



Molecular characterization of *Amblyomma geoemydae* using CO1 mitochondrial gene to validate phenotypic taxonomical evaluation

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Abstract

Animal ectoparasites are linked to the spread of serious medical and veterinary important pathogens. Our research intends to close the knowledge gap concerning the numerous ectoparasites that inhabit animals in Wayanad. Ectoparasites in animals brought to the veterinary dispensaries in Wayanad were retrieved and identified morphologically and molecularly. Using a high-quality stereomicroscope, the taxonomic features of the four following species were examined and identified: *Haemaphysalis bispinosa*, *Rhipicephalus annulatus*, *R. microplus*, and *Amblyomma geoemydae*. The important disease vector *A. geoemydae* was reported for the first time in Kerala. The important phenotypic characters of the highlighted species *A. geoemydae* are the edge of the basis capituli is circular without cornua, and the hypostomal dental formula is 2/2. The taxonomically identified four species were subjected to CO1 gene sequence analysis. The evolutionary relationship was inspected through the neighbour-joining method, and the phylogenetic tree was built through the Maximum Likelihood method. The present study has also estimated the diversity index of *R. microplus*, *R. annulatus*, *H. bispinosa*, and *A. geoemydae*. Among them, *R. microplus* 0.36638 have reported with the maximum diversity index score. The significance of the study is the presence of Lyme disease vector *A. geoemydae*, in the Wayanad District of Kerala, and it is the first report of the species from where an outbreak of Lyme disease occurred in 2013.

Keywords Lyme disease vector · *Amblyomma geoemydae* · mtCO1 gene · Phylogenetic analysis · Kerala

Introduction

In an account of their propensity to transmit pathogens, including bacteria, viruses, and protozoan parasites, ticks rank second among arthropods in tropical regions behind mosquitoes (Ariyaratne et al. 2016). Only a few of the 106 tick species (Bandaranayaka et al. 2022) discovered in India

serve as carriers of disease pathogens. In Kerala, at least 24 different species of ticks have been reported belonging to the genera *Amblyomma*, *Dermacentor*, *Haemaphysalis*, and *Rhipicephalus*. The *Amblyomma* genus is the third largest group within the Ixodidae family, with 138 valid and one fossil species globally (Guglielmone et al. 2010). From this genus, 14 species were recorded from India. Species of this genus are competent vectors for various bacterial, spirochete, and viruses causing severe diseases such as Lyme disease, rocky-mountain spotted fever, and novel relapsing fever in wild and domestic animals and also humans (Aenishaenslin et al. 2022; Baneth et al. 2022; Martin 2022). The increasing rate of disease transmission is a major public health problem due to the abundance and widespread of tick vectors in human settlements. The microbial transmission from tick species to hosts is a major complication in the developing world. The vectorial capacity of Ixodidae, microbial inhabitation and tick-borne diseases are listed in Table 1.

Hard ticks have a two- to three-year life cycle. They go through the egg, six-legged larva, eight-legged nymph, and adult stages of development (Hoffman et al. 2023). The ticks

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Table 1 An inventory of diseases transmitted by ticks and the agents responsible for them, all from India

Species	Tick-borne diseases	Causative agents	References
<i>Amblyomma integrum</i>	Kyasanur Forest Disease	KFDV	Pattnaik (2006)
<i>Haemaphysalis spinigera</i>	Kyasanur forest disease	KFDV	Naren Babu et al. (2019)
<i>Amblyomma hebraeum</i>	Crimean congo haemorrhagic fever	<i>Ehrlichia ruminantium</i> , <i>Theileria mutans</i> and <i>T. velifera</i>	Whitehouse (2004)
<i>Amblyomma gervaisi</i>	Q—fever, Lyme diseases	<i>Coxiella burnetii</i> , <i>Borrelia turcica</i>	Inoue et al. (2011)
<i>Hyalomma marginatum</i> , <i>Rhipicephalus bursa</i>	Congo hemorrhagic fever	Congo hemorrhagic fever virus	Acha and Szyfres (2003)
<i>Amblyomma cajennense</i>	Rocky mountain spotted fever	<i>Rickettsia rickettsii</i> ,	Nava et al. (2014)
<i>Amblyomma ovale</i>	Lyme diseases	<i>Rickettsia bellii</i>	Khan et al. (2022)
<i>Amblyomma parvum</i>	Ehrlichioses	<i>Ehrlichia canis</i> , <i>Ehrlichia chaffeensis</i> , <i>Ehrlichia ewingii</i> , and <i>Ehrlichia minascensis</i>	Sebastian et al. (2017)
<i>Amblyomma auricularium</i>	Rickettsiosis	<i>Rickettsia amblyommi</i>	Saraiva et al. (2013)
<i>Amblyomma americanum</i>	Human monocytic ehrlichiosis	<i>Ehrlichia chaffeensis</i>	Ahmed et al. (2002)
<i>Amblyomma testudinarium</i>	Canine granulocytic ehrlichiosis	<i>Ehrlichia ewingii</i>	Rikihisu et al. (1992)
<i>Amblyomma maculatum</i>	American tick bite fever	<i>Rickettsia parkeri</i>	Jensenius et al. (2003)

