



Diagnostic nucleotide variation among *Hyalomma* (Acari: Ixodida) species, and intraspecific COI diversity of *Hyalomma anatolicum* from Iran and neighboring countries

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ABSTRACT

Accurate discrimination of *Hyalomma* species is important for taxonomy and vector surveillance. Although mitochondrial COI has been widely used for tick identification, nucleotide variation relevant to species discrimination and regional genetic diversity remain insufficiently investigated. This study evaluated the discriminatory power of COI by identifying informative nucleotide positions among *Hyalomma* species and assessing the phylogeographic diversity of *H. anatolicum* at nucleotide as well as amino acid level. Because *H. anatolicum* provided the largest number of suitable sequences, 71 sequences from Iran, Iraq, Pakistan and Türkiye were selected for phylogenetic analysis, estimation of genetic diversity, neutrality tests and construction of a TCS haplotype network. The haplotype network showed patterns broadly consistent with genetic differentiation among the species examined, although it does not independently validate species boundaries. Within *H. anatolicum*, phylogenetic trees revealed no clear grouping by country of origin. Eleven haplotypes were detected, with moderate haplotype diversity ($H_d = 0.648$) and nucleotide diversity ($\pi = 0.0709$). A significantly negative Tajima's D value (-2.444 , $P < 0.01$) indicated departure from neutral expectations. The network also showed haplotype sharing among sequences from different countries, although interpretation of geographic structure was limited by the uneven geographic sampling. Overall, the results support the utility of COI for molecular identification of *Hyalomma* species and for population genetic studies of *H. anatolicum*. The candidate informative nucleotide positions may provide a reference for future validation and development of molecular assays for epidemiological and surveillance applications in Iran.

KEYWORDS

COI, haplotype network, *Hyalomma anatolicum*, Iran, phylogenetic analysis

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INTRODUCTION

Correct identification of tick species is essential for reliable ecological, epidemiological and evolutionary studies, as well as for understanding their role in the transmission of tick-borne pathogens (Nabian *et al.* 2024). Among hard ticks (family Ixodidae), the genus *Hyalomma* includes several species of considerable veterinary and medical importance. These species differ in their ecological preferences and vector competencies (Nabian *et al.* 2009). Members of the genus are widely distributed across Africa, southern Europe, the Middle East, and large parts of Asia, where they parasitize a broad range of domestic and wild vertebrates (Rahbari *et al.* 2007; Nabian and Rahbari 2008; Hekimoglu and Ozer 2017; Celina *et al.* 2023; Hussain *et al.* 2024a; Zhou *et al.* 2025). *Hyalomma* ticks have been associated with several pathogens of veterinary and public health significance, including *Theileria* spp., *Babesia* spp., *Anaplasma*



spp., *Rickettsia* spp. and *Coxiella burnetii* (Moshaverinia *et al.* 2009; Hekimoglu and Ozer, 2017; Abdoli *et al.* 2021; Choubdar *et al.* 2021). Most notably, they are recognized as the principal vectors of Crimean-Congo haemorrhagic fever virus (CCHFV), which places them among the most epidemiologically important tick genera worldwide (Sharifinia *et al.* 2015; Kumar *et al.* 2020; Maghsood *et al.* 2020).

Species discrimination within *Hyalomma* remains difficult. Many taxa share overlapping morphological characters, and reliable identification is particularly challenging in immature stages or damaged specimens (Estrada-Peña *et al.* 2018). Even in adult ticks, species discrimination often requires detailed examination of subtle morphological characters, such as the basis capituli, scutum, and genital aperture (Shi *et al.* 2025). Misidentification can distort estimates of species distribution, ecological associations and the interpretation of vector-related studies. Molecular markers have therefore become valuable complementary tools. Mitochondrial genes such as mitochondrial *cytochrome c oxidase subunit I* (COI) and 16S rDNA, together with the nuclear ribosomal marker ITS2, are now widely used for species discrimination and phylogenetic analysis in this genus (Shi *et al.* 2025). In Iran, molecular evidence has also helped resolve taxonomic uncertainties within *Hyalomma* spp. complexes (Hosseini-Chegeni *et al.* 2019). Therefore, combining morphological observations with molecular data represents a more reliable approach for resolving species boundaries and improving tick surveillance (Shi *et al.* 2025).

Among the available markers, the COI gene is widely regarded as the primary DNA barcoding region because of its high discriminatory power and broad applicability. COI sequences have been used successfully to support morphological identifications, to separate closely related species and to reconstruct phylogenetic relationships in ticks (Hernández-Triana *et al.* 2026). Comparative studies have suggested that COI often provides higher resolution for species-level discrimination than some other mitochondrial markers, including 16S and 12S rDNA, supporting its widespread application in molecular identification (Low *et al.* 2015; Abouelhassan *et al.* 2019). At the same time, geographically structured lineages and previously unrecognized evolutionary relationships have been reported in several tick taxa, underlining the need for regional COI reference datasets (Zheng *et al.* 2024).

However, available molecular data on *Hyalomma* spp. from Iran and neighboring countries remain limited and have not yet been integrated into a comprehensive comparative framework. Previous studies have not yet combined species-level analysis of COI sequences among Iranian *Hyalomma* species with an evaluation of genetic diversity and phylogenetic relationships in *H. anatolicum* populations across Iran and neighboring countries. The present study therefore aimed to characterize informative COI nucleotide positions among *Hyalomma* species reported from Iran, and to examine the phylogenetic relationships, haplotype diversity and intraspecific genetic variation of *H. anatolicum* across Iran and neighbouring countries. *Hyalomma anatolicum* was selected for the intraspecific geographic analysis based on the availability of the largest number of eligible COI sequences and the broadest geographic coverage in the dataset.

MATERIAL AND METHODS

Data retrieval and sequence filtering

All publicly available mitochondrial *cytochrome c oxidase subunit I* (COI) sequences of the genus *Hyalomma* were retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI) on 17 June 2026. The search included all available COI records from Iran and neighboring countries (Iraq, Türkiye, Pakistan, Turkmenistan, Azerbaijan, Armenia, and Afghanistan). Retrieved records were subsequently screened based on predefined inclusion and exclusion criteria. An overview of the study workflow is presented in Figure 1.

