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Phylogenetic analysis of some hard ticks from India using mitochondrial 16s rDNA

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ABSTRACT

The present study was conducted to analyze inter-relationships between Indian hard tick using mitochondrial 16s rDNA. The sequence alignment consisted of 85 sequences, 32 sequences of 16s rDNA belonging to 7 species of two genera viz. *Rhipicephalus* and *Hyalomma* generated from PCR amplified products and 53 sequences of hard ticks from India available in genbank database. The NJ analyses conducted in MEGA6 revealed that Haemaphysalinae is basal to the clade of Rhipicephalinae + Hyalomminae in the metastriate lineage while Ixodinae was basal in Ixodidae. There were two large clades, one clade of *Hyalomma anatolicum*, *Hy. excavatum*, *Hy. hussaini* and *Hy. brevipunctata* and second clade of *Rhipicephalus* (*Rhipicephalus*) + *Rhipicephalus* (*Boophilus*). The results provide evidence for the contention of polyphyly of *Rh.* (*Rh.*) *sanguineus* and species complex status of *Rh.* (*B.*) *microplus*. A further molecular study from wider distributional area using more genetic markers is needed to confirm these observations.

1. INTRODUCTION

India is predominantly an agricultural country with about 70% of its population dependent on income from agriculture [1]. The livestock sector especially the dairy sector comprising of approximately 199 million cattle and 105 million buffaloes in India is an important part of the rural agribusiness Indian economy [1]. As tick species parasitizing buffaloes and cattle are similar so the threat of tick borne diseases carried from cattle to buffalo and vice versa is possible and equal attention to the health care of cattle and buffalo should therefore be taken [2]. Ghosh and Nagar [1] have reviewed the various tick borne diseases threatening livestock in India and the recent report of spread of Kyasanur forest disease (KFD) from endemic regions in South India to other regions [3] is very alarming so it is all the more pertinent to have knowledge of tick distribution, speciation and evolution. Ticks are classified in the sub-order Ixodida of the order Parasitiformes, one of the two orders of mites (Acari) consisting of about 900 species divided into two major families: Argasidae Canestrini, 1890 of soft ticks and Ixodidae Murray, 1877 of hard ticks. The third family Nuttalliellidae Schulze, 1935 contains only a single species *Nuttalliella namaqua* Bedford, 1931 in a single genus *Nuttalliella* Bedford, 1931. In the last two decades, molecular phylogenetics has revolutionized the phylogeny and systematics of Arthropods [4], especially Arachnids [5] and Insects [6]. The family Ixodidae of prostriate and metastriate hard ticks has 6 subfamilies Ixodinae, Bothriocrotoninae, Amblyomminae, Haemaphysalinae, Hyalomminae and Rhipicephalinae [7-11]. 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 [12, 13]. 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 [14-16]. The traditional morphology based identification is sometimes problematic due to variations caused by blood meal [17] and chances of geographical strains of tick species having different vectorial capacity [18-20], genetic introgression, fertile hybrids [21-22], and resistance to acaricides [23] are always there. During the past two decades several molecular markers have been used to resolve relationships and solve problems facing systematics of hard ticks in the family Ixodidae [24-40]. In this context, there is lack of any study on molecular analysis of hard ticks from the India [2, 41]. This crucial gap in information related to hard ticks prompted us to carry out molecular investigation on members of the family Ixodidae from India using mitochondrial 16s rDNA sequences.

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2. MATERIAL AND METHODS

2.1Material

The hard ticks infesting cattle and buffalo hosts were field collected from different animal farms located in the state of Haryana (India) (Table 1). After initial separation of hard ticks identification up to species level was done by using standard identification keys available [42-46]. The ticks were photographed using a trinocular stereo-zoom microscope (Labomed™) and subsequently preserved in 100% ethanol in a -20°C deep freezer (Bluestar).

2.2DNA 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.

2.3PCR 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') [47]. 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 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).

