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Arthur F. Sands
Stellenbosch University

Dmitry A. Apanaskevich
Georgia Southern University

Sonja Matthee
Stellenbosch University

Ivan G. Horak
University of Pretoria

Alan Harrison
University of Pretoria

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Effects of tectonics and large scale climatic changes on the evolutionary history of *Hyalomma* ticks

Arthur F. Sands[1][†], Dmitry A. Apanaskevich[2], Sonja Matthee[3], Ivan G. Horak[4], Alan Harrison[5,6], Shahid Karim[7], Mohammad K. Mohammad[8], Kosta Y. Mumcuoglu[9], Rupika S. Rajakaruna[10], Maria M. Santos-Silva[11], and Conrad A. Matthee[1][*]

[1]Evolutionary Genomics Group, Department of Botany and Zoology, Stellenbosch University, Matieland, South Africa.

[2]United States National Tick Collection, The James H. Oliver, Jr. Institute for Coastal Plain Science, Georgia Southern University, Statesboro, Georgia, United States of America.

[3]Department of Conservation Ecology and Entomology, Stellenbosch University, Matieland, South Africa.

[4]Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa.

[5]Department of Zoology and Entomology, University of Pretoria, Hatfield, Pretoria, South Africa.

[6]KSU Mammals Research Chair, Department of Zoology, King Saud University, Riyadh, Saudi Arabia.

[7]Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, Mississippi, USA

[8]Iraq Natural History Research Centre and Museum, University of Baghdad, Bab Al-Muadham, Baghdad, Iraq.

[9]Parasitology Unit, Department of Microbiology and Molecular Genetics, The Kuvin Centre for the Study of Infectious and Tropical Diseases, The Hebrew University-Hadassah Medical School, Jerusalem, Israel.

[10]Department of Zoology, University of Peradeniya, Peradeniya, Sri Lanka.

[11]Centro de Estudos de Vectores e Doenças Infecciosas Doutor Francisco Cambournac, Instituto Nacional de Saúde Doutor Ricardo Jorge, Aguas de Moura, Portugal.

[*] Corresponding author E-mail: cam@sun.ac.za

[†]Current address: Department of Animal Ecology and Systematics, Justus Liebig University, Giessen, Germany.

Abstract:

Hyalomma Koch, 1844 are ixodid ticks that infest mammals, birds and reptiles, to which 27 recognized species occur across the Afrotropical, Palearctic and Oriental regions. Despite their medical and veterinary importance, the evolutionary history of the group is enigmatic. To investigate various taxonomic hypotheses based on morphology, and also some of the mechanisms involved in the diversification of the genus, we sequenced and analysed data derived from two mtDNA fragments, three nuclear DNA genes and 47 morphological characters. Bayesian and Parsimony analyses based on the combined data (2,242 characters for 84 taxa) provided maximum resolution and strongly supported the monophyly of *Hyalomma* and the subgenus *Euhyalomma* Filippova, 1984 (including *H. punt* Hoogstraal, Kaiser and Pedersen, 1969). A predicted close evolutionary association was found between morphologically similar *H. dromedarii* Koch, 1844, *H. somalicum* Tonelli Rondelli, 1935, *H. impeltatum* Schulze and Schlottke, 1929 and *H. punt*, and together they form a sister lineage to *H. asiaticum* Schulze and Schlottke, 1929, *H. schulzei* Olenev, 1931 and *H. scupense* Schulze, 1919. Congruent with morphological suggestions, *H. anatolicum* Koch, 1844, *H. excavatum* Koch, 1844 and *H. lusitanicum* Koch, 1844 form a clade and so also *H. glabrum* Delpy, 1949, *H. marginatum* Koch, 1844, *H. turanicum* Pomerantzev, 1946 and *H. rufipes* Koch, 1844. Wide scale continental sampling revealed cryptic divergences within African *H. truncatum* Koch, 1844 and *H. rufipes* and suggested that the taxonomy of these lineages is in need of a revision. The most basal lineages in *Hyalomma* represent taxa currently

confined to Eurasia and molecular clock estimates suggest that members of the genus started to diverge approximately 36.25 Million years ago (Mya). The early diversification event coincides well with the collision of the Indian and Eurasian Plates, an event that was also characterized by large scale faunal turnover in the region. Using S-Diva, we also propose that the closure of the Tethyan seaway allowed for the genus to first enter Africa approximately 17.73 Mya. In concert our data support the notion that tectonic events and large scale global changes in the environment contributed significantly to produce the rich species diversity currently found in the genus *Hyalomma.*

Keywords: *Hyalomma*, phylogenetics, systematics, tick evolution, dispersal, Ixodidae

1. Introduction

Bont-legged ticks belonging to the genus *Hyalomma* Koch, 1844 are obligate haematophagous ectoparasites of mammals, birds and reptiles. They occur widespread across the Afrotropical, Palearctic and Oriental zoogeographic regions (Guglielmone et al., 2014) and the oldest fossil remains resembling *Hyalomma* were found in the Baltic region (dating back to 35-50 million years ago (Mya): de la Fuente, 2003). *Hyalomma* are important ectoparasites from a medical and veterinary perspective (Aktas et al., 2004; Bente et al., 2013; Formosinho and Santos-Silva, 2006; Heisch et al., 1962; Norval and Horak, 2004; Taboada and Merchant, 1991; Shyma et al., 2012) and are known vectors for various pathogens such as *Nairovirus* (the agent of Crimean-Congo haemorrhagic fever in humans: Bente et al., 2013; Swanepoel and

Burt, 2004) and *Theileria annulata* (causative agent of bovine tropical theileriosis in cattle: Morrison, 2015). Despite the fact that they are one of the most common ectoparasites on domesticated animals, the most comprehensive molecular comparisons among members of the genus merely includes five of the 27 recognized species (Bakheit et al., 2012; Barker and Murrell, 2004; Guglielmone and Nava, 2014; Zhang and Zhang, 2014). In addition, the difficulties of sampling throughout their wide range, identifying all the life stages of each species correctly, the clear-cut sexual dimorphism in adults, and the preponderance of intraspecific morphological variation in *Hyalomma* have made it nearly impossible to create an accurate and well resolved morphological phylogeny for extant members of the genus (Apanaskevich and Horak, 2008a, 2008b, 2005).

At the subgeneric level, Schulze (1930) recognized three assemblages: 1) *Hyalommasta* Schulze, 1930 with a single species *H. aegyptium* (Linnaeus, 1758); 2) *Hyalommina* Schulze, 1919 with *H. brevipunctatum* Sharif, 1928*, H. kumari* Sharif, 1928 and *H. hussaini* Sharif, 1928 from the Indian subcontinent, and the extralimital Near Eastern/North African *H. rhipicephaloides* Neumann, 1901; 3) *Hyalomma* s. str. with all the remaining species known at that time. Later, the subgeneric nomenclature was revised by Filippova (1984), who synonymized the monotypic subgenus *Hyalommasta* with *Hyalomma* s. str. and proposed the subgenus *Euhyalomma* Filippova, 1984. Since this revision the taxonomic content of subgenera has not changed, except that more species of *Hyalommina* and *Euhyalomma* were recognized and added over time.

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At the species level the taxonomy is equally obscured: *Hyalomma excavatum* Koch, 1844 has been considered either a synonym or a subspecies of *H. anatolicum* Koch, 1844 (Delpy, 1949; Hoogstraal, 1956; Hoogstraal and Kaiser, 1959) but Apanaskevich (2003) and Apanaskevich and Horak (2005) afforded *H. excavatum* a full species status closely related to *H. anatolicum*. Similarly, *H. isaaci* Sharif, 1928, *H. rufipes* Koch, 1844 and *H. turanicum* Pomerantzev, 1946 were treated as subspecies of *H. marginatum* Koch, 1844 (Hoogstraal and Kaiser, 1960; Kaiser and Hoogstraal, 1963; Pomerantzev, 1946). After a thorough morphological revision, Apanaskevich and Horak (2008a) recognized *H. isaaci*, *H. rufipes* and *H. turanicum* as full species. Moreover, Apanaskevich and Horak (2006) also re-established *H. glabrum* Delpy, 1949 that was then considered a synonym of *H. turanicum* (Hoogstraal, 1956). The full species status of morphologically near similar *H. albiparmatum* Schulze, 1919 from central-East Africa (that has an ivory-coloured parma in males), *H. nitidum* Schulze, 1919 from West Africa (that shows a reduction in clarity of ivory-colored bands on the segments of the legs in both sexes and the external cuticular preatrial fold of the genital aperture of females are convex) and the widely distributed *H. truncatum* Koch, 1844 have also been a subject of much debate (see Apanaskevich and Horak, 2008b; Camicas et al., 1998; Feldman-Muhsam, 1962; Hoogstraal, 1979, 1956; Tomassone et al., 2005; Walker, 1974). Finally, at the intraspecific level, evidence also exists that species occupying wide geographic ranges such as *H. truncatum* and *H. rufipes* show cryptic speciation (Cangi et al., 2013; Sands et al., 2017).

