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Article

The parasitism of Persian jird by immature stages of *Hyalomma asiaticum* (Acari: Ixodidae) and its identification using molecular approaches in Iran

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ABSTRACT
In the present study, occurrence of *Hyalomma asiaticum* on wild rodents was explored. Rodents were trapped using Sherman traps. The tick specimens were collected by forceps from the rodents. Overall, one larva and 59 nymphs of immature ticks were collected on 23 *Meriones persicus* from three different locations in western Iran. A 408 bp length fragment of nuclear *5.8S*/internal transcribed spacer 2 (*ITS2*) genes was amplified in 60 examined tick specimens using PCR, of which one sample was sequenced, successfully. The BLAST results showed 99% similarity between a new haplotype from this study and two sequences of *H. asiaticum* from GenBank and. Therefore, we conclude that immature stages of *H. asiaticum* live on *M. persicus* and/ or their burrows. This finding helps us to better understand tick's ecology and control tick borne diseases.

KEY WORDS: *Hyalomma asiaticum*; nymph; *Meriones persicus*; Lorestan; Iran.

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INTRODUCTION

Hyalomma asiaticum (Acari: Ixodidae), so-called Asiatic *Hyalomma*, was first described by Schulze and Schlottke, 1929 as *H. dromedarii asiaticum* subspecies (Schulze and Schlottke 1929). Many years later, it was raised to species level by Schulze (1935). This species was accepted as a synonym of *H. dromedarii* (Delpy 1949). Three subspecies were described for *H. asiaticum*, namely; *H. a. asiaticum*, *H. a. caucasicum* and *H. a. kozlovi* by the former Soviet tick researchers (Pomerantzev 1950). Today, only *H. asiaticum* is a valid species and other subspecies are considered as junior synonyms (Guglielmone *et al.* 2014). *Hyalomma asiaticum* is closely related to *H. anatolicum* and *Hyalomma dromedarii* (Mazlum 1968). *Hyalomma asiaticum* is widely distributed in desert-like habitats in Asia from China to minor Asia, Russia, Turkey and Middle East (Vatansever 2017). Immature individuals of many tick species seem to have a predilection for