require blood meals at every stage of development after the eggs hatch to remain alive (Makwabela et al. 2023). Ixodidae can feed on mammals, birds, reptiles, and amphibians (Rodriguez et al. 2023). Ticks locate their hosts by smelling animal breath and body scents or detecting body heat, moisture, and vibrations (Ebert et al. 2023). Some species can even identify a shadow (Ortiz-Baez et al. 2023). Ticks also decide where to wait by locating well-travelled routes (Koual et al. 2023). They then wait for a host while resting on grass and shrub tips. Several tick species remain in a “questing” position even though ticks cannot fly or jump (Szcotko et al. 2023). Ticks use their third and fourth pairs of legs to grip onto grass and leaves while they are digging (McCoy et al. 2023). They grip the first set of extended legs as they climb the host. A tick is anchored on the host as soon as the host brushes the area where it is hiding (Stanilov et al. 2023). While some ticks attach immediately, others scurry around in search of spots with thinner skin, like the ear or other spots (Brandenburg et al. 2023). During the blood meal uptake, the microbial content is transferred from one body to another, and there are two types of microbial transmission in ticks (Grandi et al. 2023) before being transmitted to vertebrates, diseases acquired by larval ticks must first be transstadially transmitted to the nymph stage (Ravindran et al. 2023). The vertical transmission of parasites from an infected vector to its descendants is known as transgenerational transmission (Ebert et al. 2023; Parola and Raoult 2001; Ravindran et al. 2023; Reis et al. 2011). The transstadially and transovarially transmitting ticks are analyzed through mitochondrial genes.

Chao et al., (2017) compared the 16S mitochondrial DNA sequences recovered from the province of Taiwan (Chao et al. 2017) to determine the genetic identity of the Lyme disease vector *A. geoemydae* (Cutler et al. 2017). Moreover,

a comparable study was conducted in Malaysia (Che Lah et al. 2022). Earlier scientific studies had a limited knack for recognizing *A. geoemydae* species based on nucleotide sequence (Kaenkan et al. 2020). In earlier research, house-keeping gene analysis revealed the presence of *Rickettsia*, *Ehrlichia*, and *Borrelia* spp. from *A. geoemydae*, conventional morphology was the sole method used to confirm the tick species (Che Lah et al. 2022). More research on the various tick vectors and their molecular characterization is required to ascertain the significance of ticks and the infections they carry for humans and animals.

Materials and methods

Study area

The present study was carried out in the Wayanad wildlife sanctuary of Western Ghats Kerala, India, from March to October 2021. During the dry season, juvenile ticks from different genera are distributed on the soil. Tick samples were collected in three seasons from the deciduous forest of the Wayanad part of the Western Ghats. Geographic parameters ranged from a latitude of 11.7154812, a longitude of 76.260733, and an altitude of 813.69 m. Wayanad is one of the major tourist spots in the North-Eastern part of Kerala, having lush-green forests with high species diversity. An area of 2131 covers part of the Western Ghats in Wayanad Wildlife sanctuary (WWLS). This region experienced moderate temperatures and the annual humidity range is approximately 60%, as it receives most of the rainfall and has very low evaporation in the cloudy areas. The dense vegetational zone and diversity of wild creatures provide a favorable environment for tick viability.

Collection and identification

The questing ticks were collected from the forest area of Wayanad, Kerala, and they were mostly found on the tip of leaves, where they patiently waited for their hosts. Early morning and afternoons are the foremost time for tick collection because ticks activate their hematophagous behavior during that time. For the large-scale collection of tick fauna, 1 m² flannel cloth was attached to a 1.2 m wooden stick. Then 3 m rope was tied on both ends and led to dragging; a total of 352 tick specimens were collected and the collected samples were placed in a separate vial containing 70% ethanol. At the same zoogeographic references, data regarding the site selection and sample collection was noted in the vial. The tick specimens were observed under a high-resolution ZEISS microscope for morphological identification. The external morphology of the tick body consisted of gnathosoma and idiosoma with prominent regions, including a hypostome with several rows and columns, associated chelicera, and a pair of palps. Idiosoma is composed of four pairs of legs, and each coxa has many spurs that are the most prominent phenotypic character for conventional morphological identification. The morphological identification was done based on the standard taxonomic keys of Chao et al. (2022a), DA Apanaskevich (Apanaskevich et al. 2016). However, the mitochondrial cytochrome oxidase 1 gene was amplified, sequenced, and analyzed for accurate confirmation of species.

Mitochondrial cytochrome oxidase c subunit 1 gene extraction from tick specimens

The nucleic acid samples were prepared through DNA isolation using a kit before the segregation, and the selected tissue was placed in a 1.5 ml micro-centrifuge tube, 180 µl of T1 buffer and 25 µl of proteinase K were supplemented to the tube and incubated at 56 °C in a water bath until the tissue was completely lysed. After this procedure, the DNA was extracted using a NucleoSpin® Tissue kit (Macherey–Nagel).