A better understanding of the mechanisms involved in the diversification of tick species can facilitate a more stable taxonomic framework for *Hyalomma*. In general, host association and host movement are regarded as important facilitators of tick dispersal across the landscape (Araya-Anchetta et al., 2015; Blouin et al., 1995; Engelbrecht et al., 2016; Sands et al., 2017). Life history, ecology and environmental factors off the host are however equally important (Araya-Anchetta et al., 2015; Johnson et al., 2010; Maze-Guilmo et al., 2016). In ticks, deciphering the mechanisms responsible for speciation is particularly complex since members of the group are characterized by complex life cycles (Sonenshine, 1991; Walker et al., 2014). They are often dependent on various hosts for part of their life cycle (Sonenshine, 1991; Walker et al., 2014), and they are also sensitive to environmental influences that can severely affect the survival of free-living stages off the host (Walker et al., 2014). Since tick species also differ in time spent off the host, it is to be expected that the effect of external environmental conditions will be more important for tick species that spend a larger portion of the life cycle off the host (two- and three-host ticks) compared to those that spend less (one-host ticks) (Sonenshine, 1991).

Because the majority of *Hyalomma* species are multi-host parasites with wide host ranges (Apanaskevich, 2004), we predict that host association and host diversification *per se* will play a less important role in the speciation process of *Hyalomma* (Cumming, 1999; McCoy et al., 2013). It is more likely that the majority of the recent diversifications of *Hyalomma* species are driven by a combination of ecological interactions between the parasites and their hosts (Cangi et al., 2013) and the abiotic biogeographic conditions in the

environment (Klompen et al., 1996; McCoy et al., 2013). The latter also specifically includes the effects that biogeography can have on host movement and distribution (Sands et al., 2017).

Based on fossil evidence, the origin of *Hyalomma* potentially dates back to 50 Mya (de la Fuente, 2003). Since members of the genus are distributed across continents, it is possible that deeper divergences among lineages may coincide with tectonic events and large scale climatic changes that were responsible for the formation and disappearances of land bridges throughout geological time periods (Rögl, 1999). In particular, restricted dispersal imposed on multiple hosts (vicariance) could have resulted in allopatric speciation processes within the genus (Sands et al., 2017). The formation of land bridges in turn could have facilitated intercontinental exchanges of host species between the Afrotropics and Paleartic/Oriental regions (Popov et al., 2004; Portik and Papenfuss, 2015; Rögl, 1999). Particularly pertinent to this study, the Arabian plate separated from Africa approximately 30 Mya and this was followed by rifting in the southern Red Sea at approximately 27 Mya (Bohannon, 1986; Bosworth et al., 2005; Jolivet and Faccenna, 2000). This resulted in the formation of the Tethyan seaway which only temporarily closed again at approximately 19 Mya (forming of the so-called *Gomphotherium* land bridge: Cox, 2000; Popov et al., 2004; Rögl, 1999). At 15 Mya, a more permanent land bridge was formed between Africa and Arabia when the Arabian plate fused with Eurasia (Bosworth et al., 2005). These geologic activities had severe effects on the faunal biogeography in the region

(Fernendes et al., 2006; Portik and Papenfuss, 2015) and we predict that it may have also affected the evolution of *Hyalomma* in a similar way.

The aim of this study was to investigate some of the mechanisms involved in the diversification of *Hyalomma,* by constructing a dated phylogeny based on molecular data sets. In addition, we aimed to test some of the numerous taxonomic hypotheses by generating various phylogenetic trees based on mtDNA, nuclear DNA and morphological data. The geographic variation present in some of the more widespread taxa was addressed by using comprehensive taxonomic sampling. The study improves our current understanding of the evolutionary relationships among the species or genetic lineages within the genus (which is important from a disease perspective). Moreover, by using a molecular clock and a dispersal-vicariance analysis, we also provide additional evidence to explain the role of plate tectonics and the associated large scale climatic changes on tick evolution.

2. Materials and methods

2.1 Sampling design

Data were gathered from the 27 known species, sampled from 26 different countries. A morphological data matrix was constructed for all species, whereas 24 of the recognized species (78 individuals) were included in the molecular analyses (Table 1). Cytochrome c oxidase subunit I (COI) DNA sequences for eight *Hyalomma* species were also obtained from GenBank to validate and cross check the species identifications across studies (Appendix

Table 1. Sampling localities for the 27 *Hyalomma* species and outgroups used in the present study. Genbank accession numbers for various gene fragments sequenced herein are also given.

