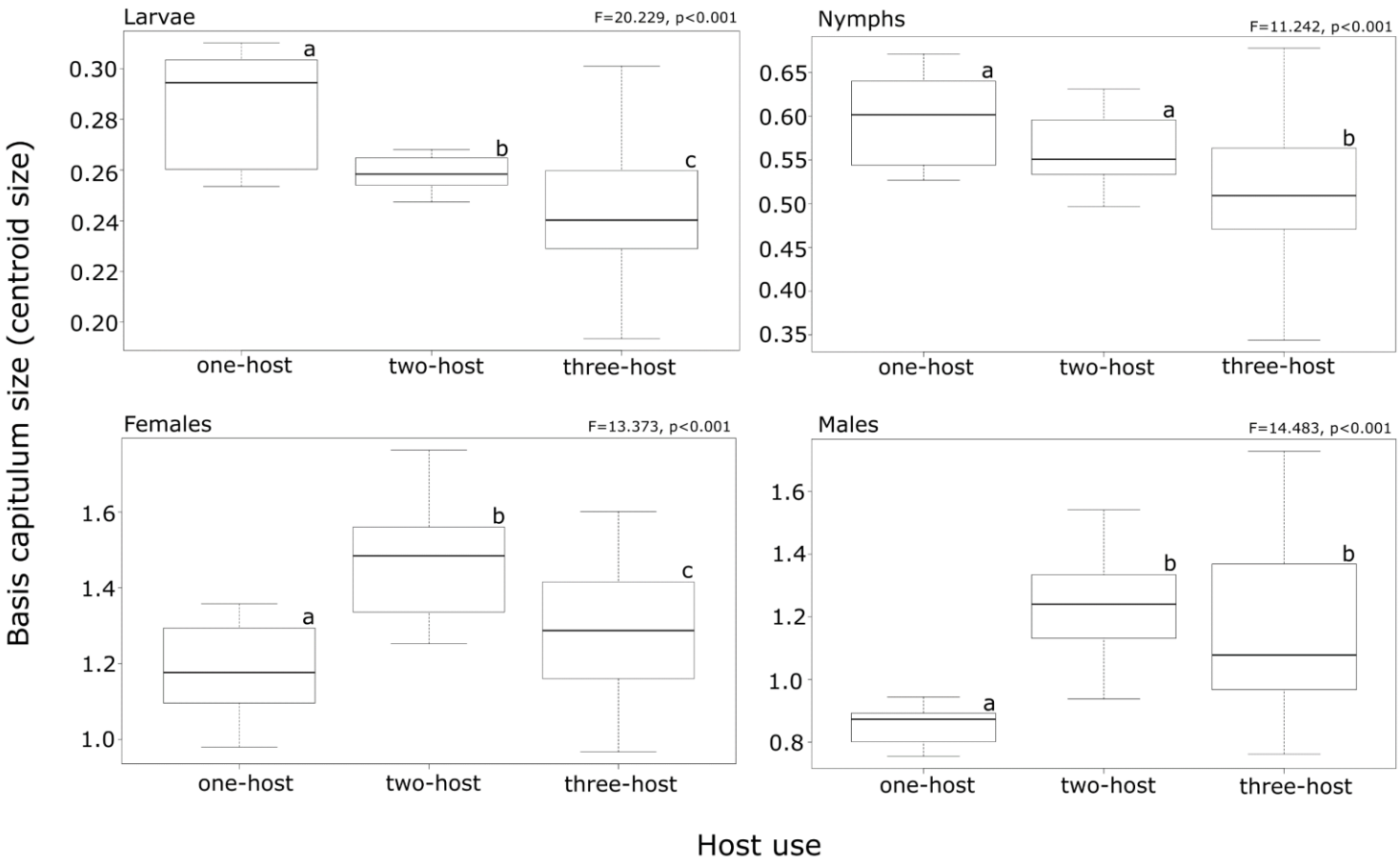

Figure 3.5. Box plots for centroid size among per life stage/sex of *Rhipicephalus* basis capituli grouped by host-use. Results of ANOVA for host-use (F and p-values) are indicated. Significance levels from Tukey HSD tests between centroid sizes among host-use indicated by 'a', 'b' or 'c'.

Discussion

Selection and Evolution

Shape data indicate that basis capituli increase in overall size throughout development as expected (Figs. 3.3, 3.5: y-axes) with the addition of cornua and lateral extensions (Fig. 3.2). Anterior convexities of the basis capitulum-hypostomal fusion are distinct at early life stages, but recede during development, suggesting they are no longer as important in adults as in immatures (Fig. 3.2). Basis capituli house structures that are used for the cutting-boring action of the chelicerae and hypostome into host skin when taking a bloodmeal (Richter et al., 2013). Skin thickness presents a challenge for ticks taking a blood meal by way of stiffness and density, probably involving collagen or keratin crosslinking in the extracellular matrix of epidermal cells that resist the cutting-boring actions of the hypostome (Jonsson et al., 2014; Maiorano et al., 2016; Piper et al., 2008; Wang et al., 2007). Larger animals tend to have greater skin stiffness as a function of greater skin thickness (Wei et al., 2017), suggesting large hosts provide a greater challenge for tick attachment than small hosts. Host-truncate species have greater sagittal length (anterior-posterior) and distinct anterior convexities as

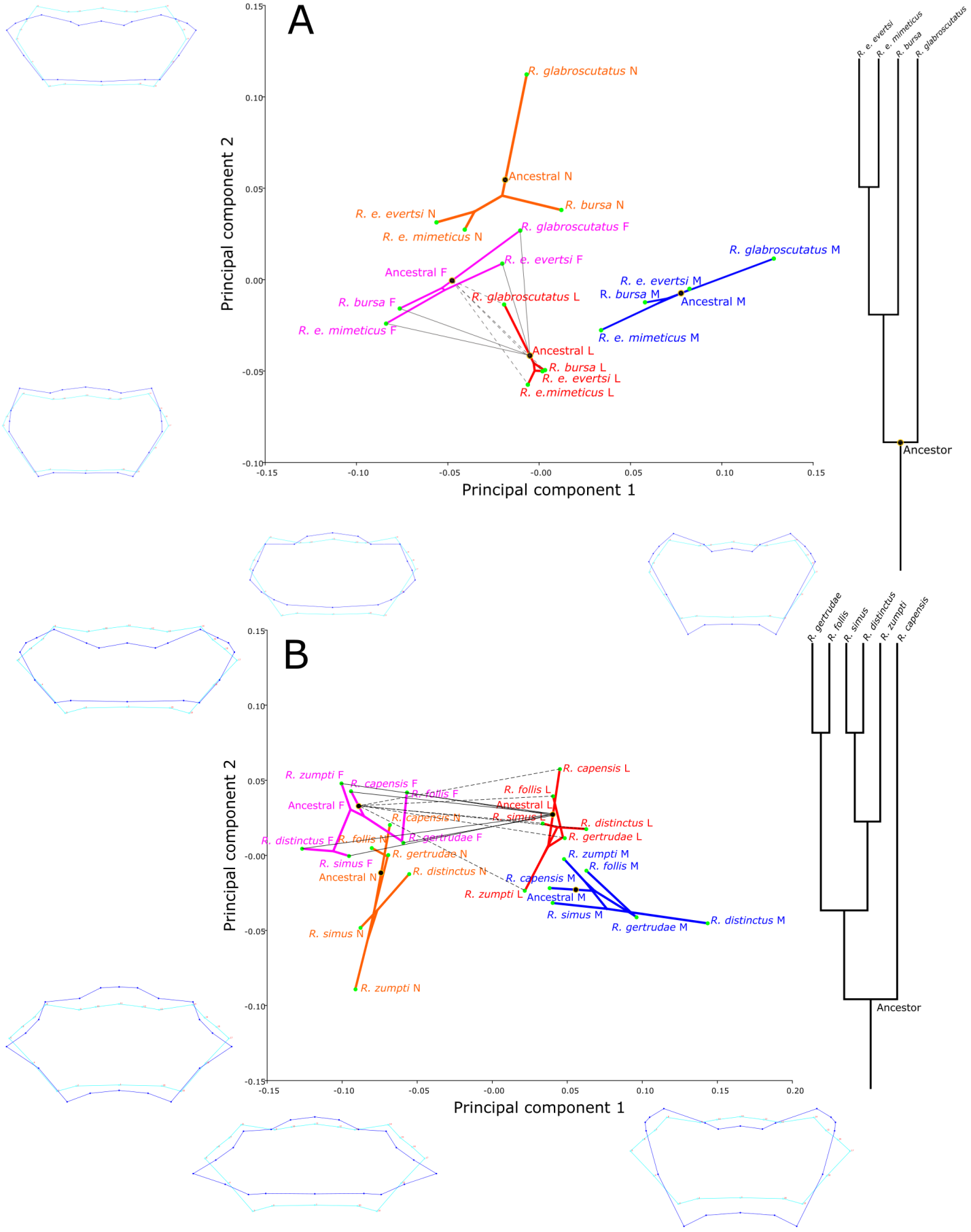

Figure 3.6. Principal component analysis of subgenus *Digineus* (A) and simus+follis+capensis gr. (B) mapped onto phylogeny for each life stage/sex as exemplars for the method underlying tests of evolutionary-developmental modification. Signal is present for paedomorphosis (A) or no evolutionary-developmental modification (B). Solid black lines represent Procrustes distances for the paedomorphosis hypothesis, and dotted black lines represent Procrustes distances for the peramorphosis hypothesis. Colours represent life stages (red: larvae, orange: nymph, purple: adult female, blue: adult male). Shape changes along PC (Principal Component) axes are to scale at minimum and maximum extents. Light blue traces represent the mean shape and dark blue traces represent deviation from the mean shape at the given extent.

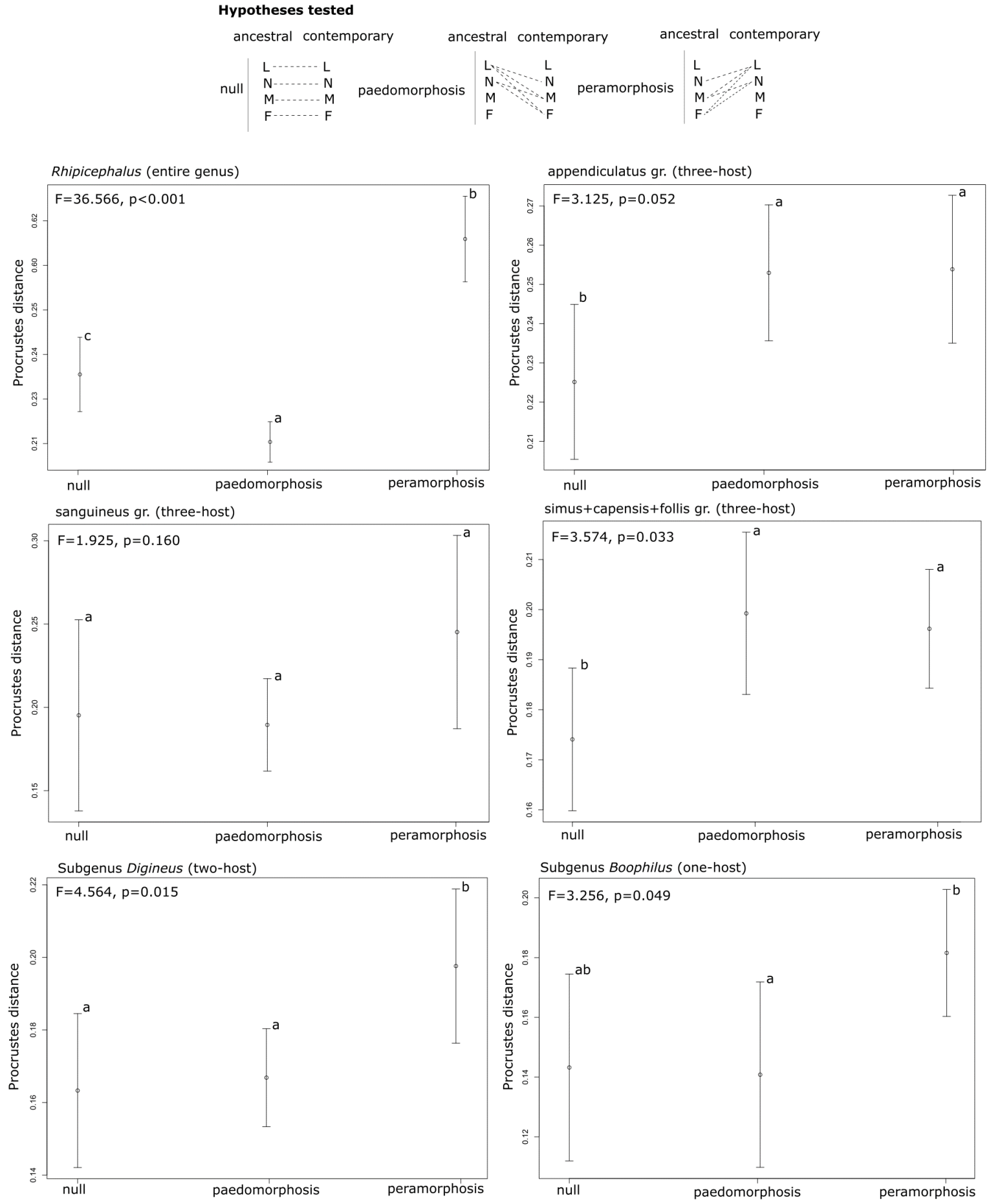

Figure 3.7. Tests of evolutionary-developmental modification for paedomorphosis, peramorphosis and no evolutionary-developmental modification (null) between life stages. Smaller Procrustes distances represent higher signal for a given hypothesis, based on Procrustes distances in principal component scores between contemporary taxa and estimated ancestral shapes (for examples, see Fig. 3.6). Values of ANOVA for evolutionary-developmental hypothesis (F and p-values) are indicated. Bars represent 95% confidence intervals. Significance levels from Tukey HSD tests between hypotheses indicated by 'a', 'b' and 'c'.

immatures (Fig. 3.2), which also contribute to increased centroid size of basis capituli (Fig. 3.5). These species use large hosts with greater than 50 kg adult body mass at early life stages, which may suggest an influence of host skin thickness/stiffness using host size as a proxy (Fig. 3.4). Conversely, sagittal length and anterior convexities are reduced in most immatures of three-host species (Fig. 3.2). These species use small hosts at early life stages (Horak et al., 2018; Walker et al., 2000), suggesting they are less suited for boring into stiff skin of large animals (Wei et al., 2017). Exceptions exist however, where some three-host species have greater sagittal length and distinct anterior convexities at early life stages (Figs 3.2, 3.4, 3.5), but these species also use large hosts as immatures (Horak et al., 2018; Walker et al., 2000). Increased volume of the hydrostatic cavity is afforded by greater sagittal length and distinct anterior convexities that enlarge basis capituli (Fig. 3.1F). These greater volumes contain more fluid that may facilitate increased hydrostatic pressure applied to the hypostome and chelicerae to provide greater force for protraction.

Significant correlations were recovered between host size and basis capitulum shape at early life stages, but not among adults (Fig. 3.4, Table 3.2). This may suggest that sagittal length and anterior convexities present in immature basis capituli among host-truncate and some three-host species that use large hosts at early life stages, represent an adaptive response to bore into large hosts with stiff skin. This assertion is based on the mechanism whereupon hydrostatic cavities facilitate generation of protraction force by hydrostatic pressure (Richter et al., 2013). Hypothetically, enlargement of these cavities by means of anterior convexities and increased sagittal length will facilitate greater protraction force. As such, using large hosts at early life stages may initiate selection pressure among *Rhipicephalus* immatures where selection smay favour basis capituli shapes that facilitate attachment to large hosts with stiff skin. Correlations between host size and immature basis capituli were generally weak however (Table 3.2), which may indicate selection pressure is reduced by generalist host-use strategies among *Rhipicephalus* where individuals can move to find a more appropriate host or predilection site if they are unable to attach initially (Tahir et al., 2020). Alternatively, weak correlations may be due to using host size data as an approximation for skin thickness and stiffness at predilection sites. The lack of correlation with host size among adult basis capituli shapes (Table 3.2) may suggest sagittal length and anterior convexities are not as important for adults, and that adults may experience reduced selection pressure for basis capitulum morphology. Adult basis capituli are larger than those of immatures (Fig. 3.5: y-axes), and increase in size as a result of overall body size development. Hypothetically, overall body size development should enable sufficient hydrostatic cavity volumes without the need for specific shapes in order to produce adequate force for protraction of chelicerae into the stiff skin of large hosts. Indeed, almost all

Rhipicephalus adults use large hosts (Horak et al., 2018; Walker et al., 2000). This could indicate that a threshold of basis capitulum size and shape exists, which mediates protraction of chelicerae into stiff skin. Adults develop past this threshold and are able to attach to large hosts with little to no directional selection on basis capitulum phenotypes. Immatures require additional basis capitulum shape features however (greater sagittal length and anterior convexities), in order to increase basis capitulum size and concomitant hydrostatic cavity volume (Figs 3.1, 3.2). Moreover, basis capituli of one-host species adults are small relative to adults of other species (Fig. 3.5), which may indicate they are just above the putative threshold of basis capitulum size and shape required for attaching to the stiff skin of large hosts (Fig. 3.5).

Hosts are beneficial for ticks in that they buffer individuals from dehydrating in harsh off-host conditions that are especially detrimental to immatures due to their small body size which increases surface volume to area ratios (Hoogstraal, 1978; Leal et al., 2020; Yoder et al., 2006). However, this is counterbalanced by the time spent on hosts, which limits this advantage for one-host species. Large hosts can also withstand tick parasitism in greater abundances, whereas small hosts easily succumb to ex-sanguination when under high infestation rates of adult ticks (Jellison and Kohls, 1938). These factors exert selection pressure on ticks, whereby individuals able to parasitize large hosts at early life stages may mitigate the combined adverse effects of dehydration, predation/grooming and ex-sanguination. Additionally, subgenus *Boophilus* have significantly smaller adult body size compared with other *Rhipicephalus* species, which reduces on-host visibility and subsequent predation/grooming (Mooring and Hart, 1997; Petney and Kok, 1993; Snowball, 1956). This may function as an additional adaptation to avoid predation/grooming given their longer periods of host attachment. Alternatively, their smaller adult size may result from selection against incurring developmental costs associated with significant body shape changes during moulting due to reduced host changes and off-host periods. As such, all life stages would be subject to similar selection pressures. Taken together, selective pressures of dehydration, predation/grooming and ex-sanguination provide an evolutionary challenge to ticks, which has hypothetically been met by the response of using large hosts at early life stages and also by truncating host-use during life cycles. However, this response would have been accompanied by the additional challenge to attach to the stiff skin of large hosts at early life stages while only having smaller basis capituli (Figs 3.3-3.5). This would create an adaptive threshold for basis capitulum size and shape among immatures, which would mediate the niche of using large hosts at early life stages with the associated survival advantages. Nevertheless, using small hosts at early life stages is prevalent among other *Rhipicephalus* clades, and is not without survival advantages. Small hosts have higher activity patterns due to increased

metabolic rates, and this provides increased opportunity for tick attachment (Brunner and Ostfeld, 2008; Clarke et al., 2010; Kowalski et al., 2015). Moreover, small hosts are generally more abundant in the environment (Cotgreave, 1993). These factors are especially important for immatures given higher dehydration risk (Hoogstraal, 1978; Leal et al., 2020; Yoder et al., 2006). The thin skins of small hosts may allow ticks to bypass the selective pressure for attaching to stiff skin as for large hosts. This would allow many individuals to attach successfully, with reduced selection against basis capitulum phenotypes. Instead, selection is expected to favour reduced consumption of developmental resources, producing smaller and less complex phenotypes (DeWitt et al., 1998). Indeed, immatures of three-host species that use small hosts at early life stages have basis capituli with reduced sagittal length and anterior convexities (Figs. 3.2-3.5). As such, during *Rhipicephalus* diversification, using small hosts at early life stages offered another viable niche alongside using large hosts at early life stages. Furthermore, the niche of using large hosts at early life stages may have provided the starting point for the evolution of host-truncate life cycles via preadaptation of larval basis capituli.

Sites of attachment on hosts may also play a role. Tick attachment sites on host skin can vary in stiffness and thickness, and different life stages of the same species often attach at different sites if present on the same host (Horak et al., 2018; Walker et al., 2000). On cattle, larvae tend to attach to areas where skin is thin (Riek, 1962). This is an example of ontogenetic niche partitioning, and allows all stages to feed, while also minimizing intraspecific competition (Field et al., 2005; Werner and Gilliam, 1984; Zimmerman et al., 2009). However, exceptions to this do exist where all life stages feed at similar sites on their host, such as in one-host ticks *Rhipicephalus* subgenus *Boophilus* (Matthee et al., 1997), which may add further selection pressure for optimised phenotypes through competitive interactions (Bournez et al., 2015; Cangi et al., 2013; Sutherst, 1987). One example of ontogenetic niche partitioning is in two-host ticks, *R. e. eversti*, where all stages parasitize cattle, sheep and goats, with immatures preferring attachment sites with softer skin, such as between hooves and external ear canals, while adults prefer attachment in areas with thicker and stiffer skin such as perianal, and inner groin areas (Horak et al., 2018; Walker et al., 2000). In this case, selection to attain advantages of feeding on large hosts would still apply, but ontogenetic niche partitioning pertains to the number of different attachment sites, in place of number of different hosts used. As such, these species become adapted to certain predilection sites and associated skin traits, rather than to host species.

Gross skin characteristics such as thickness have previously been proposed as candidates for tick resistance, however thicker skin was shown as ineffective to reduce infestation of *R. microplus* and *R. decoloratus* in some studies on cattle (Riek, 1956; Spickett et al., 1989; Wagland, 1978). Given the present findings, this is likely because these ticks are

adapted to bore into thicker skin. Moreover, *R. microplus* have been demonstrated to only penetrate up to 50 µm into host skin (Moorhouse and Tatchell, 1966). This indicates the critical factor for tick attachment may be densities of crosslinked collagens and keratins in the outermost layer of epidermal skin which provide rigidity and stiffness, rather than skin thickness which provides depth. Developing tick resistance by means of skin characteristics will likely require enhancing skin stiffness beyond normal levels. Moreover, this would only preclude attachment of immature stages, but may reduce tick abundance if applied consistently in an area over time to break tick life cycles. Additionally, hair length may play a role where shorter hairs tend to preclude tick attachment in Nguni cattle (Marufu et al., 2011).

Characteristics of basis capitulum shape and size in all life stages correlate significantly with phylogeny (Table 3.2, Figs 3.2, 3.4). Notably, two clades of host-truncate species have basis capituli with the greatest sagittal length and distinct anterior convexities at immature life stages (Figs 3.2, 3.4). This indicates basis capitulum shapes of immatures among host-truncate species have a phylogenetic basis, and may suggest the functionality of boring into stiff skin is linked to their success in combination with host-truncation as a 'key innovation' (Gavrilets and Vose, 2005). Greater sagittal length and distinct anterior convexities at early life stages are present in basal species of *Rhipicephalus* (Chapter 2), suggesting these features represent the ancestral condition and not a synapomorphy of host-truncate species. Moreover, this phenotype was retained in derived groups that use large hosts at early life stages, some of which went on to evolve host-truncate life cycles. This may suggest these groups were preadapted to using large hosts at early life stages and that host-truncation may have been facilitated by selection for individuals with prolonged expression of juvenile features (neoteny). The remaining groups retained three-host life cycles, but some of these adapted to using small hosts at early life stages (Chapter 2), and consequently lost putative basis capitulum adaptations for boring into stiff skin (Figs. 3.2, 3.4). This may have resulted from the absence of selection for boring into stiff skin, where reduced consumption of developmental resources are favoured instead (DeWitt et al., 1998). As such, reduction of these basis capitulum features and using small hosts at early life stages represents a derived condition that arose around the same time as host-truncate life cycles, but in a different *Rhipicephalus* clade. Three-host life cycles represent the ancestral condition for host-use in *Rhipicephalus* (Barker and Murrell, 2004, chapter 2), and the six species in appendiculatus and pulchellus groups that have basis capituli adapted for boring into stiff skin are phylogenetically closest to the origin of crown-*Rhipicephalus* (Chapter 2; Barker and Murrell, 2002; Coimbra-Dores et al., 2018). This suggests extant *Rhipicephalus* originated from progenitors that utilised large hosts at early life stages, which is consistent with the hypothesis for *Rhipicephalus* initial diversification on early Ungulata, about 52 Mya (Chapter 2). Moreover, putative extinction in

Rhipicephalus progenitors, possibly related to *Rhipicentor* (Chapter 2), would have eliminated transitionary forms that underwent the host switch in immature life stages from Afroinsectivora to early Ungulata (Chapter 2). If so, using small hosts at early life stages would have arisen subsequently, followed by host-truncate life cycles in other groups that continued to use large hosts at early life stages.

Evolutionary-Developmental Modification

Tests for evolutionary-developmental modification, based on ancestral shape estimates of basis capituli, indicate adults of host-truncate species more closely resemble ancestral immatures than ancestral adults (Figs 3.6A, 3.7). Moreover, these similarities are approximately equal in magnitude to shape distances expected at same life stages across phylogeny (same stage comparisons of ancestral and contemporary species). This is not the case for the three clades of three-host species however, where expected shape distances (between same stages) most closely resemble one another to the exclusion of both paedomorphosis and peramorphosis (appendiculatus group and simus + capensis + follis group), or with no significant differences compared with paedomorphosis and peramorphosis (sanguineus group) (Fig. 3.7). These results provide signal for paedomorphosis in host-truncate species, and suggest their developmental evolution proceeded by neotenic selection that favoured retention of immature phenotypes through development (Gould, 1977; Reilly et al., 1997). Larval features of basis capitulum morphology that were presumably adapted for attachment to large hosts ancestrally, were retained into nymphal and adult stages during the evolution of host-truncate species. Nymphs and adults that retained paedomorphic basis capitulum shapes through development would probably have gained a selective advantage that expanded their set of possible predilection sites to include areas of stiffer host skin. This would have given paedomorphic individuals a selective advantage, which eventually resulted in diversification of host-truncate species. Indeed, all three-host species lose anterior convexities as nymphs and adults (Fig. 3.2). However, basis capituli represent only one aspect of morphology under neotenic selection. The possibility remains that additional immature features are retained during development in species with host-truncate life cycles. Adults of subgenus *Boophilus* have additional paedomorphic features such as small size, pale colouration and circular spiracles (Abdel-Shafy et al., 2013; Arthur, 1960; Horak et al., 2018). These features may be paedomorphic as byproducts (‘spandrels’ *sensu* Gould and Lewontin, 1979) of neotenic evolutionary-developmental modification rather than linked to adaptation themselves. However, smaller adult size may provide an adaptive advantage to reduce visibility to predators while on hosts given *Boophilus* never leave their host until mating. An alternative hypothesis to consider for *Boophilus* neoteny overall, is that selection to undergo

costly developmental transitions may be reduced by the lack of host changes and off-host periods during life cycles, such that all life stages are subject to similar selection pressures. Nevertheless, neoteny explain the highly divergent morphology of *Boophilus* that is phylogenetically among *Rhipicephalus* based on nucleotide sequence data (Murrell et al., 2000; Murrell and Barker, 2003, Chapter 2)..

One-host species start post-egg development with sagittally large basis capituli that also have distinct anterior convexities (Fig. 3.2), but undergo slowed development during all moults (Fig. 3.3), making development neotenic (Klingenberg, 1998). Moreover, adults have small basis capituli relative to other species and cornua typical of *Rhipicephalus* adults never develop, making their basis capituli resemble those of immature life stages (Figs. 3.2, 3.5). As such, these species have similar trajectories between early and late development (Fig. 3.3). In contrast, two- and three-host species have distinct trajectories between early and late development (Fig. 3.3). Generally, accelerated development occurs before the first off-host/questing period for a given life cycle (Fig. 3.3). Basis capitulum development is an approximation for overall development of body size, given that tick growth proceeds by moults which make stage correlated with size (Mcnamara and Mckinney, 2005). As such, accelerated development of basis capituli reflect accelerated development in overall body size. Small body size increases dehydration risk for ticks in off-host environments (Hoogstraal, 1978; Leal et al., 2020; Yoder et al., 2006). Consequently, selection may favour rapid development to produce larger individuals that can better retain water balance in the stage that is first subjected to off-host/questing conditions. However, this is not the case for three-host species that use small hosts at early life stages. Instead, development before the nymphal stage is slow and accelerates later (Fig. 3.3 – despite divergent trajectories: $R^2 = -0.0304$, $p = 0.548$). In these ticks, dehydration risk may be instead mitigated by increased opportunities for tick attachment provided by small hosts with high activity and abundance (Brunner and Ostfeld, 2008; Clarke et al., 2010; Cotgreave, 1993; Kowalski et al., 2015). Alternatively, this may also be due to three-host immatures questing in humid microclimates closer to the ground given their host preference. Nevertheless, selection for phenotypes that can tolerate dehydration only comes into effect during the adult off-host/questing phase, where preceding development is accelerated (Fig. 3.3). A third possibility considers small size in three-host nymphs as important to facilitate higher on-host abundance, given competition for fewer attachment sites on small hosts due to smaller host body size. This may place additional selection pressure on three-host species that use small hosts at early life stages to favour slowed development producing small body sizes. Three-host species that use large hosts at early life stages are phylogenetically closest to basal among *Rhipicephalus* (Chapter 2), and best represent the ancestral trajectory of development for *Rhipicephalus*. As such, developmental trajectories of

three-host species that use small hosts at early stages are post-displaced (Klingenberg, 1998). In one-host species, no off-host period is present which entirely negates dehydration risk. However, longer on-host periods increase risk of predation/grooming for large individuals, and this may result in selection to retain immature features such as small size into late development. Following this, greater sagittal length and anterior convexities that enlarge basis capituli in immatures, are especially important to maintain the ability to attach to large hosts with stiff skin throughout development. Size and shape disparity between species vary the most in all life stages of three-host species (Figs 3.3, 3.5), and linear models were unable to adequately represent developmental trajectories of three-host species that use small hosts at early life stages (R^2 = -0.0304, p = 0.548). However, developmental trajectories for three-host species that use large hosts at early life stages were adequately modelled (R^2 = 0.5769, p = 0.0025). This may suggest that divergent developmental trajectories are present among three-host species that use small hosts at early life stages, possibly due to the wide range of phylogenetic diversity (three major clades) that likewise use a wide range of hosts, including small mammals and carnivores (Chapter 2).