2.4Sequence details and analysis

A total of 32 sequences were generated for 16s rDNA of 7 species of two hard tick genera during the present study while 53 sequences of hard ticks of various species from India available in the genbank database were retrieved and used for deriving phylogenetic relationships (Table 2). Multiple sequence alignment of eighty five 16s rDNA sequences was generated with Muscle software tool executed in MEGA6 phylogenetic analysis software [48]. The alignment was manually edited to remove any alignment errors and exported as mega and fasta format files.

Table 1: The details of hard tick populations analysed during the present study from Haryana (India).

S. No	Place of Collection	Species Identified	Species code used	Longitude and Latitude
1	NDRI Karnal	*Rhipicephalus (R) microplus* (Canestrini, 1888)	RBM	29.703504 76.983218
2	Chopra Colony, Rohtak Road, Gohana	*Rhipicephalus (B) microplus* (Canestrini, 1888)	RBM	29.127408 76.698571
3	Nuran Khera, Jind Road, Gohana	*Hyalomma brevipunctata* Sharif, 1928	HB	29.203916 76.581767
4	Gharaunda City	*Rhipicephalus (B) decoloratus* (Koch, 1844)	RBD	29.539130 76.967769
5	Hansi Road, Karnal	*Hyalomma excavatum* Koch, 1844	HE	29.687237 76.974474
6	Arainpura, Gharaunda, Karnal	*Rhipicephalus (B) microplus* (Canestrini, 1888)	RBM	29.548281 77.016225
7	Village Thuska Mahra, Rohtak Road, Gohana	*Rhipicephalus (R) sanguineus* (Latreille, 1806)	RRS	29.095420 76.688218
8	Ashok Vihar, Sonepat	*Hyalomma excavatum* Koch, 1844	HE	28.995288 77.006662
9	Garhi Brahmanan, Sonepat	*Hyalomma anatolicum* Koch, 1844	HA	28.994380 76.994348
10	Gangana, Jind Road, Gohana	*Hyalomma anatolicum* Koch, 1844	HA	29.236950 76.614468
11	Badthal, Nilokheri	*Rhipicephalus (B) microplus* (Canestrini, 1888)	RBM	29.867340 76.872346
12	Kaimla, Gharaunda	*Hyalomma hussaini* Sharif, 1928	HH	29.505624 76.997015
13	Kurana, Israna, Panipat	*Rhipicephalus (B) decoloratus* (Koch, 1844)	RBD	29.275754 76.718506
14	Namaste Chowk, Karnal	*Rhipicephalus (B) decoloratus* (Koch, 1844)	RBD	29.670994 76.990600

Table 2: Accession numbers of 16s rDNA sequences submitted to the GenBank nucleotide database in the study on hard ticks from Haryana (India).

