Research Article

Mitochondrial 16s rDNA based Analysis of some Hard Ticks Belonging to Genus *Hyalomma* Koch, 1844 (Acari: Ixodidae)

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Abstract | A molecular analysis of mitochondrial 16s rDNA sequences of hard ticks belonging to genus *Hyalomma* from Haryana (India) and those available in genbank database was done so as to resolve inter-relationships between members of genus *Hyalomma*. For this a total of eighty one 16s rDNA sequences belonging to 16 taxa were subjected to molecular and phylogenetic analysis which was conducted in MEGA6 and Beast 1.8.0 software. Phylogenetic relationships were inferred using the bayesian, maximum likelihood (ML) and neighbor joining (NJ) methods by means of Tamura three parameter model + unequal frequency + gamma distribution (TPMuf+G). The analysis revealed 249 variable sites, 232 conserved sites, and 137 parsimony informative sites in the alignment. Results of the phylogenetic analysis provide support for monophyletic origin of genus *Hyalomma*. Further our results assert that *H. anatolicum*, *H. excavatum*, *H. marginatum*, *H. lusitanicum*, *H. hussaini* and *H. brevipunctata* represent closely related but rapidly diverging taxa and also substantiate *H. asiaticum* as a species complex. The molecular clock results exemplify divergence time of subfamily Hyalomminae from the common ancestor of subfamily Rhipicephalinae to be 61.99 mya and the origin of family Ixodidae to be 86.79 mya. A basic phylogenetic relationship tree is also provided to help future studies for phylogenetics of family Ixodidae especially for subfamily Hyalomminae.

Keywords | *Hyalomma*, Phylogeny, Ixodidae, 16s rDNA, Hard ticks

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INTRODUCTION

The family Ixodidae of prostriate and metastriate hard ticks has 6 subfamilies Ixodinae, Bothriocrotoninae, Amblyomminae, Haemaphysalinae, Hyalomminae and Rhipicephalinae (Klompen et al., 1997, 2002; Horak et al., 2002; Barker and Murrell, 2004, 2007). Recently, there have been reports of prevalence of sibling/cryptic species in different hard tick genera viz. *Rhipicephalus*, *Rhipicephalus* (*Boophilus*), *Haemaphysalis* and *Hyalomma* Koch, 1844 (Liu et al., 2013; Burger et al., 2014a). Thus there can be possible difficulties in the study of disease transmission and vector control as proper identification of the vector and understanding of the relationships between closely related species is a must for devising any effective control strategy (Klompen et al., 1996; Jongejan and Uilenberg, 2004; Ghosh et al., 2007a). The traditional morphological character based identification of closely related tick species is sometimes problematic which is attributable to polymorphic characters and variations owing to engorgement on blood meal (Lv et al., 2014a). Besides there is always a possibility of geographical strains of tick species having different vectorial capacity for transmission of pathogens (Hoogstraal and Aeschlimann, 1982; Gou et al., 2013; Lu et al., 2013), chances of genetic introgression, possible fertile hybrids (Rees et al., 2003; Labruna et al., 2009), and different acaricidal resistance (Shyma et al., 2012).

To resolve relationships and solve problems facing systematics of hard ticks in the family Ixodidae several molecu-

lar markers have been used during the past two decades (Black et al., 1997; Black and Piesmann, 1994; Mangold et al., 1998a,b; Dobson and Barker, 1999; Murrell et al., 2001, 1999; Norris et al., 1999; Beati and Keirans, 2001; Guglielmone et al., 2003; Chitimia et al., 2010; Abdigoudarzi et al., 2011; Song et al., 2011; Burger et al., 2012, 2014b; Kulakova et al., 2014; Lv et al., 2014b). In this context, understanding the position of genus *Hyalomma* Koch, 1844 has long been considered necessary (Hutcheson et al., 2000). Likewise there is lack of any study on molecular analysis of hard ticks from the state of Haryana (India) (Miranpuri, 1988; Ghosh et al., 2007b). This crucial gap in information related to hard ticks prompted us to carry out molecular investigation on members of the genus *Hyalomma* using mitochondrial 16s rDNA sequences.

MATERIAL AND METHODS

The hard ticks infesting cattle and buffalo hosts were field collected from different animal farms located in the state of Haryana (India) (Figure 1, Table 1). After initial separation of hard ticks identification up to species level was done by using standard identification keys available for *Hyalomma* ticks (Kaiser and Hoogstraal, 1964; Geevarghese and Dhanda, 1987; Keirans and Litwak, 1989; Petney and Keirans, 1995; Walker et al., 2003). The ticks were photographed using a trinocular stereo-zoom microscope (Labomed™) and subsequently preserved in 100% ethanol in a -20˚C deep freezer (Bluestar).

Table 1: Collection Details of Four *Hyalomma* species from Haryana, India sequenced during the present study

S. No	Place of collection	Code used	Latitude Longitude	Species Identified
1.	Village Nuran Khera, Jind road, Gohana	NK	29.204 76.582	*Hyalomma brevipunctata* Sharif, 1928
2.	Hansi road, Karnal	HR	29.687 76.974	*Hyalomma excavatum* Koch, 1844
3.	Ashok vihar, Sonepat	Ashok Vihar	28.995 77.007	*Hyalomma excavatum* Koch, 1844
4.	Garhi brahmanan, Sonepat	Garhi	28.994 76.994	*Hyalomma anatolicum* Koch, 1844
5.	Village Gangana, Jind road, Gohana	Gangana	29.237 76.614	*Hyalomma anatolicum* Koch, 1844
6.	Village Kaimla, Gharaunda, Karnal, Haryana	Kaimla	29.506 76.998	*Hyalomma hussaini* Sharif, 1928

DNA Extraction

DNA was extracted from individual hard ticks using DNeasy® DNA isolation kit (Qiagen). For this, individual ticks were crushed with sterile glass pestle in liquid nitrogen and subsequently DNA was extracted by following the protocol provided with the kit. The DNA was quantified using Tecan's Infinite® NanoQuant and stored at 4˚C. Quality of DNA was checked by resolving on 0.8% Agarose gels using standard procedure.

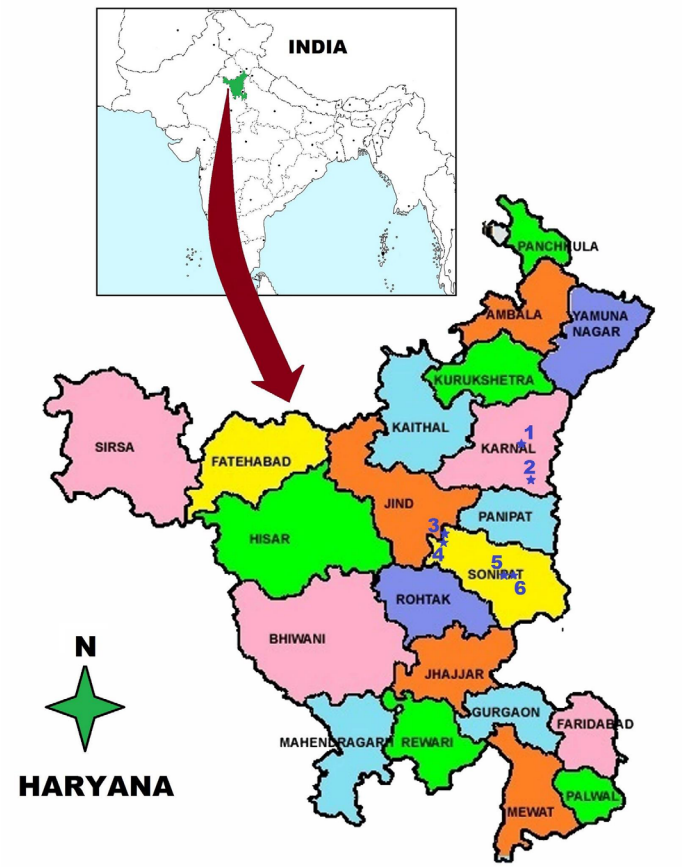

Figure 1: Map showing the populations of 4 species of genus *Hyalomma* sequenced during the present study from the state of Haryana, India
1) Hansi Road Karnal; **2**) Village Kaimla; **3**) Village Gangana; **4**) Village Nuran Khera; **5**) Village Garhi Brahmanan; **6**) Ashok Vihar Sonepat (Map not to scale) (For details see Table 1)

PCR Amplification and Sequencing

PCR was performed to amplify 16s rDNA from individual hard tick DNA samples with the following primer pairs: S16S FP (5'-CTGCTCAATGAATATTTAAATTGC-3') and S16S RP (5'-CGGTCTAAACTCAGATCATGTAGG-3') (Tian et al., 2011). PCR reactions were performed in 25µl reaction mixture that had 100ng DNA template and 1.5U of Taq Polymerase (GeNei™) per reaction along with standard reaction ingredients. The PCR cycling conditions set in the program were as follows: initial denaturation at 94˚C for 3 min followed by 30 cycles of 94˚C for 30 sec (denaturation), 50˚C for 40sec (annealing), 72˚C for 40 sec (extension) and a final extension step of 72˚C for 5 min. PCR products were resolved on 2% Agarose gels and compared with 100bp

Table 2: Taxonomic details of species and groups studied

Group in family Ixodidae	Subfamily	Genus	Species group*	Member species	Code used
Metastriata	Hyalomminae	*Hyalomma*	Anatolicum group	*anatolicum*	HA
				excavatum	HE
				lusitanicum	HL
				marginatum	HM
				hussaini	HH
				brevipunctata	HB
			Asiaticum group	*asiaticum asiaticum, asiaticum kozlovi*	HAS
				dromedarii	HD
				detritum	HDD
	Rhipicephalinae	*Dermacentor*	Out-group	*albipictus*	*Dermacentor*
	Rhipicephalinae	*Rhipicephalus*		*sanguineus, microplus*	*Rhipicephalus*
	Haemaphysalinae	*Haemaphysalis*		*bispinosa*	*Haemaphysalis*
	Amblyomminae	*Amblyomma*		*aureolatum*	*Amblyomma*
Prostriata	Ixodinae	*Ixodes*		*ricinus*	*Ixodes*

*These groups are not having formal taxonomic status but have been created by authors for analysis based on relationships derived during the present study.

DNA standard ladder as the expected product size was in range of 420-440bp. PCR products were purified by using Geneipure™ Quick PCR Purification kit (GeNei™) and sent for commercial DNA sequencing to 1st base sequencing service (Malaysia).