DNA amplification by polymerase chain reaction (PCR)

From our investigation, the collected samples allowed for DNA extraction, and then the extracted product was amplified through PCR thermal cycle using universal primer sequences deposited in the NCBI gene-bank database. According to the literature survey, CO1 primer was evident for the tick species confirmation, so this investigation preferred COX1 forward 5'-GGTCAACAAATCATAAAGATA TTGG-3' and reverse 5'-TAAACTTCAGGGTGACCAAAA AATCA-3' primers for the DNA proliferation of extracted

nucleotide sequences. Initial denaturation at 98 °C for the 30 s is followed by denaturation and the annealing phase, which happen at 98 °C and 45 °C, in the PCR thermal cycle. The amplified product was electrophoresed in a 1.2% agarose gel prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide, 1 µl of 6X loading dye with 4 µl of PCR product. The gel was visualized using a UV trans illuminator (Genei) and the image was captured under UV light using a Gel documentation system (Anoopkumar et al. 2019). ABI 3500 DNA sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1.

Alignment of amplified sequences and phylogenetic analysis

An advanced version of MEGA (MEGA Version 11) software (Anoopkumar et al. 2019) was employed for the alignment of the mitochondrial oxidase c subunit 1 gene sequence. The evolutionary history of collected tick specimens was inferred by using the Neighbor-joining and Maximum Likelihood method by the Tamura-Nei model (Tamura and Nei 1993). This analysis involved 25 nucleotide sequences and codon positions, including 1st + 2nd + 3rd + Noncoding (Tamura et al. 2021).

Statistical analysis

The Statistical Package for Social Sciences Version 23 (IBM SPSS Statics 23, USA) software was used to analyze and describe the raw data after which it was transferred into the Microsoft Excel 2016 computer application.

Result

Morphological identification

A total of 352 ticks were collected from the study sites and were morphologically and molecularly identified under three genera, 118 *Rhipicephalus microplus*, 67 *R. annulatus*, 7 *A. geoemydae*, and 160 *Haemaphysalis bispinosa*. The sample size of the collected ticks was demonstrated in Fig. 1.

Further, the images of collected specimens were captured via a high-resolution ZEISS microscope (Discovery. V20). The morphological evaluation was based on the descriptions from the literature (Apanaskevich et al. 2016) (Fig. 2, Table 2). The morphologically identified species were allowed for further analysis by using the mitochondrial cytochrome oxidase 1 subunit gene.

The overall tick specimens were collected from the study sites from three season's pre-monsoon, monsoon, and

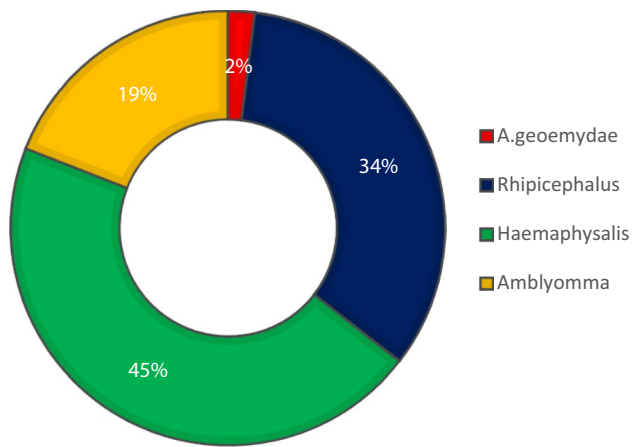


Fig. 1 Sample size of the collected tick species *A. geoemydae* and *Rhipicephalus*, *Haemaphysalis* and *Amblyomma* species from study site Wayanad

post-monsoon and are illustrated in Fig. 3. During each season, the number of tick specimens varied due to the different factors. The dispersal and abundance of Ixodid tick species depend on the host's availability and environmental parameters. Temperature and humidity play a significant role in the survival of hard tick species. So the atmospheric temperature variation drastically affected the tick population; the present study revealed a seasonal abundance of *A. geoemydae* from Wayanad Wild Life Sanctuary. Pre-monsoon is adequate for the survival, and population growth of *A. geoemydae* due to the increased rate of environmental temperature and availability of hosts, 57% of *A. geoemydae* were collected during this season. The life cycle of *A. geoemydae* and other Ixodid species have also been threatened by certain kinds of climatic factors associated with the rainy seasons; the

temperature-dependent characteristics feature may primarily be recognized as the major factor in this regard. So the number of tick specimens from the monsoon season was low, about 14% of *A. geoemydae* was reported. Moreover, environmental parameters in the post-monsoon were moderate for the viability of *A. geoemydae*. Compared to pre-monsoon, in post-monsoon 29% of tick species were collected from this investigation; we observed that environmental parameters significantly influence the abundance of tick species.