S.NO	DETAILS OF SEQUENCE	POPULATION	SPECIES	Sequence ID
1.	lcl|1573839 T44 16SR	Badthal	RBM	KP210071
2.	lcl|1573838 E2 16SR	Namaste chowk	RBD	KP210070
3.	lcl|1573837 E1 16SR	Namaste chowk	RBD	KP210069
4.	lcl|1573828 A3 16SF	Gharonda city	RBD	KP210068
5.	lcl|1573827 A2 16SF	Gharonda city	RBD	KP210067
6.	lcl|1573826 A1 16SF	Gharonda city	RBD	KP210066
7.	lcl|1st BASE 1573836D3	Kaimla	HH	KP210065
8.	lcl|1st BASE 1573835D2 16SR	Kaimla	HH	KP210064
9.	lcl|1573834 D1 16SR	Kaimla	HH	KP210063
10.	lcl|1573833 C3 16SF	Kurana	RBD	KP210062
11.	lcl|1573832 C2 16SF	Kurana	RBD	KP210061
12.	lcl|1573831 C1 16SF	Kurana	RBD	KP210060
13.	lcl|1573830 B2 16SF	Arainpura	RBM	KP210059
14.	lcl|1573829 B1 16SF	Arainpura	RBM	KP210058
15.	lcl|1st BASE 1551253 K4 16SF	Nurankhera	HB	KP210057
16.	lcl|1st BASE 1547348 K1616SR	Badthal	RBM	KP210056
17.	lcl|1st BASE 1547347 K1516SR	Badthal	RBM	KP210055
18.	lcl|1st BASE 1547346 K1416SR	NDRI	RBM	KP210054
19.	lcl|1st BASE 1547345 K1316SR	NDRI	RBM	KP210053
20.	lcl|1st BASE 1547344 K1216SR	NDRI	RBM	KP210052
21.	lcl|1st BASE 1547343 K1116SR	NDRI	RBM	KP210051
22.	lcl|1st BASE 1547342 K1016SR	Thuska mahra	RRS	KP210050
23.	lcl|1st BASE 1547341 K9 16SR	Chopra colony	RBM	KP210049
24.	lcl|1st BASE 1547340 K8 16SF	Hansi road	HAE	KP210048
25.	lcl|1st BASE 1547339 K7 16SF	Hansi road	HAE	KP210047
26.	lcl|1st BASE 1547338 K6 16SF	Nurankhera	HB	KP210046
27.	lcl|1st BASE 1547337 K5 16SF	Nurankhera	HB	KP210045
28.	lcl|1st BASE 1547335 K3 16SF	Ashok Vihar	HAE	KP210044
29.	lcl|1st BASE 1547334 K2 16SF	Ashok Vihar	HAE	KP210043
30.	lcl|1st BASE 1547333 K1 16SF	Ashok Vihar	HAE	KP210042
31.	lcl|1st BASE 1364793 P4 16SF	Gangana	HA	KP210041
32.	lcl|1st BASE 1364792 P3 16SF	Garhi brahmanan	HA	KJ912623

2.5Phylogenetic analysis

Phylogenetic analysis was performed in MEGA6 [48] using neighbor joining (NJ) method. The best fit model to derive relationships based on the current data set was estimated by using the model test tool in MEGA6 [48] which supported the - Tamura three parameter model + unequal frequency + gamma distribution (TPMuf+G). 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: nucleotide substitution model - Tamura 3-parameter model [49], test of phylogeny - bootstrap method, 1000 replications, substitutions to include – d: transitions + transversion, rates among sites – gamma distributed, pattern among lineages – different (heterogeneous) [50].

3.RESULTS

3.1Tick diversity

In this context, the culmination of this study from the state of Haryana (India) resulted in identification of seven Ixodid tick species belonging to two genera viz. *Hyalomma* Koch, 1844 and *Rhipicephalus* Koch, 1844. Seven tick species that were identified belonging to these two genera are- *Hyalomma anatolicum* Koch, 1844, *Hyalomma excavatum* Koch, 1844, *Hyalomma hussaini* Sharif, 1928, *Hyalomma brevipunctata* Sharif, 1928, *Rhipicephalus* (*Rhipicephalus*) *sanguineus* (Latreille, 1806), *Rhipicephalus (Boophilus*) *microplus* (Canestrini, 1888), and *Rhipicephalus (Boophilus*) *decoloratus* (Koch, 1844). The study revealed that *Hyalomma anatolicum* and *Rhipicephalus (B.*) *microplus* are the most common vector species that infest the buffalo and cattle in Haryana. *Hyalomma excavatum* Koch, 1844, *Hyalomma brevipunctata* Sharif, 1928, and *Rhipicephalus (Boophilus*) *decoloratus* (Koch, 1844) have been reported during the present study for the first time from the state of Haryana, India.