Conclusions

Ticks face a complex set of evolutionary problems that are defined by taking bloodmeals while avoiding dehydration in off-host environments, and avoiding predation/grooming and ex-sanguination in on-host environments. Early life stages are the most sensitive to off-host conditions, and *Rhipicephalus* species have evolved to use either small or large hosts at early life stages. Small hosts are highly active and abundant and consequently mitigate dehydration risk, but are more susceptible to ex-sanguination, and increase visibility of attached ticks which likewise increase predation/grooming risk. Large hosts mitigate these effects, but tick immatures must be able to attach to the stiffer skin of large hosts and are limited by small basis capituli with small hydrostatic cavities, that generate limited chelicerae protraction force. Selection that favours sagittally enlarged basis capituli with distinct anterior convexities increases the volume of the internal hydrostatic cavity in tick immatures that utilise large hosts. These features recede less during development of species that use large hosts at early life stages (host-truncate and some three-host species). This may enable certain clades of *Rhipicephalus* to apply additional force for protraction of chelicerae into thick and stiff host skin as immatures, and mediates the niche of using large hosts at early life stages. These basis capitulum features of increased sagittal length and distinct anterior convexities represent the ancestral condition among *Rhipicephalus*, while reduction of these features at early life stages is a derived condition, and associated with using small hosts with thin skin at early life stages.

Host truncation of life cycles in *Rhipicephalus* ticks is proposed as an evolutionary adaptive response to selective pressures and survival advantages associated with using large hosts at early stages and for longer periods of time (across life stages). Hypothetically, this response is mediated by an adaptive threshold in basis capitulum size and shape to attach to stiff skin. Moreover, response to these selection pressures in host-truncate species includes neotenic selection to retain features of immature morphology during tick life cycles, such as basis capitulum morphology and small body size. Neotenic selection is especially pronounced in subgenus *Boophilus* where additional features display paedomorphosis, such as pale colouration and circular spiracles. However, an alternative hypothesis for neotenic selection is that selection to incur costs associated with developmental transitions may be reduced by reduced host changes and off-host periods. Off-host periods may modulate development in two- and three-host species, that instead favour accelerated growth to produce larger body sizes better able to avoid dehydration during the first off-host/questing period for a given life cycle (adults in two-host species, nymphs in three-host species that use large hosts at early life stages). In contrast, three-host species that use small hosts at early life stages undergo acceleration during late development, instead of during early development as expected due to nymphal stages being exposed to off-host environments. In these species, dehydration risk may be instead mitigated by highly active and abundant hosts that increase opportunities for attachment. Moreover, small size may also be selected for in early life stages to facilitate higher on-host abundance on small hosts. This would produce small phenotypes that incur less developmental costs. Furthermore, phylogenetic placements indicate the ancestral developmental trajectory is approximated by three-host species that use large hosts at early life stages. As such, early development of three-host species that use small hosts at early life stages is post-displaced.

A main factor which produces morphological variation in the evolution of developmental systems is chance mutations in genes and developmental regulatory networks. This forms the set of developmental phenotypes from which drift may fix certain phenotypes by chance or selection pressures may favour successful phenotypes. In the case of *Rhipicephalus*, variations in developmental phenotypes must meet the combined requirements of different sets of selection pressure at different life stages in on- and off-host environments. These developmental phenotypes go on to contribute to future progeny in their respective environments, and increase the fitness of these developmental phenotypes. This has produced paedomorphic adult phenotypes in subgenus *Boophilus*, as well as composite developmental phenotypes in two- and three-host *Rhipicephalus* species that have early or late development either slowed or accelerated. Taken together, these findings suggest tick evolution is influenced by a complex of trade-offs between tick size in off-host environments

(dehydration risk), and size in on-host environments (risk for predation/grooming, competition and ex-sanguination). These interact with host-use where basis capitulum morphology is constrained to be sagittally enlarged with distinct anterior convexities, when hosts or predilection sites with stiff skin are utilised – especially at early life stages when ticks are small. Moreover, the number of off-host periods modulate these trade-offs and introduce ticks to different sets of selection pressure. Additionally, these trade-offs interact with the effects of temperature variation gradients on differential niche partitioning, as well as immature and adult host-use on geneflow, dispersal and geographic ranges (Chapter 2).

These findings provide clarity for the suggestion that cattle with thicker skin should be less prone to tick infestation, at least insofar as tick immatures are concerned. Previous investigation into tick resistance have shown no effect of skin thickness (Riek, 1956; Spickett et al., 1989; Wagland, 1978). Given the present findings, this is true for some ticks, particularly those with host-truncate life cycles, because they are adapted for boring into the thick and stiff skin of large animals. This is important for agriculture as it indicates the value of further research to enhance skin thickness and stiffness in key attachment sites for ticks, either by means of genetic manipulation or selective breeding (Maiorano et al., 2016; Piper et al., 2008; Shyma et al., 2013; Wang et al., 2007). Reduction of tick attachment at immature life stages will also reduce the overall tick abundance in that area or farm, as progressively more immatures are unable to attach and proceed through their life cycles. Future research should study the association between immature tick attachment and skin thickness and stiffness in greater detail to determine the threshold that precludes tick attachment. In this way, the selective pressure applied to tick immatures from host skin traits, which drove the evolutionary adaptation of host truncation, can be turned to the advantage of agriculture.

SUPPLEMENTARY INFORMATION:

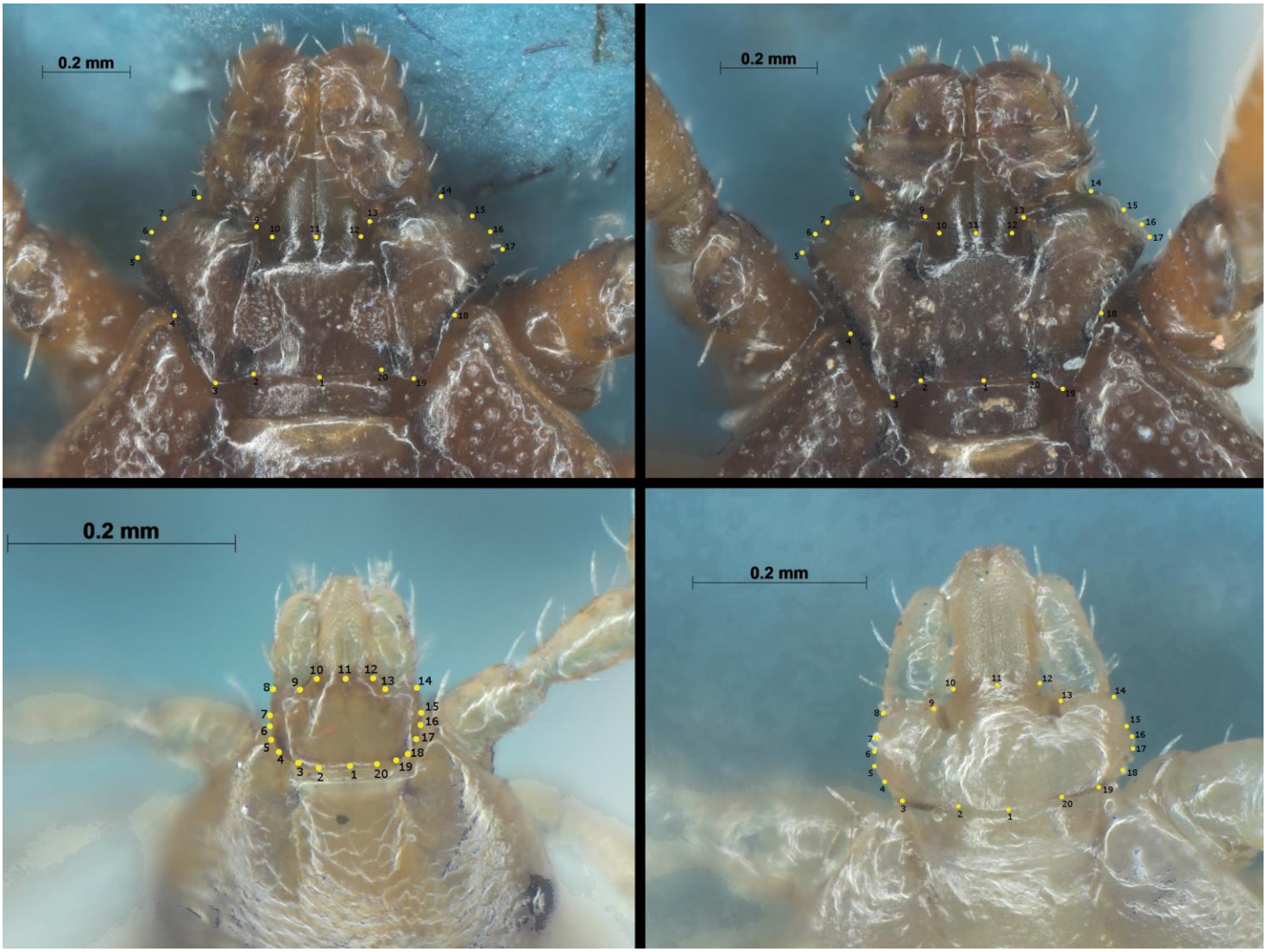

Figure S3.1. Landmarks used for basis capitulum shapes measured in this study. Exemplar specimens are *R. e. evertsi*.

Table S3.1: Accession numbers for GTTM specimens photographed in this study.

GTTM accession/individual/species/stage-sex

OP2496x__aBDEF	JBWCAPJONaCAPF	JBW3263A_aEVEF	OP2577iv_aPULL
OP2496x__aBDEL	JBWCAPJONaCAPL	JBW3263A_aEVEL	OP2577iv_bPULL
OP2496x__aBDEM	JBWCAPJONaCAPM	JBW3263A_aEVEM	OP2577iv_cPULL
OP2496x__aBDEN	JBWCAPJONaCAPN	JBW3263A_aEVEN	OP2577iv_dPULL
OP2496x__bBDEF	JBWCAPJONbCAPF	JBW3263A_bEVEF	OP2577iv_ePULL
OP2496x__bBDEL	JBWCAPJONbCAPL	JBW3263A_bEVEL	OP2577v_aPULN
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OP2496x__bBDEN	JBWCAPJONbCAPN	JBW3263A_bEVEN	OP2577v_cPULN
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OP2496x__cBDEL	JBWCAPJONcCAPL	JBW3263A_cEVEL	OP2577v_ePULN
OP2496x__cBDEM	JBWCAPJONcCAPM	JBW3263A_cEVEM	OP2577vi_aPULF
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OP2496x__dBDEN	JBWCAPJONdCAPN	JBW3263A_dEVEN	OP2577vi_cPULM
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OP2999i__bBANM	OP2796iiiaDISM	JBWFOL___dFOLL	JBWsang3_bSANM
OP2999i__bBANN	OP2796iiibDISF	JBWFOL___dFOLN	JBWsang3_bSANN
OP2999i__cBANF	OP2796iiibDISM	JBWFOL___eFOLL	JBWsang3_cSANF
OP2999i__cBANL	OP2796iiicDISF	JBWFOL___eFOLN	JBWsang3_cSANL
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OP2656iiieAPPL	JBWno3349cEVML	JBWkochi2_bKOCF	JBWL37___bSULL
OP2657i__aAPPF	JBWno3349cEVMN	JBWkochi2_bKOCL	JBWL37___bSULM
OP2657i__bAPPF	JBWno3349dEVML	JBWkochi2_bKOCM	JBWL37___cSULF
OP2657i__cAPPF	JBWno3349dEVMN	JBWkochi2_bKOCN	JBWL37___cSULL
OP2657i__dAPPF	JBWno3349eEVML	JBWkochi2_cKOCF	JBWL37___cSULM
OP2657i__eAPPF	JBWno3349eEVMN	JBWkochi2_cKOCL	JBWL37___dSULF
JBWKENYA_aAPPM	OP2470i__aEVMF	JBWkochi2_cKOCM	JBWL37___dSULL
JBWKENYA_bAPPM	OP2470i__bEVMF	JBWkochi2_cKOCN	JBWL37___dSULM
JBWKENYA_cAPPM	OP2470i__cEVMF	JBWkochi2_dKOCF	JBWL37___eSULF
JBWKENYA_dAPPM	OP2470i__dEVMF	JBWkochi2_dKOCL	JBWL37__bSULN
JBWKENYA_eAPPM	OP2470i__eEVMF	JBWkochi2_dKOCM	JBWL37__cSULN
OP6243iiiaAPPN	OP2470iiiaEVMM	JBWkochi2_dKOCN	JBWL37__dSULN
OP6243iiibAPPN	OP2470iiibEVMM	JBWkochi2_eKOCF	JBWL37__eSULN
OP6243iiicAPPN	OP2470iiicEVMM	JBWkochi2_eKOCL	JBWL37___eSULL
OP6243iiidAPPN	OP2470iiidEVMM	JBWkochi2_eKOCM	JBWL37___eSULM
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OP2049i__bBMIL	JBWEXOP__aEXON	JBWmuehl1bMUEL	JBWtsitsiaZUML
OP2049i__cBMIL	JBWEXOP__bEXOL	JBWmuehl1cMUEL	JBWtsitsiaZUMM
OP2049i__dBMIL	JBWEXOP__bEXON	JBWmuehl1dMUEL	JBWtsitsiaZUMN
OP2049i__eBMIL	JBWEXOP__cEXOL	JBWmuehl1eMUEL	JBWtsitsibZUMF
OP3063i__aBMIF	JBWEXOP__cEXON	JBWmuehl2aMUEN	JBWtsitsibZUML
OP3063i__aBMIM	JBWEXOP__dEXOL	JBWmuehl2bMUEN	JBWtsitsibZUMM
OP3063i__aBMIN	JBWEXOP__dEXON	JBWmuehl2cMUEN	JBWtsitsibZUMN
OP3063i__bBMIF	JBWEXOP__eEXOL	JBWmuehl2dMUEN	JBWtsitsicZUML
OP3063i__bBMIM	JBWEXOP__eEXON	JBWmuehl2eMUEN	JBWtsitsicZUMM
OP3063i__bBMIN	OP2749iv_aEXOF	OP3033v__aMUEF	JBWtsitsicZUMN
OP3063i__cBMIF	OP2749iv_aEXOM	OP3033v__aMUEM	JBWtsitsidZUML
OP3063i__cBMIM	OP2749iv_bEXOF	OP3033v__bMUEF	JBWtsitsidZUMM
OP3063i__cBMIN	OP2749iv_bEXOM	OP3033v__bMUEM	JBWtsitsidZUMN
OP3063i__dBMIF	OP2749iv_cEXOF	OP3033v__cMUEF	JBWtsitsieZUML
OP3063i__dBMIM	OP2749iv_cEXOM	OP3033v__cMUEM	JBWtsitsieZUMM
OP3063i__dBMIN	OP2749iv_dEXOF	OP3033v__dMUEF	JBWtsitsieZUMN
OP3063i__eBMIF	OP2749iv_dEXOM	OP3033v__dMUEM	TickbookaZUMF
OP3063i__eBMIM	OP2749iv_eEXOM	OP3033v__eMUEF	TickbookbZUMF
OP3063i__eBMIN	OP2749iv_eEXOF	OP3033v__eMUEM	TickbookcZUMF
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JBW3317__aBURN	FransDeonbGERL	JBWniten1bNITL	JBW3258a_bSIML
JBW3317__bBURL	FransDeoncGERL	JBWniten1cNITL	JBW3258a_cSIML
JBW3317__bBURN	FransDeondGERL	JBWniten1dNITL	JBW3258a_dSIML
JBW3317__cBURL	FransDeoneGERL	JBWniten1eNITL	JBW3258a_eSIML
JBW3317__cBURN	FransDeonaGERN	JBWniten2aNITN	JBWBushp_aSIMF
JBW3317__dBURL	FransDeonbGERN	JBWniten2bNITN	JBWBushp_aSIMM
JBW3317__dBURN	FransDeoncGERN	JBWniten2cNITN	JBWBushp_bSIMF
JBW3317__eBURL	FransDeondGERN	JBWniten2dNITN	JBWBushp_bSIMM
JBW3317__eBURN	FransDeoneGERN	JBWniten2eNITN	JBWBushp_cSIMM

OP2576v__bBURF	OP3496___aGERF	JBWniten3aNITF	JBWBushp_dSIMM
OP2576v__cBURF	OP3496___aGERM	JBWniten3aNITM	JBWBushp_eSIMM
OP2576v__dBURF	OP3496___bGERF	JBWniten3bNITF	JBWswazi_aSIMF
OP2576vi_aBURF	OP3496___bGERM	JBWniten3bNITM	JBWswazi_bSIMF
OP2576vi_aBURM	OP3496___cGERF	JBWniten3cNITF	JBWswazi_cSIMF
OP2576vi_bBURM	OP3496___cGERM	JBWniten3cNITM	OP3052___aSIMN
OP2576vi_cBURM	OP3496___dGERF	JBWniten3dNITF	OP3052___bSIMN
OP2576vi_dBURM	OP3496___dGERM	JBWniten3dNITM	OP3052___cSIMN
OP2576vi_eBURM	OP3496___eGERF	JBWniten3eNITF	OP3052___dSIMN
OP2576vv_eBURF	OP3496___eGERM	JBWniten3eNITM	OP3052___eSIMN
JBWRGPL66aCAMF	JBWglabro_aGLAF	JBWpravu1aPRAL	JBW3400__aZAMF
JBWRGPL66aCAML	JBWglabro_aGLAL	JBWpravu1aPRAN	JBW3400__aZAMM
JBWRGPL66aCAMM	JBWglabro_aGLAM	JBWpravu1bPRAL	JBW3400__aZAMN
JBWRGPL66aCAMN	JBWglabro_aGLAN	JBWpravu1bPRAN	JBW3400__bZAMF
JBWRGPL66bCAMF	JBWglabro_bGLAF	JBWpravu1cPRAL	JBW3400__bZAMM
JBWRGPL66bCAML	JBWglabro_bGLAL	JBWpravu1cPRAN	JBW3400__bZAMN
JBWRGPL66bCAMM	JBWglabro_bGLAM	JBWpravu1dPRAL	JBW3400__cZAMF
JBWRGPL66bCAMN	JBWglabro_bGLAN	JBWpravu1dPRAN	JBW3400__cZAMN
JBWRGPL66cCAMF	JBWglabro_cGLAF	JBWpravu1ePRAL	JBW3400__dZAMF
JBWRGPL66cCAML	JBWglabro_cGLAL	JBWpravu1ePRAN	JBW3400__dZAMN
JBWRGPL66cCAMM	JBWglabro_cGLAM	JBWpravu2aPRAF	JBW3400__eZAMF
JBWRGPL66cCAMN	JBWglabro_cGLAN	JBWpravu2aPRAM	JBW3400__eZAMM
JBWRGPL66dCAMF	JBWglabro_dGLAF	JBWpravu2bPRAF	JBW3401__aZAML
JBWRGPL66dCAML	JBWglabro_dGLAL	JBWpravu2bPRAM	JBW3401__bZAML
JBWRGPL66dCAMM	JBWglabro_dGLAM	JBWpravu2cPRAF	JBW3401__cZAML
JBWRGPL66dCAMN	JBWglabro_dGLAN	JBWpravu2cPRAM	JBW3401__dZAML
JBWRGPL66eCAMF	JBWglabro_eGLAF	JBWpravu2dPRAF	JBW3401__eZAML
JBWRGPL66eCAML	JBWglabro_eGLAL	JBWpravu2dPRAM	JBW3400__cZAMM
JBWRGPL66eCAMM	JBWglabro_eGLAM	JBWpravu2ePRAF	JBW3400__dZAMM
JBWRGPL66eCAMN	JBWglabro_eGLAN	JBWpravu2ePRAM	JBW3401_aZAMN

Table S3.2: Host size data for three primary hosts per tick species.

	Immatures		Adults	
	Host	Mass (g)	Host	Mass (g)
R. appendiculatus	*Syncerus caffer*	592665.98	*Tragelaphus angasii*	87616.76
	Bos taurus	618642.42	*Bos taurus*	618642.42
	Tragelaphus strepsiceros	206056.41	*Tragelaphus strepsiceros*	206056.41
R. annulatus	*Bos taurus*	618642.42	*Bos taurus*	618642.42
R. decoloratus	*Tragelaphus strepsiceros*	206056.41	*Tragelaphus strepsiceros*	206056.41
	Bos taurus	618642.42	*Bos taurus*	618642.42
	Equus quagga	400000	*Aepyceros melampus*	52591.69
R. microplus	*Bos taurus*	618642.42	*Bos taurus*	618642.42
R. bursa	*Bos taurus*	618642.42	*Bos taurus*	618642.42
	Ovis aries	39097.89	*Ovis aries*	39097.89
	Capra hircus	47386.47	*Capra hircus*	47386.47
R. camicasi	"mouse-like rodents"	150	*Bos taurus*	618642.42
	-		*Ovis aries*	39097.89
	-		*Camelus dromedarius*	492714.47
R. capensis	*Otomys sp.*	135.418	*Bos taurus*	618642.42
	-		*Taurotragus oryx*	562592.69
	-		*Equus caballus*	403598.53
R. distinctus	*Procavia capensis*	2952.48	*Procavia capensis*	2952.48
	Micaelamys namaquensis	57.1	-	
	Lepus saxatilis	2593.63	-	
R. e. evertsi	*Ovis aries*	39097.89	*Ovis aries*	39097.89
	Equus quagga	400000	*Equus quagga*	400000
	Aepyceros melampus	52591.69	*Bos taurus*	618642.42
R. e. mimeticus	*Bos taurus*	618642.42	*Bos taurus*	618642.42
	Equus quagga	400000	*Equus quagga*	400000
	Ovis aries	39097.89	*Ovis aries*	39097.89
R. exophthalmos	*Elephantulus edwardii*	49.69	*Bos taurus*	618642.42
	Lepus saxatilis	2593.63	*Capra hircus*	47386.47
	Macroscelides proboscideus	38.64	*Tragelaphus strepsiceros*	206056.41
R. follis	*Rhabdomys pumilio*	40.73	*Bos taurus*	618642.42
	Micaelamys namaquensis	57.1	*Taurotragus oryx*	562592.69
	Otomys irroratus	114.45	*Diceros bicornis*	995940.54
R. gertrudae	*Rhabdomys pumilio*	40.73	*Canis lupus familiaris*	15000
	Micaelamys namaquensis	57.1	*Bos taurus*	618642.42
	Otomys irroratus	114.45	*Ovis aries*	39097.89
R. glabroscutatus	*Tragelaphus strepsiceros*	206056.41	*Tragelaphus strepsiceros*	206056.41
	Capra hircus	47386.47	*Capra hircus*	47386.47
	Redunca fulvorufula	29352.66	*Bos taurus*	618642.42
R. kochi	*Aepyceros melampus*	52591.69	*Aepyceros melampus*	52591.69
	Tragelaphus angasii	87616.76	*Tragelaphus angasii*	87616.76
	Tragelaphus scriptus	43250.39	*Tragelaphus scriptus*	43250.39
R. muehlensi	*Tragelaphus angasii*	87616.76	*Tragelaphus angasii*	87616.76
	Cephalophus natalensis	12724.51	*Tragelaphus scriptus*	43250.39

	Petrodromus tetradactylus	201	*Syncerus caffer*	592665.98
R. nitens	*Damaliscus pygargus*	77784.55	*Damaliscus pygargus*	77784.55
	Ovis aries	39097.89	*Ovis aries*	39097.89
	Pelea capreolus	22731.33	*Antidorcas marsupialis*	33571.24
R. pravus	*Elephantulus rufescens*	52.78	*Capra hircus*	47386.47
	Lepus capensis	2047.11	*Bos taurus*	618642.42
	Lepus saxatilis	2593.63	*Giraffa camelopardalis*	964654.73
R. pulchellus	*Eudorcas thomsonii*	22907.43	*Diceros bicornis*	995940.54
	Lepus capensis	2047.11	*Equus burchellii*	279160.65
	Equus quagga	400000	*Taurotragus oryx*	562592.69
R. sanguineus s.l. "tropical lineage"	*Canis lupus familiaris*	15000	*Canis lupus familiaris*	15000
R. simus	*Lepus saxatilis*	2593.63	*Canis lupus familiaris*	15000
	Panthera leo	158623.93	*Panthera leo*	158623.93
	Aethomys chrysophilus	80.85	*Phacochoerus africanus*	82499.99
R. sulcatus	???		*Lepus* spp.	2500
	???		*Canis mesomelas*	8247.3
	???		*Equus quagga*	400000
R. zambeziensis	*Tragelaphus strepsiceros*	206056.41	*Tragelaphus strepsiceros*	206056.41
	Lepus saxatilis	2593.63	*Panthera leo*	158623.93
	Aepyceros melampus	52591.69	*Aepyceros melampus*	52591.69
R. zumpti	???		*Potamochoerus larvatus*	69063.79
	???		*Canis lupus familiaris*	15000
	???		*Diceros bicornis*	995940.54

4 Integrative taxonomy and species delimitation of *Rhipicephalus turanicus* (Acari: Ixodida: Ixodidae)

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Abstract

Rhipicephalus turanicus ticks are widely distributed across the Palearctic and Afrotropics. These two continental populations display differences in morphological characters that raise the question of a potential species boundary. However, the taxonomic status of these morphologically divergent lineages is uncertain because *R. turanicus* from Cyprus and Zambia have been shown to interbreed and produce fertile hybrids. We employ integrative taxonomy that considers data from mtDNA sequences (12S and 16S rDNA), geographic distribution, traditional (qualitative) morphology, as well as shape outlines of female spiracles and male adanal plates measured in a geometric morphometric framework (quantitative morphology) to resolve this taxonomic issue. Molecular lines of evidence (12S and 16S rDNA) support taxonomic separation between ticks sampled in the Afrotropics and the Palearctic. This is corroborated by qualitative and quantitative morphology. Within the Palearctic, two sub-lineages were recovered based on sequence data that loosely correspond to southern Europe and the Middle East/Asia. One new species, *Rhipicephalus afranicus* n. sp. is described from South Africa with a geographic distribution that extends into eastern Africa. This leaves *R. turanicus* sensu lato comprised of two lineages located in southern Europe and the Middle East/Asia. The type locality for *R. turanicus* is in Uzbekistan, thus the Middle East/Asia lineage is considered *R. turanicus* sensu stricto. Detailed descriptions are provided for *R. afranicus* n. sp. and *R. turanicus* sensu stricto together with high resolution images. Speciation is attributed to recent Sahara desert expansion that formed a natural barrier to dispersal approximately 5-7 million years ago. However, reproductive potential between these two species suggests that divergence time and mode of speciation were not sufficient for the development of reproductive isolation. We suggest speciation was complicated by divergence and population reintegration events driven by oscillating climatic conditions contributing to reticulate evolution and maintenance of compatibility between reproductive mechanisms. This study represents an integrative (iterative) approach to delimiting *Rhipicephalus* spp., and provides the first application of shape outlines for female spiracles and male adanal plates measured in a geometric morphometric framework, applied to testing species boundaries between ticks.

Introduction

The Rhipicephalinae represents one of six subfamilies of hard-bodied ticks within the family Ixodidae (Guglielmone et al., 2010). Within Rhipicephalinae, the genus of brown ticks, *Rhipicephalus* Koch, 1844, is comprised of ~75 species, mostly confined to the Afrotropics. Taxonomy and evolutionary history of the genus is not well-resolved and *Rhipicephalus*

turanicus Pomerantsev, 1940 is a case in point. Morphologically, this species closely resembles *Rhipicephalus sanguineus* (Latreille, 1806) and *Rhipicephalus sulcatus* Neumann, 1908 (Walker et al., 2000). Together these species form part of the *R. sanguineus* group which is comprised of both Afrotropical and non-Afrotropical species including *Rhipicephalus camicasi* Morel, Moucheti & Rodhain, 1976, *Rhipicephalus leporis* Pomerantsev, 1946, *Rhipicephalus pumilio* Schulze, 1935, *Rhipicephalus pusillus* Gil Collado, 1936, *Rhipicephalus rossicus* Yakimov & Kol-Yakimova, 1911, *Rhipicephalus schulzei* Olenev, 1929 and *Rhipicephalus guilhoni* Morel & Vassiliades, 1963.