Only sequences meeting the predefined inclusion criteria were included in this study. Eligible sequences were required to correspond to the COI gene (annotated as COI, CO1, or COX1), contain complete information on species identification and country of origin, be of sufficient sequence quality, and have adequate length for multiple sequence alignment. Records corresponding to complete mitochondrial genomes, whole genomes, sequences containing multiple genetic markers, ribosomal genes

(16S, 12S, 18S, and 28S rRNA), nuclear markers (ITS1, ITS2, and 5.8S), other mitochondrial genes (including CytB, ND4, ND5, ATP6, and COII), records lacking essential information (e.g. unidentified species or country of origin), excessively short sequences, low-quality sequences, and duplicate records were excluded. For *H. anatolicum*, 96 sequences met the initial inclusion criteria. Before the final intraspecific analyses, 25 sequences were further excluded because of exact or near identical sequence duplication (21 sequences; one representative was retained from each identical group) or insufficient sequence length and overlap for reliable multiple sequence alignment (4 sequences: MZ411453.1, MZ411456.1, MZ411457.1, and MZ411458.1). Thus, 71 *H. anatolicum* sequences were retained for the final intraspecific analyses. The accession numbers and specific exclusion criteria for the 25 excluded sequences are provided in Table 1. To ensure the reliability of the geographic and taxonomic information associated with the retrieved GenBank records, the corresponding published articles of the accession numbers were also examined. Details of the verified sequences, including their accession numbers, country, and associated references, are provided in Table 2. The species identity and country of origin of the retrieved sequences were accepted as reported in the corresponding GenBank records. No independent morphological or molecular identification was performed in the present study. The retrieved sequences were subsequently included in the phylogenetic analysis to assess their relationships among the examined *Hyalomma* species (Supplementary File 3). For the interspecific analysis, all eligible COI sequences reported from Iran were retained without additional subsampling. Based on the predefined inclusion and exclusion criteria, 33 sequences representing nine *Hyalomma* species were included. Thus, the number of sequences included for each species reflected the total number of eligible sequences available for that species in the Iranian dataset, rather than a selected representative subset. Only the nine *Hyalomma* species represented by eligible COI sequences from Iran were included in the interspecific analysis; the remaining six species were represented only by sequences from neighboring countries and were therefore not included in this analysis. In addition, because *H. anatolicum*, which had the largest number of sequences and the broadest geographic representation in the dataset, was selected for intraspecific analysis, including phylogenetic reconstruction, genetic diversity assessment, and haplotype network analysis. Finally, the eligible sequences were exported in FASTA format for subsequent analyses.

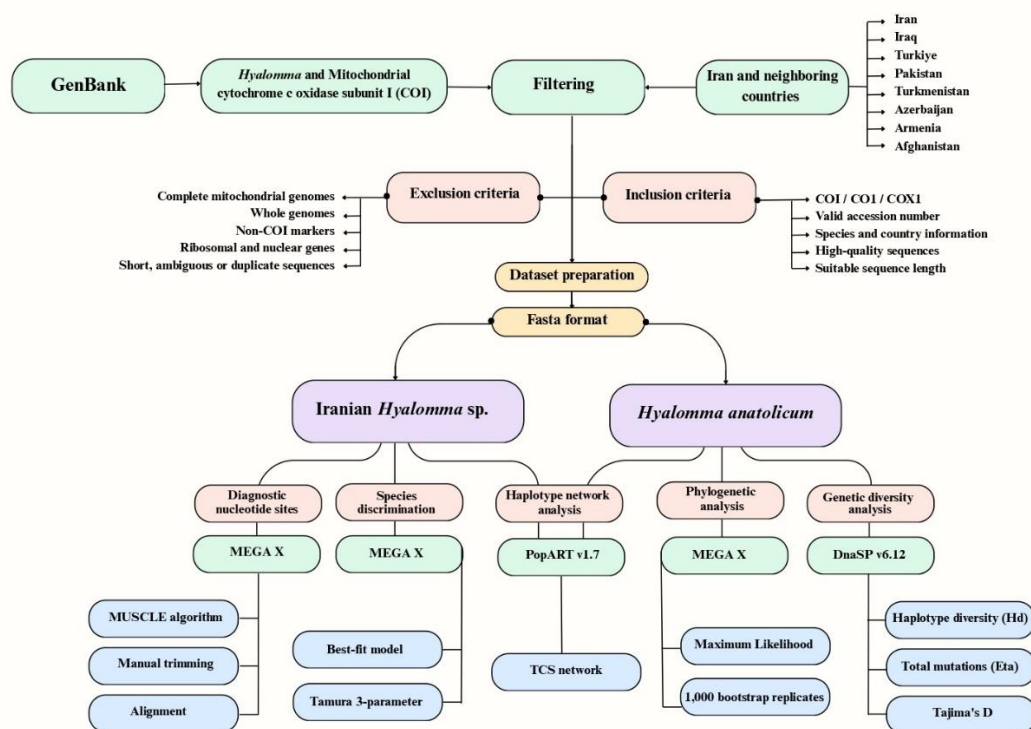


Figure 1. Workflow of the study.

Sequence alignment and identification of discriminatory sites

The selected sequences were imported into MEGA X version 10.0.4 (Kumar *et al.* 2018), and multiple sequence alignment was performed using the MUSCLE algorithm with default parameters (Edgar 2004). Alignment quality was manually inspected, and terminal regions containing multiple gaps or segments present in only a limited number of sequences were manually trimmed to retain a homologous region shared among all sequences. The final trimmed alignment was subsequently used for phylogenetic reconstruction, genetic diversity analysis, haplotype network analysis, and identification of representative discriminatory nucleotide positions. The representative nucleotide positions were selected based on informative nucleotide variation and their potential contribution to species discrimination among the examined species. For each nucleotide position, Discrimination (%) was calculated as the percentage of pairwise comparisons among the nine *Hyalomma* species that yielded different nucleotide states (total possible pairs = 36). Positions represented by species with ≤ 2 sequences were regarded as preliminary observations only.

Sequence quality control

As a quality control step, the aligned COI sequences were translated using the invertebrate mitochondrial genetic code and examined for premature stop codons and apparent translation anomalies. Where necessary, the reading frame was adjusted by correcting one or two leading nucleotides based on alignment with homologous COI sequences. The sequences were then retranslated and checked for premature stop codons before downstream analyses. The aligned sequences were manually inspected for conspicuous sequence quality anomalies or unusual alignment patterns before the final alignment was used for downstream analyses. The phylogenetic placement of the sequences was also examined to identify any obvious anomalous or outlier sequences. Sequence divergence alone was not considered sufficient evidence for misidentification or the presence of a nuclear mitochondrial DNA segment (NUMT).