3.2Molecular Phylogeny

The molecular methods were employed to perform phylogenetic analysis of ticks infesting cattle from Haryana using mitochondrial 16S ribosomal DNA. The sequences obtained during the present study were compared with the sequences from India which resulted in some interesting inferences having consequent implications on the tick molecular evolution and systematics. When the dataset of eighty-five 16s rDNA sequences belonging to members of family Ixodidae from India was analysed it revealed 180 variable sites, 304 conserved sites, 117 parsimony informative sites and 57 singleton sites. When the evolutionary history was inferred for 16s rDNA sequences of the members of the family Ixodidae from India by the Neighbor-Joining method it resulted in a bootstrap consensus tree inferred from 1000 replicates where branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed (Figure 1a, b, c). The evolutionary distances were computed using the Tamura 3-parameter method in which rate variation among sites was modelled with a gamma distribution.

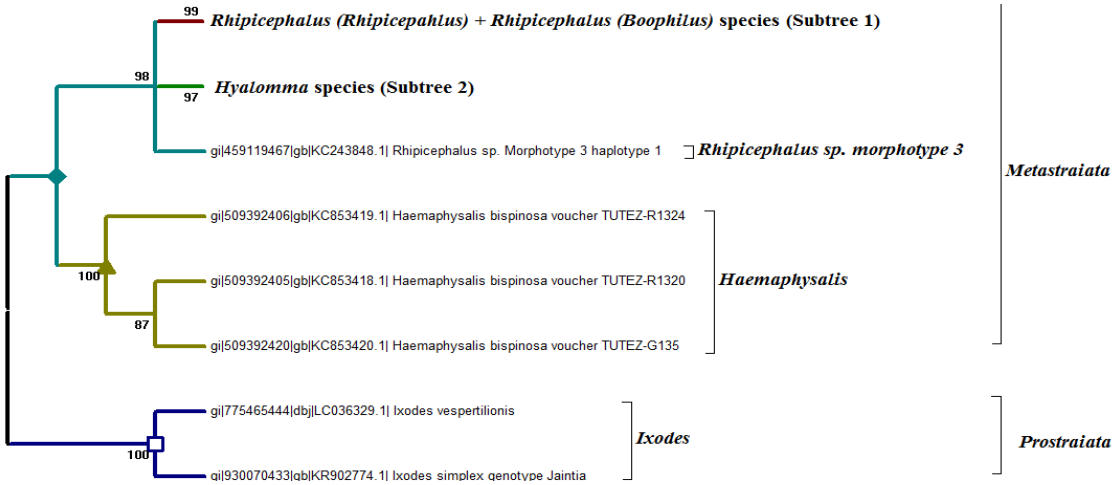

Fig. 1: A-Phylogenetic tree (50% consensus) generated by Neighbor-Joining method based on T3PM+G model with 1000 bootstrap replicates (values on branches are bootstrap support) for 16s rDNA sequences of hard tick species from India.

