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Article

Multiplex-PCR differentiation of two *Hyalomma* and two *Haemaphysalis* species (Acari: Ixodidae)

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ABSTRACT
Hyalomma and *Haemaphysalis* are two most important genera of ticks (Acari: Ixodidae). The rapid and accurate identification of field collected specimens is crucial in faunal works, laboratory assays, anti-tick vaccine experiments, etc. The present study was designed to introduce a rapid and more sensitive method for the differentiation of closely related *Hyalomma* and *Haemaphysalis* species namely the pairs *Hy. anatolicum-Hy. asiaticum* and *Ha. punctata-Ha. sulcata*, as the main vectors of different animal and human pathogenic agents. Tick specimens were collected from domestic animals in Ardabil, Hormozgan, Khuzestan, Kurdistan, Lorestan, and Mazandaran and identified according to the taxonomic keys. DNA was extracted by the Phenol-Chloroform method. Then, PCR was carried out in a single PCR reaction tube using three pairs designed primers (one forward and two reverse) adapted from the internal transcribe spacer 2 (ITS2) and cytochrome oxidase subunit 1 (COI) genes for *Hyalomma* and *Haemaphysalis*, respectively. In the present study, results of Multiplex-PCR revealed that the pairs *Hy. anatolicum-H. asiaticum* and *Ha. punctata-Ha. sulcata* could be well differentiated on gel electrophoresis. Morphological misidentification of *Hy. anatolicum-Hy. asiaticum* and *Ha. punctata-Ha. sulcata* could be reduced significantly after using Multiplex-PCR.

KEY WORDS: Closely related species; COI, ITS2; *Hyalomma*; *Haemaphysalis*; Identification.

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INTRODUCTION

The worldwide public health impact and economic losses caused by ticks and tick-borne diseases (TBD) to human and livestock health in terms of mortality and morbidity is undisputed (de Castro 1997; Piper 2011). The epidemiologic surveillance of tick transmitted diseases basically is relying on the correct identification of tick vectors (Black and Munstermann 2005). *Hyalomma* and *Haemaphysalis* are amongst important genera of ixodid ticks as vectors of many tick borne animal and human pathogenic agents (Hosseini-Chegeni *et al.* 2013, 2014). Morphological identification of *Hyalomma* and *Haemaphysalis* species using taxonomical keys is a conventional method of tick identification; however, misidentification may occur during this process. Morphologically, the

representative specimens of these species are easily identified but occurrence of some inter-population variations can complicate tick identification (Filippova 1997; Hosseini *et al.* 2011). Similarly, the differentiation of some closely related species is really difficult and trained and experienced tick taxonomists are required. Moreover, the determination of female *Hyalomma* species (except few species) and immature stages is not possible only based on morphology (Hosseini-Chegeni 2015). The reliable, rapid and simple identification of tick vectors involved in tick borne diseases may be a valuable work in epidemiological surveillance even in small geographic areas (Rumer *et al.* 2011). Therefore, an alternative method for rapid and accurate identification is needed, especially for verification of specimen identification for different research purposes. Systematics has been revolutionized after the invention of PCR by Kary Mullis in 1983, and the impact of PCR on biological and medical research has been like a supercharger in an engine, dramatically speeding the rate of progress of the study of genes and genome (Mcpherson and Moller 2006). Molecular tools rather than morphological characteristics can therefore provide a better understanding of the epidemiology of target tick vectors. Similarly, the correct identification can be obtained without the need of highly trained taxonomists (Black and Munstermann 2005). Multiplex-PCR is a convenient and reliable approach for multi-target identification of species in a single PCR reaction tube (Elnifro *et al.* 2000; Markoulatos *et al.* 2002). Mitochondrial and nuclear DNA has been used extensively for the identification of ixodid and argasid ticks (Caporale *et al.* 1995; Mtambo *et al.* 2007; Lempereur *et al.* 2010; Rumer *et al.* 2011; Brahma *et al.* 2014; Hosseini-Chegeni *et al.* 2015). Similarly, a Multiplex-PCR study was conducted on metastriate tick immature stages (Anderson *et al.* 2004). The present study was designed to introduce a rapid and more sensitive method for the differentiation of closely related *Hyalomma* and *Haemaphysalis* species namely *Hy. anatolicum-Hy. asiaticum* and *Ha. punctata-Ha. sulcata*, all are the main vectors of different animal and human pathogen agents in Iran (Hosseini-Chegeni *et al.* 2013, 2014; Telmadarraiy *et al.* 2015).

