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Identifying ticks of genus *Hyalomma* using the *COI* gene from preserved old specimens – a significant approach for controlling zoonotic diseases†

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Ticks are vectors for a range of human and animal diseases. Accurate species identification is a crucial step for effective pest management, as each species plays host to specific parasites. Species identification based on morphological characteristics is prone to error in cryptic species. Molecular techniques have been used in recent times for accurate species identification; however, few studies are available on Indian tick species. The present study aims to bridge this gap in species identification of *Hyalomma* ticks from India using conventional morphological and recent molecular methods. We also studied the evolutionary relationships between species using a phylogenetic approach. The study included historical samples (*N* = 14) representing four species obtained from the National Zoological Collection of the Zoological Survey of India, Kolkata. Genetic analysis was done using universal barcoding with COI primers. The results indicate a 99–100% match between the genetic and morphological analyses for the four samples of *Hyalomma* species collected, i.e. *Hyalomma hussaini*, *Hyalomma aegyptium*, *Hyalomma kumari* and *Hyalomma anatolicum*. The findings were also supported by phylogenetic and evolutionary tree analyses. The present study is helpful in identifying tick species using integrated approach, interpreting evolutionary relationships between different species, and solving taxonomic problems.

Keywords: Accurate species identification, evolutionary divergence, genetic analysis, *Hyalomma*, phylogenetic tree, zoonotic diseases.

TICKS are arthropod (class Arachnida) vectors of various animal and human diseases (e.g. Rocky Mountain spotted fever, tick-borne relapsing fever, Q fever and Lyme disease) and significantly impact the health of human beings, livestock and wild animals[1,2]. They are divided into two major families – Ixodidae (hard ticks; *n* = 650 species) and Argasidae (soft ticks; *n* = 150 species) with a global distribution. The genus *Hyalomma* of family Ixodidae includes about 30 species and subspecies that inhabit areas with the long dry seasons in Asia, Europe and Africa[3]. *Hyalomma hussaini*, *Hyalomma aegyptium*, *Hyalomma kumara* and *Hyalomma anatolicum* are of significant human and animal health importance[3–6]. *Hyalomma* tick fauna of India is characterized by the presence of several distinct endemic species that have small-sized individuals. The genus includes several medically important species that act as vectors of Crimean–Congo hemorrhagic fever (CCHF) in parts of Europe, Asia and Africa[7–12]. In 2011, Gujarat was the first state to confirm CCHF in India[13] and *H. anatolicum* was being identified as the vector. Ticks are also pests of livestock such as camels, cattle, sheep and goat[14,15]. Therefore, accurate species identification plays a critical role in the management of tick-borne zoonotic diseases.

Conventionally species identification of ticks has primarily relied on morphological characters, which are typically restricted by sampling conditions, high hybridization rates and morphological similarity. Due to its adverse impact on human as well as livestock health, a rapid and dependable identification technique is necessary[16]. Over the past several years, molecular technology has evolved to provide methods for rapidly and accurately identifying tick species[17–19]. DNA barcoding-based approach has been widely accepted for its efficacy in discriminating ticks at species level[19,20]. Several molecular markers have been utilized to resolve the problems related to taxonomy of the family Ixodidae[21–23].

†The data that support the findings of this study are available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/; accession number MW587123-MW587126).

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The importance of identification of this tick genus for human and animal health and limited knowledge of the accurate identification methods prompted us to undertake the present study using a DNA barcoding approach in combination with conventional morphological methods. The study focused on four species belonging to the genus *Hyalomma*, viz. *H. aegyptium*, *H. kumari*, *H. hussaini* and *H. anatolicum* collected from different states of India around 80 years ago.

A total of 14 samples of four tick species from India were collected from the reference repository of the Zoological Survey of India, Kolkata and stored as dried/fresh tissue in 70% alcohol in cryo vials. Four species, i.e. *H. kumari* ($n = 4$), *H. hussaini* ($n = 2$) and *H. anatolicum* ($n = 4$) and *H. aegyptium* ($n = 4$) were used in this study (Table 1).

The morphological identification to species level was done with the aid of a stereomicroscope at 100× magnification (M205A). Diagnostic characteristics of the different parts of ticks (genital aperture, mouth parts and body appearance) were carefully observed and photographed. Fourteen specimens (four males and ten females) were used for molecular studies.