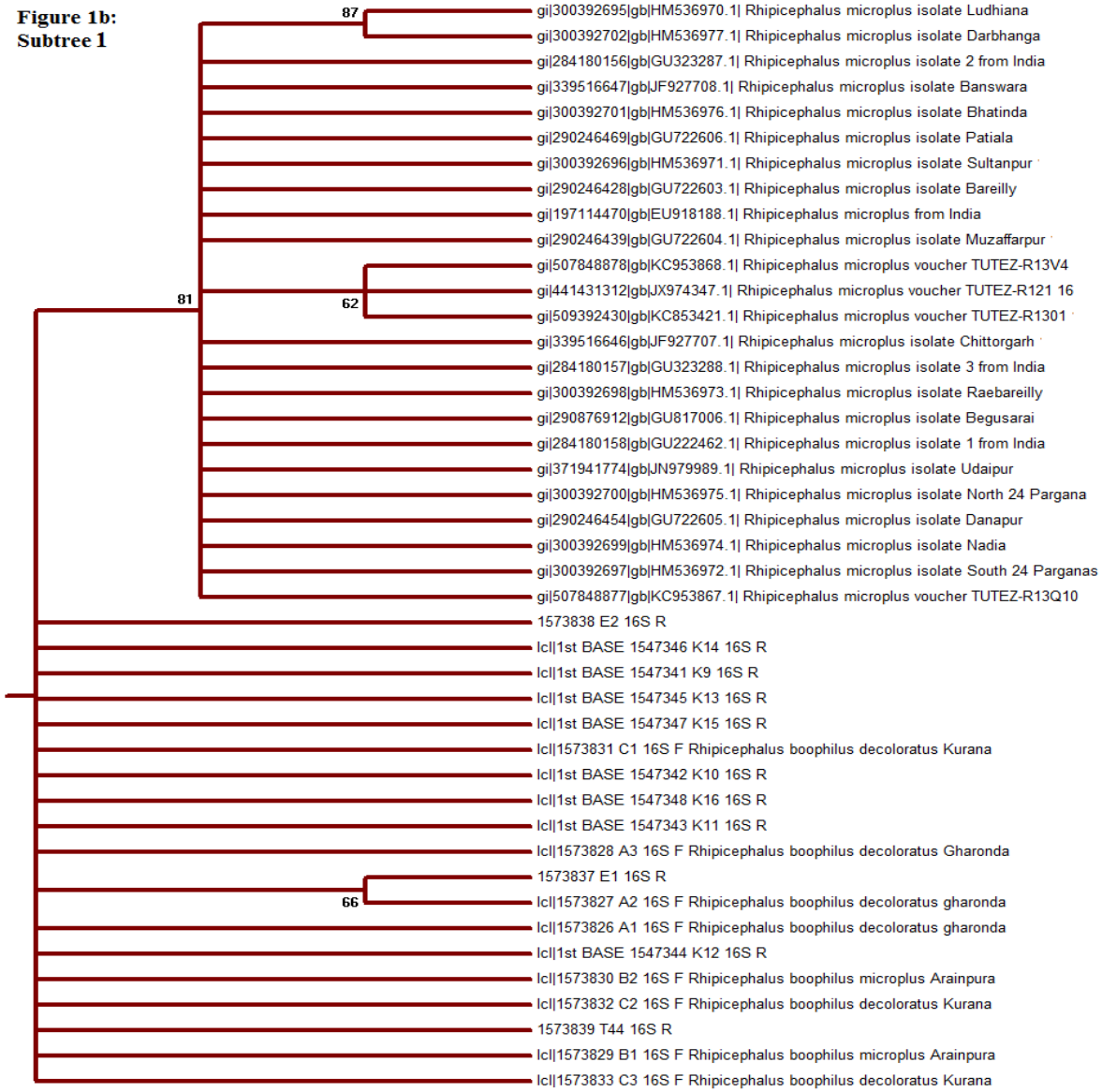

Fig. 1: B-Subtree 1 having *Rhipicephalus* (*Rhipicephalus*) + *Rhipicephalus* (*Boophilus*) species sequences.

