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Biogeographical Patterns in the Hard-Tick Genus Amblyomma Koch 1844 (Acari: Ixodidae)

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BIOGEOGRAPHICAL PATTERNS IN THE HARD-TICK GENUS AMBLYOMMA KOCH 1844 (ACARI: IXODIDAE)

by

MATTHEW HUNTER SEABOLT

(Under the Direction of Lorenza Beati)

ABSTRACT

Amblyomma Koch is a genus of hard-ticks with approximately 130 species. Its geographical range is typical for organisms with a Gondwanan origin. A majority of these species are endemic to the Neo- and Afrotropical regions, with the remaining taxa dispersed throughout Southeast Asia, Australia and the Pacific islands. Based on this distribution, we hypothesize that the genus dispersal patterns will mirror the fragmentation and continental drift of the Gondwanan supercontinent. Maximum parsimony, maximum likelihood, Bayesian inference and node-dating analyses of nuclear 18S rDNA gene sequences reveal a more recent origin and radiation patterns within the genus and suggest that *Amblyomma*'s evolutionary history is more complex than simple continental drift (ancient Gondwanan vicariance) hypotheses.

INDEX WORDS: ticks, phylogenetics, *Amblyomma*, systematics, biogeography

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DEDICATION

The pursuits of graduate studies are never a simple undertaking. Oftentimes, I second-guessed my decision to return to school, yet in the end, I always came back to my senses reinvigorated and with greater resolve. I could never have achieved my successes without the support of my family and my boyfriend, Matt, each and every step along the way. You have never failed to offer encouraging thoughts or a helping hand, whichever I needed. Without you I would never have braved the first steps on this journey, much less seen them through to completion. This work is dedicated first to you.

Secondly, without an advisor, a graduate student is lost. I would like to dedicate this thesis next to Dr. Lorenza Beati, who has been a wonderful advisor and teacher as well as a delightfully rigorous critic of my thoughts, writing, and professional growth during my time as her student. Lorenza is always eager to share a laugh, followed soon after by urging – sometimes even tormenting – me to get back to work. The greatest contribution she made to this project was to knock me down a few pegs when I needed it, reminding me how much I still have to learn.

Lastly, I dedicate this to Dr. Melanie Link-Pérez, my mentor and a woman whom I have no doubt will be a lifelong friend and collaborator. Dr. L-P is the one who started it all by taking me on as a naïve undergraduate student and opening my eyes to the joys of collections-based science. Without her guidance, I would not have discovered my appetite for biological wonder. She is still one of my strongest supporters and I owe her a great deal more than I can ever express for the insight she has provided over the years. At times, she probably knew me better than I knew myself.

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"Thus, from the war of nature, from famine and death, the most exalted object which we are capable of conceiving, namely, the production of the higher animals, directly follows. There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved."

--Charles Darwin, *The Origin of Species*

INTRODUCTION

Amblyomma Koch (1844) is a genus of ticks in the family Ixodidae, members of which parasitize all classes of terrestrial vertebrate hosts. Approximately 130 species are currently recognized (Guglielmone 2010). Traditionally, the subfamily Amblyomminae included two genera, *Amblyomma* Koch (1844) and *Aponomma* Neumann (1899). Camicas et al. (1998) and Travassos Santos-Dias (1974) recognized subgenera of both genera delimited primarily by geographic distribution and morphological characters, although the groups are never clearly defined. Morphologically, *Aponomma* species were described as *Amblyomma*-like ticks "lacking eyes" (Neumann 1899). The genus *Aponomma* underwent a major revision by Kaufmann (1972), who identified three main groups within the genus: the "endemic Australian" taxa, the "primitive" taxa, and the "typical" *Aponomma*.

The earliest cladistic analyses of tick relationships, based on morphology and life history, placed the Amblyomminae basal to the rest of the Metastriate tick lineages (hard ticks minus the genus *Ixodes*) (Hoogstraal & Aeschlimann 1982), a position which has not always been consistently found by other molecular studies (Mans et al. 2012, Burger et al. 2012, Klompen et al. 2002).

Phylogenetic analyses using molecular data showed *Aponomma* to be polyphyletic (Dobson & Barker 1999, Burger et al. 2012). The *Aponomma* subgenus *Bothriocroton* Keirans, King, and Sharrad, 1994, which was comprised of the five "endemic Australian" species of Kaufmann (Kaufmann, 1972), was elevated to generic rank and the remaining *Aponomma* species were transferred to a revised genus *Amblyomma* by Klompen et al. (2002). Uncertainty over the taxonomic placement of three former "primitive" *Aponomma* species (*A. transversale,* *A. elaphense,* and *A. sphenodonti*) remains unresolved, although it is now clear that these taxa do

not belong to the genus *Amblyomma* or to *Bothriocroton* (Klompen et al. 2002; Miller et al. 2007; Burger et al. 2013).

The genus *Amblyomma* exhibits a typical Gondwanan distribution, spread throughout the southern continents (excluding Antarctica) and a few localities in the northern hemisphere. The majority of the diversity is found in the New World (around 60 species), with a further 30 species in Africa, and the remaining 40 species distributed in India, Madagascar, Seychelles, Australia, and Southeast Asia. This distribution pattern, combined with the fossil evidence of ticks in Caribbean and Burmese amber (Keirans et al. 2002; Poinar & Brown 2003; Poinar & Buckley 2008), suggests that the ancestors of modern *Amblyomma* species were widespread throughout the Gondwanan supercontinent prior to the Mesozoic tectonic activity which resulted in its breakup.

Evidence in support of this can be drawn from a molecular study incorporating node-dating techniques done by Mans et al. (2012), in which mitochondrial genomes and nuclear 18S rDNA sequence data were used to assign dates for the divergence times between the tick families Nuttalliellidae, Argasidae, and Ixodidae. Further, the same authors provide an estimated date for the split between the Prostriata (genus *Ixodes*) and Metastriata (all other genera in the Ixodidae) at (249 ± 23 Mya; Late Permian/Early Triassic), and the subsequent initial metastriate diversification around 124 ± 17 Mya (Early Cretaceous).

Fossil evidence from amber deposits also corroborates the hypothesis that *Amblyomma* has been widely distributed for more than 100 Mya. An *Amblyomma* tick larva has been recovered from Burmese amber deposits dated to the Late Cretaceous period (ca. 100 Mya) (Klompen in Grimaldi 2002, pg. 27), a finding which indicates that the genus was already present in that area by the late Mesozoic. Other tick fossils from Burmese amber have been

assigned to different extinct genera, *Cornupalpatum* and *Compluriscutula*, which are considered to be more closely related to *Amblyomma* than any other extant hard tick genus (Poinar & Brown 2003, Poinar & Buckley 2008). Other *Amblyomma* fossils have been found in Dominican amber and dated to the Miocene (approx. 16 Mya). These ticks are recognizable as an extant species, *A. dissimile*, which is still commonly found on reptiles and amphibians in the Caribbean and Neotropics (Keirans et al. 2002).

Due to the number of taxa included in *Amblyomma*, many other studies have focused on small subsets of species, often very closely related taxa, or those reported from a defined locality (Beati et al. 2013, Labruna et al. 2009, Nava et al. 2007). Relevant to this study are those publications which have used molecular markers to clarify relationships at the lower (sub-familial) taxonomic ranks. Labruna et al. (2009) used a combination of mitochondrial markers (12S and 16S rDNA) to demonstrate the validity of *Amblyomma parkeri*, as a separate taxon from two closely related species, *A. longirostre* and *A. geayi*. Two new species, *Amblyomma boeroi* (Nava et al. 2009) and *A. hadanii* (Nava et al. 2014), were described using a very similar methodology (18S rDNA and 16S rDNA sequence variation) to provide support for the establishment of both new species. Beati et al. (2013) used a set of rapidly-evolving markers (mitochondrial 12S rDNA, control-region "D-Loop", cytochrome oxidase II and nuclear internal transcribed spacer region "ITS2") to reassess the taxonomic status of species within the *Amblyomma cajennense* complex and to hypothesize an age of approximately 13-16 Mya for the earliest divergence of those taxa.