small animals such as rodents, cats, dogs, and ground-inhabiting birds, whereas adults seem to prefer to feed on cattle, horses and a variety of large and wild mammals (Service 1980). *Hyalomma asiaticum* is an exophilic three-host tick (Vatansever 2017). Adult ticks are found on Artiodactyla such as; Bovidae and Camelidae (Guglielmone *et al.* 2014). Immature stages infest burrowing mammals, especially rodents, and also hedgehogs, hares, and carnivores preying on rodents. The great gerbil; *Rhombomys opimus* Lichtenstein 1823, ground squirrel; *Spermophilopsis leptodactylus* Lichtenstein 1823 and yellow ground squirrel; *Spermophilus fulvus*; Lichtenstein 1823, are common hosts of the immatures (Hoogstraal 1979). Numerous studies on this species were performed by the former Soviet tick researchers in terms of systematics, anatomy, biology, ecology and experimental infection (Berdyev 1972; Balashov 1979; Dayter 1979; Leonovich 1981; Leonovich 1986; Filippova *et al.* 1995; Apanaskevich 2001). Although *Hyalomma asiaticum* is a non-nidicolous (exophilic) species, i.e., ticks dispersed throughout the open landscape and attacking passing hosts, all developmental stages are strongly associated with rodent burrows and their surroundings (Vatansever 2017). Owing to complex developed adaptations, *H. asiaticum* is one of the few ixodid tick species that has been adapted to spring-summer existence on desert soil surfaces (Balashov 1960). The amounts of blood imbibed by immatures and adults of *H. asiaticum* are among the greatest of any ixodid species in the Palearctic fauna (Hoogstraal 1979). *Hyalomma asiaticum* were found to be infected with *Theileria annulata* (Theileriidae), agent of bovine theileriosis (Meng *et al.* 2014), a novel *Cardiovirus* (Picornaviridae) virus (Lvov *et al.* 2014b), Wad Medani (Reoviridae, *Orbivirus*) virus (Alkhovskiĭ *et al.* 2014), as well as Tamdy (Bunyaviridae, *Nairovirus*) virus (Lvov *et al.* 2014a). Moreover, Asiatic tick is the main vector of Crimean Congo hemorrhagic fever virus (Kayedi *et al.* 2015; Telmadarraiy *et al.* 2015) and *T. annulata* (Mazlum 1968) in Iran. *Hyalomma asiaticum* is widely distributed in Iran, except for the coastal Caspian sea (Hosseini-Chegeni *et al.* 2013). The rodents and the associated infections (rodent-borne diseases) pose a heavy burden on public health in the tropical and semitropical regions of the world (Gratz 2006). Rodents play a significant role in enzootic cycles of tick-borne pathogens such that recently seven diseases were attributed to *Meriones persicus* in Iran (Rabiee *et al.* 2018). In this respect, *M. persicus* was considered as a reservoir of numerous zoonotic diseases in Iran (Shojaei and Mohebali 2005; Mahdavi 2009; Akhoundi *et al.* 2013; Esamaeili *et al.* 2013; Fallah *et al.* 2013; Zarei *et al.* 2016). The study of tick life cycle in natural conditions may be a key interacting component in order to better the understanding of tick ecology and epidemiology of tick transmitted diseases (Mulenga 2014). A considerable amount of tick research is being carried out by people with little or no training in acarine taxonomy and morphology, thus misidentification of immature tick species can simply lead to incorrect results (Anderson *et al.* 2004). Thus applying molecular data is a key step forward to eradicate these taxonomic impediments. During a large scale investigation conducted by Pasteur Institute of Iran in collaboration with Lorestan University of Medical Sciences on Rodentia collected from Lorestan province, many immatures of a hard tick species were found that we were not able to identify at species level. Thus this study was designed to identify for immature stages of *Hyalomma* species infesting *Meriones* (Rodentia) species in this region using molecular methods.

MATERIALS AND METHODS

Rodent capture, tick collection and generic identification of tick specimens

Rodents were entrapped using Sherman traps placed in entrance hole of rodent's nests during 2016–2018. Traps were baited with preferred foods of rodents. Altogether, 60 ticks (one larva and 59 nymphs) were collected by forceps from live trapped *Meriones persicus* rodents in three regions located at Lorestan province, western Iran (Fig. 1, Table 1). A tick infested animal is shown in Fig. 2. All ticks were stored at 70% ethanol for preservation and were transported to the laboratory of Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences for further

species identification. Ticks were identified to genus level under a light stereomicroscope (SZX12-Olympus®, Japan) based on Bregetova *et al*. (1955). Tick voucher specimens used in this study are retained in Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences (Lorestan, Iran). Finally, the tick specimens from different areas were selected for differential molecular assay and identification to the species level.