Molecular evaluation of mt COX1 gene sequences

The unique approach to verifying ticks genetic identification is through the analysis of the mitochondrial CO1 gene. In the current study, four tick species were identified under the three genera including *A. geoemydae*, *H. bispinosa*, *R. annulatus*, and *R. microplus*. In Kerala, there have been no studies on the phylogenetic analysis of *A. geoemydae* or mitochondrial gene sequencing. This is the first molecular confirmation and evolutionary implications of the Wayanad region's *A. geoemydae* nymph. The Lyme disease vector *A. geoemydae* was the main focus of this work. For the evolutionary relationship, the amplified PCR sequences of species were searched through BLAST, and 98.9% sequence similarity with *A. geoemydae* (WD202130). The percentage similarity and dissimilarity of *Amblyomma* species are illustrated in Fig. 4. The DNA sequence of *A. geoemydae* provides evidence for the genetic relationship of some species. Among these *A. testudinarium*, *A. marmoreum*, and *A. pattoni* share more sequence similarities while *A. dissimili*, *A. calcaratum*, and *A. coelebs* show less sequence similarity with *A. geoemydae*.

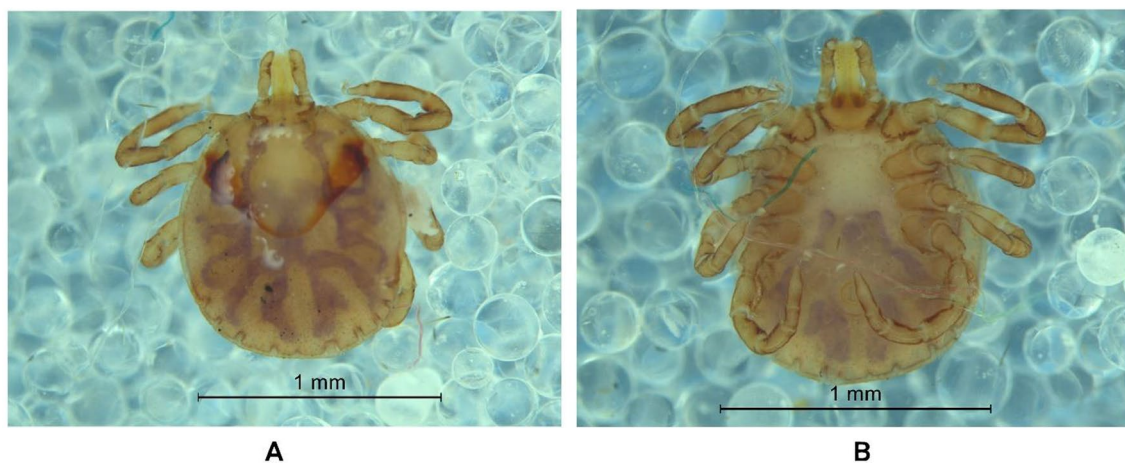


Fig. 2 Nymphal stage of *A. geoemydae*, **A** Dorsal view: angular scutum, edge of the capituli without cornua. **B** Ventral view: distinct and indistinct spurs on coxa 1–4

Table 2 Overview of the phenotypic traits of isolated species from the research location Wayanad

Phenotypic characters	<i>A. geoemydae</i>	<i>H. bispinosa</i>	<i>R. microplus</i>	<i>R. annulatus</i>
Brown colour oval shape body without any patterns	×	✓	×	×
Cylindrical body possess numerous small hairs on the dorsal side	×	×	✓	✓
Inornate scutum possess short indistinct cervical grooves	✓	✓	×	×
Light yellow to pale color angular shape scutum with large and small numerous punctuations clustered over	×	×	×	×
Cervical groove depression is well-marked and extends up to the posterior of the scutum	×	×	×	×
Round shape scutum possess large and deep punctuations, distributed regularly	×	×	×	×
Triangular shape basis capituli with well -marked cornua	✓	×	×	×
Hexagonal shape basis capituli along with moderately developed cornua projecting downward	×	✓	✓	×
Hexagonal shape basis capituli with well-developed cornua	×	×	×	×
Hypostomal dental formula is 4/4	×	✓	✓	✓
Hypostomal dental formula 2/2	✓	×	×	×
Porose areas shape is a broad oval	×	×	×	✓
Absence of eyes	×	×	✓	✓
Presence of eyes	✓	✓	×	×
Inornate scutum with convex eyes on the lateral edge of the scutum	×	×	✓	✓
The second segment of the palp extending laterally, and the third segment has a small conical projection dorsally and ventrally	×	✓	×	×
Palp segment 1 internal margin has no protuberance and is long and slightly concave	×	×	×	✓
Genital aperture is positioned in between in the third leg	×	✓	×	×
Genital aperture is positioned in between in the second leg	×	×	✓	✓
Y-shaped anal groove situated posterior to the anus	✓	✓	✓	✓
Presence of well-marked 11 festoons separated by large festoon grooves	×	✓	×	×
Presence of 9 festoons without festoon grooves	✓	×	×	×
Festoon grooves are narrow but clearly marked	✓	×	×	×
1st pair of coxa has a small pointed single internal spur and coxa 2–4 have an indistinct spur	✓	✓	×	×
1st pair of coxa has indistinct spurs and are absent in 2 and 3	✓	×	×	✓
1st pair of coxa have single internal spur and coxa 2–4 have short external spurs	×	×	×	×
Coxa 1 spurs are distinct, 2 and 3 have small spurs and coxa 4 haven't any spurs	×	×	✓	×
1st pair of coxa has unequal-sized internal and external spurs. Coxa 2–4 have small triangular external spurs	×	×	×	×
Presence of caudal parma and pair of adanal and accessory glands situated on both side of the anus	×	×	×	×
Sub-oval shape spiracular plate with round macula arranged at the anterior of the spiracle	✓	×	×	×
Spiracular plates are large and coma shape with numerous spiracular goblets closely arranged over the spiracle. The elongated macula extended from anterior to posterior of the spiracle	✓	×	×	×