A study of the phylogeographic relationships within the whole genus *Amblyomma* has yet to be undertaken. Based on what we know of the present distribution of *Amblyomma* species (illustrated in Fig. 1), and knowing that the genus is monophyletic (Mans et al. 2012) after the

"primitive" *Aponomma* species were definitively removed from it, we can formulate a hypothesis supposing that the distributional pattern we observe is the product of ancient vicariance caused by the break-up of Gondwana. The ancient Gondwanan vicariance scenario carries with it the assumptions that *Amblyomma* must be old enough to predate the beginning of the tectonic rifting which fragmented Gondwana (approximately 180 Mya) and subsequent nodal branching must approximately coincide with events which led to vicariance between clades. If this hypothesis is supported, we would expect to see monophyletic clades emerge from the main continental groups (African, South American, and Australian) (Fig. 1 inset). The African, Indian, and Malagasy landmasses, as we know them now, separated from Gondwana first and the last land bridge between Africa and South America was cleaved by approx. 90-100 Mya (McGloughlin 2001, Storey et al. 1996). South America and Australia were linked through a cool-temperate Antarctica until the latter became totally isolated and started cooling down around 30-50 Mya (Reguero et al. 2013, Clarke & Crame 1989). Also, if vicariance accounts for the present distribution of *Amblyomma* species, the occurrence of the genus in Asia can be explained by additional scenarios: the genus could have reached what is now Southeast Asia with the Sibumasu terrane, a Gondwana fragment that drifted from Gondwana in the late Early Permian from Australia (approx. 280 Mya) (Dunlop 2014, Metcalfe 2011) or when India moved away from East Gondwana (130 Mya) and collided with Laurasia (43 Mya) (McGloughlin 2001).

A species-level phylogeny encompassing species from all extant continents is necessary to test the monophyly of the current circumscription of *Amblyomma* and to provide a framework for testing biogeographical hypotheses. To accomplish these goals, we generated a phylogeny using nuclear 18S ribosomal DNA, a gene which is known to be conserved and informative at the basal intrageneric level (Klompen et al. 2007, Beati et al. 2008, Mans et al. 2012).

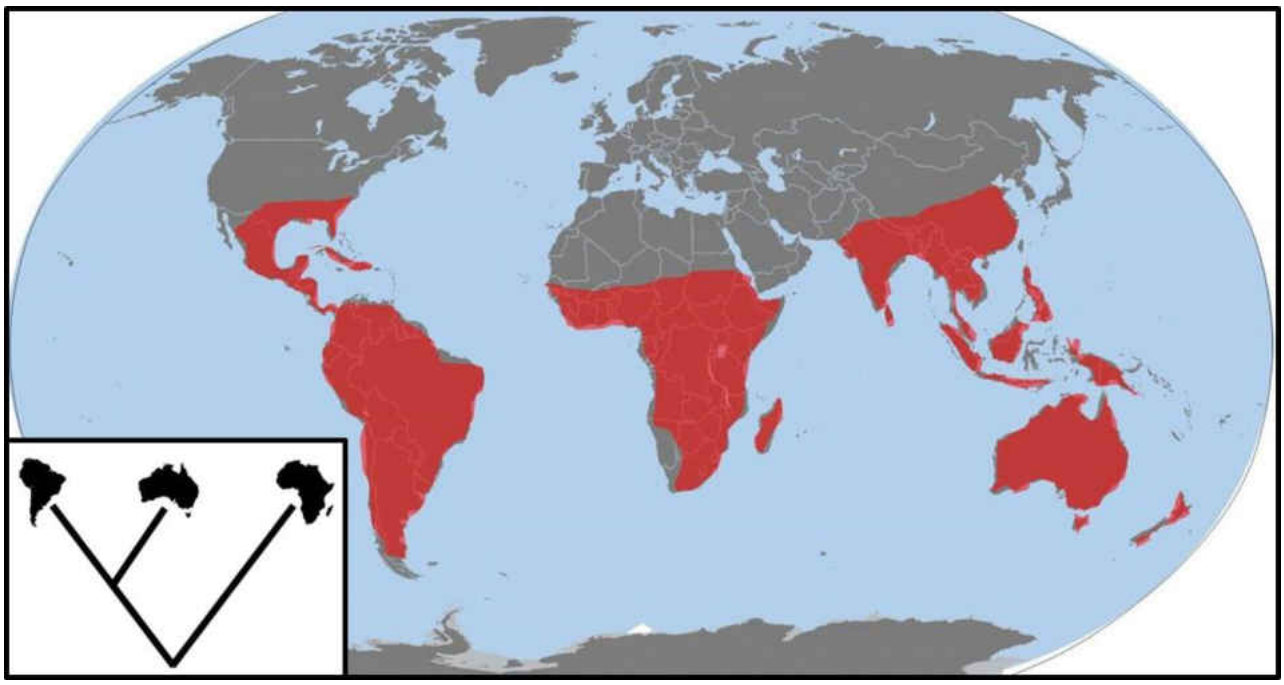

Fig. 1 – Distribution map of *Amblyomma* (in red). **Inset**: Initial hypothesis predicting phylogenetic structure of *Amblyomma*.

MATERIALS AND METHODS

Sampling

The ticks, their geographical origin, and sequences used in this study are listed in Appendix 1. Outgroups were selected from a variety of other ixodid genera, based on availability in GenBank and our laboratory database. For outgroups, we included sequences from at least two different species in the Metastriata subfamilies Ixodinae, Rhipicephalinae, Haemaphysalinae, and Bothriocrotoninae and from different geographical origins corresponding to the Gondwanan constituent landmasses plus North America and the Paleartic.

DNA Extraction, PCR, and Sequencing

The tick tissues were lysed at 56°C overnight in 180 µL ATL lysis buffer (Qiagen DNEasy Blood and Tissue kits; Qiagen, Valencia, CA) and 40 µL (14.3 mg/ml) Proteinase K (Roche Applied Sciences, Indianapolis, IN) by following a protocol that allows for preservation of the exoskeletons of the ticks as morphological voucher specimens (Beati & Keirans 2001, Beati et al. 2013). All tick exoskeletons were retained and preserved in 70% EtOH and DNA was preserved in molecular grade H_2O at 4°C.

PCR was carried out in 25 µL reactions with 2 µL of template DNA and reagents from MasterTaq Extender kits (5-Prime, Gaithersburg, MD). Master mixes were assembled using 14.5 µL of molecular-grade water, 2.5 µL of 10 x Extender Buffer, 2.5 µL of 2.5 mM MgCl solution (25 mM), 0.25 µL of dNTPs (10 mM each), 1.25 µL of each primer (10 µM) (Table 1), and 0.25 µL of Taq polymerase (5U/µl). PCR conditions as follows amplified 1780 bp of nuclear 18SrDNA: 5 min of initial denaturation at 94°C; 8 touchdown cycles with 25 sec denaturation at 94°C , 25 sec annealing at 62°C - 1°C/cycle, 1 min 45 sec elongation at 72°C; an additional 30 cycles with annealing at 54°C and elongation at 72°C, and a final elongation at

72°C for 5 min. Products were visualized by electrophoresis on 1% agarose gels with ethidium bromide staining. Successful amplicons were purified and sequenced at the High-Throughput Genomics Unit (HTGU, University of Washington, Seattle, WA). Because, in a majority of cases, we were unable to amplify the complete target, nested PCRs were performed by diluting the PCR products with molecular-grade water at a ratio of 1:9 and using 5 µL of this dilution as template for further attempts at amplification. Primers NS1-NS4 and NS5.8-NS8 were used separately to reamplify portions of the original target of 1120 bp and 697 bp, respectively. The locations of described primers along the targeted sequence are shown in Figure 2. PCR conditions for amplifying both smaller fragments differed from the total length 18SrDNA conditions only in annealing temperature (T_A=60°C - 1°C/cycle for 8 touchdown cycles, and a constant T_A=52°C for an additional 30 cycles). This modification was to both NS1-NS4 and NS5.8-NS8 primer pairs.