No.	Species	Country/origin	GenBank accesion no.	No.	Species	Country/origin	GenBank accesion no.
	Hyalomma			1	*H. nitidum*	Benin	KU130449, KU130531, KU130613, KU130698, KU130778
1	*H. aegyptium*	Israel	KU130407, KU130490, KU130573, KU130656, KU130737	2	*H. nitidum*	Benin	KU130450, KU130532, KU130614, KU130699, KU130779
2	*H. aegyptium*	Israel	KU130408, KU130491, KU130574, KU130657, KU130738	3	*H. nitidum*	Benin	KU130451, KU130533, KU130615, KU130700, KU130780
3	*H. aegyptium*	Israel	KU130409, KU130492, KU130575, KU130658, KU130739	1	*H. punt*	Somalia	KU130452, KU130534, KU130616, KU130701, KU130781
1	*H. albiparmatum*	Kenya	KU130410, KU130493, KU130576, KU130659, KU130740	2	*H. punt*	Somalia	KU130453, KU130535, KU130617, KU130702, KU130782
2	*H. albiparmatum*	Kenya	KU130411, KU130494, KU130577, KU130660, KU130741	3	*H. punt*	Somalia	KU130454, KU130536, KU130618, KU130703, KU130783
3	*H. albiparmatum*	Kenya	KU130412, KU130495, KU130578, KU130661, KU130742	1	*H. rhipicephaloides*	Israel	KU130456, KU130538, KU130620, KU130705, KU130785
1	*H. anatolicum*	Iraq	KU130413, KU130496, KU130579, KU130662, KU130743	1	*H. rufipes*	Senegal	KU130457, KU130539, KU130621, KU130706, KU130786
2	*H. anatolicum*	Pakistan	KU130414, KU130497, KU130580, KU130663, KU130744	2	*H. rufipes*	Senegal	KU130458, KU130540, KU130622, KU130707, KU130787
3	*H. anatolicum*	Pakistan	KU130415, KU130498, KU130581, KU130664, KU130745	3	*H. rufipes*	Nigeria	KU130459, KU130541, KU130623, KU130708, KU130788
1	*H. arabica*	Saudi Arabia	KU130416, KU130499, KU130582, KU130665, KU130746	4	*H. rufipes*	Burkina Fuso	KU130460, KU130542, KU130624, KU130709, KU130789
2	*H. arabica*	Saudi Arabia	KU130417, KU130500, KU130583, KU130666, KU130747	5	*H. rufipes*	Somalia	KU130543, KU130625, KU130710, KU130790
3	*H. arabica*	Saudi Arabia	KU130418, KU130501, KU130584, KU130667, KU130748	6	*H. rufipes*	Namibia	KU130461, KU130544, KU130626, KU130711, KU130791
1	*H. asiaticum*	Turkmenistan	KU130419, KU130502, KU130585, KU130668, KU130749	7	*H. rufipes*	Namibia	KU130462, KU130545, KU130627, KU130712, KU130792
2	*H. asiaticum*	Turkmenistan	KU130420, KU130503, KU130586, KU130669, KU130750	8	*H. rufipes*	Mozambique	KU130463, KU130546, KU130628, KU130713, KU130793
3	*H. asiaticum*	Turkmenistan	KU130421, KU130504, KU130587, KU130670, KU130751	9	*H. rufipes*	Mozambique	KU130464, KU130547, KU130629, KU130714, KU130794
1	*H. brevipunctatum*	India	Morphological data only	10	*H. rufipes*	South Africa	KU130465, KU130548, KU130630, KU130715, KU130795
1	*H. dromedarii*	Iraq	KU130422, KU130505, KU130588, KU130671, KU130752	1	*H. schulzei*	Iraq	KU130466, KU130549, KU130631, KU130716, KU130796
2	*H. dromedarii*	Pakistan	KU130423, KU130506, KU130589, KU130672, KU130753	2	*H. schulzei*	Iraq	KU130467, KU130550, KU130632, KU130717, KU130797
3	*H. dromedarii*	Saudi Arabia	KU130424, KU130507, KU130590, KU130673, KU130754	1	*H. scupense*	Russia	KU130468, KU130551, KU130633, KU130718, KU130798
4	*H. dromedarii*	Senegal	KU130425, KU130508, KU130591, KU130674, KU130755	2	*H. scupense*	Pakistan	KU130469, KU130552, KU130634, KU130719, KU130799
1	*H. excavatum*	Israel	KU130426, KU130509, KU130592, KU130675, KU130755	3	*H. scupense*	Tunisia	KU130470, KU130553, KU130635, KU130720, KU130800
2	*H. excavatum*	Israel	KU130427, KU130510, KU130593, KU130676, KU130756	4	*H. scupense*	Iran	KU130471, KU130554, KU130636, KU130721, KU130801
3	*H. excavatum*	Tunisia	KU130428, KU130511, KU130594, KU130677, KU130757	1	*H. somalicum*	Somalia	KU130472, KU130555, KU130637, KU130722, KU130802
4	*H. excavatum*	Israel	KU130429, KU130512, KU130595, KU130678, KU130758	2	*H. somalicum*	Somalia	KU130473, KU130556, KU130638, KU130723, KU130803
1	*H. franchinii*	Egypt	Morphological data only	1	*H. truncatum*	Benin	KU130474, KU130557, KU130639, KU130724, KU130804
1	*H. glabrum*	South Africa	KU130430, KU130513, KU130596, KU130679, KU130759	2	*H. truncatum*	Kenya	KU130475, KU130558, KU130640, KU130725, KU130805
2	*H. glabrum*	South Africa	KU130431, KU130514, KU130597, KU130680, KU130760	3	*H. truncatum*	Senegal	KU130476, KU130559, KU130641, KU130726, KU130806
3	*H. glabrum*	South Africa	KU130432, KU130515, KU130598, KU130681, KU130761	4	*H. truncatum*	Mali	KU130477, KU130560, KU130642, KU130727, KU130807
1	*H. hussaini*	Pakistan	KU130433, KU130516, KU130682, KU130762	5	*H. truncatum*	South Africa	KU130478, KU130561, KU130643, KU130728, KU130808
1	*H. hystricis*	India	Morphological data only	6	*H. truncatum*	Namibia	KU130479, KU130562, KU130644, KU130729, KU130809
1	*H. impeltatum*	Senegal	KU130434, KU130517, KU130599, KU130683, KU130763	7	*H. truncatum*	Somalia	KU130563, KU130645, KU130730, KU130810
2	*H. impeltatum*	Saudi Arabia	KU130435, KU130518, KU130600, KU130684, KU130764	1	*H. turanicum*	Iraq	KU130480, KU130564, KU130646, KU130731, KU130811
3	*H. impeltatum*	Senegal	KU130436, KU130519, KU130601, KU130685, KU130765	2	*H. turanicum*	Iraq	KU130481, KU130565, KU130647, KU130732, KU130812
1	*H. impressum*	Benin	KU130437, KU130520, KU130602, KU130686, KU130766	3	*H. turanicum*	Iraq	KU130482, KU130566, KU130648, KU130733, KU130813
2	*H. impressum*	Benin	KU130438, KU130521, KU130603, KU130687, KU130767	4	*H. turanicum*	Pakistan	KU130483, KU130649, KU130734, KU130814
1	*H. isaaci*	Pakistan	KU130439, KU130522, KU130604, KU130688, KU130768		*Amblyomma*		
2	*H. isaaci*	Sri Lanka	KU130440, KU130523, KU130605, KU130689, KU130769	1	*A. hebraeum*	South Africa	Morphological data only
3	*H. isaaci*	Pakistan	KU130441, KU130524, KU130606, KU130690, KU130770	1	*A. variegatum*	Senegal	KU130401, KU130484, KU130567, KU130650
1	*H. kumari*	Pakistan	KU130442, KU130525, KU130607, KU130691, KU130771	2	*A. variegatum*	Senegal	KU130402, KU130485, KU130568, KU130651
2	*H. kumari*	Pakistan	KU130443, KU130526, KU130608, KU130692, KU130772	3	*A. variegatum*	Nigeria	KU130403, KU130486, KU130569, KU130652
1	*H. lusitanicum*	Portugal	KU130444, KU130527, KU130609, KU130693, KU130773	4	*A. variegatum*	Nigeria	KU130404, KU130487, KU130570, KU130653
2	*H. lusitanicum*	Italy	KU130445, KU130694, KU130774		*Nosomma*		
1	*H. marginatum*	Ukraine	KU130446, KU130528, KU130610, KU130695, KU130775	1	*N. monstrosum*	Sri Lanka	KU130405, KU130488, KU130571, KU130654, KU130735
2	*H. marginatum*	Portugal	KU130447, KU130529, KU130611, KU130696, KU130776	2	*N. monstrosum*	Sri Lanka	KU130406, KU130489, KU130572, KU130655, KU130736
3	*H. marginatum*	Russia	KU130448, KU130530, KU130612, KU130697, KU130777				

Table A.1). *Amblyomma hebraeum* Koch, 1844, *A. variegatum* (Fabricius, 1794) and *Nosomma monstrosum* (Nuttall and Warburton, 1908) were used as outgroups based on the work of Murrell et al. (2001, 2000) (Table 1).

2.2 Morphological data matrix

The morphological data matrix comprised 47 characters (Table 2) and were similar to those used in the more recent *Hyalomma* species revisions (Apanaskevich, 2003; Apanaskevich et al., 2010, 2008a, 2008b; Apanaskevich and Horak, 2010, 2009, 2008a, 2008b, 2007, 2006, 2005). Characters were scored using a stereoscopic microscope and included larval, nymph and adult life stages. All scoring was done against known voucher specimens or descriptions thereof (Table 2). Each morphological character was treated as independent (Beati and Keirans, 2001). Where data were not available for a particular life stage, or the character state could not be established, the character was treated as missing (indicated by "-" in Table 2).

2.3 Molecular data generation

All freshly collected specimens were placed in 100% ethanol and stored until DNA extraction. The CTAB technique (Winnepenninckx et al., 1993) was used to extract total genomic DNA. Extractions were performed by placing the entire animal in the extraction buffer. Specimens were incubated at 55°C for 48 hours in a heat block to allow for maximum digestion. After the extraction, exoskeletons of specimens were removed and stored in 100% ethanol to form part of a reference collection at the Evolutionary Genomics Group, Stellenbosch University.

Table 2. Morphological character states (n = 47) derived from 27 *Hyalomma* species and the outgroup taxon *Amblyomma hebraeum*.

