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Molecular Characterisation using 16S rRNA and COI Gene Sequences in Hard Ticks of Gwalior, India

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ABSTRACT

Tick infestation in humans and animals represents a global threat for different tick-borne diseases. In the present study, the ticks from the Gwalior region of India have been mapped to create a database of tick diversity. We explored 773 ticks collected from domestic animals and vegetation in Gwalior. Animals were screened visually, and ticks were collected manually, whereas the flag-drag method was used to collect ticks from the vegetation. The 16S rRNA and cytochrome oxidase I (COI) genes of tick samples were amplified and purified for sequencing and respective phylogenetic trees were constructed. The ticks were morphologically identified using taxonomical keys, revealing the presence of five genera in the region: *Hyalomma*, *Haemaphysalis, Rhipicephalus, Boophilus,* and *Nosomma*. *Hyalomma* spp. (*Hy. annatolicum and Hy. marginatum)* were the most abundant accounting for 69.598% of the total sample, followed by *Rhipicephalus sanguineus* (17.335%), *Rhipicephalus microplus (*7.115%), *Haemaphysalis* sp. (5.692%), and *Nosomma monstrotum* (0.258%). The tick sequences were submitted to the GenBank database. Phylogenetic analysis confirmed the morphological identification at the species level. The combination of molecular and morphological analyses of the ticks supported the result obtained with each method, thus providing more reliable estimates for continued surveillance studies.

Keywords: Ticks; Diversity; 16S rDNA; COI; Surveillance; Phylogenetics

1. INTRODUCTION

Ticks are the primary vectors of several infectious diseases affecting humans, including rickettsiosis, ehrlichiosis, anaplasmosis, and borreliosis. Ticks and tick-borne diseases influence approximately 80% of the global cattle population[1]. Several rickettsial outbreaks have been reported in numerous parts of India[2,3,4]. Rickettsial diseases, including Indian tick typhus, Crimean-Congo haemorrhagic fever, and Kyasanur forest disease, have been continuously reported in India[5,6,7,8,9]. As tick-borne rickettsiosis is a re-emerging disease entity, its prevalence and potential outbreak need to be considered and carefully monitored by Indian medical organisations in different geographical areas[10]. Therefore, it is crucial to study the prevalence of ticks at regular intervals.

Ticks themselves can be used as a tool for the early diagnosis of disease, which involves the initial identification of the ticks to the species level. Indeed, precise identification of a tick species is essential to develop a potential tick control program[11]. Traditionally, taxonomical studies of organisms have relied on the use of morphological keys. However, many morphological similarities are present among different members of a species, which has caused confusion among researchers, making morphological-based identification a cumbersome task to accomplish[12]. In contrast, several molecular methods using different markers have recently been developed to identify ticks, which can facilitate identification in cases in which the tick specimens are damaged or engorged. Cytochrome oxidase I (COI) and 16S rRNA were found to be the most suitable genetic markers for the species identification of ticks, and therefore a DNA barcoding system based on these genes was developed for this purpose[13]. In the present study, we used 16S rRNA and COI sequences for identification of tick species. Moreover, we used an integrated operational taxonomic unit (IOTU) approach, which combines different data sources such as morphological, ecological, and molecular data for taxonomic studies[14]. In particular, combination of morphological and molecular analysis provides clearer and more reliable interpretation of the results of species identification than a single approach, and therefore this combined approach was adopted for tick species identification in the present study.

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2. MATERIALS AND METHODS

2.1 Study area

Ticks were collected in different areas of Gwalior, including Morar, Raura, and Tekanpur (26.22 N 78.18 E).

2.2 Tick Sampling

Ticks were collected during 2015–2016 directly from hosts, including cows, buffalo, goats, sheep, and dogs. The flag-drag method was used to collect the ticks from vegetation.