TABLE 1 - List of primers used in PCR amplification

Primer Name	Marker	Reference	Sequence
NS1	18S rDNA	Black et al. 1997	5'-GTAGTCATATGCTTGTCTC-3'
NS4	18S rDNA	Black et al. 1997	5'-CTTCCGTCAATTCCTTTAAG-3'
NS5.8	18S rDNA	Black et al. 1997	5'-GATACCGCCCTAGTTCTAACC-3'
NS8	18S rDNA	Black et al. 1997	5'-TCCGCAGGTTCACCTACGGA-3'

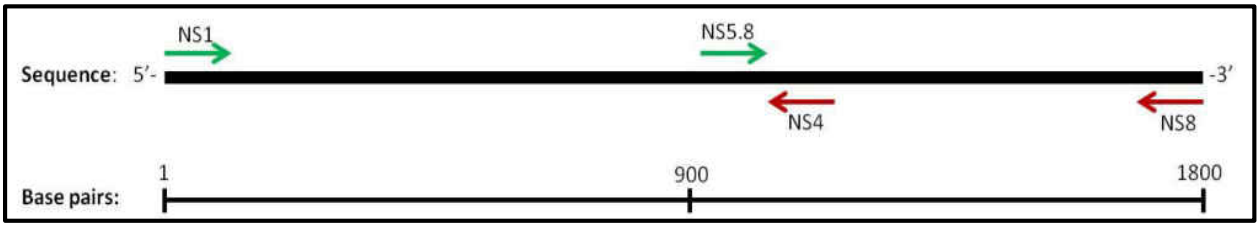

Fig. 2 – Location of 18S primers along template strand.

Phylogenetic Analysis

Sequences were assembled and manually corrected using Sequencher v.4.5 (GeneCodes Corporation, Ann Arbor, MI). Additional sequences generated previously in our laboratory or obtained from GenBank were also included in our analyses.

Sequences were manually aligned using Mesquite v3.04 (Maddison & Maddison 2015) and by considering secondary structure. Gap positions created by single sequences were treated as being phylogenetically uninformative and excised from the alignment. The matrix was imported into DNAsp 5.10 (Librado & Rozas 2009) to detect identical sequences. A matrix with only unique sequences was used for phylogenetic analyses.

Maximum Parsimony Analysis – Maximum parsimony (MP) analyses were performed using PAUP* v.4.0a147 (Swofford 2002). The heuristic search, using the TBR algorithm with equal weight given to all characters, was run by treating gaps in the alignments as missing data and by limiting the maximum of trees retained to 2000, for computational brevity. Tree scores were recorded. Branch support was assessed by bootstrap resampling using 100 replicates. The strict consensus tree was saved and branch support recorded.

Maximum Likelihood Analysis – The best-fitting substitution model for the data was estimated using jModelTest 2 (Darriba et al. 2012). We applied the model with the best AIC score and conducted a maximum likelihood (ML) heuristic search in PAUP*. Branch support (100 bootstrap replicates) was carried out using the online PHYML service hosted at www.phylogeny.fr (Guindon & Gascuel 2003, Dereeper et al. 2008).

Bayesian Inference Analysis -- Bayesian analyses were conducted using MrBayes v.3.2.1 (Ronquist et al 2012). Two simultaneous MCMC runs, with four chains each, saved trees every 100th generation for a total of 5,000,000 generations. Chain temperature was set to 0.2 with uniform priors. We interpreted an average standard deviation of split frequencies of < 0.01 to indicate that convergence had been reached. The initial 25% of saved trees were discarded as burn-in and the remaining trees were used to calculate posterior probability (PP) values for branch support and generate a 50% majority-rule consensus tree.

Molecular Clock Estimates for Node-Dating

DAMBE was used to test the molecular clock hypothesis (least-squares method with TN93 as substitution model) in order to establish whether or not our lineages mutated at similar rates (Xia 2013). Following this, estimates of divergence dates were calculated using the BEAST v2.1.2 software package (Bouckaert et al. 2014, Heled & Drummond 2012, Drummond et al. 2006). A relaxed molecular clock model was used with an uncorrelated log-normal distribution and Yule Process priors. Three different calibration schemes were tested using previously published node age estimates by Mans et al. (2012) and Beati et al. (2013) (Table 2) to estimate the mean divergence time between nodes and generate a 95% confidence interval for the variability of the dates from the mean. MCMC chains were set to 1,000,000 generations and sampled every 1000th generation. Using TreeAnnotator v2.1.2, the initial 10% of trees were

discarded and a maximum clade credibility tree calculated. FigTree v1.4.2 visualized the trees with approximate ages based on mean divergence times.

Table 2 - Node Dating Calibration Schemes

Calibration Node	"codename"	Mean	Sigma	Reference
Australian *Ixodes* - All other *Ixodes*	Pro	217	12	Mans et al. 2012
Metastriata	Meta	124	8.5	Mans et al. 2012
Am. cajennense + *Am. americanum* group	Caj	25	2.5	Beati et al. 2013

RESULTS

Sampling

Complete sequences were obtained from 64 species. Sequences from *Amblyomma nuttalli* were identical to *A. sparsum* (with *A. sparsum* retained), and were eliminated from analysis of this marker. Similarly, *A. dissimile, A. scutatum,* and *A. sabanerae* sequences were identical to *A. rotundatum* (*A. rotundatum* retained), and *A. mixtum* and *A. patinoi* were identical to *A. cajennense* (*A. cajennense* retained). The final 1669 bp alignment included 58 unique sequences.

Phylogenetic Analyses

Maximum parsimony -- Parsimony analysis of the total length of target 18SrDNA included 58 species, and a total of 1669 characters, of which 142 were parsimony-informative. The heuristic search found 2000 trees (the specified limit) with (length: 442; CI = 0.624, RI = 0.845, HI = 0.376). The MP reconstruction identified monophyletic (100% support) Prostriata (subdivided into Australian and non-Australian groups) (Fig 3). The Metastriata (clade A) was also strongly supported (100%). Bootstrap resampling identified all outgroup genera with strong support (clades B, C, and D), but their basal radiation pattern was not resolved. These lineages arose from a polytomy, which also included the so-called "primitive" *Aponomma*: *A. transversale, A. sphenodonti,* and *A. elaphense*. All remaining taxa assigned to *Amblyomma* formed a monophyletic ingroup (clade E) with strong bootstrap support (99%). Within the polytomic ingroup, two main clusters were monophyletic, one involving all Australian taxa (100%), and one including species from South America, Asia, and Africa (clade G; 87%). The position of three species from Argentina (*A. boeroi*, *A. neumanni,* and *A. parvitarsum*) was

unresolved within the same polytomy. Within clade G, there was little resolution with monophyletic lineages not clustering by geographical origin.

Maximum likelihood – (Fig. 4) The model with the lowest AIC score selected by jModelTest2 was the General Time Reversible model (nst=6) with proportion of invariable sites (I = 0.739) and a gamma distribution (Γ = 0.709). The resulting tree had a log-likelihood of (-5137.919). The overall structure and support of the ML tree was very similar to that of the MP strict consensus tree. A few additional lineages were supported within the polytomic *Amblyomma* (clade E), most notably a group containing all taxa sampled from Asia (clade I). Two additional supported groups arose from the polytomic node: one cluster contains *A. maculatum, A. cajennense, A. internandinum, A. americanum, A. parvum, A. pseudoparvum,* and *A. auricularium* while the other comprises *A. nodosum, A. hadanii,* and *A. dubitatum*.