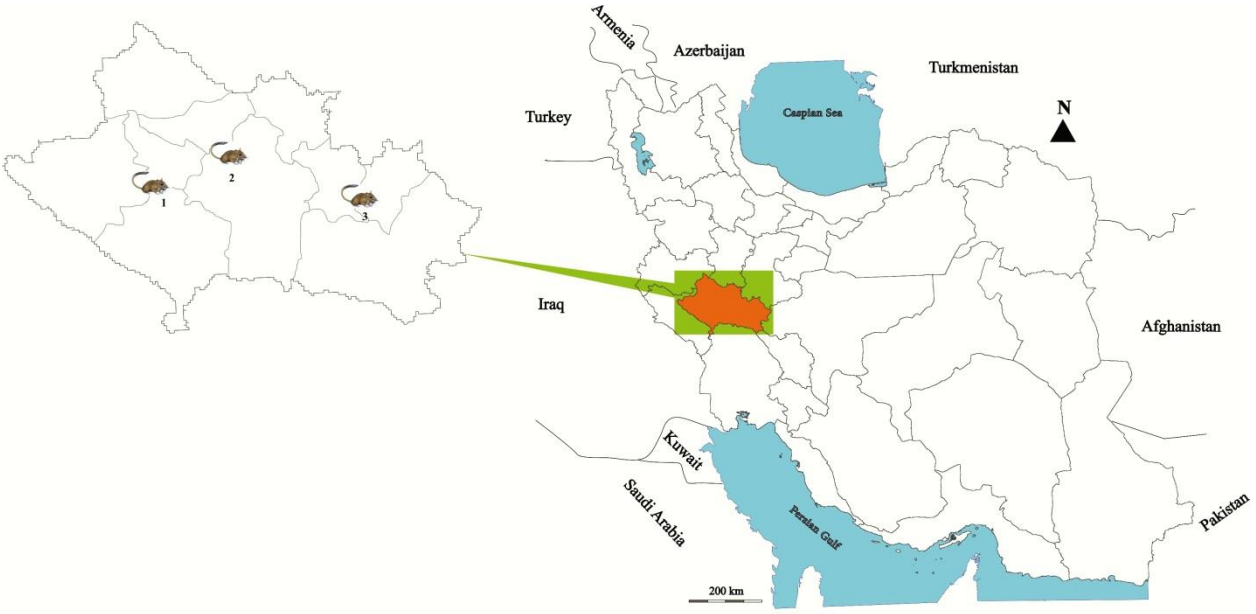

Figure 1. Three rodent collection sites located in Lorestan province western Iran inlcuding: 1. Khorramabad-Kuhdasht road, Zamzam village; 2. Khorramabad-Tehran road, LUMS Campus; 3. Dorud-Azna road, Zarnan village.

Figure 2. A rodent specimen infested by a number of immature ticks (red arrow) (animal is restrained through the ear by forceps and treated by insecticied for killing of ectoparasites).

PCR and sequencing

The multiplex-PCR protocol was performed according to Hosseini-Chegeni *et al.* (2017). Here the procedure is mentioned briefly. A 408 bp fragment of *5.8S/ITS2* gene was amplified by PCR using three primers; Fanas (forward for both *Hyalomma anatolicum* and *H. asiaticum*): 5′- TCT AAG CGG TGG ATC AC -3′, Ran (reverse for *H. anatolicum*): 5′- CTC GAA CCG TCT CAT AGA -3′ and Ras (reverse for *H. asiaticum*): 5′- CTT TCT TCC CCA GCG G -3′. PCR reactions were then carried out in thermocycler, MyCycler™ Thermal Cycler-Bio-Rad® (U.S.) by initial denaturation (5 min. at 95°C), 34 cycles of denaturation (60 sec. at 95°C), annealing (60 sec. at 58°C), extension (1 min. at 72°C), and a final extension step (5 min. at 72°C). PCR for each 25 µl final volume reaction was done using 12.5 µl RedMaster® PCR 2X, 1 µl from each forward and reverse primers (10 µM), 4 µl gDNA template (50–100 ng/µl), 5.5 µl deionized water.

Amplifications were seen by one percent agarose gel electrophoresis. To confirm multiplex PCR species identification, a single desired band of *H. asiaticum* was purified using GeneAll Expin™ Combo GP Kit (South Korea). Then purified PCR product was submitted for sequencing to Faza-Biotech® Company (Iran). One sequenced specimen was edited manually using FinchTV® software. The corrected sequence was compared with the sequences deposited in GenBank using BLASTn protocol (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally, our new sequence from Iran was submitted to GenBank and an accession number (MH270663) was assigned.