Evolutionary model selection

The best-fit nucleotide substitution model was determined using the Find Best DNA/Protein Models (ML) function implemented in MEGA X. Candidate models were evaluated based on the Bayesian Information Criterion (BIC) and the corrected Akaike Information Criterion (AICc). The model with the lowest BIC value was selected as the optimal substitution model for phylogenetic reconstruction. Accordingly, the Tamura 3-parameter (T92) model was selected for all subsequent phylogenetic analyses (Tamura 1992).

Amino acid sequence analysis

The final COI nucleotide alignment was translated into amino acid sequences using the invertebrate mitochondrial genetic code implemented in MEGA X. The translated amino acid sequences were aligned using the MUSCLE algorithm. Amino acid residues corresponding to the selected discriminatory nucleotide positions and variable nucleotide positions identified within each *Hyalomma* species were examined to determine whether the observed nucleotide substitutions resulted in synonymous or non-synonymous changes. Nucleotide positions were numbered according to the final multiple-sequence alignment. For the positions reported in Table 2, the alignment-column numbers were identical to the corresponding nucleotide coordinates in the partial COI reference sequence KX911989.1 (*H. excavatum*). These positions do not represent coordinates within the complete COI coding sequence.

Phylogenetic analysis

Phylogenetic relationships among the selected sequences were reconstructed using the Maximum Likelihood (ML) method implemented in MEGA X under the Tamura 3-parameter (T92) substitution model. Branch support was assessed using 1,000 bootstrap replicates (Kumar *et al.* 2018).

Genetic diversity analysis

To evaluate genetic diversity, the trimmed sequence alignment was imported into DnaSP version 6.12. The following genetic diversity indices were calculated: number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), number of polymorphic sites (S), total number of mutations (Eta), average number of nucleotide differences (k), and Tajima's D statistic (Tamura 1992). The statistical

significance of Tajima's D was assessed using the built-in significance test implemented in DnaSP (Rozas *et al.* 2017). All analyses were performed using the final trimmed alignment.

Haplotype network analysis

To investigate haplotype relationships, the trimmed sequence alignment was imported into PopART version 1.7. Information on the country of origin for each sequence was incorporated into the analysis using a traits file. A haplotype network was constructed using the Templeton Crandall Singh (TCS) algorithm (Clement *et al.* 2002). In the resulting network, the size of each circle represented haplotype frequency, whereas the color of each segment indicated the country of origin of the sequences comprising each haplotype (Leigh and Bryant 2015).

RESULTS

Distribution of retained COI sequences among *Hyalomma* species

The distribution of mitochondrial COI sequences retained after the initial sequence filtering step is summarized in Table 1. *Hyalomma anatolicum* was represented by the largest number of sequences ($n = 96$), whereas *H. excavatum* showed the widest geographical distribution among the investigated countries. Because *H. anatolicum* was represented by the largest number of sequences and provided broad geographic coverage, it was selected for subsequent intraspecific analyses.

Table 1. Distribution of mitochondrial COI sequences of *Hyalomma* species retained after initial sequence filtering from Iran and neighboring countries, retrieved from GenBank.

<i>Hyalomma</i> species	Iran	Türkiye	Iraq	Pakistan	Turkmenistan	Total
<i>H. marginatum</i>	9	26	3	3	0	41
<i>H. asiaticum</i>	8	0	1	4	3	16
<i>H. anatolicum</i>	7	4	37	48	0	96
<i>H. rufipes</i>	3	1	1	0	0	5
<i>H. excavatum</i>	2	4	11	18	1	36
<i>H. scupense</i>	0	0	4	16	0	20
<i>H. detritum</i>	1	1	4	3	0	9
<i>H. dromedarii</i>	1	1	4	15	0	21
<i>H. schulzei</i>	1	0	2	0	0	3
<i>H. aegyptium</i>	1	13	0	0	0	14
<i>H. turanicum</i>	0	0	8	4	0	12
<i>H. truncatum</i>	0	0	2	0	0	2
<i>H. impeltatum</i>	0	2	1	0	0	3
<i>H. isaaci</i>	0	0	0	8	0	8
<i>H. kumari</i>	0	0	0	3	0	3
Total	33	52	78	122	4	289

Species discrimination based on informative nucleotide sites

Representative informative nucleotide positions were identified from the mitochondrial COI alignment that may contribute to species discrimination among the examined Iranian *Hyalomma* species (Table 2). Position 327 exhibited a three-state polymorphism, with *H. anatolicum* and *H. excavatum* sharing C, *H. asiaticum* and *H. schulzei* sharing A, and the remaining species predominantly showing T. Positions 347 and 356 showed nucleotide states that were unique to *H. aegyptium*, with C and G, respectively. Position 389 showed an informative nucleotide pattern that partitioned the taxa into three nucleotide-defined groups: *H. anatolicum* and *H. excavatum* (C), *H. asiaticum*/*H. schulzei* (A), and the remaining species (T). Additional informative positions, including positions 288, 290, 365, 371, and 411, showed variation that may contribute to the discrimination of closely related species. Importantly, *H. anatolicum* and *H. excavatum* had identical nucleotide states at all 10 selected positions. Therefore, these positions should be considered candidate informative sites for future molecular assay development rather than definitive diagnostic characters, and their diagnostic utility requires independent validation.

Table 2. Informative COI nucleotide positions observed among *Hyalomma* species represented in the Iranian sequence dataset.

Species	Position in alignment (KX911989.1)										N*
	288	290	327	347	353	356	365	371	389	411	
<i>H. anatolicum</i>	T	A	C	A	A	A	T	A	C	A	(7)
<i>H. marginatum</i>	T	A	T	A	A	A	C	A	T	T	(9)
<i>H. asiaticum</i>	C	T	A	A	C	A	T	C	A	A	(8)
<i>H. excavatum</i>	T	A	C	A	A	A	T	A	C	A	(2)
<i>H. dromedarii</i>	C	T	T	A	A	A	C	A	T	A	(1)
<i>H. rufipes</i>	T	A	T	A	A	A	C	A	T	T	(3)
<i>H. detritum</i>	C	T	T	A	A	A	C	C	T	A	(1)
<i>H. schulzei</i>	C	T	A	A	C	A	T	C	A	A	(1)
<i>H. aegyptium</i>	C	T	T	C	T	G	C	A	T	A	(1)
Discrimination (%)	55.56	55.56	66.67	22.22	55.56	22.22	55.56	50.00	66.67	38.89	(33)

N*: Number of eligible Iranian COI sequences included in the interspecific analysis for each species.