	CHARACTER	0	1	2	H. aegyptium	H. albiparmatum	H. anatolicum	H. arabica	H. asiaticum	H. brevipunctata	H. dromedarii	H. excavatum	H. franchinii	H. glabrum	H. hussaini	H. hystricis	H. impeltatum	H. impressum	H. isaci	H. kumari	H. lusitanicum	H. marginatum	H. nitidum	H. punt	H. rhipicephaloides	H. rufipes	H. schulzei	H. scupense	H. somalicum	H. truncatum	H. turanicum	A. hebraeum
1	Shape of eyes (all stages)	Roundish	Oval		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
2	Shape of eyes (adults)	Flat	Spherical		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
3	Position of eyes (adults)	Marginal	Orbital		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
4	Pigmentation on scutum or conscutum (adults)	Intensive	Marbled	Absent	2	2	2	2	2	2	2	1	1	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	0
5	Number of ventro-median setae on I segment of palpi (adults)	< 5	< 5		1	1	1	0	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	0
6	Spurs of coxa I (adults)	Separated	Juxtaposed		0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
7	Median spur of coxa I (adults)	Absent	Present		1	1	1	0	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	0
8	Coloration of leg segments (adults)	Uniformly brown	Ivory-coloured pigment diffused	Ivory pigment forming rings and strips	0	2	1	0	2	0	2	1	1	2	0	0	2	2	2	0	1	2	2	2	0	2	2	2	2	2	2	2
9	Number of anal shields (male)	2 pairs	3 pairs	Absent	1	1	1	0	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	-
10	Ratoi length : width of adanal schield (male)	< 1.5	< 1.5		0	1	1	0	1	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	-
11	Ventral lateral projections of basis capituli (male)	Absent	Present		0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
12	Festoons (male)	11	4 or 5		0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	1	1	1	1	1	1	0
13	Caudal field (male)	Not pronounced	Pronounced		0	1	1	0	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	1	1	1	1	1	1	0
14	Cervical grooves (male)	Pit-like	Furrow-like		0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0
15	Cervical grooves (male)	Short (≤ 1/3 of conscutum)	Long (≥ 1/2 of conscutum)		0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
16	Lateral grooves (male)	Long (reach midlength of conscutum)	Short (don''t reach midlength of conscutum)		-	0	1	1	1	0	1	0	0	0	0	-	1	0	0	0	1	0	0	1	1	0	1	1	1	0	0	1
17	Posterio-median margin of adanal shields (male)	Straight	Curved		0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	1	0	-
18	Parma (male)	Always absent	Present (at least sometimes)		0	1	1	0	1	0	1	1	1	0	0	1	1	0	0	1	0	0	0	0	1	1	1	0	0	0	0	0
19	Median projection of adanal shields (male)	Poor developed	Absent	Well developed	1	2	2	1	2	1	2	2	2	1	1	2	2	2	1	2	2	2	0	1	2	2	2	2	2	2	2	-
20	Ratio l : w of basis capituli (male)	< 1.7	1.9 <		0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
21	Marginal grooves (male)	Present	Absent		1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
22	Narrowing of conscutum in area of spiracular plates (male)	Not clear	Clear		0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0
23	Ventral lateral projections of basis capituli (female)	Absent	Present		0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0
24	Genital operculum (female)	Wide	Narrow		0	0	0	0	1	1	1	0	0	0	0	1	1	1	0	1	0	0	0	1	1	0	0	0	1	0	0	1
25	Genital operculum (female)	High arc (U)	V-shaped (high)	Low arc	0	0	2	0	0	0	1	2	2	-	0	0	0	0	2	0	2	2	0	0	0	2	0	0	1	0	2	1
26	Vestibular part of vagina (female)	Swollen	Not swollen		0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
27	Ratio l : w of basis capituli (female)	Approximately ≤2	Approximately 2.5		0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0
28	Ratio l : w of gnathosoma (adults)	l > w	l = w		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29	Ratio l of palpi (II and III articles) : l of basis capituli (adults)	Palpi > basis capituli	Palpi <= basis capituli		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	Ratio l : w of gnathosoma (female)	l > w in 2 times and more	l < w	l > w in 1.3 to 1.6 times	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0
31	Lateral projections of basis capituli (female)	Absent	Almost unpronounced from lateral edge	Clearly pronounced from lateral adge	1	1	1	2	1	2	1	1	1	2	1	1	1	1	2	1	1	1	1	2	1	1	1	1	1	1	1	0
32	Position of lateral projections of basis capituli (female)	Absent	Anteriorly	Middle or posterior	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0
33	Base of capituli (nymph)	Hexagonal	Triangular	Rectangular	0	0	0	0	0	1	0	0	0	0	1	-	0	0	0	1	0	0	0	-	0	0	0	0	0	0	0	0
34	Lateral projections of basis capituli (nymph)	Midlength	Posterior		1	1	1	1	1	1	1	1	1	1	1	-	1	1	1	1	1	1	1	-	1	1	1	1	1	1	1	0
35	Hypostome (nymph)	Strong dentated	Poorly dentated		1	0	1	1	1	1	1	1	1	0	1	-	1	0	0	1	1	0	0	-	1	0	1	1	1	0	0	1
36	Scutum ratio l : w (nymph)	l ≥ w	l < w		1	1	1	0	1	0	1	1	1	1	0	-	1	1	1	0	1	1	1	-	0	1	1	1	1	1	1	0
37	Setae of alloscutum (nymph)	With dentation	Without dentation		0	0	1	-	1	0	1	1	1	1	0	-	1	0	1	0	0	1	0	-	1	1	1	1	1	1	1	0

#	Character	State 0	State 1	State 2	Data
38	Spurs of caxae II-IV (nymph)	*Moderate*	*Large*	*Reduced (fold-like)*	0 0 0 0 0 0 0 0 1 0 - 0 0 1 0 0 1 0 - 0 1 0 2 0 0 1 1
39	Coxal pore (nymph)	*Present*	*Absent*		0 0 1 - 0 0 1 1 0 1 0 - 0 0 1 0 0 1 0 - 1 1 1 1 0 0 1 0
40	Ratio II : III segments of palpi (nymph)	*II < III more than 2 times*	*II < III less than 2 times*		0 0 0 0 0 0 0 0 0 0 0 - 0 0 0 0 0 0 0 - 0 0 0 0 0 0 0 0
41	Spurs of coxae I (nymph)	*Separated*	*Close together*		0 0 0 0 1 0 0 0 0 1 - 0 0 0 1 0 0 0 - 0 0 0 0 0 0 0 0
42	Spurs of coxae I (nymph)	*Narrow*	*Broad*		0 0 0 0 0 0 0 0 1 0 - 0 0 0 0 1 1 0 - 0 1 0 0 0 0 1 0
43	Posteromedian spur of coxae Ish	*Shorter than posterolateral*	*Subequal to posterolateral*		0 1 1 1 1 1 1 1 0 1 1 - 1 1 1 1 1 1 1 - 0 1 1 0 1 1 1 0
44	Scutum (larva)	*Short (portion of scutum posterior to eyes ≤ 1/4 of scutal length)*	*Moderately long (portion of scutum posterior to eyes ± 1/3 of scutal length)*	*Long (portion of scutum posterior to eyes ± 1/2 of scutal length)*	2 2 1 0 0 0 1 2 2 0 - 0 1 2 0 2 2 2 - 0 2 0 2 0 2 2 2
45	I segment of palpi (larva)	*Present*	*Reduced*		0 0 0 0 0 0 0 0 0 0 0 - 0 0 0 0 0 0 0 - 0 0 0 0 0 0 0 0
46	Eyes (larva)	*Undivided*	*Divided*		1 1 1 1 1 1 1 1 1 1 1 - 1 1 1 1 1 1 1 - 1 1 1 1 1 1 1 0
47	Basis capituli dorsally (larva)	*Hexagonal*	*Triangular*	*Rectangular*	0 0 0 0 0 1 0 0 0 0 1 - 0 0 0 1 0 0 0 - 0 0 0 0 0 0 0 0

Table 3. Gene regions sequenced for *Hyalomma.* Primer names, primer sequences and the edited sequence length for each fragment are given. The optimal annealing temperatures of the primer pairs and the sources of the primers used are also indicated.

