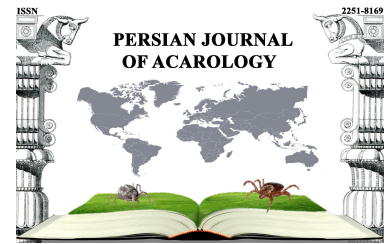




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Article

The Iranian *Hyalomma* (Acari: Ixodidae) with molecular evidences to understand taxonomic status of species complexes

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ABSTRACT

The identification of *Hyalomma* is a challenging issue in the systematics of ixodid ticks. Here, we examined 960 adult males of *Hyalomma* tick from 10 provinces of Iran using morphological and molecular methods. PCR was carried out on 60 samples to amplify an *ITS2* fragment of nuclear and a *COI* fragment of mitochondrial genomes. Nine species, namely *H. aegyptium*, *H. anatolicum*, *H. asiaticum*, *H. scupense*, *H. dromedarii*, *H. excavatum*, *H. marginatum*, *H. rufipes* and *H. schulzei* were identified. The validity of *H. rufipes* and *H. excavatum* can be challenged. We concluded that these species should be regarded as *H. marginatum* and *H. anatolicum* complexes, respectively. Furthermore, the taxonomic status of the two closely related *H. anatolicum* and *H. asiaticum* was confirmed as distinct species.

KEY WORDS: *Hyalomma*; phylogenetic relationships; taxonomy; variation; Iran.

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INTRODUCTION

Ticks of the genus *Hyalomma* Koch, 1844 (Acari: Ixodidae), are well-known ectoparasites of livestock. Their geographical distribution is limited to Afro-tropical, Palearctic and Oriental regions (Guglielmone *et al.* 2014). They are competent vectors of several pathogens causing disease in humans and animals (Sonenshine 1993), making them veterinary and medically important species; however, their systematics remains largely unknown. So far the subgenera *Hyalomma*, *Hyalommasta*, *Hyalommina* and *Euhyalomma* have been introduced to the *Hyalomma* genus (Kaiser and Hoogstraal 1964; Filippova 1984). The identification of *Hyalomma* species is still a challenging issue in the systematics of ixodid ticks (Apanaskevich and Horak 2005). Taxonomic uncertainties are prevalent within the *Hyalomma* genus, and identifying individual specimens can be a demanding task. Morphological variability of characters in a single species, occurrence of hybrid, intraspecific and intermediate forms, and impossible specific identification of most females are reasons behind the

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chaotic state associated with *Hyalomma* nomenclature (Cwilich and Hadani 1963; Keirans 1992; Rees *et al.* 2003; Hosseini *et al.* 2011; Tavakoli *et al.* 2012). Up to 146 names have been proposed for only 10 so-called species including *H. aegyptium* Linnaeus, 1758, *H. brumpti* Delpy, 1946, *H. detritum* Schülze, 1919, *H. dromedarii* Koch, 1844, *H. excavatum* Koch, 1844, *H. impressum* Koch, 1844, *H. rufipes* Koch, 1844, *H. savignyi* Gervais, 1844, *H. schulzei* Olenov, 1931 and *H. transiens* Schülze, 1919 in terms of Delpy (1949a). Moreover, a given character state, such as parma, central festoon, cervical and lateral grooves can be present in more than one *Hyalomma* species, meaning various states are shown in taxonomical characters of closely related species (Hosseini-Chegeni *et al.* 2013). The identification of females is more problematic than of males since reliable character states are few and diagnostic features are very similar (such as scutum characteristics) in many *Hyalomma* species (Pomerantzev 1950). Possible hybridization ability suggests the occurrence of interspecific crossings among *Hyalomma* species (Dalal *et al.* 2007). However, the reproductive capability of hybrid individuals has yet to be fully known. Delpy (1946) believed that hybrid forms are infertile, although crossbreeding among *Hyalomma* species seems to occur frequently. This implies that species complexes in the genus could be more difficult to taxonomically resolve than previously thought. *Hyalomma* is a phylogenetically young group of Asian origin (Balashov 1994; Murrell *et al.* 2001; De La Fuente 2003; Filippova 1984). Kolonin (2007) believed that the domestication of animals, development of cattle breeding and passing of ticks to new hosts serve as factors for the evolutionary transformations of this genus. As a matter of fact, evolutionary transformations still occur, resulting in a great number of intraspecific forms (Kolonin 1983).

Today, molecular methods are suitable tools for identifying species. Hebert and Gregory (2005) claimed that DNA barcoding delivers species level resolution in 95 to 97% of the cases. They further suggested a portion of cytochrome oxidase subunit I (*COXI* or *COI*) as a DNA barcode standard for animals. *COI* barcode has been widely employed for the identification or the study of the taxonomic status and phylogeny of many *Hyalomma* taxa at species or higher levels (Murrell *et al.* 2000; Rees *et al.* 2003; Lv *et al.* 2013, 2014; Zhang and Zhang 2014; Vial *et al.* 2016; Sands *et al.* 2017). However, DNA-based taxonomy is not limited to the *COI* gene markers in ticks, and other genes, especially internal transcribed spacer 2 (*ITS2*) and ribosomal DNA, are extensively used for the identification of species, examination of taxonomic status, study of phylogenetic relationships and phylogeographic patterns in ticks including *Hyalomma* species (Labruna *et al.* 2002; Rees *et al.* 2003; Dergoussoff and Chilton 2007; Barker and Murrell 2008; Ganjali *et al.* 2011; Cangi *et al.* 2013; Lv *et al.* 2014; Grech-Angelini *et al.* 2016; Alsarraf *et al.* 2017; Sands *et al.* 2017).

The species status of *H. asiaticum* Schulze and Schlottke, 1929, *H. anatolicum* Koch, 1844, *H. excavatum*, *H. marginatum* Koch, 1844 and *H. rufipes* (species occurring also in Iran) has been verified based on morphological criteria (Apanaskevich and Horak 2005, 2008, 2010). However, morphological-based differentiation of *Hyalomma* species is often very difficult to interpret, and there has been no comprehensive molecular study for molecular identification, re-evaluation of morphological species and/or determining the phylogenetic relationship among the taxa of the *Hyalomma* genus.

