

Molecular and morphological characterization of hard ticks (Acari: Ixodidae) infesting rodents in Southern Iran: evidence of a cryptic *Haemaphysalis* species

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Abstract

Background: Hard ticks are important vectors of various zoonotic diseases. Identifying closely related tick species using morphological methods alone can be challenging. This study aimed to assess the taxonomic status of hard ticks infesting rodents in southern Iran using morphological and molecular approaches.

Methods: Ticks were collected from the ear bases and rears of rodents and identified morphologically. Genomic DNA was extracted, and the cytochrome oxidase subunit 1 (*COXI*) and internal transcribed spacer 2 (*ITS2*) genes were amplified via polymerase chain reaction (PCR). Sequencing and phylogenetic analyses were performed using assembled DNA sequences and reference sequences from GenBank.

Results: A total of 62 rodents from seven species were examined. Collected ticks were identified as *Rhipicephalus turanicus* and *Haemaphysalis* spp. Phylogenetic analysis revealed that the *Haemaphysalis* samples were genetically distinct from 29 congeneric species recorded in the Barcode of Life Data System (BOLD) and GenBank. No sequence matches were found with known *Haemaphysalis* species in Iran, except for *H. caucasica* and *H. aponommoides*, which lack sequence data in these databases.

Conclusions: This study represents the first molecular characterization of *Haemaphysalis* and *Rhipicephalus* ticks infesting rodents in Iran. Our findings confirm the presence of *Rhipicephalus turanicus* on *Rattus* species. More significantly, we identified a distinct *Haemaphysalis* lineage parasitizing *Calomyscus cf. bailwardi*, a rodent endemic to the Zagros Mountains. The considerable genetic divergence observed in both the *COXI* and *ITS2* genes suggests that this *Haemaphysalis* lineage may represent a cryptic species or a unique ecotype adapted to this specific host and geographic location.

Key words: Acarina; ixodid; Iran; nucleotide sequences; PCR; rodents; taxonomy.

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Background

Rodent ectoparasites may have medically important pathogens that can be transferred to humans or domestic animals [1-3]. Therefore, evaluating ectoparasites on rodents across different zoogeographical zones seems indispensable due to the impact of rodent-associated diseases on human and livestock health [4-6]. On the other hand, since the evolutionary relationship or coevolution of host/ parasite is very important in the speciation process and the origin of a new species [7,8], it is crucial to know both the host and parasite fauna in each geographical region. *Haemaphysalis* Koch, 1844, is the second-largest genus (after *Ixodes*) of hard ticks (Acari: Ixodidae), comprising 166 species [9,10]. Based on a recent study [11] on the Iranian *Haemaphysalis* taxon, ten species, including *H. caucasica*, *H. concinna*, *H. erinacei*, *H. inermis*, *H. aponommoides*, *H. kashmirensis*, *H. kopetdaghica*, *H. parva*, *H. punctata*, and *H. sulcata*, have been recorded from this region.

Accurate identification of tick species, often through morphological characteristics, is of great importance to public and veterinary health and for controlling associated tick-borne diseases [12-14]. Traditional morphologic identification of ticks is difficult due to variations within larval and nymphal life stages, when ticks are engorged, or when specimens have been disfigured [15,16]. Molecular data can also estimate genetic variation in specific genes directly from the taxa examined and discriminate among closely related species [17-19]. A DNA-based approach to tick species-level specimen identification is thus advantageous in these cases. The 5' region of the cytochrome oxidase subunit one (*COXI*) gene is the standard marker for DNA barcoding of animals, as a large number of *COXI* sequences from animals have been deposited in the Barcode of Life Data (BOLD) System [20-23]. There are few molecular investigations on the systematic status of rodents' hard tick

ectoparasites, especially *Haemaphysalis* species from Iran. A molecular study on the immature stages of *Hyalomma asiaticum* (Acari: Ixodidae) collected from the Persian jird was done by Chegeni *et al.* (2018) [4]. So far, no molecular study has been performed on *Haemaphysalis* and *Rhipicephalus* ticks infesting rodents in Iran. Based on their mitochondrial and nuclear DNA sequences, only two recent molecular studies have been conducted on the *Haemaphysalis* species collected from livestock, hedgehogs, and red foxes [11,24]. In this study, *Haemaphysalis* ticks were collected from brush-tailed calomyscid rodents (Rodentia: Calomyscidae), endemic to the Zagros Mountains in southern Iran. These rodents have a vast distribution range in the Iranian plateau [25-30], from which *Calomyscus cf. bailwardi* is endemic to the Zagros mountains of Iran [25,26]. Based on a recent molecular phylogeographic study of this species, it is further believed that the calomyscid rodent from the two adjacent provinces of Fars and Kerman in southern Iran should be regarded as a new species [29,30].