Before DNA extraction, the preserved samples were rinsed twice with distilled water and kept at room temperature for drying. Complete genomic DNA was extracted using the commercially available forensic DNA isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. After DNA extraction, the fragment of universal cytochrome *C* oxidase subunit I (COI) was amplified using a thermal cycler (Quanta Biotech)[24]. The master mix preparation and amplification condition were according to Kumar *et al.*[25]. Thereafter, 4 µl of PCR products were subjected to electrophoresis on 1.8% agarose gel to detect amplification of the selected region visualized over a trans-illuminator. Throughout the procedure, blanks were included in the analysis to monitor cross-contamination. Bidirectional sequencing (commercially) of the *COI* gene was performed (BigDye Terminator Cycle Sequencing Kit VR v.3.1)[26].

Table 1. Details of samples used in the present study

Species	Locality (no. samples)	Accession no.
Hyalomma kumari	India ($n = 4$)	MW587123-MW587126
	Pakistan	KU130608.
Hyalomma aegyptium	India ($n = 4$)	MW587127-MW587130
	Australia	
	Romania	JX394190.1-JX394193.1
Hyalomma anatolicum	India ($n = 4$)	MW587131-MW587134
	China	JQ737067.1
		KJ912622.1
	Iraq	KM235698.1-KM235699.1
		KM235700.1-KM235703.1
		KM235710.1-KM235711.1
Hyalomma hussaini	India ($n = 2$)	MW587135-MW587136
	Spain	MN728998.1-MN728200.1

The sequences obtained from this study have been submitted to NCBI GenBank.

The sequence data produced were examined using the Sequencing Analysis software v. 5.2 (Applied Biosystems, CA, USA) and the individual direction (reverse and forward) of each sample was visually checked, trimmed and combined individually in a single super matrix using Chromas 2.6.4 (http://www.technelysium.com.au). Thereafter, multiple sequence alignment (MSA) was performed utilizing CLUSTAL W implemented in Bio Edit v 7.0.9.0 for further analysis[27]. The intraspecies and interspecies separations were determined employing the Kimura two-parameter (K2P) model[28], as suggested by the Consortium for Barcode of Life (CBOL, http://www.barcoding.si.edu/protocols.html) using MEGA 7.0 program[29]. Then the species-specific variable sites assessment was performed, and the phylogenetic tree (neighbor-joining (NJ) method) was constructed[30] with 1000 bootstrap replicates[31] using MEGA 7.0. The exact species success rate was determined using hereditary separation and BLAST techniques (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Initially, ticks were identified morphologically using a stereomicroscope at 100× magnification (M205A)[32]. The identification was based on genital aperture, mouthparts and body appearance. The following morphological characteristics were used to differentiate the species and their sex.

Sexual dimorphism and other characteristic features of the species *H. hussaini* males have palps about three times as long as broad and are club-shaped, while the female scutum is slightly longer than wide. In *H. kumara*, the male scutum is sayal-brown in colour with some darker areas in the neighbourhood of the eyes, while in the female, the cervical grooves are at first deep and convergent; they then diverge and reach the posterior margin of the scutum. In *H. aegyptium*, the cervical grooves in males are at first broad, deep and convergent, but become narrow and divergent towards their posterior ends. The female scutum is sub-hexagonal. In *H. anatolicum* males have convex, lightly punctuated scutum with short but distinct lateral grooves, while females have a shallow scapular grooves profile (grooves reach the posterior margin of the scutum) (Figure 1).

The isolated genomic DNA concentrations ($n = 14$ samples) was of good quality and ranged between 20.90 and 50.84 ng/µl. Universal CO1 mitochondrial fragments were effectively amplified in all the samples, the DNA sequencing result was 100%, and finally obtained readable sequence was 546 bp. The final sequence (546 bp) of four tick species consisted of 408 conserved regions, 122 parsimony informative sites, 138 variable sites, 16 singleton sites and the nucleotide composition: A-30.4%, T-37%, G-17.7% and C-17.9% (Figure 2). BLAST analysis result of the obtained COI query sequences of the four different species showed that they all had 99% similarity with their respective *Hyalomma* species (Table 2A). Since no Indian-origin sequences of *Hyalomma* species were available in the public domain database, the query sequences showed 99%

Figure 1. *Hyalomma aegyptium* Linnaeus, 1758, male. ***a***, Cervical groove female. ***b***, Scutum subhexagonal; *Hyalomma anatolicum* Koch, 1844, male. ***c***, Scutum female. ***d***, Scapular grooves; *Hyalomma hussaini* Sharif, 1928, male. ***e***, Palps female. ***f***, Scutum; *Hyalomma kumari* Sharif, 1928 male. ***g***, Scutum female. ***h***, Cervical groove.