Region	Gene	F/R	Primer name	Primer sequence (5'-3')	Edited sequence length (bp)	Optimal annealing temperature (^{0} C)	Source
mtDNA	16S	Forward	16+1	CTGCTCAATGATTTTTTAAATTGCTGTGG	378	52^0 C	Black and Piesman (1994)
		Reverse	16S-1	CCGGTCTGAACTCAGATCAAGT			Black and Piesman (1994)
	CO I	Forward	AR-U-COIa	AAACTRTKTRCCTTCAAAG	664	45^0 C	Cangi et al. (2013)
		Reverse	AR-L-COIa	GTRTTAAARTTTCGATCSGTTA			Cangi et al. (2013)
nDNA	ITS II	Forward	RIB-8	GTCGTAGTCCGCCGTC	273	62^0 C	Rees et al. (2003)
		Reverse	RIB-11	GAGTACGACGCCCTACC			Rees et al. (2003)
	28S	Forward	28v	AAGGTAGCCAAATGCCTCG	632	55^0 C	Hillis and Dixon (1991)
		Reverse	28x	GTGAATTCTGCTTCACAATGATAGGA			Hillis and Dixon (1991)
	H3	Forward	HyH3F	GTGGATGGCRCAMARGTTGG	268	56.5^0 C	Sands et al. (2017)
		Reverse	HyH3R	GCAAGAGYACCGGWGGVAAR			Sands et al. (2017)

Polymerase Chain Reaction (PCR) amplifications on a GeneAmp PCR 2700 thermal cycler (Applied Biosystems) and sequencing of selected gene regions were performed using previously published primers (Table 3). DNA sequences for two mitochondrial genes (mtDNA); Cytochrome c oxidase subunit I (COI) and ribosomal 16S rRNA (16S), and three nuclear fragments (nDNA); Histone 3 (H3), ribosomal 28S rRNA (28S) and the Internal Transcribed Spacer 2 region (ITS II) were generated (Table 3). Different annealing temperatures were used for the different loci (Table 3) and the remainder of the PCR cycling conditions were according to Sands et al. (2015). Aliquots of PCR products were separated by gel electrophoresis using 1% agarose and successful PCR products were purified using a BioFlux, Biospin Gel Extraction Kit (Bioer Technology Co., Ltd.). Sequences were generated by the Central Analytical Facility of Stellenbosch University using BigDye Chemistry and an ABI 3730 XL DNA Analyzer (Applied Biosystems).

2.4 Sequence editing and alignment

Sequence calls were visually confirmed and corrected where needed using BioEdit 7.1.3.0 (Hall, 1999). Data were aligned using CrustalW (Thompson et al., 1994), and to limit missing data, ends were trimmed. Alignment gaps were inserted in the 16S, 28S and ITS II regions (where appropriate the secondary structures of the regions were also considered to aid in the alignment) (Hillis and Dixon, 1991). A total of 32bp (between positions 152bp and 186bp in the published alignment) could not be aligned with certainty and were removed from the 16S data set before analyses.

2.5 Phylogenetic relationships and divergence dating

Since COI mtDNA sequence distances, subjected to Automatic Barcode Gap Discovery (ABGD), proved useful in defining the taxonomic rank of tick taxa (Zhang and Zhang, 2014), we calculated COI sequence distances between all *Hyalomma* taxa used herein. With a few exceptions, interspecific COI divergences in ticks usually exceeded 8.0% while intraspecific divergences of most species did not exceed 2.0% (Zhang and Zhang 2014).

Phylogenies were first generated for each data type (morphology and DNA) and gene fragment separately (to explore conflict among phylogenies). To further explore congruence between mitochondrial and nuclear gene trees, we conducted combined analyses of all nDNA fragments and all mtDNA fragments, respectively. Since morphological characters have been shown to generally increase resolution for closely related tick lineages (Klompen et al., 2000; Murrell et al., 2001), and the combination of molecular data tend to increase nodal support where branches are short (Matthee et al., 2004; Willows-Munro et al., 2005), the phylogenetic relationships were also derived from a supermatrix of all available data (COI, 16S, 28S, H3, ITS II and 47 distinct morphological characters).

Phylogenetic reconstructions were based on unweighted Parsimony (MP) and model informed Bayesian inference (BI). The MP analysis was performed in PAUP 4.0b10 (Swofford, 2001), using the heuristic search option, with TBR branch swapping and 100 random taxon additions. In instances where multiple equally parsimonious trees were retrieved, only 1,000 equally

parsimonious trees were saved during each replicate. The robustness of nodes was assessed by 1,000 bootstrap replicates and values ≥ 70% were considered supported (Felsenstein, 1985). For the partitioned BI analyses, jModelTest 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008) and the Akaike Information Criterion (AIC) (Akaike, 1973) were used to determine the best-fit models to define as the priors for each gene fragment (Posada and Buckley, 2004). The Standard Discrete Model was used for the morphological dataset (Ronquist and Huelsenbeck, 2003). To determine the posterior probability (PP) of nodes, MrBayes 3.2.5 (Ronquist et al., 2012) was used. Two parallel Markov Chain Monte Carlo (MCMC) simulations used five chains run for 5,000,000 generations, saving one tree in every 1,000 generations. Burnin of 10% of the total generations was determined via parameter convergence in Tracer 1.5 (Rambaut and Drummond, 2007) and this was also confirmed by the standard deviation (SD) of split frequencies that reached stationarity at this level.

Divergence dating was performed using a log-normal relaxed molecular clock approach (Drummond et al., 2006) in BEAST 1.8.2 (Drummond et al., 2012). Input files were generated in BEAUti 1.8.2 (Drummond et al., 2012) using the fossil calibrated divergence date and the best-fit models as determined by jModelTest 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008). Early runs were evaluated in Tracer 1.5 (Rambaut and Drummond, 2007) to optimize run-time parameters. The fossil date suggesting that a *Hyalomma* spp. was present between 35-50 Mya was used in the calibration (see de la Fuente, 2003). This date is not in conflict with the idea that the origin of ticks date back

to the Cretaceous and that most of the evolution and dispersal of the modern hard tick genera occurred during the Tertiary, after 65 Mya (see de la Fuente, 2003 and references therein). Since *Haylomma* is most likely a derived member of the Ixodidae (Barker and Murell, 2002, Mans et al., 2012), and the fossil specimen does not clearly represent any of the extant *Hyalomma* species, the fossil calibration date was regarded to represent the crown of *Hyalomma*. We used exponential priors for the established divergence date calibration (Offset: 34.75; Mean: 5.1). Hard minimum and relaxed maximum bounds were set so that 95% of the probability was contained around the specified divergence date. Data were analysed as partitioned and the Birth-Death Process was used as tree prior. The MCMC simulation ran for 100,000,000 generations, sampling every 10,000 generations. Validation of convergence and mixing was assessed in Tracer 1.5 (Rambaut and Drummond, 2007) and all effective sample size (ESS) values were > 200. TreeAnnotator 1.8.2 (Drummond et al., 2012) was used to summarize trees, after discarding 2,000 samples as burn-in.

To determine the likely geographic origin of *Hyalomma* lineages, the dated consensus tree was evaluated in RASP 3.2 (Yu et al., 2015), where the current zoogeographic distribution of extant taxa was used to plot likely ancestral distributions at each node. The software employed Statistical Dispersal-Vicariance Analysis (S-DIVA) (Yu et al., 2010) to circumvent impossible ranges. Unfortunately the exact distributions of many *Hyalomma* species are not well defined, rendering the identification of the species specific ranges challenging. To allow for a broader, and probably more

accurate perspective, the distributions of species were linked to major zoogeographic regions (Holt et al., 2013; Kreft and Jetz, 2010; Procheş and Ramdhani, 2012), since these have also been used to test biogeographical hypotheses with success (Beck et al., 2006; Davis et al., 2002; Kreft and Jetz, 2010; Smith, 1983).

3. Results

3.1 Sequence divergence among Hyalomma *taxa*

The COI sequence divergence values between recognized *Hyalomma* species range from 0.44% (SD ± 0.16%) between *H. marginatum* and *H. turanicum* to 16.62% (SD ± 0.00%) between *H. somalicum* Tonelli Rondelli, 1935 and *H. impressum* Koch, 1844 (Appendix Table A.2 and A.3). The average COI sequence divergence between 23 *Hyalomma* species (*H. hussaini* failed to amplify with COI primers despite numerous attempts) is 11.46% (SD ± 2.47%) and average intraspecific diversity is 0.50% (SD ± 0.57%) (Appendix Table A.3). In sharp contrast to the high interspecific sequence divergence values found among most of the recognized species, *H. marginatum* and *H. turanicum* are only 0.44% (SD ± 0.16%) different (Appendix Table A.2 and A.3). On the other side of the spectrum, an intraspecific sequence divergence of 10.22% (SD ± 0.38%) separated northern African and southern African *H. truncatum* (Appendix Table A.2 and A.3).