Despite the increasing number of faunistic reports on ixodid ticks in Iran, molecular data on rodent-associated *Haemaphysalis* and *Rhipicephalus* species, particularly from southern Iran, remain extremely limited. This lack of DNA-based evidence hampers accurate species identification and obscures the detection of cryptic or closely related lineages. Moreover, reliance on morphological identification alone is often insufficient for immature stages or damaged specimens, which are common among rodent-associated ticks. Therefore, an integrative taxonomic approach that combines morphological and molecular markers is essential for resolving taxonomic uncertainties and improving our understanding of tick

diversity and host associations in this region. In the present study, we aimed to evaluate the taxonomic status of *Haemaphysalis* and *Rhipicephalus* ticks collected from rodents in the south of Iran based on the morphological characters and molecular data sets of the mitochondrial *COXI* and nuclear ITS2 genes.

Methods

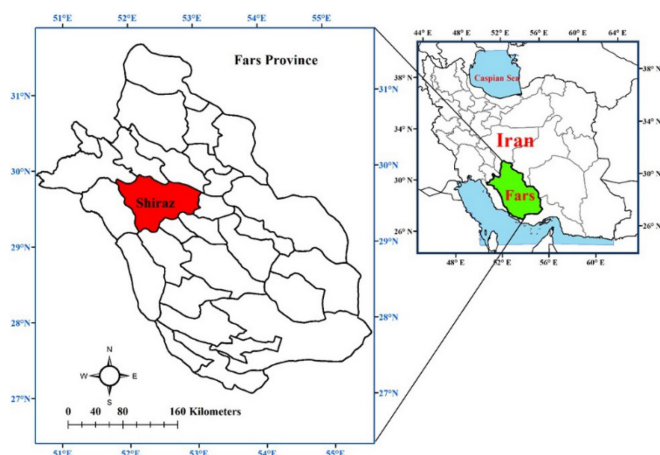
Study Area and Sample Collection

Rodents were trapped near the settler houses in Shiraz city, Fars province of Iran (Figure 1). Rodent trapping was conducted at multiple peri-urban and mountainous sites around Shiraz. Trapping locations were separated by several kilometers. At each site, 20–30 Sherman live traps were deployed per night for 2–3 consecutive nights. Shiraz is the third-largest and fifth-most-populous city in Iran, and the capital of Fars province. With an average elevation of 1500 m, this city is in an NW–SE elongated valley bounded by the Zagros mountains. Sherman traps captured rodents as part of a research program on rodent pathogens during the spring and summer of 2021-2023. We used cucumber, walnut, and cheese puffs as bait for sampling rodents. The traps were placed in the evening and collected early the following day. Captured rodents were transferred to the Shiraz University of Medical Sciences (SUMS) animal laboratory for further examination. Bodies of collected rodents were brushed after general anesthesia with CO₂ to remove ectoparasites on a white tray filled with water. Collected tick specimens were preserved in 75% ethanol for further processing. All *Haemaphysalis* specimens collected in the present study were immature stages (nymphs), whereas *Rhipicephalus* specimens consisted of adult males and females. Morphological identification was performed using standard taxonomic keys; however, independent verification by a second taxonomist was not conducted, which is acknowledged as a limitation of the study. Ticks were photographed, viewed under a microscope and identified morphologically to genus level, and compared to other species using proper valid dichotomous keys [24,31-35].

DNA Extraction, Polymerase Chain Reaction (PCR) and Sequencing

Genomic DNA was extracted from the leg tissues of 11 ticks with minimal damage to the specimens using the FavorPrep™ tissue genomic DNA extraction mini kit (Favorgene Biotech Corp, Taiwan), following the

Figure 1. Map of Iran and Fars province showing the geographical position of the sampling area (Shiraz).



manufacturer's instructions. A total of 11 tick specimens were processed for molecular analyses, including two *Haemaphysalis* and nine *Rhipicephalus* specimens. The *COX1* partial mitochondrial gene of ticks was amplified by polymerase chain reaction (PCR) using the two universal primers of LCO1490 (5'-gggtcaacaaatcataaagatattgg-3') and HC02198 (5'-taaaacttcagggtgacacaaaaatca-3') [36] to amplify approximately 710 bp DNA fragments of *COX1*. PCR reactions were conducted in a 50 µL reaction volume containing one µL of template DNA (100 ng/µL), 15 µL of *Taq* DNA polymerase (2x red master mix) (Amplicon, Odense, Denmark), 2.5 µL of each primer (10 pmol/µL), and 29 µL of sterile ddH₂O. PCR conditions involved one cycle of 95°C for 5 minutes, followed by 35 cycles of 94°C for one minute, 40°C for one minute, and 72°C for one and a half minutes, and a final extension at 72°C for 7 minutes. Double-distilled water (DDW) was included in each experiment run instead of template DNA as a negative control. PCR products were separated on 2% agarose gel by electrophoresis and stained with GelRed nucleic acid gel stain (Biotium, USA) for visualization under a BioDoc gel documentation System. The partial nuclear ITS2 gene (1350 bp) was amplified by PCR using the two primers of 5'-CTGCGAGACTTGGTGTGAATTG-3' and 5'-GCAGAGACTGCGAAGCAC-3' [11]. Touchdown PCR was employed to enhance amplification specificity for the relatively long ITS2 fragment and to reduce non-specific products. The PCR program for this nuclear gene was as follows: 5 min. at 94°C, touchdown temperature profile of 11 cycles [45 s at 94 °C, 50 s at 60–50 °C, 90 s at 72 °C], followed by 20 cycles [45 s at 94 °C, 50 s at 45°C, 90 s at 72 °C], and finally a step at 72 °C for 5 minutes. Seven PCR products were sequenced in both directions using the same primers used for PCR by the Pishgam Company, Tehran, Iran.