The genetically similar tick species in the genus, *R. microplus*, and *R. annulatus* are widely distributed throughout the study site, and Kyasanur forest disease vector *H. bispinosa* easily scattered from one habitat to another, diversity of hard tick species was high in this region. The estimated diversity of species from Wayanad is represented in Table 3. The cattle tick *R. microplus* and the Kyasanur forest disease vector *H. bispinosa* show the highest diversity, the index value of *R. microplus* is 0.36638 and *H. bispinosa* 0.35838,

followed by *R. annulatus* 0.31576. And the overall diversity index of collected species is 1.11844.

Phylogenetic analysis of *A. geoemydae*

The phylogenetic relationship was evaluated based on mitochondrial CO 1 gene sequences aligned in CLUSTAL W, to demonstrate the genetic similarity of *A. geoemydae*, concerning the retrieved sequences of *Amblyomma* spp. from

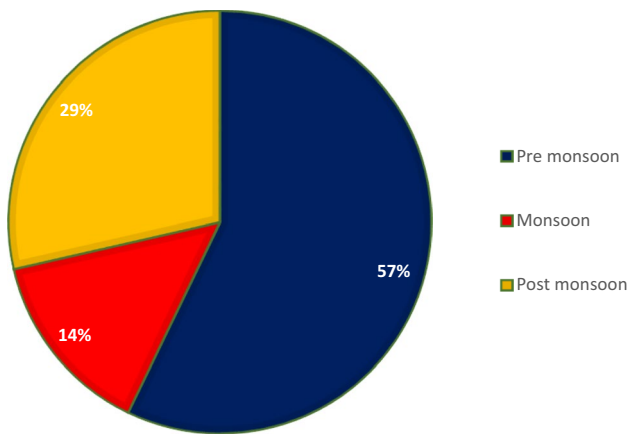


Fig. 3 Season-wise variation in the abundance of *A. geoemydae* collected from Wayanad

NCBI. The evolutionary tree was constructed using the Neighbor-joining and Maximum likelihood method (Fig. 5). According to our findings, we provide the genetic relationship of *Amblyomma* sp. to *A. geoemydae*, with these, are *A. testudinarium*, *A. pattoni*, and *A. marmoreum*, and forming a monophyletic relationship with *A. geoemydae*. The remaining species *A. dissimili* and *A. calcaratum* and *A. coelebs* in the phylogenetic tree did not form a distinctive clade. The clades of the phylogenetic tree provide evidence that the species from the present study show more congruity with *A. geoemydae* sequences deposited in NCBI. The scale length in the second clade is less, which indicates that the nucleotide sequence of collected species is similar to the sequence of *A. geoemydae* retrieved. Moreover, *A. dissimili*, *A. calcaratum*, and *A. coelebs* have long scale lengths in clades, showing sequence divergence. The constructed phylogenetic

Fig. 4 Percentage of sequence similarity and dissimilarity of collected tick species and sequences retrieved from NCBI