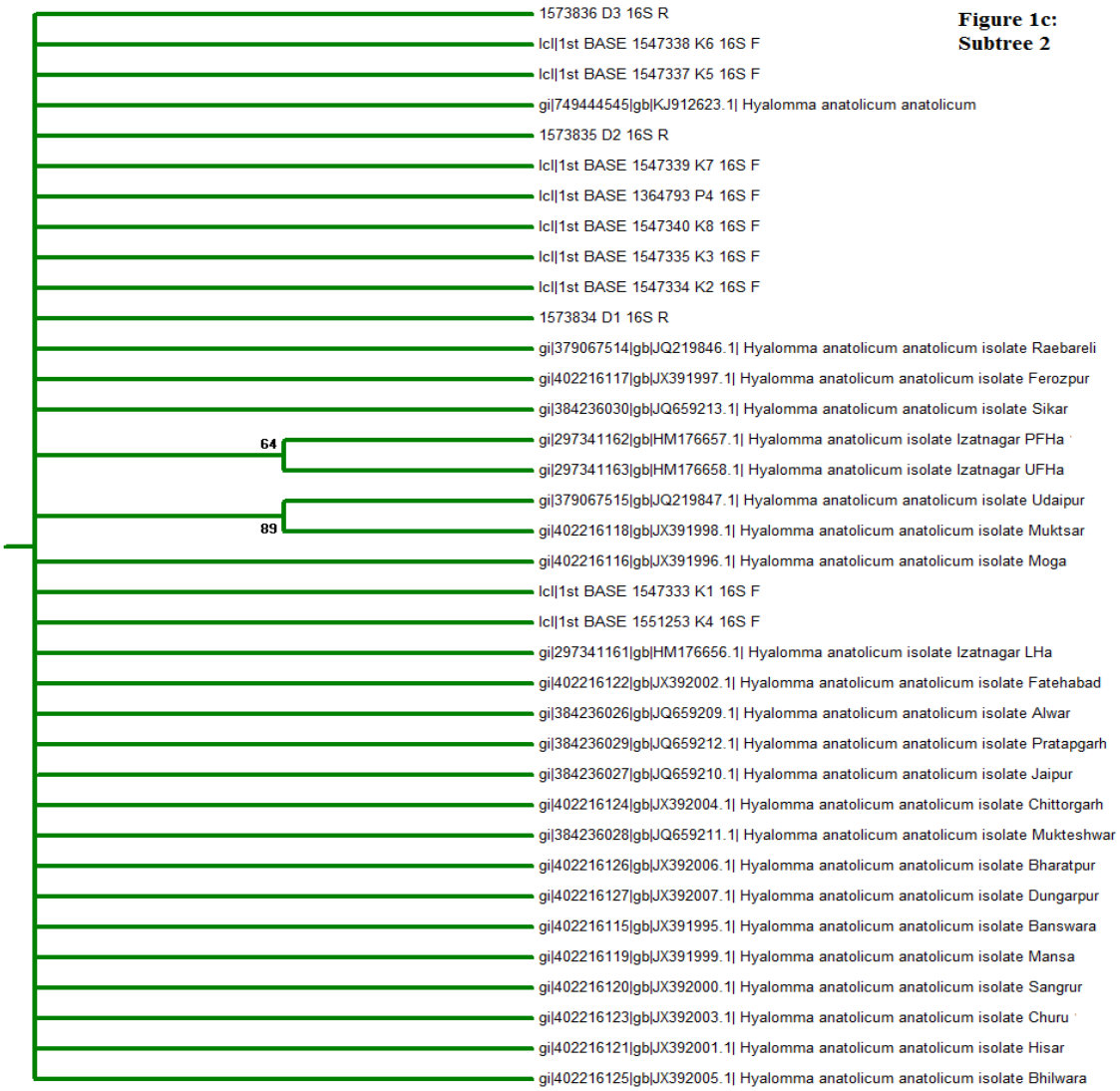

Fig. 1: C-Subtree 2 having *Hyalomma* species sequences

In the final analysis all positions with less than 90% site coverage were eliminated resulting in a total of 501 positions in the final dataset. The results revealed that genus *Ixodes* as the out-group was most basal. *Rhipicephalus (Boophilus) microplus* formed clade having 98% BT support with *R. (B). decoloratus*. Species of genus *Hyalomma* formed a separate clade with 97% BT support. Species *R. morphotype* was paraphyletic to the clade of *Hyalomma* and *Rhipicephalus*. *Haemaphysalis bispinosa* was paraphyletic to all the above with 100% BT support.

4. DISCUSSION

4.1Inter-relationships within Ixodidae

A review of literature reveals that there is no explicit study to infer relationships within hard ticks from India but some of the investigations had a few members from India using various molecular markers. The result of this first molecular study from India have thrown open some important points for discussion as detailed hereunder that will be helpful for future studies on tick distribution, epidemiology, control, and in understanding the tick evolution and systematics. Based on the present 16S based phylogeny following points of importance could be inferred viz. subfamily Haemaphysalinae is basal in Metastriate while *Rhipicephalus* is paraphyletic to *Hyalomma* and *Rhipicephalus* (*Rhipicephalus*) forma a clade with *Rhipicephalus* (*Boophilus*).