Bayesian Inference -- (Fig. 5) Bayesian analysis revealed a topology that is largely congruent with the MP and ML trees, with overall slightly increased branch support. *Bothriocroton* formed the sister clade to all the other Metastriata. *A. elaphense* was well supported as sister to *Haemaphysalis* taxa. The placement of *A. sphenodonti* was resolved as an independent lineage sister to the remaining groups. The next radiation was polytomic, with three identifiable lineages arising from it: *A. transversale,* the Rhipicephalinae, and *Amblyomma* (clade F). *Amblyomma* was again recovered as a well supported monophyletic lineage (1.0 posterior probability). Australian and southern South American lineages again diverge first from the basal *Amblyomma* polytomy. The basal radiation pattern of clade I was unresolved. however, it involved only South American mammal-associated taxa. From this Neotropical backbone, a monophyletic clade included South American species mostly associated with reptiles, with the exception of clade L, members of which parasitizes endemic South American mammals. Within

this clade all Asian taxa cluster in a monophyletic lineage, while African *Amblyomma* are paraphyletic.

Molecular Clock Hypothesis and Node-Dating Estimates

The molecular clock hypothesis was not rejected in 85 of the 100 datasets generated by DAMBE, with an AICu score for the molecular clock at -9.72, and for non-molecular clock at -11.88, indicating that the evolutionary rate along branches was clock-like. We used, nevertheless, the relaxed log-normal molecular clock model, which allows for rate variability among branches, to estimate node dating. The mean dating corresponding to the three different calibrations are listed in Table 3 for all relevant and supported nodes (from I to X) obtained in the Bayesian phylogenetic analysis performed in BEAST (Fig. 6).

Calibration 3, based on a fairly recent radiation event (25Mya) appeared to provide the most divergent set of node date estimates, while calibrations 1 and 2 were fairly congruent. Overall it appears that the ancestors of *Amblyomma* started radiating approximately 80 Mya (shown in Fig. 6). The basal split within *Amblyomma* (approx. 74-60 Mya, node IV) involved two main groups of species: the first encompassing Australian taxa and taxa that are now found in southern South America, whereas the second group included everything else. The two lineages further separated approx. 46-48 Mya (nodes V and VII). The Australian clade dated back to approx. 34-19 Mya (node VI). Between 31 and 48 Mya the remaining *Amblyomma* diversified in the Neotropics, with an Asian cluster appearing 24-16 Mya, and African lineages diversifying repeatedly and independently from Neotropical origins.

Table 3 - Estimated divergence dates (Mya) by calibration scheme			
Clade	**Calibration 1**	**Calibration 2**	**Calibration 3**
Ixodidae (node I)	250.5 ± 53.8	231.5 ± 34.1	266.4 ± 189.6
Australian *Ixodes* – other *Ixodes* (node II)	234.3 ± 38.2	215.4 ± 21.8	157.7 ± 125.8
Metastriata (node III)	139.8 ± 34.4	124.3 ± 17.6	212.8 ± 161.0
***Amblyomma* (Amblyomminae) (node IV)**	73.7 ± 53.0	60.2 ± 26.2	119.4 ± 87.8
Australia + South-South America (node V)	63.5 ± 49.9	48.0 ± 21.7	92.0 ± 67.2
Australia (node VI)	34.0 ± 30.9	18.9 ± 14.5	37.0 ± 30.9
Other *Amblyomma* (node VII)	60.8 ± 47.9	45.6 ± 17.6	78.5 ± 55.2
All-American clade (node VIII)	43.8 ± 38.3	32.1 ± 4.1	25.2 ± 4.1
Node IX	48.3 ± 39.4	31.2 ± 11.8	66.3 ± 43.0
Asia (node X)	24.8 ± 17.2	16.6 ± 9.6	34.7 ± 25.7
	Pro+Meta	**Pro+Meta+Caj**	**Caj only**

DISCUSSION

Molecular methods have primarily being used to reassess phylogenetic relationships and taxonomy of ticks (Dobson and Barker 1999, Klompen et al. 2000, Nava et al. 2008, 2009, Beati et al. 2013). More rarely have they been used as a phylogenetic framework for testing biogeographical hypotheses (Klompen et al. 2000, Murrell et al. 2001, Beati et al. 2012; Beati et al. 2013). Our goal was to explore the phylogeographical evolutionary patterns within the genus *Amblyomma.*

The choice of 18SrDNA gene sequences for this study was justified by the fact that this slowly evolving gene had already been used successfully by other authors dealing with generic- and intra-generic level rankings (Black et al. 1997, Klompen et al. 2000, Beati et al. 2008, Mans et al. 2012). The samples we obtained are representative of all regions occupied by *Amblyomma*: South America, Africa, Australia, North America, and Asia. The analyzed gene sequences proved to be effective markers when resolving early diverging events within *Amblyomma* and between *Amblyomma* and other genera. They were slightly less informative at inferring more recent evolutionary patterns.

Our 18SrDNA phylogenetic reconstruction confirms the monophyly of the genus *Amblyomma*, which had also been established by other authors (Klompen et al. 2002, Burger et al. 2012, 2013). The topology of our phylogenetic tree reveals that the first radiation events within the genus involve species that are endemic to either Australia or the southern part of South America. The following diversifying lineages are South American. From this Neotropical backbone, a rapidly evolving polytomic radiation emerged which contained samples from three continents (South America, Africa, and Asia). The condition that *Amblyomma* must be sufficiently old to predate the Gondwanan rifting events was rejected, as we estimated the oldest

branching patterns to be near the very end of the Cretaceous or early Paleogene (around 60-70 Mya). In light of these results, we can deduce that Africa had not yet been colonized by ancestors of the genus when the continent started drifting away from Gondwana (130 Mya). Alternatively, if some *Amblyomma* lineages occurred on the African landmass before it was completely isolated (90 Mya), they might have gone extinct, possibly during the End Cretaceous extinction event (Benton 1995).

Following the isolation of Africa, however, tree topology and node dating supported an evolutionary history consistent with the vicariance events that shaped the southernmost part of the earth (Fig. 6).

The resolution in clade G is not the same for all our trees. Nevertheless, its basal subdivision consistently identified one Australian branch somewhat related to southern South American taxa (Fig. 5, clade H) and clade I. This radiation supposedly happened 74-60 Mya, a date consistent with the occurrence of the high-latitude Scotia land bridge (Fig. 7).

Between 80-45 Mya, this land bridge connected the southern tip of South America to the Antarctic Peninsula. At the end of the Cretaceous/early Paleogene, Antarctica supported a rich fauna and extensive forests (Reguero et al. 2013, Clarke and Crame 1989, McGloughlin 2001). The discovery of Paleocene and Eocene mammal fossils (marsupials and Gondwanatheres) indicated that these groups occupied the Antarctic landmass for some time prior to the glaciations of that continent (Reguero et al. 2013, Case 1989). These hosts may have been the mechanism of tick migration between Australia and South America while the land bridge was still in existence. Also, our data indicated that the origin of the genus might be located in what became South America, as the Australian clade arose predominantly from South American lineages (apporx. 64-48 Mya). This would not be in agreement with other authors who