Sequence Details

A total of 12 sequences generated for *Hyalomma anatolicum* Koch, 1844, *Hyalomma excavatum* Koch, 1844, *Hyalomma hussaini* Sharif, 1928 and *Hyalomma brevipunctata* Sharif, 1928 during the present study and 63 sequences available in GenBank database for seven *Hyalomma* species viz. *H. anatolicum*, *H. lusitanicum* Koch, 1844, *H. marginatum* Koch, 1844, *H. asiaticum asiaticum* Schulze & Schlottke, 1930, *H. asiaticum kozlovi* Olenev, 1931, *H. dromedarii* Koch, 1844, and *H. detritum* Schulze, 1919 were used for deriving phylogenetic relationships (Table 1 and Supplementary Table 1). In addition, one sequence each for *Rhipicephalus sanguineus* (Latreille, 1806), *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1888), *Dermacentor albipictus* (Packard, 1869), *Haemaphysalis bispinosa* (Neumann, 1897), *Amblyomma aureolatum* (Pallas, 1772), and *Ixodes ricinus* (Linnaeus, 1758) were used to infer relationships of genus *Hyalomma* with other genera of family Ixodidae and to root the trees (Supplementary Table 2).

Sequence Alignment and Analysis

Multiple sequence alignment of eighty one 16s rDNA sequences was generated with Muscle software tool executed in MEGA6 phylogenetic analysis software (Tamura et al., 2013). The alignment was manually edited to remove any alignment errors and exported as mega and fasta format files. The readseq program available at European Bioinformatics Institute (EBI) server link http://www.ebi.ac.uk/Tools/sfc/readseq/ was used for sequence conversion to nexus format before performing bayesian analysis. The sequences were segregated into groups (Table 2), and analysed for GC% (Figure 2, Supplementary Table 1), maximum likelihood estimate of transition/transversion bias (Table 3), estimates of evolutionary divergence over sequence pairs between groups (Tables 4, 5 and 6), and Tajima's neutrality test (Tajima, 1989) (Table 7) using MEGA6 phylogenetic and sequence analysis software (Tamura et al., 2013).

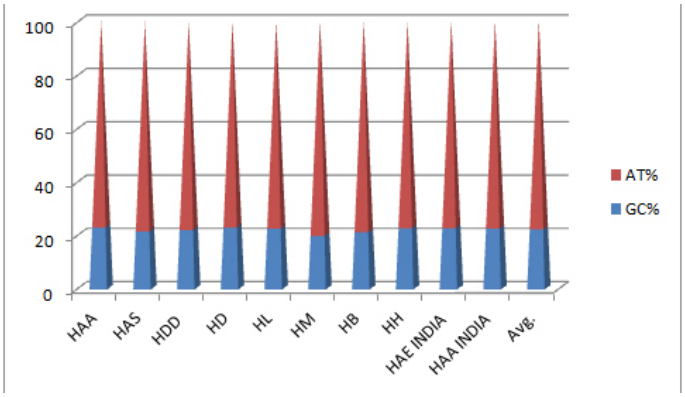

Figure 2: GC/AT percentage among various species groups of genus *Hyalomma* and out-groups

Phylogenetic Analysis

Phylogenetic analysis was performed using the bayesian, maximum likelihood (ML) and neighbor joining (NJ)

methods. The best-fit model to derive relationships based on the current data set was estimated by using the jmodeltest software (Guindon and Gascuel, 2003; Darriba et al., 2012) as well as model test tool in MEGA6. Both the model testing approaches supported the same model viz. Tamura three parameter model + unequal frequency + gamma distribution (TPMuf+G) having BIC, AICc and log likelihood values of 6964.73, 6433.27 and -2966.88 in jmodeltest respectively. ML and NJ analysis was performed using MEGA6 and bayesian analysis was done using Bayesian Evolutionary Analysis Sampling Trees Version v1.8.0 (Beast 1.8.0) (Designed and developed by Alexei J. Drummond, Andrew Rambaut and Marc A. Suchard). In all the analysis gaps and missing data were treated as partial deletion with 90% site coverage cut-off. The phylogenetic trees were constructed using the below mentioned parameters for each analysis method:

ML analysis: nucleotide substitution model - Tamura 3-parameter model (Tamura, 1992), test of phylogeny - bootstrap method (Felsenstein, 1985), 100 replications, rates among sites – gamma distributed, tree inference methodology – nearest-neighbor-interchange (NNI) method, branch swap filter – very strong.

NJ analysis: nucleotide substitution model - Tamura 3-parameter model (Tamura, 1992), test of phylogeny bootstrap method (Felsenstein, 1994), 1000 replications, substitutions to include – d: transitions + transversion, rates among sites – gamma distributed, pattern among lineages – different (heterogeneous) (Tamura and Kumar, 2002).

Bayesian analysis: A relaxed clock bayesian analysis to estimate the topology and divergence times simultaneously was performed where evolutionary rates along branches followed an uncontrolled log normal distribution and a Yule speciation process was imposed for all analysis. One Marked Chain Monte Carlo (MCMC) run was performed of 50 million generations with sampling every 500 generations. Adequate sampling and convergence of the chain to stationary distribution were confirmed by inspection of MCMC samples using Tracer v1.6 (Available from http://beast.bio.ed.ac.uk/Tracer). The sampled posterior trees were summarized using tree annotator 1.8.0 to generate a maximum clade credibility tree (Max posterior probability (PP)) after removal of 10% trees as burn-in. The mean node ages and PP values for each node were also calculated. The tree topology was visualized and annotated for publication quality with Fig Tree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Calibration point and molecular clock: Two hard tick fossils were used as calibration points, the most recent common ancestor (MRCA) of the out-group *Ixodes ricinus* was based on amber *Ixodes succineus* (35-40 mya) (Weidner, 1964) while the MRCA of the clade including all the *Hyalomma* species was based on amber *Hyalomma* sp (35-50 mya) (De La Fuente, 2003).

RESULTS

The dataset used in the present analysis includes 81 sequences of 16s rDNA - 75 sequences belonging to ten species of genus *Hyalomma* and 6 sequences from other genera from family Ixodidae (as detailed earlier). All sequences have been aligned and annotated to have a final alignment length of 498bp which when analysed reveal 249 variable sites, 232 conserved sites, 137 parsimony informative sites and 110 singleton sites.

Nucleotide Composition

The analysis of nucleotide composition of 16s rDNA sequences demonstrates them to be AT rich having base composition percentage in decreasing order of A>T>G>C except for *Amblyomma* where it is T>A>G>C (Supplementary Table 1). The average GC% is 22.47, while it is lowest in *Hyalomma marginatum* of 19.93% and highest in *Hyalomma anatolicum* of 23.09% (Figure 2).

Maximum Likelihood Estimate of Transition/Transversion Bias and Substitution Matrix

The estimated transition/transversion bias (R) for whole dataset of the genus *Hyalomma* is estimated to be 0.52 indicating no bias as its very close to expected value of 0.5 but there is marked deviation from the observed and expected ti/tv bias when individual species groups are considered which is 0.40 for *H. anatolicum*, 0.11 for *H. asiaticum*, 0.67 for *H. detritum*, 0.69 for whole of anatolicum clade and 0.55 for asiaticum clade.

Table 3: The transition and transversion substitutions obtained by maximum likelihood estimate of substitution matrix in Mega 6.0 (Rates of different transition substitutions are shown in bold and those of transversion substitutions are shown in italics)

	A	T/U	C	G
A	-	*11.01*	*3.06*	**4.75**
T/U	*11.01*	-	**4.75**	*3.06*
C	*11.01*	**17.12**	-	*3.06*
G	**17.12**	*11.01*	*3.06*	-

When rates of different transitional substitutions (shown in bold in Table 3) and those of transversion substitutions (shown in italics in Table 3) are studied the rate of transition are found to be more than transversion for all base substitutions. A→G and T→C transition are found to have 17.12 probability values while C→T and G→A transition have a probability value of 4.75 only. As for the transversion T→A, A→T, C→A and G→T have a

Table 4: Matrix showing estimates of evolutionary divergence over Sequence Pairs between different genera (divergence is shown in the lower diagonal while the standard error is shown in upper diagonal)

Genus	Divergence↓	S.E.→				
Hyalomma		1.88%	2.83%	1.88%	1.98%	2.56%
Dermacentor	14.49%		3.36%	2.11%	1.98%	2.63%
Ixodes	26.13%	26.21%		3.47%	2.52%	3.53%
Rhipicephalus	19.57%	17.96%	32.70%		2.21%	3.10%
Haemaphysalis	17.39%	14.39%	22.03%	21.84%		2.57%
Amblyomma	21.39%	20.75%	24.35%	25.43%	18.39%	

Table 5: Matrix showing estimates of percentage evolutionary divergence over sequence pairs between groups for genus *Hyalomma*. (Divergence is shown in the lower diagonal while the standard error values are shown in upper diagonal) (For species codes see Table 2)

Species code	Divergence↓	S.E.→							
HA		1.55	1.42	1.55	1.12	0.99	0.58	0.43	0.75
HAS	8.76		1.49	1.37	1.80	1.55	1.80	1.68	1.76
HDD	7.34	7.84		1.32	1.76	1.65	1.60	1.50	1.75
HD	8.72	7.66	7.95		1.63	1.72	1.82	1.67	1.86
HL	4.75	10.27	9.96	9.33		1.38	1.12	1.12	1.25
HM	2.83	7.34	7.56	8.14	4.83		1.26	1.09	1.20
HH	1.82	10.30	8.29	9.82	4.56	3.85		0.54	0.88
HE	1.68	10.10	8.22	9.56	4.67	3.56	1.60		0.75
HB	4.90	13.80	12.04	13.19	7.01	5.37	4.80	4.18	

probability value of 11.01 while A→C, T→G, C→G and G→C have a probability value of only 3.06.

Estimates of Evolutionary Divergence over Sequence Pairs between Groups

When divergence between prostriate and metastriate groups is observed the divergence of *Ixodes* from metastriate genera in increasing order of divergence is *Haemaphysalis* (22.02%) < *Amblyomma* (24.35%) < *Hyalomma* (26.13%)< *Dermacentor* (26.21%) < *Rhipicephalus* (32.7%), while within metastriate genera least between group evolutionary divergence is between *Haemaphysalis* and *Dermacentor* (14.39%), and *Hyalomma* and *Dermacentor* (14.48%) groups while maximum between group divergence is between *Rhipicephalus* and *Amblyomma* (25.43%), followed by *Rhipicephalus* and *Haemaphysalis* (21.83%), and *Hyalomma* and *Amblyomma* (21.39%)(Table 4).