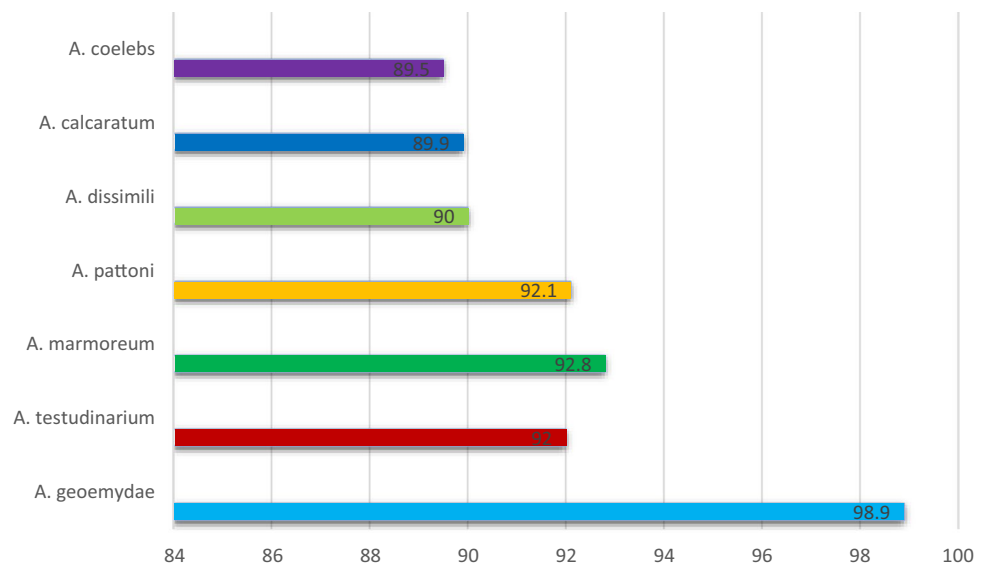


Table 3 Shannon wiener index of collected tick species from study site Wayanad

Species	No	n/N	ln(n/N)	H = nN*ln(n/N)
<i>A. geoemydae</i>	7	0.019886	- 3.91772	- 0.077909225
<i>H. bispinosa</i>	160	0.454545	- 0.78846	- 0.358389709
<i>R. microplus</i>	118	0.335227	- 1.09295	- 0.366385492
<i>R. annulatus</i>	67	0.190341	- 1.65894	- 0.315763873
Total	352	1	- 7.45806	- 1.118448299

tree provides evidence of the relationship of sequences of *Amblyomma* species supported by a bootstrap value is 100%.

Discussion

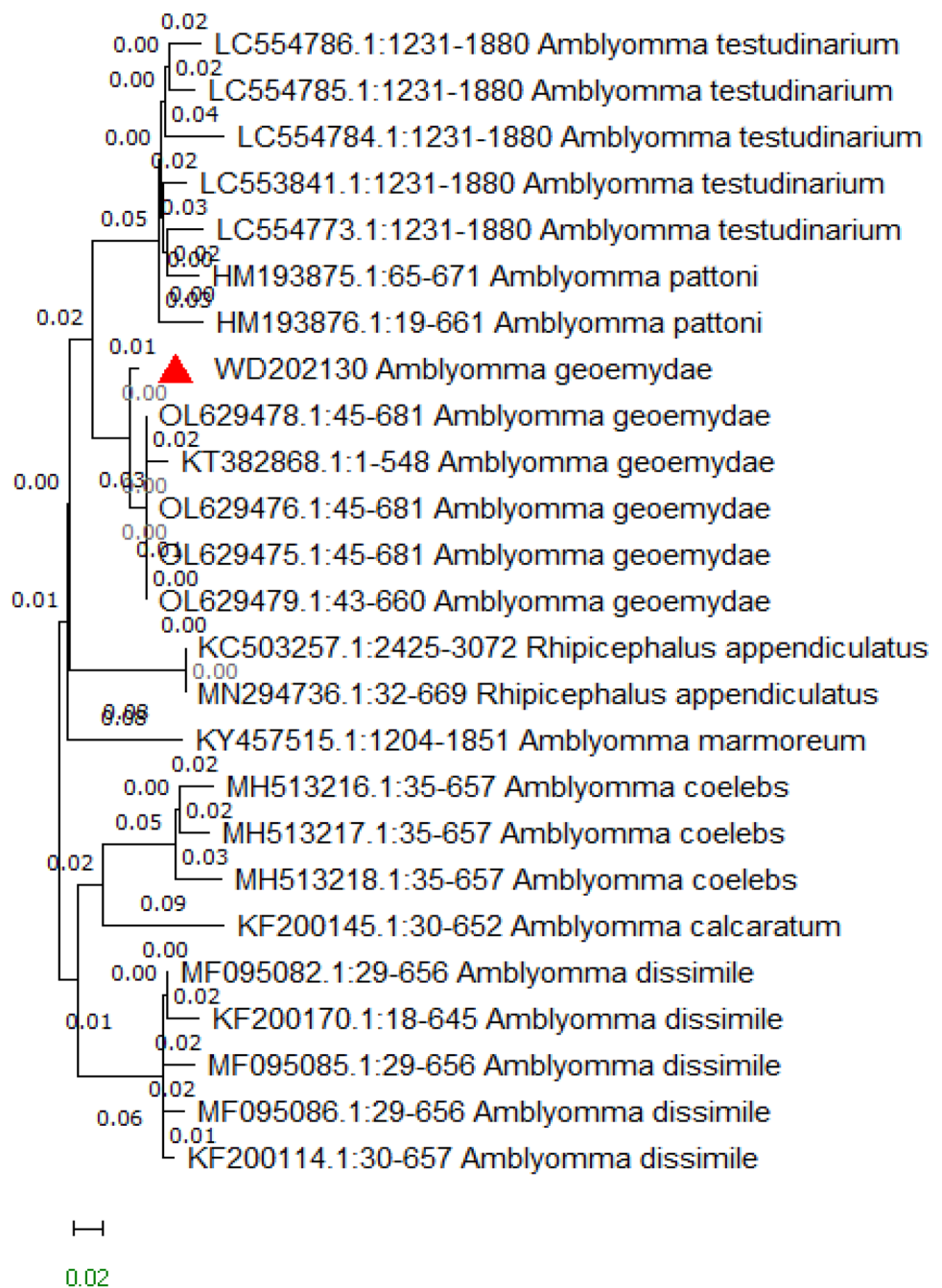
The purpose of the study was to identify species of ticks collected from the forest, four tick species were identified through morphology and molecular analysis, namely *Amblyomma geoemydae*, *Haemaphysalis bispinosa*, *Rhipicephalus annulatus* and *R. microplus*. The distribution of *A. geoemydae* from the province was not reported early, so this investigation highlighted the Lyme disease vector *A. geoemydae* from this province because in the past years (2013–2015) there was an outbreaks of Lyme disease and Kyasanur forest disease occurred in Wayanad (Sehgal and Bhatt 2021). The species of this study *H. bispinosa*, *A. geoemydae*, *R. microplus* and *R. annulatus* have been reported in different ecological niches of many countries including Taiwan (Thinnabut et al. 2023), Philippines, Japan, and Austria (Chao et al. 2022a; Simmons and Burridge 2000). Rather than this report, previous studies documented the host-tick vector relationship, and Simmons et al., in 2000