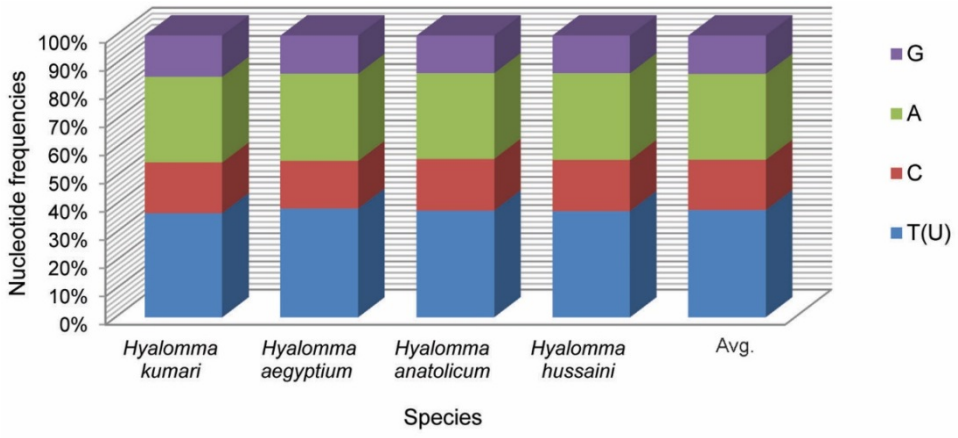

Figure 2. COI based frequencies of nucleotide composition among 4 *Hyalomma* species.

similarity. To reconfirm the BLAST finding, further analysis was done.

In the 546 bp dataset of the four *Hyalomma* species (n = 14 sequences), the *intraspecies* K2P divergence ranged from 0.1% to 1.6%, with maximum and minimum distance observed in *H. anatolicum* and *H. hussaini* respectively. In the other two species, viz. *H. kumari* and *H. aegyptium*, the divergence was 0.7% and 1.3% respectively (Table 2B).

Table 2. (A) Similarities in the COI locus between the present study sample and available sequences in GenBank using Blast Search tool. (B) Number of base differences per site from averaging over all sequence pairs within each group. Standard error estimate(s) are shown in the last column

A			B		
		Query coverage	Within group mean distance		
Sample	Species with the highest similarity	Similarity		*d* (%)	SE
H. kumari	*H. kumari*	100.0 99.63	*H. kumari*	0.7	0.002
H. aegyptium	*H. aegyptium*	100.0 99.00	*H. aegyptium*	1.3	0.003
H. anatolicum	*H. anatolicum*	100.0 99.0	*H. anatolicum*	0.4	0.001
H. hussaini	*H. hussaini*	100.0 99.0	*H. hussaini*	1.6	0.004

Table 3. Interspecies divergence among tick samples. The number of base substitutions per site from averaging over all sequence pairs between groups is shown. Analysis was done using the Kimura two-parameter model

	H. kumari	*H. hussaini*	*H. aegyptium*	*H. anatolicum*
H. kumari				
H. hussaini	11.1% ± 0.017			
H. aegyptium	12.8% ± 0.019	12.7% ± 0.013		
H. anatolicum	14.7% ± 0.018	13.4% ± 0013	11.3% ± 0.014	

The minimum interspecies K2P divergence observed was between *H. kumari* and *H. hussaini*, i.e. 11.1%, while the maximum K2P divergence was observed between *H. kumari* and *H. anatolicum*, i.e. 14.7%. The second maximum and minimum interspecies K2P divergence was between *H. hussaini* and *H. anatolicum*, i.e. 13.4%, as well as *H. aegyptium* and *H. anatolicum*, i.e. 13.4% respectively. Table 3 shows divergence among rest of the species.

The 546 nucleotide sequences obtained for the four species indicated unique species-specific variable sites ($n = 125$), which differentiated the *Hyalomma* species from each other. The species-wise distribution analysis of these unique variable sites ($n = 125$) indicated that the four nucleotide sequences of *H. anatolicum* have maximum SNPs, $n = 35$, other species of *Hyalomma* examined, i.e. *H. kumari* has 33 unique species specific variable sites and the remaining two species, i.e. *H. aegyptium* and *H. hussaini* consisted of $n = 27$ and $n = 30$ unique variable sites respectively (Table 4).