Rhipicephalus turanicus has a wide geographic distribution from the Afrotropics to the Palearctic, with its type locality in Uzbekistan (Filippova, 1997). Closely related *R. sanguineus* is cosmopolitan and previous investigations have suggested that at least three taxonomic lineages may exist – one associated with tropical climates, one with temperate climates, and a third limited to south-eastern Europe (Dantas-Torres et al., 2013; Hekimoğlu et al., 2016; Zemtsova et al., 2016; Chitimia-Dobler et al., 2017b; Coimbra-Dores et al., 2018). Apart from being used as an outgroup in phylogenetic studies of the *R. sanguineus* group (Dantas-Torres et al., 2013; Hekimoğlu et al., 2016; Zemtsova et al., 2016; Chitimia-Dobler et al., 2017b) and *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis* (Mtambo et al., 2007a, 2007b, 2007c), intraspecific sequence data for Afrotropical *R. turanicus* is limited. Nonetheless, these studies have indicated considerable divergence between Afrotropical and Palearctic *R. turanicus*. This highlights the need for more data to assess the problem of the species boundary and test monophyly in *R. turanicus* ticks.

Rhipicephalus turanicus is a three host species with an adult stage that generally parasitizes goats, dogs, cattle, sheep, lions and occasionally horses (Walker et al., 2000; Horak et al., 2018). These ticks have been implicated in transmitting *Babesia* and *Hepatozoon* that are linked to animal diseases (Walker et al., 2000; Gianelli et al., 2016). In Europe, *R. turanicus* has been implicated in transmission of *Theileria equi* (Friedhoff, 1988). Notably, South African specimens were unsuccessful in transmitting *Babesia caballi* and *Theileria equi* to horses (Potgieter et al., 1992; Walker et al., 2000). Geographically distant individuals of *R. turanicus* from Zambia (Afrotropical) and Cyprus (Palearctic) have divergent morphologies, but readily interbreed in laboratory environments and produce viable progeny with hybrid vigour, presumably by heterosis (Pegram et al., 1987b). Across all laboratory matings, 90% produced offspring, but a higher fecundity was observed in crosses between Zambian and Cypriot individuals (circa 5000 eggs in hybrid matings versus circa 4000 in same-population matings). Following this, past workers have opted to defend the *R. turanicus* species boundary based on the biological species concept (Pegram et al., 1987b; Walker et al., 2000; Horak et al., 2018). However, the biological species concept has been shown to be insufficient in certain

cases (Sokal and Crovello, 1970; De Queiroz, 2005, 2007), and ticks are no exception (Araya-Anchetta et al., 2013; Kovalev et al., 2015).

Two hypotheses of species boundary may be considered. Either *R. turanicus* in the Afrotropics and Palearctic represent a single morphologically divergent species, or two distinct species exist that are able to hybridize but would not do so in nature due to disjunct distributions. Preliminary data suggest the latter (Mtambo et al., 2007a, 2007b, 2007c; Dantas-Torres et al., 2013; Hekimoğlu et al., 2016; Zemtsova et al., 2016), and if this is true, hybrid vigour may have contributed to introgression and evolutionary reticulation between these two species. In the present study, we test the monophyly of *R. turanicus* based on integrative taxonomy that considers multiple lines of evidence including traditional qualitative morphology, quantitative morphology of shape outlines in female spiracles and male adanal plates (geometric morphometrics), 12S and 16S rDNA molecular sequence data, as well as geographic distribution patterns. Shape outlines of specific morphological features can hold useful clues for taxonomy and species delimitation, given that conspecific individuals should display more similar morphological shapes as opposed to heterospecific individuals. Excluding cases of destabilizing selection, shape outlines are expected to tend toward a central mean within a population of interbreeding individuals. Geometric morphometrics can prove especially useful to quantify such shape distributions between individuals and species given the high resolution afforded by the methods (Slice, 2007; Mitteroecker and Gunz, 2009; Klingenberg, 2010; Adams et al., 2013). These methods can measure the statistical distribution of shape variables to determine whether central tendencies overlap between a priori hypothesized species. Notably, this has been applied to morphological cryptic species complexes with demonstrated success (Pretorius and Clarke, 2001, 2000; Mutanen and Pretorius, 2007; Villemant et al., 2007; Karanovic et al., 2016; Bakkes et al., 2018b). However, convergence can introduce homoplasy to such data, and other lines of evidence that can delimit species boundaries should be employed to corroborate or refute findings. The approach adopted in this study assumes a contemporary formulation of integrative taxonomy that tests a species boundary hypothesis along an iterative framework against multiple lines of evidence (Yeates et al., 2011; Skoracka et al., 2015; Dantas-Torres, 2018).

Methods

Samples and qualitative morphology

Fresh adult specimens were collected from a range of localities by dragging (Table S4.1: not included in dissertation, see link under data availability). Additional specimens of all life stages were obtained from the Gertrud Theiler Tick Museum (GTTM), Agricultural Research Council-Onderstepoort Veterinary Research, South Africa, and were used for

qualitative and quantitative morphology as well as sequencing in some cases (Table S4.1). Specimens were identified a priori according to established taxonomic characters (Pegram et al., 1987b; Walker et al., 2000; Horak et al., 2018), and were categorized by geographic locality. Specimens were examined under a Zeiss Discovery V20 Stereomicroscope for qualitative morphological characters. Terminology generally follows that of Horak et al. (2018). Complete specimen data are presented in the details of material examined (Table S4.1).

Phylogenetic analysis of 12S and 16 rDNA

Two legs were removed from individual samples for DNA extraction using the prepGEM extraction kit (ZyGEM, Hamilton, New Zealand) according to the manufacturer's instructions. 16S rDNA was amplified using the 16SF and 16SR primers (Black and Piesman, 1994) and 12S rDNA was amplified using the 12S+1 and 12S-1 primers (Norris et al., 1999). PCR cycles included initial denaturation: 94°C (2 min); 40 cycles of 94°C (30 s), 45±2°C (30 s), 72°C (2 min); final elongation: 72°C (2 min). PCR products were sequenced at the Central Analytical Sequencing Facility at Stellenbosch University, South Africa, following the Sanger method and by using the 16SR and 12S-1 primers. All sequences were deposited in GenBank (Accession numbers MK158971-MK159007 and MN944860-MN945345). Additional sequences for *R. turanicus*, *R. sanguineus, R. guilhoni, R. camicasi, R. pusillus, R. rossicus, R. pumilio* and outgroups *R. appendiculatus* and *Rhipicephalus evertsi evertsi* were retrieved from GenBank for analysis (Table S4.1).

Sequences were aligned using MAFFT (Q-INS-i, 200PAM / k=2, Gap opening penalty: 1.53) (Katoh et al., 2002). Optimal nucleotide substitution models were selected using BIC (Bayesian Information Criterion) calculations in W-IQ-TREE (Trifinopoulos et al., 2016). Nucleotide substitution models were determined as TPM3u+F+G4 (Kimura, 1981) for 16S rDNA and TN+F+G4 for 12S rDNA (Tamura and Nei, 1993). Optimal models and associated parameters were applied to all subsequent analyses. Phylogenetic networks were estimated in SplitsTree v4.14.3 (Huson and Bryant, 2006) using neighbour network analysis with 1000 bootstrap replicates. Phylogenetic network analyses are well suited to cryptic species problems where speciation is complex because the analysis considers reticulate networks that make reticulate evolution explicit (Nakhleh et al., 2005). Analysis for each gene was performed separately with the 12S rDNA dataset including 171 aligned sequences with 256 nucleotide sites, with 63 phylogenetically informative and 163 conserved. The 16S rDNA dataset included 157 aligned sequences with 305 nucleotide sites, with 80 phylogenetically informative and 164 conserved. Pairwise genetic distances were calculated in MEGA v7.0.14 (Kumar et al., 2016) and then converted to pairwise genetic similarity.

For combined analysis, 12S and 16S rDNA data were concatenated into a single matrix in SequenceMatrix v1.8 (Vaidya et al., 2011) and only included individuals with data for both genes. Separate 12S and 16S rDNA sequences from GenBank were concatenated into single taxa in the data matrix after confirmation that each sequence belonged to the same species and lineage with respect to the 12S and 16S rDNA topologies (Figs. 4.1, 4.2). Furthermore, these sequences were concatenated according to locality (Table S4.1). Sequences that could not be combined due to a lack of reciprocal 12S and 16S rDNA sequences from the same locality for the same species were excluded to minimize taxa with missing data. Thus, the total dataset for combined analysis consisted of 66 taxa. Maximum likelihood inference was done using 1000 bootstrap replicates followed by a thorough maximum likelihood search in RaxML v8.1.20 (Stamatakis, 2014). Bayesian phylogenetic inference was done using two Monte-Carlo Markov chains run simultaneously for 5 million iterations sampling every 200th iteration in MrBayes v3.2.6 (Ronquist and Huelsenbeck, 2003). The first 25% of trees were discarded as burn-in and a majority-rule consensus tree with posterior probabilities was calculated. Tracer v1.6 (Drummond and Rambaut, 2007) was used to assess convergence and effective sampling between all runs, and all ESS (Effective Sample Size) values were greater than 200, indicating effective sampling (Drummond et al., 2006).

Geometric morphometric analysis

The total sample for morphometric analysis consisted of landmark data for 69 male adanal plates and 76 female spiracles among nine species divided into 12 lineage groups. These included Afrotropical *R. turanicus,* Palearctic *R. turanicus, R. sanguineus* tropical lineage, *R. sanguineus* temperate lineage, *R. sanguineus* south-east lineage, *R. sulcatus,* R. *guilhoni*, *R. bergeoni*, *R. camicasi*, *R. pusillus*, *R. rossicus* and *R. pumilio*. These specimens have been stored in 96% ethanol and housed in the GTTM (Table S4.1). Some specimens used in sequencing were similarly used in morphometric analyses to iteratively test species boundaries determined from the mtDNA lineages. Note that a lack of *R. turanicus* samples to represent both Palearctic lineages prevented analysis of their morphometric data separately. Thus, we grouped Palearctic samples together as *R. turanicus* sensu lato. As such, analysis was framed in terms of distinguishing Afrotropical *R. turanicus* from *R. turanicus* s.l. in the Palearctic.

Male adanal plates and female spiracles were photographed on a rotational mount in three replicates following Bakkes (2017) (Chapter 6). Photographs were taken using a Zeiss AxioCam MRc 5 camera, and were stacked in Zeiss Axiovision v4.8. Each stacked image consisted of between 10 and 20 photographs. Outlines of female spiracles and male adanal plates were digitized using COO v41 in the CLIC package by Jean-Pierre Dujardin (available

at http://mome-clic.com/the-clic-package/) according to 15 and 13 landmarks, respectively (Fig. S4.1, S4.2: supplementary information), and were scaled to a 0.2 mm scale bar. Operational definitions for each landmark are available in Fig. S4.1 and S4.2. Replicate photographs were digitized in batches by replicate to avoid digitization bias from operator memory. Landmarks were transformed in a Procrustes fit in MorphoJ v1.06d (Klingenberg, 2011) and a covariance matrix was generated. All subsequent analyses were performed on the total 12 lineage group datasets for males and females, as well as on six lineage group datasets that focus on the two *R. turanicus*, three *R. sanguineus* and one *R. sulcatus* lineages. All lineages of *R. sanguineus* and *R. sulcatus* were selected for the focused morphometric analyses, as their morphologies are closest to, and are often confused with, *R. turanicus.*

A Procrustes ANOVA was done to measure variability in individuals with replicate as the error effect and species as the main effect. Subsequently, observations were averaged by individual. Qualitative morphological characters as well as 12S and 16S rDNA lineages recovered, directed a priori species hypotheses that were tested in a canonical variates analysis. Canonical variates transform morphospace to maximize differences between groups, and are sensitive to a priori species delimitation. This enables a test of a priori group structure based on Mahalanobis distances. In turn, Mahalanobis distance scales between-group variation by within-group variation, enabling comparison between multivariate group means. One unit in Mahalanobis distance between groups represents one unit of within-group standard deviation. This enables a test of hypothetical species group structure based on 12S, 16S rDNA data and qualitative morphology (proxies for species boundary), and serves to characterize shape changes between species (Villemant et al., 2007; Yeates et al., 2011; Karanovic et al., 2016; Bakkes et al., 2018b).

Distribution maps

Point maps of species distributions were made using co-ordinates of digitized locality data from the GTTM (Table S4.1). Co-ordinates were plotted against current data for annual precipitation in DIVA GIS v7.5.0 (available at http://www.diva-gis.org/download). This was done to determine whether distribution patterns might conform to features of physical geography and some aspects of the abiotic environment.

Data availability

All data and supplementary information associated with this work have been uploaded to Mendeley data: http://dx.doi.org/10.17632/zjmtx7ghx4.1. Sequence data have been deposited in the GenBank database (Accession numbers: MK158971-MK159007, MN944853-MN944887, MN945315-MN945352). Voucher and type specimens are housed in the Gertrud

Theiler Tick Museum (GTTM), Agricultural Research Council, South African National Museum – Iziko Museum (SAMC), and the Berlin Zoological Museum, Museum für Naturkunde (ZMB), Germany. See taxonomy and species descriptions in the results section for details about deposited specimens.

Results

Species boundary between Palearctic and Afrotropical R. turanicus

Bayesian and maximum likelihood analyses of the combined data, as well as neighbour network splits analysis for each gene, indicate two evolutionarily distinct lineages in samples previously identified as *R. turanicus* (Figs. 4.1-4.3). These two lineages correspond with geographic distribution in the Palearctic and Afrotropics. The Afrotropical lineage of *R. turanicus* (indicated as *R. afranicus* n. sp.; Figs. 4.1-4.3) forms a strongly supported monophyletic group with *R. sanguineus* (tropical lineage), *R. guilhoni* and *R. sulcatus*, and this clade excludes *R. turanicus* sampled from the Palearctic. Within the Palearctic *R. turanicus* lineage, two sub-lineages were recovered, corresponding to the Middle-East/Asia, as well as to southern Europe. For 16S rDNA, pairwise similarity between Afrotropical *R. turanicus* and both southern European and Middle Eastern/Asian *R. turanicus* lineages was 92.47% and 94.54%, respectively, and between southern European and Middle Eastern/Asian *R. turanicus* was 94.08%. For 12S rDNA, the same values were 95.84% and 95.81%, and between southern European and Middle Eastern/Asian *R. turanicus* pairwise genetic similarity was 99.7%.

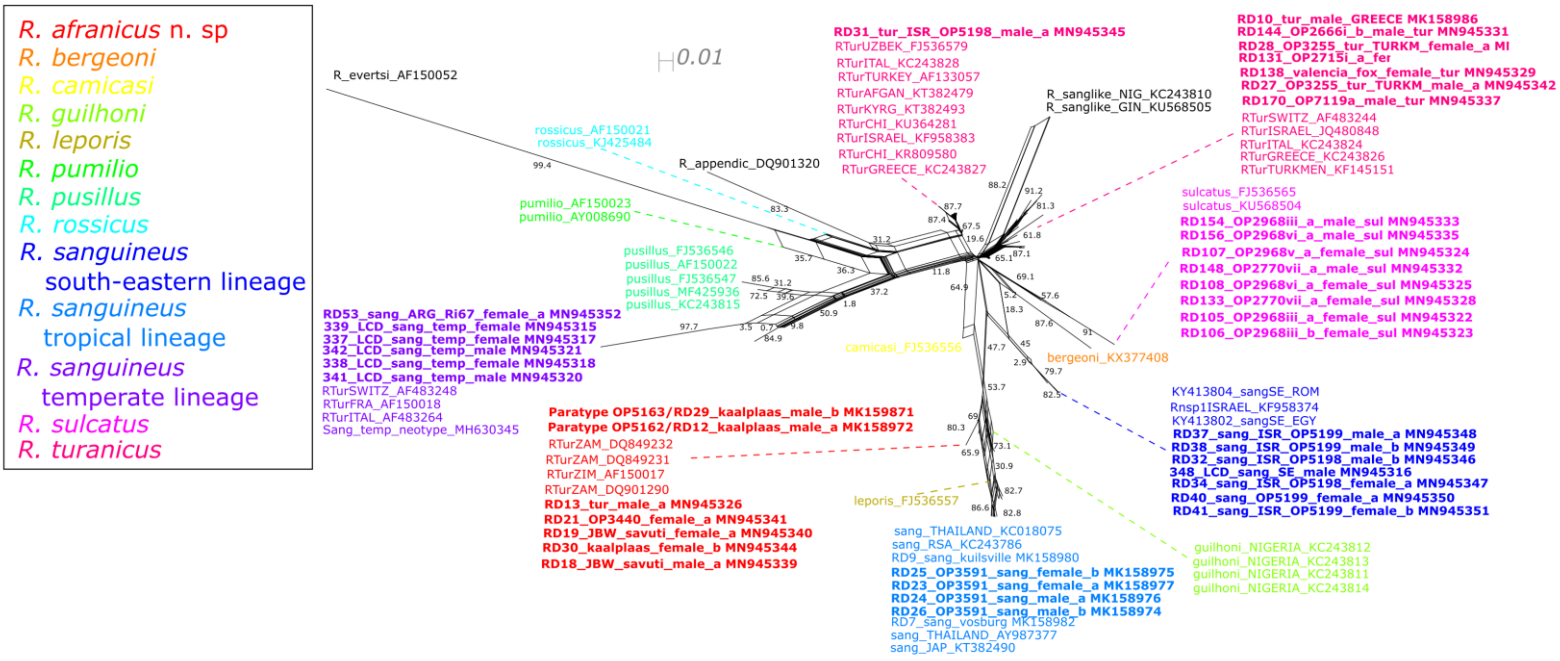


Figure 4.1. Phylogenetic splits tree analysis of the *Rhipicephalus turanicus* group 16S rDNA gene using the TPM3u+F+G4 nucleotide substitution model. Indicated are species and lineage names, GenBank accession numbers or study codes (reflected in Table S4.1), country of origin and neighbour network bootstrap support values. Bolded samples were used iteratively in morphometric analyses. Note that between 10 and 12 sequence labels per clade were retained for this figure to increase readability. See Fig. S4.3 for the fully labelled figure.

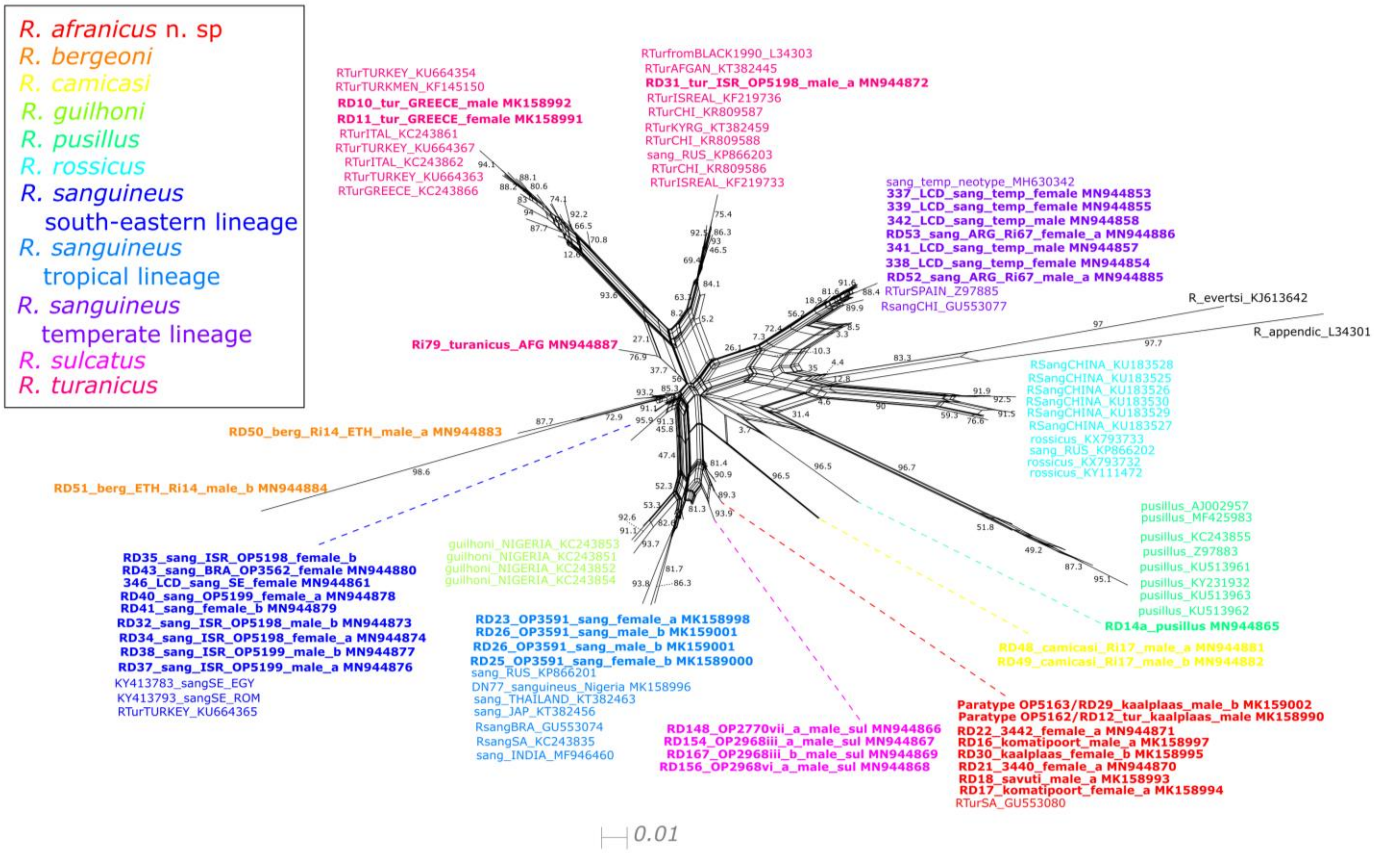


Figure 4.2. Phylogenetic splits tree analysis of the *Rhipicephalus turanicus* group 12S rDNA gene using the TN+F+G4 nucleotide substitution model. Indicated are species and lineage names, GenBank accession numbers or study codes (reflected in Table S4.1), country of origin and neighbour network bootstrap support values. Bolded samples were used iteratively in morphometric analyses. Note that between 10 and 12 sequence labels per clade were retained for this figure to increase readability. See Fig. S4.4 for the fully labelled figure.

Morphometric analyses reveal distinct shape differences between Palearctic and Afrotropical *R. turanicus* samples. For female spiracles, comparison of multivariate means indicated Mahalanobis distances between Palearctic *R. turanicus* and Afrotropical *R. turanicus* females were large compared with the other species comparisons (Fig. 4.4B), and ware significantly large to distinguish groups (Mahalanobis distance (M.dist.) = 6.1214, $p < 0.0001$). Misclassification by cross-validation between these groups was small (12.1% of comparisons). Procrustes ANOVA showed the effect of individual was 8.41 times greater than replicate error, indicating that rotational error was negligible, and that species variation was 4.55 times greater than individual variation. Canonical variate I conclusively distinguished Afrotropical *R. turanicus* from all *R. sanguineus* lineages, but did not provide distinction between Afrotropical *R. turanicus*, Palearctic *R. turanicus* and *R. sulcatus*. Shape change was attributable to (i) width of the base of the dorsal and (ii) width of the dorsal prolongation tip. Canonical variate II provided clear distinction for Afrotropical *R. turanicus* from *R. sulcatus* and Palearctic *R. turanicus,* and provided moderate distinction between Afrotropical *R. turanicus* and all *R. sanguineus* lineages. Shape change was attributable to (i) the width of dorsal prolongation, (ii) the vertical angle of dorsal prolongation, and (iii) excavation of the dorsal margin leading to dorsal prolongation.

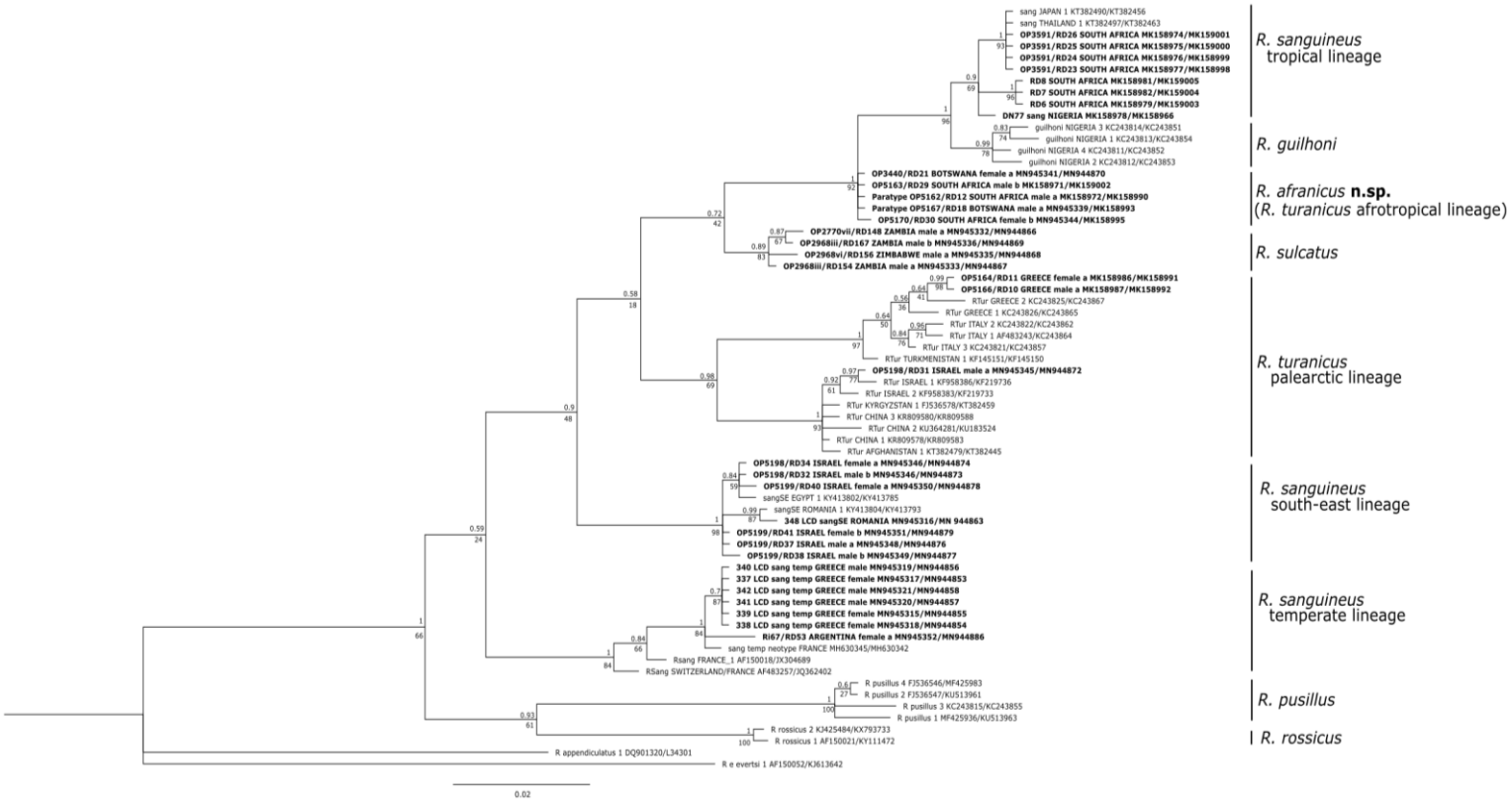


Figure 4.3. Consensus tree recovered from Bayesian analysis of the combined *Rhipicephalus turanicus* group dataset. Indicated are species and lineage names, GenBank accession numbers or study codes (left: 12S rDNA, right: 16S rDNA) and country of origin. Nodal support values represent posterior probability (top) and maximum likelihood bootstrap (bottom). Samples in bold refer to sequences generated in this study.

For male adanal plates, comparison of multivariate means indicated Mahalanobis distances between Palearctic *R. turanicus* and Afrotropical *R. turanicus* males was small compared with the other species comparisons (Fig. 4.5B). However, this distance was significantly large to distinguish groups (M.dist. = 3.6959, $p < 0.0001$). Misclassification by cross-validation between these groups was small (13.5% of comparisons). Procrustes ANOVA showed the effect of individual was 5.62 times greater than replicate error, indicating that rotational error was negligible, and that species variation was 3.42 times greater than individual variation. Canonical variate I conclusively distinguished Afrotropical *R. turanicus* from *R. sulcatus* and *R. sanguineus* south-eastern and tropical lineages. Canonical variate I provided moderate distinction between Afrotropical *R. turanicus* and Palearctic *R. turanicus*. However, canonical variate I did not provide distinction between Afrotropical *R. turanicus* and *R. sanguineus* temperate lineages. Shape change was attributable to (i) projection of the posteromedial corner, (ii) overall width of the posterior third, and (iii) excavation of the medial scallop. Canonical variate II provided clear distinction for Afrotropical *R. turanicus* from *R. sanguineus* temperate lineages and provided moderate distinction between Afrotropical *R. turanicus* and *R. sanguineus* tropical lineages. Canonical variate II did not provide distinction between Afrotropical *R. turanicus* and *R. sulcatus*, Palearctic *R. turanicus* and *R. sanguineus* south-eastern lineages. Shape change was attributable to (i) projection of the posterolateral corner, (ii) overall width of the posterior third, and (iii) excavation of the medial scallop.