Note: Positions are 1-based alignment column numbers in the final multiple-sequence alignment (Supplementary File 3). Because the reference sequence KX911989.1 (*H. excavatum*) was placed at the beginning of the alignment without leading gaps, these column numbers are identical to the nucleotide coordinates within the partial COI sequence of KX911989.1. They do not correspond to positions in the complete COI coding sequence. For species represented by very few sequences, the observed nucleotide states were treated as preliminary observations and not as species-level diagnostic characters.

Amino acid conservation at informative COI sites

Translation of the representative informative COI nucleotide sites showed that amino acid residues were conserved at most informative sites among the examined *Hyalomma* species (Table 3). Of the 10 representative informative nucleotide sites, eight corresponded to synonymous nucleotide substitutions. In contrast, only two sites (reference nucleotide positions 327 and 411) resulted in amino acid substitutions. At nucleotide position 327 (amino acid position 109), leucine (L) was substituted by methionine (M) in *H. asiaticum* and *H. schulzei*. Similarly, at nucleotide position nucleotide 411 (amino acid position 137), methionine (M) was substituted by leucine (L) in *H. marginatum* and *H. rufipes*. These findings indicate that the selected nucleotide variation within the COI barcode region is largely conserved at the protein level.

Table 3. Correspondence between representative informative COI nucleotide sites and translated amino acid residues in *Hyalomma* species reported from Iran.

Position in alignment (KX911989.1)	Nucleotide position	288	290	327	347	353	356	365	371	389	411
	Codon (reference)	286–288	289–291	325–327	346–348	352–354	355–357	364–366	370–372	388–390	409–411
	AA position	96	97	109	116	118	119	122	124	130	137
<i>H. anatolicum</i>	L	L	L	L	T	W	T	P	L	H	M
<i>H. marginatum</i>	L	L	L	L	T	W	T	P	L	H	L
<i>H. asiaticum</i>	L	L	M	T	W	T	P	L	H	M	M
<i>H. excavatum</i>	L	L	L	T	W	T	P	L	H	M	M
<i>H. dromedarii</i>	L	L	L	T	W	T	P	L	H	M	M
<i>H. rufipes</i>	L	L	L	T	W	T	P	L	H	L	L
<i>H. detritum</i>	L	L	L	T	W	T	P	L	H	M	M
<i>H. schulzei</i>	L	L	M	T	W	T	P	L	H	M	M
<i>H. aegyptium</i>	L	L	L	T	W	T	P	L	H	M	M
AA conservation	C	C	V	C	C	C	C	C	C	C	V

Abbreviations: AA, amino acid; C, conserved; L, leucine; M, methionine; T, threonine; W, tryptophan; P, proline; H, histidine; V, variable. Conserved indicates identical amino acid residues across all examined *Hyalomma* species, whereas Variable indicates amino acid replacement in one or more species. Synonymous indicates a nucleotide substitution that does not alter the encoded amino acid, whereas non-synonymous indicates a nucleotide substitution that results in an amino acid replacement. Positions refer to the alignment column numbers, which are identical to the coordinates in the reference sequence KX911989.1.

The translated amino acid sequences showed that the majority of informative nucleotide substitutions did not alter the encoded amino acid residues, whereas only two positions produced conservative amino acid substitutions. The corresponding amino acid patterns are presented in Table 4.

Table 4. Intraspecific nucleotide variation and corresponding amino acid changes identified within *Hyalomma* species in the Iranian COI dataset.

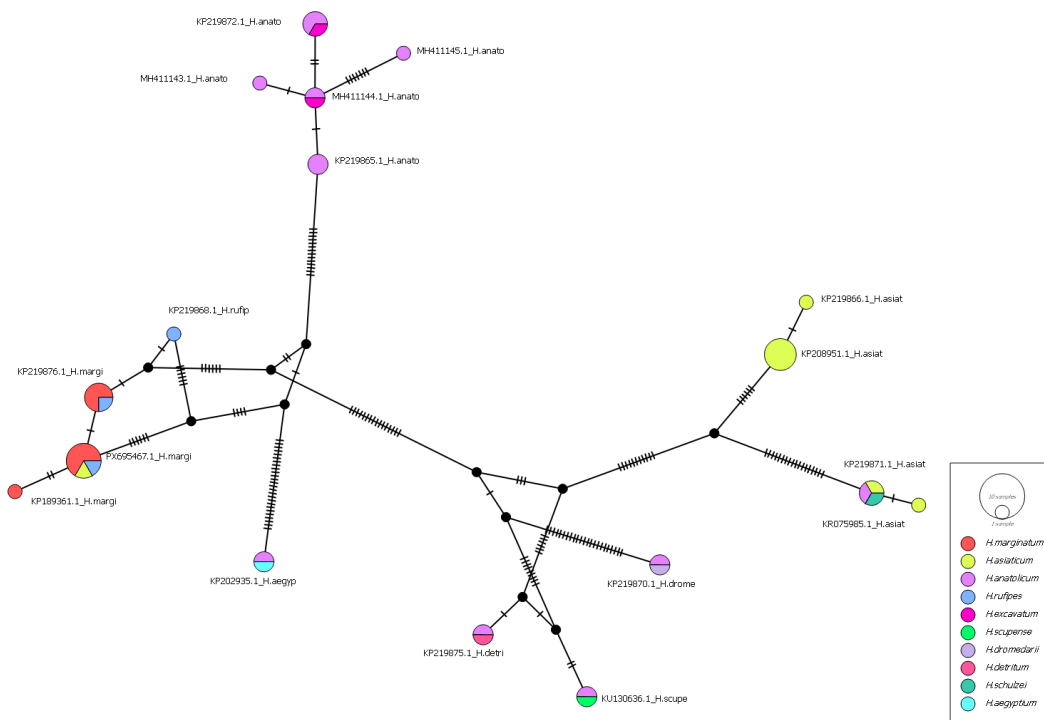
Species	Nt Pos.	Seq- IDs	V(N)	Seq- IDs	M(N)	AA Pos.	M(AA)	V(AA)
<i>H. anatolicum</i> (nt 285-716)	314	HAN1	T	HAN2-7	A	105	L	-
	326	HAN6,7	T	HAN1-5	C	109	L	-
	341	HAN1	A	HAN2-7	G	114	A	-
	359	HAN4,5	G	HAN1-3,6,7	A	120	V	-
	368	HAN1	C	HAN2-7	T	123	P	-
	410	HAN4,5	C	HAN1-3,6,7	T	137	M	-
	416	HAN1	A	HAN2-7	C	139	I	-
	440	HAN1	T	HAN2-7	C	147	A	-
	479	HAN1	C	HAN2-7	T	160	I	-
	581	HAN1	A	HAN2-7	T	194	L	-
	593	HAN3	G	HAN1,2,4-7	A	198	I	-
	629	HAN1	C	HAN2-7	T	210	S	-
	650	HAN1	C	HAN2-7	T	217	G	-
	656	HAN1	A	HAN2-7	T	219	D	-
	659	HAN5	C	HAN1-4, 6,7	T	220	P	-
	671	HAN3	C	HAN1,2,4-7	T	224	Q	-
	695	HAN3-5	A	HAN1,2,4,6,7	G	232	H	-
<i>H. marginatum</i> (nt 285-464)	285	HM7	C	HM1-6, 8-9	T	95	W	R
	300	HM7	C	HM1-6, 8-9	T	100	S	P
	590	HM7	A	HM1-6, 8-9	G	197	A	-
<i>H. asiaticum</i> (nt 270-688)	302	HAS5,6	A	HAS1-4,7,8	T	101	L	-
	305	HAS7,8	T	HAS1-6	C	102	F	-
	332	HAS7	C	HAS1-6,8	T	111	E	-
	335	HAS5,6	G	HAS1-4,7,8	A	112	S	-
	338	HAS5,6	A	HAS1-4,7,8	G	113	G	-
	344	HAS5,6	C	HAS1-4,7,8	T	115	G	-
	350	HAS5,6	C	HAS1-4,7,8	T	117	G	-
	398	HAS5,6	A	HAS1-4,7,8	T	133	P	-
	404	HAS5,6	T	HAS1-4,7,8	A	135	V	-
	407	HAS5,6	A	HAS1-4,7,8	G	136	D	-
	422	HAS5,6	C	HAS1-4,7,8	T	141	S	-
	440	HAS5,6	T	HAS1-4,7,8	C	147	A	-
	443	HAS5,6	T	HAS1-4,7,8	C	148	S	-
	449	HAS5,6	C	HAS1-4,7,8	T	150	I	-
	483	HAS5,6	G	HAS1-4,7,8	A	161	I	V
	485	HAS5,6	C	HAS1-4,7,8	T	162	N	-
	506	HAS5,6	G	HAS1-4,7,8	A	169	T	S
	508	HAS5,6	G	HAS1-4,7,8	C	170	M	-
	533	HAS6	T	HAS1-5,7,8	C	178	W	-
	557	HAS5,6	T	HAS1-4,7,8	C	186	L	-
	564	HAS5,6	C	HAS1-4,7,8	T	188	L	-
	566	HAS5,6	T	HAS1-4,7,8	A	189	L	-
	575	HAS5,6	T	HAS1-4,7,8	C	192	P	-
	578	HAS5,6	A	HAS1-4,7,8	G	193	V	-
	587	HAS5,6	T	HAS1-4,7,8	C	196	G	-
	603	HAS5,6	T	HAS1-4,7,8	C	201	L	-
	623	HAS5,6	T	HAS1-4,7,8	C	208	N	-
	659	HAS5,6	T	HAS1-4,7,8	C	220	P	-
<i>H. rufipes</i> (nt 270-728)	275	HR1	C	HR2,3	T	92	M	-
	281	HR2	C	HR1,3	A	94	F	-
	296	HR2	C	HR1,3	T	99	P	-
	314	HR2	A	HR1,3	G	105	L	-
	338	HR2	G	HR1,3	A	113	G	-