Since this method only yielded a very small numbers of ticks, we focus mainly on the results obtained from the domestic animals herein. Ticks were collected in different seasons, including winter (November to January), spring (February to April), summer (May to July), and monsoon season (August to October). The different hosts, including buffalo, goats, sheep, cow, and dogs, were screened to check for the presence of ticks only after obtaining verbal consent from the owners. The presence of ticks on the animal's coat was checked manually and the ticks were carefully collected using blunt forceps while ensuring not to damage their mouthparts. The collected ticks were stored with proper labelling of the sites and associated host of collection. The majority of the ticks were collected in glass vials containing 70 % ethanol, whereas a few ticks were stored in 90 % ethanol and preserved at –20°C for molecular analysis. The tick specimens were cleaned with double distilled water, dried, and preserved in 70 % ethanol until use. For identification, unfed ticks from the vials were air-dried and viewed under a stereo zoom microscope (Leica EZ4D and Leica M205A) coupled to a DFC500 camera. The ticks were identified by examining their fine morphological characteristics using taxonomical keys[15,16].

2.3 Amplification of 16S rRNA and COI Genes

Fifteen adult ticks preserved in 90% ethanol solution at –20 °C were selected for molecular identification. Before DNA extraction, each tick was washed separately in double-distilled water on a sterile plate. Genomic DNA was then extracted using the DNeasy blood and tissue kit (Qiagen) from a single adult tick according to the manufacturer's instructions. Qualitative analysis of the extracted DNA was performed by running the sample on 1% agarose electrophoresis gels.

Amplification of the 16S rRNA gene[17] and COI gene[18] was performed with polymerase chain reaction (PCR) to yield a product of 460 bp and 750 bp, respectively. All PCRs were performed in an iCycler thermal cycler (Biorad, USA) and the PCR products were obtained using the Qiagen PCR kit. PCR amplification was performed in a reaction mixture with a final volume of 100 µl per tube, containing 1X assay buffer, 2.5 mM $MgCl_2$, dNTPs (10 mM each), forward and reverse primers, 1 U Taq polymerase, and sterile distilled water to make up the final volume. The PCR master mix and the extracted DNA sample were mixed in individual tubes, and PCR was performed with a negative control (including the PCR mix but without the DNA template).

2.4 Genetic Analysis of the Amplified Genes

The amplified 16S rRNA and COI genes were sequenced by Sanger sequencing performed at Genotypic Technology Pvt. Ltd. (Bangalore). Low-quality sequences were removed from both ends and the obtained sequences were submitted to National Center for Biotechnology Information (NCBI) BankIt. The obtained tick sequences were then compared with the available sequences in the GenBank database using the NCBI online tool BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

For phylogenetic analysis, 20 sequences for each representative tick species collected from India or other countries were used for comparison based on sequence similarity with the tick species obtained in the current study. Similarity between sequences was checked using nucleotide BLAST and the sequences were downloaded from GenBank. Multiple sequence alignment was performed using the MUSCLE algorithm in MEGA 10 software. Molecular phylogenetic analysis was performed using the maximum-likelihood method based on the Tamura 3-parameter model.

3. RESULTS AND DISCUSSION

3.1 Identification of Tick Species using Taxonomical Keys

A total of 773 ticks were collected from different hosts and vegetation across Gwalior. Among the collected ticks, 538 were identified as *Hyalomma* sp., 134 as *Rhipicephalus* sp., 55 as *Boophilus* sp., 2 as *Nosomma* sp., and 44 as *Haemaphysalis* sp. according to morphological characters using standard taxonomical keys. Representative stereoscopic images of the collected ticks are shown in Fig. 1. The highest level of tick infestation was observed in buffalo, followed by goat, dog, cow, and sheep.