Phylogenetic tree

A single *ITS2* sequence of this study (327 bp with 5.8S gene in length) besides 32 sequences obtained from GenBank (including *Hyalomma asiaticum* submitted from China and Iran (Acc. Nos.: HQ123320, JQ737103, JQ737106, JX845147-8, KC203371-89, KP231212 and KU364298-9) and closely related *Hyalomma* species such as; *H. scupense*, *H. anatolicum*, *H. marginatum*, *H. rufipes* and *H. dromedarii*) were aligned using SeaView4 software (Gouy *et al*. 2010). *Rhipicephalus sanguineus* was used as out-group. Genetic distances among the analyzed sequences were calculated using Maximum Composite Likelihood (MCL) model in the MEGA7 software (Kumar *et al*. 2016). The final alignment of 34 sequences with 173 positions (including gaps) were analyzed using BEAST® software (version 2.4.8) (Bouckaert *et al.* 2014) to construct phylogenetic relationships among analyzed tick species based on the Bayesian Inference (BI) method that uses Markov chain Monte Carlo (MCMC) algorithms for Bayesian phylogenetic inference with Jukes-Cantor (JC) substitution model with score 970 –lnL performing in model selection using jModeltest software (version 2.1.7) (Darriba *et al.* 2012). The constructed clades of phylogenetic trees were reorganized based on 99% support posterior probability values and the reasonable genetic distances within and between the clade members.

RESULTS

Generic identification of ticks and molecular confirmation

Collected ticks were identified at the genus level as *Hyalomma* Koch, 1844 (Acari: Ixodidae: Rhipicephalinae) (Fig. 3). Successful DNA extraction and multiplex-PCR amplification was done for all collected tick specimens. Primers could accurately amplify a 408 bp *5.8S-ITS2* target gene fragment in all examined tick specimens (Fig. 4). A *5.8S/ITS2* target fragment was sequenced from a nymph specimen collected in Khorramabad-Kuhdasht road, Zamzam village. The BLAST showed 99% similarity between the only sequence of this study with two sequences of *H. asiaticum* obtained from China (JQ737103-JQ737106).

Table 1. Data related to tick specimens collected from *Meriones persicus*.

Number	Locality	GPS coordinates	No. of collected rodents	Tick life stage (No.)
1	Khorramabad-Kuhdasht road, Zamzam village	33° 31' 20.6" N 47°48'48.5" E	4	Nymph (30) Larva (1)
2	Khorramabad-Tehran road, LUMS Campus	33° 28' 32.46" N 48° 24' 0.62" E	2	Nymph (21)
3	Dorud-Azna road, Zarnan village	33° 28' 47.45" N 49° 21' 52.48" E	17	Nymph (8)

LUMS: Lorestan University of Medical Sciences

Figure 3. Immatures of *Hyalomma asiaticum* collected from *Meriones persicus* in western Iran (nymphal stage): left: dorsal and right: ventral views.

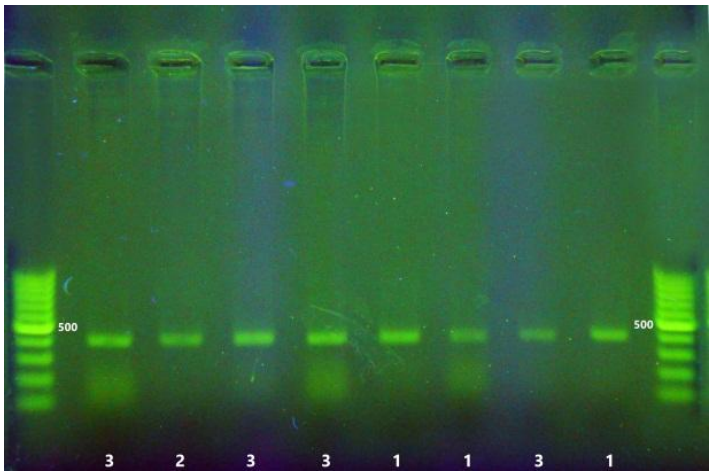

Figure 4. One percent agarose gel electrophoresis stained with Cyber Safe® showing *5.8S/ITS2* gene fragments amplified using Fanas/Ras for *H. asiaticum* with amplicon size 408 bp (100 bp DNA ladder). Numbers below bands representing location of collected specimens according to Fig. 4.