Table 4. Continued.

Species	Nt Pos.	Seq- IDs	V(N)	Seq- IDs	M(N)	AA Pos.	M(AA)	V(AA)
<i>H. rufipes</i> (nt 270-728)	275	HR1	C	HR2,3	T	92	M	-
	281	HR2	C	HR1,3	A	94	F	-
	296	HR2	C	HR1,3	T	99	P	-
	314	HR2	A	HR1,3	G	105	L	-
	338	HR2	G	HR1,3	A	113	G	-
	383	HR2	T	HR1,3	C	128	L	-
	404	HR2	G	HR1,3	A	135	V	-
	410	HR2	C	HR1,3	T	137	L	-
	428	HR2	G	HR1,3	A	143	H	-
	431	HR2	C	HR1,3	T	144	L	-
	458	HR2	T	HR1,3	G	153	A	-
	461	HR2	T	HR1,3	C	154	I	-
	599	HR1,2	T	HR3	C	200	M	-
	605	HR2	A	HR1,3	G	202	L	-
	626	HR2	C	HR1,3	T	209	T	-
	641	HR2	T	HR1,3	C	214	P	-
	717	HR2	T	HR1,3	A	239	I	-

Abbreviations: Nt Pos., nucleotide position; M(N), major nucleotide state; V(N), variant nucleotide state; Seq-IDs, coded sequence identifiers of sequences carrying the variant nucleotide; AA Pos., amino acid position; M(AA), major amino acid residue; V(AA), variant amino acid residue. Sequence ID codes: HAN, *H. anatolicum*; HM, *H. marginatum*; HAS, *H. asiaticum*; HR, *H. rufipes*. Sequence IDs correspond to the listed GenBank accession numbers in the same order: HAN1-HAN7 (MH411145.1, MH411144.1, MH411143.1, KP219872.1, KP219867.1, KP219865.1, KP219862.1); HM1-HM9 (PX695467.1, PX695466.1, MH411142.1, KP219876.1, KP219874.1, KP219863.1, KP189361.1, MG557555.1, KP100418.1); HAS1-HAS8 (KP208951.1, PQ838316.1, KP219864.1, KP219869.1, KP219871.1, KR075985.1, KP219866.1, KP219860.1); HR1-HR3 (KP231210.1, KP219868.1, KP219873.1).

Furthermore, the haplotype network constructed from Iranian *Hyalomma* COI sequences revealed that, although many haplotypes were species-specific, some haplotypes were shared among species (Fig. 2).



Figures 2. TCS haplotype network showing interspecific relationships among Iranian *Hyalomma* species based on mitochondrial COI sequences.