Nine species of *Hyalomma* have been reported in Iran, including *H. aegyptium*, *H. anatolicum*, *H. asiaticum*, *H. scupense* (syn. *H. detritum*), *H. dromedarii*, *H. excavatum*, *H. marginatum*, *H. rufipes* and *H. schulzei* (Hosseini-Chegeni *et al.* 2013). *Hyalomma asiaticum*, *H. excavatum* and *H. marginatum* are recognized as the three morphologically distinct species groups in Iran. They are considered as the main vectors of *Theileria* and *Babesia* (Piroplasmida: Theileriidae, Babesidae), *Anaplasma* and *Ehrlichia* (Rickettsiales: Anaplasmataceae), *Hemolivia mauritanica* (Apicomplexa: Haemogregarinidae), as well as Crimean Congo hemorrhagic fever virus and a novel rhabdovirus reported from Iran (Dilcher *et al.* 2015; Javanbakht *et al.* 2015; Kayedi *et al.* 2015; Telmadarraiy *et al.* 2015; Tajedin *et al.* 2016; Jafar Bekloo *et al.* 2017, 2018). The members of these species groups cannot be easily differentiated using taxonomic keys due to intraspecific variation and existence of

intermediate forms (Hoogstraal and Kaiser 1959; Feldman-Muhsam 1960; Keirans 1992; Kolonin 2007; Tavakoli *et al.* 2012). The present study was designed to identify *Hyalomma* species occurring in Iran using *COI* and *ITS2* gene fragments. We also aimed to analyze the phylogenetic relationship among *Hyalomma* species.

MATERIALS AND METHODS

Tick collection

Tick specimens were collected from 10 provinces of Iran, namely Azarbaijan-e Sharqi, Kerman, Kermanshah, Khorasan-e Razavi, Khuzestan, Kurdistan, Lorestan, Semnan, Sistan-o Baluchistan and Tehran (Fig. 1, Table 1). Specimens were collected from cattle, camels, sheep and goats as well as a terrestrial turtle species. The live tick specimens were kept in freezer or freshly transported to the laboratory. Few specimens in this study were obtained as gifts (collected by M. Tavakoli, housed in Lorestan Agricultural and Natural Recourses Research Center, Lorestan, Iran). The representative tick specimens of many species utilized in this study are retained in the collection of Natural History Museum of Guilan University, Iran.



Figure 1. Collection sites of *Hyalomma* tick species in the provinces of Iran.

Morphological study

A total of 960 adult males of *Hyalomma* spp. were identified based on species level under a light stereomicroscope (GXM-C2A-Australia) according to taxonomic keys, including Delpy (1946), Adler and Feldman-Muhsam (1948), Delpy (1949b), Pomerantzev (1950), Hoogstraal (1956), Kaiser and Hoogstraal (1963), Kaiser and Hoogstraal (1964), Estrada-Peña *et al.* (2004), Hosseini-Chegeni *et al.* (2013); additional taxonomic literature included Hoogstraal and Kaiser (1959), Filippova *et al.*

(1995) and Apanaskevich and Horak (2008). In order to show detailed morphological variation in taxonomic characters of *H. anatolicum*, *H. asiaticum*, *H. marginatum* and *H. rufipes*, the representative specimens of each species were drawn with a drawing tube (Olympus SZH-Japan) connected to a light stereomicroscope (Olympus) and then redrawn using Corel Draw Graphics Suite (version X7). Taxonomical characters of representative *H. excavatum* specimens and the morphological variations of *H. asiaticum*, *H. marginatum* and *H. rufipes* were photographed by a camera.

Table 1. Data of *Hyalomma* specimens included in *COI* and *ITS2* analysis.

Species	GB Acc. Nos.		Host	Locality (province)	GPS Coordinates
	<i>COI</i>	<i>ITS2</i>			
<i>H. aegyptium</i>	KP202935	KP231214	Land turtle	Kurdistan	36° 5' 32.00" N, 46° 2' 11.00" E
<i>H. anatolicum</i>	MH411145	-	Sheep	Lorestan	33° 16' 23" N, 48° 25' 40" E
	MH411144	-	Sheep	Lorestan	33° 16' 23" N, 48° 25' 40" E
	MH411143	-	Sheep	Lorestan	33° 16' 23" N, 48° 25' 40" E
	KP219862	KP208955	Sheep	Sistan-o Baluchistan	31° 04' 40" N, 61° 32' 29" E
	KP219865	KP208957	Cattle	Lorestan	33° 51' 53.60" N, 48° 9' 39.50" E
	KP219867	KP208959	Camel	Khorasan-e Razavi	-
	KP219872	KP208962	Cattle	Kerman	27° 23' 33" N, 57° 32' 05" E
<i>H. asiaticum</i>	KP208951	KP231212	-	Semnan	-
	KP219860	-	Sheep	Lorestan	33° 38' 25" N, 47° 50' 16" E
	KP219864	KP208956	-	Azarbaijan-e Sharqi	-
	KP219866	KP208958	Sheep	Lorestan	33° 24' 39.84" N, 48° 3' 41.49" E
	KP219869	KP208960	-	Kurdistan	-
	KP219871	KP208961	-	Kurdistan	-
	KP219870	KP231216	Camel	Semnan	35° 29' 57" N, 53° 24' 23" E
<i>H. excavatum</i>	KP219861	KP231213	Sheep	Lorestan	33° 24' 29" N, 48° 30' 42" E
<i>H. marginatum</i>	MH411142	-	Sheep	Lorestan	33° 16' 23" N, 48° 25' 40" E
	KP189361	KP208954	Sheep	Tehran	35° 23' 47.34" N, 51° 48' 12.80" E
	KP100418	KP231211	Sheep	Lorestan	33° 17' 38.43" N, 48° 12' 26.37" E
	KP219863	KP208953	Buffalo!	Azarbaijan-e Sharqi	-
	KP219874	KP208964	-	Lorestan	29° 51' 27" N, 52° 59' 19" E
	-	KP208967	-	Khuzestan	-
	KP219876	KP208968	Sheep	Kermanshah	34° 38' 9.58" N, 45° 57' 55.01" E
<i>H. rufipes</i>	-	KP208952	Cattle	Lorestan	33° 16' 3.13" N, 48° 42' 48.37" E
	KP231210	-	-	Lorestan	-
	KP219868	KP231215	Goat	Lorestan	33° 13' 52.81" N, 48° 33' 37.52" E
	KP219873	KP208963	Cattle	Kerman	27° 23' 33" N, 57° 32' 05" E
	-	KP208966	-	Khuzestan	-
<i>H. schulzei</i>	KP241853	KP241854	Camel	Semnan	35° 30' 41" N, 53° 23' 11" E
<i>H. scupense</i>	KP219875	KP208965	-	Khuzestan	-