feigned acquaintanceship between the host and *A. geoemydae*, which is predominantly inhabited in turtles (*Pyxidea mouhotii* and *Geomyda spengleii*). Furthermore, some studies documented the pathogenicity of *A. geoemydae*, *H. bispinosa* in different zones of India (Molaei et al. 2021). Our investigation discovered the dispersal of *A. geoemydae* from the study sites, which is relevant because they harbour vector-borne agents like *Ehrlichia*, *Francisella*, (Qiu et al. 2021a), and *Borrelia lonestari*. (James et al. 2001). These are the causative agents of Lyme disease and relapsing fever (Kambayashi et al. 2021). The disease-causing agents have high efficacy in inducing health disparities in animals and humans, including skin lesions, erythema migrans, arthralgias, myalgias, and extreme weakness are common, and lymphadenopathy moreover may occur (Kambayashi et al. 2021). And also affect in the quality of animal products, like meat, milk, eggs, etc. (Bekere et al. 2022). Severe infectious disease leads to eventuate infirmity in economic status (Gugnani 2022). Finding these developing stage (larva, nymph, and adult) microbial vector *R. microplus*, *R. annulatus*, and *H. bispinosa* from the tick-contaminated areas are necessary for implementing sophisticated and progressive tick control strategies. *Rhipicephalus microplus* and *R. annulatus* were widely distributed in southern India, while *A. geoemydae* was rarely documented (Kanduma et al. 2020; Okely and Al-Khalaf 2022). However, the rate of ticks and tick-pathogens dispersal continues to gradually increase, opening a great chance of infestation of Lyme disease to human beings. Flattering environmental parameters from the lush green forest of several states of India provide ample opportunity for the ixodid ticks to parasitize the human population and the domestic animals, which negatively impacts valuable resources of creatures (Goswami et al. 2022). For the establishment of new strategies against the complications mentioned above many research works was carried out on aspects of host tick vector relationship, diversity, and its prevalence was documented. From South-East Asia *A. geoemydae* nymphs were collected and identified, which are instantly distinguished from other amblyommines due to their ornate scutum (Oda et al. 2023). In the province, this reptilian tick was the second ectoparasite of *Heosemys depressa* (Robbins and Platt 2011). The juvenile tick *A. geoemydae* population is abundant in soil and vegetational zone during the summer season, so they easily reach the preferred host, Chelonia, Squamata. In the absence of a favourable host, they can survive in new hosts under the same group, such as Coraciiformes (Martínez-Sánchez et al. 2020) and Carnivora, and few on Galliformes (Rassouli et al. 2016), Ciconiiformes (Labruna et al. 2007), Passeriformes, Lagomorpha (Saraiva et al. 2012), and Artiodactyla (Maturano et al. 2015). The immature chelonian tick *Amblyomma marmoreum* infest on tortoises (Allan et al. 1998). In our findings, the evolutionary tree demonstrated

genetic similarities between, *A. geoemydae* shows 92% to *A. testudinarium*, (Fig. 3). The species confirmation was done not solely from the nucleotide sequence analysis, but also a conventional phenotypic examination before the amplified PCR product was evaluated for the confirmation of species. The nymph of *A. americanum* resemble *A. geoemydae*, having a single external spurs from coxa 1–4 and lateral margins of the basis capituli (Holscher 1981). Large numerous deep punctuations are distributed on the lateral sides of the scutum in *A. americanum* (Vasil'eva, 2011) but *A. geoemydae* possesses few punctuations on scutum followed by little more punctuations scattered over alloscutum. *Amblyomma longirostre* (Ogrzewalska et al. 2010), *A. testudinarium*, *A. sculptum* (Suzin et al. 2022), and *A. marmoreum* display relatively short cervical groove length although cervical field depression is apparent in *A. sculptum* and non-apparent in *A. geoemydae* and *A. longirostre* (Luz et al. 2017). Eyes are flat and slightly convex in *A. naponense* (Szabó et al. 2007), *A. brasiliense* and *A. geoemydae* however, differ in the number and width of festoon grooves. The shape of the posterior edge of the scutum is narrow in *A. rotundatum* (Luz et al. 2018), *A. brasiliense*, and *A. geoemydae* but they have unequal paired external and internal spurs on 1st coxa and a single external spurs on coxa 2–4. Moreover, this essential feature is also observed in *A. neumanni* (Martins et al. 2014). Other than the morphological characters according to evolutionary consideration, *A. geoemydae* reveals more sequence similarity with *Amblyomma testudinarium*, *A. marmoreum*, *A. pattoni* (Zhang and Zhang 2014). Here we found that the isolated sequences under investigation have primarily exhibited close resemblance to the sequences retrieved from NCBI.