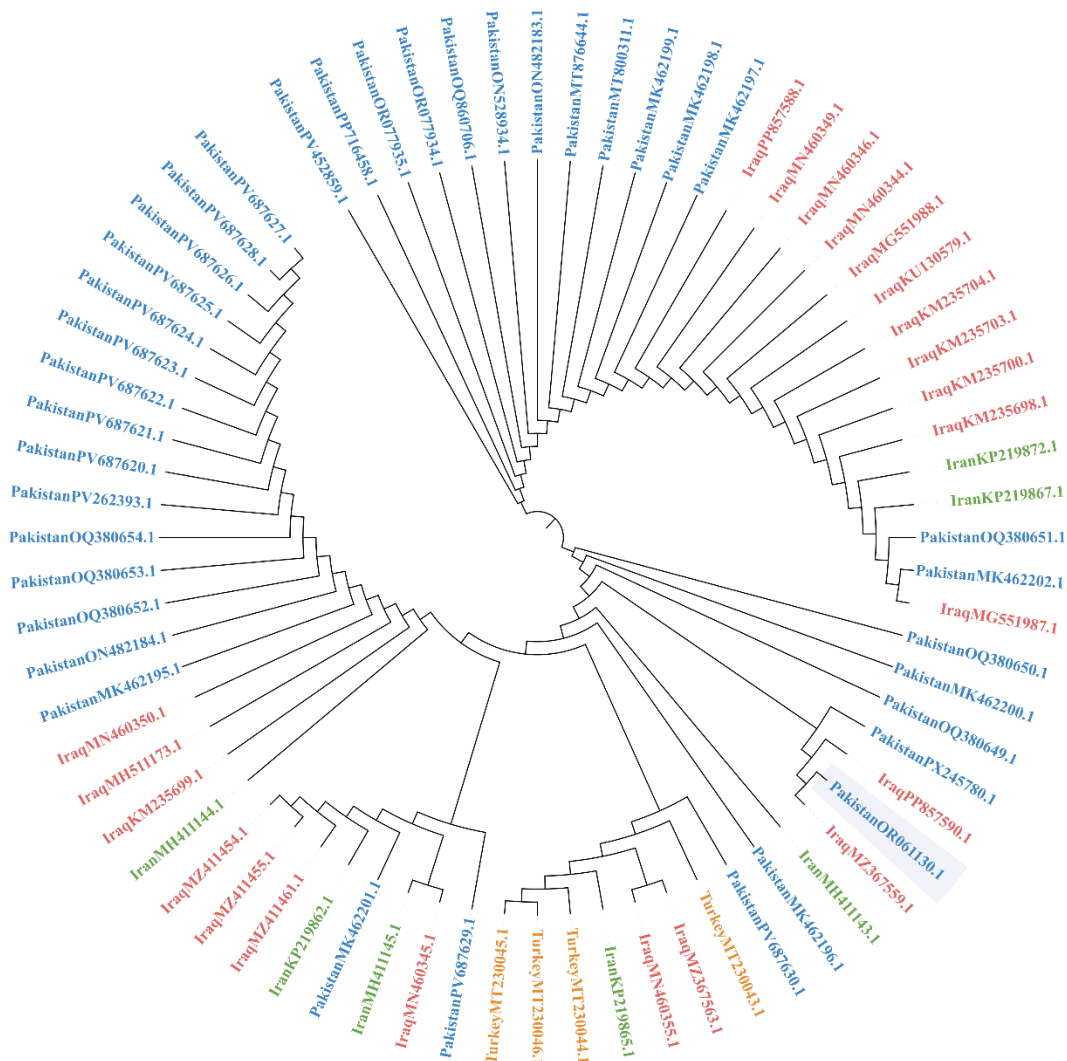
Each circle represents a unique haplotype, with circle size proportional to the number of sequences. Connecting lines indicate mutational steps between haplotypes. Colors represent the different Iranian *Hyalomma* species included in the analysis.

Characteristics of the final *H. anatolicum* dataset

After applying the inclusion and exclusion criteria, a total of 71 COI sequences of *H. anatolicum* were retained for subsequent analyses. These sequences originated from four countries, namely Iran, Iraq, Pakistan, and Türkiye. Multiple sequence alignment followed by manual trimming generated a final alignment of 747 bp sequences in length. Within this alignment, 131 polymorphic sites were identified.

Phylogenetic analysis

The Maximum Likelihood phylogenetic tree reconstructed under the Tamura 3-parameter (T92) substitution model resolved the analyzed sequences into several distinct clades (Figure 3). However, no clear clustering pattern according to the country of origin was observed. Sequences from Iran, Iraq, Pakistan, and Türkiye were intermingled within multiple clades, indicating the absence of an obvious geographic genetic structure among the analyzed populations.



Figures 3. Maximum Likelihood phylogenetic tree of *H. anatolicum* based on mitochondrial COI sequences.

The phylogenetic tree was reconstructed using the Tamura 3-parameter (T92) substitution model. Bootstrap support values (1,000 replicates) are shown at the nodes. Sequence labels indicate the GenBank accession number and country of origin.

Genetic diversity of *H. anatolicum*

Genetic diversity analysis identified 11 haplotypes among the 71 analyzed sequences. The haplotype diversity (H_d) and nucleotide diversity (π) were estimated at 0.648 and 0.0709, respectively. Tajima's D was significantly negative ($D = -2.44$, $P < 0.01$), indicating a significant departure from neutrality. The remaining genetic diversity indices are summarized in Table 5.

Table 5. Genetic diversity indices of *H. anatolicum* based on mitochondrial COI sequences.

Parameter	Value
Number of sequences (n)	71
Sequence length (bp)	747
Number of polymorphic sites (S)	131
Total mutations (Eta)	175
Number of haplotypes (h)	11
Haplotype diversity ($H_d \pm SD$)	0.648 ± 0.036
Nucleotide diversity (π)	0.07091
Average nucleotide differences (k)	10.636
Tajima's D	-2.444
P-value	<0.01

Haplotype network analysis

Consistent with the genetic diversity analysis, the TCS haplotype network identified 11 haplotypes among the analyzed *H. anatolicum* sequences (Fig. 4). Two major haplotypes comprised sequences from multiple countries, whereas the remaining haplotypes were represented by relatively few sequences (mostly singletons). The two dominant haplotypes were shared among sequences from Iran, Iraq, Pakistan, and Türkiye, while some rare haplotypes were restricted to individual countries. Overall, the haplotype network was consistent with the phylogenetic tree and showed no clear geographic clustering of sequences by country of origin.