- : Sequencing was failed; - : No data available

DNA extraction and PCR

Genomic DNA was extracted by the Phenol-chloroform method according to Sambrook and Russell (2001) with minor modifications. An *ITS2* fragment of nuclear genome was amplified by polymerase chain reaction (PCR) using primers that were designed by Telmadarraiy *et al.* (2016): TAH-*ITS2*-F (5' GCG TGC TCG ATG GGA GAT G 3') and reverse; TRH-*ITS2*-R (5' TCT TCG GGA CGG CGA CTG 3'). A *COI* fragment of mitochondrial genome was amplified by PCR using the following primers: C1-J-1718 (5' GGA GGA TTT GGA AAT TGA TTA GTT CC 3') and reverse primer C1-N-2191 (5' CCC GGT AAA ATT AAA ATA TAA ACT TC 3') designed by Simon *et al.* (1994). PCR reactions for both target genes were carried out in a thermocycler (MyGenie, Bioneer®, South Korea) based on touchdown temperature profile for *ITS2* gene by 5 min at 95 °C, 20X [60 s at

94 °C, 60 s at 65 °C, and 90 s at 72 °C], followed by 20X [60 s. at 94 °C, 60 s. at 45 °C, 90 s. at 72 °C], 10 min at 72 °C). For *COI* gene, by 5 min at 95°C, 34X [60 s. at 94 °C, 60 s. at 54 °C, 60 s. at 72 °C], followed by 10 min at 72 °C. PCR for each 25 µl final volume reaction was done using 2.5 µl PCR buffer Bioflux[®]-10X, 1 µl MgCl₂ Bioflux[®]-50 mM (which can be increased up to 2 µl), 0.5 µl dNTPs Bioflux[®]-10 mM, 1 µl from each primer 10 µM, 0.3 µl Taq DNA polymerase enzyme Bioflux[®]-5 U/µl, 4 µl gDNA template (50-100 ng/µl) and 14.6 µl D.D. water. The PCR products were visualized by 1% agarose gel electrophoresis and the desired bands were purified using GeneJET Gel Extraction Kit[®]; purified PCR products were then submitted for sequencing to Sequetech[®] Company using an ABI 3730XL DNA sequencer. *COI* sequences were examined for stop codons using GeneDoc[®] software (Nicholas *et al.* 1997). Finally, all sequences were submitted in GenBank and accession numbers were assigned.

Phylogenetic study

The sequences were aligned using SeaView4 software (Gouy *et al.* 2010), and the genetic distances among the sequences were calculated through the use of Maximum Composite Likelihood (MCL) model in the MEGA7 software (Kumar *et al.* 2016). To construct *COI* and *ITS2* phylogenetic trees, the aligned sequences of each gene were analyzed using BEAST[®] software (version 2.5.0) (Bouckaert *et al.* 2014) based on the Bayesian Inference (BI) method. This method employs Markov chain Monte Carlo (MCMC) algorithms for Bayesian phylogenetic inference. For this purpose, 80 and 53 taxa sequences (comprising in- and out-group) were used to construct *COI* and *ITS2* phylogenetic trees, respectively. The phylogenetic trees were compared in order to clarify phylogenetic relationships among the Iranian *Hyalomma* species. The clades of constructed phylogenetic tree were reorganized based on both criteria posterior probability values higher than 0.99 support (except for the clade of *H. asiaticum* in the *COI* phylogenetic tree with 0.86 posterior probability value support) and morphological characteristics of each species. Taxa from the genera *Dermacentor* and *Rhipicephalus* were examined as out-groups in both *COI* and *ITS2* phylogenetic trees.

RESULTS

Morphological studies

A total of nine *Hyalomma* species were identified: *H. aegyptium*, *H. anatolicum*, *H. asiaticum*, *H. scupense*, *H. dromedarii*, *H. excavatum*, *H. marginatum*, *H. rufipes* and *H. schulzei* (Fig. 1, Table 1). Figures 2–4 show taxonomic characters and their variations in *H. anatolicum*, *H. asiaticum*, *H. marginatum* and *H. rufipes*. Taxonomical characters of *H. excavatum* are shown in Figure 5. The most reliable taxonomic characters of *H. anatolicum* and *H. asiaticum* were cervical grooves and posterior margin of basis capituli (Fig. 2). However, cervical grooves, and the form and color of parma can be variable in different populations (Fig. 3). A unique character of *H. asiaticum* is spiracular plate with considerable variation in outline and shape (Fig. 3). *Hyalomma marginatum* and *H. rufipes* were differentiated by scutal punctations, spiracular plate tail and circumspiracular setae (Fig. 4).

Molecular studies

The target electrophoretic bands of *COI* and *ITS2* gene fragments were observed in all *Hyalomma* species with 523 bp and 653 bp size, respectively. A total of 53 PCR products (27 *COI* and 24 *ITS2*), out of more than 60 reactions were successfully sequenced. Table 1 demonstrates species, host of specimens, locality, GPS coordinates of collection places and assigned GenBank accession numbers for each sequence. The aligned sequences of partial *ITS2* ranged in length from 403 bp (*H. scupense*), 408 bp (*H. aegyptium*), 429 bp (*H. schulzei*), 430 bp (*H. marginatum*-*H. rufipes*), 432–433 bp (*H.*

anatolicum), 435-437bp (*H. asiaticum*) to 442 bp (*H. dromedarii*). The aligned sequences of partial *COI* were 459 bp in length.