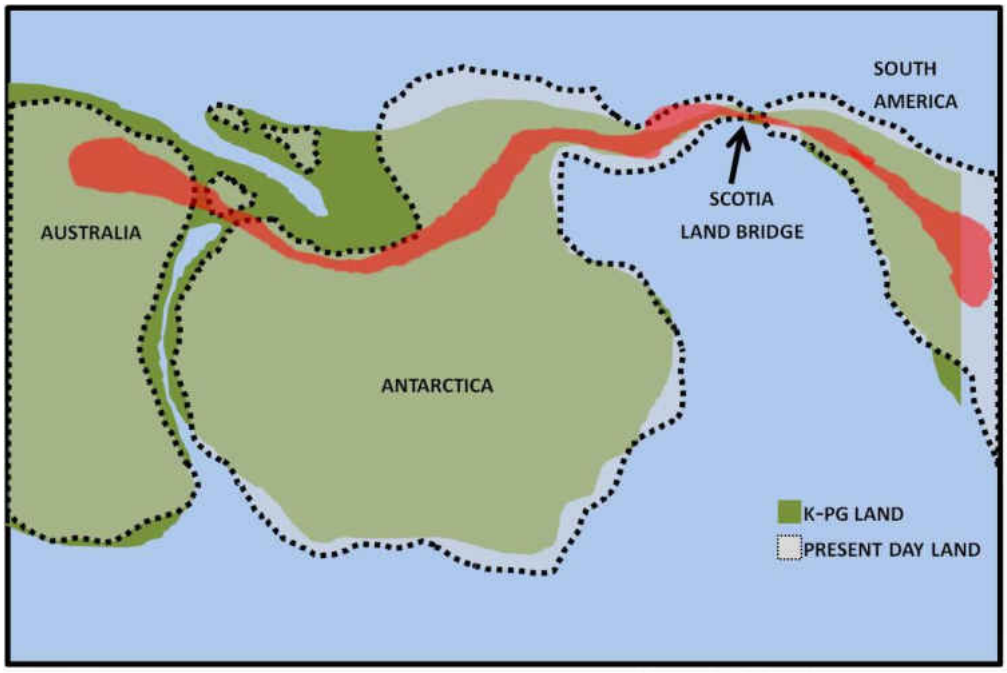

Figure 7: Map of southern Gondwana ca. Late Cretaceous-Early Paleogene (approx. 80-50 mya). Dispersal corridor between Australia and South America shown in red. Abbreviations: "K" = Cretaceous; "Pg" = Paleogene.

hypothesized that all ticks originated in Australia (Dobson and Barker 1999, Barker et al. 2014), unless Australian lineages underwent early extinction events. It would rather suggest that ticks migrated over the land bridge towards Australia as did marsupials, carnivores, and fleas (Case 1988, Reguero et al. 2013, Zhu et al. 2015).

Almost simultaneously (61-46 Mya) the remaining lineages (beginning with node VII in Fig. 6; clade I in Fig. 5) started diversifying (Fig. 8). All taxa in the "American" group (Node VIII in Fig 6; shown in purple in Fig. 8), which began to diversify around 44-31 Mya, are associated with various mammals and birds. Species in this group have a combined range from equatorial latitudes in South America up to temperate North America. This implies northward migration events. Exactly how or when this happened is unclear due to poor resolution of our phylogeny at this point.

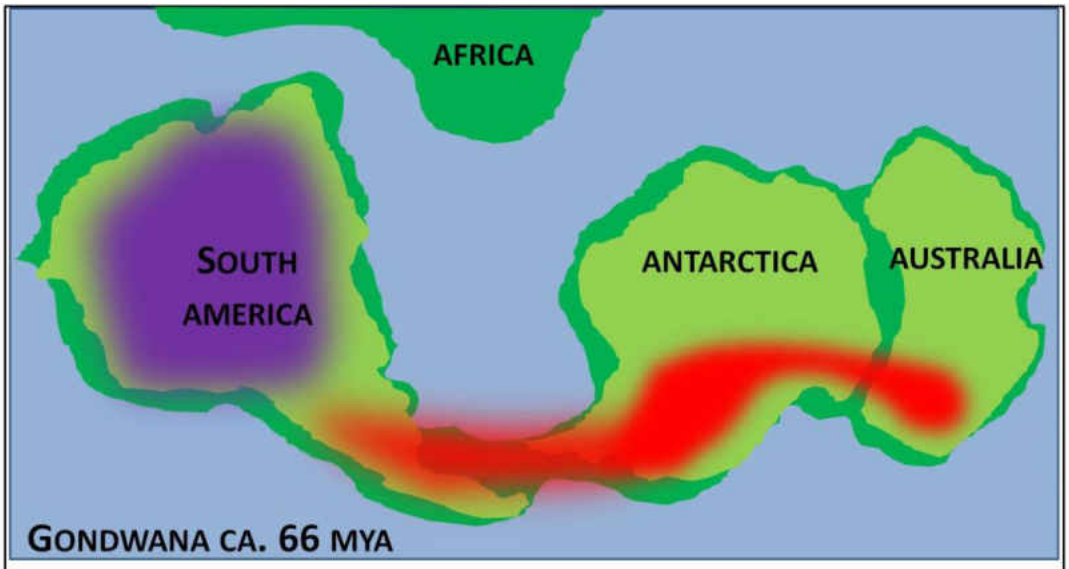

Fig. 8 – map of gondwana ca. 66 mya (k-pg boundary). Australian + southern south american lineages (red); all remaining lineages (purple).

Recent re-evaluations of the timing of the closure of the Panama Isthmus, traditionally placed 7-3.5 Mya, have shown that the seaway between the Caribbean and the Pacific Ocean may have been at least partially closed as early as 20 Mya (Montes et al. 2012, 2015; Bacon et al. 2015), thus allowing some biotic interchanges. In addition, immature *Amblyomma* ticks are known to often parasitize birds (Camicas et al. 1998, Nava et al. 2008, 2010) that could have easily flown over relatively narrow seaways.

From the polytomic node (Fig. 6, Node IX), which stemmed from a South American stock around 48-31 Mya, several unresolved lineages (the Caribbean, several from the Americas and from Africa) and a well-supported endemic Asian lineage arose. This suggests that these geographically heterogeneous branches radiated very rapidly and almost simultaneously.

The African lineages are not monophyletic and their origin clearly post-dated the split between Africa and the rest of Gondwana by 60 million years. This indicated that African *Amblyomma* reached the continent by means other than vicariance, through either overland or trans-oceanic migration.

De Oliviera et al. (2009) have proposed the existence of (now submerged) large continental islands (up to 500km long) between the northeastern tip of South America and northwestern edge of Africa between 50-35 Mya. These islands would have allowed biotic interchange across the spreading Atlantic Ocean due to much shorter distances between viable landmasses. *Amblyomma* may have travelled between the two continents by using these islands. Other organisms (geckos, amphisbaenians, rodents, primates, the plant genus *Manilkara*) are known to have crossed the Atlantic around 20-30 Mya (Gamble et al. 2010, Vidal et al. 2008, de Oliviera et al. 2009, Armstrong et al. 2014). These examples are thought to have migrated from Africa to South America by floating on rafts with oceanic currents or by being carried by wind. However, large, flightless ratite birds have been shown to have travelled in the opposite direction (Mourer-Chauviré et al. 2011), indicating that biotic movements were not unidirectional. It is not unlikely to imagine that ticks might have crossed the ocean repeatedly while the distance between the continents increased progressively by travelling on migratory birds, similar to how *Amblyomma* may have reached North America over the Caribbean seaway. A Gondwanan origin of Neoaves during the Cretaceous has been proposed, which might lend support to this supposition (Cracraft 2001). However, the overall lack of resolution within the South American-African-Asian clade might obscure the real evolutionary radiation pattern of these taxonomic units, which should be considered with caution.

Alternatively, an overland dispersal across another landmass before reaching Africa is also a possibility. The Bering land bridge, still climactically temperate at that time, could have supported migration of ticks on vertebrate hosts, as was the case for reptiles and some mammals (Eberle and Greenwood 2012, Guo et al. 2012). Nevertheless, our analyses show a likely rapid radiation from the polytomic node, indicating that the ticks colonized new areas in quick

succession. As the African continent was isolated from all others during this time, we consider a trans-oceanic mechanism, possibly on birds, far more likely than an overland migration on terrestrial fauna due to the much greater distances *Amblyomma* would have needed to cross in a very short period of time (travelling from South America to North America, and from there, to Asia and then to Africa).

Within the same polytomy**,** all Asian species cluster together and arise nowhere else on the tree. This would undermine the hypothesis of the ancestors of the present Asian species reaching the northern hemisphere with the Gondwana-derived Sibumasu landmass in Burma (approx. 250 Mya, Late Permian; Metcalfe 2011). This would have required the Asian lineage to radiate before the Australian and South American (trans-Antarctic) lineages diverged prior to 100 Mya. Therefore, we can deduce that the fossils found in the Amber deposits of Burma belong to extinct lineages.