When divergence between groups within genus *Hyalomma* is studied it reveals that maximum divergence is between *H. asiaticum* and *H. brevipunctata* (13.8%), followed by *H. dromedarii* and *H. brevipunctata* (13.19%), *H. detritum* and *H. brevipunctata* (12.04%), *H. asiaticum* and *H. hussaini* (10.30%), *H. asiaticum* and *H. lusitanicum* (10.27%), and *H. asiaticum* and *H. excavatum* (10.10%) while the least divergence is between *H. hussaini* and *H. excavatum* (1.60%), *H. anatolicum* and *H. excavatum* (1.68%), *H. anatolicum* and *H. excavatum* (1.68%), *H. anatolicum* and *H. hussaini* (1.82%), *H. anatolicum* and *H. marginatum* (2.83%) (Table 5).

Table 6: Results of Tajima's Neutrality Test performed in Mega6 for the current dataset (For group details see Table 2)

Group name	m	S	Ps	T	P	D
Complete dataset	81	203	0.49	0.099	0.06	-1.226
Metastriata	80	186	0.45	0.091	0.06	-1.164
Out-groups	6	107	0.40	0.177	0.19	0.617
Hyalomma	75	113	0.27	0.056	0.04	-0.398
Anatolicum group	43	84	0.19	0.045	0.01	-2.175
H. anatolicum	30	39	0.08	0.022	0.007	-2.461
Asiaticum group	32	49	0.11	0.029	0.02	-0.741
H. asiaticum	27	10	0.023	0.006	0.003	-1.512
H. detritum	4	5	0.011	0.006	0.005	-0.796
Metastriata out-group only	5	89	0.334	0.161	0.17	0.511

Tajima's Neutrality Test

The test results reveal that the value of D for the whole dataset is -1.22 with a p value of 0.06 (Table 6). When the out-groups are removed to test only for the genus *Hyalomma* the value of D comes out to be -0.39 with p value of 0.04 while for out-groups it comes out to be 0.61 with p value of 0.19. Within genus *Hyalomma* the D value for the Anatolicum group is -2.17 with p value of 0.018 while the D value for Asiaticum group is -1.51 with p value of 0.003.

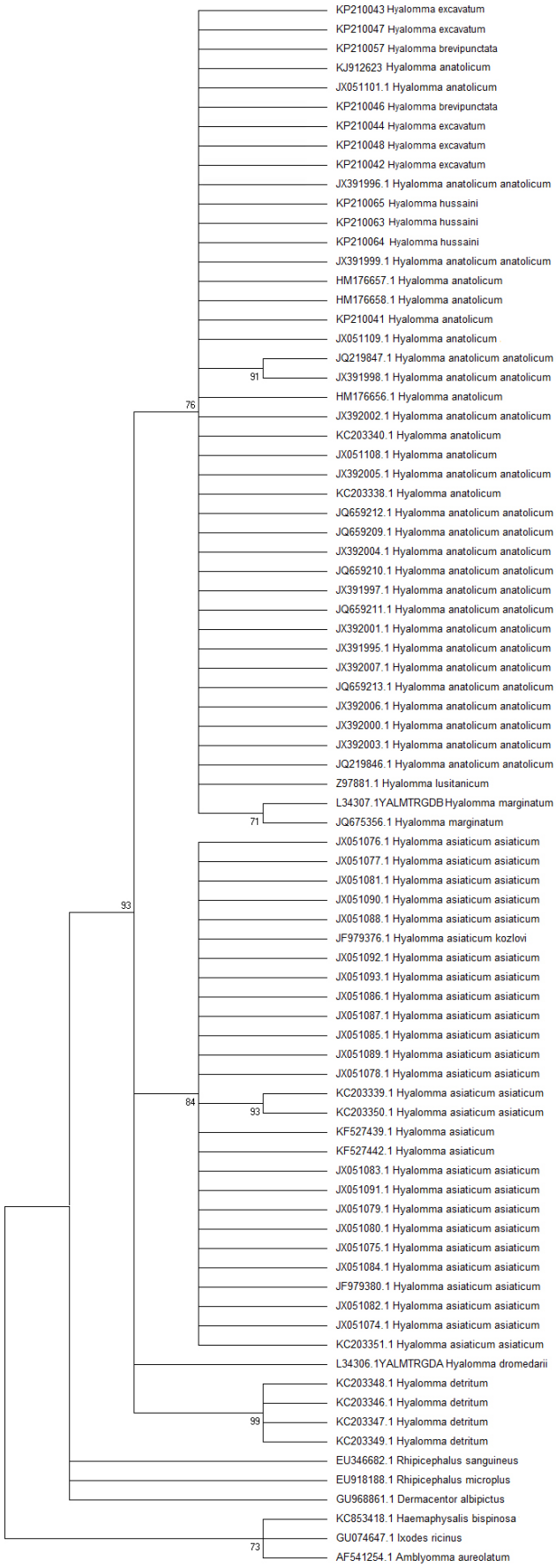

Figure 3: Phylogenetic tree (70% consensus) generated by Maximum Likelihood method based on T3PM+G model with 100 bootstrap replicates (values on branches are bootstrap support)

Phylogenetic analysis

The analysis of 16s rDNA sequences belonging to hard ticks of genus *Hyalomma* and outgroups results in a phylogeny with good statistical support using all three approaches having some topology variations but majority of important clades differ only in bootstrap/PP support (Table 7). *Ixodes ricinus* has been used to root all the trees. The relationships between hard ticks obtained using different approaches are detailed here under:

ML analysis

In the evolutionary tree, inferred by using the ML method based on the T3P model using 100 bootstrap pseudo-replicates, branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed (Figure 3) and the values are shown next to the branches. There is 76% support for the Anatolicum group i.e. (*Hyalomma anatolicum* + *Hyalomma excavatum* + *Hyalomma lusitanicum* + *Hyalomma marginatum* + *Hyalomma hussaini* + *Hyalomma brevipunctata*), 84% support for the Asiaticum species complex (*Hyalomma asiaticum asiaticum* and *Hyalomma asiaticum kozlovi*) and 99% support for *Hyalomma detritum*. The monophyletic clade of genus *Hyalomma* is supported by 93% bootstrap values. The out-groups assembled together with Genus *Hyalomma* paraphyletically ((*Rhipicephalus* + *Rhipicephalus* (*Boophilus*)) + *Dermacentor*) + *Haemaphysalis*) + *Amblyomma*).

NJ Analysis

In the evolutionary tree, inferred by using the NJ method based on 1000 bootstrap pseudo-replicates, branches corresponding to partitions reproduced in less than 70% bootstrap pseudo-replicates are collapsed (Figure 4) and bootstrap support values are shown next to the branches. There is 97% support for the Anatolicum group i.e. (*Hyalomma anatolicum* + *Hyalomma excavatum* + *Hyalomma lusitanicum* + *Hyalomma marginatum* + *Hyalomma hussaini* + *Hyalomma brevipunctata*), 99% support for Asiaticum species complex (*Hyalomma asiaticum asiaticum* and *Hyalomma asiaticum kozlovi*), and 99% support for *Hyalomma detritum*. The monophyletic clade of genus *Hyalomma* is supported by 94% bootstrap support. The out-groups formed paraphyletic assemblage (*Haemaphysalis* + *Amblyomma*) with 84% support and ((*Rhipicephalus* + *Rhipicephalus* (*Boophilus*)) + *Dermacentor*) having weak BT support that shows polytomy in 70% consensus tree.

Bayesian analysis

In the maximum clade credibility tree the Posterior Probability (PP) values of replicate trees in which the associated taxa clustered together in bayesian analysis is shown above the branches while the node ages are shown against the nodes (Figure 5). There is very strong PP support of 1.0 for the Asiaticum species complex (*Hyalomma asiaticum asiaticum+Hyalomma asiaticum kozlovi*) and *Hyalom*

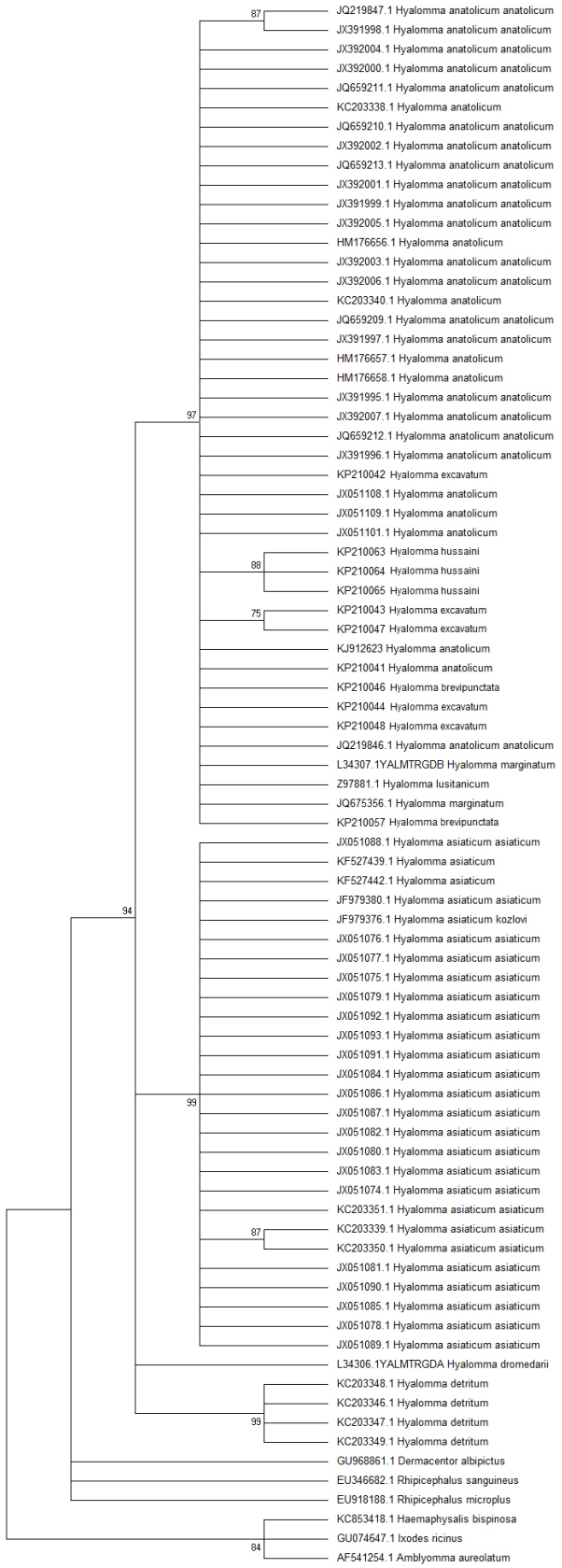

Figure 4: Phylogenetic tree (70% consensus) generated by Neighbor-Joining method based on T3PM+G model with 1000 bootstrap replicates (values on branches are bootstrap support)

ma detritum while Anatolicum group i.e. (*Hyalomma anatolicum* + *Hyalomma excavatum* + *Hyalomma lusitanicum* +*Hyalomma marginatum* + *Hyalomma hussaini* + *Hyalomma brevipunctata*) is supported by 0.89 PP. The monophyletic clade of genus *Hyalomma* is having a support of 0.93 PP. The out-groups which showed strong PP support were *Rhipicephalus* with 0.96 PP and *Dermacentor* + *Rhipicephalus* + *Rhipicephalus* (*Boophilus*) clade with 0.91 PP.