The combined present study sequences and another closest sequence retrieved from GenBank of *Hyalomma* phylogenetic (NJ) relatedness examination of COI sequences segregated the *Hyalomma* into particular species groups by their congeners and conspecifics with strong bootstrap values (99% or 100% bootstrap upheld) (Figure 3). In tree geography, the four *Hyalomma* species nested in two significant clades (A and B) (Figure 2). Clade A was represented by *H. anatolicum* and *H. aegyptium* with strong bootstrap values, whereas clade B was represented by *H. kumari* and *H. hussaini* was strongly supported by bootstrap values. Within the tree, all Indian samples clustered together and were separated from samples of congeneric species from different geographical areas. The complete nucleotide sequences of calreticulin (1408 bp) and internally transcribed spacer region-2 (ITS-2), (1285 bp), encoding genes of *Hyalomma dromedarii* (tick species collected from one-humped camels of Bikaner, Rajasthan, India) were amplified by polymerase chain reaction. Gene analysis revealed that the calreticulin gene of *Hy. dromedarii* from India shared 96.59% identity at the nucleotide level with *Hy. excavatum* (*Hy. anatolicum excavatum*). With other genera of the family Ixodidae, *Hyalomma dromedarii* from India showed 72.54–88.54% nucleotide identity. However, nucleotide sequence analysis of *ITS-2* gene revealed that *Hy. dromedarii* from India shared 81.92% nucleotide identity with that of *Hy. dromedarii* isolate Gansu from China. *Hy. dromedarii* from India exhibited 66.12–82.11% and 41.36–42.74% identity with the species of genus *Hyalomma* and species of genus *Rhipicephalus* respectively at the nucleotide level. Thus calreticulin and ITS-2 encoding genes can be used in the phylogenetic analysis of ticks of Ixodidae[33]. The present study also highlights the use of *COI* gene to identify the genus *Hyalomma*.

Species diagnosis is a crucial step in controlling the transfer of pathogens from animals to humans through developing species-specific pest management strategies. The integrated option is the best approach to identifying *Hyalomma* tick species. The present study is an initiative to solve the taxonomic problems and ambiguity faced by the group. In this study, the species-specific nucleotide sequences obtained using COI marker of four *Hyalomma* tick species were

Table 4. Observed variable sites in the COI sequences among all four species of *Hyalomma*. Shaded box shows the species-specific nucleotide sites

Variable sites

Nucleotide position / species	2	3	8	11	12	14	27	31	32	33	34	44	47	53	71	72	77	80	86	95	98	101	122	128	134	137	146	290	293	296	299	155	156	161	165	170	173
H. kumari	T	T	T	A	T	A	T	G	G	C	A	A	A	G	T	G	C	T	T	T	T	G	T	A	G	A	T	C	C	C	A	A	C	T	T	C	T
H. aegyptium	C	A	A	A	C	A	T	C	C	C	A	T	A	A	T	G	T	A	C	T	T	A	C	A	A	A	T	T	T	A	A	T	G	C	T	T	A
H. anatolicum	A	A	A	T	T	A	T	C	A	A	G	A	T	T	C	C	T	C	G	T	T	A	T	A	A	T	T	T	T	T	A	A	C	T	T	C	C
H. hussaini	T	T	A	A	T	G	C	C	T	C	A	A	A	T	T	G	T	A	A	C	C	A	A	G	A	A	C	T	T	T	T	T	C	T	C	A	T

Nucleotide position / species	185	194	210	216	218	224	227	230	239	240	241	251	254	255	263	266	269	272	275	278	284	287	305	317	323	326	329	330	335	338	341	347	350	353	354	356	359
H. kumari	A	A	A	C	T	T	A	A	G	T	T	C	A	T	A	C	G	A	A	T	A	A	A	T	T	G	A	T	A	T	G	T	C	G	T	A	C
H. aegyptium	A	A	T	C	T	T	T	T	A	T	T	A	A	T	A	A	A	T	C	T	G	A	C	A	T	A	T	T	A	T	G	T	T	A	T	A	T
H. anatolicum	A	T	T	T	A	C	T	T	A	C	A	A	C	C	A	A	G	A	A	C	A	G	T	C	C	A	T	T	T	C	A	C	T	T	T	A	C
H. hussaini																																					
H. kumari	T	A	T	C	T	T	C	A	A	T	T	T	A	T	G	A	G	A	A	T	A	A	T	T	T	C	C	A	A	T	A	T	T	A	C	T	C

Nucleotide position / species	360	362	365	368	374	383	392	398	401	407	422	425	431	432	446	450	464	467	470	473	476	478	488	491	494	497	500	509	512	518	524	527	534	539
H. kumari	T	A	A	A	T	C	C	T	T	T	T	T	A	A	T	A	G	T	C	A	T	A	A	A	T	C	T	A	G	C	A	C	C	
H. aegyptium	T	A	G	C	A	T	T	C	T	T	A	A	T	T	T	C	A	T	T	T	C	A	A	G	T	A	A	T	T	T	A	T	A	
H. anatolicum	C	T	A	C	A	T	T	T	C	T	G	A	A	T	T	C	A	T	C	A	T	T	A	A	T	A	T	T	A	T	C	T	A	
H. hussaini	T	A	A	T	T	T	T	C	T	C	T	A	G	A	A	T	A	C	T	A	T	A	C	A	C	C	T	T	A	T	A	T	A	