Phylogenetic Analyses

The raw nucleotide sequences and chromatograms in both the forward and reverse directions were viewed and analyzed using the Chromas program implemented in BioEdit version 7.2.5 [37]. Consensus sequences were assembled and aligned using ClustalW in BioEdit. The final sequences were submitted to GenBank (NCBI) with accession numbers (ac.nu.) of MW831028-29 (*COX1*), PP301377 (*COX1*), and PP192773-74 (ITS2). The final aligned sequences were converted into FASTA format for further analysis in MEGAX software [38]. Consensus sequences were compared with homologous sequences in the GenBank database using the BLAST algorithm [39].

Phylogenetic relationships between the present samples and 24 *Haemaphysalis* species from NCBI were reconstructed using a Bayesian inference (BI) method in MRBAYES, version 3.1.2 [40], and also the Neighbor-Joining (NJ) method in MEGA X [38] for *COX1* sequences. Bayesian inference was performed with two simultaneous runs and four search chains within each run (three heated chains and one cold chain) for 10⁷ generations, sampling trees every 1000 generations using the Markov chain Monte Carlo method. Phylogenetic relationships among *Haemaphysalis* species based on the nuclear gene ITS2 were reconstructed using the maximum likelihood (ML) method implemented in PhyML v2.4.4. Evolutionary relationships of *Rhipicephalus* species (*COX1* gene) were inferred using the Neighbor-Joining method in MEGA X [38]. The reliability of nodes was assessed using Bayesian posterior probability for the Bayesian analysis [41] and bootstrap method (10000 generations) for other phylogenetic trees (NJ and ML). For distance analysis, estimates of evolutionary divergence between *Haemaphysalis* species sequences were obtained using the Kimura 2-parameter (K2P) model [42] in MEGAX [38]. *Hyalomma* and *Haemaphysalis* sequences were used as outgroups for

Table 1. Host species, collection site, and GenBank accession numbers of tick specimens analyzed in this study.

Tick ID	Tick genus / species	Life stage	Host species	Collection site	COX1 GenBank accession no.	ITS2 GenBank accession no.
T1	<i>Haemaphysalis</i> sp.	Nymphs	<i>Calomyscus cf. bairdardi</i>	Shiraz (Fars Province)	MW831028	PP192773
T2	<i>Haemaphysalis</i> sp.	Nymphs	<i>Calomyscus cf. bairdardi</i>	Shiraz (Fars Province)	MW831029	PP192774
T3	<i>Rhipicephalus turanicus</i>	Adult	<i>Rattus norvegicus</i>	Shiraz (urban area)	PP301377	–
T4	<i>Rhipicephalus turanicus</i>	Adult	<i>Rattus norvegicus</i>	Shiraz (urban area)	PP301377	–
T5	<i>Rhipicephalus turanicus</i>	Adult	<i>Rattus norvegicus</i>	Shiraz (urban area)	MW831028	–
T6	<i>Rhipicephalus turanicus</i>	Adult	<i>Rattus norvegicus</i>	Shiraz (urban area)	MW831028	–
T7	<i>Rhipicephalus turanicus</i>	Adult	<i>Rattus norvegicus</i>	Shiraz (urban area)	MW831028	–
T8	<i>Rhipicephalus turanicus</i>	Adult	<i>Rattus norvegicus</i>	Shiraz (urban area)	MW831028	–
T9	<i>Rhipicephalus turanicus</i>	Adult	<i>Rattus norvegicus</i>	Shiraz (urban area)	MW831028	–
T10	<i>Rhipicephalus turanicus</i>	Adult	<i>Rattus norvegicus</i>	Shiraz (urban area)	MW831028	–
T11	<i>Rhipicephalus turanicus</i>	Adult	<i>Rattus norvegicus</i>	Shiraz (urban area)	MW831028	–

the phylogenetic trees of *Haemaphysalis* and *Rhipicephalus*, respectively.