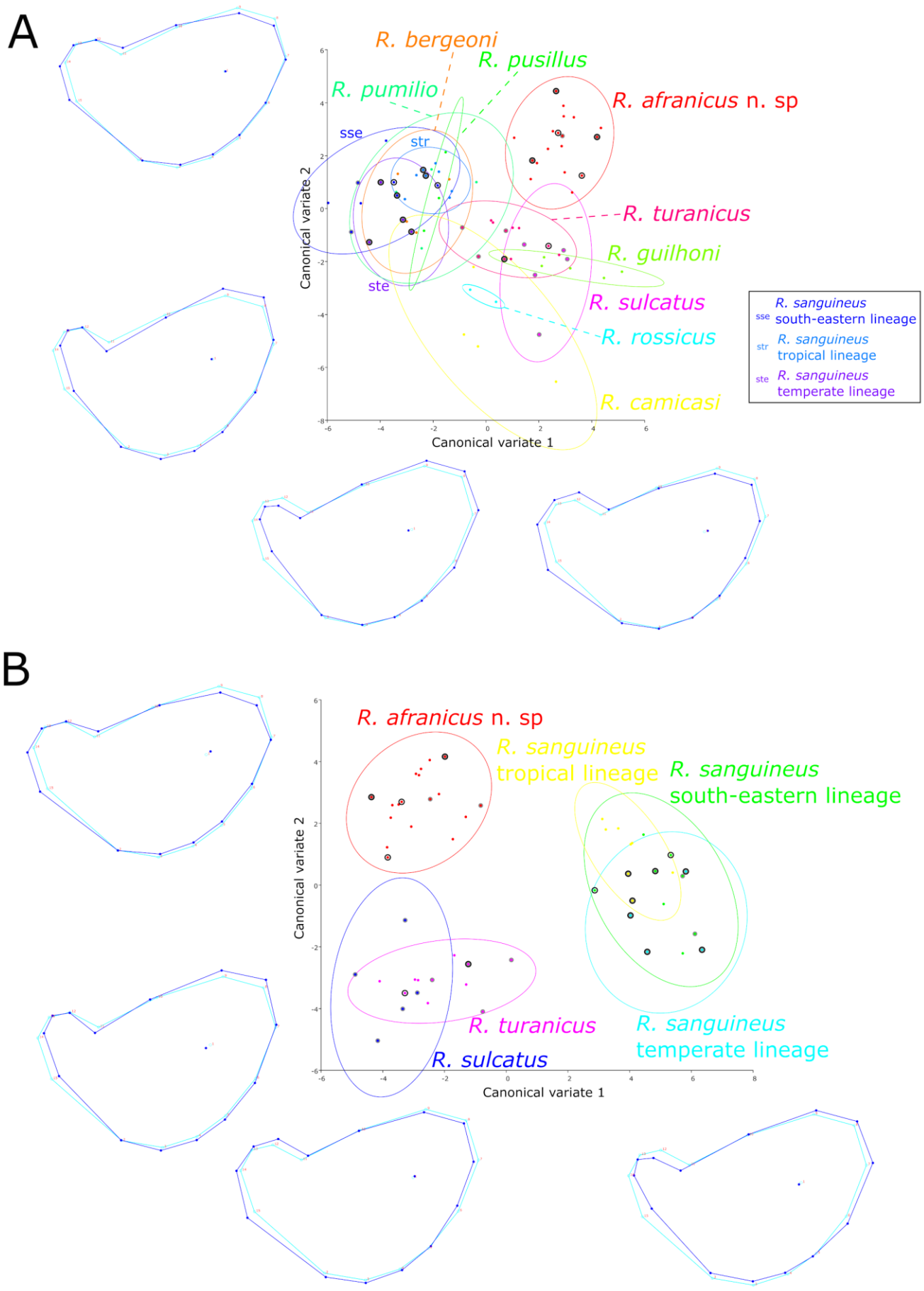


Figure 4.4. Canonical variates analysis of female *R. turanicus* group spiracle shape data for the (A) total 12 lineage group and (B) six lineage group datasets. Indicated are axes for canonical variates I and II. Dots represent averages for single specimens. Black and grey rings represent specimens used in 16S or 12S rDNA phylogenetic analysis, respectively. Ellipses represent 95% confidence intervals. Shape changes along PC (Principal Component) axes are to scale at minimum and maximum extents. Light blue traces represent the mean shape and dark blue traces represent deviation from the mean shape at the given extent.

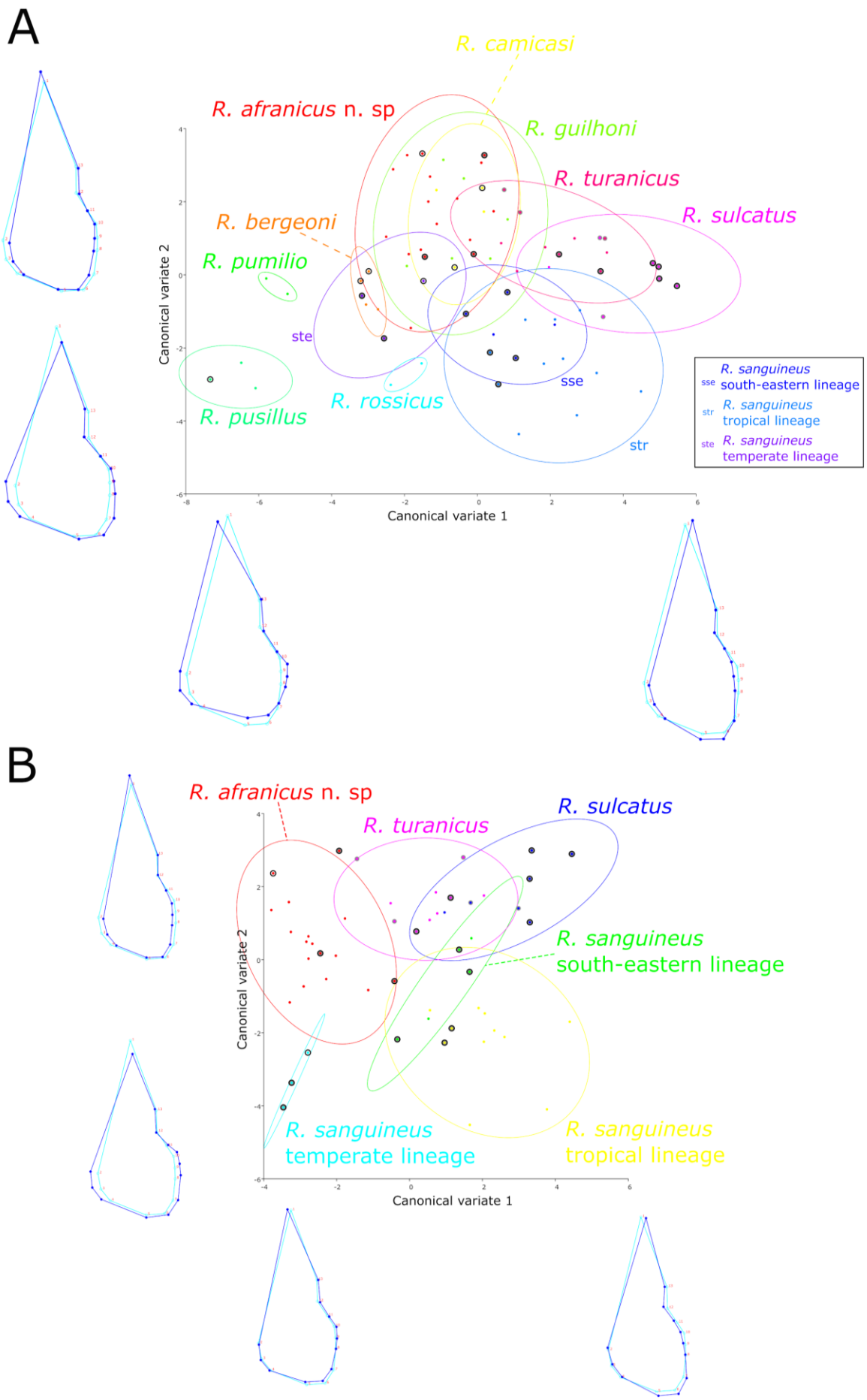


Figure 4.5. Canonical variates analysis of male *R. turanicus* group adanal plate shape data for the (A) total 12 lineage group and (B) six lineage group datasets. Indicated are axes for canonical variates I and II. Dots represent averages for single specimens. Black and grey rings represent specimens used in 16S or 12S rDNA phylogenetic analysis, respectively. Ellipses represent 95% confidence intervals. Shape changes along PC (Principal Component) axes are to scale at minimum and maximum extents. Light blue traces represent the mean shape and dark blue traces represent deviation from the mean shape at the given extent.

Distribution patterns indicate that Afrotropical *R. turanicus* is limited to regions with 400 mm to 1500 mm annual precipitation. In contrast, Palearctic *R. turanicus* lineages are limited to regions with 100 mm to 1000 mm annual precipitation (Middle Eastern/Asian lineage: 100 mm to 500 mm; southern European lineage: 200 mm to 1000 mm) (Fig. 4.6). The Sahara desert forms a natural barrier of uninhabitable land between the two species.

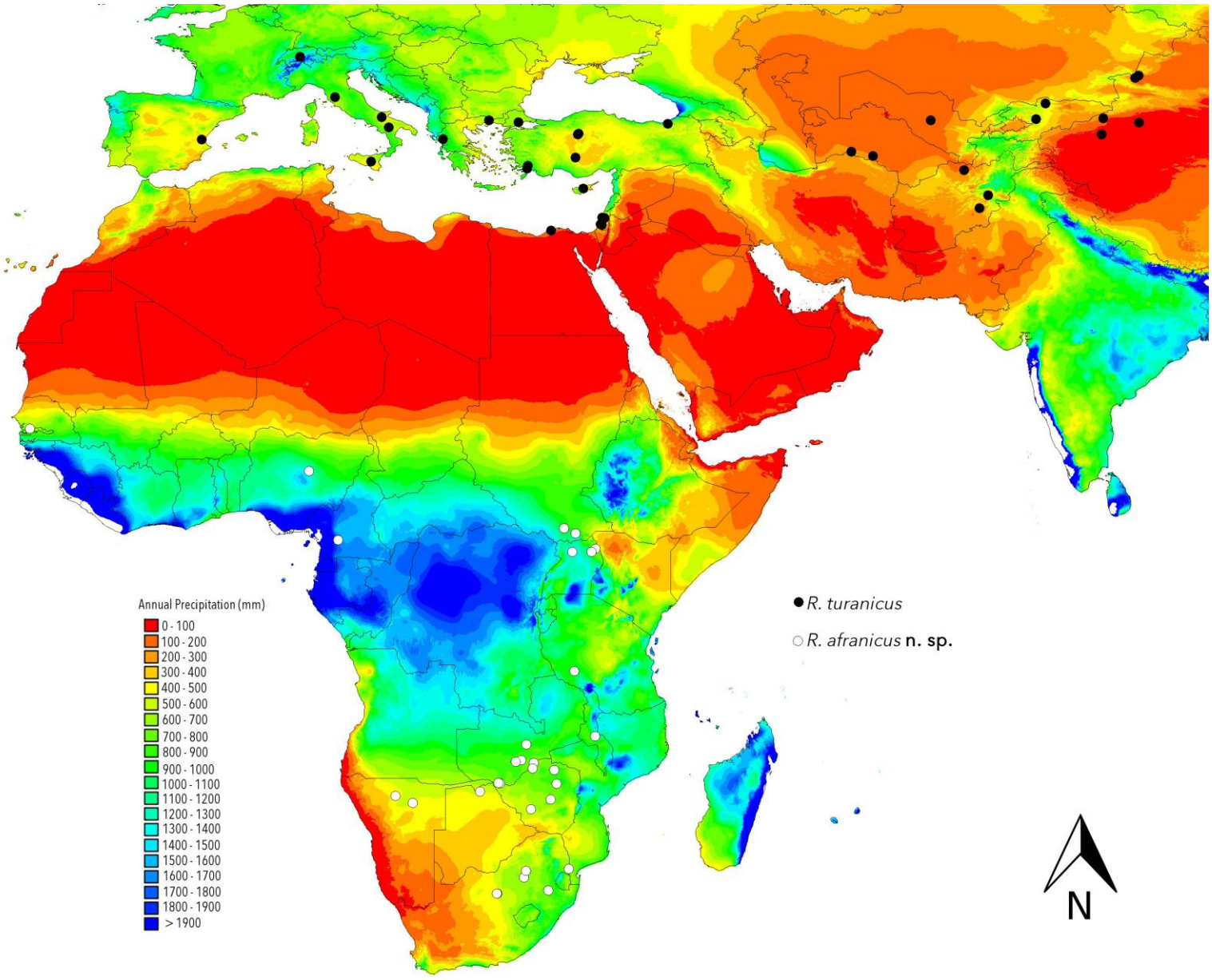


Figure 4.6. Point map distribution of *Rhipicephalus afranicus* n. sp. (white) and *Rhipicephalus turanicus* sensu lato (black) against annual precipitation data based on samples studied (Table S4.1).

Taxonomy and species descriptions

Family IXODIDAE Koch, 1844

Genus ***Rhipicephalus*** Koch, 1844

Rhipicephalus afranicus Bakkes **n. sp.**

(Figs. 4.7-4.15)

ZooBank LSID: zoobank.org:act:B4417059-1998-41D5-AA4C-841571751094

Synonymy.

Rhipicephalus turanicus Pomerantsev, 1940, pro parte.

Type depository: GTTM, Holotype: Male

Type locality: Kaalplaas, Onderstepoort, South Africa (25° 37' 38.0172" S 28° 8' 49.2576" E)

Etymology. From geographic distribution in the Afrotropics, Latin –*icus*, adjective (belonging to, derived from), as well as from similarity to *R. turanicus* in morphology.

Material examined. Forty-seven specimens from South Africa, Zimbabwe, Botswana, Namibia, Zambia, Malawi, Tanzania, Uganda, Sudan, Cameroon and Nigeria (Table S4.1).

Type material

Holotype ♂ (deposited in Gertrud Theiler Tick Museum, Onderstepoort, South Africa (GTTM); OP5172) designated here. Allotype ♀ (deposited in GTTM; OP5173) designated here. Holotype and Allotype not sequenced in order to preserve specimen integrity.

Paratype series designated here: OP5162/RD12 (♂ deposited in GTTM) Genbank: 12S rDNA - MK158972, 16S rDNA - MK158990; OP5163/RD29 (♂ deposited in GTTM) Genbank: 12S rDNA - MK158971, 16S rDNA - MK159002; OP5174 (2♂2♀, deposited in South African National Museum, Iziko Museum, Cape Town (SAMC)); OP5175 (2♂2♀, deposited in Berlin Zoological Museum, Museum für Naturkunde, Berlin (ZMB)).

Specimen data for all types: 'Kaalplaas, Onderstepoort, South Africa / -25.627227 28.147016 / iii.2018 / dragged from vegetation / Lidia Chitimia-Dobler & Deon Bakkes.

Description.

Males (voucher numbers: OP5172, OP5162, OP5163, OP5174, OP5175)

Length 2.6 to 3.2 mm, width 1.6 to 2.1 mm

Basis capitulum hexagonal, short, wide. Lateral angles slightly obtuse projecting at anterior third of length. Cornua present, short, broadly triangular. Palps short, sub-triangular.

Body reddish brown. Conscutum elongate, ovate, broadest at coxae IV with anterolateral margin convex. Lateral idiosoma expanded when engorged. Eyes flat, dorsally bordered by a row of medium punctations. Cervical grooves convergent and deep anteriorly, shallow and divergent posteriorly. Cervical fields slightly sunken with numerous small punctations present imparting a rugose appearance, lateral grooves bordered by a row of large, confluent punctations. Marginal grooves enclosing first festoon and reaching level of coxa III, bordered by a row of sparse, large punctations. Posteromedial groove short, oval, rugose. Posterolateral grooves approximately half the length of posteromedial groove, rugose. Small punctations numerous, a few large punctations sparsely scattered in four irregular rows as well as on scapulae.

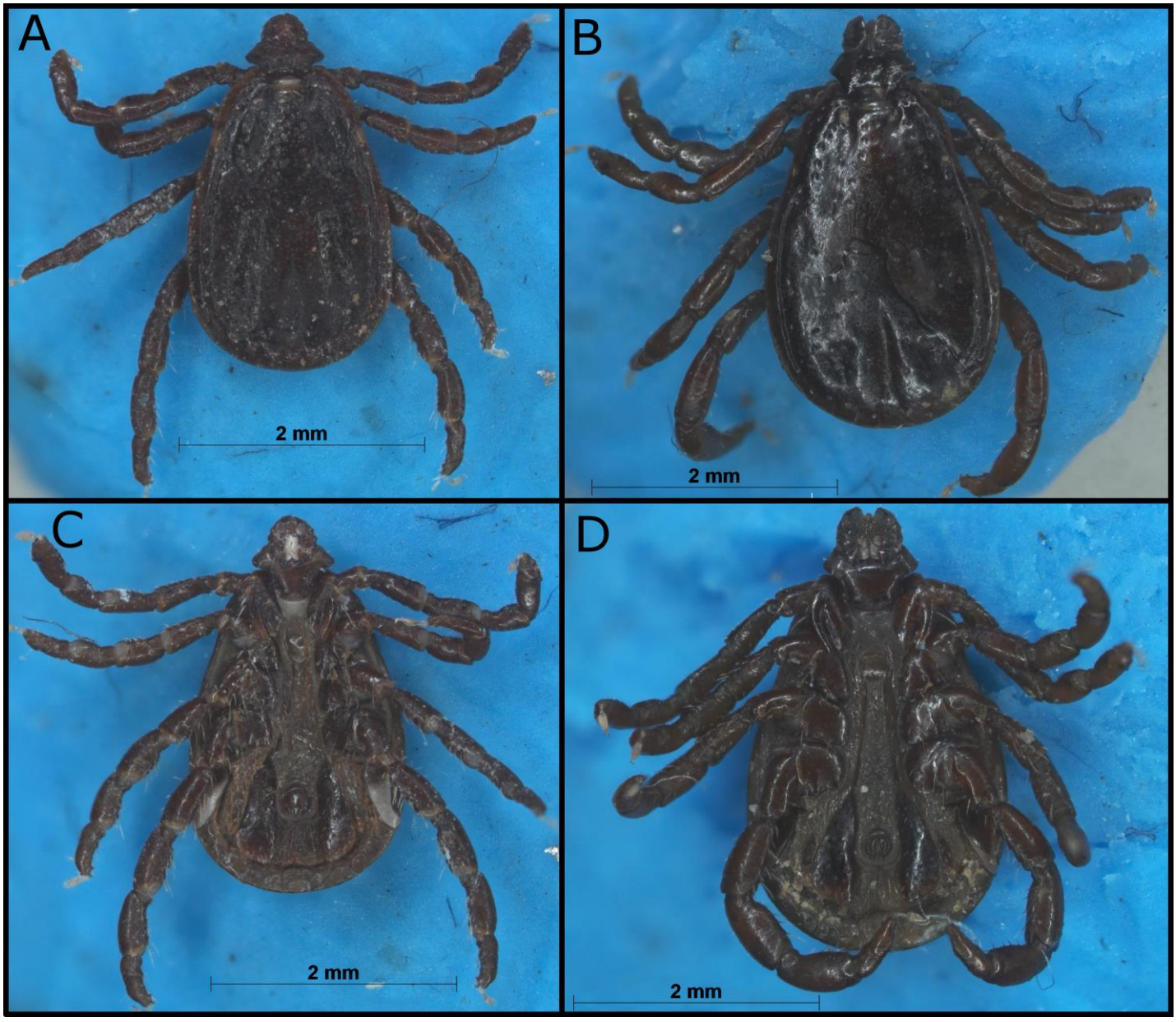


Figure 4.7. Dorsal and ventral habitus photos of male *Rhipicephalus afranicus* n. sp. (A, C) and *Rhipicephalus turanicus* sensu stricto (B, D). Specimen data: *R. afranicus* n. sp. (A, C) – OP5172 Holotype, Kaalplaas, vegetation, March 2018; *R. turanicus* sensu stricto (B, D) – OP3255/RD27, Turkmenistan, *Canis lupus familiaris* (Dog), April 1988.

Legs reddish brown, slender, slightly thicker posteriorly. Coxae approximately equal in size. Coxa I elongate with small anterior process and posteromedial spur broad with triangular tip, posterolateral spur narrow, tapering to rounded tip. Coxa II sub-triangular with posteromedial spur minute, broad, flange-like and posterolateral spur moderate size, broad, triangular. Coxa III sub-rectangular with posteromedial spur broad, flange-like and posterolateral spur short, narrow, triangular. Coxa IV sub-rectangular with posteromedial spur minute, triangular and posterolateral spur short, narrow, triangular. Adanal plates elongate, triangular with slight medial concavity and posterolateral convexity. Posterior third slightly more wide than long, imparting a stunted appearance. Accessory plates short, reaching level of posterolateral convexity. Spiracles elongate, sub-ovate with dorsal prolongation short, broad.

Females (description voucher numbers: OP5173, OP5174, OP5175)
Length 2.6 to 3.1 mm, width 1.5 to 2.2 mm (Unengorged)

Basis capitulum hexagonal, short, wide. Lateral angles rectangular, projecting at mid-length. Cornua present, short, broadly triangular. Porous areas sub-ovate, small, separated by a distance slightly less than twice their width. Palps short, sub-triangular, stalked on article I. Body reddish brown. Scutum elongate, ovate, broadest at mid-length with anterolateral margin convex, posterior margin sinuous with most posterior point slightly pronounced. Eyes flat, dorsally bordered by a row of medium punctations. Cervical grooves convergent and deep anteriorly, shallow and divergent posteriorly. Cervical fields slightly sunken with numerous confluent punctations, not reaching posterolateral scutal margins. Lateral grooves distinct, bordered by a row of large punctations. Small punctations numerous, large punctations moderately scattered across scutum.

Legs reddish brown, slender, slightly thicker posteriorly. Coxae approximately equal in size. Coxa I elongate with posteromedial spur broad with triangular tip, posterolateral spur narrow, tapering to pointed tip. Coxa II sub-rectangular with posteromedial spur short, broad, flange-like and posterolateral spur moderate size, triangular. Coxa III sub-rectangular with posteromedial spur short, broad, flange-like and posterolateral spur short, narrow, triangular. Coxa IV sub-rectangular with posteromedial spur minute, arcuate and posterolateral spur short, broad, triangular. Genital aperture U-shaped with lateral margins slightly diverging anteriorly. Spiracles broadly sub-triangular with rounded angles, dorsal prolongation short, thick proximally, tapering to rounded tip distally.

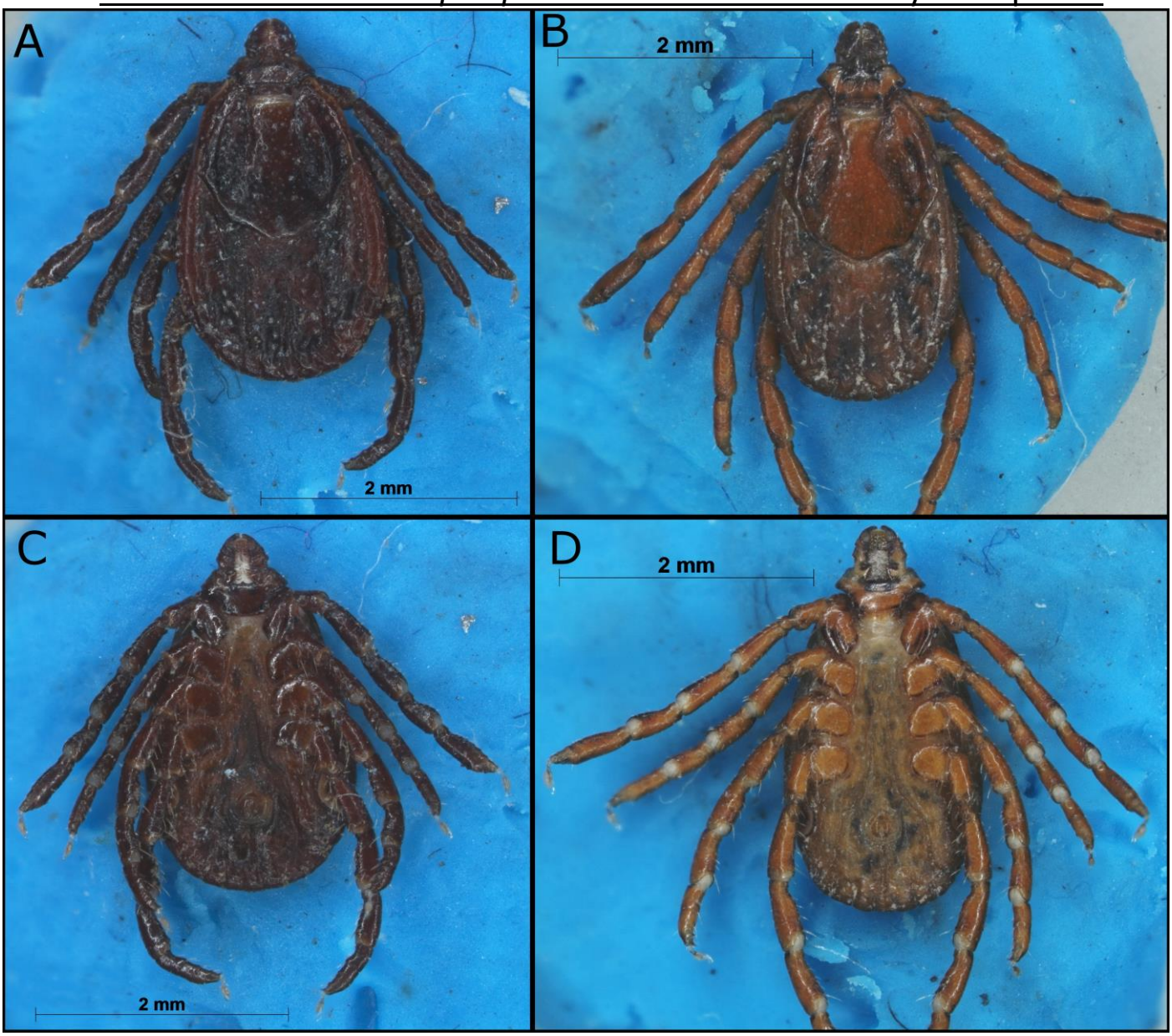


Figure 4.8. Dorsal and ventral habitus photos of female *Rhipicephalus afranicus* n. sp. (A, C) and *Rhipicephalus turanicus* sensu stricto (B, D). Specimen data: *R. afranicus* n. sp. (A, C) – OP5173 Allotype, Kaalplaas, vegetation, March 2018; *R. turanicus* sensu stricto (B, D) – OP3255/RD28, Turkmenistan, *Canis lupus familiaris* (Dog), April 1988.

Nymphs (description voucher numbers: 5 specimens from OP5194)

Length 0.7 to 1.1 mm, width 1.0 to 1.2 mm (Unengorged)

Basis capitulum hexagonal, almost flat, wide. Lateral angles acute, projecting at mid-length. Cornua absent. Palps short, sub-triangular. Body yellowish brown. Scutum elongate, ovate, broadest at posterior third with anterolateral margin convex, posterior margin rounded. Eyes flat, located at posterior third of scutum. Cervical grooves convergent and deep anteriorly, shallow and divergent posteriorly. Cervical fields, narrow, distinctly sunken, not reaching posterolateral scutal margins. Lateral grooves distinct.

Legs transparent brown, slender. Coxae approximately equal in size. Coxa I sub-rectangular with spurs moderately separated. Posteromedial spur short, broad, triangular and

posterolateral spur long, broad, triangular. Coxa II sub-rectangular with posteromedial spur absent and posterolateral spur moderate size, broad, triangular. Coxa III sub-rectangular with posteromedial absent and posterolateral spur short, narrow, triangular. Coxa IV sub-rectangular with spurs absent.

Larvae (description voucher numbers: 5 specimens from OP5194)
Length 0.4 to 0.6 mm, width 0.3 to 0.5 mm (Unengorged)

Basis capitulum hexagonal, almost flat, width almost equal to posterior margin of scutum. Lateral angles acute, projecting at mid-length. Cornua absent. Palps short, sub-triangular. Body yellowish brown. Scutum narrow, triangular, widest posteriorly with anterolateral margin convex, posterior margin rounded. Eyes flat, located at posterior corner of scutum. Cervical grooves convergent and deep anteriorly, shallow and divergent posteriorly. Cervical fields, narrow, not distinctly sunken, not reaching posterolateral scutal margins. Lateral grooves indistinct.