In this context, Hoogstraal and Aehlimann [18] grouped Haemaphysalinae + Hyalomminae + Rhipicephalinae based on shared presence of hard hooking devices (Spines and Hooks on legs and mouthparts) and the results of the present study are in lines with their hypothesis. Further, the first molecular study based on 16s rDNA conducted to infer phylogenetic relationships of hard and soft ticks by Black and Piesmann [24] revealed that the members of subfamily Hyalomminae cladded with members of

subfamily Rhipicephalinae and over the years different studies have supported monophyly of Metastriata including the present study [7, 25-28, 30, 51].

Similar to present study, Barker and Murrell [10] while reviewing phylogeny of Ixodidae presented a working hypothesis in which the subfamily Haemaphysalinae claded with (Rhipicephalinae + Hyalomminae). Recently, Burger *et al.,* [37] based on their mitochondrial genome analysis also reported paraphyly of genera *Rhipicephalus* + *Hyalomma*. In this context, Mans *et al.,* [52] based on their 18s Bayesian analysis also contended that there is very strong support for Metastriata and the clade of Haemaphysalinae + (Rhipicephalinae + Hyalomminae).

4.2 Status of different species groups:

When the individual species groups were considered for the sequences from India the majority of sequences belonged to three species groups' viz. Sanguineus group, Microplus group and Anatolicum group. These are individually discussed hereunder:

4.2.1 Sanguineus group

Recently, there has been considerable debate about the status of *Rhipicephalus sanguineus* sensu stricto as it has been considered as a species complex of 17 closely related species [53, 54, 55], however, there is no consensus [56] and the morphological similarities among ticks belonging to the *Rh. (Rh.) sanguineus* group make their identification a difficult task.

Based on the analysis of present data *Rh.* (*Rh.*) *morphotype* 3 is paraphyletic with *Rh.* (*Rh.*) *sanguineus* from Mahra (Haryana) which is suggesting that the former might be a different cryptic/sibling species and that *Rh.* (*Rh.*) *sanguineus* species complex might be polyphyletic.

4.2.2Microplus group

Rhipicephalus (*Boophilus*) *microplus* is a cattle parasite with a one host life cycle. It is thought to have originated in the Indian sub-continent but has now been introduced into many parts of the world including South East Asia with its Host [57-58]. *Rhipicephalus* (*Boophilus*) *annulatus* (Say, 1821) and *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1888) have long been thought to be sister species on the basis of morphology and molecular markers [10]. Labruna *et al.* [22] analyzed *Rh.* (*B.*) *microplus* from different geographical regions using 12s and 16s sequences and according to their hypothesis at least two different species share the name of *Rh.* (*B.*) *microplus*. According to them *Rh.* (*B.*) *microplus* from India and Nepal have been shown to be highly divergent from *Rh.* (*B.*) *microplus* from the Americas and Africa [22]. However, the phylogenetic placement of *Rh.* (*B.*) *microplus* from India and Nepal was not strongly resolved, though *Rh.* (*B.*) *microplus* from India clustered with *Rh.* (*B.*) *annulatus* in the 16S rRNA analysis of Labruna *et al.,* [22].

In this context, Estrada-Peña *et al.,* [59] reinstated as a separate species the cattle ticks from Australia previously known as *Rh.* (*B.*) *microplus* as *Rhipicephalus* (*Boophilus*) *australis* (Fuller, 1899). Interestingly, *Rh.* (*B.*) *decoloratus* during the present study claded with from *Rh.* (*B.*) *microplus* suggesting that *Rh.* (*B.*) *microplus* is a species complex of at least four species viz. *Rh.* (*B.*) *microplus*, *Rh.* (*B.*) *annulatus*, *Rh.* (*B.*) *australis*, and *Rh.* (*B.*) *decoloratus* and the strains from India and Nepal need to be studied using several molecular markers to discern the sibling/cryptic species and inter-relationships within this important species complex.