Collection plays an integral role for the surveillance of vectors to determine the dominant species according to different

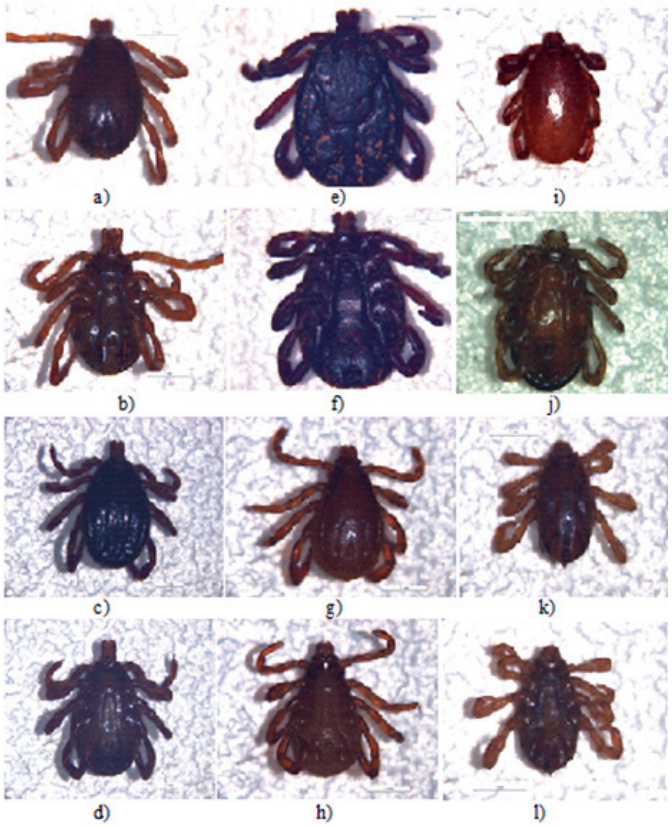


Figure 1. Stereomicroscopic image of collected ticks (a, b) *Hyalomma annatolicum* dorsal and ventral views, (c, d) *Hyalomma marginatum* dorsal and ventral views, (e, f) *Nosomma monstrotum* dorsal and ventral views, (g, h) *Rhipicephalus sanguineus* dorsal and ventral views, (i, j) *Haemaphysalis* sp. dorsal and ventral views, and (k, l) *Rhipicephalus microplus* dorsal and ventral views.

hosts and seasons, thus yielding detailed knowledge on the occurrence of different vector-borne diseases. *Hyalomma anatolicum, Hyalomma marginatum isacci, Rhipicephalus microplus,* and *Rhipicephalus haemaphysaloides* ticks are widely distributed in almost all states of India[19,20].

However, in a study conducted in Lucknow, Uttar Pradesh, *Rh. microplus* was reported to be predominant over other tick species based on screening of cattle[21]. In our study, *Hyalomma* sp. was the most prevalent tick species observed overall. *Rh. microplus* mostly favours humidity and hot climatic conditions, whereas *Hy. anatolicum* prefers a dry environmental condition[22]. Although *Nosomma monstrotum* has not yet been reported from the north eastern states, central zones, Rajasthan, and southern states except for Arunachal Pradesh and Kerala[19], we here provide the first record of detection of an *N. monstrotum* tick in Madhya Pradesh, which was collected from a buffalo host.

Different macro- and micro-climatic conditions affect the distribution and prevalence of ticks in different areas[22]. The distribution of ticks collected at different seasons is shown in Fig. 2.

The highest prevalence of ticks was found in the rainy (monsoon) season compared to the other three seasons. A similar observation of a higher tick prevalence during the rainy season was reported from another study conducted in India[23]. *Hy. annatolicum* was the most prevalent species identified overall, followed by *Rh. sanguineus, Rh. microplus, Haemaphysalis* sp., *Hy. marginatum,* and *N. monstrotum* during the summer and rainy seasons, whereas *Rh. sanguineus* was the most prevalent, followed by *Rh. microplus, Hy. annatolicum, Haemaphysalis* sp., *Hy. marginatum,* and *N. monstrotum* during the winter and spring seasons.