This study showed that the isolated *Amblyomma geoemydae* nucleotide sequences have exhibited extensive genetic similarity towards the nucleotide sequences with accession numbers KT382870, KT382869, KT382868, KT382867, KT382866, and KT382865 representing *Amblyomma* sp. We found that molecular characterization principally confirmed the morphometrically described tick species *Amblyomma geoemydae*. The nucleotide sequence with accession number KY457515 representing *A. marmoreum* exhibits a relatively considerable range of genetic similarity to the isolated nucleotide sequences of *A. geoemydae*. Similar to our discovery scientists, previously examined the phylogenetic assessment of *Amblyomma pattoni* and *A. nitidum* in response to the molecular characterization (Qiu et al. 2021b). And the above-mentioned pattern has been similar for the remaining nucleotide sequences retrieved from NCBI which are *A. marmoreum*, and *A. pattoni*. Previously, Chao Li et al. (2022a) examined the genetic identity by comparing the 16S mitochondrial DNA gene sequences isolated from 11 adult *A. geoemydae* from the province of Taiwan (Chao et al. 2022b). Similar studies were conducted in Malaysia,

Fig. 5 The evolutionary tree of *A. geoemydae* tick based on the mitochondrial CO1 gene was inferred by using the Maximum Likelihood method and the Tamura-Nei model. Pairwise distances are estimated using the Tamura-Nei model and then selecting the topology with a superior log-likelihood value. This analysis involved 25 nucleotide sequences. There were a total of 672 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 software



which validated the *A. geoemydae* utilizing the perusal of the CO1 gene (Che Lah et al. 2022). Since Kerala, molecular characterization of *Haemaphysalis bispinosa*, *Rhipicephalus microplus* and *R. annulatus* was documented in the past years (Csordas et al. 2016; Nusrat et al. 2017) but *A. geoemydae* was lack in previous literature, making this is the first report of mitochondrial cytochrome oxidase subunit 1 gene. Recently more studies were documented in molecular analysis of tick-borne pathogens without genetic confirmation of tick species. Studies were reported on the presence of

Rickettsia, *Ehrlichia*, and *Borrelia* spp. from *A. geoemydae* via the analysis of housekeeping genes (*gltA*, *ompA*, *ompB*, *sca4*, 16SrRNA, *flaB*, *glpQ*, and *groEL*) for the experimental purpose the tick species was vindicated only via conventional morphology (Kaenkan et al. 2020). Prevalence and molecular characterization of Ixodid ticks provide directions to the research area as well as species diversity, vector competence, microbial specificity on tick vectors, and also advanced preventive strategies and vaccine development.

Conclusion

Our investigation described three existing species of hard ticks *H. bispinosa*, *R. microplus* and *R. annulatus* and the first report of *A. geoemydae* from the forest of WWLS Kerala. The collected ticks were examined through a stereomicroscope, and the important morphological characteristics such as length of palps, hypostomal dental formula, number of spurs on coxa, and the shape of the anal groove were identified in this morphological analysis. Moreover, the molecular technique was employed for further accurate and precise species identification. The mitochondrial CO 1 gene was used to confirm *A. geoemydae*, *H. bispinosa*, *R. microplus*, and *R. annulatus*. This investigation highlighted the phylogenetic analysis of Lyme disease vector *A. geoemydae*; which has previously been known to instigate severe impacts over India including the study area. According to our findings, the current study is relevant in the context of vector-borne diseases because the morphological characteristics of nymphs and adults have always produced less variation. However, while considering their significance in disease transmission, it was perceived that both of them have an equal role. Moreover, the juvenile stage can potentially convey microbes to the host, including people. Within the current situation, the huge exacerbation of tick-borne infections transmitted through the tick-life cycle gives a major public well-being imbroglio. Therefore, species affirmation is vital for the discoveries of advanced vector control techniques.

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Author contribution KKA was involved in data collection and analysis and prepared the manuscript, carried out molecular analysis, and created a phylogenetic tree. EMA and KVA took part in the editorial section, maintenance, and supervision of research work.

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Data availability The datasets used in this article are available from the corresponding author upon reasonable request.

Declarations

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

Ethical approval and consent to participate Not applicable.

Consent for publication Not applicable.

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