Interestingly, associations between Neotropical and Asian taxa, similar to what we observe here, have been observed in other taxonomic groups with assumed Gondwanan origins (Hormuridae scorpions, Monod and Prendini 2014; Zalmoxidae harvestmen, Sharma and Giribet 2012; and in Chironomoidae flies, Krosch et al. 2011). These studies have led to several hypotheses being proposed to explain disjunct distributions of phylogenetically closely-associated taxa**.**

One alternate scenario encompasses recent trans-oceanic dispersal (such as in the cases for *Ixodes uriae* and *I. eudyptidis*) by travelling on birds across the Pacific Ocean (Glyfe et al. 2001, Moon et al. 2015). The distances involved in trans-Pacific dispersal are vast, but collection records from the USNTC (unpublished) show the presence of *Amblyomma* species in Japan, eastern China, Taiwan, the Philippines, Borneo, Indonesia and several Pacific

archipelagos as far as Guam. Additionally, endemic *Amblyomma* in the Galapagos clearly demonstrate that the capacity of these species to colonize remote islands.

Overland migration routes could also be considered. As outlined above, Beringia was a viable land bridge for ticks requiring warmer climates during the Eocene prior to global cooling events (Reguero et al. 2013). Taxa on/near the Asian mainland in China, Taiwan and Japan may be the remnants of such a southward migration from Beringia towards the southern rainforests. It is unlikely that the Asian lineages reached their present distribution range by hitch-hiking through Africa, after the latter was completely isolated from South America and the rest of Gondwana approx. 90 Mya. Monod and Prendini (2014) found evidence for a "Eurogondwana" migration route for Hormuridae scorpions occupying an almost identical distribution as *Amblyomma*. They suggest that, from Africa, the scorpions migrated north onto the European continent and then southeast towards tropical Asia. Nevertheless, we found no evidence supporting an African origin for the Asian clade.

Despite good geographic coverage of our representative taxa, it would have been preferable to obtain data from additional *Amblyomma* species. Unfortunately, the DNA of the additional 63 *Amblyomma* species we attempted to amplify did not yield complete sequences probably because our samples were fairly old and their preservation method possibly not optimal. Given the fact that 18SrDNA gene sequences are very conserved with relatively little polymorphism, we decided to use only sequences for which the whole length was available, sacrificing broader taxonomic coverage for a greater number of phylogenetically informative characters to support our analysis. While complete taxon coverage was not possible, we were able to obtain data from 49 of the 136 recognized *Amblyomma* species (including identical sequences removed from phylogenetic analyses) (Appendix 1), which constitutes the widest

breadth of coverage in *Amblyomma* phylogenetic studies so far (Burger et al. 2012, Klompen et al. 2000, Murrell et al. 2001, Mans et al. 2012).

The occurrence of fossils closely related to *Amblyomma* in Burmese amber deposits indicates that the genus is probably older than our estimates let us believe and that the extant species might be the survivors of the K-Pg extinction event from 66 Mya. In order to include these extinct taxa in a phylogenetic analysis, an extensive morphological data matrix would have to be generated and analyzed parallel to the molecular dataset. Zhu et al. (2015) found a similar pattern with fleas (Siphonaptera), where molecular clock estimates placed the major radiation of lineages just after/around the End Cretaceous extinction events. They observed a trans-Antarctic pattern similar to ours among the basal branches and interpret this to be evidence of a rapid radiation at the beginning of the Paleogene. Additional 18S rDNA sequences of *Amblyomma* species (particularly from Asia) are necessary to further evaluate the early branching pattern of lineages, as well as additional molecular and/or morphological data sets which would allow inclusion of the fossils.

In summary, *Amblyomma* exhibits a phylogenetic structure showing a complex history involving Gondwanan vicariance and recent dispersal events. Our phylogeny and node-dating estimates support the evolution of *Amblyomma* in the southern part of Gondwana after Africa broke off from the main landmass. The genus subsequently underwent radiation first through vicariance (Scotia land bridge), which resulted in some of the southernmost American lineages appearing alongside or clustering with Australian taxa. The following evolutionary history of *Amblyomma* appears to indicate that all subsequent diverging events originated from a Cenozoic South American cluster that later dispersed to Africa and Asia, although the progression of these migration patterns is difficult to substantiate based on our sequences.

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APPENDIX I - List of taxa included in the concatenated matrix and associated sequences used		
Amblyomma species	Accession/GenBank No.†	Origin
APPENDIX I (cont)		
A. chabaudi Rageau	RML 122817	Africa
A. compressum Macalister	CRT 467	Africa
A. flavomaculatum Lucas	RML 125057	Africa
A. gemma Dönitz	RML 123111	Africa
A. hebraeum Koch	RML 120106	Africa
A. latum Koch	L76347	Africa
A. nuttalli Dönitz	RML 119232	Africa
A. sparsum Neumann	RML 122024	Africa
A. tholloni Neumann	RML 35553	Africa
A. variegatum Fabricius	L76346†	Africa
A. crassipes Neumann	RML 122475	Asia
A. fimbriatum Koch	AF018644†	Asia
A. gervaisi Lucas	RML 119828	Asia
A. javanense Supino	USNMENT 00714871	Asia
A. albolimbatum Neumann	RML 83002	Australia
A. glauerti Keirans, King and Sharrad	AF115372†	Australia
A. limbatum Neumann	RML 122622	Australia
A. triguttatum Koch	AF018641†	Australia
A. vikirri Keirans, Bull and Duffield	AF018642†	Australia
A. antillorum Koch	RML 118422	Neotropics
A. argentinae Neumann	RML 124060	Neotropics
A. aureolatum Pallas	RML 124023	Neotropics
A. auricularium Conil.	FJ464426†	Neotropics
A. boeroi Nava et al.	FJ464426†	Neotropics
A. cajennense Fabricius	Rondonia 3	Neotropics
A. dissimile Koch	RML 119249	Neotropics
A. dubitatum Neumann	IBSP 7429	Neotropics
A. hadanii Nava et al.	USNMENT 00957446	Neotropics
A. interandinum Beati, Nava, Caceres	RML 124076	Neotropics
A. longirostre Koch	RML 125522	Neotropics
A. maculatum Koch	L76344†	Neotropics
A. neumanni Ribaga	FJ464424†	Neotropics
A. nodosum Neumann	RML 125529	Neotropics
A. parvitarsum Neumann	FJ464423†	Neotropics
A. parvum Aragão	AMPARV_AP1A	Neotropics
A. pseudoparvum Guglielmone, Mangold & Keirans	RML 123652	Neotropics
A. rotundatum Koch	RML 123652	Neotropics
A. sabanerae Stoll	RML 124516	Neotropics

A. scutatum Neumann	RML 123041	Neotropics
A. tenellum Banks	RML 46324	Neotropics
A. torrei Pérez Vigueras	RML 123609	Neotropics
A. americanum L.	M60487†	North America
A. tuberculatum Marx	L76345†	North America
Outgroup taxa		
A. transversale Lucas	RML 121579	Africa
Hyalomma dromedarii	L76348†	Africa
Haemaphysalis flava	JX573120†	Asia
Haemaphysalis longicornis	JQ346681†	Asia
Bothriocroton concolor	AF018643†	Australia
Bothriocroton glebopalma	AF115371†	Australia
Bothriocroton hydrosauri	F115370†	Australia
Bothriocroton oudemansi	DQ668033†	Australia
Bothriocroton undatum	AF018645†	Australia
Ixodes tasmani	AF115368†	Australia
Ixodes uriae	AF115369†	Australia
Rhipicephalus microplus	KC769615†	Cosmopolitan
Rhipicephalus sangineus	L76342†	Cosmopolitan
A. sphenodonti Dumbleton	RML 123589	New Zealand
A. elaphense Banks	JN863721†	North America
Dermacentor andersoni	DCN18SR†	North America
Ixodes affinis	IXO18SR†	North America
Ixodes persulcatus	AY274888†	Paleartic
Ixodes ricinus	GU07470†	Paleartic