Estimating Divergence Time

Most recent common ancestor (MRCA) of various nodes has the following divergence times when the molecular clock analysis results are studied: node labelled as A in Figure 5 (((((*Ixodes*) + ((*Haemaphysalis* + *Amblyomma*) + (((*Dermacentor* + (*Rhipicephalus* + *Rhipicephalus Boophilus*)) + (*Hyalomma*)))) has the divergence time of 86.79 mya, (*Haemaphysalis* + *Amblyomma*) and *Rhipicephalinae* node labelled B – 68.37 mya, *Dermacentor* and *Rhipicephalus* split node labelled C– 54.68 mya, *Rhipicephalus* + *Rhipicephalus* (*Boophilus*) split node labelled D-36.59 mya, *Dermacentor* + *Rhipicephalus* and *Hyalomma* split node labelled E– 61.99 mya and *Hyalomma asiaticum* node labelled F- 21.46 mya, Anatolicum group clade node labelled G – 31.63 mya, and *Hyalomma* clade labelled H– 40.45 mya.

DISCUSSION

Nucleotide Composition, Sequence Divergence, and Relationships within Genus *Hyalomma*

The nucleotide differences in the 16s rDNA between various members of genus *Hyalomma* especially ti/tv bias and variation in the tajima's test of neutrality values is indicative of population expansion or a selective sweep. The values are significant for different species groups within genus *Hyalomma* at 0.05 or 0.01 p values (Tables 2 and 6) especially Anatolicum group and Asiaticum species complex. Consequently, when the sequence divergence between groups is studied with the intention of estimating inter-specific variation within genus *Hyalomma* then it reveals that *H. anatolicum*, *H. excavatum*, *H. marginatum*, *H. lusitanicum*, *H. hussaini* and *H. brevipunctata* are having less than 5% sequence divergence indicating a recent evolutionary split. In contrast the *H. asiaticum* is more departed having 7.3% to 13.8% divergence from these species (Table 5). As for *H. dromedarii* and *H. detritum* that cluster with *H. asiaticum* in the phylogenetic trees, *H. asiaticum* shows 7.65% and 7.84% divergence which is in agreement with between species differences reported earlier for *Rhipicephalus* (Liu et al., 2013) and *Ixodes* species (Song et al., 2011; Apanaskevich et al., 2011; Chao et al., 2009).

In this context, a review of literature reveals that there is no explicit study to infer relationships within genus *Hyalomma* but some of the investigations had a few members of *Hyalomma* analyzed using various markers. Specifically, *H. anatolicum anatolicum* and *H. anatolicum excavatum* were subspecies until Apanaskevich and Horak (2005) raised those to the rank of species namely *Hyalomma ana*

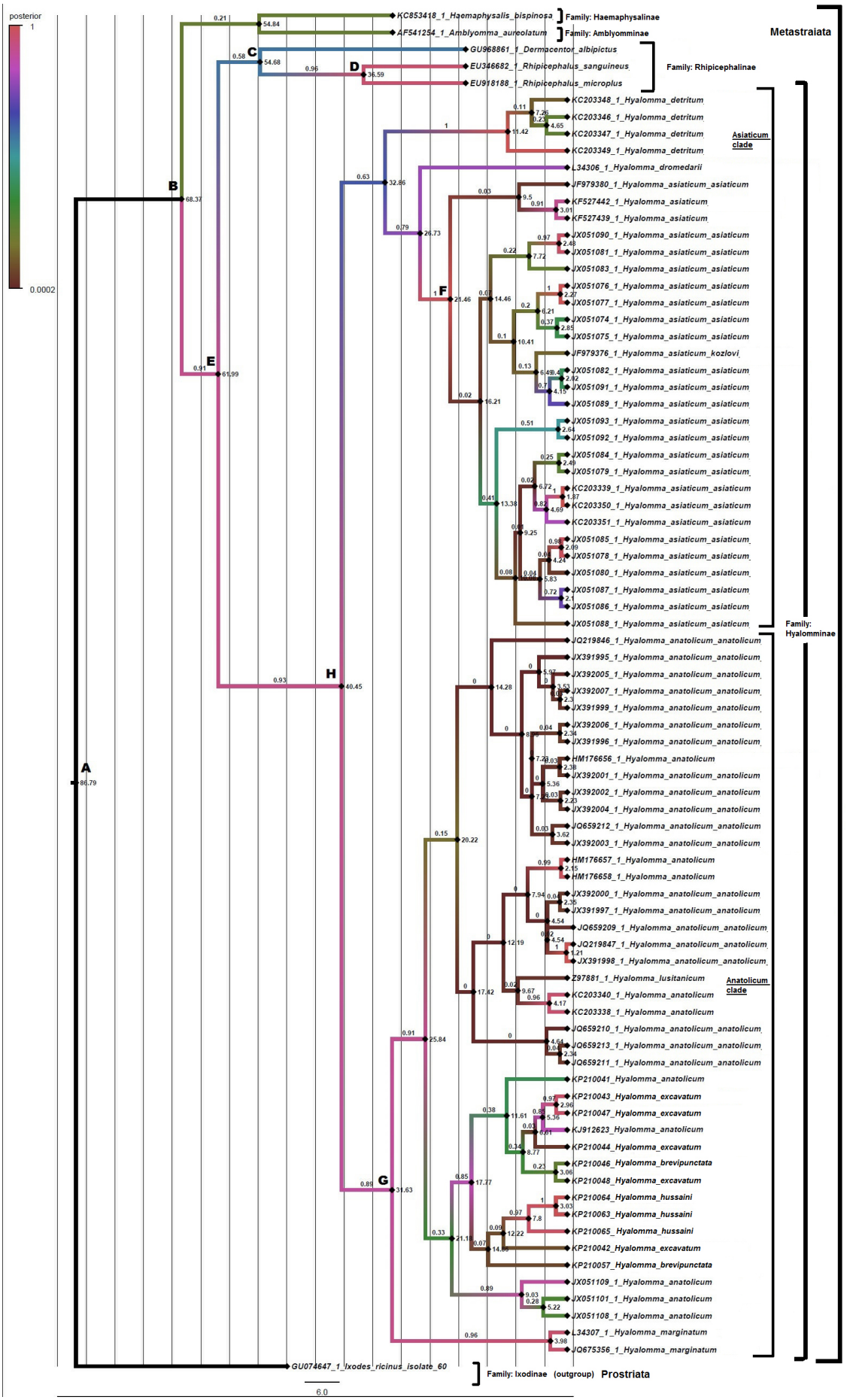

Figure 5: Phylogenetic tree generated by Bayesian method based on T3PM+G model executed in Beast (the values on nodes are node ages in million years while the values on bar are Posterior Probability values)

Table 7: Bootstrap support, Posterior Probability, and Posterior age distributions of the major clades of hard ticks using ML, NJ and Bayesian analysis

Node	Genus/Clades/Node details	Bootstrap support		Posterior probability	Posterior age distributions
		ML	NJ		Mean node age in mya
A	*Prostraiata+Metastriata* i.e *Ixodes* + all others	-	-	-	86.79
B	(*Haemaphysalis+Amblyomma*) and *Rhipicephalinae* node labelled B	65	63	0.21	68.37
C	*Dermacentor* and *Rhipicephalus* split	73	84	0.58	54.68
D	*Rhipicephalus* + *Rhipicephalus* (*Boophilus*) split	60	55	0.96	36.59
E	*Dermacentor+Rhipicephalus* and *Hyalomma* split	38	-	0.91	61.99
F	Asiaticum group	84	99	1	21.46
G	Anatolicum group	76	97	0.89	31.63
H	Genus *Hyalomma*	93	94	0.93	40.45

tolicum Koch 1844 and *Hyalomma excavatum* Koch 1844 based on morphological characters. Based on the present molecular analysis *Hyalomma anatolicum* and *H. excavatum* form a single clade, it might be possible to generate molecular discrimination keys for these closely resembling problematic species using multiple genetic markers. Similarly, *H. asiaticum* was revealed to be a sibling species complex (Apanaskevich and Horak, 2010), while there is ambiguity about the status of *H. detritum* Schulze, 1919 (recently it has been synonymised with *H. scupense* (Schulze, 1919; (Apanaskevich et al., 2010)).

Based on our phylogenetic analysis by three different computational approaches (ML, NJ and Bayes) genus *Hyalomma* appears to be monophyletic with strong bootstrap and PP supports. Further the results reveal strong support for groups of *H. anatolicum* and related species labelled here as Anatolicum group (Table 2) that comprises *H. anatolicum*, *H. excavatum*, *H. marginatum*, *H. lusitanicum*, *H. hussaini* and *H. brevipunctata* taxa and Asiaticum species complex having *H. asiaticum asiaticum* and *H. asiaticum kozlovi*. The results of sequence divergence and phylogenetic analysis provides credence to the fact that the Anatolicum group of species in the genus *Hyalomma* has undergone speciation during the recent evolutionary times which might still be going on. Further, detailed population genetic studies have to be carried out on populations and species from broader geographical localities to better illuminate the taxonomy and population genetic structures of these hard tick species of genus *Hyalomma* some of which are important vectors in their areas of geographical distribution.

Relationships with other Members of Family Ixodidae

Filippova (1993) based on the structure of the ventral skeleton of male Ixodid hard ticks suggested that subfamily Hyalomminae be included in the subfamily Rhipicephalinae. Later molecular studies of Black and Piesman (1994), Klompen et al. (1996, 1997), Black et al. (1997), Mangold et al. (1998a, 1998b) further provided evidence that *Hyalomma* species are found within the monophyletic Rhipicephalinae clade, whereas *Dermacentor* was placed either basal to Rhipicephalinae or within that clade. In the study of Dobson and Barker (1999) based on 18s rDNA the Haemaphysalinae species formed a monophyletic group with high bootstrap (98%) support, and there was also strong support for the clade containing the *Rhipicephalus* and *Hyalomma* species (100%) but there was no substantial bootstrap support for the sister group relationship of *Dermacentor* with the *Rhipicephalus* + *Hyalomma* clade. In the analysis of Murrell et al. (2001) the following clade had common ancestor (*Dermacentor* + ((*Hyalomma* + *Nosomma*) + (*Boophilus* + *Rhipicephalus*))). Labruna et al. (2009) using 16s and 12s rDNA gene reported *Hyalomma* and *Dermacentor* to be paraphyletic and basal to subfamily Rhipicephalinae which itself was a polyphyletic assemblage thus countering the earlier hypothesis.