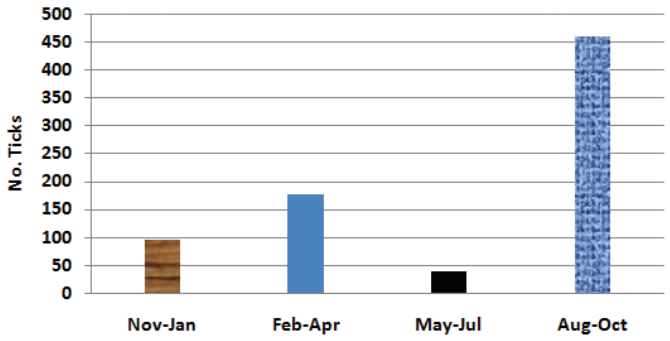
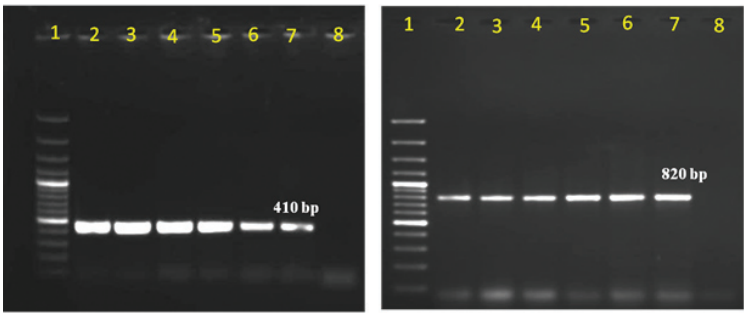


Figure 2. Seasonal distribution of ticks.

3.2 Molecular Identification of Ticks

After DNA extraction, PCR was performed for amplification of the 16S rRNA and COI genes, resulting in expected PCR products of 410 bp and 820 bp, respectively (Fig 3(a), 3(b)). The five sequences obtained for the COI and 16S rRNA genes each were submitted to NCBI under accession number MH765330, MH765331, MH765332, MH765333, and MH765334, and MH765335, MH765336, MH765337, MH765338, and MH765339, respectively. This result of molecular identification corresponded with the morphological identification of ticks using taxonomical keys.



(a) (b)

Figure 3. Agarose gel electrophoresis of amplified PCR products from collected tick species.

(Lane 1: 100-bp DNA Ladder; Lanes 2-3: *Rhipicephalus sanguineus*: Lanes 4-5: *Rhipicephalus microplus*; Lane 6: *Hyalomma annatolicum*; Lane 7: *Hyalomma marginatum*; Lane 8: negative control)

3.3 Phylogenetic Analysis

All the sequences obtained in the present study were used along with the 20 available sequences of the closest relatives in NCBI GenBank for constructing the phylogenetic trees. For intraspecies evolutionary analysis, a phylogenetic tree was constructed for each species that was identified separately for each gene (see supplementary Figs. S1-S4).

3.3.1 16S rRNA Gene

The phylogenetic analysis for *Rh. microplus* was based on 22 available 16S rRNA nucleotide sequences (Fig. 4). There were a total of 426 positions in the final dataset. *Rh. microplus* from the present study (accession numbers MH765332 and MH7653354) showed the closest relationships to each other and were also more closely related to other *Rh. microplus* collected from India as expected, followed by the same tick species collected from China. As shown in Fig. 5, the *Rh. sanguineus* phylogenetic tree was constructed from 22 sequences, in which 282 positions were present in the final dataset. *Rh. sanguineus* from the present study (accession number MH765331) was in the same clade with the tick species identified from Taiwan and Kinmen Island, followed by the tick identified from the Assam state of India as the next closest relative. However, the other *Rh. sanguineus* tick identified in this study (accession number MH765330) formed a clade with the species identified from Assam. The phylogenetic tree for *Hy. anatolicum* (Fig. 6) involved 21 nucleotide sequences with a total of 403 positions in the final dataset. *Hy. anatolicum* from this study (accession number MH765333) formed a distinct clade on its own.