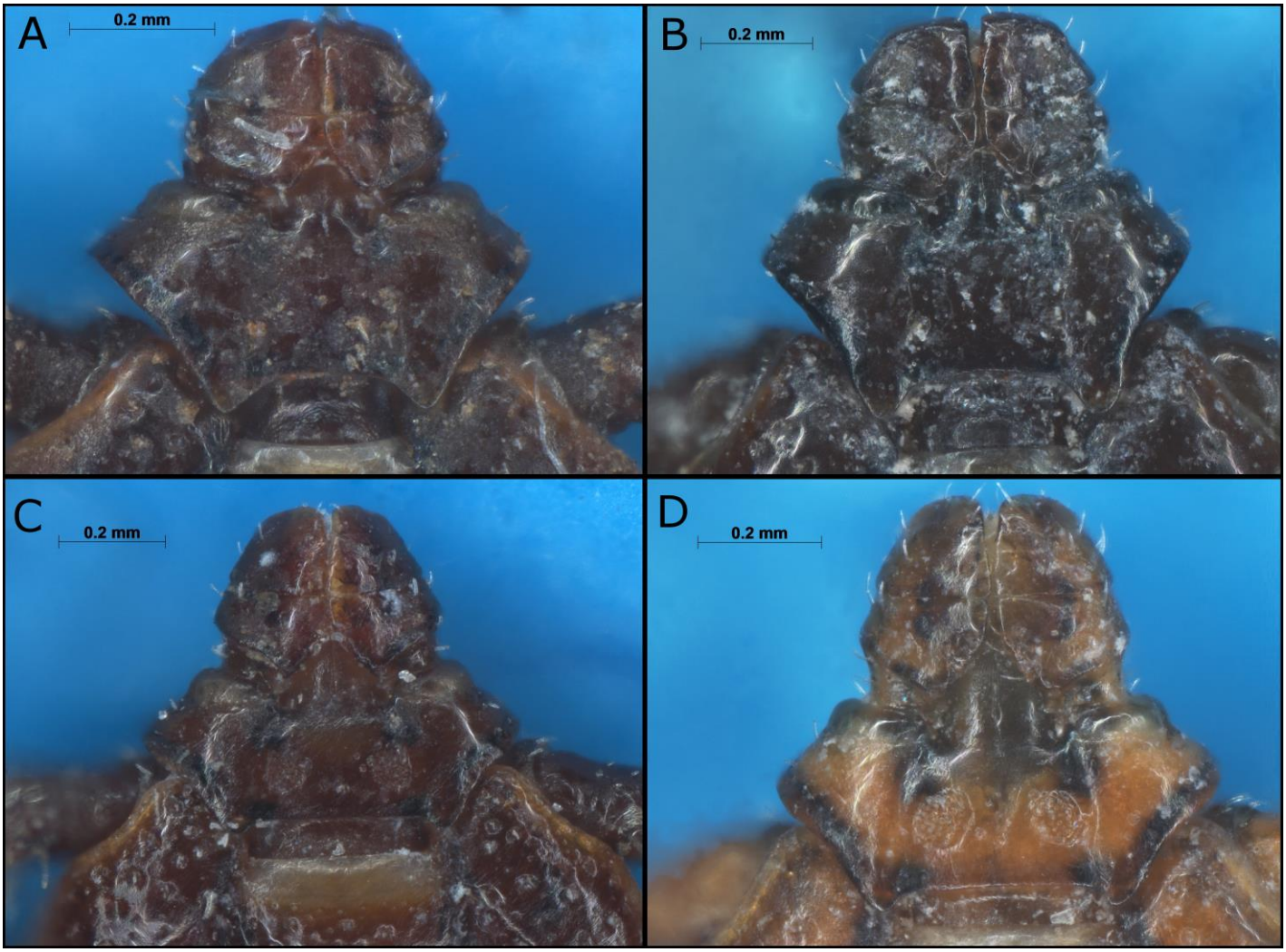


Figure 4.9. Comparative morphology of basis capituli in male and female *Rhipicephalus afranicus* n. sp. (A, C) and *Rhipicephalus turanicus* sensu stricto (B, D). Specimen data: *R. afranicus* n. sp. male (A) – OP5172 Holotype, Kaalplaas, vegetation, March 2018; *R. turanicus* sensu stricto male (B) – OP3255/RD27, Turkmenistan, *Canis lupus familiaris* (Dog), April 1988. *R. afranicus* n. sp. female (C) – OP5173 Allotype, Kaalplaas, vegetation, March 2018; *R. turanicus* sensu stricto female (D) – OP3255/RD28, Turkmenistan, *Canis lupus familiaris* (Dog), April 1988.

Legs transparent brown, slender. Coxae approximately equal in size. Coxa I sub-rectangular with posteromedial spur moderate size, broad, flange-like and posterolateral spur absent. Coxa II sub-rectangular with posteromedial spur short, broad, flange-like and posterolateral spur absent. Coxa III sub-rectangular with posteromedial spur minute, triangular and posterolateral spur absent.

Biogeography. Afrotropical (Fig. 4.6). Most collections are from southern and east Africa with limited records from west Africa. Generally, occurring in regions with annual precipitation between 400 mm and 1500 mm.

Hosts. Three host life cycle. Host data from Walker et al. (2000), Horak et al. (2018) and GTTM records. Black-backed Jackal (*Canis mesomelas*), Black-footed Cat (*Microfelis nigripes*), Hare (*Lepus* sp.), Leopard (*Panthera pardus*), Red Lechwe (*Kobus leche*), Cattle (*Bos taurus*), Stanley's Bustard (*Ardeotis denhami jacksoni*), Marabou Stork (*Leptoptilos crumeniferus*), Grant's Gazelle (*Nanger granti*), Common Ostrich (*Struthio camelus*), Spotted Thick-knee (*Burhinus capensis*), Domestic Dog (*Canis lupus familiaris*), Serval Cat (*Leptailurus serval*), Sheep (*Ovis aries*), Mountain Zebra (*Equus zebra*), Plains Zebra (*Equus quagga*), Genet (*Genetta* sp.), Side-striped Jackal (*Canis adustus*), Lion (*Panthera leo*), African Wildcat (*Felis silvestris*), Eland (*Taurotragus* sp.), Goat (*Capra aegagrus hircus*), Horse (*Equus caballus*), Cat (*Felis catus*), Cheetah (*Acionyx jubatus*), African Wild Dog (*Lycaon pictus*), Crested Francolin (*Dendroperdix sephaena*), African Grass Owl (*Tyto capensis*), Aardwolf (*Proteles cristatus*), Bat-eared Fox (*Otocyon megalotis*), Black-bellied Bustard (*Lissotis melanogaster*), Southern Pale Chanting Goshawk (*Melierax canorus*), Secretary Bird (*Sagittarius serpentarius*), Greater Kudu (*Tragelaphus strepsiceros*), Gemsbok (*Oryx gazella*), Red Hartebeest (*Alcelaphus caama*), Cape Fox (*Vulpes chama*), African Aardvark (*Orycteropus afer*), Kori Bustard (*Ardeotis kori*).

Rhipicephalus turanicus Pomerantsev, 1940

(Figs. 4.7-4.15)

Synonymy.

Rhipicephalus turanicus Pomerantsev, 1936 *nomen nudum*

Rhipicephalus secundus Feldman-Muhsam, 1952

Rhipicephalus sulcatus Morel and Vassiliades, 1963 *pro parte*

Rhipicephalus turamicus Uzakov 1964 *nomen nudum, lapsus*

Type depository: ZIAC, Lectotype: Male

Type locality: Uzbekistan

Etymology. From geographic distribution in the Turan (Persian, meaning the region north of the Amu-Darya river and east of the Caspian Sea - Uzbekistan and Turkmenistan). Latin –*icus*, adjective (belonging to, derived from).

Material examined. Thirty-six specimens from Turkey, Israel, Egypt, Greece, Afghanistan, Turkmenistan (Table S4.1).

Type material

Types not examined due to museum collection inaccessibility, but samples from Turkmenistan and Afghanistan are taken to represent *R. turanicus* sensu stricto (sensu Filippova, 1997) as they are closest to the type locality in Uzbekistan.

Redescription.

Males (description voucher numbers: OP3255/RD27, OP5198/RD31)
Length 2.8 to 3.0 mm, width 1.6 to 2.2 mm

Basis capitulum hexagonal, short, wide. Lateral angles slightly obtuse projecting at anterior third of length. Cornua present, medium length, rounded. Palps short, sub-triangular. Body reddish-brown. Conscutum elongate, ovate, broadest at coxae IV with anterolateral margin convex. Lateral idiosoma expanded when engorged. Eyes flat, dorsally bordered by a row of medium punctations. Cervical grooves convergent and deep anteriorly, shallow and divergent posteriorly. Cervical fields slightly sunken with few small punctations imparting a smooth appearance, lateral grooves bordered by a row of large, punctations. Marginal grooves enclosing first festoon and reaching level of coxa III, bordered by a row of sparse, large punctations. Posteromedial groove short, oval, rugose. Posterolateral grooves approximately half the length of posteromedial groove, rugose. Small punctations few imparting a smooth appearance, a few large punctations sparsely scattered in four irregular rows as well as on scapulae.

Legs reddish brown, slender, slightly thicker posteriorly. Coxae approximately equal in size. Coxa I elongate with small anterior process and posteromedial spur broad with triangular tip, posterolateral spur narrow, tapering to rounded tip. Coxa II sub-triangular with posteromedial spur broad, flange-like and posterolateral spur moderate size, triangular. Coxa III sub-rectangular with posteromedial spur broad, flange-like and posterolateral spur minute, triangular. Coxa IV sub-rectangular with posteromedial spur moderate size, triangular and posterolateral spur moderate size, narrow, triangular. Adanal plates elongate triangular with slight medial concavity centrally and convexity posterolaterally. Posterior third slightly more long than wide, imparting a slender appearance. Accessory plates short, reaching level of posterolateral convexity. Spiracles elongate, sub-ovate with dorsal prolongation short, broad.

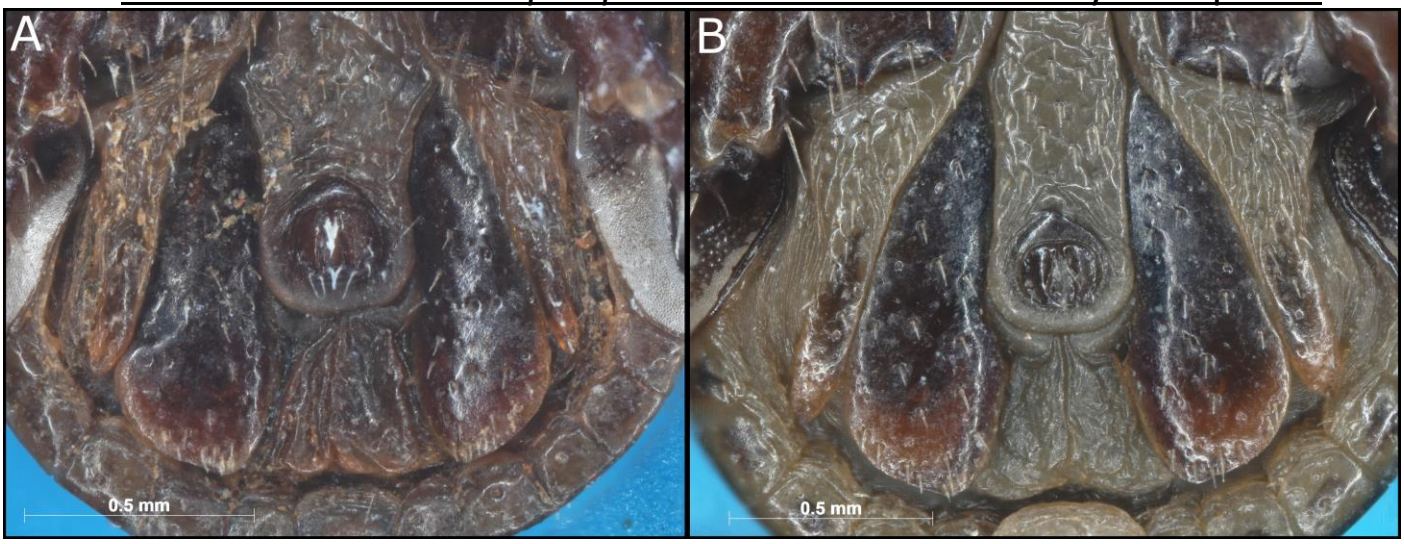


Figure 4.10. Comparative morphology of adanal plates in male *Rhipicephalus afranicus* n. sp. (A) and *Rhipicephalus turanicus* sensu stricto (B). Specimen data: *R. afranicus* n. sp. (A) – OP5172 Holotype, Kaalplaas, vegetation, March 2018; *R. turanicus* sensu stricto (B) – OP3255/RD27, Turkmenistan, *Canis lupus familiaris* (Dog), April 1988.

Females (description voucher numbers: OP3255/RD28, OP5198/RD34)

Length 2.7 to 3.1 mm, width 1.5 to 2.3 mm (Unengorged)

Basis capitulum hexagonal, short, wide. Lateral angles rectangular, projecting at mid-length. Cornua present, short, broadly triangular. Porous areas sub-ovate, moderate size, separated by a distance approximately equal to their width. Palps short, sub-triangular, stalked on article I. Body reddish brown. Scutum elongate, ovate, broadest at mid-length with anterolateral margin convex, posterior margin sinuous with most posterior point distinctly pronounced. Lateral idiosoma expanded when engorged. Eyes flat, dorsally bordered by a row of medium punctations. Cervical grooves convergent and deep anteriorly, shallow and divergent posteriorly. Cervical fields slightly sunken with numerous confluent punctations, not reaching posterolateral scutal margins. Lateral grooves distinct, bordered by a row of large punctations. Small punctations numerous, large punctations sparse across scutum.

Legs reddish brown, slender, slightly thicker posteriorly. Coxae approximately equal in size. Coxa I elongate with posteromedial spur broad with triangular tip, posterolateral spur narrow, tapering to pointed tip. Coxa II sub-rectangular with posteromedial spur short, broad, flange-like and posterolateral spur short, broad, triangular. Coxa III sub-rectangular with posteromedial spur short, broad, flange-like and posterolateral spur minute, narrow, triangular. Coxa IV sub-rectangular with posteromedial spur minute, arcuate and posterolateral spur minute, broad, triangular. Genital aperture widening U-shape with lateral margins distinctly diverging anteriorly. Spiracles broadly sub-triangular with rounded angles, dorsal prolongation short, moderate thickness proximally, tapering to rounded tip distally.

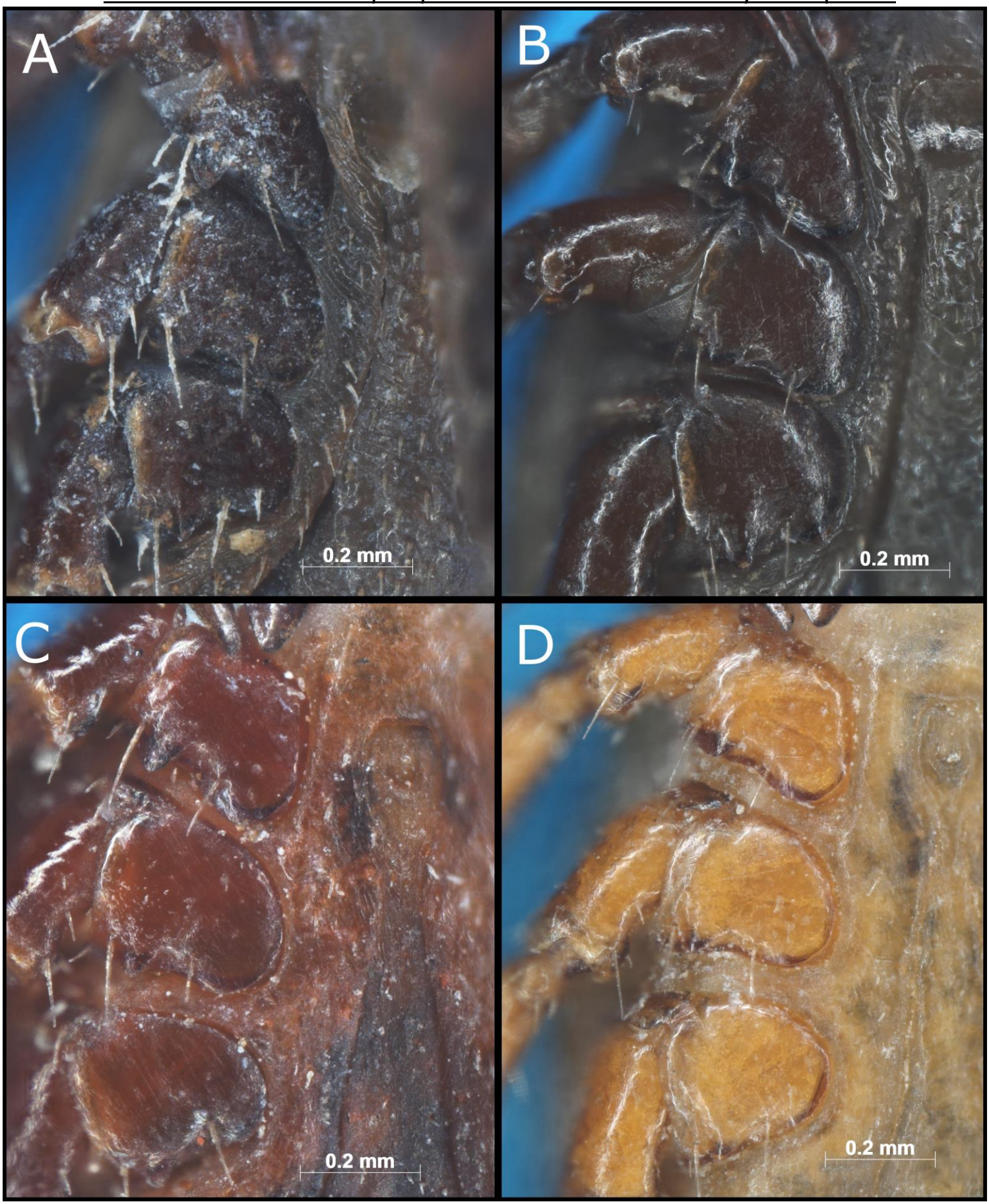


Figure 4.11. Comparative morphology of coxae II-IV in male and female *Rhipicephalus afranicus* n. sp. (A, C) and *Rhipicephalus turanicus* sensu stricto (B, D). Specimen data: *R. afranicus* n. sp. male (A) – OP5172 Holotype, Kaalplaas, vegetation, March 2018; *R. turanicus* sensu stricto male (B) – OP3255/RD27, Turkmenistan, *Canis lupus familiaris* (Dog), April 1988. *R. afranicus* n. sp. female (C) – OP5173 Allotype, Kaalplaas, vegetation, March 2018; *R. turanicus* sensu stricto female (D) – OP3255/RD28, Turkmenistan, *Canis lupus familiaris* (Dog), April 1988.

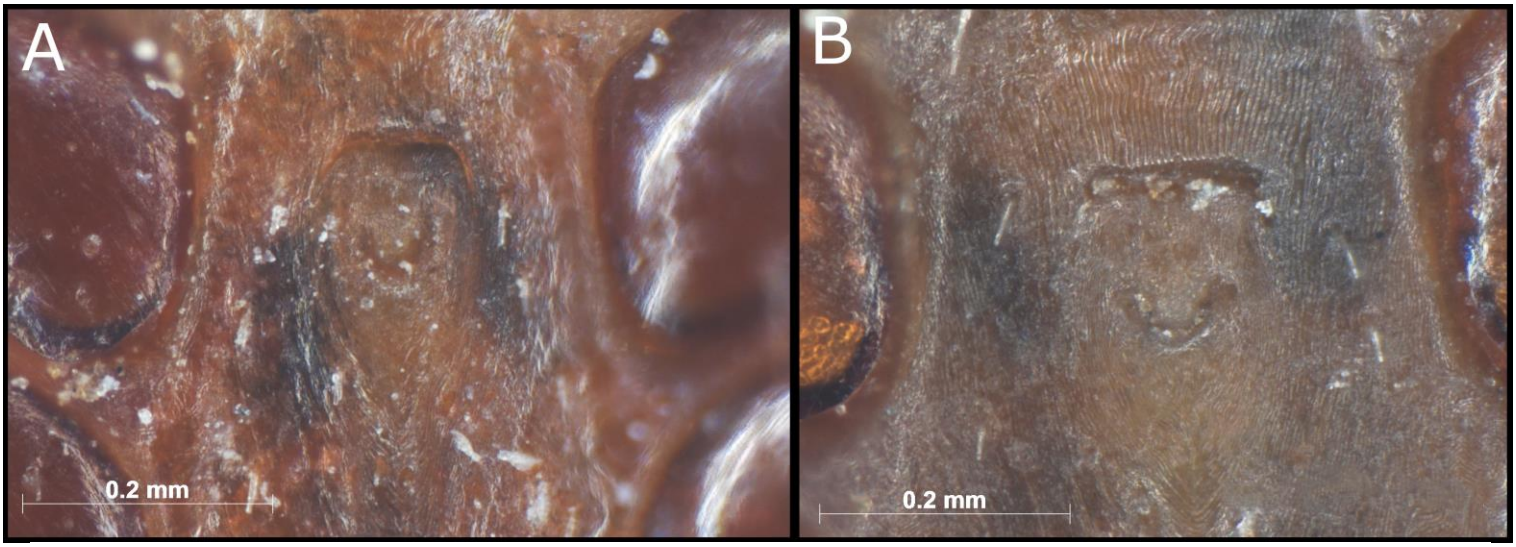


Figure 4.12. Comparative morphology of genital aperture in female *Rhipicephalus afranicus* n. sp. (A) and *Rhipicephalus turanicus* sensu stricto (B). Specimen data: *R. afranicus* n. sp. (A) – OP5173 Allotype, Kaalplaas, vegetation, March 2018; *R. turanicus* sensu stricto (B) – OP3255/RD28, Turkmenistan, *Canis lupus familiaris* (Dog), April 1988.

Nymphs (description voucher numbers: 5 specimens from OP5195)
Length 0.9 to 1.2 mm, width 0.8 to 1.0 mm (Unengorged)

Basis capitulum hexagonal, almost flat, wide. Lateral angles acute, projecting at mid-length. Cornua absent. Palps short, sub-triangular. Body yellowish brown. Scutum broad, circular, widest at posterior third with anterolateral margin convex, posterior margin rounded. Eyes flat, located at posterior third of scutum. Cervical grooves convergent and deep anteriorly, shallow and divergent posteriorly. Cervical fields, broad, slightly sunken, not reaching posterolateral scutal margins. Lateral grooves distinct.

Legs transparent brown, slender. Coxae approximately equal in size. Coxa I sub-rectangular with spurs distinctly separated. Posteromedial spur short, broad, triangular and posterolateral spur long, broad, triangular. Coxa II sub-rectangular with posteromedial spur absent and posterolateral spur minute, triangular. Coxa III sub-rectangular with spurs absent. Coxa IV sub-rectangular with spurs absent.

Larvae (description voucher numbers: 5 specimens from OP5195)
Length 0.4 to 0.6 mm, width 0.3 to 0.5 mm (Unengorged)

Basis capitulum hexagonal, almost flat, width distinctly shorter than posterior margin of scutum. Lateral angles acute, projecting at mid-length. Cornua absent. Palps short, sub-triangular. Body yellowish brown. Scutum broad, triangular, widest posteriorly with anterolateral margin convex, posterior margin rounded. Eyes flat, located at posterior corner of scutum. Cervical grooves convergent and deep anteriorly, shallow and divergent posteriorly. Cervical fields, broad, distinctly sunken, not reaching posterolateral scutal margins. Lateral groove indistinct.

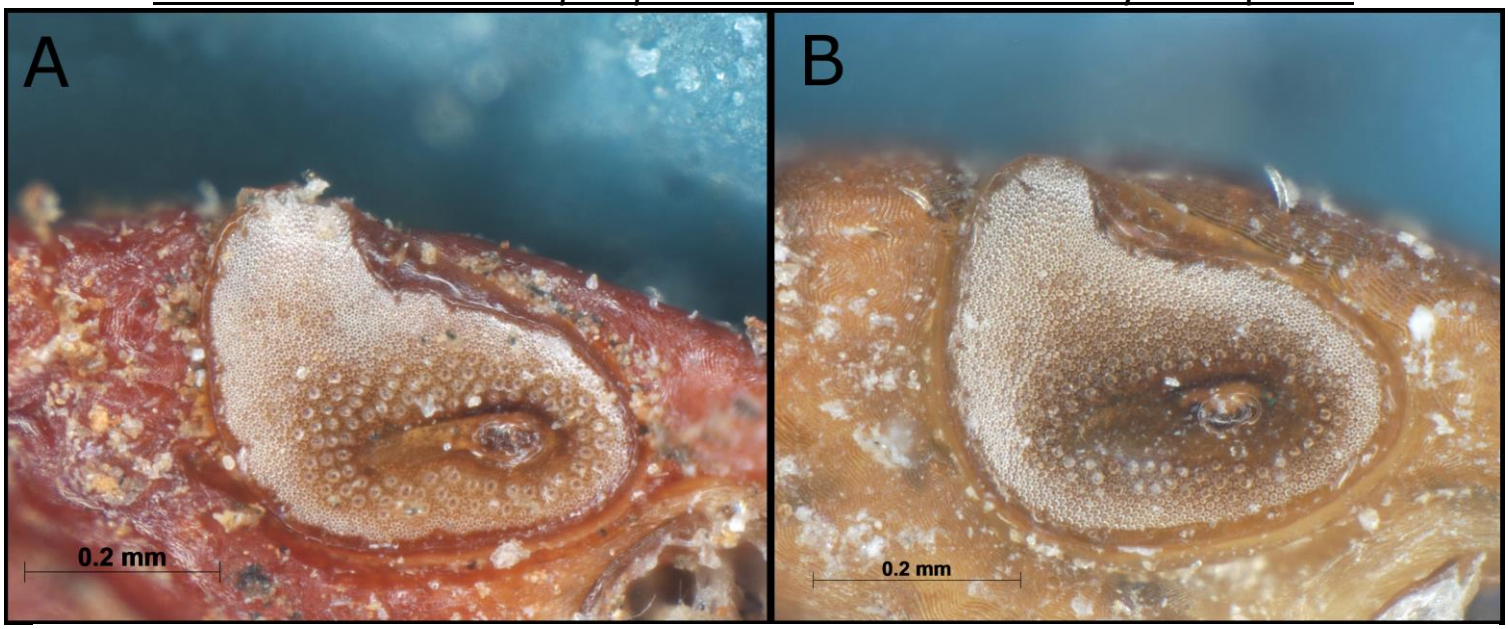


Figure 4.13. Comparative morphology of spiracles in female *Rhipicephalus afranicus* n. sp. (A) and *Rhipicephalus turanicus* sensu stricto (B). Specimen data: *R. afranicus* n. sp. (A) – OP5173 Allotype, Kaalplaas, vegetation, March 2018; *R. turanicus* sensu stricto (B) – OP3255/RD28, Turkmenistan, *Canis lupus familiaris* (Dog), April 1988.

Legs transparent brown, slender. Coxae approximately equal in size. Coxa I sub-rectangular with posteromedial spur moderate size, broad, flange-like and posterolateral spur absent. Coxa II sub-rectangular with posteromedial spur short, broad, triangular and posterolateral spur absent. Coxa III sub-rectangular with posteromedial spur short, triangular and posterolateral spur absent.

Biogeography. Palearctic (Fig. 4.6). Generally, occurring in regions with annual precipitation between 100 mm and 1000 mm. This wide range may be a result of differential arid tolerance in the two lineages observed in *R. turanicus*.

Hosts. Three host life cycle. Host data from Filippova (1997), Walker et al. (2000) and GTTM records. Cattle (*Bos taurus*), Domestic Dog (*Canis lupus familiaris*), Sheep (*Ovis aries*), Goat (*Capra aegagrus hircus*), Cat (*Felis catus*), Donkey (*Equus africanus*), Pig (*Sus scrofa domesticus*), Bactrian Camel (*Camelus bactrianus*), Red Deer (*Cervu elaphus*), Markhor (*Capra falconeri*), East Caucasian Tur (*Capra caucasia cylindricornis*), Urial (*Ovis orientalis vignei*), Black-tailed Gazelle (*Gazella subgutturosa*), Onager (*Equus hemionus*), Wild Boar (*Sus scrofa*), Grey Wolf (*Canis lupus*), Golden Jackal (*Canis aureus*), Red Fox (*Vulpes vulpes*), European Badger (*Meles meles*), Amur Leopard (*Panthera pardus orientalis*), European Wildcat (*Felis silvestris*), Jungle Cat (*Felis chaus*), Marbled Polecat (*Vormela peregusna*), European Hedgehog (*Erinaceus europaeus*), Southern White-breasted Hedgehog (*Erinaceous concolour*), Long-eared Hedgehog (*Hemiechinus auritus*), European Hare (*Lepus europaeus*), Cape Hare (*Lepus capensis*), Long-clawed Ground Squirrel (*Spermophilus leptodactylus*), Yellow Ground Squirrel (*Spermophilus fulvus*), Egyptian Vulture (*Neophron percnopterus*), Short-eared Owl (*Asio flammeus*), Rook (*Corvus frugilegus*),

Hoopoe (*Upupa epops*), Calandra Lark (*Melanocorypha calandra*), Common Blackbird (*Turdus merula*), Brown Rat (*Rattus norvegicus*), Turkestan Rat (*Rattus pyctoris*), Short-tailed Bandicoot Rat (*Nesokia indica*), House Mouse (*Mus musculus*), Wood Mouse (*Apodemus sylvaticus*), Yellow-necked Mouse (*Apodemus flavicollis*), European Snow Vole (*Chionomys nivalis*), Social Vole (*Microtus socialis*), European Water Vole (*Arvicola terrestris*), Grey Dwarf Hamster (*Cricetulus migratorius*), Great Gerbil (*Rhombomys opimus*), Libyan Jird (*Meriones libycus*), Tristram's Jird (*Meriones tristrami*), Persian Jird (*Meriones persicus*), Small Five-toed Jerboa (*Allactaga elater*), Brandt's Hedgehog (*Paraechinus hypomelas*), Gueldenstaedt's Shrew (*Crocidura gueldenstaedtii*), Common Bent-wing Bat (*Miniopterus schreibersii*), Crested Lark (*Galerida cristata*), European Green Lizard (*Lacerta viridis*), Sand Lizard (*Lacerta agilis*), Indian Crested Porcupine (*Hystrix indica*).