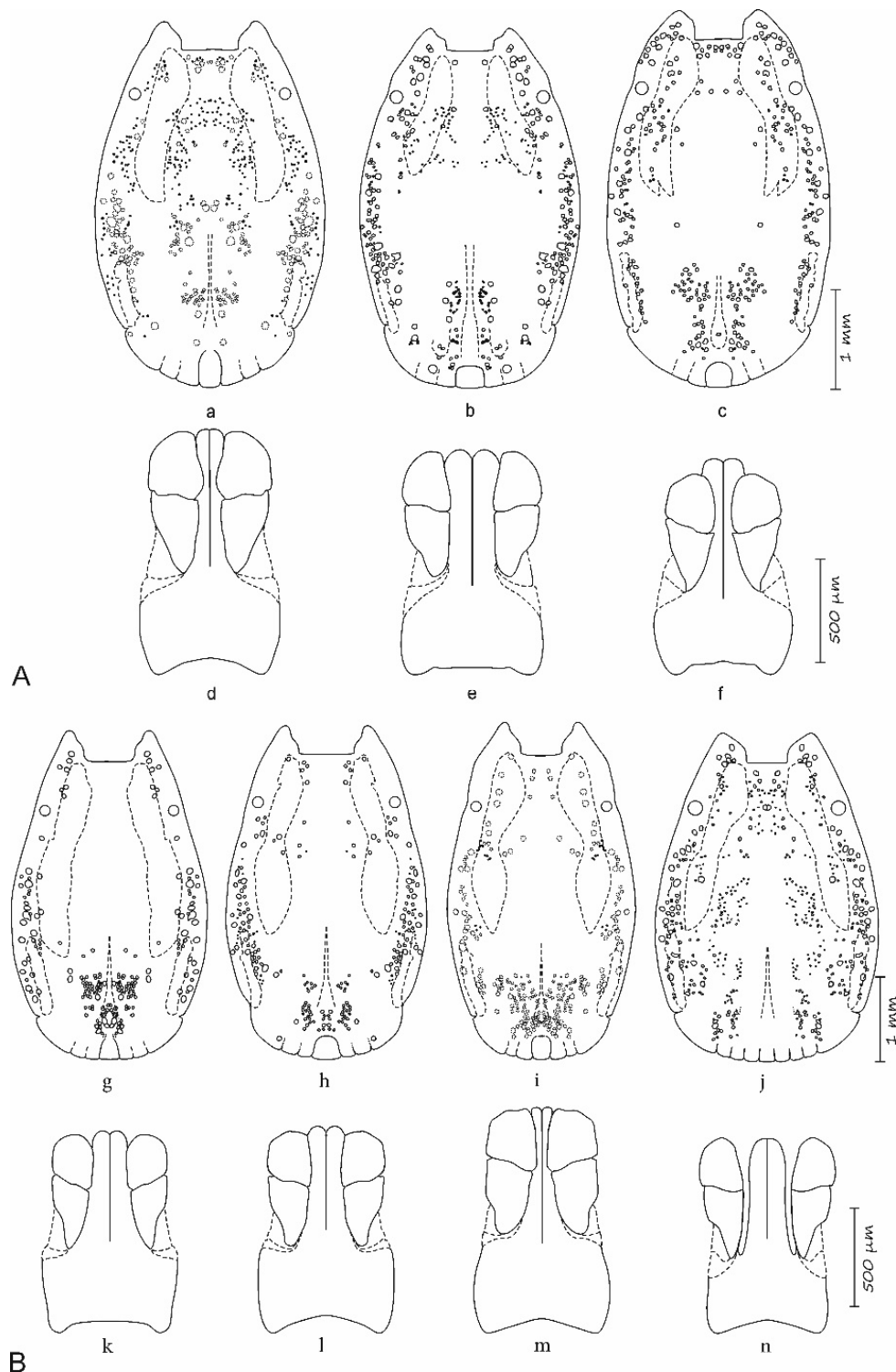


Figure 2. Taxonomic characters of two closely related *Hyalomma* tick – *H. anatolicum* (A); short and superficial cervical grooves (a–c), short lateral grooves with slight variation (as line of punctations), variation in parma size, shallow dorsal posterior margin of basis capituli with slight variation (d–f). *H. asiaticum* (B); long and depressed cervical grooves (g–j), short lateral grooves with slight variation (as line of punctations) (g–j), variation in size (g–i) and shape of parma (j), depth of dorsal posterior margin of basis capituli (k–n).