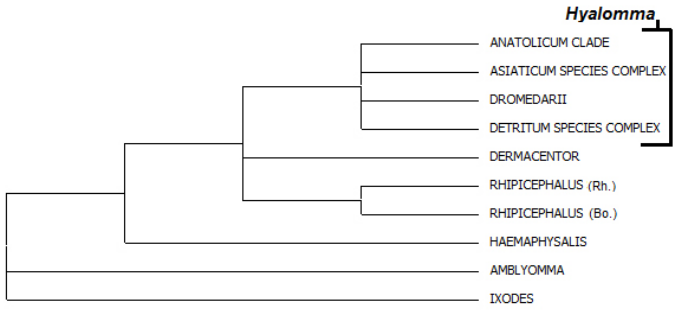

Figure 6: Condensed Phylogenetic tree summarising the results of the present study recommended as a Key for future works

In relation to this context, based on the present 16S based phylogeny following points of importance could be inferred viz. subfamily Amblyomminae is most basal in Metastriate after subfamily Haemaphysalinae but the weak support to this node is in lines with the recent contention of Burger et al. (2012) that it is a polyphyletic assemblage. *Dermacentor* and *Rhipicephalus* are paraphyletic to *Hyalomma* with *Dermacentor* older than the other two genera. In all the three

phylogenetic trees *Rhipicephalus* (*Rhipicephalus*) and *Rhipicephalus* (*Boophilus*) clade together thus justifying the recent subgeneric status of *Boophilus*. Although the main aim of our study was to provide a phylogenetic tree as the basis for further comparative studies of inter-relationships within the genus *Hyalomma* subfamily Hyalomminae rather than a detailed analysis of phylogenetic relationships with other genera within family Ixodidae, still our preliminary results does not support merging the subfamily Hyalomminae within subfamily Rhipicephalinae. This hypothesis needs to be tested using more sequences from other genes covering whole of the metastriate lineage.

Origin of the subfamily Hyalomminae

Estimating the divergence time plays an important role as indicator of speciation events that happened in the past evolutionary history of organisms (Hayakawa et al., 2008; Ricklefs and Outlaw, 2010). Recently Gou et al. (2013) have reviewed the divergence times of hard ticks based on three Dominica amber samples estimating that hard ticks began to diverge during the cretaceous approx 86mya (95% HPD 61.11-104-16mya) which is later than earlier estimates (90-120mya) (Mans et al., 2002; Klompen et al., 2005). In our analysis subfamily Hyalomminae seems to have diverged 61.99 mya from the common ancestor of subfamily Rhipicephalinae, while the divergence time of prostriate and metastriate ticks comes out to be 86.79 mya. It can be surmised that all the genera began to form during the early Eocene-late Pleistocene but separation of *Hyalomma* from the basal stalk happened at the beginning of Oligocene in the warm but cooling climate which coincided with the rapid evolution and diversification of the mammalian hosts of these important ectoparasites.

CONCLUSIONS

The results of the present study have confirmed some of the existing morphological and molecular hypotheses about *Hyalomma* phylogeny. Furthermore, information about the inter-relationships of taxa not previously included in any phylogenetic study has been provided. In conclusion, the molecular evidence presented here suggests that *H. anatolicum*, *H. excavatum*, *H. marginatum*, *H. lusitanicum*, *H. hussaini* and *H. brevipunctata* represent closely related but rapidly diverging taxa and confirms *H. asiaticum* as a species complex. The subfamily Hyalomminae diverged from the common ancestor of subfamily Rhipicephalinae approx 62 mya at the beginning of Oligocene. Apart from this, a basic phylogenetic tree is provided (Figure. 6) to help any future phylogenetic studies on members of family Ixodidae. Further study covering other members of genus *Hyalomma* from different geographical regions needs to be done using multiple genes to corroborate findings and shed light on questions raised in the present study.

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CONFLICT OF INTEREST

There exists no conflict of interest.

AUTHOR'S CONTRIBUTION

Harpreet Kaur helped in planning of research, guidance, writing and revision of manuscript. Jainder Singh Chhilar contributed in data analysis, and manuscript preparation. Shivani Chhillar contributed in Sample collection, Experimental design and execution along with manuscript preparation and revision.

REFERENCES

- Abdigoudarzi M, Noureddine R, Seitzer U, Ahmed J (2011). rDNA-ITS2 identification of *Hyalomma*, *Rhipicephalus*, *Dermacentor* and *Boophilus* spp. (Acari: Ixodidae) collected from different geographical regions of Iran. Adv. Stud. Biol. 3(5): 221-238.
- Apanaskevich DA, Horak IG (2005). The genus *Hyalomma* Koch, 1844. II. Taxonomic status of *H.* (*Euhyalomma*) *anatolicum* Koch, 1844 and *H.* (*E.*) *excavatum* Koch, 1844 (Acari, Ixoididae) with redescription of all stages. Acarina. 13(2): 181-197.
- Apanaskevich DA, Horak IG (2010). The genus *Hyalomma*. XI. Redescription of all parasitic stages of *H.* (*Euhyalomma*) *asiaticum* (Acari: Ixodidae) and notes on its biology. Exp. Appl. Acarol. 52:207-220. http://dx.doi.org/10.1007/s10493-010-9361-0
- Apanaskevich DA, Filippova NA, Horak IG (2010). The genus *Hyalomma* Koch, 1844. X. Redescription of all parasitic stages of *H.* (*Euhyalomma*) *scupense* Schulze, 1919 (= H. detritum Schulze) (Acari: Ixodidae) and notes on its biology. Folia Parasitol. 57(1): 69–78. http://dx.doi.org/10.14411/fp.2010.009
- Apanaskevich DA, Horak IG, Matthee CA, Matthee S (2011). A new species of *Ixodes* (Acari: Ixodidae) from South African mammals. J. Parasitol. 97: 389-398. http://dx.doi.org/10.1645/GE-2366.1
- Barker SC, Murrell A (2004). Systematics and evolution of ticks with a list of valid genus and species names. Parasitology. 129: S15-S36. http://dx.doi.org/10.1017/S0031182004005207
- Barker SC, Murrell A (2007). Phylogeny, evolution and historical zoogeography of ticks: a review of recent progress. Exp. Appl. Acarol. 28: 55-68. http://dx.doi.org/10.1023/A:1025333830086
- Beati L, Keirans JE (2001). Analysis of the systematic relationships among ticks of the genera *Rhipicephalus* and *Boophilus* (Acari: Ixodidae) based on mitochondrial 12s ribosomal DNA gene sequences and morphological characters. J. Parasitol. 87(1): 32-48. http://dx.doi.