MATERIALS AND METHODS

Tick specimens

Tick specimens were collected from livestock within the provinces including *Hy. anatolicum* from Hormozgan (Minab, Bashagard), Khuzestan (Shush), Kurdistan (Bijar), Lorestan (Khorramabad), Ardabil (unknown region); *Hy. asiaticum* from Lorestan (Khorramabad), Ardabil (Meshginshahr); *Ha. sulcata* from Lorestan (Zagheh), Mazandaran (Tonekabon) and *Ha. punctata* from Mazandaran (Ramsar). The tick specimens were kept at −20°C or alive then transported to the laboratory. Five male and five female specimens of each species collected from different geographical locations were examined for molecular assay. These specimens were selected from at least one hundered specimens except tens number of *Ha. punctata* specimens.

Morphological studies

Tick specimens were identified at species level under the light stereomicroscope (SZX12-Olympus®, Japan) using taxonomic keys by Hosseini-Chegeni *et al.* (2013) and Hosseini-Chegeni *et al.* (2014) for *Hyalomma* and *Haemaphysalis* species, respectively. Then, photos were taken from some representative specimens by the camera Nikon® Coolpix connected to a stereomicroscope (Olympus®).

Molecular studies: Primer design

For designing primers, previously published sequence data from GenBank were aligned using MEGA6 Ver. 6 (Tamura *et al.* 2013), SeaView Ver. 4.5.4 (Gouy *et al.* 2010) and GeneDoc Ver. 2.7.0 (Nicholas *et al.* 1997). Then desired oligonucleotides were selected and analysed using online software Oligoanalyzer (version 3.1) (*www.eu.idtdna.com/analyzer/applications/oligoanalyzer*) and

then their physicochemical characteristics including GC percentage, melting temperature, secondary structures (hairpin, self and heterodimer) were measured. The candidate oligonucleotides were synthesized through the SinaClon Inc., Iran.

Molecular studies: Polymerase chain reaction (PCR)

Genomic DNA was extracted using the Phenol-Chloroform method according to Hosseini-Chegeni (2015). Two different size ITS2 fragments of nuclear genome of *Hy. anatolicum* and *H. asiaticum* were amplified by PCR using designed primers including forward primer (Fanas: 5′ TCT AAG CGG TGG ATC AC 3′; positions 4-20 within 5.8s gene), reverse primers (Ran: 5′ CTC GAA CCG TCT CAT AGA 3′; positions 581-598 within ITS2 gene and Ras: 5′ CTT TCT TCC CCA GCG G 3′; positions 241-257 within ITS2 gene). As well as, two different size COI fragments of mitochondrial genome the pairs *Ha. punctata-Ha. sulcata* were amplified by PCR using forward primer (Fsul: 5′ CTT ATT GGA AAC GAT CAA ACT TAC 3′; positions 1606-1629 within COI gene) and reverse primers (Rsul: 5′ GCC TGG AAG AAT CAG AAT G 3′; positions 2199-2217 and Rpun: 5′ GAA AAA TGA AGT GTT AAA GTT TCG G 3′; positions 2103-2127 both within COI gene). PCR reactions for the COI gene were carried out in Corbett® thermocycler (Australia) 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), a final extension step (5 min. at 72°C). PCR for each 25 µl final volume reaction was done using 12 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 confirmed by 1% agarose gel electrophoresis.

Test of generality

To determine generality of primers in terms of working, several tick specimens from different parts of Iran were tested.