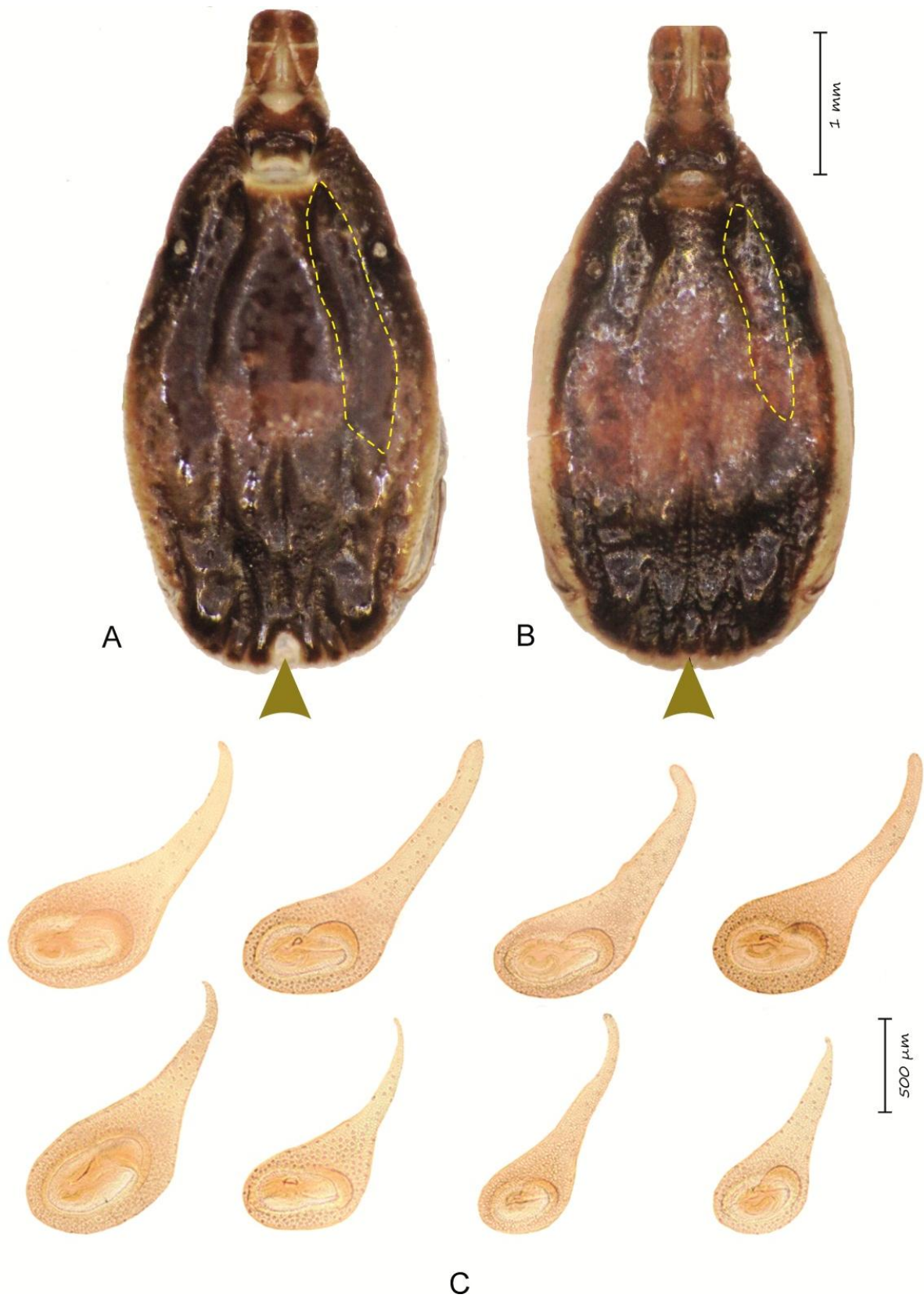


Figure 3. The variable forms of parma, cervical groove and spiracular plate in *Hyalomma asiaticum*: parma present in A (arrow) and parma absent (as central festoon) in B (arrow); variation in length of cervical grooves (surrounded area by yellow dot line) as long (A) and slightly short (B). Variation in form of spiracular plate (C).

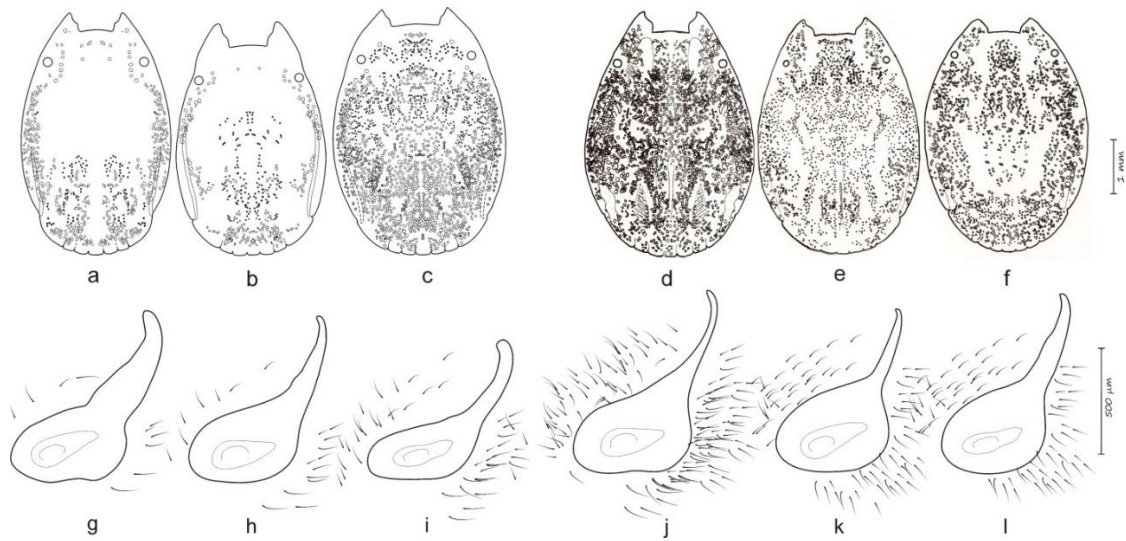


Figure 4. Variation in scutal punctations, spiracular plate tail and circumspiracular setae of closely related *Hyalomma marginatum* and *Hyalomma rufipes* – *H. marginatum* with scutal punctations (a–c) as **a**) very sparse and small; **b**) moderately dense and small; **c**) dense and small. *H. marginatum* with spiracular plate tail and circumspiracular setae (g–i) as **g**) very broad tail and very sparse setae; **h**) moderately broad tail and sparse setae; **i**) moderately narrow and moderately dense setae. *H. rufipes* with scutal punctations (d–f) as **d**) very dense and large; **e**) very dense and small; **f**) very dense and small with smooth regions in middle scutum. *H. rufipes* with spiracular plate tail and circumspiracular setae (j–l) as **j**) very narrow tail and very dense setae; **k**) very narrow tail and dense setae; **l**) moderately broad and dense setae.

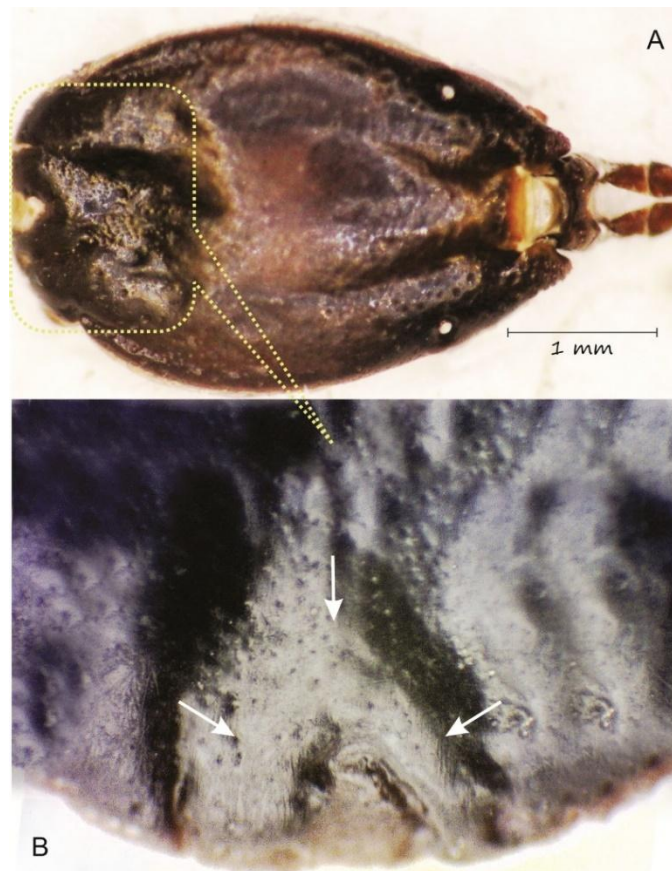


Figure 5. Taxonomic characters of *Hyalomma excavatum* – A) Arch and elevated ridges in scutal caudal field; B) arch (white arrows) in a larger view.