org/10.2307/3285173
•Black WCIV, Klompen JSH, Keirans JE (1997). Phylogenetic relationships among tick subfamilies (Ixodida: Ixodidae: Argasidae) based on the 18s nuclear rDNA gene. Mol. Phyl. Evol. 7(1): 129-144. http://dx.doi.org/10.1006/mpev.1996.0382
•Black WCIV, Piesman J (1994). Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. P.N.A.S., U.S.A. 91 (21): 10034-10038. http://dx.doi.org/10.1073/pnas.91.21.10034
•Burger TD, Shao R, Beati L, Miller H, Barker SC (2012). Phylogenetic analysis of ticks (Acari: Ixodida) using mitochondrial genomes and nuclear rDNA genes indicates that the genus *Amblyomma* is polyphyletic. Mol. Phyl. Evol. 64: 45-55. http://dx.doi.org/10.1016/j.ympev.2012.03.004
•Burger TD, Shao R, Barker SC (2014a). Phylogenetic analysis of mitochondrial genome sequences indicates that the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, contains a cryptic species. Mol. Phyl. Evol. 76: 241-253. http://dx.doi.org/10.1016/j.ympev.2014.03.017
•Burger TD, Shao R, Labruna MB, Barker SC (2014b). Molecular phylogeny of soft ticks (Ixodida: Argasidae) inferred from mitochondrial genome and nuclear rDNA sequences. Ticks Tick Borne Dis. 5: 195-207. http://dx.doi.org/10.1016/j.ttbdis.2013.10.009
•Chao LL, Wu WJ, Shih CM (2009). Molecular analysis of *Ixodes granulatus*, a possible vector tick for *Borrelia burgdorferi* sensu lato in Taiwan. Exp. Appl. Acarol. 48: 329-344. http://dx.doi.org/10.1007/s10493-009-9244-4
•Chitimia L, Lin RQ, Cosoroaba I, Wu XY, Song HQ, Yuan ZG, Zhu XQ (2010). Genetic characterization of ticks from southwestern Romania by sequences of mitochondrial cox1 and nad5 genes. Exp. Appl. Acarol. 152: 305-311. http://dx.doi.org/10.1007/s10493-010-9365-9
•Darriba D, Taboada GL, Doallo R, Posada D (2012). jModelTest 2: more models, new heuristics and parallel computing. Nature Methods. 9(8): 772. http://dx.doi.org/10.1038/nmeth.2109
•De La Fuente J (2003). The fossil record and the origin of ticks (Acari: Parasitiformes: Ixodida). Exp. Appl. Acarol. 29(3-4): 331-344. http://dx.doi.org/10.1023/A:1025824702816
•Dobson SJ, Barker SC (1999). Phylogeny of the hard ticks (Ixodidae) inferred from 18s rDNA indicates that the genus *Aponomma* is Paraphyletic. Mol. Phyl. Evol. 11(2): 288-295. http://dx.doi.org/10.1006/mpev.1998.0565
•Felsenstein J (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 39: 783-791. http://dx.doi.org/10.2307/2408678
•Filippova NA (1993). Ventral skeleton of male of ixodid ticks of the subfamily Amblyomminae, its evolution and role for supergeneric taxonomy. Parasitologia. 27: 3–18.
•Geevarghese G, Dhanda V (1987). The Indian *Hyalomma* ticks (Ixodoidea: Ixodidae), Indian Council of Agricultural Research, New Delhi.
•Ghosh S, Azhahianambi P, Yadav MP (2007a). Upcoming and future strategies of tick control: A review. J. Vector Borne Dis. 44: 79-89.
•Ghosh S, Bansal GC, Gupta SC, Ray D, Khan MQ, Irshad H, Shahiduzzaman Md, Seitzer U, Ahmed JS (2007b). Status of tick distribution in Bangladesh, India and Pakistan. Parasitol. Res. 101 (Suppl 2): s207-s216. http://dx.doi.org/10.1007/s00436-007-0684-7
•Gou H, Guan G, Liu A, Ma M, Chen Z, Liu Z, Ren Q, Li Y, Yang J, Yin H, Luo J (2013). Coevolutionary analysis of the relationships between piroplasmids and their hard tick hosts. Ecol. Evol. 3(9): 2985-2993. http://dx.doi.org/10.1002/ece3.685
•Guglielmone AA, Estrada-Pena A, Mangold AJ, Barros-Battesti DM, Labruna MB, Martins JR, Venzal JM, Arzua M, Keirans JE (2003). *Amblyomma aureolatum* (Pallas, 1772) and *Amblyomma ovale* Koch, 1844 (Acari: Ixodidae): hosts, distribution and 16S rDNA sequences. Vet. Parasitol. 113 (3-4): 273-288. http://dx.doi.org/10.1016/S0304-4017(03)00083-9
•Guindon S, Gascuel O (2003). A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Syst. Biol. 52: 696-704. http://dx.doi.org/10.1080/10635150390235520
•Hayakawa T, Culleton R, Otani H, Horii T, Tanabe K (2008). Big bang in the evolution of extant malaria parasites. Mol. Biol. Evol. 25: 2233–2239. http://dx.doi.org/10.1093/molbev/msn171
•Hoogstraal H, Aeschlimann A (1982). Tick–Host Specificity. Bulletin de la Socie´te´ Entomologique Suisse. 55: 5–32.
•Horak IG, Camicas JL, Kierans JE (2002). The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida): a world list of valid tick names. Exp. Appl. Acarol. 28: 27–54 http://dx.doi.org/10.1023/a:1025381712339
•Hutcheson HJ, Klompen JSH, Keirans JE, Norris DE, Barker SC, Black WCIV (2000). Current progress in tick molecular systematics. In: M. Kazimirova, et al. (eds), Proceedings of 3rd International Conference on Ticks and Tick-borne Pathogens, Slovakia Institute of Zoology, Slovak Academy of Sciences, Bratislava, 11–19.
•Jongejan F, Uilenberg G (2004). The global importance of ticks. Parasitology. 129: S3–S14. http://dx.doi.org/10.1017/S0031182004005967
•Kaiser MN, Hoogstraal H (1964). The *Hyalomma* ticks (Ixodoidea, Ixodidae) of Pakistan, India and Ceylon, with keys to subgenera and species. Acarologia. 6 (2): 257-286.
•Keirans JE, Litwak TR (1989). Pictorial Key to the Adults of Hard Ticks, Family Ixodidae (Ixodida: Ixodoidea), East of the Mississippi River. J. Med. Entomol. 26(5): 435-448. http://dx.doi.org/10.1093/jmedent/26.5.435
•Klompen J, Black WC, Keirans JE, Norris DE (2005). Systematics and biogeography of hard ticks, a total evidence approach. Cladistics. 16: 79–102. http://dx.doi.org/10.1111/j.1096-0031.2000.tb00349.x
•Klompen JSH, Black WCIV, Keirans JE, Norris DE (2000). Systematics and biogeography of hard ticks, a total evidence approach. Cladistics. 16: 79–102. http://dx.doi.org/10.1006/clad.1999.0126
•Klompen JSH, Oliver-Jr JH, Keirans JE, Homsher PJ (1997). A re-evaluation of relationships in the metastraiata (Acari: Parasitiformes: Ixodidae). Syst. Parasitol. 38: 1-24. http://dx.doi.org/10.1023/A:1005815925466
•Klompen, JSH, Black WCIV, Keirans JE, Oliver JHJr (1996). Evolution of ticks. Ann. Rev. Entomol. 41: 141–161. http://dx.doi.org/10.1146/annurev.en.41.010196.001041
•Kulakova NV, Khasnatinov MA, Sidorova EA, Adel`shin RV, Belikov SI (2014). Molecular identification and phylogeny of *Dermacentor nuttalli* (Acari: Ixodidae). Parasitol. Res. 113 (5): 1787-1793. http://dx.doi.org/10.1007/s00436-014-3824-x
•Labruna MB, Naranjo V, Mangold AJ, Thompson C, Estrada-Pena A, Guglielmone AA, Jongejan F, de la Fuente J (2009).

Allopatric speciation in ticks: genetic and reproductive divergence between geographic strains of *Rhipicephalus* (*Boophilus*) *microplus*. BMC Evol. Biol. 9: 46. http://dx.doi.org/10.1186/1471-2148-9-46

•Liu GH, Chen F, Chen YZ, Song HQ, Lin RQ, Zhou DH (2013). Complete mitochondrial genome sequence data provides genetic evidence that the brown dog tick *Rhipicephalus sanguineus* (Acari: Ixodidae) represents a species complex. Int. J. Biol. Sci. 9(4): 361-369. http://dx.doi.org/10.7150/ijbs.6081

•Lu X, Lin XD, Wang JB, Qin XC, Tian JH, Guo WP, Fan FN, Shao R, Xu J, Zhang YZ (2013). Molecular survey of hard ticks in endemic areas of tick borne diseases in China. Ticks Tick Borne Dis. 4(4): 288-296. http://dx.doi.org/10.1016/j.ttbdis.2013.01.003

•Lv J, Wu S, Zhang Y, Chen Y, Feng C, Yuan X, Jia G, Deng J, Wang C, Wang Q, Mei L, Lin X (2014a). Assessment of four DNA fragments (COI, 16S rDNA, ITS2, 12S rDNA) for species identification of the Ixodida (Acari: Ixodida). Parasit. Vectors. 7 (1): 93. http://dx.doi.org/10.1186/1756-3305-7-93

•Lv J, Wu S, Zhang Y, Zhang T, Feng C, Jia G, Lin X (2014b). Development of a DNA barcoding system for the Ixodida (Acari: Ixodida). Mitochondr. DNA. 25 (2): 142-149. http://dx.doi.org/10.3109/19401736.2013.792052

•Mangold AJ, Bargues MD, Mas-coma S (1998a). 18S rDNA gene sequences and phylogenetic relationships of European hard-tick species (Acari: Ixodidae). Parasitol. Res. 60: 31–37.

•Mangold JJ, Bargues MD, Mas-coma S (1998b). Mitochondrial 16S rDNA sequences and phylogenetic relationships of species of *Rhipicephalus* and other tick genera among Metastriata (Acari: Ixodidae). Parasitol. Res. 84: 478–484. http://dx.doi.org/10.1007/s004360050433

•Mans BJ, Louw AI, Neitz AWH (2002). Evolution of hematophagy in ticks: common origins for blood coagulation and platelet aggregation inhibitors from soft ticks of the genus *Ornithodoros.* Mol. Biol. Evol. 19: 1695– 1705. http://dx.doi.org/10.1093/oxfordjournals.molbev.a003992

•Miranpuri GS (1988). Ticks parasitizing the Indian buffalo (*Bubalus bubalis*) and their possible role in disease transmission. Vet. Parasitol. 27: 357-362. http://dx.doi.org/10.1016/0304-4017(88)90050-7

•Murrell A, Campbell NJ, Barker SC (1999). Mitochondrial 12S rDNA indicates that the Rhipicephalinae (Acari: Ixodida) is paraphyletic. Mol. Phyl. Evol. 12: 83–86. http://dx.doi.org/10.1006/mpev.1998.0595

•Murrell A, Campbell NJH, Barker SC (2001). A total evidence phylogeny of ticks provides insights into the evolution of life cycles and biogeography. Mol. Phyl. Evol. 21(2): 244-258. http://dx.doi.org/10.1006/mpev.2001.1018

•Norris DE, Klompen JSH, Black WCIV (1999). Comparison of the mitochondrial 12S and 16S Ribosomal DNA genes in resolving phylogenetic relationships among hard ticks (Acari: Ixodidae). Ann. Entomol. Soc. Am. 92: 117–129. http://dx.doi.org/10.1093/aesa/92.1.117

•Petney TN, Keirans JE (1995). Ticks of the genera *Amblyomma* and *Hyalomma* from South-east Asia. Trop. Biomed. 12: 45-56.

•Rees DJ, Dioli M, Kirkendall LR (2003). Molecules and morphology: evidence for cryptic hybridization in African *Hyalomma* (Acari: Ixodidae). Mol. Phyl. Evol. 27: 131-142. http://dx.doi.org/10.1016/S1055-7903(02)00374-3

•Ricklefs RE, Outlaw DC (2010). A molecular clock for malaria parasites. Science. 329: 226–229. http://dx.doi.org/10.1126/science.1188954

•Shyma KP, Kumar S, Sharma AK, Ray DD, Ghosh S (2012). Acaricide resistance status in Indian isolates of *Hyalomma anatolicum.* Exp. Appl. Acarol. 58 (4): 471-481. http://dx.doi.org/10.1007/s10493-012-9592-3

•Song S, Shao R, Atwell R, Barker S, Vankan D (2011). Phylogenetic and phylogeographic relationships in *Ixodes holocyclus* and *Ixodes cornuatus* (Acari: Ixodidae) inferred from COX1 and ITS2 sequences. Int. J. Parasitol. 41: 871-880. http://dx.doi.org/10.1016/j.ijpara.2011.03.008

•Tajima F (1989). Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. Genetics. 123: 585-595.

•Tamura K (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. Mol. Biol. Evol. 9: 678-687.

•Tamura K, Kumar S (2002). Evolutionary distance estimation under heterogeneous substitution pattern among lineages. Mol. Biol. Evol.19: 1727-1736. http://dx.doi.org/10.1093/oxfordjournals.molbev.a003995

•Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30: 2725-2729. http://dx.doi.org/10.1093/molbev/mst197

•Tian Z, Xie J, Yin H, Luo J, Zhang L, Zhang P, Luo J (2011). Discrimination between *Haemaphysalis longicornis* and *H. qinghaiensis* based on the partial 16S rDNA and the second internal transcribed spacer (ITS-2). Exp. Appl. Acarol. 54: 165–172. http://dx.doi.org/10.1007/s10493-010-9423-3

•Walker AR, Bouattour A, Camicas JL, Estrada-Pena A, Horak IG, Latif AA, Pegram RG, Preston PM (2003). Ticks of domestic animals in Africa: A guide to identification of species, Bioscience Reports, Edinburgh, Scotland, U.K.

•Weidner H (1964). Eine Zecke, *Ixodes succineus* sp. n. im baltischen Bernstein. Veroff Uberseemus Bremen. 3: 143–151.