3.3.2 COI

The phylogenetic tree for *Rh. microplus* based on the COI sequence (Fig. S1) included 21 nucleotide sequences with 807 positions in the final dataset. *Rh. microplus* from the present study (accession number MH765338) was most closely related to a tick identified from Pakistan. The *Hy. marginatum* tree (Fig. S2) included 21 nucleotide sequences. *Hy. marginatum* formed a separate clade from the other sequences used in constructing the tree. There were a total of 777 positions in the final dataset. *Rh. sanguineus* from the present study (accession number MH765337) formed a single clade along with the ticks

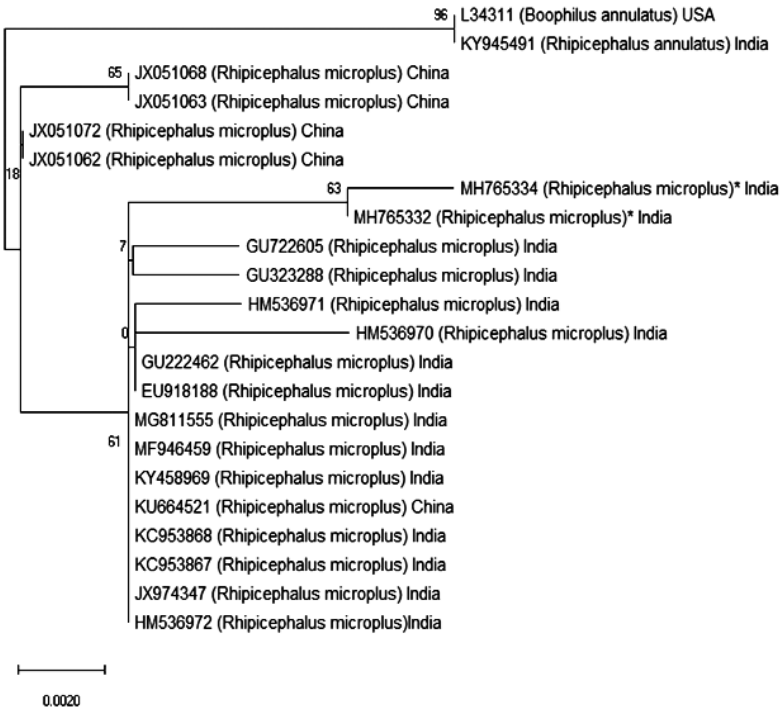


Figure 4. Maximum-likelihood tree inferred from 16S rRNA gene partial sequences of *Rh. microplus* tick specimens collected in the present study (*) and sequences obtained from GenBank.

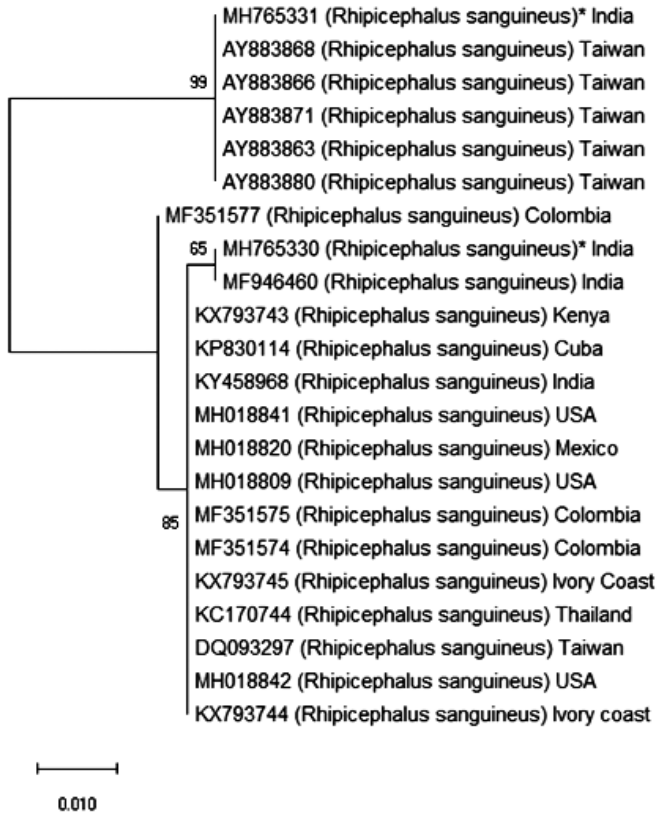


Figure 5. Maximum-likelihood tree inferred from 16S rRNA gene partial sequences of *Rh. sanguineus* tick specimens collected in the present study (*) and sequences obtained from GenBank.

from Brazil (Fig. S3). The tree was constructed using 21 nucleotide sequences with a total of 723 positions in the final dataset. The phylogenetic tree of *Hy. annatolicum* (Fig. S4) was constructed using 22 nucleotides with a total of 518 positions in the final dataset, which formed an different clade with *Hy. annatolicum, Hy. excavatum,* and *Hy. marginatum* ticks.