3.2 Phylogenetic associations

Phylogenetic analyses of the individual gene fragments were not equally informative in resolving the evolutionary history of *Hyalomma*. The faster evolving COI and 16S mtDNA gene trees separately (data not shown) and combined (Appendix Fig. B.1) showed a higher degree of resolution than the morphological data set (Appendix Fig. B.2) and also the individual (data not shown) and combined nDNA data sets (Appendix Fig. B.3). The combined mtDNA topology supported the monophyly of 20 of 24 *Hyalomma* species (two species were represented by single individuals) and 17 of these had significant PP and or 70% or more BS support. The combined nDNA only supported the monophyly of 11 *Hyalomma* species and two internal nodes displayed significant PP and BS support higher than 70 (Appendix Fig. B.3). The only higher level relationship that was supported by both methods (MP and BI) and both molecular data sets was the sister taxon relationship between *H. arabica* Pegram et al., 1982 and *H. rhipicephaloides*. Interestingly a further two nodes are supported by either BS or PP: The node supporting the monophyly of *H. hussaini* and *H. kumari* and the node supporting the monophyly of *H. asiaticum* Schulze and Schlottke, 1929, *H. dromedarii* Koch, 1844, *H. impeltatum* Schulze and Schlottke, 1929, *H. punt* Hoogstraal, Kaiser and Pedersen, 1969, *H. schulzei* Olenev, 1931, H. scupense Schulze, 1919 and *H. somalicum*. In instances where associations among trees were different in the independent analysis (morphology, mtDNA and nDNA), none of the conflicting nodes had high bootstrap or significant posterior probability values (Appendix Figs. B.1-3).

The combined data set comprised 2,242 characters, for 84 taxa (*A. hebraeum, H. brevipunctatum, H. franchinii* Tonelli Rondelli, 1932 and *H. hystricis* Dhanda and Raja, 1974 were omitted due to a lack of DNA data). The optimal prior model specification for the BI analysis was nst = 6, rates = gamma for all five gene regions. This same data set revealed 545 parsimony informative characters and the MP analysis obtained >1,000 equally parsimonious trees of 1,680 steps. All the branch swapping among equally parsimonious trees were confined to associations among individuals within the same species. Combining the data in a single matrix (total evidence approach) increased the support for nodes (de Queiroz and Gatesy, 2007; Klompen et al., 2000; Murrell et al., 2001). For example, the unsupported monophyletic clustering of *H. lusitanicum* Koch, 1844*, H. excavatum* and *H. anatolicum* and the placement of *H. aegyptium* is significantly supported in the combined analyses. More resolution is also obtained regarding the more basal placement of a clade containing *H. arabica* and *H. rhipicephaloides,* and finally a clade containing *H. glabrum*, *H. marginatum*, *H. turanicum* and *H. rufipes* (Fig. 1).

Based on the combined analyses, the monophyly of *Hyalomma* was well supported (BS = 100%, PP = 1.00: Fig. 1) and within the genus, 5 higher level clades and six subclades were identified (Fig. 1). A total of 21 of the 29 interspecific nodes were supported by ≥ 70% bootstrap and significant posterior probabilities (≥ 95 PP: Fig. 1). Five of the nodes, not supported by our criteria, were related to species complexes where the species status of these lineages has been questioned (three nodes reflecting the associations

between *H. nitidum, H. truncatum* and *H. impressum* and two nodes describing the relationships among *H. marginatum* and *H. turanicum*: Fig. 1). The monophyly of all the other *Hyalomma* species (where multiple individuals were included) is supported by significant PP and ≥ 70% BS (Fig. 1).
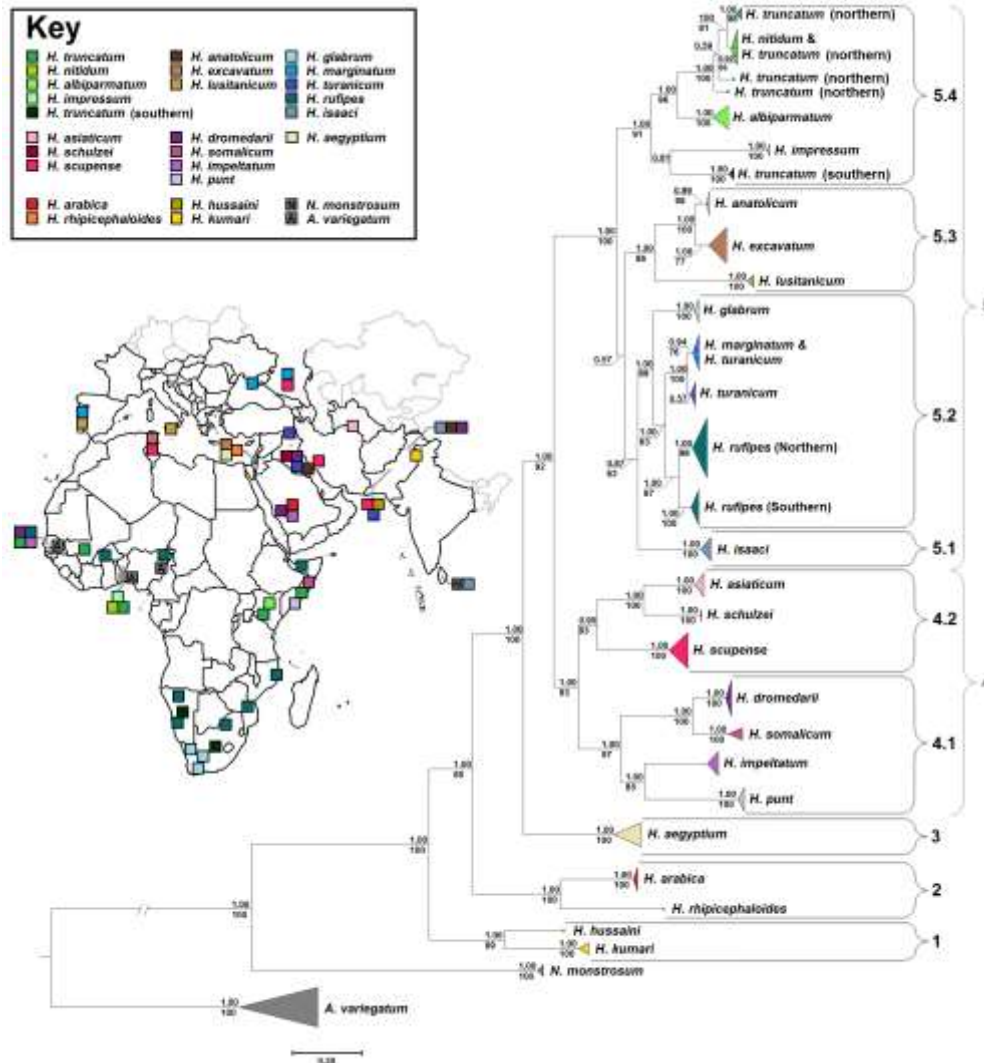

Figure 1. *Hyalomma* Bayesian tree based on a supermatrix of COI, 16S, 28S, ITS II, H3 gene regions and 47 morphological characters (Outgroup taxa from bottom to top: *Amblyomma variegatum* and *Nosomma monstrosum*). Maximum parsimony bootstrap values are indicated below and Bayesian posterior- probabilities are above branches. Branch lengths represent the number of base-pair changes. Color squares on the map indicate the approximate location at which specimens were sampled. The key indicates the respective colors associated to species on the map and tree.