ITS2 phylogenetic tree

The constructed Bayesian tree is shown in Figure 5. It seems that *H. asiaticum* consists of three distinct clades; Clade I (China and Iran), Clade II (China), and Clade III (our new sequence from Lorestan province). Our results showed close relationships bwtween our new sequence from western Iran and Clade II encompassing the sequences belong to *H. asiaticum* and *H. kozlovi* from

China (JQ737103, JX845147-8, KC203371-89 and KU364298-9). Clade I consisting of sequences labaled as *H. asiaticum* (JQ737106, KP231212) from China and Iran and one sequence of *H. anatolicum* (HQ123320) from Iran appeared as a sister clade of *H. dromedarii*. The genetic distances between the three clades of *H. asiaticum* are shown in Table 2. in which, remarkable divergence separated Clade III from the clade II (7%) and Clade I (4%).

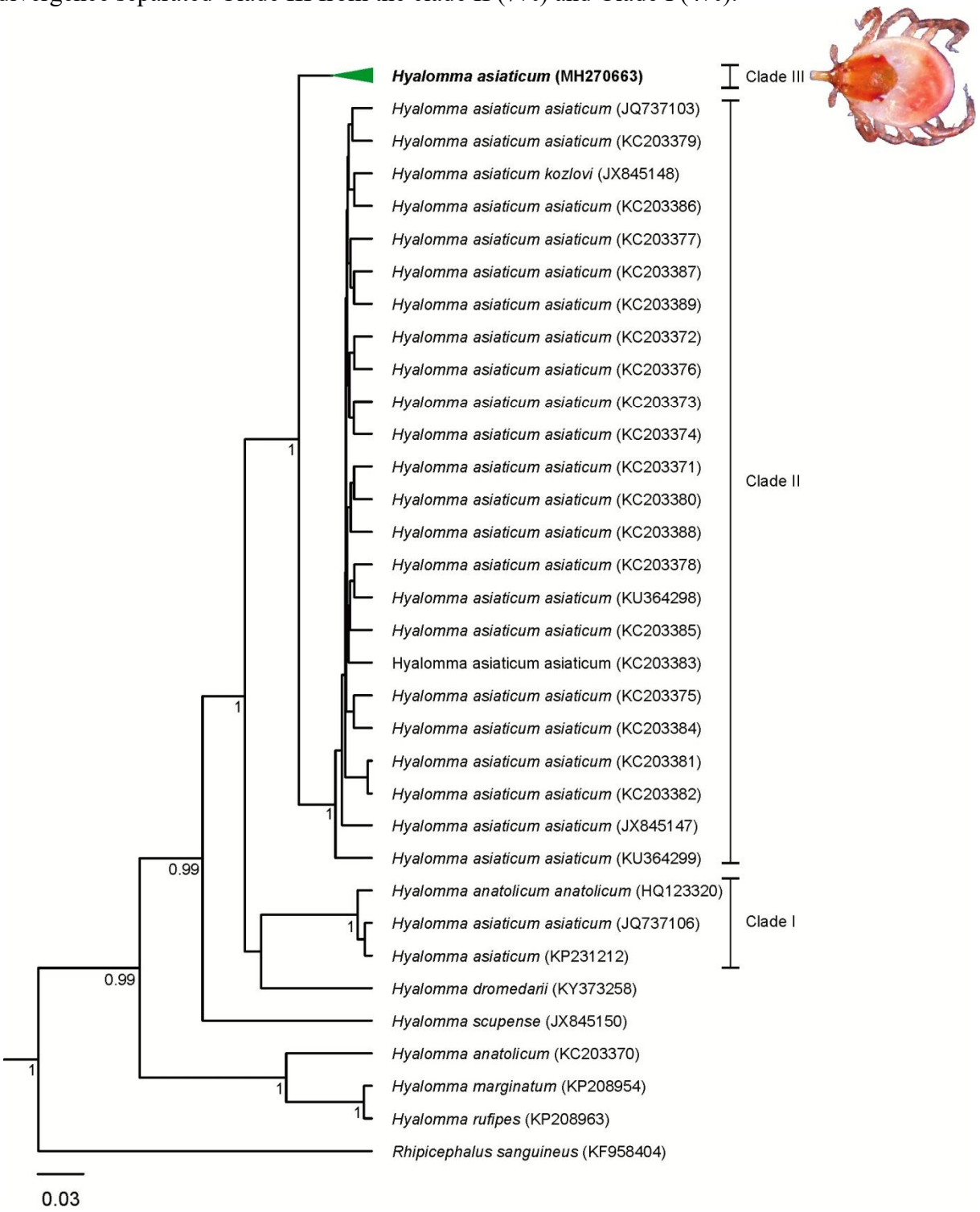

Figure 5. Phylogenetic tree of *Hyalomma asiaticum* ticks inferred from *ITS2* sequence data constructed using Bayesian Inference (BI) method. Nodes indicated with posterior probability values. Branch lengths are proportional to evolutionary changes (substitutions/site). Tree was rooted by *Rhipicephalus sanguineus*.

Table 2. Percentage of MCL genetic distances between the three clades of *H. asiaticum* inferred from *ITS2* gene. Within-clade divergences are shown as diagonal (in bold).

Clades	1	2	3
1. Clade I (China-Iran)	**1**		
2. Clade II (China)	7	**0**	
3. Clade III (Lorestan)	4	7	**-**

DISCUSSION