FIG. 3 - MAXIMUM PARSIMONY STRICT CONSENSUS TREE

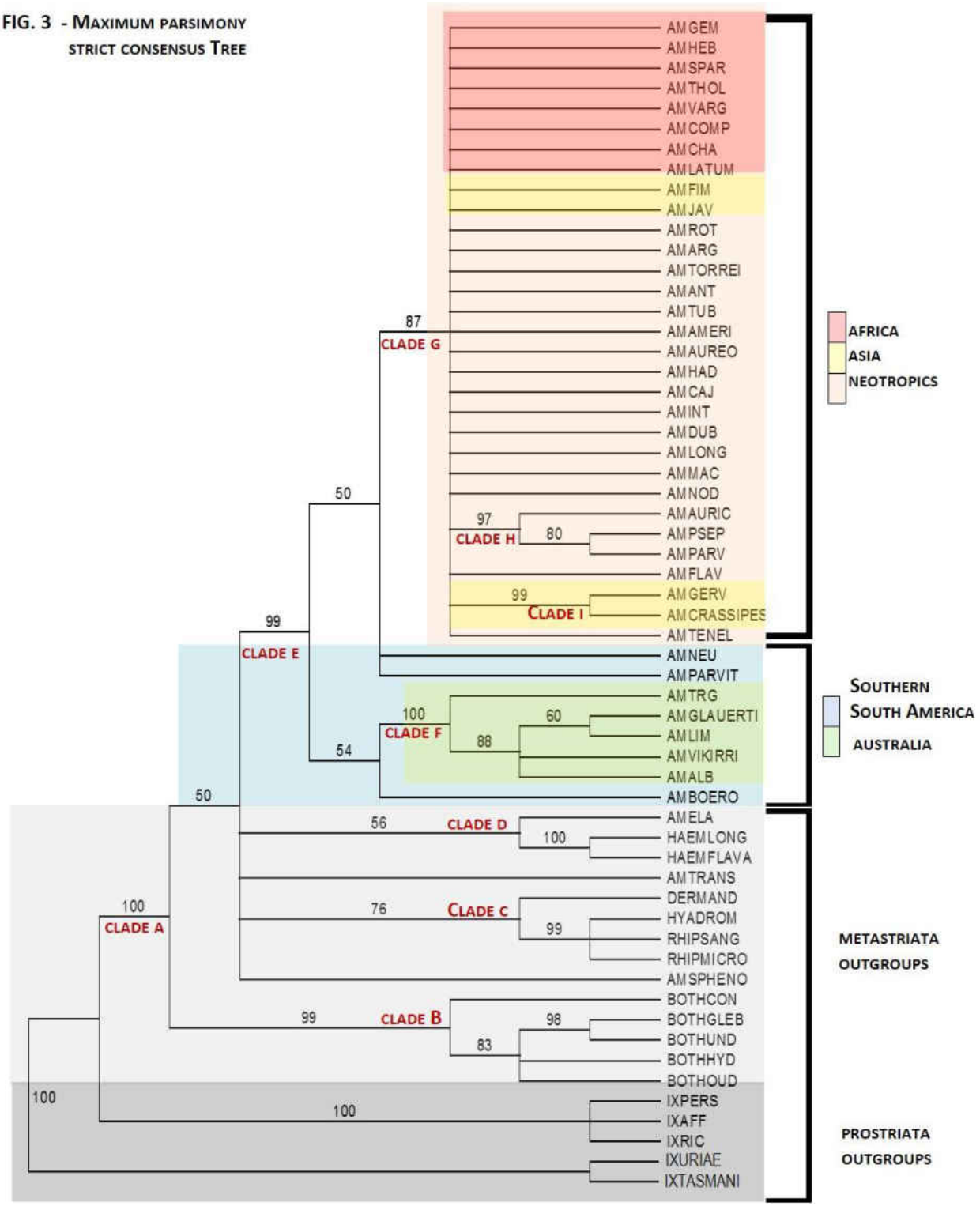
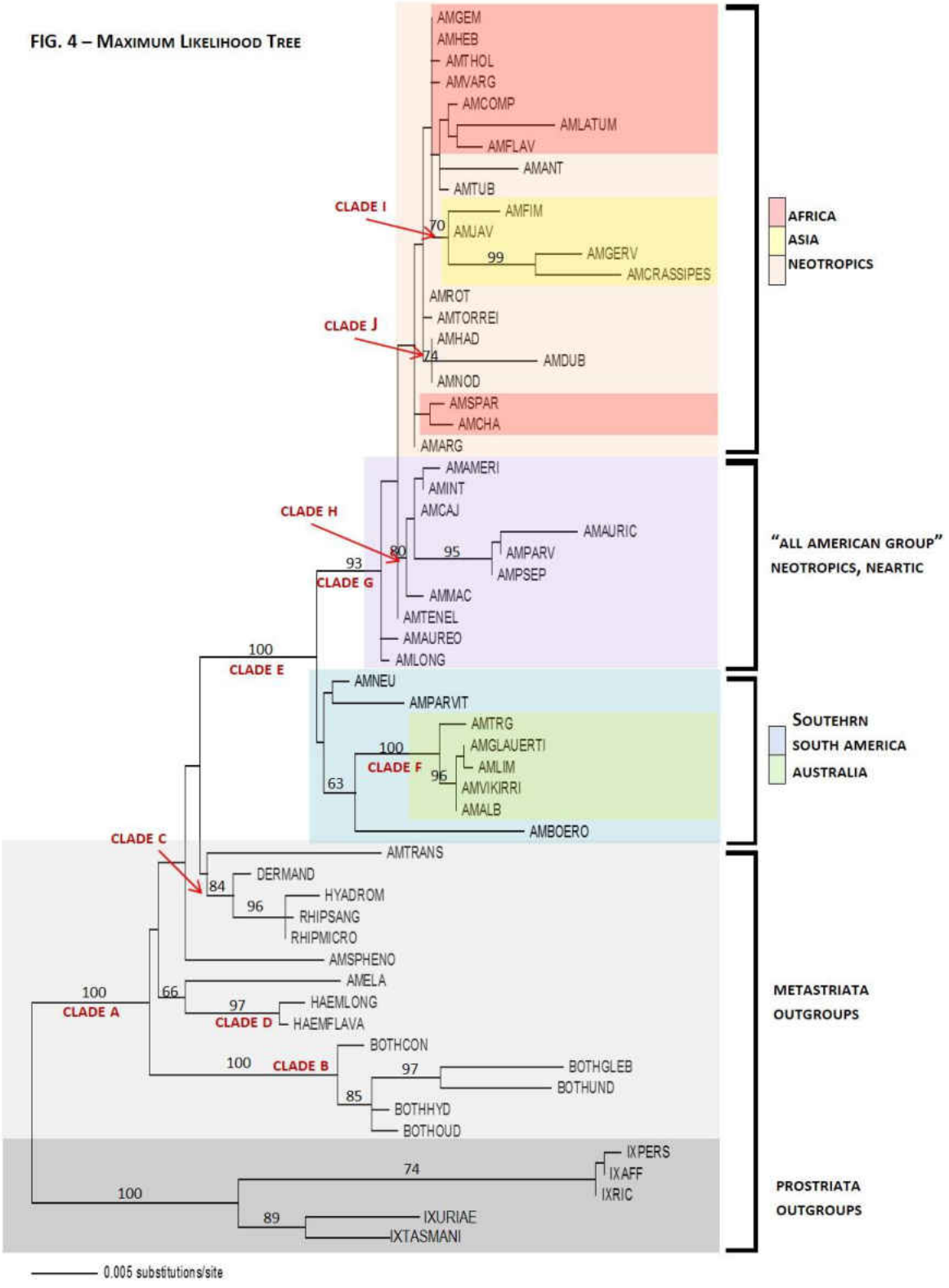