3.3 Divergence dating

The BEAST topology (Fig. 2), based on molecular data only, differs in two respects from the total topology described above (Fig. 1). The first of these differences concerns the placement of *H. isaaci* (subclade 5.1), which moves to a more basal position but still within clade five (Figs. 1 and 2). The second difference concerns the relationship between southern *H. truncatum* and *H. impressum*. Importantly, however, none of these nodes had high support in the combined analysis where all data were used. The exact biogeographic interpretation and dating of these two nodes are thus inconsistent, and regarded as speculative.

Molecular clock estimates suggest that the divergence among *Hyalomma* species likely occurred around 36.25 Mya (95% HPD = 34.75-39.80 Mya) separating ticks occurring in the Oriental and Palearctic regions (Fig. 2). Since this point, members of the genus diverged repeatedly at regular intervals giving rise to the present species composition and ranges (Fig. 2). From the Palearctic and Oriental regions, the first signs of an expansion into Africa likely occurred between 22.69 Mya (95% HPD = 16.79-25.08 Mya) and 17.73 Mya (95% HPD = 13.09-20.65 Mya). After this period, several intercontinental exchanges occurred such as the recolonization of the Oriental region by species such as *H. anatolicum*, *H. dromedarii*, *H. isaaci* and *H. scupense* (Fig. 2). In addition, *H. excavatum* and *H. anatolicum* dispersed back into the Afrotropical region, as the ancestral lineage likely became restricted in the Palearctic region approximately 10.82 Mya (95% HPD = 8.03-14.06 Mya)

(Fig. 2). Finally, parts of the African Palearctic region have been recolonized by *H. dromedarii* and *H. impeltatum* as they likely have an Afrotropical origin.
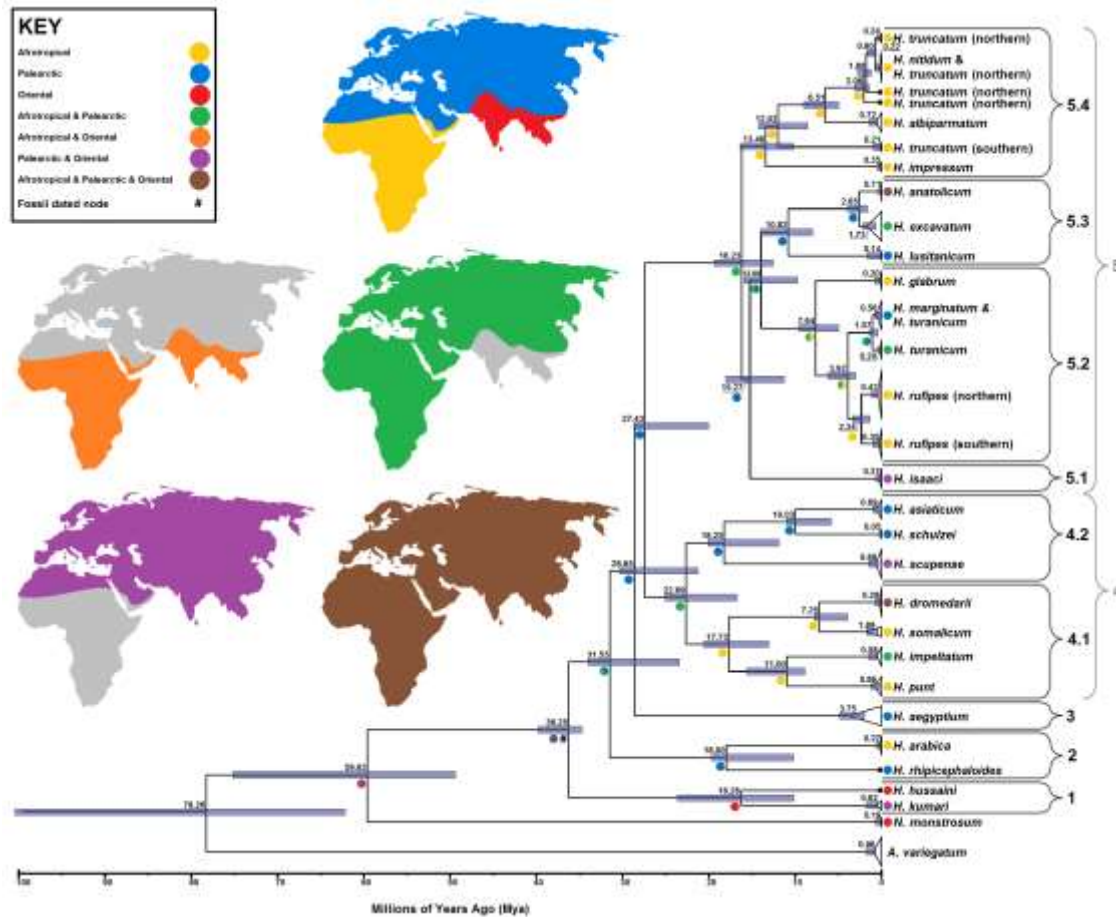

Figure 2. Dated tree for *Hyalomma* obtained from the fossil-calibrated BEAST analysis (Outgroup taxa from bottom to top: *Amblyomma variegatum* and *Nosomma monstrosum*). The Fossil-calibrated node is indicated by the BLACK "#" (Oldest fossil record of *Hyalomma*: 35–50 Mya: de la Fuente, 2003). Values at each node represent dates in millions of years since present. The BLUE bars at each node represent the 95% confidence intervals around divergence dates. Colour pies represent the optimal zoogeographic placement of current and ancestral taxa as calculated through S-Diva analysis in RASP.

4. Discussion

The well resolved *Hyalomma* phylogeny presented herein allows for the clarification of several of the taxonomic uncertainties currently pertaining in this genus. Despite the reported limitations when using morphological data for tick systematics, such as gynandromorphisms (Buczek et al., 2014), structural changes in characters during feeding (Lv et al., 2014) and hybridization (Rees et al., 2003), there is a marked congruence between the species tree recovered herein and the morphological subgeneric associations proposed nearly a century ago (Schulze, 1930, 1919). With the exception of the clustering of the African *H. punt,* the morphological defined subgenus *Hyalommina* all have a distribution outside of the African continent (*H. kumari, H. hussaini, H. rhipicephaloides* and *H. arabica* and possibly *H. brevipunctatum,* that shares 46 of the 47 morphological characters compared in this study with *H. hussaini* and *H. kumara*: see Table 2; Appendix Fig. B.2) and all cluster in a basal position separate from a strongly supported monophyletic group containing the remaining species in the subgenus *Euhyalomma* (including *H. punt*). The placement of *H. punt* within the subgenus *Euhyalomma* is also supported by unpublished morphological observations (the basis capituli has slight dorso-lateral projections in the female, coxae I has a median projection, legs are characterized by white enamelling and there are non-humped tarsi in both sexes). Interestingly, the tortoise adapted, *H. aegyptium,* also sometimes classified as a distinct subgenus (*Hyalomma* s. str.) is the most basal evolutionary distinct lineage within *Euhyalomma.* The morphological distinction between *Hyalommina* and

Euhyalomma lineages (Appendix Fig. B.2) are also partly supported by the analyses based on the more conservative nuclear DNA only (Appendix Fig. B.3).

The findings of the present study furthermore refine the taxonomy of *Hyalomma* at the interspecific level and specifically within the more recently diverging species rich *Euhyalomma* subgenus. *Hyalomma dromedarii*, *H. somalicum*, *H. impeltatum* and *H. punt* form a monophyletic assemblage (see subclade 4.1: Fig. 1) and based on the RASP analysis this clade likely had an African origin (Fig. 2). This lineage is sister to a clade comprising the previously unresolved *H. scupense* together with *H. asiaticum* and *H. schulzei* (clade 4: Fig. 1). This total evidence finding is congruent with hypotheses derived from morphology only (Apanaskevich et al., 2010, 2008b; Apanaskevich and Horak, 2010) and assisted to resolve the taxonomic placement of *H. scupense*. The close evolutionary relationships between the widely distributed *H. anatolicum*, *H. excavatum* and *H. lusitanicum* (Appendix Fig. B.2) are also corroborated in our study (subclade 5.3: Fig. 1) (Apanaskevich and Horak, 2005; Hoogstraal and Kaiser, 1959) and so are the reportedly close relationships between *H. glabrum*, *H. marginatum*, *H. turanicum* and *H. rufipes* (subclade 5.2: Fig. 1) (Apanaskevich and Horak, 2009, 2008a). "The strong bootstrap and posterior probability support for the monophyletic sister taxon relationship between *H. rufipes* and the lineage comprising *H. marginatum* + *H. turanicum*, provide further evidence for the recognition of the morphologically distinct *H. rufipes* as a species separate

from *H. marginatum* and *H. turanicum* (subclade 5.2: Fig. 1) (Apanaskevich and Horak, 2009, 2008a)."