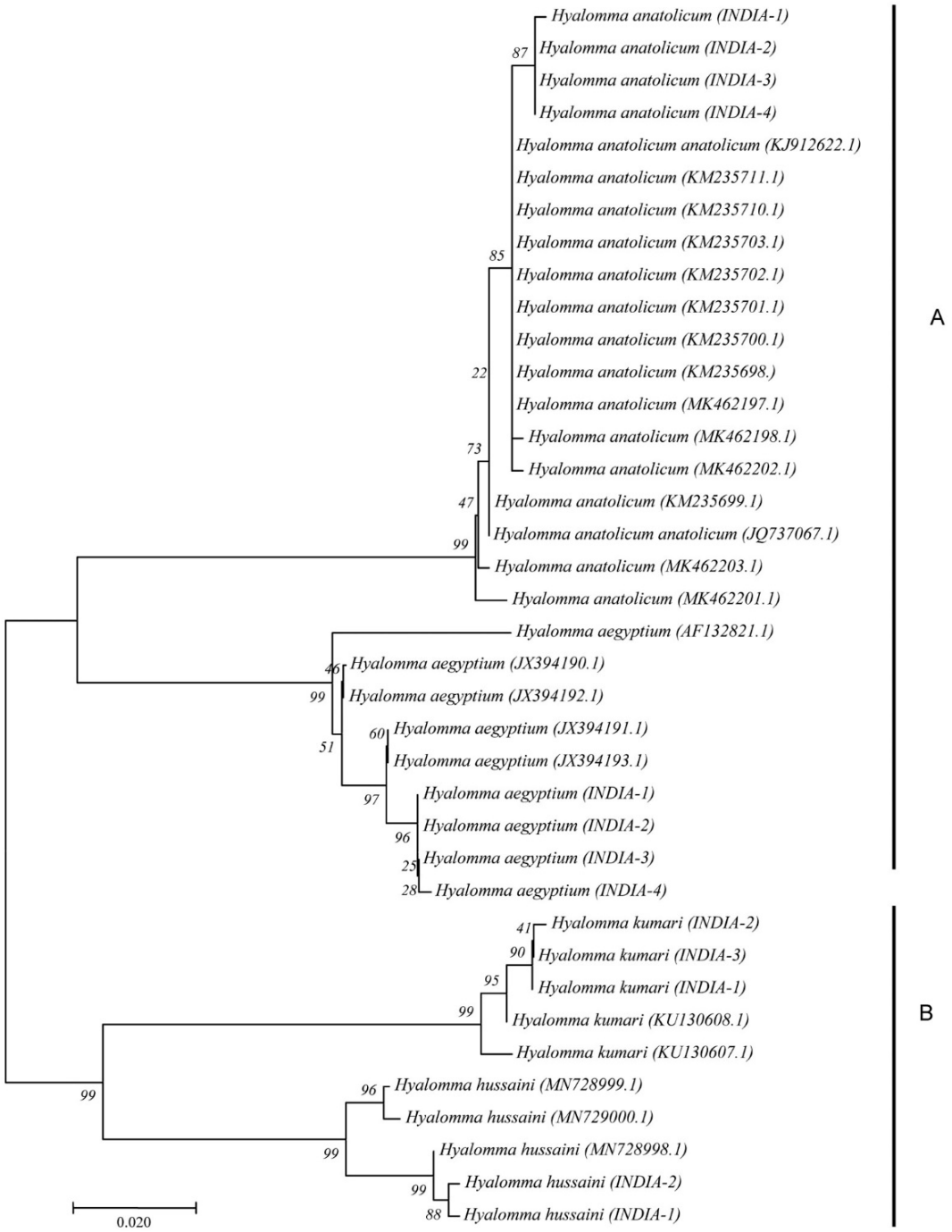

Figure 3. Evolutionary relationships among four species of *Hyalomma* based on COI sequence. The evolutionary history was inferred using the Neighbor-Joining method. With 10000 bootstrap test.

found to be effective in species identification. This is the first step towards an integrated approach in the identification of tick species using old preserved specimens. It may help in developing DNA barcodes of other species of *Hyalomma* and other tick genera using preserved specimens. Further studies to fill the gap in the characterization of other tick species may generate an authentic genetic reference library.

Conflict of interest: The authors declare that they have no conflict of interest.

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