4.2.3 Anatolicum group

H. anatolicum anatolicum and *H. anatolicum excavatum* were subspecies until Apanaskevich and Horak [60] raised those to the rank of species namely *Hyalomma anatolicum* Koch 1844 and *Hyalomma excavatum* Koch 1844 based on morphological characters. In this context, a recent study of Hosseini *et al.,* [61] using morphometric methods on male specimens of *Hyalomma anatolicum* have shown polymorphism in the important taxonomic characters and have recommended more studies on related species for proper identification of species. According to the present analysis there is very strong support for Anatolicum clade of *Hy. anatolicum* + *excavatum* + *hussaini* + *brevipunctata* with 97%BT support supporting the contention of Kaur *et al.,* [62] that it might be a group of species.

5. CONCLUSIONS

The results of the present study have confirmed some of the existing morphological and molecular hypotheses about hard tick phylogeny. A condensed phylogenetic tree is provided as a reference standard for any future studies from India (Figure 2). 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. hussaini* and *H. brevipunctata* represent closely related but rapidly diverging taxa, confirms that *Rh.* (*B.*) *microplus* is a species complex of at least four species, and asserts that *Rh.* (*Rh.*) *sanguineus* species complex might be polyphyletic.

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 hard ticks from India 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.

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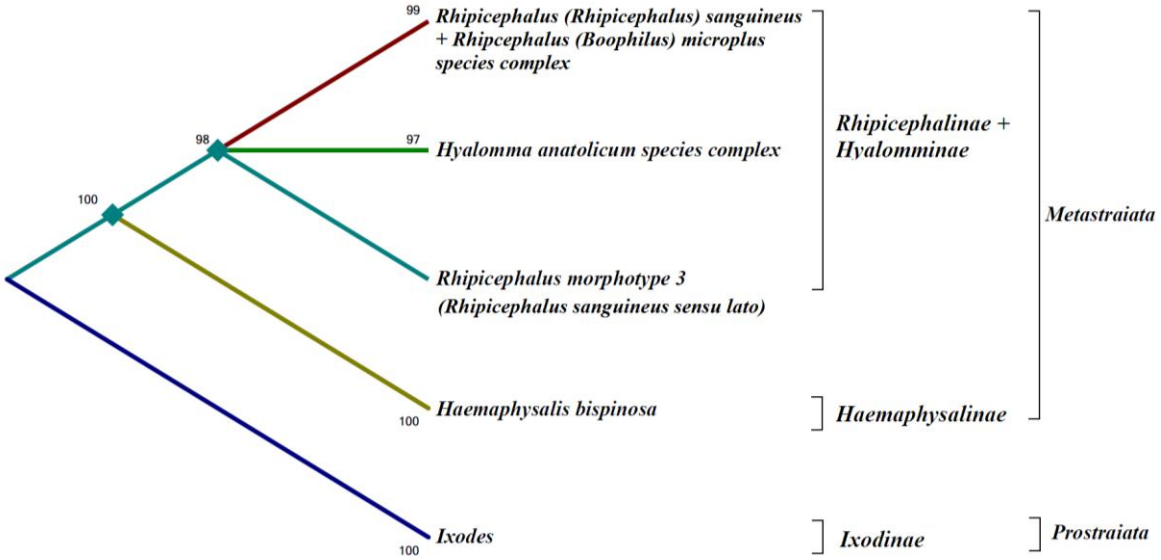

Fig. 2: Condensed Phylogenetic tree summarising the results of the present phylogenetic study on hard tick species from India recommended as a Key for future works

7. CONFLICT OF INTEREST

There exists no conflict of interest.

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