Table 2. Genetic distance (%) among *COI* sequences of *Hyalomma* of this study.

Clades (No. of sequences)	Interspecies*							
	<i>H. aegyptium</i>	<i>H. anaticum</i> – <i>H. excavatum</i>	<i>H. asiaticum</i>	<i>H. dromedarii</i>	<i>H. marginatum</i> – <i>H. rufipes</i>	<i>H. scupense</i>	<i>H. rufipes</i> **	<i>H. schulzei</i> – <i>H. asiaticum</i> ***
<i>H. aegyptium</i> (1)								
<i>H. anaticum</i> (7) – <i>H. excavatum</i> (1)	13	1						
<i>H. asiaticum</i> (5)	15	17	0					
<i>H. dromedarii</i> (1)	15	20	14					
<i>H. marginatum</i> (6) – <i>H. rufipes</i> (3)	12	8	15	17	1			
<i>H. scupense</i> (1)	13	15	7	11	11			
<i>H. rufipes</i> **	12	8	15	17	4	12		
<i>H. schulzei</i> (1) – <i>H. asiaticum</i> *** (1)	16	18	7	15	16	11	16	0

*: Calculation of 349 bp with 13% as the mean of interspecies variation; **: Lorestan specimen; ***: Kurdistan specimen. Tentative clade name; *H. anaticum* – *H. excavatum*: ***H. anaticum***, *H. marginatum* – *H. rufipes*: ***H. marginatum***, *H. schulzei* – *H. asiaticum***: ***H. schulzei***. Within-clade intraspecies divergences are shown as diagonal (in **bold**).

Table 3. Genetic distance (%) among *ITS2* sequences of *Hyalomma* of this study.

Clades (No. of sequences)	Interspecies*							
	<i>H. marginatum</i> – <i>H. rufipes</i>	<i>H. asiaticum</i>	<i>H. asiaticum</i> **	<i>H. anaticum</i> – <i>H. excavatum</i>	<i>H. scupense</i>	<i>H. aegyptium</i>	<i>H. schulzei</i>	<i>H. dromedarii</i>
<i>H. marginatum</i> (6) – <i>H. rufipes</i> (4)								
<i>H. asiaticum</i> (4)	11	1						
<i>H. asiaticum</i> ** (1)	14	3						
<i>H. anaticum</i> (4) – <i>H. excavatum</i> (1)	6	13	16	1				
<i>H. scupense</i> (1)	11	6	9	14				
<i>H. aegyptium</i> (1)	17	12	16	19	9	0		
<i>H. schulzei</i> (1)	16	8	9	17	10	17		
<i>H. dromedarii</i> (1)	14	8	10	15	8	16	13	

*: Calculation of 464 bp (gaps as fifth base) with 13% as the mean of interspecies variation, **: Code 60. Tentative clade name; *H. marginatum* – *H. rufipes*: ***H. marginatum***, *H. anaticum* – *H. excavatum*: ***H. anaticum***. Within-clade divergences are shown as diagonal (in **bold**).