Apart from supporting many of the morphological hypotheses (see above) the genetic data provide new insights into more recent evolutionary events that have some implications for the taxonomy of *Hyalomma*. For example, low levels of genetic differentiation, similar to intraspecific divergences (Zhang and Zhang, 2014), were detected between *H. marginatum* and *H. turanicum* (at the mtDNA COI level = 0.44%, SD ± 0.16%). In addition, a *H. turanicum* individual included from Pakistan clustered closer to *H. marginatum* (from Portugal and Russia) than to conspecifics from Iraq (Fig. 2). These two species are, however, characterized by distinct morphological features (such as the scutal punctuation pattern, thickness of dorsal prolongation of the spiracular plates, density of circumspiracular setae and coloration of leg segments: Apanaskevich and Horak, 2008a). The low sequence divergences and non-monophyletic clustering suggest that the divergence between the lineages are very recent (since the non-monophyly of *H. turanicum* could either be attributed to incomplete lineage sorting between the two species or alternatively recent introgression among these two species). Clearly the taxonomic status of *H. marginatum* and *H. turanicum* are in need of further investigation and this should include sufficient geographic sampling throughout the range of both taxa.

The present study also provides information to support cryptic speciation within single species. The total phylogeny presented herein confirmed that *H.*

truncatum comprise two genetically distinct geographic lineages that fail to cluster together as a monophyletic group (*H. truncatum* southern and *H. truncatum* northern: Fig. 1). These two separate monophyletic lineages are characterized by an average COI mtDNA sequence divergence of 10.28% (SD ± 0.38) which is in the same order or even higher than that separating well defined *Hyalomma* species (Sands et al., 2017; Zhang and Zhang, 2014). It is proposed that the divergence of these two lineages are more than likely the result of host vicariance on the African continent and that differential seasonal breeding cycles probably act as a premating isolating mechanism keeping them apart (Sands et al., 2017). Interestingly, in Africa, *H. truncatum* is not the only species showing a South – North divide. Cryptic divergences among geographic assemblages is also present in *H. rufipes* showing a southern (Namibia, Mozambique and South Africa) and a northern lineage (Somalia, Burkina Fuso, Nigeria, Senegal and northern Mozambique) (Fig. 1). A similar trend was also recorded for *Amblyomma hebraeum* (Cangi et al., 2013) and in total these findings support the role of host movement and biogeographic barriers as important mechanisms shaping the evolution of ticks.

The discovery of the cryptic diversity in *H. truncatum,* and the fact that the two genetic lineages are not monophyletic, poses a serious problem for the recognition of *H. albiparmatum* and *H. nitidum* as valid species. The validity of *H. nitidum* and *H. albiparmatum* has been a subject of much debate (Apanaskevich and Horak, 2008b; Camicas et al., 1998; Feldman-Muhsam, 1962; Hoogstraal, 1979, 1956; Tomassone et al., 2005; Walker, 1974). In

short, only male *H. albiparmatum* can be distinguished from *H. truncatum* by having an ivory-colored parma, while the females and immature stages of the two species are virtually identical (Apanaskevich and Horak, 2008b). Hoogstraal (1979) also proposed the re-instatement of *H. nitidum* without a formal description and more recently Tomassone et al. (2005) suggested two morphological characters to distinguish between *H. nitidum* and *H. truncatum* (the reduction in clarity of ivory-colored bands on the segments of the legs in both males and females and the external cuticular preatrial fold of the genital operculum that differs in shape in females only between the two species). The genetic evidence presented in this study suggest that either *H. truncatum* is an incredibly diverse single species (rendering the species status of *H. nitidum* and *H. albiparmatum* invalid) or that at least three valid species exist comprising *H. truncatum* northern lineage, *H. truncatum* southern lineage and *H. albiparmatum*.

The total evidence phylogenetic tree presented herein supports a notion that closely related species (with large distribution ranges) often overlap in parts of their distribution (for example subclade 5.4: Fig. 1), while distantly related species (with restricted distributions) are found on different continents (for example *H. hussaini* versus *H. punt*: Fig. 1). The sympatry of closely related species supports the hypothesis that speciation in ticks is partly driven by competition between closely related taxa (Cangi et al., 2013) and or abiotic conditions that affect ticks during the long off-host period as part of their life cycle (Klompen et al., 1996; McCoy et al., 2013; Sands et al., 2017). The allopatric distribution of distantly related taxa suggests that large scale

environmental changes such as those associated with rifting and continental drift, are equally important in being responsible for the diversity found in *Hyalomma.*

The dated phylogeny and the S-Diva analyses are particularly informative to highlight whether any correlations exist between the evolution of *Hyalomma* and the reported tectonic events (also associated with large scale climatic changes on earth). Although RASP fail to reveal the continental origin of *Hyalomma* (Fig. 2), it is important to realize that the majority of the most basal nodes exclude a strict Afrotropical ancestral distribution. The Palearctic/Oriental origin for the genus is further supported by the fossil record for *Hyalomma* (de la Fuente, 2003). The earliest diversification among the *Hyalomma* subgenera is estimated to have started round 36.25 Mya (95% HPD = 34.75-39.80 Mya) and this event coincides well with the collision of the Indian and Eurasian Plates 35-40 Mya and the subsequent uplift of the Himalayas (Molnar and Tapponnier, 1977; Najman et al., 1994; Rashid, 2014). This period also marks the transition between the Eocene Optimum to the Eocene-Oligocene (dated at 34 Mya) and was characterized by a marked drop in global temperatures (Barret, 2006; Zachos et al., 2001). In Asia specifically, it has been proposed that this period was associated with faunal turnover changes from large-size perissodactyl-dominant fauna to small rodent/lagomorph-dominant fauna (Sun et al., 2014). It is thus possible to postulate that these large scale changes in host availability, and the drastic changes in the off host environment, could have brought about differential selection pressures on a once widespread Eurasian *Hyalomma,* separating

the ancestors of *H. hussaini* and *H. kumari* with an Oriental origin (Fig. 2) from ancestral *H. arabica* and *H. rhipicephaloides* within the Palearctic region. A second clear indication of the importance of land bridge formation and tectonics on the evolution of *Hyalomma* can be found when the connections between Eurasia and Africa are considered. The earliest dispersal into Africa is represented by the ancestor of *H. punt, H. impeltatum, H. somalicum,* and *H. dromedarii* and this is dated to have occurred between 22.69 Mya and 17.73 Mya. This date coincides very well with the temporary closing of the Tethyan seaway at approximately 19 Mya, forming of the so-called *Gomphotherium* land bridge (Cox, 2000; Popov et al., 2004; Rögl, 1999) which allowed for the first connectivity between Africa and Arabia. Interestingly, it has been hypothesized that the formation of the *Gomphotherium* land bridge also created the first opportunity for hosts of *Hyalomma* (bovids) to enter Africa (Cox, 2000). Since this time, more frequent two-way continental exchanges of mammals occurred throughout the Miocene (Vrba, 1985; Matthee and Davis, 2001; Harzhauser et al., 2007; Lindsay et al., 2013), and we propose that these exchanges also resulted in the pattern of dispersion back from Africa to Eurasia (for example see *H. dromedarii* and *H. impeltatum*: Fig. 2). Given that there is little indication of direct dispersal from the Afrotropical region to the Oriental region, and vice versa, it seems reasonable to conclude that the speciation events in *Hyalomma* have been mostly influenced by local vicariance events that caused disruptions in gene flow in previously more widely distributed species. In concert, the results from the latter confirms that tectonic events and the associated large scale global

changes in the environment contributed significantly towards producing the rich diversity currently found in *Hyalomma.*

Acknowledgments

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