Results

Rodents and tick identification

A total of 62 rodents were caught and identified based on the external and dental morphological characteristics, including 29 (46.77%) *Rattus rattus*, 15 (24.2%) *Mus musculus*, 10 (16.12%) *Calomyscus cf. bailwardi*, three (4.8%) *Rattus norvegicus*, three (4.8%) *Meriones libycus*, one (1.61%) *Apodemus witherbyi* and one (1.61%) *Meriones persicus*. Recent molecular studies have shown that *Calomyscus* species from Fars province represent a new species distinct from *C. bailwardi* [30]. Therefore, this species was considered *Calomyscus cf. bailwardi*.

Eleven attached ticks were collected on the ear base and back of the infested rodents. Details of each tick specimen, including host species, collection site, developmental stage, and corresponding GenBank accession numbers, are summarized in Table 1. The low number of ticks collected likely reflects low infestation intensity in the examined rodent populations and limits the generalizability of the results. All ticks were identified as *Rhipicephalus turanicus*, collected from two *R. norvegicus*, except one female nymph and a bloody male nymph collected from two brush-tailed calomyscid rodents. These nymphs were identified as belonging to the genus *Haemaphysalis* based on the morphological criteria for this taxon (Figure 2).

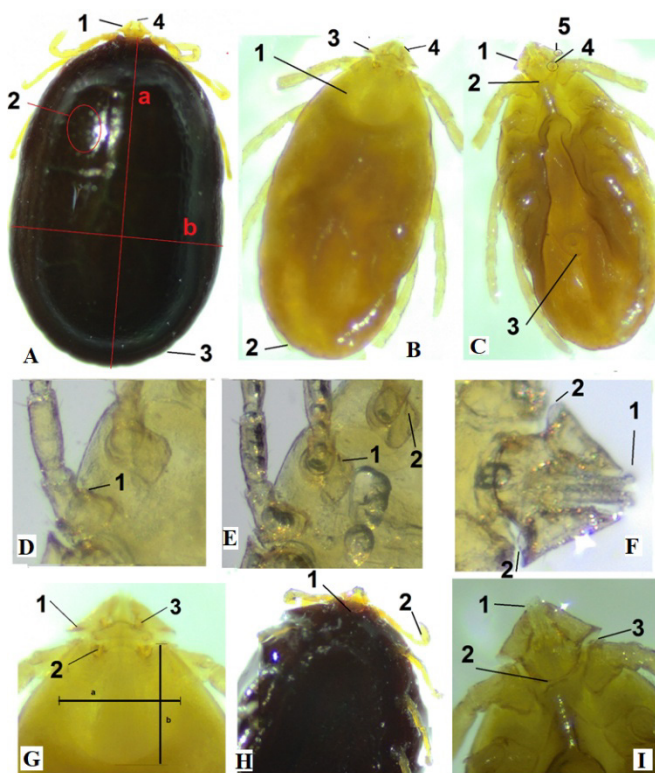
Morphological features of *Haemaphysalis* specimens

Briefly, the characteristics representing the genus *Haemaphysalis* include absence of eyes; festoons are present (Figures 2A, 2B); size of unfed ticks small comprising mouthparts (Figure 2B); no ornamentation on scutum (Figure 2B); a distinct anal groove embracing the anus posteriorly (Figure 2C); legs are slender and pulvilli are always present (Figure 2H); coxa 1 has a single spur (Figure 2D).

Other morphological features of these specimens are: the palp is conical with narrow tips (width of palp broader than that of basis capitulum), with the posterolateral margins of article 2 clearly projecting from the lateral margin of the basis capitulum (Figures 2A, 2C); coxa 1 basal spur is small (Figure 2D); spurs of coxa 2 and coxa 3 approximately medium in size (Figure 2E); spur on coxa 4 was not observed in the present nymphs; the punctuation distribution is dense (Figure 2A); palp article 2 dorsal spur is present (Figure 2C); anterolateral margin of basis capitulum with a small spur (Figure 2C); lateral side of palp segment 2

with angle (Figure 2C); breadth and length of scutum is equal, and outline broadly-rounded (Figure 2G) (although, like in adult *H. erinacei*, scutum may become oval *i.e.* length of scutum longer than its width); conscutum ca 1.7 times as long as broad (Figure 2A); hypostome slightly longer than palps in female nymph (Figures 2F, 2I), or approximately as long as palps in male (Figure 2A); basis capitulum dorsally sub-rectangular; palp segment 2 dorsal spur is present (Figure 2G); basis capitulum dorsally with prominent posteriorly-located medium cornua (Figure 2G); segment 3 triangular with acute apex shorter than segment 2 (Figure 2B). There is a backward pointing spur in the form of an angular projection from the posterior margin of the dorsal surface of the second

Figure 2. Morphology of *Haemaphysalis* sp. nymphs from Iran.