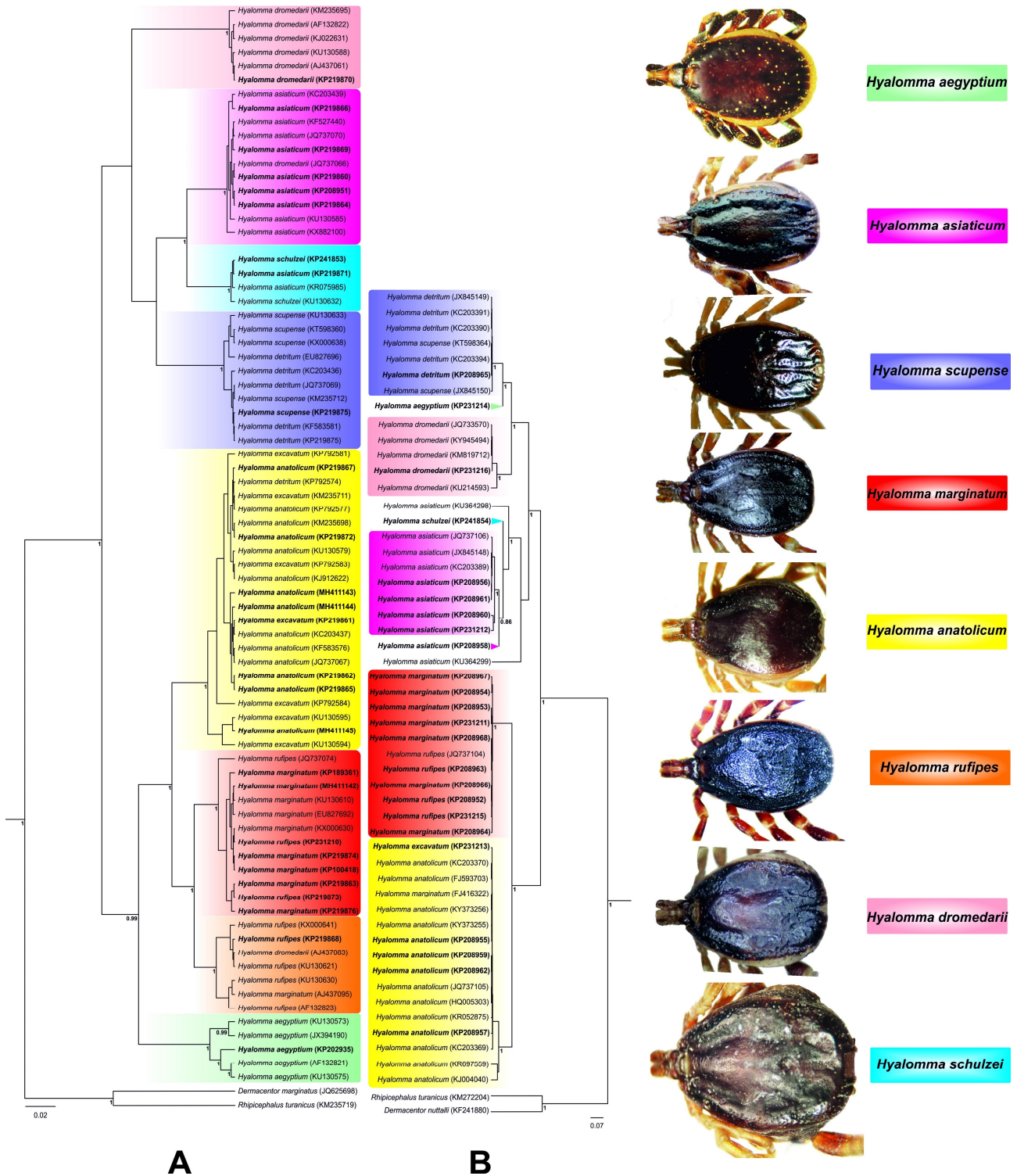


Figure 6. Phylogenetic tree inferred from *COI* (A) and *ITS2* (B) sequence data of *Hyalomma* species of this study and GenBank data constructed using Bayesian Inference method; main clades of tree separated with colored rectangular shapes. Taxa of the present study are bold as species name and GenBank accession number. Nodes indicated with posterior probability value. Branch lengths are proportional to evolutionary changes. *Rhipicephalus turanicus*, *Dermacentor marginatus* and *D. nuttalli* are inserted as out-groups.

Phylogenetic studies

The genetic distances (inter- and intra-species variation) among *COI* and *ITS2* sequences of

Hyalomma species are shown in Tables 2 and 3, respectively. The *COI* and *ITS2* phylogenetic trees were constructed using Bayesian inference (BI) method (Fig. 6). *Dermacentor marginatus*, *D. nuttalli* and *Rhipicephalus turanicus* Pomerantsev, 1936 were located as out-group. Both phylogenetic trees were seen as fully resolved trees as they were genus *Hyalomma* as one ancestry in tree topology, dichotomous branching and full posterior probability values in nodes places. In both phylogenetic trees, the genetic affinity of clades *H. anatolicum*/*H. marginatum* and *H. asiaticum*/*H. scupense*/*H. dromedarii*/*H. schulzei* is evident. The condition of *H. aegyptium* cannot be interpreted because it is a paraphyletic clade in the *COI* phylogenetic tree having a common root with *H. scupense*, *H. dromedarii* clades in the *ITS2* phylogenetic tree.

DISCUSSION

Phylogenetic trees generated from the sequence data consistently supported the monophyly of *Hyalomma*. The presented phylogeny indicates that the genus *Hyalomma* involves several distinct clades well supported with posterior probability values well separated by high levels of genetic difference.

Hyalomma aegyptium (subgenus *Hyalommasta*) is a common ectoparasite of land tortoise (*Testudo* species) occurring in Iran and other parts of the world (Tavassoli *et al.* 2007). *Hyalomma aegyptium* clade is a more distinct evolutionary lineage in *COI* phylogenetic tree (as ancestral, basal or as a sister group) compared with other *Hyalomma* taxa; however, it is closely correlated with *H. scupense* and *H. dromedarii* clades in *ITS2* phylogenetic tree. Phylogenetically, *H. aegyptium* forms a polyphyletic clade in which an unexpected genetic distance (up to 4%) was observed among the members from Romania (JX394190), Belgium (AF132821) and Palestine (KU130575, KU130573) in the *COI* phylogenetic tree. This may be due the scattered geographic distribution pattern of *Testudo* species as the preferred host of *H. aegyptium* (Široký *et al.* 2006), so that genetic variations among different *H. aegyptium* populations associated with *Testudo* species can occur. However, amino acids translation of all *COI* sequences of *H. aegyptium* shows no variation among these different sequences (data not shown).

Hyalomma anatolicum (subgenus *Hyalomma*) is a common ectoparasite of livestock in Iran (Nabian *et al.* 2009), and includes an abroad geographical range from China to Africa; therefore, it is only natural that intraspecific morphological variation and intermediate forms occur (Adler and Feldman-Muhsam 1948; Hoogstraal and Kaiser 1959, 1960; Kaiser and Hoogstraal 1963; Hosseini *et al.* 2011; Tavakoli *et al.* 2012; Hosseini-Chegeni *et al.* 2013). In the present study, morphological variation was not confirmed with genetic variability because only 1% genetic distance was found within *ITS2* and *COI* sequences of *H. anatolicum*. However, 2% to 4% genetic difference was found among *COI* sequences of *H. anatolicum* clade including sequences in this study and those submitted from Turkmenistan, Iraq, India, and China (Lv *et al.* 2014). Interestingly, 19% and 16% genetic distance variation was found between *H. anatolicum* and *H. asiaticum* in their *ITS2* and *COI* sequences, respectively. Thus, the taxonomic status of two closely related species, *H. anatolicum* and *H. asiaticum*, was confirmed as full distinct species. *Hyalomma anatolicum* and *H. marginatum* clades form a strongly supported monophyletic clade in both *COI* and *ITS2* phylogenetic trees. Taxonomical identity of *H. excavatum* is a matter of controversy (Pervomaisky 1954) and its validity is subject to debates (Feldman-Muhsam 1962; Kaiser and Hoogstraal 1964). Hoogstraal and Kaiser (1959), Camicas *et al.* (1998) and Horak *et al.* (2002) considered *H. excavatum* as a subspecies of a polymorphic *H. anatolicum*, whereas Guglielmone *et al.* (2014) listed *H. excavatum* as a valid species. In the present research, genetic evidence showed that *H. excavatum* is not a separate species from *H. anatolicum* according to *COI* and *ITS2* sequences.