Supplementary Table 1: Species and sequence details for various species of genus *Hyalomma* and out-groups

S. No	Species	Sequence Accession Number	Place Of Collection	Isolate	bp	GC%	Reference
1.	*Hyalomma anatolicum*	HM176656.1	India: Izatnagar	LHa	449	23.16	Shyma et al., 2012
2.	*Hyalomma anatolicum*	HM176657.1	India: Izatnagar	PFHa	449	23.39	Shyma et al., 2012
3.	*Hyalomma anatolicum*	HM176658.1	India: Izatnagar	UFHa	449	23.39	Shyma et al., 2012

4.	*Hyalomma anatolicum*	JX051101.1	China: Xinjiang province	XJ063	446	23.54	Lv et al., 2014a
5.	*Hyalomma anatolicum*	JX051108.1	China: Xinjiang province	XJ074	431	22.74	Lv et al., 2014a
6.	*Hyalomma anatolicum*	JX051109.1	China: Xinjiang province	XJ076	438	22.83	Lv et al., 2014a
7.	*Hyalomma anatolicum*	KC203338.1	China: Xinjiang province	XJ187	442	22.40	Lv et al., 2014b
8.	*Hyalomma anatolicum*	KC203340.1	China: Xinjiang province	XJ190	440	22.27	Lv et al., 2014b
9.	*Hyalomma anatolicum anatolicum*	JQ219846.1	India: Raebareli	-	449	22.94	Shyma et al., unpublished
10.	*Hyalomma anatolicum anatolicum*	JQ219847.1	India: Udaipur	-	449	23.16	Shyma et al., unpublished
11.	*Hyalomma anatolicum anatolicum*	JQ659209.1	India: Alwar	-	449	23.16	Shyma et al., unpublished
12.	*Hyalomma anatolicum anatolicum*	JQ659210.1	India: Jaipur	-	449	23.16	Shyma et al., unpublished
13.	*Hyalomma anatolicum anatolicum*	JQ659211.1	India: Mukteshwar	-	449	23.16	Shyma et al., unpublished
14.	*Hyalomma anatolicum anatolicum*	JQ659212.1	India: Pratapgarh	-	449	23.16	Shyma et al., unpublished
15.	*Hyalomma anatolicum anatolicum*	JQ659213.1	India: Sikar	-	449	23.16	Shyma et al., unpublished
16.	*Hyalomma anatolicum anatolicum*	JX391995.1	India: Banswara	-	449	23.16	Shyma et al., unpublished
17.	*Hyalomma anatolicum anatolicum*	JX391996.1	India: Moga	-	449	23.16	Shyma et al., unpublished
18.	*Hyalomma anatolicum anatolicum*	JX391997.1	India: Ferozpur	-	449	23.16	Shyma et al., unpublished
19.	*Hyalomma anatolicum anatolicum*	JX391998.1	India: Muktsar	-	449	23.16	Shyma et al., unpublished
20.	*Hyalomma anatolicum anatolicum*	JX391999.1	India: Mansa	-	449	23.16	Shyma et al., unpublished
21.	*Hyalomma anatolicum anatolicum*	JX392000.1	India: Sangrur	-	449	23.16	Shyma et al., unpublished
22.	*Hyalomma anatolicum anatolicum*	JX392001.1	India: Hisar	-	449	23.16	Shyma et al., unpublished
23.	*Hyalomma anatolicum anatolicum*	JX392002.1	India: Fatehabad	-	449	23.16	Shyma et al., unpublished
24.	*Hyalomma anatolicum anatolicum*	JX392003.1	India: Churu	-	449	22.94	Shyma et al., unpublished
25.	*Hyalomma anatolicum anatolicum*	JX392004.1	India: Chittorgarh	-	449	23.16	Shyma et al., unpublished
26.	*Hyalomma anatolicum anatolicum*	JX392005.1	India: Bhilwara	-	449	23.16	Shyma et al., unpublished
27.	*Hyalomma anatolicum anatolicum*	JX392006.1	India: Bharatpur	-	449	23.16	Shyma et al., unpublished
28.	*Hyalomma anatolicum anatolicum*	JX392007.1	India: Dungarpur	-	449	23.16	Shyma et al., unpublished
29.	*Hyalomma asiaticum*	KF527439.1	China: Xinjiang Yuli	Hasinor1	457	22.76	Chen et al., unpublished
30.	*Hyalomma asiaticum*	KF527442.1	China: Xinjiang Yuli	Hasigyn1	457	22.76	Chen et al., unpublished

31.	*Hyalomma asiaticum asiaticum*	JF979380.1	China	Xinjiang	416	20.91	Chen et al., 2012
32.	*Hyalomma asiaticum asiaticum*	JX051074.1	China: Inner Mongolia	IM034	445	21.57	Lv et al., 2014a
33.	*Hyalomma asiaticum asiaticum*	JX051075.1	China: Inner Mongolia	IM035	445	21.57	Lv et al., 2014a
34.	*Hyalomma asiaticum asiaticum*	JX051076.1	China: Inner Mongolia	IM036	445	21.35	Lv et al., 2014a
35.	*Hyalomma asiaticum asiaticum*	JX051077.1	China: Inner Mongolia	IM037	432	20.83	Lv et al., 2014a
36.	*Hyalomma asiaticum asiaticum*	JX051078.1	China: Inner Mongolia	IM038	441	21.54	Lv et al., 2014a
37.	*Hyalomma asiaticum asiaticum*	JX051079.1	China: Inner Mongolia	IM039	444	21.62	Lv et al., 2014a
38.	*Hyalomma asiaticum asiaticum*	JX051080.1	China: Inner Mongolia	IM041	441	21.54	Lv et al., 2014a
39.	*Hyalomma asiaticum asiaticum*	JX051081.1	China: Inner Mongolia	IM042	442	21.49	Lv et al., 2014a
40.	*Hyalomma asiaticum asiaticum*	JX051082.1	China: Inner Mongolia	IM043	446	21.97	Lv et al., 2014a
41.	*Hyalomma asiaticum asiaticum*	JX051083.1	China: Inner Mongolia	IM044	444	21.85	Lv et al., 2014a
42.	*Hyalomma asiaticum asiaticum*	JX051084.1	China: Inner Mongolia	IM045	443	21.67	Lv et al., 2014a
43.	*Hyalomma asiaticum asiaticum*	JX051085.1	China: Inner Mongolia	IM046	442	21.72	Lv et al., 2014a
44.	*Hyalomma asiaticum asiaticum*	JX051086.1	China: Inner Mongolia	IM047	444	21.40	Lv et al., 2014a
45.	*Hyalomma asiaticum asiaticum*	JX051087.1	China: Inner Mongolia	IM048	445	21.57	Lv et al., 2014a
46.	*Hyalomma asiaticum asiaticum*	JX051088.1	China: Inner Mongolia	IM049	445	21.80	Lv et al., 2014a
47.	*Hyalomma asiaticum asiaticum*	JX051089.1	China: Inner Mongolia	IM050	419	21.24	Lv et al., 2014a
48.	*Hyalomma asiaticum asiaticum*	JX051090.1	China: Inner Mongolia	IM051	453	22.52	Lv et al., 2014a
49.	*Hyalomma asiaticum asiaticum*	JX051091.1	China: Inner Mongolia	IM053	445	21.80	Lv et al., 2014a
50.	*Hyalomma asiaticum asiaticum*	JX051092.1	China: Inner Mongolia	IM054	444	21.85	Lv et al., 2014a
51.	*Hyalomma asiaticum asiaticum*	JX051093.1	China: Inner Mongolia	IM055	444	21.85	Lv et al., 2014a
52.	*Hyalomma asiaticum asiaticum*	KC203339.1	China: Xinjiang Province	XJ189	433	21.25	Lv et al., 2014b
53.	*Hyalomma asiaticum asiaticum*	KC203350.1	China: Xinjiang Province	XJ191	435	21.38	Lv et al., 2014b
54.	*Hyalomma asiaticum asiaticum*	KC203351.1	China: Xinjiang Province	XJ192	435	21.38	Lv et al., 2014b
55.	*Hyalomma asiaticum kozlovi*	JF979376.1	China	Neimeng	415	20.72	Chen et al., 2012
56.	*Hyalomma detritum*	KC203346.1	China: Xinjiang Province	XJ142	444	22.30	Lv et al., 2014b
57.	*Hyalomma detritum*	KC203347.1	China: Xinjiang Province	XJ144	436	21.79	Lv et al., 2014b

58.	*Hyalomma detritum*	KC203348.1	China: Xinjiang Province	XJ145	434	22.35	Lv et al., 2014b
59.	*Hyalomma detritum*	KC203349.1	China: Xinjiang Province	XJ150	447	22.15	Lv et al., 2014b
60.	*Hyalomma dromedarii*	L34306.1	-	YALM TRGDA	455	23.08	Black and Piesman, 1994
61.	*Hyalomma lusitanicum*	Z97881.1	Spain: Alcalá de los Gazules, Sierra de Cádiz.	-	380	22.63	Mangold et al., unpublished
62.	*Hyalomma marginatum*	L34307.1	-	YALM TRGDB	453	22.96	Black and Piesman, 1994
63.	*Hyalomma marginatum*	JQ675356.1	-	clone_12	272	16.91	Movila et al., 2013
64.	*Dermacentor albipictus*	GU968861.1	Canada: Alberta, Dillberry	hapltype _2.14 SL3474	404	22.64	Leo et al., 2010
65.	*Ixodes ricinus*	GU074647.1	France	60	438	25.80	Noureddine et al., 2011
66.	*Rhipicephalus sanguineus*	EU346682.1	Brazil: Porto Velho, Rondonia	RO1	316	24.05	Burlini et al., unpublished
67.	*Rhipicephalus microplus*	EU918188.1	India	-	454	23.13	Labruna et al., unpublished
68.	*Haemaphysalis bispinosa*	KC853418.1	India	TU-TEZ-R1320	421	23.28	Brahma et al., unpublished
69.	*Amblyomma aureolatum*	AF541254.1	Brazil	-	371	24.26	Guglielmone et al., 2003
70.	*Hyalomma anatolicum*	KP210041	India: Gangana, Gohana, Haryana	1364793_P4	426	22.59	This publication
71.	*Hyalomma hussaini*	KP210063	India: Kaimla, Gharaunda, Haryana	1573834_D1	424	22.88	This publication
72.	*Hyalomma hussaini*	KP210064	India: Kaimla, Gharaunda, Haryana	1573835_D2	423	22.93	This publication
73.	*Hyalomma hussaini*	KP210065	India: Kaimla, Gharaunda, Haryana	1573836_D3	424	22.64	This publication
74.	*Hyalomma excavatum*	KP210042	India: Ashok Vihar, Sonipat, Haryana	1547333_K1	424	22.70	This publication
75.	*Hyalomma excavatum*	KP210043	India: Ashok Vihar, Sonipat, Haryana	1547334_K2	428	22.72	This publication
76.	*Hyalomma excavatum*	KP210044	India: Ashok Vihar, Sonipat, Haryana	1547335_K3	425	22.88	This publication
77.	*Hyalomma anatolicum*	KJ912623	India: Garhi Brahmanan, Sonipat, Haryana	1364792_P3	400	22.65	This publication
78.	*Hyalomma brevipunctata*	KP210046	India: Nuran Khera, Gohana, Haryana	1547338_K6	425	22.88	This publication
79.	*Hyalomma brevipunctata*	KP210057	India: Nuran Khera, Gohana, Haryana	1551253_K4	429	19.86	This publication
80.	*Hyalomma excavatum*	KP210047	India: Hansi Road, Karnal, Haryana	1547339_K7	431	23.02	This publication
81.	*Hyalomma excavatum*	KP210048	India: Hansi Road, Karnal, Haryana	1547340_K8	426	22.82	This publication