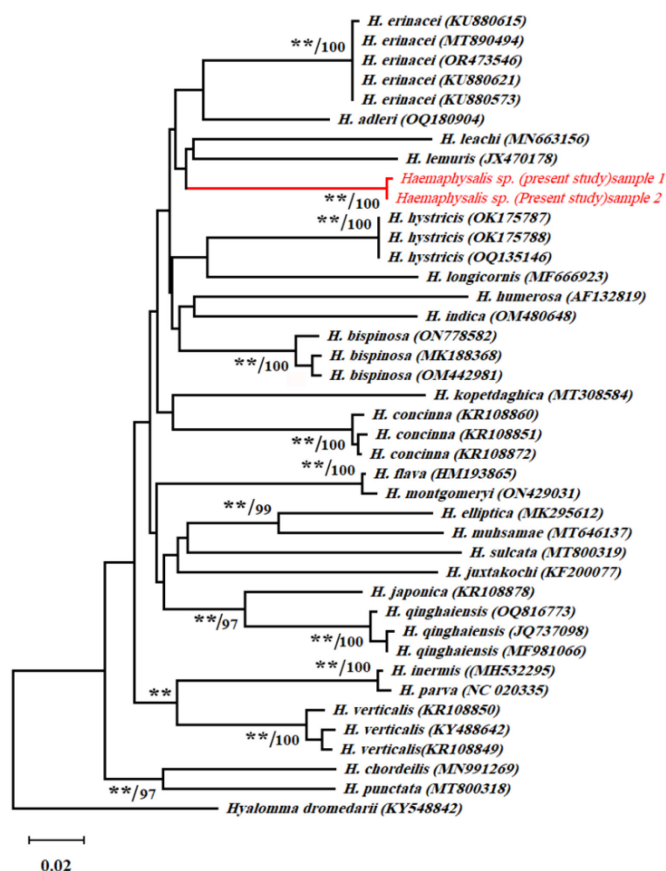
A. bloody male, dorsal view: 1- posterolateral margins of article 2 projection, 2- punctuation distribution, 3- festoons, 4- hypostome of mouthparts almost as long as palpi, a- conscutum length, b- conscutum breadth; **B.** female, dorsal view, eyes are absent: 1- scutum with no ornamentation, 2- festoons, 3- backward pointing spur of dorsal surface of palp segment 2, 4- palp segment 3; **C.** female, ventral view: 1- lateral side of palp segment 2 with angle, 2- basis capitulum, 3- anal groove embracing the anus posteriorly, 4- anterolateral margin of basis capitulum with a small spur, 5- tiny spur on lateroventral surface of palp segment 2; **D.** 1- small spur on coxa 1; **E.** 1- coxa 2 spur, 2- coxa 3 spur; **F.** 1- hypostome slightly longer than palps, 2- ventral spur on palp segment 1; **G.** 1- backward pointing spur of dorsal surface of the second article of the palp, 2- cornua, 3- dorsal spur of palp segments 2, a- scutum width, b- scutum length; **H.** 1- ventral side of basis capitulum, 2- pulvillus; **I.** 1- hypostome slightly longer than palps, 2- ventral side of basis capitulum, 3- ventral spur on palp segment 1.

article of palps (Figures 2B, 2G); ventral spur on palp segment 1 had a triangular profile (Figures 2F, 2I).

Molecular analysis

The length of *COX1* and ITS2 sequences used for the molecular analyses were 673 and 1255 base pairs (bp) respectively. BLAST analysis results of the *COX1* sequences of *Haemaphysalis* sp., in the present study, showed 86.63 to 87.31% identity (E value = 0.0, 97% query coverage) to the published sequences of *Haemaphysalis* species, including *H. erinacei*, *H. bispinosa*, *H. concinna*, and *H. turturis*. BLAST analysis of ITS2 sequences of *Haemaphysalis* sp. showed 99.2% homology with the sequence of a *Haemaphysalis* sp. originating from Iran (ac.nu.: MH414962) and a percentage identity of 93.94% with a sequence of *H. erinacei* (ac.nu.: KU364288) from China.

Figure 3. Neighbor-Joining tree reconstructed for *COX1* gene sequences of *Haemaphysalis* species.

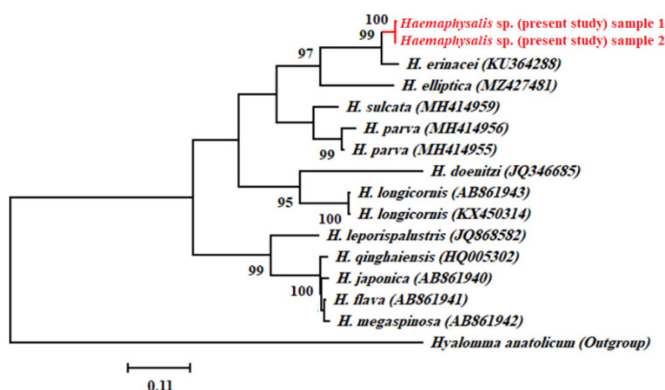


Values of the bootstrap test (10,000 replicates) are more than 97%, and they are shown next to the branches. Posterior probability values for the same clade in the Bayesian analysis are indicated at the > 99% (**) and > 95% (*) significance levels. *Hyalomma dromedarii* was considered as outgroup.