3.4 Combining Morphological and Molecular-based Identification

Most IOTU studies now adopt a combinatorial approach, taking both the classical taxonomy and molecular characteristics for identification[14]. In the present study, to achieve precise identification of tick species, we performed both morphological and molecular analyses. Molecular identification of ticks was conducted using the mitochondrial 16S rRNA and COI genes, which identified *Rh. microplus*, *Hy. anatolicum*, *Rh. sanguineus,* and *Hy. marginatum*. These might be the first sequences submitted for *Hy. marginatum* in India. Both COI and 16S rRNA have their pros and cons as molecular markers for species identification; the ease of alignment is better for COI, whereas the quality of sequencing is much better with 16S rRNA[13]. For species-level identification of ticks, the suitability of nuclear markers remains controversial[11].

The tick *Rh. sanguineus* has been reported to be the most predominant ectoparasite of dogs[24]. In the phylogenetic tree based on the 16S rRNA gene in the present study, *Rh. sanguineus* ticks formed two lineages; one formed an independent lineage along with the ticks of Taiwan, whereas the other formed a tropical lineage with ticks of India, the USA, Taiwan, Ivory Coast, and Mexico. Similar results were obtained by Dantas-Torres[25], who reported two different lineages, a temperate and tropical lineage. However, the Indian haplotype was also present in the northern lineage (i.e., the tropical lineage), along with one clade comprising the ticks of India and Pakistan, which was predicted to form a separate species. Hence, the second clade formed with Taiwan ticks may be the same independent clade that was previously described[25], or the clade consisting of ticks from the temperate region, since similar coexistence in close vicinity has been reported for both lineages in Chile[26]. In contrast to a previous study[27] indicating the absence of these two lineages from the same location, the presence of both the temperate and tropical lineages of ticks was also detected in San Diego, USA[28]. Kaur and Chillar[29] also reported that *Rh. sanguineus* ticks of India likely represented a cryptic species. With comparison of COI gene sequences, *Rh. sanguineus* formed a sister clade with the ticks of Brazil. Morphological similarities within the *Rh. sanguineus* group highlight the difficulty in tick identification in spite of experience in taxonomical studies; therefore, the use of molecular tools is essential to achieve the precise identification of *Rhipicephalus* sp. As *Rh. sanguineus* cannot be considered a single species[30], more studies are needed to disclose the diversity of *Rh. sanguineus* ticks.

Hy. marginatum ticks included in the present study formed a separate group with the ticks of France and Spain,