Notes. Two lineages are observed among *R. turanicus*. One lineage is distributed in the Middle East/Asia, while the other is in southern Europe (Figs. 4.3, 4.6). These two lineages seem to correspond to differential arid tolerance in these environments based on annual precipitation (Fig. 4.6). These represent separately evolving lineages that may be shown as different species upon further investigation. If true, *R. turanicus* sensu stricto (sensu Filippova, 1997) would be represented by the Middle Eastern/Asian lineage. It is possible that the synonym *R. secundus* Feldman-Muhsam, 1952 represents the southern European lineage because samples associated with this name have been collected from Italy, Israel, Iraq, Turkey, Serbia and France (Feldman-Muhsam, 1953; Feldman-Muhsam, 1952; Hoogstraal, 1956; Paperna and Giladi, 1974).

Differential diagnosis. Males of *R. afranicus* **n. sp.** may be distinguished from *R. turanicus* by (1) adanal plates slightly shorter with posterior third more wide than long [*R. turanicus* = longer with posterior third more long than wide, (2) adanal plates with posteromedial tip indistinct [*R. turanicus* = posteromedial tip slightly distinct], (3) conscutum more punctate [*R. turanicus* = less punctate], and (4) cornua short, broadly triangular [*R. turanicus* = medium length, rounded].

Females of *R. afranicus* **n. sp.** may be distinguished from *R. turanicus* by (1) spiracles with dorsal prolongation thick proximally [*R. turanicus* = thin proximally], (2) spiracles with excavation between dorsal prolongation and spiracle dorsal margin indistinct [*R. turanicus* = excavation distinct], (3) spiracles with dorsal prolongation angled slightly posteriorly [*R. turanicus* = angled near-perpendicularly], (4) scutum with sinuous posterior margin bearing mild pronouncement [*R. turanicus* = distinct pronouncement], and (5) scutum bearing many large punctations among numerous small punctations [*R. turanicus* = few large punctations].

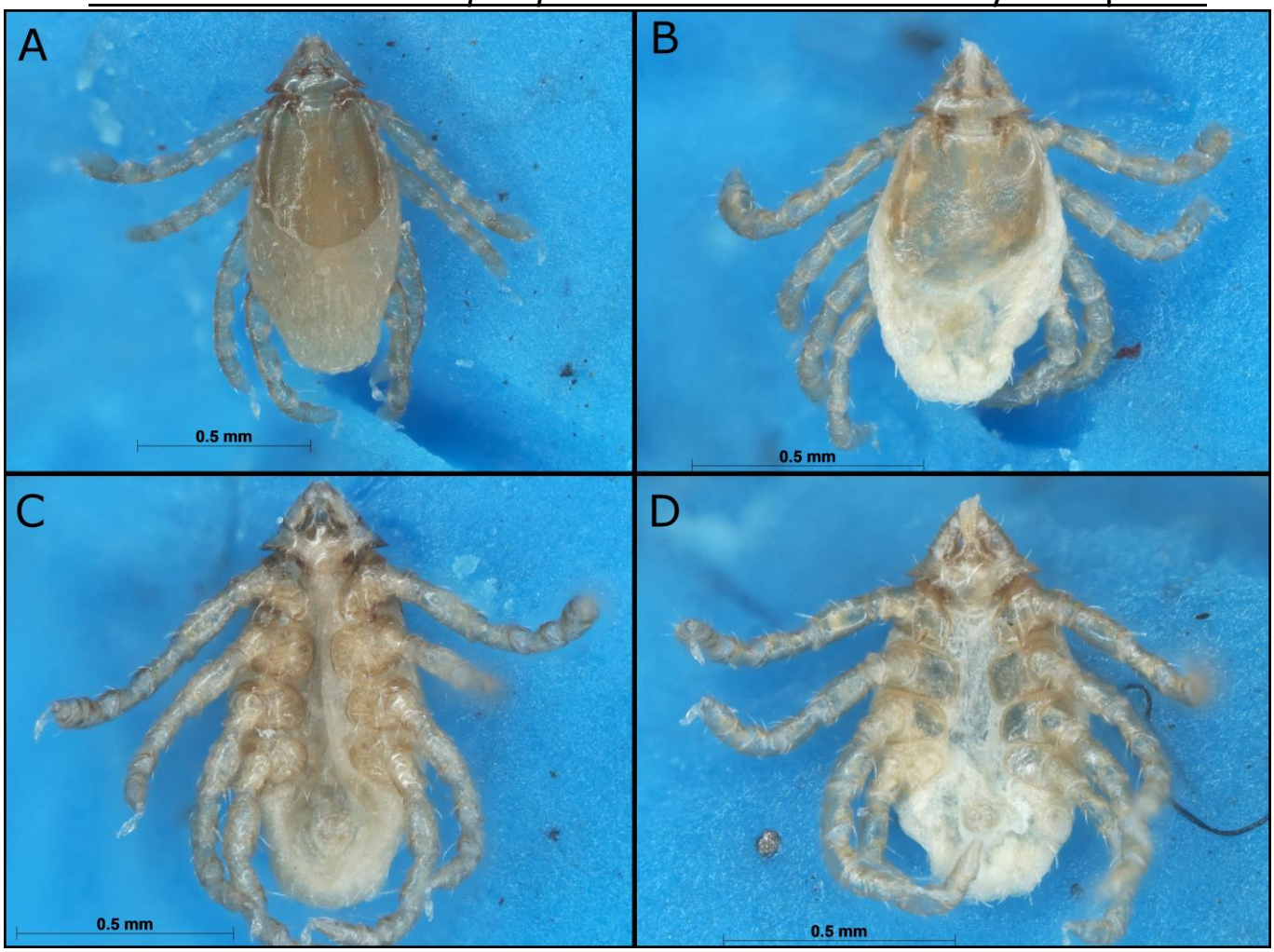


Figure 4.14. Dorsal and ventral habitus photos of nymphal *Rhipicephalus afranicus* n. sp. (A, C) and *Rhipicephalus turanicus* sensu stricto (B, D). Specimen data: *R. afranicus* n. sp. (A, C) – OP5194, Mukulaikwa, Zambia, laboratory reared; *R. turanicus* sensu stricto (B, D) – OP5195, Cyprus, Greece, laboratory reared.

Nymphs of *R. afranicus* **n. sp.** may be distinguished from *R. turanicus* by (1) scutum elongate, ovate [*R. turanicus* = broad, ovate], (2) cervical fields narrow, distinctly sunken [*R. turanicus* = broad, slightly sunken], and (3) coxa I with spurs moderately separated [*R. turanicus* = distinctly separated].

Larvae of *R. afranicus* **n. sp.** may be distinguished from *R. turanicus* by (1) basis capitulum width almost equal to posterior margin of scutum width [*R. turanicus* = distinctly shorter than posterior margin], (2) scutum narrow triangular [*R. turanicus* = broad triangular], and (3) cervical fields narrow, slightly sunken [*R. turanicus* = broad, distinctly sunken].

Differential disease relationships.

Rhipicephalus afranicus **n. sp. –** Experimentally shown as a vectors of *Babesia trautmanni* to domestic pigs in South Africa (Lopez-Rebollar & De Waal, 1994), referring to *R. afranicus* **n. sp.**. However, *R. afranicus* **n. sp.** do not readily parasitize pigs in Africa, making this finding potentially more applicable to *R. turanicus* in Europe, but requires further vector competency testing.

Rhipicephalus turanicus sensu lato **–** suspected vector of *Babesia caballi* and *Theileria equi* to horses in Europe (Enigk, 1943; Friedhoff, 1988). South African ticks were unsuccessful in transmitting infections to horses (Potgieter et al., 1992). As such, this finding refers to *R. turanicus* sensu lato in Europe. Experimentally shown as a vector of *Babesia canis* to domestic dogs in India (Achuthan et al., 1980), and of *Hepatozoon canis* to dogs in Italy (Gianelli et al., 2016). Implicated as a vector of Q fever and Siberian tick typhus in Europe and the Middle-East (Balashov & Daiter, 1973; Berdyev, 1980). Whether these refer to the southern European, Middle-Eastern/Asian or both lineages in each case is uncertain.

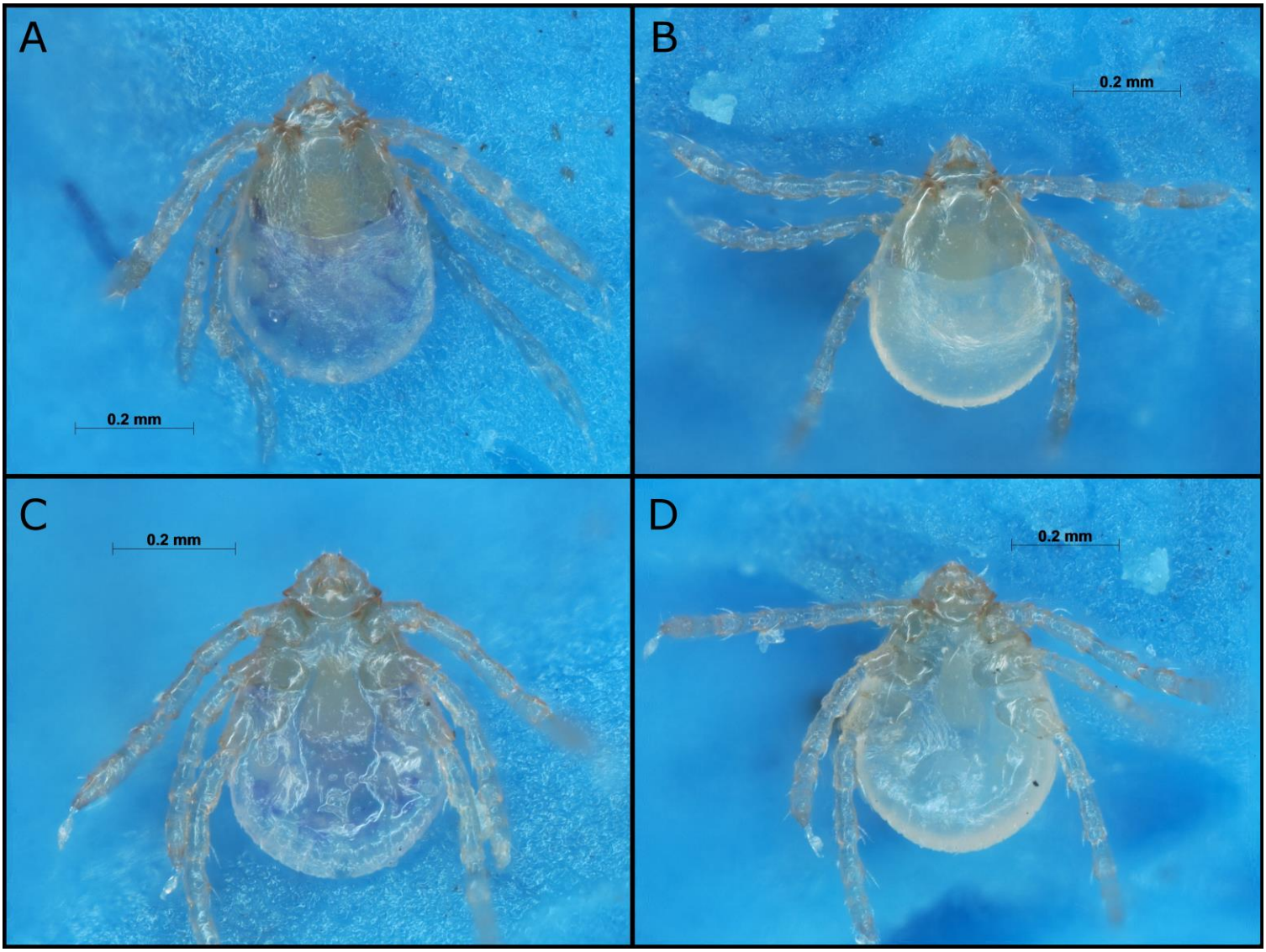


Figure 4.15. Dorsal and ventral habitus photos of larval *Rhipicephalus afranicus* n. sp. (A, C) and *Rhipicephalus turanicus* sensu stricto (B, D). Specimen data: *R. afranicus* n. sp. (A, C) – OP5194, Mukulaikwa, Zambia, laboratory reared; *R. turanicus* sensu stricto (B, D) – OP5195, Cyprus, Greece, laboratory reared.

Discussion

Phylogenetic relationships based on 12S and 16S rDNA sequence data provided evidence for at least two distinct lineages among what are traditionally known as *R. turanicus* (Figs. 4.1-4.3). As such, the hypothesis of *R. turanicus* as a single monophyletic species is refuted. To deal with the resulting paraphyly, a new taxon, *R. afranicus* n. sp., is established to represent all Afrotropical *R. turanicus*. Pairwise genetic distance data for both 12S and 16S rDNA are below 95% similarity for these two clades. This value is generally considered a threshold of conspecificity for these genes in ticks (Chitimia-Dobler et al., 2017b; Bakkes et al., 2018b; Lado et al., 2018; Li et al., 2018; Mans et al. 2019). More comprehensive sampling of *R. turanicus* in the current study corroborates the observations of Dantas-Torres et al. (2013) and Coimbra-Dores et al. (2018) based on similar molecular data. This species boundary was tested against qualitative morphology (traditional) and quantitative analysis of shape outlines in female spiracles and male adanal plates (Figs. 4.4, 4.5). All of these lines of evidence confirmed *R. afranicus* n. sp. as a distinct species. Furthermore, two lineages among *R. turanicus* were recovered (Figs. 4.1-4.3) that appear to correlate with two different regions, as well as two regimes of annual rainfall in southern Europe and the Middle East/Asia (Fig. 4.6). These may prove to be distinct species upon further investigation and may refer to the synonym *R. secundus*. Samples associated with the name *R. secundus* have been collected from Italy, Israel, Iraq, Turkey, Serbia and France (Feldman-Muhsam, 1952, 1953; Hoogstraal, 1956; Paperna and Giladi, 1974). For the purpose of this study however, the focus is testing the species boundary of Afrotropical populations of *R. turanicus* against Palearctic *R. turanicus* sensu lato, thus we refrain from examining the possibility of two species within Palearctic *R. turanicus*. We provide full descriptions for both *R. afranicus* n. sp. and *R. turanicus* sensu stricto (Middle-Eastern/Asian lineage).

Disjunct distribution patterns between *R. afranicus* n. sp. and *R. turanicus* sensu lato (Fig. 4.6) support a species boundary based on allopatric speciation (Mayr, 1947). An obvious geographic barrier separating these two tick lineages is the Sahara desert which probably served as the vicariant agent for speciation approximately 5-7 million years ago (Zhang et al., 2014; Schuster et al., 2006). However, this region is characterized by humid and arid cycles, and the oscillating climatic history of the Sahara can result in complex evolutionary patterns (Gonçalves et al., 2018). Indeed, generalist tick species (e.g. *Hyalomma*) that utilize a variety of hosts for dispersal, do not show consistent vicariance patterns across the Sahara desert and, instead, several intercontinental dispersals between the Afrotropics and Palearctic have been shown (Sands et al., 2017b). If this holds true for *R. afranicus* n. sp. and *R. turanicus* sensu lato, it implies the initial expansion of the Sahara desert (7-5 million years ago) acted

as a catalyst for initial divergence, followed by population re-integration events driving reticulate evolution as a result of oscillating climatic history.

Reproductive potential between *R. turanicus* from Cyprus and *R. afranicus* n. sp. from Zambia under laboratory conditions (Pegram et al., 1987b) challenges the species boundary proposed here. Fertile hybrids display marked heterosis and increased fecundity which produces approximately 20% more offspring than same-population matings do (Pegram et al., 1987b). However, it is uncertain whether or not these were bona fide *R. turanicus* samples (Guglielmone et al., 2014). The morphology of *R. afranicus* n. sp. is an exact match with Zambian *R. turanicus* in Pegram et al. (1987b), but the morphology between *R. turanicus* sensu stricto (Middle East/Asia) is a close, but not exact, match with Cypriot *R. turanicus* in Pegram et al. (1987b) (Figs. 4.7-4.10, 4.12-4.15). These Cypriot *R. turanicus* likely refer to the southern European lineage recovered among *R. turanicus* sensu lato (Figs. 4.1-4.3). As such, further investigation is required to determine the exact species status of *R. turanicus* sensu lato in the Palearctic. Nevertheless, hybridization between *R. afranicus* n. sp. from Zambia and at least one lineage of *R. turanicus* sensu lato has been demonstrated (Pegram et al., 1987b).

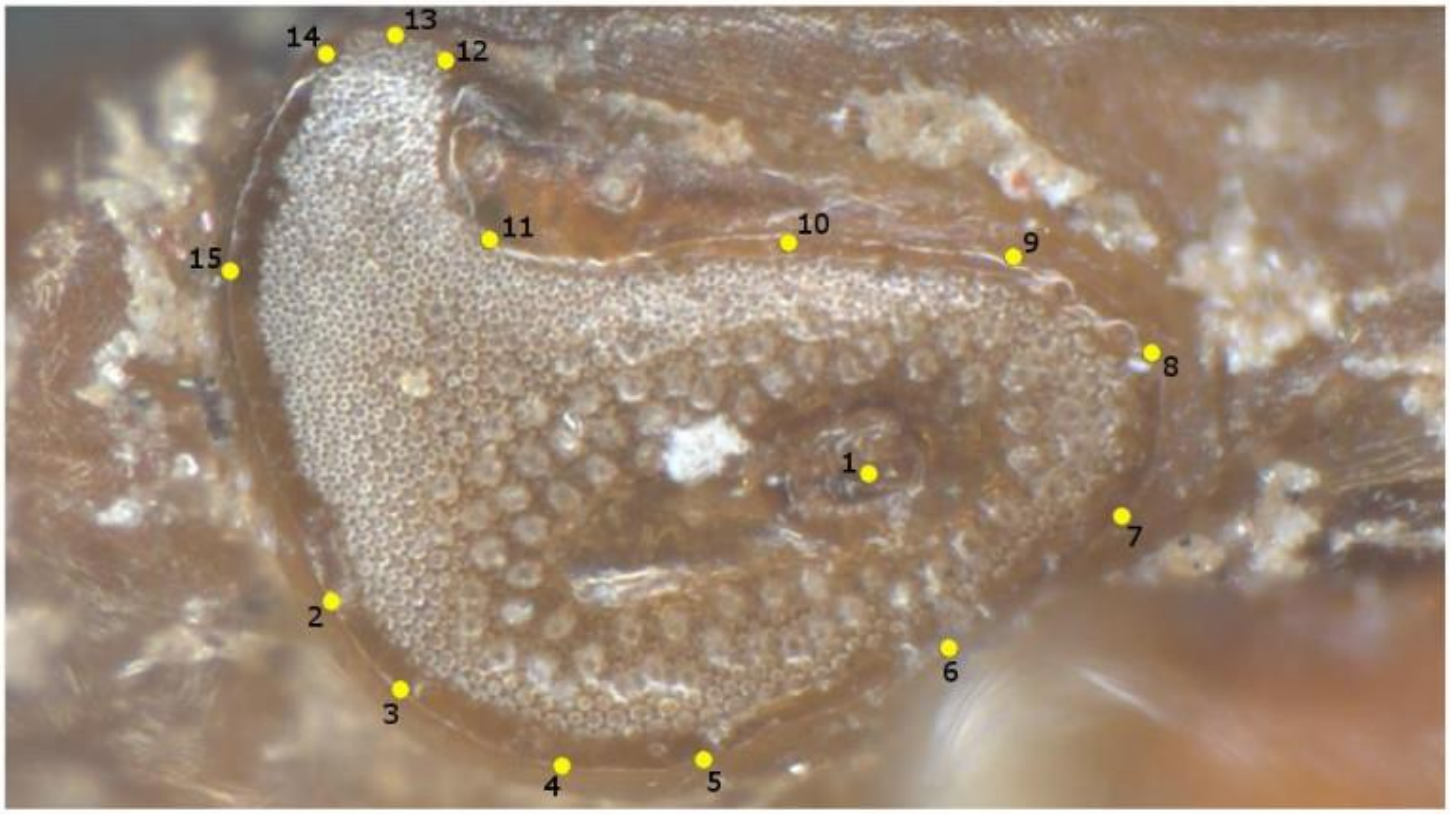
Hybridization in ticks is not well understood, yet fertile hybrids between closely related tick species outside of the *R. sanguineus* group have been documented before (Zivkovic et al., 1986; Rees et al., 2003; Kovalev et al., 2015). Within the *R. sanguineus* group, fertile hybrids have been observed in matings between lineages from North America and the Mediterranean, but these lineages produce infertile hybrids when mated with Afrotropical lineages (Levin et al., 2012). Similarly, lineages from Argentina and Brazil produce infertile hybrids (Szabó et al., 2005), as do Brazilian and French lineages (Nava et al., 2018). These reproductive incompatibilities are in line with distinct *R. sanguineus* group lineages distributed in temperate and tropical regions where Brazilian and African samples are closely related, and conversely Argentinian, North American and European samples are closely related (Szabó et al., 2005; Coimbra-Dores et al., 2018). Moreover, *R. turanicus* and the closely related *R. sulcatus* produce infertile hybrids (Pegram et al., 1987b). This makes fertile hybrids produced by *R. afranicus* n. sp. from Zambia and *R. turanicus* sensu lato from Cyprus highly unusual for the *R. sanguineus* group. Such anomalous hybridization suggests recombination of these two geographically distant and genetically distinct lineages leads to hybrid vigour (Edmands, 2002; Matter et al., 2014). However, for this process to cross distinctly divergent species boundaries is puzzling (Figs. 4.1-4.3), and suggests there may be unknown pre- or post-zygotic reproductive mechanisms occurring at the cellular level that remain compatible between these lineages despite evolutionary divergence. This warrants further investigation into gamete

compatibility, oogenesis and cytogenetics that form pre- and post-zygotic reproductive mechanisms between these species.

Given corroboration of the four independent lines of evidence in this study (mtDNA, male adanal plates, female spiracles and disjunct distribution), it is reasonable to consider *R. afranicus* n. sp. as a distinct and diagnosable species (De Queiroz, 2007) which is able to hybridize with at least one lineage among *R. turanicus* sensu lato under laboratory conditions. Pre- and post-zygotic reproductive isolation is expected to evolve faster when driven by direct selection rather than neutral evolution and drift (Edmands, 2002). As such, an alternative explanation for these two species must consider their hybridization potential due to (i) insufficient evolutionary time for development of pre- or post-zygotic reproductive barriers, and (ii) a lack of direct selection against hybrids that would differentiate pre- and post-zygotic reproductive mechanisms (Edmands, 2002; De Queiroz, 2007). These may be due to allopatric speciation, driven by Sahara desert expansion, which would form an environmental barrier to the formation of hybrids before a physiological barrier would evolve, limiting selection against hybrids during speciation (Edmands, 2002; Douady et al., 2003). An additional consideration is the oscillating climatic history in the Sahara desert (Gonçalves et al., 2018) which would have facilitated population reintegration events after initial divergence. Taken together, a lack of direct selection against hybrids to conserve reproductive mechanisms following rapid allopatry (Douady et al., 2003) likely acted in concert with fluctuating allopatric divergence and population re-integration events driven by environmental oscillation (Gonçalves et al., 2018). This would drive reticulate evolution between *R. afranicus* n. sp. and *R. turanicus* sensu lato to maintain compatibility of reproductive mechanisms and facilitate hybridization potential even after divergence.

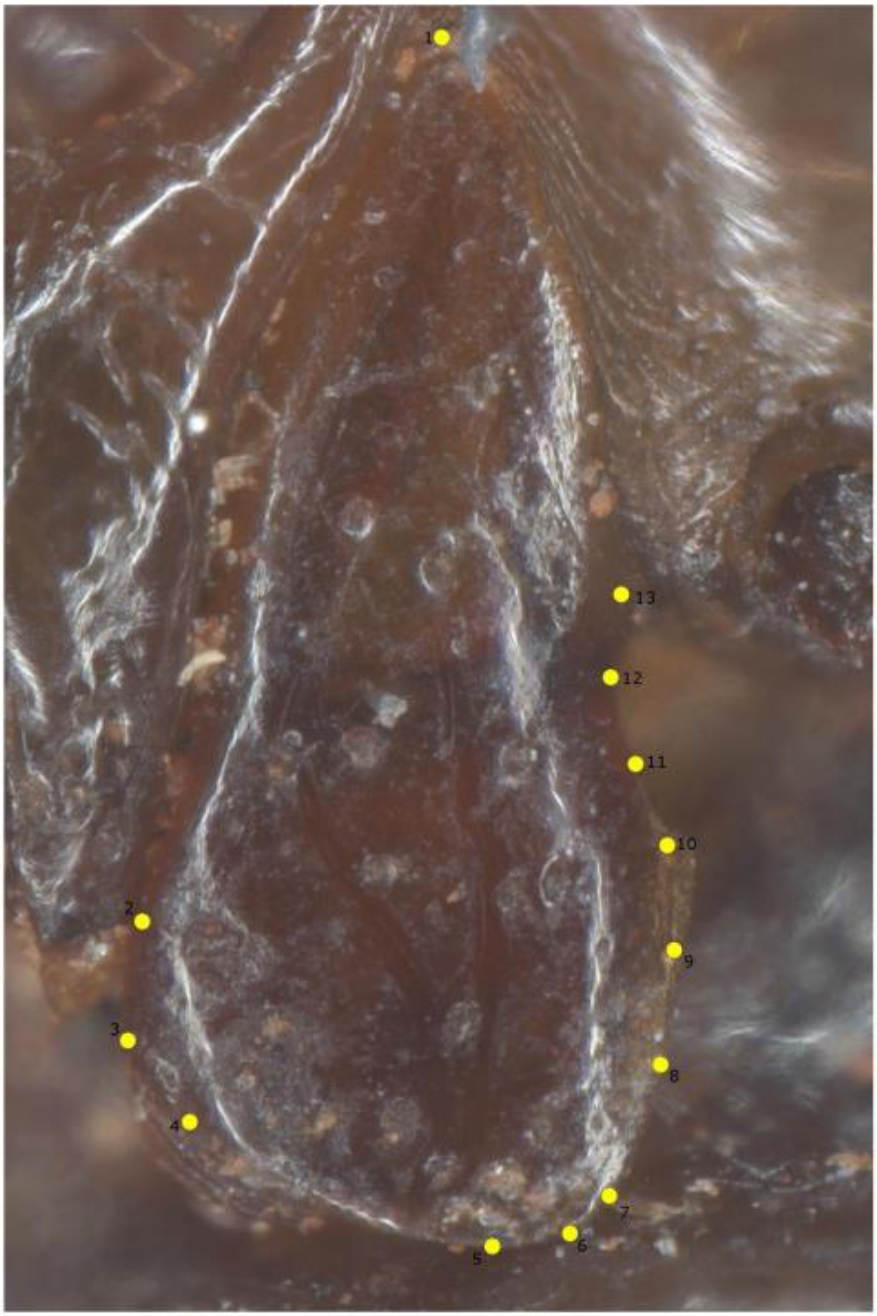
These findings indicate that speciation in ticks might be complicated by localized historical introgression events during divergence (Maddison, 1997; Arnold, 2004). As such, evolution may have been reticulate, where lineages diverged and recombined due to rapid changes in external factors such as climate and host shifts before divergence finalized (incomplete lineage sorting). More recently, global human-mediated movement of *R. sanguineus* group ticks associated with dogs might also play a role (Gray et al., 2013). In either case, introgression (both recent and historical) might explain current confusion associated with morphologically cryptic species among ticks that have highly structured lineages. As demonstrated in this paper, future studies on *R. sanguineus* group taxonomy should employ multiple independent lines of evidence in combination with tracing causality of evolutionary events in order to stabilize the taxonomy of this group.

SUPPLEMENTARY INFO:



1- Centre of operculum
2- Left edge of spiracle plate, along horizontal axis from operculum
3- Start of curve on lower left of spiracle plate
4- Inflection of curve on lower left of spiracle plate
5- Termination of curve on lower left of spiracle plate
6- Lower edge of spiracle plate, along vertical axis from operculum
7- Start of curve on right of spiracle plate
8- Inflection of curve on right of spiracle plate
9- Termination of curve on right of spiracle plate
10- Upper edge of spiracle plate, along vertical axis from operculum
11- Inner inflection of dorsal tail
12- Tip of dorsal tail, start of curve
13- Tip of dorsal tail
14- Tip of dorsal tail, termination of curve
15- Outer inflection of dorsal tail

Figure S4.1. Operational definitions for female spiracle landmarks.