All phylogenetic trees revealed that the present *Haemaphysalis* tick samples from Iran belonged to the clade of a cryptic *Haemaphysalis* species (*Haemaphysalis* sp.) (Figure 3 and 4). A monophyletic origin for this clade was supported with 100% Bayesian posterior probability, and also 100% bootstrap (Figure 3 and 4). Based on K2P genetic distance, this samples were more similar to *H. erinacei* (K2P = 12.9%). Although no universal genetic divergence threshold exists for species delimitation in ticks, COX1 divergences exceeding 10% have been widely used as indicative of cryptic or distinct species in Ixodidae. Still, each phylogenetic tree yielded different results, and bootstrap or Bayesian posterior probability did not support the relations. ML tree based on ITS2 sequences showed the close relation of the present samples to *H. erinacei* (Figure 4). The percentage of K2P distance (COX1 gene) between the 25 *Haemaphysalis* species from NCBI ranged from 9.1 to 22.5%, while between *Haemaphysalis* sp. and other species ranged from 12.9 to 23.5% (S.1). The percentage of K2P distance based on ITS2 sequences between *Haemaphysalis* sp. and *H. erinacei* was 5%. Surprisingly, the K2P distances on percentages between the four taxa deposited as *H. flava* (ac.nu.: AB861941), *H. japonica* (ac.nu.: AB861940), *H. megaspinoso* (ac.nu.: AB861942), *H. qinghaiensis* (ac.nu.: HQ005302) in GenBank (NCBI) was one or two percent that may be due to misidentification by authors. Such unusually low genetic distances among distinct *Haemaphysalis* taxa may indicate misidentification or mislabeling of sequences in public databases, which can complicate phylogenetic interpretation.

All *COX1* sequences BLAST analysis for *COX1* sequences (n = 5, one haplotype) showed that

Figure 4. Phylogenetic tree reconstructed for nuclear ITS2 gene sequences of *Haemaphysalis* species using the ML method. *Hyalomma anatolicum* was considered an outgroup. Nodal supports presented above the nodes indicate ML bootstrap values (1000 replicates).



Rhipicephalus samples had 100% homology with *Rhipicephalus turanicus* from Egypt (ac.nu.: OM984989, OM984992-93), Iran (ac.nu.: KT313117), and China (ac.nu.: MT237705). Phylogenetic analysis using the Neighbor-Joining method clustered these samples within the Rh *clade. turanicus* with a nodal bootstrap support of 99% (Figure 5).

Discussion

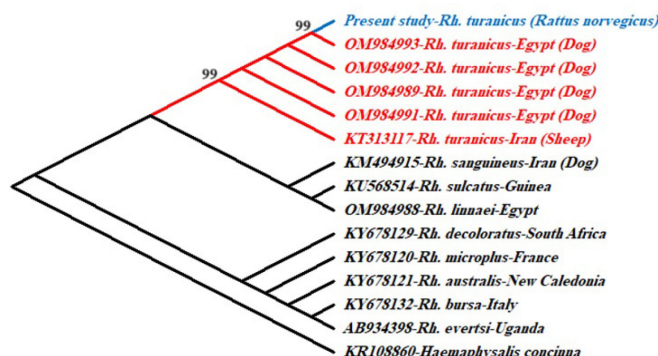
Hard ticks are vectors of several tick-borne diseases of livestock, wild animals, pets, and humans [43-46]. Rodents are implicated as reservoirs of many diseases such as Lyme disease, babesiosis, anaplasmosis, relapsing fever, and ehrlichiosis, and their hard tick ectoparasites are considered as vectors of some pathogenic agents to humans [46-48]. Hence, distribution range, host diversity, and taxonomic study of these parasites are essential to control tick-borne diseases. However, no molecular systematic study has been conducted on *Haemaphysalis* and *Rhipicephalus* ticks infesting rodents in Iran.

In the present study, the taxonomic status of these species was evaluated based on some visible morphological characteristics and the partial sequences of *COX1*, being used as a prime candidate in the DNA barcode methodology for species discovery and delineating cryptic taxa [20,25,49] in diverse taxonomic groups [16,21,50-52], including species in the family Ixodidae [16,18,53]. Lv *et al.* (2014a) showed that *COX1* was more effective at delineating hard tick species as a single gene identification system than 16S or 18S.