In the present study, immature stages of *Hyalomma asiaticum* from Persian jird were diagnosed to genus level, morphologically. Consequently, for the first time, immature tick specimens were identified by a sensitive multiplex PCR approach and confirmed using DNA sequencing. Ticks were collected in three regions located in western Iran. The method of multiplex-PCR frequently was used to differentiation of tick and tick borne diseases (Anderson *et al.* 2004; Chan *et al.* 2013; Rodríguez *et al.* 2015; Hosseini-Chegeni *et al.* 2017). We set out to link unknown immature stages with the corresponding adult stages. Filippova *et al.* (1976) reported infested *M. persicus* with immatures of *Ixodes*, *Haemaphysalis*, *Dermacentor* and *Rhipicephalus* in Iran. *Meriones persicus* has frequently been reported to be infested by various tick species in Iran. Different rodent species such as; *M. persicus* were examined for tick infestation from some localities of Iran (Shayan and Rafinejad 2006; Kia *et al.* 2009; Moravvej *et al.* 2015; Hamidi *et al.* 2016). Moravvej *et al.* (2015) reported a number of tick specimens (without species name) from *M. persicus* in Khorasan-e Razavi province, northeast of Iran. In a study conducted in our study area (Lorestan Province) larval stage of *Haemaphysalis* tick was already found on *M. persicus* (Shayan and Rafinejad 2006). *Meriones persicus* has also been introduced as a novel host for *Haemaphysalis punctata* and *Ixodes trianguliceps* in northeast of Iran (Hamidi *et al.* 2016). Based on our results we confirmed that immatures of *H. asiaticum* live on *M. persicus* in rodent burrows. This helps us better our understanding of ecology and biology (life cycle) *H. asiaticum* in order to control tick borne diseases associated to tick vectors. Further studies are needed to investigate microclimatic conditions on the tick-host interaction, tick's seasonal dynamics and predictions of the spatial and temporal variation in parasite transmission by this common vector.