1- Upper tip of adanal plate
2- Left edge of adanal plate, start of curvature
3- Left edge of adanal plate, inflection of curvature
4- Left edge of adanal plate, termination of curvature
5- Lower right edge of adanal plate, start of curvature
6- Lower right edge of adanal plate, inflection of curvature
7- Lower right edge of adanal plate, termination of curvature
8- Right edge of adanal plate, start of convex curvature
9- Right edge of adanal plate, inflection of convex curvature
10- Right edge of adanal plate, termination of convex curvature
11- Right edge of adanal plate, start of concave curvature
12- Right edge of adanal plate, inflection of concave curvature
13- Right edge of adanal plate, termination of concave curvature

Figure S4.2. Operational definitions for male adanal plate landmarks.

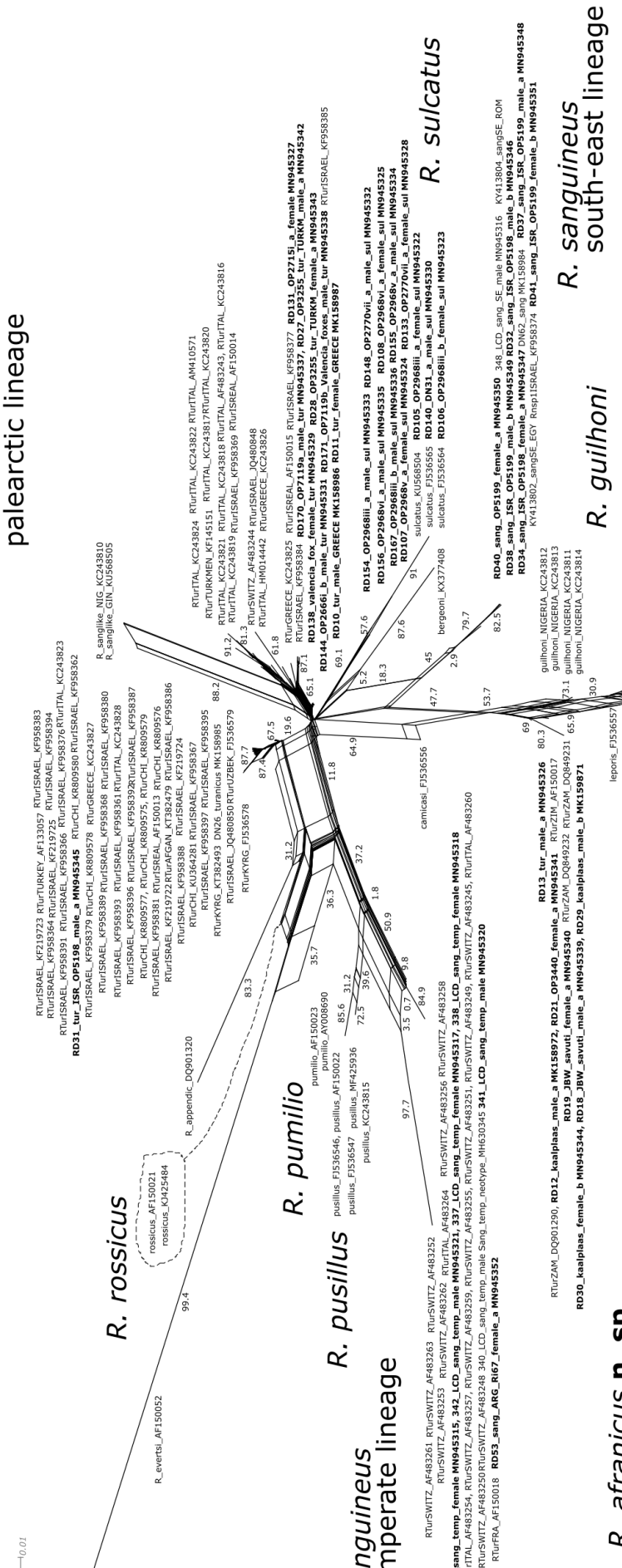


Figure S4.3. Fully labelled phylogenetic splits tree analysis of the *Rhipicephalus turanicus* group 12S rDNA gene using the TN+F+G4 nucleotide substitution model. Indicated are species and lineage names, GenBank accession numbers or study codes (reflected in Supplementary Table S1), country of origin and neighbour network bootstrap support values. Bolded samples were used iteratively in morphometric analysis.

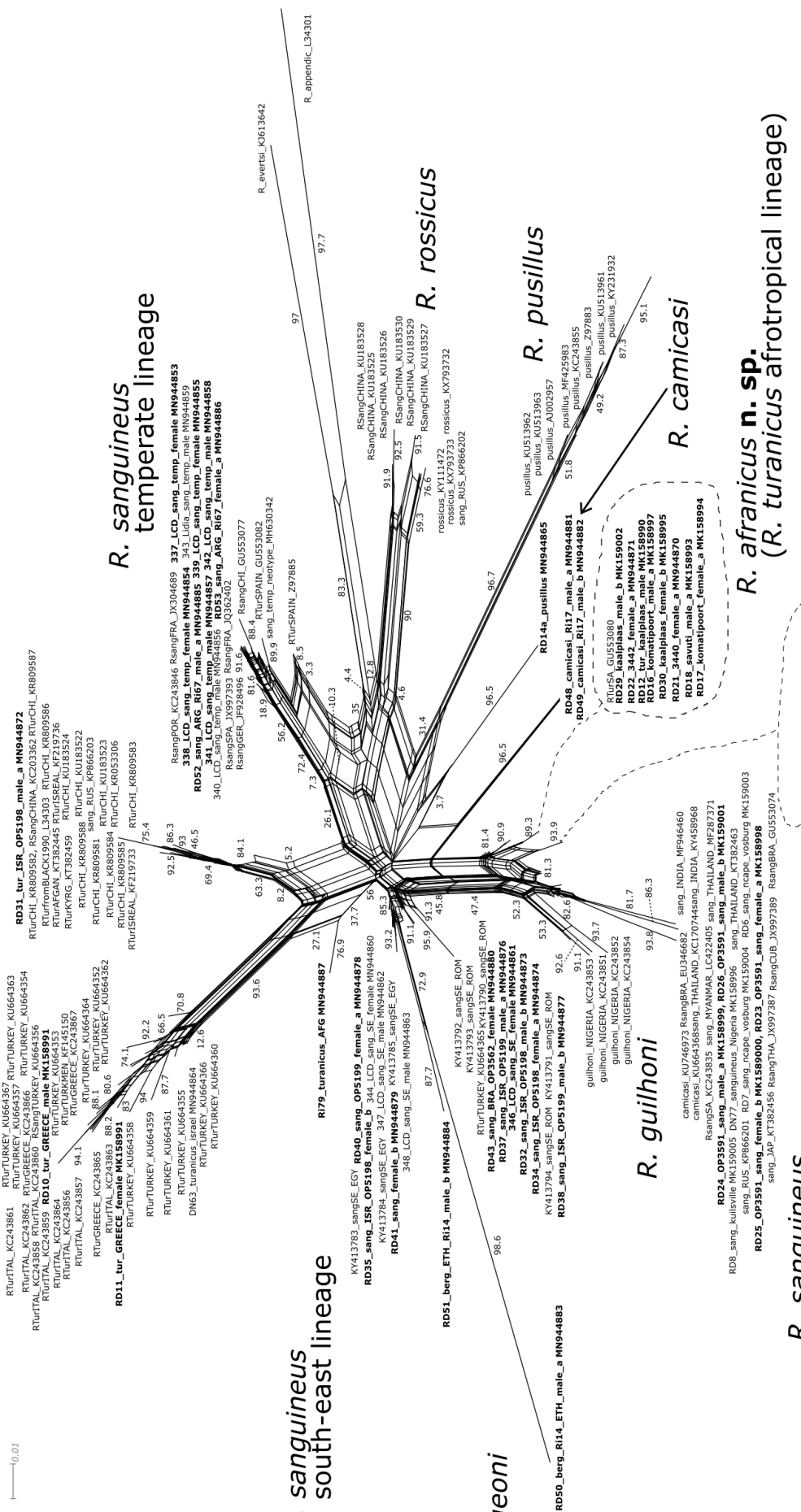


Figure S4.4. Fully labelled phylogenetic splits tree analysis of the *Rhipicephalus turanicus* group 16S rDNA gene using the TPM3u+F+G4 nucleotide substitution model. Indicated are species and lineage names, GenBank accession numbers or study codes (reflected in Supplementary Table S1), country of origin and neighbour network bootstrap support values. Bolded samples were used iteratively in morphometric analysis.

5 General Discussion, Key Questions Answered and Future Research

Rhipicephalus evolution is defined by distinct elements of morphology, development, ecology and biology (specifically life cycles for the purpose of this study) that are interrelated in a complex manner, and united by a phylogeny based on descent with modification (Darwin, 1859). This work integrates some key elements that define this process in order to reconstruct evolutionary history such that we may understand some of the main themes present in *Rhipicephalus* evolution. Key questions were defined to investigate *Rhipicephalus* evolution and morphological shape ontogeny (development), and their answers highlight important themes for *Rhipicephalus* evolution, development and ecology. These are listed below:

Chapter Two – *Rhipicephalus* Phylogeny and Historical Biogeography:

1) What are the phylogenetic relationships between *Rhipicephalus* species and groups?

Answer: Phylogenetic relationships are mostly congruent with Murrell *et al.* (2001), however pulchellus group species are considered basal given their position in the basal polytomy of *Rhipicephalus*, their short branch length from stem-*Rhipicephalus* to extant species, and their similar morphology to sister genera *Rhipicentor*, *Cosmiomma* and *Dermacentor*.

2) Are the nine sub-generic groups of Walker et al. (2000) monophyletic?

Answer: The nine species groups are generally monophyletic, with exceptions being pulchellus group species that are distinct from appendiculatus group species (these two groups are monophyletic *sensu* Walker et al. 2000), as well as *R. duttoni* and *R. nitens* that are closer to pravus group species than to appendiculatus group species (these species are monophyletic *sensu* Walker et al. 2000).

3) Do ancestral area and divergence time estimates indicate an African origin for *Rhipicephalus*?

Answer: Divergence time estimates post-date the breakup of Gondwanaland (Beati and Klompen, 2019; Mans et al., 2019), and ancestral area estimates place the most likely region of *Rhipicephalus* origin in central Africa based on geographic ranges of species in basal clades.

4) Does (i) host-use, (ii) climate or (iii) geography correlate best with *Rhipicephalus* phylogeny?

Answer: *Rhipicephalus* phylogeny is correlated with a complex set of factors. These include, but are likely not limited to, four climatic variables of temperature variation, host-use patterns at immature and adult life stages (including off-host periods), and geography by means of host-linked dispersal.

5) To what degree are climate niches conserved in *Rhipicephalus*?

Answer: Climatic niches are conserved at subgeneric/species group level, and with four variables pertaining to temperature variation.

Chapter Three – Evolutionary-Developmental Modification in *Rhipicephalus*:

1) Does selection affect post-embryonic basis capitulum evolutionary-development?

Answer: Selection factors are present in basis capitulum evolutionary-development. The primary factor is host skin stiffness that initiates selection pressure for different basis capituli sizes and shapes at different life stages. Large basis capituli, relative to other species at the same life stage, have large hydrostatic cavities that are required to bore into the stiff skin of large hosts. A secondary factor is overall body size that has a concomitant effect on the size of basis capituli. Overall body size is partially determined by selection to avoid dehydration in off-host environments, and to avoid predation/grooming in on-host environments.

2) Is there signal for paedomorphosis (neoteny), peramorphosis (recapitulation) or no evolutionary-developmental modification between life stages among the 24 species of *Rhipicephalus* studied?

Answer: Signal for paedomorphosis (neoteny) is present in two clades that have host-truncate life cycles. Signal for no evolutionary-developmental modification is predominant in all other clades. As such, no signal for peramorphosis was recovered, indicating recapitulation is not present in post-embryonic life stages of *Rhipicephalus*.

Chapter Four – *Rhipicephalus turanicus* species delimitation:

1) Do molecular markers support a monophyletic *R. turanicus*?

Answer: 12S and 16S rDNA markers support two distinct clades of *R. turanicus* in Afrotropical and Palearctic regions, with two sub-lineages among Palearctic *R. turanicus*.

2) Do female spiracle and male adanal plate shapes support a monophyletic *R. turanicus*?

Answer: Shape variation in female spiracles and male adanal plates support two distinct clades of *R. turanicus*, one in the Afrotropical region and another in the Palearctic region.

General Discussion

This work has demonstrated that *Rhipicephalus* evolution is underpinned by a complex set of factors owing to the life cycles of ticks that subject them to a large variety of conditions and selection pressures. These include host-use patterns at immature and adult life stages that are important for dispersal events, and which emerged as responses to novel ecological opportunities that arose with mammal radiations and dispersals. Host switches may have been facilitated by off-host periods, which probably provided flexibility in host-use and proved crucial for tick survival as host groups appeared or went extinct. Basis capitulum mouthpart morphology that enables attachment to stiff host skin of large hosts at early life stages mediates host-use patterns in some *Rhipicephalus* clades. Attachment to large hosts at early life stages may have offered survival advantages to reduce predation/grooming and ex-sanguination, and which also facilitated dispersal and geneflow given large hosts are generally

more mobile. Some of these clades went on to evolve host-truncate life cycles where immatures develop completely or partially on their hosts instead of dropping off to moult. Host-truncate life cycles provide survival advantages that further reduce dehydration risk, as well as facilitate dispersal and geneflow. However, this is an evolutionary specialisation that may limit host-use flexibility in the event of emergent ecological change. Host-truncate life cycles may have been facilitated by retention of immature features throughout development, or until the first off-host period, which produces paedomorphosis (Klingenberg, 1998) and enabled ticks to attach to large hosts. If true, this would have provided selective advantages by increasing the set of possible attachment sites to include areas of stiff host skin (selection for large basis capituli), as well as reducing predation/grooming risk on-host (selection for small body size). The latter may be especially pronounced in subgenus *Boophilus*, and likely explains their distinct morphology among *Rhipicephalus* (Barker and Murrell, 2004). After the first off-host moult however, two- and three-host species that use large hosts at early life stages, must quest and risk exposure to dehydration. Hypothetically, selection favours large individuals that can better maintain water balance, and accelerated development (Klingenberg, 1998) was selected for in later life stages of these species. Accumulation of these numerous survival advantages for host-truncate species may explain their success and wide geographic ranges. In contrast, a second evolutionary response also emerged which involved using small hosts at early life stages. These hosts probably emerged as a viable niche for three-host tick species because higher host activity and abundance reduces dehydration risk of immatures. Moreover, thinner host skin may bypass selection pressure for large basis capitulum phenotypes that were selected for in other species that use large hosts at early life stages. In some unique cases, this strategy resulted in some *Rhipicephalus* species becoming specially adapted for using only small hosts at all life stages (such as *Rhipicephalus distinctus*).

Host-linked dispersal may have proved crucial for *Rhipicephalus* diversification and speciation where dispersing progenitors established populations in novel environments along gradients in annual, seasonal and diurnal temperature variation that initiate niche partitioning by differential approximation to the ancestral niche (Kozak and Wiens, 2010). If true, this would have partially driven divergence between populations and lineages (along with host-use patterns and competition), that eventually formed distinct species. Hosts for adult life stages may be more important for this process because adults generally attach to large hosts that are geographically mobile (Horak et al., 2018; Walker, 2014; Walker et al., 2000). If true, this would be especially important among three-host species that use small and less mobile hosts at early life stages. However, in clades that attach to large hosts at early life stages (including host-truncate clades), the effect of host-linked dispersal is probably enhanced and facilitates

range expansion to establish wide geographic populations. Moreover, increased host mobility facilitates gene flow (Matthee, 2020), which may counteract the effect of niche partitioning and limit speciation in these groups. Indeed, clades that use large hosts at early life stages tend to be less species-diverse, and have wider geographic ranges. In contrast, clades that use small hosts at early life stages tend to be more species-diverse and have small geographic ranges. Moreover, in host-truncate species, these geneflow and host-linked dispersal processes are enhanced even further due to reduced exposure to off-host environments. Additionally, competitive interactions between sister species modulate geographic ranges (Cangi et al., 2013) that are partially produced by these combined processes, and may reinforce niche partitioning to drive additional divergence between populations to eventually form distinct species.

The test for phylogenetic recapitulation in post-embryonic basis capituli of *Rhipicephalus* ticks indicated no signal for peramorphosis, which is a prerequisite for diagnosing phylogenetic recapitulation (Chapter 1). This refutes the possibility of any condensing selection to produce developmental phenotypes that resemble ancestral adults (Clune et al., 2012; Gould, 1977). Instead, morphological variation through development was linked to selection pressures in on- and off-host environments. Nevertheless, it remains possible that phylogenetic recapitulation occurs in the background of developmental evolution during post-embryonic life stages, but is obfuscated by the action of selection which favours certain phenotypes for certain environments. As such, it may be worthwhile to investigate this hypothesis using similar methods in embryonic life stages instead (Domazet-Lošo and Tautz, 2010; Kalinka and Tomancak, 2012). Alternatively, it may be worthwhile to consider higher phylogenetic order comparisons which involve greater phylogenetic distances, given that condensing selection and phylogenetic recapitulation is expected to occur after significantly long periods of evolution. Moreover, future investigations should consider other morphological features that are less functional and expected to undergo less selection, such as spiracles or scutae.

Comparative studies based on phylogeny place biodiversity within a phylogenetic context and highlight phylogenetic structure that may have been passed over previously (Dayrat, 2005; Padial et al., 2010). This treatment of biological variation takes species-relative variation into account and integrates data across multiple lines of evidence to facilitate clear delimitation of groups that form fundamental building blocks of biodiversity (Yeates et al., 2011). This was illustrated in the investigation for species boundary among Afrotropical and Palearctic *R. turanicus*. Multiple lines of evidence detected congruent patterns between populations that suggest a natural break in reproducing lineages, indicating a species boundary between Afrotropical and Palearctic *R. turanicus*. As such, a new species, *R.*

afranicus, was described to represent Afrotropical elements. The species boundary was geographically situated in the Sahara Desert, suggesting allopatric divergence drove speciation as a result of expansion of the Sahara Desert. However, interbreeding is possible between these species (Pegram et al., 1987b), indicating the biological species boundary is semi-permeable (Harrison, 1990). This may have occurred as a result of population divergence and re-integration events during speciation, due to oscillating climatic conditions (Gonçalves et al., 2018). Lines of evidence that delimit species were based on modern methods used to study molecular, morphological and geographic variation. It is important to delimit and describe species that form biodiversity in a way that accurately represents reproducing lineages along phylogenetic branches (De Queiroz, 2007). Biological processes tend to become shared within clades as a result of descent with modification in phylogenetic hierarchy. As such, adopting this approach to species delimitation and description ensures predictive power of taxonomic names is maximised such that biological processes are accurately represented. As such, taxonomic names can serve as the ‘keys’ to unlock doors to information that are stored in an ordered manner to enable effective downstream usage.

ADDENDUM

6 Evaluation of Measurement Error in Rotational Mounting of Larval *Rhipicephalus* (Acari: Ixodida: Ixodidae) Species in Geometric Morphometrics

Published in *Zoomorphology,* 136(3): 403-410.

Abstract

Geometric morphometric analysis, in combination with modern computational power, allows for precise measurements of morphological shape to study even minimal variation. One key challenge however, is 2D photographs which serve as proxies for 3D structures that introduce measurement error into a dataset by omitting variability in the Z-dimension. The ease of application of 2D proxies means we should optimise them however, rather than limit studies by enforcing expensive or complex protocols. This may be done by explicitly measuring and reporting error. A rotational mounting surface should be capable of reducing measurement error associated with positioning (rotation). This study empirically quantified positioning error in basis capituli of three closely related *Rhipicephalus* species larvae. This study investigated symmetrical and asymmetrical components of variation, in addition to the number of replicates required to obtain confident results. Asymmetry showed rotational error along the roll axis (side to side) which had a large effect that confounded species variation. In contrast, symmetrical variation showed much less rotational error, only along a pitch axis (forwards and backwards), that was much smaller than species variation. Some systematic error was noted in roll axis variation that caused the left sides of specimens to be slightly raised. However, this error was small and avoidable by only considering symmetrical variation. Confidence of differences in multivariate means between groups increased drastically with three replicates, and slightly more with five. This study demonstrates that variation at and below the individual level is out of scope for rotational mounting, and will provide unreliable results. However, this study validates this mounting method for questions pertaining to species variation and taxonomy.

Introduction

The combination of geometric morphometric analysis protocols and modern computational power has revived the study of morphology in contemporary biology (Mitteroecker and Gunz, 2009). Precise measurements of morphological shape features can be obtained to generate detailed datasets for studying even minimal shape changes and their drivers (Adams et al., 2013; Klingenberg, 2010; Slice, 2007). Methods to obtain these data usually include photographs where landmarks are placed on 2D images to measure morphological features in multivariate space. Photographs are currently the most common method facilitating ease of use and rapid data collection. A significant advantage is that larger sample sizes may be sought which increases statistical power in analysis. Methods to obtain

3D models instead of images are also used, however less often, and these include digitizers (such as MicroScribe), laser scanners, photogrammetry and computed tomography (CT scanning). These methods have the advantage of measuring morphological features such as skulls more precisely, but data collection is slower and equipment more expensive.

A key challenge is the issue of 2D photographs serving as proxies for 3D structures. These proxies 'miss' the Z-dimension of depth variability, introducing additional noise into a dataset (Fig. 6.1; Cardini, 2014). A numbesr of workers have expressed potential pitfalls of excluding the Z-dimension in analyses of shape for certain research questions (Álvarez and Perez, 2013; Cardini, 2014; Cardini and Thorington, 2006; Fruciano, 2016; Openshaw et al., 2016). Crania seem to be particularly problematic due to their generally spherical shape that increases the tendency to miss Z-dimension information (Cardini, 2014; Cardini and Thorington, 2006). Structures that have an approximately flat shape, which can be placed in a single plane, should be less problematic however (Álvarez and Perez, 2013). One solution is to employ 3D methods as mentioned above (Katz and Friess, 2014; Webster and Sheets, 2010), and another solution considers simultaneous study of dorsal, ventral and lateral aspects to maximise coverage and quantify intraspecific variation (Openshaw et al., 2016). However, these solutions are either costly or complex. Hence, simplicity of 2D proxies means that they will continue to be used well into the future (Cardini, 2014; Openshaw et al., 2016). Rather than limiting research output by enforcing expensive or complex analytical protocols, we could aim to optimise shape analysis of 2D proxies, and explicitly measure and report error (Fruciano, 2016).

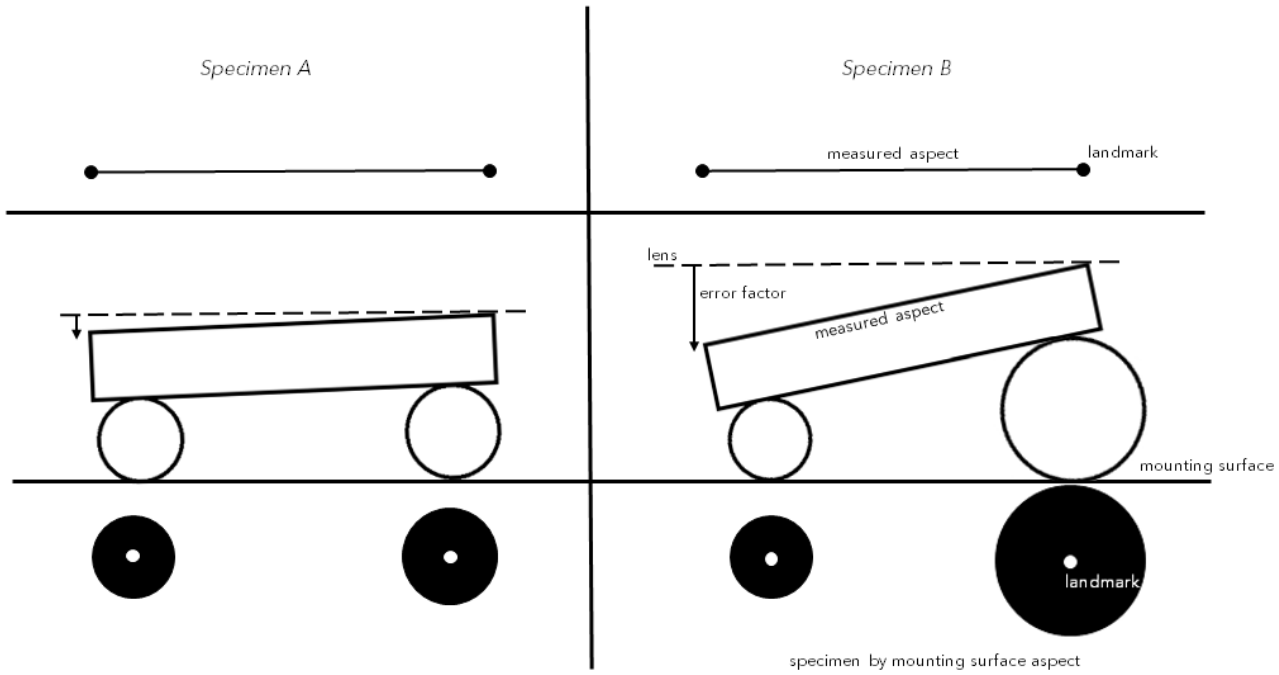


Figure 6.1. Line diagram showing the confounding effect of positioning error on measured aspect. In specimen B, the interaction of the specimen-by-mounting surface is equal to that of specimen A. The contact points (white dots) are in the same positions in both specimens, however the measured aspect is shorter due to tilt according to an error factor.

Rotational Mounting Error - Chapter 6 (Addendum)

Measurement error can obscure the variance of a dataset (Palmer, 1994), and 2D proxies are a source of measurement error. This is not often accounted for explicitly in studies of shape however (Cardini, 2014). Limiting measurement error is crucial to obtain accurate and precise 2D proxies of shape to answer questions of biological variation. Measurement error can be random or non-random (systematic), and may result from positioning (presentation) and digitization (landmarking) of the specimen studied (Arnqvist and Martensson, 1998; Fruciano, 2016). Positioning specimens in a field of view for capture, typically comprises laying them on a flat mounting surface in an attempt to standardise the aspect being measured. However, this may confound standardization in certain cases. In dorsoventrally flattened organisms, the ventral surface of the specimen interacts with the mounting surface and this interaction becomes somewhat standardized – instead of the dorsal aspect (measured plane) of the specimen being standardized (Fig. 6.1). Irregularities, such as outgrowths or variably preserved appendages, interact with the mounting surface in a standardized manner, but leave the measured plane to vary unpredictably according to an unknown error factor (Fig. 6.1). It is reasonable to argue for standardizing the measured aspect, and rather allow variation in the ventral aspect that interacts with the mounting surface. This allows comparison of corresponding portions of 3D structures, and ensures landmarks have an approximate one-to-one correspondence in an approximately flat plane. The problem of 2D to 3D shape proxy error is reasonably assumed as greater when comparisons are made between individuals that do not show distinct differences (Cardini, 2014). Geometric morphometric analysis has previously provided insight into the taxonomy of tick species that are difficult to separate (Pretorius and Clarke, 2001, 2000). *Rhipicephalus* species are notoriously difficult to identify (Walker et al., 2000), and have many indistinct species - this is especially true for immature specimens (for example see Fig. 6.2).

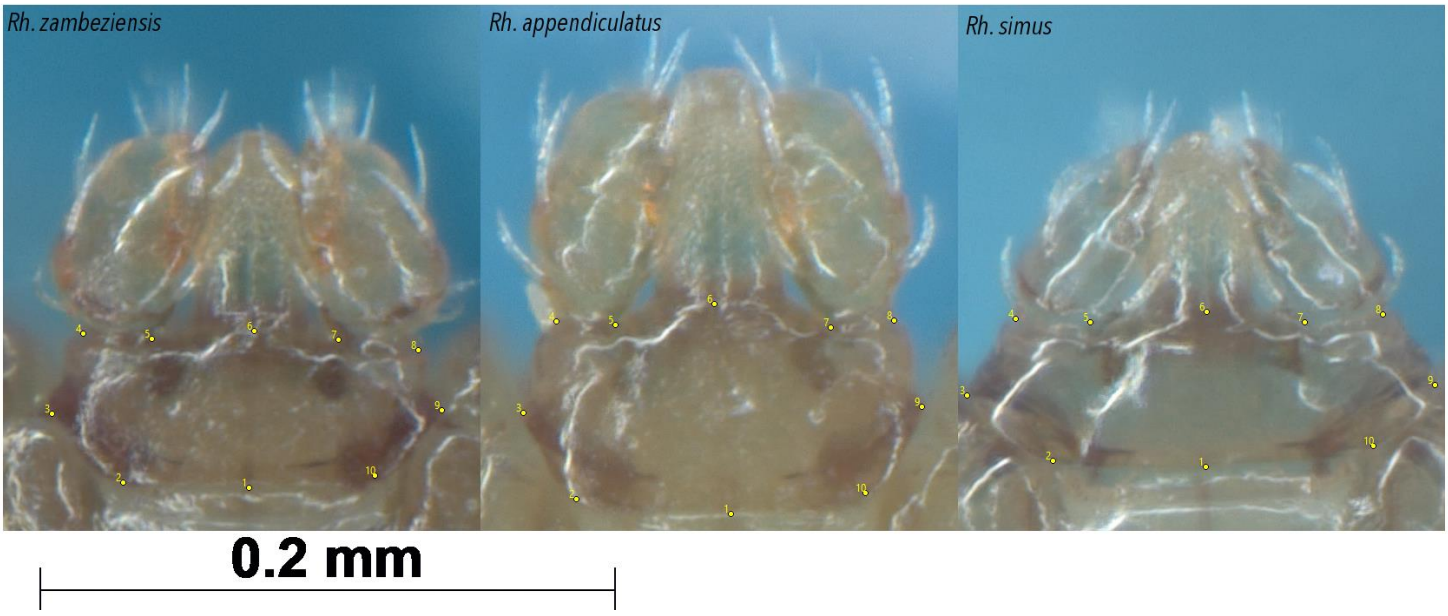


Figure 6.2. Photographs of dorsal aspect of basis capituli for larval *Rh. appendiculatus, Rh. zambeziensis* and Rh. *simus* with the ten landmarks used in this study plotted on each image.