Supplementary Table 2: Nucleotide Composition Data for 16s rDNA sequences used in this study

Name of taxon	T(U)	C	A	G	Total
HM176656.1 Hyalomma anatolicum isolate Izatnagar LHa	37.6	9.4	39.2	13.8	449.0
HM176657.1 Hyalomma anatolicum isolate Izatnagar PFHa	37.4	9.6	39.2	13.8	449.0
HM176658.1 Hyalomma anatolicum isolate Izatnagar UFHa	37.4	9.6	39.2	13.8	449.0
JX051101.1 Hyalomma anatolicum isolate XJ063	37.0	9.2	39.5	14.3	446.0
JX051108.1 Hyalomma anatolicum isolate XJ074 16S	36.9	9.3	40.4	13.5	431.0
JX051109.1 Hyalomma anatolicum isolate XJ076	37.2	9.1	40.0	13.7	438.0
KC203338.1 Hyalomma anatolicum isolate XJ187	38.2	8.8	39.4	13.6	442.0
KC203340.1 Hyalomma anatolicum isolate XJ190	38.2	8.9	39.5	13.4	440.0
JQ219846.1 Hyalomma anatolicum anatolicum isolate Raebareli	37.9	9.1	39.2	13.8	449.0
JQ219847.1 Hyalomma anatolicum anatolicum isolate Udaipur	37.2	9.4	39.6	13.8	449.0
JQ659209.1 Hyalomma anatolicum anatolicum isolate Alwar	37.6	9.4	39.2	13.8	449.0
JQ659210.1 Hyalomma anatolicum anatolicum isolate Jaipur	37.6	9.4	39.2	13.8	449.0
JQ659211.1 Hyalomma anatolicum anatolicum isolate Mukteshwar	37.6	9.4	39.2	13.8	449.0
JQ659212.1 Hyalomma anatolicum anatolicum isolate Pratapgarh	37.6	9.4	39.2	13.8	449.0
JQ659213.1 Hyalomma anatolicum anatolicum isolate Sikar	37.6	9.4	39.2	13.8	449.0
JX391995.1 Hyalomma anatolicum anatolicum isolate Banswara	37.6	9.4	39.2	13.8	449.0
JX391996.1 Hyalomma anatolicum anatolicum isolate Moga	38.1	9.4	38.8	13.8	449.0
JX391997.1 Hyalomma anatolicum anatolicum isolate Ferozpur	37.6	9.4	39.2	13.8	449.0
JX391998.1 Hyalomma anatolicum anatolicum isolate Muktsar	37.2	9.4	39.6	13.8	449.0
JX391999.1 Hyalomma anatolicum anatolicum isolate Mansa	37.6	9.4	39.2	13.8	449.0
JX392000.1 Hyalomma anatolicum anatolicum isolate Sangrur	37.6	9.4	39.2	13.8	449.0
JX392001.1 Hyalomma anatolicum anatolicum isolate Hisar	37.6	9.4	39.2	13.8	449.0
JX392002.1 Hyalomma anatolicum anatolicum isolate Fatehabad	37.6	9.4	39.2	13.8	449.0
JX392003.1 Hyalomma anatolicum anatolicum isolate Churu	37.9	9.1	39.2	13.8	449.0
JX392004.1 Hyalomma anatolicum anatolicum isolate Chittorgarh	37.6	9.4	39.2	13.8	449.0
JX392005.1 Hyalomma anatolicum anatolicum isolate Bhilwara	37.6	9.4	39.2	13.8	449.0
JX392006.1 Hyalomma anatolicum anatolicum isolate Bharatpur	37.6	9.4	39.2	13.8	449.0
JX392007.1 Hyalomma anatolicum anatolicum isolate Dungarpur	37.6	9.4	39.2	13.8	449.0
HAA	**37.6**	**9.3**	**39.3**	**13.8**	**447.3**
KF527439.1 Hyalomma asiaticum isolate Hasinor1	37.2	8.8	40.0	14.0	457.0
KF527442.1 Hyalomma asiaticum isolate Hasigyn1	37.2	8.8	40.0	14.0	457.0
JF979380.1 Hyalomma asiaticum asiaticum isolate Xinjiang	37.5	7.9	41.6	13.0	416.0
JX051074.1 Hyalomma asiaticum asiaticum isolate IM034	38.0	8.3	40.4	13.3	445.0
JX051075.1 Hyalomma asiaticum asiaticum isolate IM035	38.0	8.3	40.4	13.3	445.0
JX051076.1 Hyalomma asiaticum asiaticum isolate IM036	38.2	8.3	40.4	13.0	445.0
JX051077.1 Hyalomma asiaticum asiaticum isolate IM037	38.0	7.9	41.2	13.0	432.0
JX051078.1 Hyalomma asiaticum asiaticum isolate IM038	37.4	8.2	41.0	13.4	441.0
JX051079.1 Hyalomma asiaticum asiaticum isolate IM039	37.8	8.3	40.5	13.3	444.0
JX051080.1 Hyalomma asiaticum asiaticum isolate IM041	37.6	8.2	40.8	13.4	441.0
JX051081.1 Hyalomma asiaticum asiaticum isolate IM042	38.0	8.1	40.5	13.3	442.0
JX051082.1 Hyalomma asiaticum asiaticum isolate IM043	37.7	8.3	40.4	13.7	446.0
JX051083.1 Hyalomma asiaticum asiaticum isolate IM044	37.6	8.6	40.5	13.3	444.0
JX051084.1 Hyalomma asiaticum asiaticum isolate IM045	37.7	8.4	40.6	13.3	443.0
JX051085.1 Hyalomma asiaticum asiaticum isolate IM046	37.3	8.1	41.0	13.6	442.0
JX051086.1 Hyalomma asiaticum asiaticum isolate IM047	37.8	8.1	40.8	13.3	444.0
JX051087.1 Hyalomma asiaticum asiaticum isolate IM048	37.8	8.1	40.7	13.5	445.0

JX051088.1 Hyalomma asiaticum asiaticum isolate IM049	38.0	8.3	40.2	13.5	445.0
JX051089.1 Hyalomma asiaticum asiaticum isolate IM050	37.2	7.4	41.5	13.8	419.0
JX051090.1 Hyalomma asiaticum asiaticum isolate IM051	37.3	8.6	40.2	13.9	453.0
JX051091.1 Hyalomma asiaticum asiaticum isolate IM053	37.8	8.3	40.4	13.5	445.0
JX051092.1 Hyalomma asiaticum asiaticum isolate IM054	37.6	8.6	40.5	13.3	444.0
JX051093.1 Hyalomma asiaticum asiaticum isolate IM055	37.6	8.6	40.5	13.3	444.0
KC203339.1 Hyalomma asiaticum asiaticum isolate XJ189	37.4	7.9	41.3	13.4	433.0
KC203350.1 Hyalomma asiaticum asiaticum isolate XJ191	37.2	7.8	41.4	13.6	435.0
KC203351.1 Hyalomma asiaticum asiaticum isolate XJ192	37.5	8.0	41.1	13.3	435.0
JF979376.1 Hyalomma asiaticum kozlovi isolate Neimeng	36.9	8.0	42.4	12.8	415.0
HAS	**37.6**	**8.2**	**40.8**	**13.4**	**440.6**
KC203346.1 Hyalomma detritum isolate XJ142	36.9	8.3	40.8	14.0	444.0
KC203347.1 Hyalomma detritum isolate XJ144	36.5	8.3	41.7	13.5	436.0
KC203348.1 Hyalomma detritum isolate XJ145	36.4	8.5	41.2	13.8	434.0
KC203349.1 Hyalomma detritum isolate XJ150	36.9	8.3	40.9	13.9	447.0
HDD	**36.7**	**8.3**	**41.2**	**13.8**	**440.3**
L34306.1YALMTRGDA Mitochondrion Hyalomma dromedarii	38.2	9.5	38.7	13.6	455.0
Z97881.1 Hyalomma lusitanicum	36.8	8.7	40.5	13.9	380.0
L34307.1YALMTRGDB Mitochondrion Hyalomma marginatum	37.7	9.3	39.3	13.7	453.0
JQ675356.1 Hyalomma marginatum clone 12	37.9	7.0	45.2	9.9	272.0
HM	**37.8**	**8.1**	**42.3**	**11.8**	**362.5**
KP210046 HB INDIA NK 1547338 K6	36.8	9.0	40.3	13.9	424.0
KP210057 HB INDIA NK 1551253 K4	38.1	7.5	42.1	12.4	428.0
HB	**37.4**	**8.2**	**41.2**	**13.1**	**426.0**
KP210063 1573834 D1 KAIMLA HH INDIA	37.5	8.7	39.6	14.2	424.0
KP210064 1573835 D2 KAIMLA HH INDIA	37.6	9.0	39.5	13.9	423.0
KP210065 1573836 D3 KAIMLA HH INDIA	37.3	8.7	40.1	13.9	424.0
HH	**37.5**	**8.8**	**39.7**	**14.0**	**423.7**
KP210042 HAE ASHOKVIHAR 1547333 K1	37.4	9.0	40.0	13.7	423.0
KP210043 HAE ASHOKVIHAR 1547334 K2	37.0	8.9	40.3	13.8	427.0
KP210044 HAE ASHOKVIHAR 1547335 K3	36.8	9.0	40.3	13.9	424.0
KP210047 HAE HR 1547339 K7	36.5	9.3	40.5	13.7	430.0
KP210048 HAE HR 1547340 K8	36.9	8.9	40.2	13.9	425.0
HAE INDIA	36.9	9.0	40.3	13.8	425.8
KJ912623 HAA Garhi 1364792 P3	36.9	9.2	40.5	13.5	415.0
KP210041 HAA GANGANA 1364793 P4	37.4	8.7	40.0	13.9	425.0
HAA INDIA	37.1	8.9	40.2	13.7	420.0
Avg.	**37.5**	**8.8**	**40.1**	**13.6**	**437.8**