BLAST and phylogenetic analyses of *COX1* sequences identified *Rhipicephalus* specimens, as they had 100% homology with *Rhipicephalus turanicus*. However, *Haemaphysalis* ticks collected from the *Calomyscus* species, endemic to the southern Zagros mountains of Iran, were 12.9 to 23.5% divergent from the other 25 *Haemaphysalis* species. This issue was also examined by the nuclear ITS2 gene showing *Haemaphysalis* sp. with 5% divergence from the close species of *H. erinacei*. ITS2 sequences (1350 bp) of this species showed 99.2 % homology with the sequence (413 bp) of a *Haemaphysalis* sp. collected from a red fox in Iran (ac. nu.: MH414962) by Faghihi *et al.* (2020), which had an 8% genetic distance from *H. erinacei*. This *Haemaphysalis* sp. ectoparasite of the red fox had shown high phylogenetic and morphological similarity with *H. erinacei* in the ITS2 phylogenetic tree [11].

Closely related species, including siblings or cryptic species in the genus *Haemaphysalis*, are hard to

Figure 5. Evolutionary relationship of *Rhipicephalus* species using the Neighbor-Joining method. Nodal supports presented above the nodes indicate bootstrap values (10000 replicates). *Haemaphysalis concinna* was considered an outgroup.



differentiate morphologically, as Faghihi *et al.* (2020) stated difficulties in identifying some haemaphysalids. For the present haemaphysalids, however, some are morphologically similar to *H. erinacei* and *H. elliptica*, a very isomorphic species to *H. leachi* [34]. In the present specimens, and also *H. leachi*, *H. elliptica*, and *H. erinacei* [24,34,54,55], palp was conical with the posterolateral margins of article 2 projecting from the lateral margin of the basis capitulum. Like in *H. leachi*, the punctuation distribution on present specimens was dense. The breadth and length of the scutum were equal. The outline was broadly rounded (Figure 2G), like *H. elliptica* (Apanaskevich *et al.* 2007), in which the scutum was irregularly circular as the ratio of length to breadth was 0.92-1.08 (1.00 ± 0.04). However, like in adult *H. erinacei*, the scutum may become oval, *i.e.*, the length of the scutum is longer than its width. The conscutum ca 1.7 times as long as broad (Figure 2A), but it is ca 1.9 times as long as broad in nymphs of *H. elliptica* [54]. The posterior margin of the basis capitulum ventrally in both *H. leachi* and *H. elliptica* is convex. Still, in the present specimens (Figure 2C, Figure 2I) and some of the specimens from Africa (Asir), it is straight [55].

The hypostome was slightly longer than palps in female nymphs (Figure 2F, Figure 2I) or approximately as long as palps in males (Figure 2A), but in nymphs of *H. elliptica*, it was slightly shorter than palps; here there is a backward-pointing spur in the form of an angular projection from the posterior margin of the dorsal surface of the second article of palps (Figure 2B, Figure 2G). This is absent from the species found in the Mediterranean region [31]. The ventral spur on palp segment 1 had a triangular profile (Figure 2F, Figure 2I), unlike that of *H. erinacei* [55]. *H. erinacei* uses various small- and medium-sized mammals as hosts,

mainly hedgehogs, jackals, rodents, and foxes. Rodents can be used as this species' main hosts of both larvae and nymphs [11,32,56]. The present nymphs of *Haemaphysalis* sp. were likewise collected from rodents.

Unidentified *Haemaphysalis* ticks from Iran have already been seen in other studies. For example, Mohebbi *et al.* (1997) collected *Haemaphysalis* sp. from *Meriones persicus* in Meshkin-Shahr county, northwest Iran. Rahdar *et al.* (2015) collected a *Haemaphysalis* tick from the rodent *Tatera indica* but were unable to identify it at species level. In another study [57], only the larval stage of *Haemaphysalis* sp. was found in rodents of Khorram-Abad district, Lorestan province of Iran, including five larvae from *C. bailwardi* (n = 5), eight larvae from *Mus musculus* (n = 15), 32 larvae from *M. persicus* (n = 64) and six larvae from *Microtus socialis* (n = 16). Mohammadi *et al.* (2020) collected 54 ticks from *M. persicus* and *Apodemus witherbyi* on an enzootic plague focus in west Iran, of which 35 were identified as *Haemaphysalis* spp. Some immature ticks were also collected from rodents, identified as *Haemaphysalis* sp., due to the lack of morphological descriptions and final subsection to molecular identification [58]. Such studies showed most *Haemaphysalis* species infesting rodents were commonly immature and were probably ditropic ticks. Therefore, molecular identification of such ditropic ticks seemed necessary.