Rotational Mounting Error - Chapter 6 (Addendum)

This study investigates a rotational mounting surface for microphotography of larval *Rhipicephalus* specimens. The use of larval *Rhipicephalus* specimens in this study tested the robustness of rotational mounting in a model taxon that presents difficulties in species identification. Additionally, small overall size increases sensitivity to handling and positioning the measured aspect of each specimen. Rotational error was empirically quantified in the symmetric and asymmetric components of shape. Symmetry should be sufficient for species level variation (Cardini 2016), but asymmetry was investigated to explore the limits of rotational mounting. Principal components were used to estimate shape changes and group structure for species variation and rotational error. The level of biological variability at which results are confident was determined by an analysis of variance for replicate, individual, side and species effect against fluctuating asymmetry. Fluctuating asymmetry is useful here because it represents a form of subtle biological variation that is normally much smaller than other sources of variation, such as individual or species (Klingenberg, 2015; Klingenberg et al., 2002). Furthermore, we investigated replicate number needed for confident results in discriminant analysis to assess misclassification, and for comparisons between multivariate means. Rotational mounting has been used for decades by entomologists to observe a common aspect in a set of specimens for traditional taxonomic comparisons. This study is the first to quantify the precision and error of such a method when used to study arthropod shape variation in Procrustes shape co-ordinates.

Methods

The rotational mounting surface is inexpensive and easy to use (the example in Fig. 6.3 was adapted from a commercial roll-on deodorant that was cut out and dried). It comprises a sphere that rotates about 360 degrees, placed in a supporting base. This facilitates standardising the measured (photographed) aspect of each specimen by varying the specimen-by-mounting surface interaction during positioning. Capture of an approximately common measured aspect across all specimens is directed by eye.

The study sample comprised 30 laboratory reared specimens, representing three species, stored in alcohol that are housed in the Gertrud Theiler Tick Museum, EPV, ARC-OVR, South Africa (Table S6.1: supplementary material). Species used were *Rh. appendiculatus* Neumann, 1901, *Rh. zambeziensis* Walker, Norval & Corwin, 1981 and *Rh. simus* Koch, 1844 larvae (ten specimens of each species). Specimens were first set into clay on the rotational mount, and then rotated into position. The measured aspect of basis capituli were oriented parallel to the photomicroscope lens and placed in the centre of the image field to avoid lens distortion. Basis capituli of the *Rhipicephalus* larvae present object symmetry (Klingenberg et al., 2002). Five replicates of each specimen were made by resetting the clay

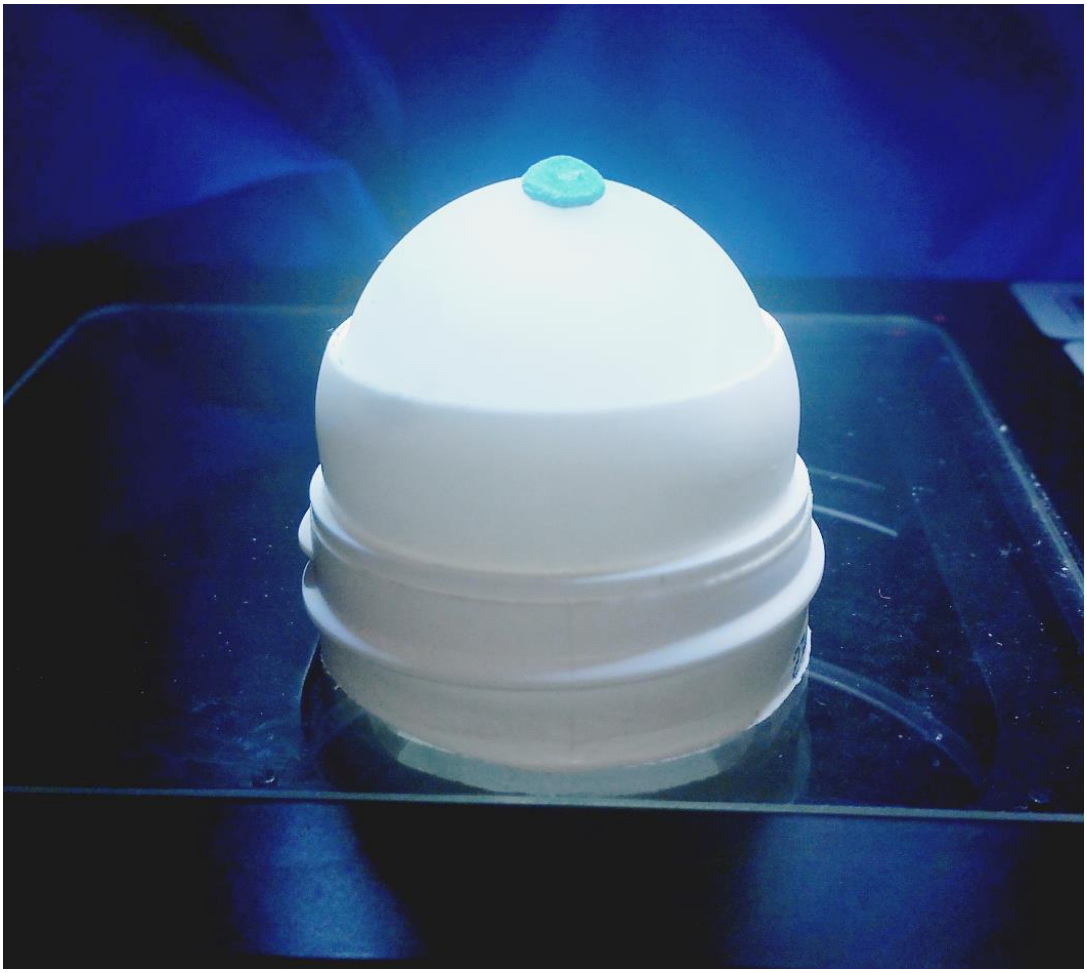


Figure 6.3. Photograph of the rotational mounting surface used in this study.

and specimen before re-photogsraphing. This made a total of 150 photographs in the dataset. Photographs were taken using a Zeiss Discovery.V20 Steromicroscope with a Zeiss AxioCam MRc 5 camera, and were stacked in Zeiss Axiovision v4.8. Each stacked image consisted of between 10 and 20 photographs taken every 3um in Z-dimension. A scale bar was placed on each stacked image.

Basis capituli were digitized using COO v41 in the CLIC package by Dujardin (available at (http://mome-clic.com/the-clic-package) according to the landmarks displayed in Fig. 6.2, and were scaled according to the scale bar. Operational definitions for each landmark are available in Table S6.2. Replicates were digitised in batches by replicate instead of individual (to avoid digitisation bias from operator memory). The landmarks were transformed in a Procrustes fit in MorphoJ v1.06d (Klingenberg, 2011) and a covariance matrix was generated for both symmetric and asymmetric components. A Procrustes ANOVA was done for individual, side and individual-by-side with replicate as the error effect and species as the main effect. Observations were averaged by individual to meet the assumption of independence, and principal component analysis was subsequently conducted on asymmetrical and symmetrical variation. Principal component scatterplots and shape changes were studied for

accuracy in representing biological reality. Minimum and maximum extent of PC axes were used to infer skewness of shape distributions. Also with averaged observations, discriminant analysis for correct classification was performed for all pairs of species in symmetric variation. Additionally, comparison of multivariate means between species groups was performed with 1 000 permutations. This was repeated on separate datasets that comprised the first replicate only, and first three replicates.

Results

Asymmetrical variation yielded a first principal component that accounted for 45.36% of variation, a second that accounted for 18.82%, and a third that accounted for 13.20%. All principal components did not show species group structure in scatterplots (Fig. 6.4 and Fig. S6.1). Shape changes of all principal components show rotational error along roll axis (side to side), because landmark displacements were such that shape changes were reciprocal about the medial axis of the basis capitulum, and do not represent a biologically real shape change (Fig. 6.2, 6.4). Principal components one, three and five had distributions slightly skewed towards the left side of the basis capitulum as larger (Fig. 6.4). Principal components seven and eight had distribution slightly skewed towards the right side as larger. Principal components two, four and six had distribution approximately equal.

Symmetrical variation yielded a first principal component that accounted for 77.86% of variation, a second that accounted for 8.77% and a third that accounted for 4.52%. The first principal component provided some group structure between species in the scatterplot (Fig. 6.5), while the remaining principal components did not (Fig. S6.1). Shape changes of principal component one show differences accounted for by species because the change in anterior-posterior length represents a biological reality that distinguishes between *R. appendiculatus* and *R. simus* + *R. zambeziensis* (Fig. 6.2). Shape changes of all remaining principal components mainly show rotational error along the pitch axis (forwards and backwards) because landmark displacements were such that shape changes were reciprocal about the transverse axis of the basis capitulum, and do not represent a biological reality (Fig. 6.5). Principal component one had distribution slightly skewed towards the flat and wide basis capituli of *R. simus*, indicating this species may have a wider range of variability in anterior-posterior length of basis capituli (Fig. 6.5). Principal component three had distribution slightly skewed towards a wider posterior margin in basis capituli (Fig. 6.5). Principal components two to eight had distribution approximately equal, indicating these represent random rotational error.

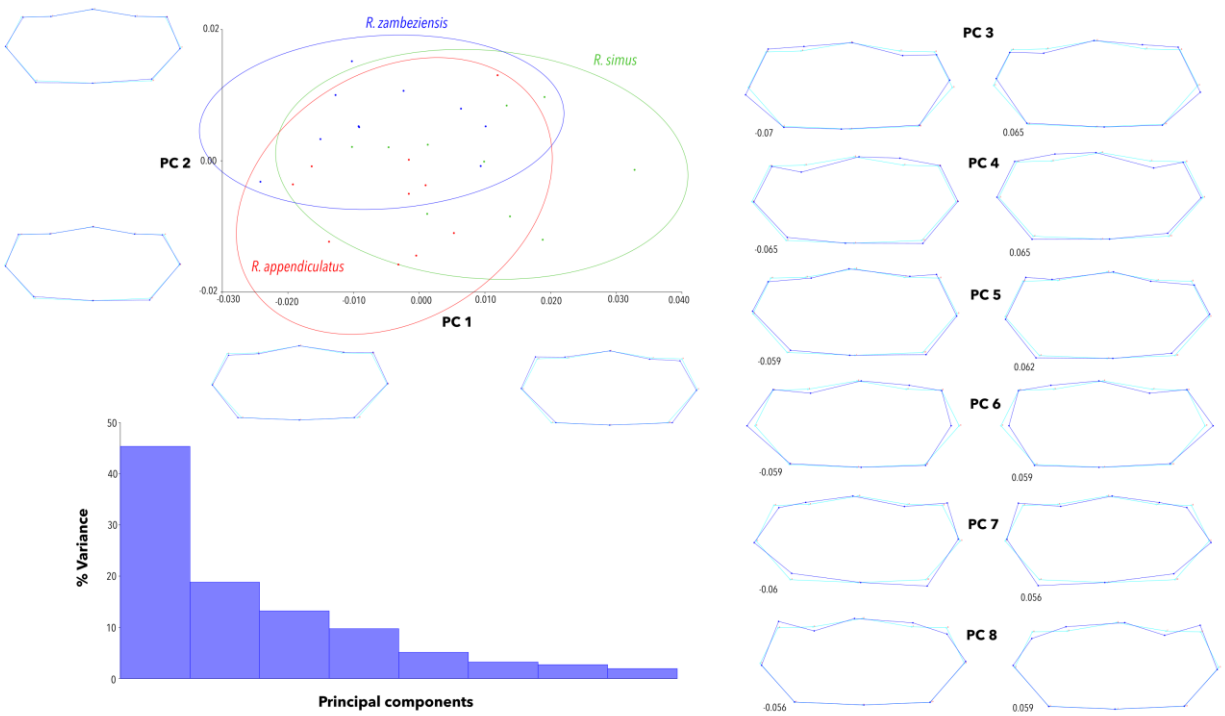


Figure 6.4. Principal components analysis of asymmetry in the five replicates dataset. Dots in scatterplot represent averaged replicates for individual in morphospace. Ellipses represent 95% confidence. Shape changes along PC axes are to scale at minimum and maximum extent respectively. The light blue trace represents the mean shape and dark blue trace represents deviation from the mean shape. Shape changes on right refer to minimum (left) and maximum (right) extents for each principal component. All principal component shape changes show general rotational error along the roll axis.

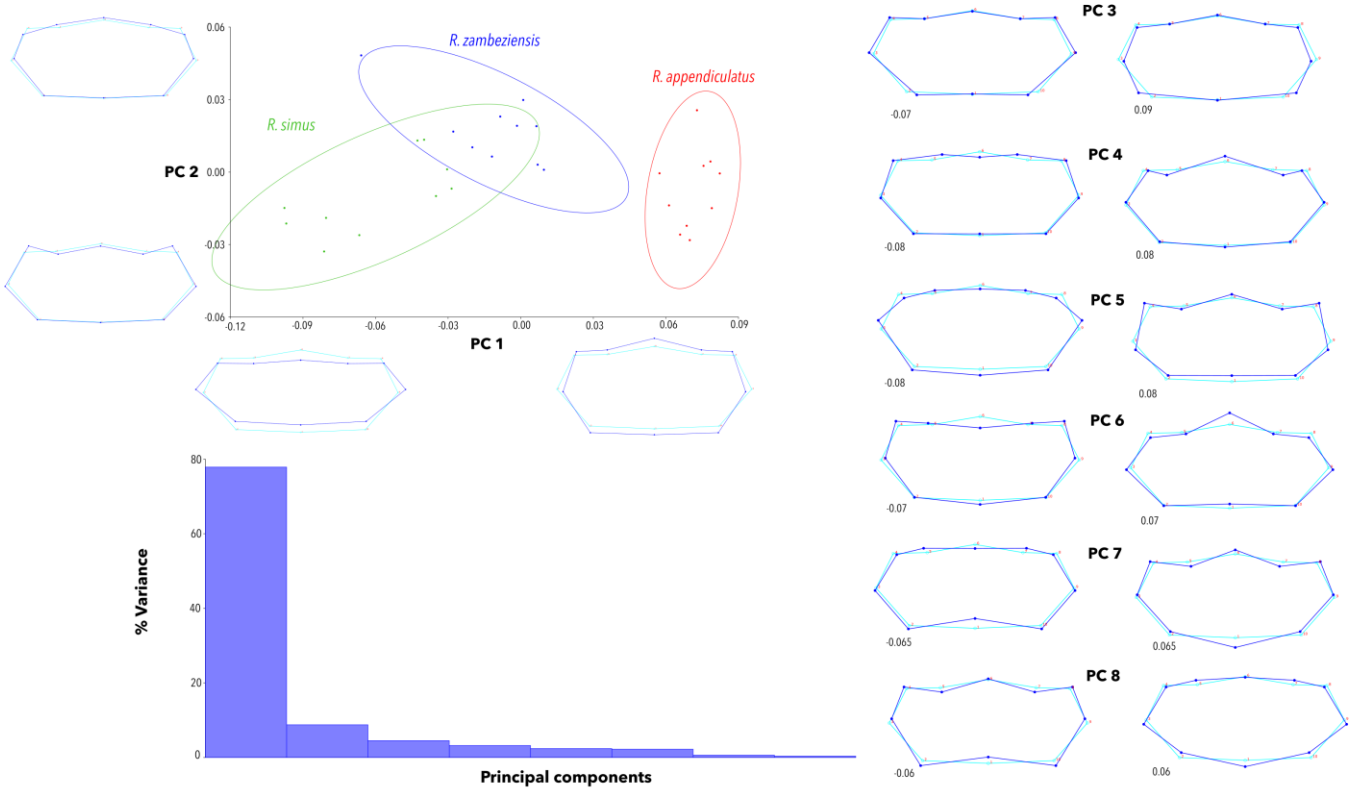


Figure 6.5. Principal components analysis of symmetry in the five replicates dataset. Dots in scatterplot represent averaged replicates for individual in morphospace. Ellipses represent 95% confidence. Shape changes along PC axes are to scale at minimum and maximum extent respectively. The light blue trace represents the mean shape and dark blue trace represents deviation from the mean shape. Shape changes on right refer to minimum (left) and maximum (right) extents for each principal component. Shape changes at principal component one show differences in species, other generally show rotational error along the pitch axis.

Table 6.1. Procrustes ANOVA of *Rhipicephalus* basis capituli shape from five replicates.

Effect	Sum of Squares	Mean Squares	Degrees of Freedom	F	p
Species	0.46942424	0.0293390152	16	36.83	<0.0001
Individual	0.17204679	0.0007965129	216	4.39	<0.0001
Side	0.02970822	0.0037135273	8	20.47	<0.0001
Individual * Side (Fluctuating Asymmetry)	0.04208924	0.0001814191	232	1.56	<0.0001
Replicate Error	0.22261333	0.0001159444	1920	-	-

Table 6.2. Results from discriminant analysis and comparison between multivariate means of *Rhipicephalus* basis capituli shape, symmetric component. *p<0.05 **p<0.0001

	Misclassification (%)			Procrustes Distance		
Replicates	5	3	1	5	3	1
Rh. simus – Rh. appendiculatus	0	0	0	0.131 (p<0.0001)	0.134 (p<0.0001)	0.127 (p<0.0001)
Rh. zambeziensis – Rh. appendiculatus	0	0	5	0.087 (p<0.0001)	0.086 (p<0.0001)	0.086 (p<0.0001)
Rh. zambeziensis – Rh. simus	15	10	25	0.058 (p=0.0002)	0.063 (p=0.0006)	0.060 (p=0.0079)

The effect of species was larger than all other effect sizes in all replicate datasets, and side was unexpectedly large, being fifteen times greater than individual (Table 6.1). However, side was approximately fifteen times smaller than the effect of species (Table 6.1). Fluctuating asymmetry was marginally greater than replicate error (Table 6.1).

Misclassification was highest when only one replicate was used (30%) and decreased by approximately 17% with three and five replicates (Table 6.2). Procrustes distances between multivariate means were largest between *R. simus* and *R. appendiculatus*, less so between *R. zambeziensis* and *R. appendiculatus* and smallest between *R. zambeziensis* and *R. simus* (Table 6.2). These were all significant, but comparisons between *R. zambeziensis* and *R. simus* were always less significant (Table 6.2). Increasing replicate number lead to increased significance in Procrustes distances between *R. zambeziensis* and *R. simus* and to fewer misclassifications (Table 6.2).

Discussion

Shape changes that reflect differences in species should fundamentally alter the symmetrical shape of basis capituli and represent biological reality. In contrast, shape changes

due to rotational error will only slightly elaborate on the fundamental structure asymmetrically, and misrepresent biological reality. Individual variation and fluctuating asymmetry will similarly modify the fundamental symmetrical structure (Klingenberg 2015). Nevertheless, rotational error will manifest in a consistent manner, and to separate it from individual variation and fluctuating asymmetry, shape changes can be inspected for the following: shapes at a given extent are (1) erroneously distorted such that biological reality is misrepresented, and (2) are distorted reciprocally across a body axis (transverse or medial) that relates to an axis of motion (pitch or roll). It may be possible to explicitly model measurement error in order to better understand their causes as has been done with fishes due to body arching (Fruciano et al. 2011; Fruciano et al. 2012; Fruciano et al. 2014; Ingram 2015; Fruciano 2016). However, this is out of scope for the present study, and instead principal shape changes are discussed in terms of what they show and why these relate to forms of rotational error.

The three species studied present a small and simple difference in shape dominated by changes in anterior-posterior length, and this was shown in symmetrical shape changes of principal component one (Fig. 6.5). Asymmetrical shape variation was most sensitive to rotational error, and all principal components showed rotational shape change characterised by landmark displacements that misrepresented the biological reality of basis capituli shape (Fig. 6.4). This variation was reciprocal about the medial axis, and thus asymmetry was most sensitive to roll axis rotational error. Moreover, fluctuating asymmetry was only marginally greater than replicate error (Table 6.1), suggesting most of the asymmetry measured was due to measurement error (Klingenberg, 2015; Klingenberg et al., 2002). In contrast, symmetrical variation was less sensitive to rotational error with only 22.14% of variation that misrepresented the biological reality (Fig. 6.5). This variation was reciprocal about the transverse axis, and thus symmetry was not sensitive to roll axis rotational error. It was instead sensitive to pitch axis rotation which was far less pronounced. The small size of these ticks and their basis capituli may have increased propensity for rotational error due to the high degree of sensitivity to positioning. It is possible that similar analyses of larger structures may be less prone to rotational error, and may present fluctuating asymmetry many times greater than measurement error.

High variability and effect size in the ‘side’ term from Procrustes ANOVA is of concern. Effect size of species was greatest, but side had a large confounding effect (Table 6.1). Moreover, the intermittent skewness of certain principal components of asymmetry indicates that an element of systematic asymmetrical error was present in rotational mounting (Fig. 6.4). This error manifests in the left side being generally raised above the right (PC1, 3, 5 = 63.66%) and less often below it (PC7, 8 = 4.61%). However, the skewness is very slight (skewed by ≤0.005 on PC axes) and is not prevalent in symmetry. In symmetry, principal components with

skewed distributions are fewer (PC3 = 4.52%, PC1 excluded here because of biologically real shape changes) and also slight (skewed by 0.02 on PC3 axis). Here, slight systematic errors in rotation were present along the pitch axis whereby the posterior margin was raised relative to the anterior. This further indicates that symmetrical variation in rotational mounting can overcome the majority of rotational error. However, it must be cautioned that this may only apply to structures that are transversely widened about the axis of symmetry. In structures that are lengthened along the axis of symmetry, roll axis error may be lower and pitch axis error greater. This remains to be tested however.

Replicating observations did well to limit error from rotational mounting. Three replicates provided a drastic increase in confidence when comparing group multivariate means, and most drastically decreased percentage of misclassifications (Table 6.2). Comparisons involving *R. zambeziensis* were generally more problematic, and most problematic when compared with *R. simus*. This is likely an effect of the similarity in width and shorter anterior-posterior length of basis capituli shape (Fig. 6.2, Fig. 6.5: PC1). This is surprising considering their placement in separate putative species groups, while *R. appendiculatus* is placed in the same group as *R. zambeziensis* (Walker et al. 2000).

Adversely, to direct positioning of specimens by eye without a rotational surface could overcome many of these sources of error in organisms larger than ticks. For example, a skull may be laid on a flat surface and photographed from an angle that approximates a common aspect among all specimens. However, small specimens present a challenge that requires microphotography. Here, the camera is not movable and sensitivity to handling is much greater. In this case, flat surfaces will presumably be inappropriate due to the specimen-by-mounting surface interaction (Fig. 6.1). Ideally, a test of error when using a flat mounting surface should be done. Furthermore, setting specimens in clay may introduce an extra source of error that is lumped into rotational error in this study. A test to separate these potential sources of variation may also prove valuable. However, error associated with setting is presumably overcome by the rotational action which aims to standardise the measured aspect of each specimen.

In conclusion, studies of intraspecific variation and fluctuating asymmetry in dorsoventrally flattened arthropods will not be reliable using rotational mounting. This may be especially true for smaller structures more prone to rotational error. However, given that rotational error can be overcome in symmetrical variation at the species level including at least three replicates, rotational mounting will provide reliable results to answer questions of variability in species and taxonomy at a level higher than the individual. Importantly, the best solution is to develop a study design that accounts for rotational error.

SUPPLEMENTARY INFORMATION:

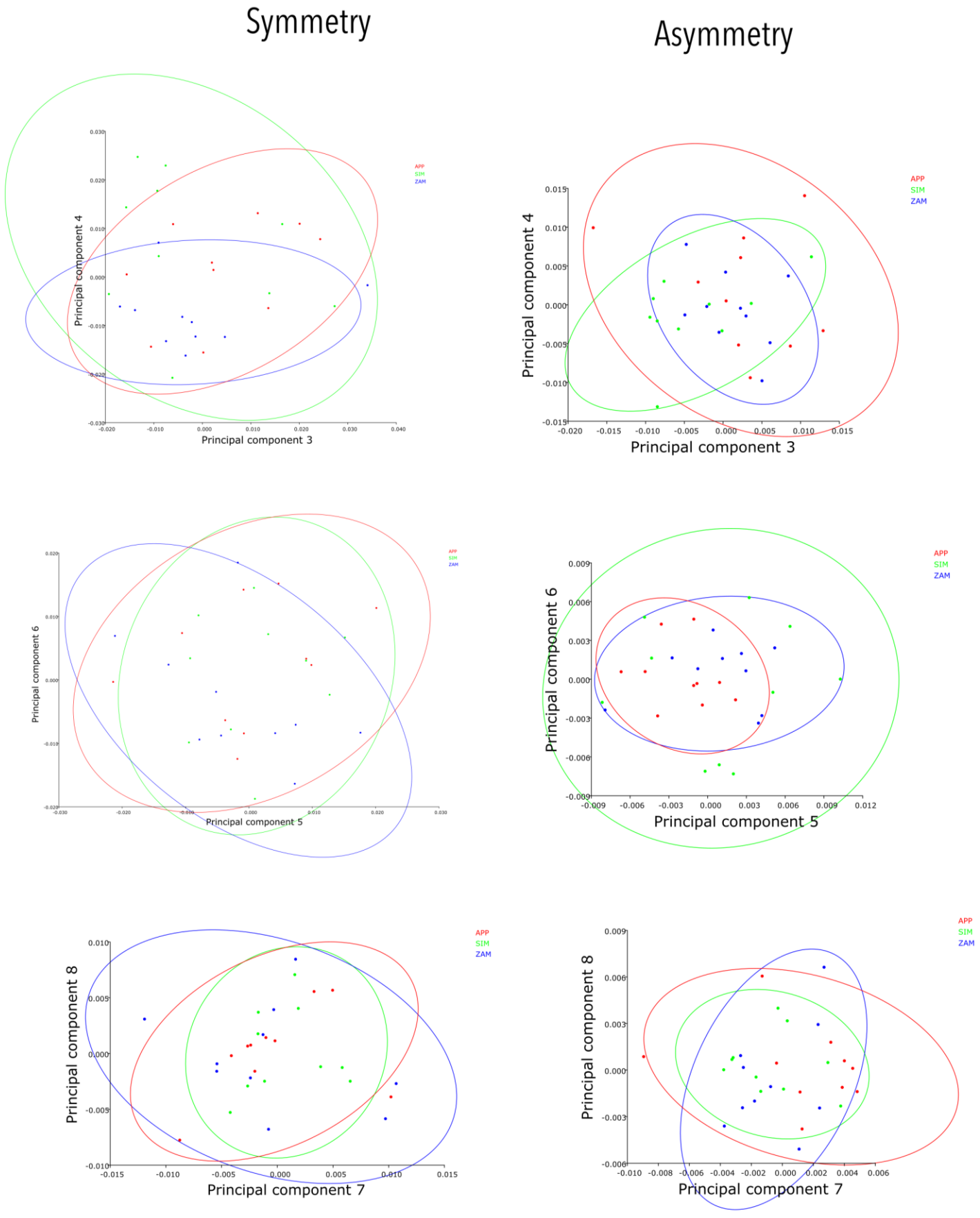


Figure S6.1. Scatterplots of principal components of symmetry and asymmetry.

Table S6.1. Accession numbers for GTTM specimens photographed in this study.

Species	Collection Accession	specimen
Rhipicephalus appendiculatus	OP2473ix	a
	OP2473ix	b
	OP2473ix	c
	OP2473ix	d
	OP2473ix	e
	OP2656iii	a
	OP2656iii	b
	OP2656iii	c
	OP2656iii	d
	OP2656iii	e
Rhipicephalus zambeziensis	JBW3400	a
	JBW3401	b
	JBW3402	c
	JBW3403	d
	JBW3404	e
	JBW3405	f
	JBW3401	a
	JBW3402	b
	JBW3403	c
	JBW3404	d
	JBW3405	e
	JBW3406	f
Rhipicephalus simus	JBW3285	a
	JBW3285	b
	JBW3285	c
	JBW3285	d
	JBW3285	e
	JBW3285	f
	JBWL67F2	a
	JBWL67F2	b
	JBWL67F2	c
	JBWL67F2	d
	JBWL67F2	e
	JBWL67F2	f

Table S6.2. Operational definitions for landmarks of larval *Rhipicephalus.*

LARVAE		
LM	Definition	Side
1	Most posterior point (centre)	-
2	Most posterlateral point	L
3	Most lateral (widest) point of basis capitulum	L
4	lateral meeting point of palp and basis capitulum	L
5	medial meeting point of palp and basis capitulum	L
6	meeting point of hypostome and basis capitulum (centre)	-
7	medial meeting point of palp and basis capitulum	R
8	lateral meeting point of palp and basis capitulum	R
9	Most lateral (widest) point of basis capitulum	R
10	Most posterlateral point	R

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