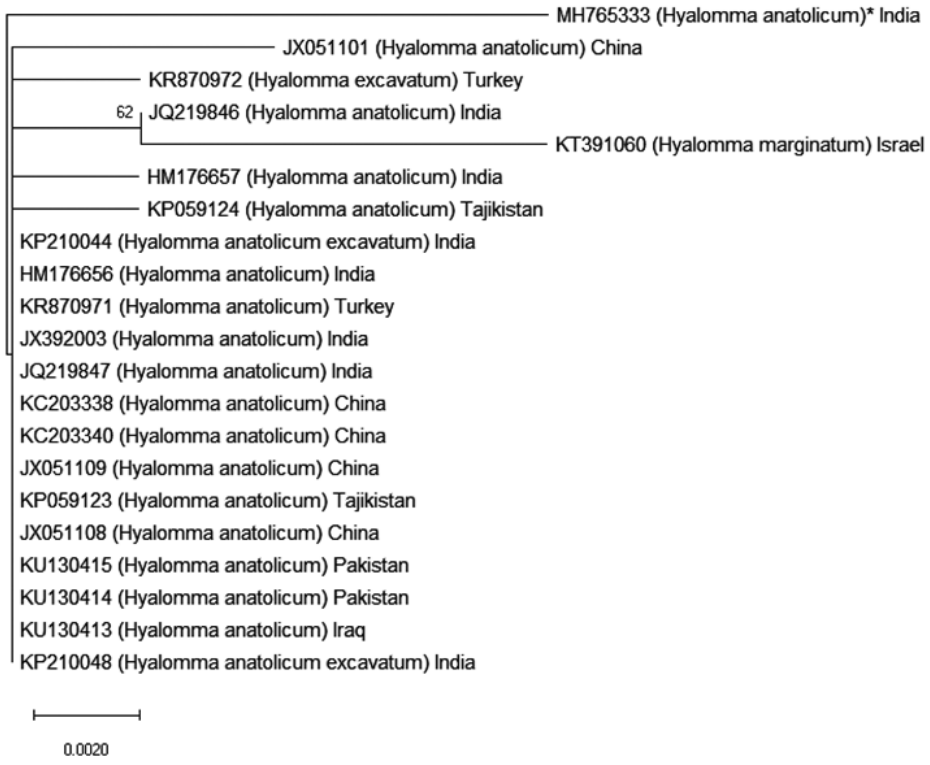


Figure 6. Maximum-likelihood tree inferred from 16S rRNA gene partial sequences of *Hy. anatolicum* tick specimens collected in the present study (*) and sequences obtained from GenBank.

resulting in the formation of two different clades. Since no other sequences for COI of *Hy. marginatum* collected from India have been reported, the phylogenetic analysis inferred in this study might be inconclusive for deducing evolutionary relationships with other *Hy. marginatum* ticks of India. In the 16S rRNA gene phylogenetic tree, *Hy. annatolicum* formed a separate single clade from other Indian ticks. A similar result was found in the case of the COI gene phylogenetic tree, in which both ticks from India formed a separate group from that of other ticks of China, France, and Israel. Morphological identification of *Rh. microplus* is a concern because of the small body size and close morphological similarity with other species in the genus[31]. The *Rh. microplus* complex consists of closely related tick species such as *Rh. australis*, *Rh. annuatus*, *Rh. microplus* clade A *sensu burg*, and *Rh. microplus* clade B *sensu burg*[31]. It is worthwhile to mention that COI and 16S rRNA have proven to be been useful in revealing phylogenetic relatedness among *Rh. microplus*[32,33]. In the present study, using the 16S rRNA gene, *Rh. microplus* ticks clustered in clade A[34] along with other Indian ticks, whereas using the COI gene, these ticks were classified as part of clade C based on their closest relatives for which there are sequences available in GenBank[32]. The virtual analysis of this study resulted in the formation of three distinct clades, similar to the results of Low[32], *et al. Rh. microplus* ticks from India and Nepal have been considered to represent a different species, which shows a much closer relationship to African and American ticks than to others[35]. Since no other *Rh. microplus* COI sequence from India of around 820 bp is available in GenBank, our sequence likely represents the first such sequence submitted from India; therefore, the relationship of this tick within *Rh. microplus* of India should be further determined owing to the paucity of sequences in GenBank.

We were unable to morphologically identify *Haemaphysalis* ticks to the species level owing to their very small size. In addition, there are very few tick sequences from India available in GenBank, which might have helped to achieve more transparency and clarity for species identification.

Given the huge diversity of ticks responsible for transmitting different tick-borne diseases, the potential distribution of each disease can be sketched out based on the distribution of the vector. Therefore, the tick ultimately serves as the principal indicator to estimate the distribution of tick-borne diseases. From this perspective, we combined both molecular and morphological analyses to obtain a better understanding of the distribution and diversity of Indian ticks in the Gwalior region of Madhya Pradesh. Further studies on tick prevalence are sorely needed in Madhya Pradesh to expand the weak knowledge of tick distribution and related pathogens in India.

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