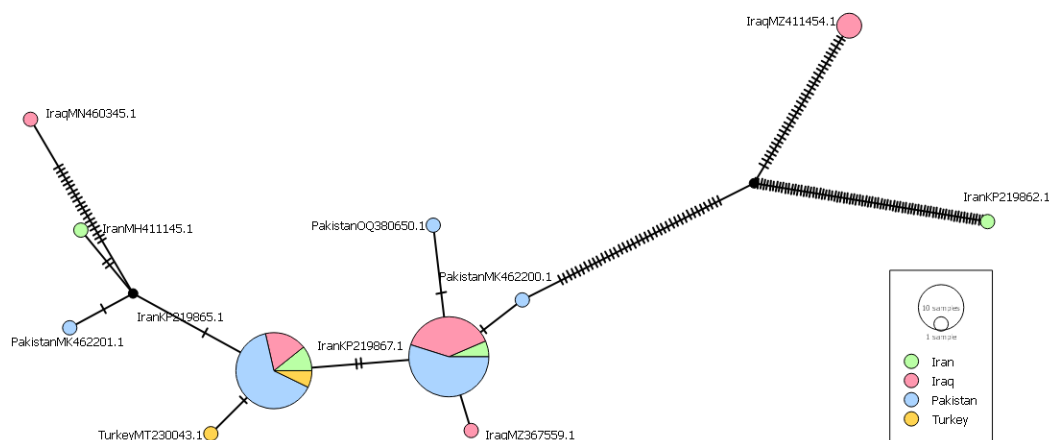


Figure 4. TCS haplotype network of *H. anatolicum* based on mitochondrial COI sequences.

Each circle represents a unique haplotype, with circle size proportional to the number of sequences. Connecting lines indicate mutational steps between haplotypes. Colors represent the country of origin of the analyzed sequences.

DISCUSSION

The results of this study indicate that the mitochondrial COI gene can provide useful information for species-level and population-level analyses in *Hyalomma*. The identification of representative informative nucleotide positions, together with phylogenetic reconstruction, haplotype network analysis, and genetic diversity assessment, provides complementary evidence for the utility of COI in molecular

and evolutionary studies of *Hyalomma* ticks in Iran and neighboring countries. Similar conclusions have been reached in recent work on *Hyalomma* (Jomli et al. 2025; Motevasseli et al. 2026). The nucleotide variation identified at the selected positions revealed informative patterns that can contribute to the discrimination of *Hyalomma* species reported from Iran, although 10 selected positions do not independently distinguish all species represented in Table 2. Notably, 10 selected positions showed identical nucleotide profiles in three species pairs: *H. anatolicum*-*H. excavatum*, *H. asiaticum*-*H. schulzei* and *H. marginatum*-*H. rufipes*. However, the limited number of available sequences for some species means that these nucleotide patterns should be considered preliminary candidates rather than evidence of species-level conservation. When these nucleotide positions were translated, most of the substitutions were synonymous and did not alter the amino acid sequence. Only two of the selected nucleotide positions resulted in non-synonymous amino acid replacements, with leucine being substituted by methionine at one position and methionine by leucine at another. Thus, most of the nucleotide variation at the selected positions did not alter the encoded amino acid residues. Overall, the present study should be regarded as a preliminary investigation that provides candidate molecular markers for *Hyalomma* species discrimination and requires further experimental validation through laboratory-based molecular assays.

A similar predominance of synonymous changes was observed among the intraspecific variable sites, with only a small number of amino-acid substitutions detected within species. The informative nucleotide patterns identified in the COI gene may represent candidate targets for future molecular assay development, including allele-specific PCR approaches, for the identification of closely related *Hyalomma* species. However, their diagnostic utility requires independent validation using larger, independently identified, and geographically diverse populations before such applications can be established. The haplotype network constructed from Iranian *Hyalomma* COI sequences showed broad genetic differentiation among species. Most species formed discrete clusters in the TCS haplotype network. Although some haplotypes were shared among different species, these shared haplotypes were limited in number and did not indicate extensive haplotype sharing across the dataset. This pattern is consistent with genetic differentiation among the sampled *Hyalomma* species; however, the TCS haplotype network alone does not independently validate species boundaries. Moreover, the haplotype network and the selected discriminatory nucleotide positions were derived from the same COI dataset and therefore should be considered complementary rather than independent lines of evidence for species delimitation. The result is consistent with the basic principle of DNA barcoding, namely that interspecific divergence in the COI gene usually exceeds intraspecific variation (Zhang and Zhang 2014).

The uneven number of available sequences among species almost certainly reflects differences in research effort and epidemiological importance rather than true biological rarity. *Hyalomma anatolicum*, one of the most common and medically significant ticks in the region, has attracted the greatest attention and therefore accounts for the largest share of sequences. *Hyalomma excavatum*, by contrast, appears across every country in the dataset, a distribution that matches its broad ecological tolerance. The higher species richness recorded in Pakistan and Iraq is probably linked to greater environmental heterogeneity, abundant hosts and a larger number of molecular studies conducted in those countries. The apparent paucity of species in Turkmenistan is more likely to result from limited sampling and fewer publicly available sequences than from genuinely lower diversity (Poulin et al. 2019; Khan et al. 2025; Motevasseli et al. 2026). Because *H. anatolicum* provided both the largest number of sequences and the widest geographic coverage, it was selected for the subsequent intraspecific analyses. These findings align with earlier studies that have demonstrated the utility of COI for DNA barcoding and species discrimination in *Hyalomma* and other taxa (Hebert et al. 2003; Roth et al. 2019; Jomli et al. 2025). Phylogenetic analysis of the available *H. anatolicum* sequences did not reveal clear clustering according to country of origin; however, this pattern should be interpreted cautiously because sequence availability was highly uneven among countries. Sequences from Iran, Iraq, Pakistan and Türkiye were largely intermixed within the same clades, suggesting weak geographic structure at the level of the mitochondrial COI marker. Similar patterns of shared haplotypes across distant localities have been reported previously for this species and are commonly interpreted as evidence of historical or ongoing gene flow (Khan et al. 2025). Nevertheless,

distinguishing between these evolutionary processes would require broader geographic sampling and the inclusion of additional genetic markers.

The 131 polymorphic sites detected among the *H. anatolicum* sequences, together with relatively high haplotype and nucleotide diversity, point to considerable genetic variation within the sampled dataset. The significantly negative Tajima's D value observed in the pooled *H. anatolicum* dataset indicates a departure from neutral expectations and should be interpreted with caution. Because the sequences were unevenly distributed among countries and were obtained from publicly available GenBank records rather than from a standardized population sampling design, this result cannot be attributed specifically to demographic expansion, purifying selection, or other evolutionary processes without further evidence. Unequal geographic sampling and population structure may also influence neutrality statistics.