FIG. 4 – MAXIMUM LIKELIHOOD TREE



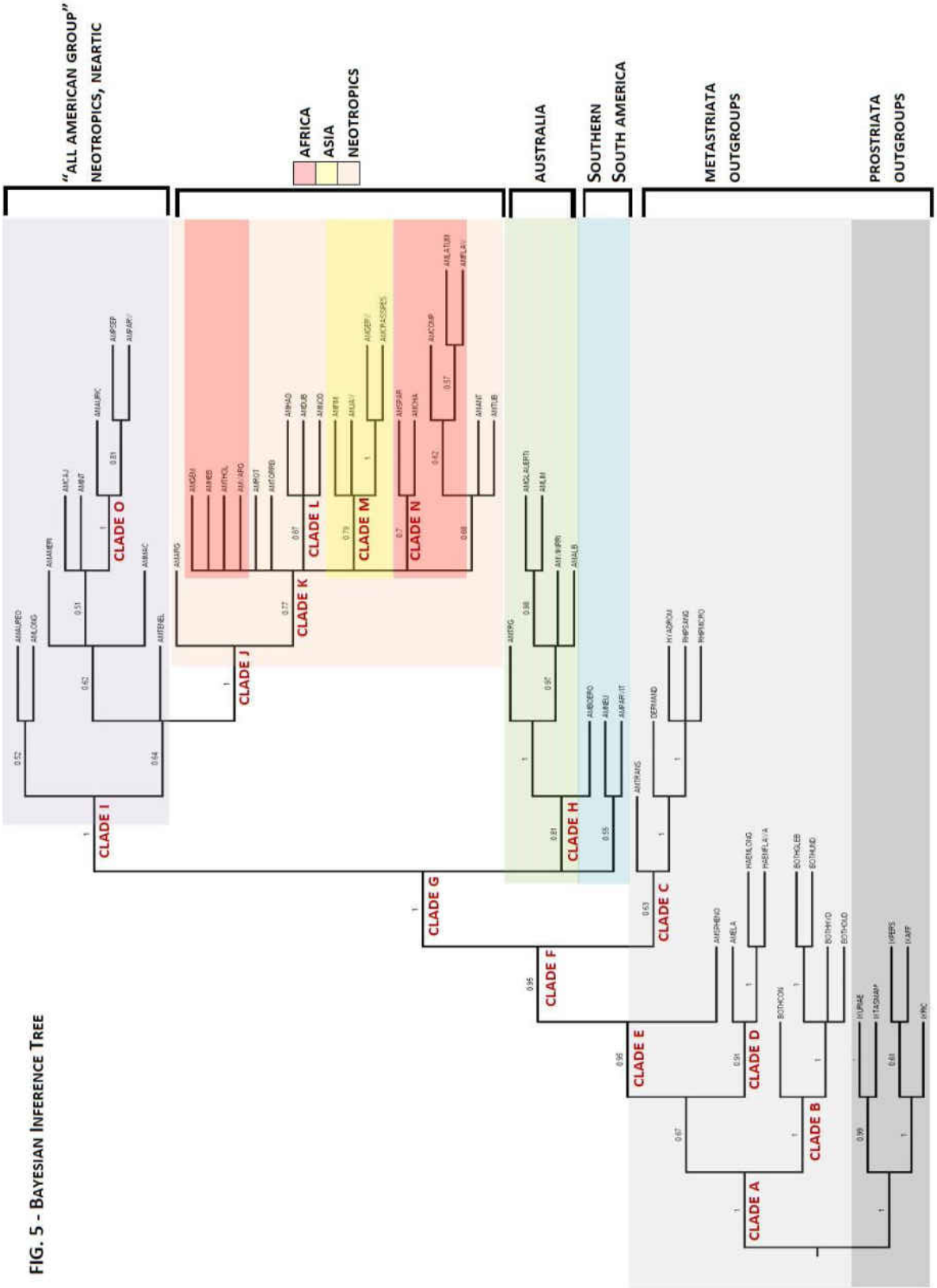


FIG. 5 - BAYESIAN INFERENCE TREE

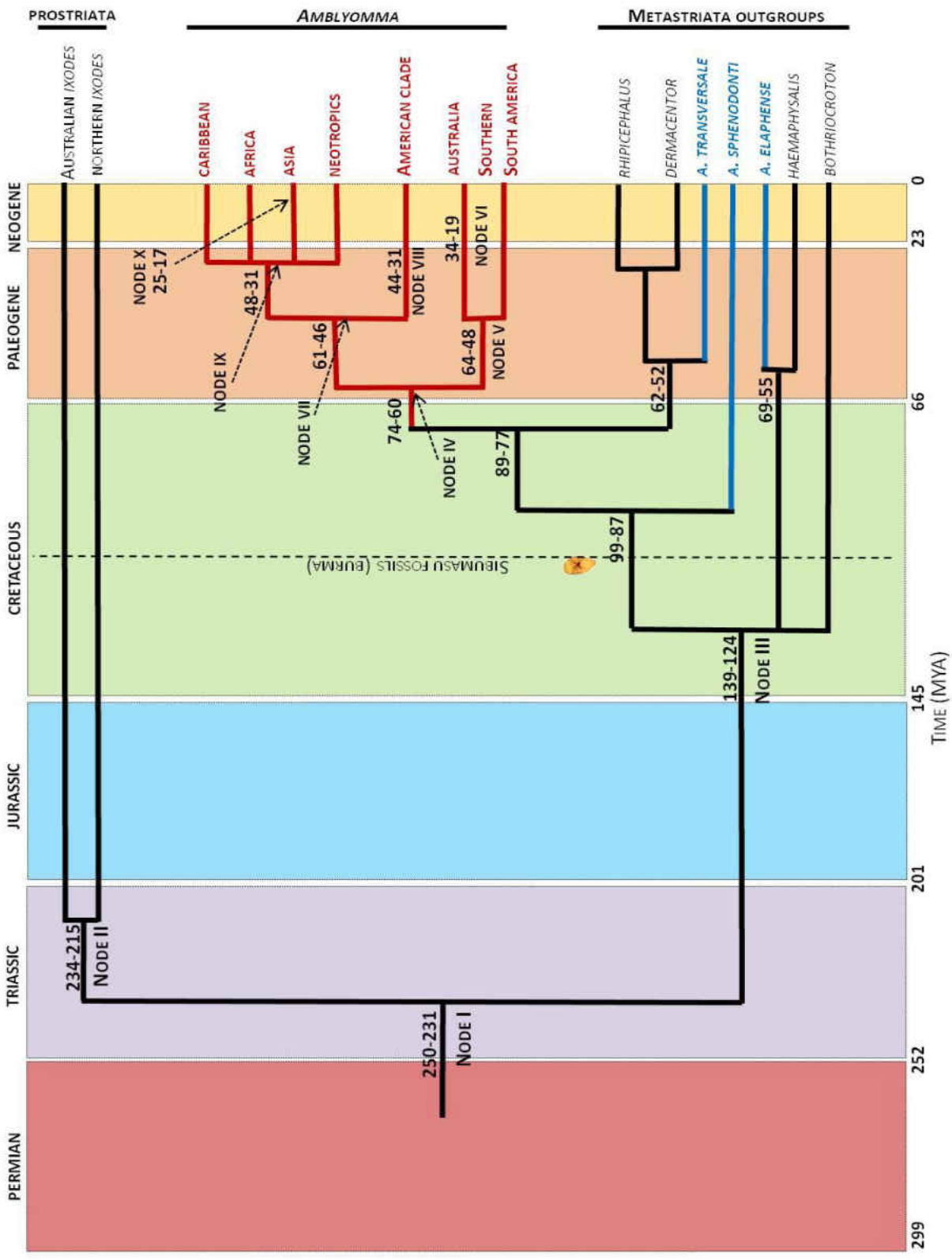


FIGURE 6: SCHEMATIC TREE OF NODE DATING ESTIMATES. NUMBERS CORRESPOND TO ESTIMATED DATES HYPOTHESIZED BY CALIBRATION SCHEMES 1 AND 2.