In traditional taxonomy, *H. asiaticum* is classified as a member of *H. asiaticum* group. This group includes: *H. dromedarii*, *H. impeltatum*, *H. schulzei* and *H. somalicum* (Apanaskevich and Horak 2010). Four subspecies namely, *H. a. kozlovi* Olenev, 1931, *H. a. citripes* Schulze, 1935, *H. a. caucasicum* Pomerantzev, 1940 and *H. a. asiaticum* Schulze and Schlottke, 1929 were described from polytypic *H. asiaticum* according to morphological criteria such as: leg, capitulum, shields and spiracle plate of male specimens (Schulze 1935; Pomerantzev 1950). Filippova *et al.* (1995) stated that all taxonomical characters on all phases of all subspecies overlap. Camicas *et al.* (1998) and Horak *et al.* (2002) accepted three subspecies *asiaticum*, *caucasicum* and *kozlovi* as valid identities. Nonetheless, none of these subspecies has been accepted in recent acarological literature and all of them were proposed to be considered as junior synonyms of a single species, *H. asiaticum* (Guglielmone *et al.* 2014; Vatansever 2017). Our results revealed three divergent *H. asiaticum* clades with high genetic distances at the level of species. Genetic differences between the three clades are equal to the distances between closely related species (4-7%). The presented *ITS2* phylogenetic analysis indicates that *H. asiaticum* ticks collected from *M. persicus* (Clade III) is closely related to Clade II originating from China. However, Clade I is a sister clade to *H. dromedarii*. Based on the main principal criteria for species boundary in various concepts (e.g., cladistics, sensu Hennig 1966) species are grouped into natural, or monophyletic groups based on sharing of synapomorphic characters. However, *H. asiaticum* appeared paraphyletic in our gene tree, which certainly points us to the presence of more than one species in this complex morphospecies. In other words, *H. asiaticum* is not a genetically homogenous species; with three

deeply divergent clades which in turn keeps all the earlier controversies alive regarding the taxonomic affinities of this complex species, and highlighting the necessity of an integrative taxonomic revision (genetic, morphology, ecology, biology, etc). In conclusion, *H. asiaticum* should be split into at least three distinct genetic entities. At the moment, we do not advocate any hypothesis regarding the species/subspecies level of divergence, but suggest providing more relevant data and comprehensive sampling for accurate taxonomic classification.

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آلودگی ژربیل ایرانی با مراحل نابالغ *Hyalomma asiaticum* (Acari: Ixodidae) و شناسایی آن با استفاده از روش‌های مولکولی در ایران

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چکیده

در مطالعه حاضر، وجود آلودگی به *Hyalomma asiaticum* روی جوندگان وحشی بررسی شده است. جوندگان با استفاده از تله شرمن شکار و سپس نمونه‌های کنه با پنس از روی جوندگان جدا شدند. در مجموع یک لارو و ۵۹ عدد پوره از روی ۲۳ سر *Meriones persicus* از سه منطقهٔ مختلف واقع در غرب ایران جمع آوری شدند. قطعه ۴۰۸ جفت بازی از ناحیه 5.8S/ITS2 با استفاده از PCR از ۶۰ نمونه کنه تکثیر شد و یک نمونه با موفقیت توالی‌یابی شد. نتایج بلاست نشان داد که ۹۹ درصد شباهت بین توالی هاپلوتایپ مطالعه حاضر و دو توالی بانک ژن دیده می‌شود. بنابراین نتیجه گرفته می‌شود که مراحل نابالغ *H. asiaticum* روی *M. persicus* و یا درون تونل‌های لانه این حیوان زندگی می‌کنند. این دستاورد برای درک بهتر اکولوژی این گونه کنه و کنترل بیماری‌های منتقله از طریق کنه کمک خواهد کرد.

واژگان کلیدی: *Hyalomma asiaticum* ؛ پوره؛ *Meriones persicus* ؛ لرستان؛ ایران.

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