Hyalomma asiaticum (subgenus *Hyalomma*) is an important ectoparasite of sheep existing all over Iran, except near the Caspian Sea border (Mazlum 1968). Formerly, *H. asiaticum* was reported

as a polymorphic species with four subspecies, namely *H. a. kozlovi* Olenev, 1931, *H. a. citripes* Schulze, 1935, *H. a. caucasicum* Pomerantzev, 1940 and *H. a. asiaticum* Schulze and Schlottko, 1929 (Schulze 1935; Pomerantzev 1950; Filippova *et al.* 1995). In traditional taxonomy, this species is classified as a member of *H. asiaticum* group. *Hyalomma asiaticum* and *H. schulzei*, two fully discrete morphological species, were classified in *H. asiaticum* group based on the morphology (Apanaskevich *et al.* 2008), which is congruent with their *COI* and *ITS2* genetic similarity (6% and 9%, respectively). Up to 5% genetic distance was calculated among *ITS2* sequences of the clade *H. asiaticum*, including sequences of this study and those from China. Two *ITS2* sequences related to *H. asiaticum* submitted from China (KU364298-9) showed more genetic distance difference, hence designated as different taxa. An unusual *ITS2* distance difference suggests that several entities can exist at the subspecies level of this polytypic species in agreement with the findings of Filippova *et al.* (1995).

Hyalomma dromedarii (subgenus *Hyalomma*) is the type species of the subgenus *Hyalomma*. This species often infects camels and is rarely found on cattle in semi-desert areas of Iran (Nabian *et al.* 2009). The evolutionary affinity of *H. dromedarii* with *H. scupense*, *H. schulzei* and *H. asiaticum* can be observed in *COI*, and, partly, in *ITS2* phylogenetic trees. This is congruent with the conventional classification of *H. dromedarii*, *H. asiaticum* and *H. schulzei* within *H. asiaticum* group (Apanaskevich *et al.* 2008). In *COI* phylogenetic tree, *H. dromedarii* is situated as ancestral (basal) toward *H. scupense*, *H. schulzei* and *H. asiaticum*.

Hyalomma marginatum group (subgenus *Hyalomma*) is one of the most complex *Hyalomma* groups in terms of taxonomy, including *H. marginatum*, *H. rufipes*, *H. isaaci*, *H. glabrum* and *H. turanicum* (Apanaskevich and Horak 2008). *Hyalomma marginatum* is the main ectoparasite of livestock in Iran (Nabian *et al.* 2009). Hoogstraal (1987) [cited in Abdigoudarzi (2004)] compiled a key for the identification of *H. m. marginatum*, *H. m. rufipes*, and *H. m. turanicum* after examining Iranian specimens. Hoogstraal and Valdez (1980) also reported *H. m. marginatum* and *H. m. turanicum* on wild sheep (*Ovis orientalis* Linnaeus, 1758) and wild goats (*Capra hircus* Linnaeus, 1758) in Iran. In the present study, only two species, *H. marginatum* and *H. rufipes* were identified according to criteria presented by Apanaskevich and Horak (2008) and Hosseini-Chegeni *et al.* (2013). Furthermore, no interspecies variation was found among *COI* and *ITS2* sequences of *H. marginatum* and *H. rufipes*, except for a 4% genetic difference between a *COI* sequence of *H. rufipes* (KP219868). In accordance with this finding, Azagi *et al.* (2017) reported about 4% divergence between *H. marginatum* and *H. rufipes* based on a fragment of *COI* gene. In the *COI* phylogenetic tree, *H. rufipes* and *H. marginatum* can be two distinct clades probably at subspecies level. Based on *ITS2* gene, *H. rufipes* and *H. marginatum* are to be considered as a single species. Ultimately, the taxonomic status of *H. rufipes* and *H. marginatum* based on *COI* and *ITS2* phylogenetic trees can be explained (i) as subspecies (according to *COI* phylogenetic tree), (ii) as a single or a unique species (according to *ITS2* phylogenetic tree).

Hyalomma scupense (subgenus *Hyalomma*) is a rare and sparsely distributed species more often than not collected from cattle and Buffalos in Iran (Mazlum 1971; Nabian *et al.* 2009). Formerly, *H. scupense* was reported as *H. detritum*, a name recently revised as a junior synonym of the valid name which is *H. scupense* (Guglielmone *et al.* 2014). In the *COI* and *ITS2* gene, 11% and 8% genetic distance was observed between this species and *H. dromedarii*, respectively. Based on the morphological identification key presented by Hosseini-Chegeni *et al.* (2013), both *H. scupense* and *H. dromedarii* are closely related species, which is partially congruent with the genetic similarity of these species in the *ITS2* gene.

CONCLUSION

Our molecular phylogeny confirmed the taxonomic status of nine morphological *Hyalomma* species;

however, the validity of *H. rufipes* and *H. excavatum* can be challenged. It is believed that these species are to be regarded as *H. marginatum* and *H. anaticum* complexes, respectively. Furthermore, the taxonomic status of the two closely related *H. anaticum* and *H. asiaticum* was confirmed as distinct species.

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گونه‌های جنس *Hyalomma* (Acari: Ixodidae) ایران و شواهد مولکولی برای درک وضعیت تاکسونومیک کمپلکس‌های گونه‌ای

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چکیده

شناسایی کنه‌های *Hyalomma* موضوعی چالش برانگیز در سیستماتیک کنه‌های سخت است. در این مطالعه، ۹۶۰ کنه نر بالغ *Hyalomma* از ۱۰ استان در کشور ایران را با روش‌های ریخت‌شناسی و مولکولی مورد بررسی قرار داده شدند. PCR روی ۶۰ نمونه به منظور تکثیر بخشی از ژن *ITS2* هسته‌ای و بخشی از ژن *COI* میتوکندریایی ژنوم انجام گرفت. نه گونه شامل *H. asiaticum*، *H. anatolicum*، *H. aegyptium*، *H. marginatum*، *H. excavatum*، *H. dromedarii*، *H. scupense*، *H. rufipes* و *H. excavatum* با چالش همراه است. بر اساس نتایج این پژوهش، این گونه‌ها بایستی به ترتیب همان دو گونه *H. marginatum* و *H. anatolicum* در نظر گرفته شوند. همچنین وضعیت تاکسونومیک دو گونه نزدیک به هم *H. asiaticum* و *H. anatolicum* به عنوان گونه‌های مستقل تأیید شد.

واژگان کلیدی: *Hyalomma*؛ روابط تبارشناختی؛ رده‌بندی؛ تنوع؛ ایران.

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