In addition, mitochondrial patterns may be affected by processes such as mitochondrial introgression or associations with maternally inherited endosymbionts. Mitochondrial introgression can result in mitochondrial haplotypes being shared across populations or even closely related species, potentially obscuring the demographic or phylogeographic history inferred from mitochondrial markers. Similarly, endosymbiotic bacteria such as *Wolbachia* can influence host mitochondrial variation through maternally linked inheritance and selective sweeps, potentially altering patterns of mitochondrial diversity and neutrality statistics (Cariou *et al.* 2017). However, the present study did not investigate mitochondrial introgression or *Wolbachia* infection directly; therefore, these processes should be considered as possible factors rather than demonstrated explanations for the negative Tajima's D value. Comparable results have been reported in earlier studies of *H. anatolicum* which also detected significantly negative Tajima's D values for COI and 16S rRNA and interpreted them as possible signatures of expansion (Moudgil *et al.* 2024; Khan *et al.* 2025). As noted by Wares (2010), however, neutrality indices alone are insufficient to distinguish demographic from selective explanations; additional markers and denser sampling are needed for firmer conclusions (Wares 2010).

The nonsynonymous substitutions identified in Table 4 were predominantly conservative. In *H. anatolicum*, the Leu-to-Met substitution at amino acid position 109 and the Met-to-Leu substitution at position 137 involve hydrophobic amino acids with similar physicochemical properties. Although COX1 is a membrane-embedded protein with functionally important regions, the specific structural or functional effects of these substitutions cannot be determined from sequence data alone. Further structural and experimental studies would be required to clarify their functional significance.

In the TCS network the majority of sequences fell into two common haplotypes, while the remaining haplotypes were rare and represented by only a few sequences. Specimens from Iran, Iraq, Pakistan and Türkiye occurred together inside the dominant haplotypes, and sequences from different countries were also scattered among the less frequent ones. Although haplotypes were shared among sequences from different countries, this pattern should not be interpreted as definitive evidence of weak geographic structure because the TCS network is sensitive to the composition and geographic balance of the sampled sequences. Similar patterns of widely shared haplotypes and weak phylogeographic signal have been described previously for this species (Khan *et al.* 2025) and for other ixodid ticks, where they are usually ascribed to host-mediated dispersal and ongoing gene flow (Kanduma *et al.* 2016; Amzati *et al.* 2018; Rain and Aslam 2025). Livestock movement and cross-border trade may therefore help maintain the genetic connectivity observed here, although direct tests of this idea will require broader sampling and data from additional mitochondrial and genome-wide markers.

Several limitations should be acknowledged. The analyses relied solely on publicly available COI sequences from GenBank, which produced uneven sample sizes across species and countries. In addition, the use of a single mitochondrial marker restricts the depth of evolutionary inference. Future work that combines further mitochondrial and nuclear loci with more extensive geographic coverage should yield a clearer picture of genetic diversity and phylogeographic structure in *Hyalomma*.

CONCLUSION

This study used publicly available COI sequences to examine molecular discrimination of *Hyalomma* species reported from Iran (up to June 2026) and to assess genetic diversity within *H. anatolicum* across

Iran and neighboring countries. Several informative nucleotide positions were identified that may contribute to species discrimination, while the corresponding amino-acid sequences remained largely conserved. Haplotype networks showed mostly species-specific clusters, consistent with genetic differentiation among the taxa examined. Within *H. anatolicum*, phylogenetic trees, diversity indices and the haplotype network all pointed to extensive sharing of genetic variation and little evidence of geographic structure across the region. The informative nucleotide positions reported here may serve as candidate targets for future SNP-based assays and as a reference for taxonomic and surveillance studies in Iran. Broader geographic sampling combined with additional mitochondrial and nuclear markers will be needed to clarify the evolutionary history and dispersal patterns of these ticks more fully.

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بررسی تمایز بین‌گونه‌ای *Hyalomma* (Acari: Ixodida) و تنوع ژنتیکی درون‌گونه‌ای *Hyalomma anatolicum* با استفاده از توالی‌های میتوکندریایی ژن COI در ایران و کشورهای همسایه

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چکیده

تفکیک دقیق گونه‌های *Hyalomma* برای مطالعات آرایه‌شناختی و پایش ناقلین اهمیت زیادی دارد. اگرچه ژن میتوکندریایی COI به‌طور گسترده برای شناسایی کتله‌ها مورد استفاده قرار گرفته است، تنوع نوکلئوتیدی مرتبط با تمایز گونه‌ای و الگوهای تنوع ژنتیکی منطقه‌ای آن کمتر مورد توجه قرار گرفته‌اند. در این مطالعه، جایگاه‌های نوکلئوتیدی تمایزدهنده در میان گونه‌های *Hyalomma* شناسایی و تنوع ژنتیکی *Hyalomma anatolicum* در سطوح نوکلئوتیدی و اسیدآمینه‌ای بررسی شد. از آنجا که *H. anatolicum* بیشترین تعداد توالی‌های مناسب را در اختیار داشت، ۷۱ توالی از ایران، عراق، پاکستان و ترکیه برای تحلیل‌های فیلوژنتیکی، برآورد تنوع ژنتیکی، آزمون خنثی بودن و ساخت شبکه هاپلوتایپی TCS انتخاب شدند. چندین جایگاه نوکلئوتیدی تمایزدهنده در میان گونه‌های *Hyalomma* گزارش شده از ایران شناسایی شد؛ با این حال، *H. anatolicum* و *H. excavatum* در هر ۱۰ جایگاه بررسی شده حالت نوکلئوتیدی یکسانی داشتند. در *H. anatolicum*، درخت تبارشناختی گروه‌بندی مشخصی بر اساس کشور مبدأ نشان نداد. یازده هاپلوتایپ شناسایی شد که با تنوع هاپلوتایپی متوسط ($Hd = 0.648$) و تنوع نوکلئوتیدی ($\pi = 0.0709$) همراه بود. مقدار منفی و معنی‌دار آمار ($Tajima's D = -2.444$, $P < 0.01$) نشان‌دهنده انحراف از فرضیه خنثی بودن بود. شبکه هاپلوتایپی نیز اشتراک هاپلوتایپ میان توالی‌های کشورهای مختلف را نشان داد، اگرچه تفسیر ساختار جغرافیایی به دلیل توزیع نابرابر توالی‌ها میان کشورها محدود بود. در مجموع، نتایج از کاربرد COI برای شناسایی مولکولی گونه‌های *Hyalomma* و مطالعات ژنتیک جمعیتی *H. anatolicum* حمایت می‌کنند. جایگاه‌های نوکلئوتیدی تمایزدهنده شناسایی شده در این مطالعه می‌توانند به‌عنوان مرجعی برای اعتبارسنجی‌های آینده و توسعه آزمون‌های مولکولی در مطالعات اپیدمیولوژیک و پایش *Hyalomma* در ایران مورد استفاده قرار گیرند.

واژگان کلیدی: COI، شبکه هاپلوتایپ، *Hyalomma anatolicum*، ایران، تحلیل تبارشناختی

دریافت

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