The molecular systematics of ticks, especially *Haemaphysalis* species associated with rodents in Iran, have been studied less and need more attention. Except for two species whose sequences had not been registered in the gene bank (*H. caucasica*, *H. aponomoides*), our samples were not similar to any of the *Haemaphysalis* species from Iran. The present study and other studies on *Haemaphysalis* ticks in Iran probably show a new cryptic species of *Haemaphysalis* infesting wild animals of Iran. If not, it can be a new record from Iran). So far, *Haemaphysalis* sp., *H. punctata*, and *Ixodes trianguliceps* were found on the calomyscid hosts [57,59], but no tick was recorded on the new species of *Calomyscus cf. bailwardi* from the southern part of the Zagros mountains. The coevolution of host and parasite is very important in the origin of new species and new tick-borne diseases [7,8].

The detection of *Rhipicephalus turanicus* on the Norway rat (*Rattus norvegicus*) in urbanized areas of Shiraz and *Haemaphysalis* spp. on the endemic rodent *Calomyscus cf. bailwardi* in mountainous peri-urban habitats suggests potentially important ecological and epidemiological intersections. *Rattus norvegicus*, a

highly synanthropic species, thrives in close proximity to human settlements, acting as a bridge host between natural and anthropogenic environments. The presence of *Rh. turanicus* on this rodent in Shiraz—a city with documented Crimean-Congo hemorrhagic fever (CCHF) virus circulation may raise concerns about urban spillover risks, as this tick species is a known vector for CCHF and other zoonotic pathogens. Meanwhile, *Calomyscus cf. bailwardi*, a native rodent adapted to mountainous terrain, likely serves as a maintenance host for *Haemaphysalis* ticks in ecotonal areas where natural habitats abut urban infrastructure. This interface between mountain ecosystems and human-populated zones facilitates tick-host interactions, potentially enabling *Haemaphysalis* spp. to transition from wildlife (e.g., rodents) to domestic animals or humans. The three-host life cycle of both *Rh. turanicus* and *Haemaphysalis* spp. further amplifies their role in pathogen transmission: immature stages feeding on rodents may molt and seek larger mammals, including livestock or humans, as adults. Evidence from livestock systems also indicates that multiple ixodid species may co-infest the same host and that non-random species associations can influence infestation intensity, which is relevant for designing effective control strategies [60]. While *Haemaphysalis* spp. in Iran are primarily associated with livestock pathogens such as *Theileria*, their presence on *Calomyscus* near urban areas underscores the need to investigate their potential role in transmitting emerging zoonoses, including rickettsial and borreliac infections. These findings emphasize the importance of peri-urban ecotones as hotspots for tick-borne disease emergence and advocate for targeted surveillance of ticks and pathogens in both urban Norway rat populations and endemic mountain rodents. Integrated strategies, including rodent population control, acaricide use in livestock, and public education on tick exposure risks, are critical to mitigate cross-species pathogen transmission in this region. Mixed infestations and non-random co-occurrence of ixodid tick species have been shown to influence infestation levels in livestock and should be considered in integrated tick control planning.

Conclusions

In conclusion, this study represents the first molecular characterization of *Haemaphysalis* and *Rhipicephalus* ticks infesting rodents in Iran. Our findings confirm the presence of *Rhipicephalus turanicus* on *Rattus* species. More significantly, we identified a distinct *Haemaphysalis* lineage parasitizing

Calomyscus cf. bailwardi, a rodent endemic to the Zagros Mountains. The considerable genetic divergence observed in both the COX1 and ITS2 genes suggests that this *Haemaphysalis* lineage may represent a cryptic species or a host- and region-specific ecotype. Further research, including comprehensive morphological and ecological studies, is warranted to fully elucidate the taxonomic status of this *Haemaphysalis* lineage and assess its potential role in the transmission of zoonotic pathogens within the region. This work underscores the importance of continued surveillance and molecular characterization of tick vectors to better understand and manage the risk of tick-borne diseases in Iran and beyond.

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Ethics approval and consent to participate

Ethics approval was obtained for all protocols from the Shiraz University of Medical Sciences ethics committee with reference / permit number of IR.SUMS.SCHEANUT.REC.1402.126.

All co-authors have seen and agreed with the manuscript's contents. All co-authors certify that the submission is an original work and is not under review with any other publications.

Availability of data and materials

The datasets generated and analyzed during the current study are available in the GenBank repository under accession numbers of MW831028-29 (*COX1*), PP301377 (*COX1*) and PP192773-74 (*ITS2*).

Authors' contributions

KA: Conceptualization, study design, validation, writing—review and editing. ZVB: laboratory work (DNA extraction, PCR, sequencing). BS: Conceptualization, study design, writing—review and editing. MDjMF: writing—review and editing, validation. AP: visualization, writing—review and editing. ZN: laboratory work (DNA extraction, PCR, sequencing). SSH: Funding acquisition, project administration, resources, data curation, fieldwork (tick collection), molecular analysis, supervision, validation, writing—original draft and review. All authors read and approved the final manuscript.

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Conflict of interest

No conflict of interest is declared.

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