RESULTS

Morphological studies

The cervical groove and dorsal posterior margin of the basis capituli are the main taxonomical characteristics for morphological differentiation of males of *Hy. anatolicum* and *Hy. asiaticum*. The cervical grooves in *Hy. anatolicum* can be seen as short and superficial with dorsal posterior margin of the basis capituli as slightly concave and without angle (Fig. 1A), in contrast to the typical specimens of *Hy. asiaticum* having long and very deep cervical grooves, and the dorsal posterior margin of the basis capituli which is concave and angular (Fig. 1B). The females of two closely related *Hy. anatolicum* and *Hy. asiaticum* cannot be recognized based on morphological characters, e.g. dorsal scutum (Fig. 3). The most considerable characteristic to separate males of *Ha. punctata* from *Ha. sulcata* was the tip of the spur of coxa IV that is directed centrally and laterally, respectively (Fig. 2). The females are more similar, morphologically however, roughly can be identified based on coxal spurs II-IV especially coxal spur IV. Since, coxal spur IV in *Ha. sulcata* is shorter than the same in *Ha. punctata* (Fig. 4).

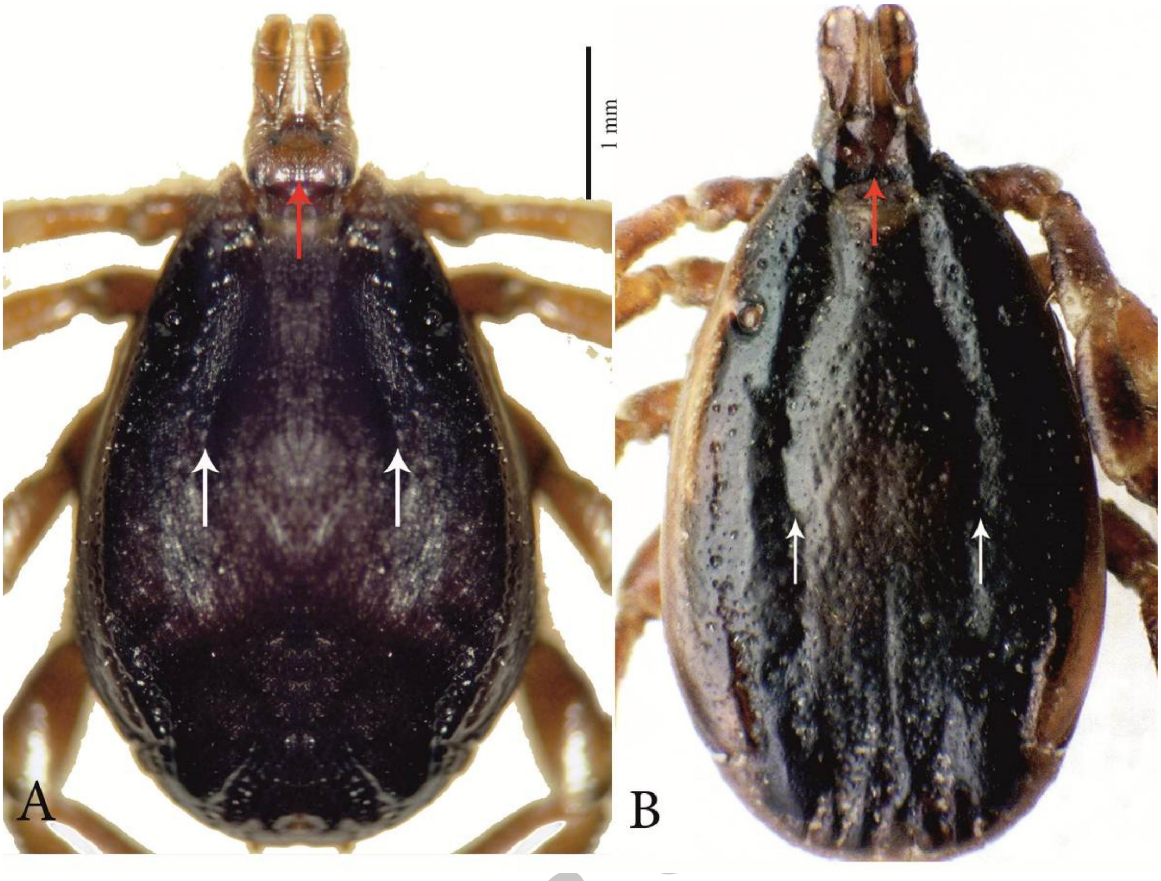

Figure 1. The dorsal morphological aspect of representative male specimens of *Hyalomma anatolicum* (A) and *Hy. asiaticum* (B) both collected from Lorestan province; white and red arrows showing position of characters cervical grooves and dorsal posterior margin of the basis capituli, respectively (size of specimens was not considered).

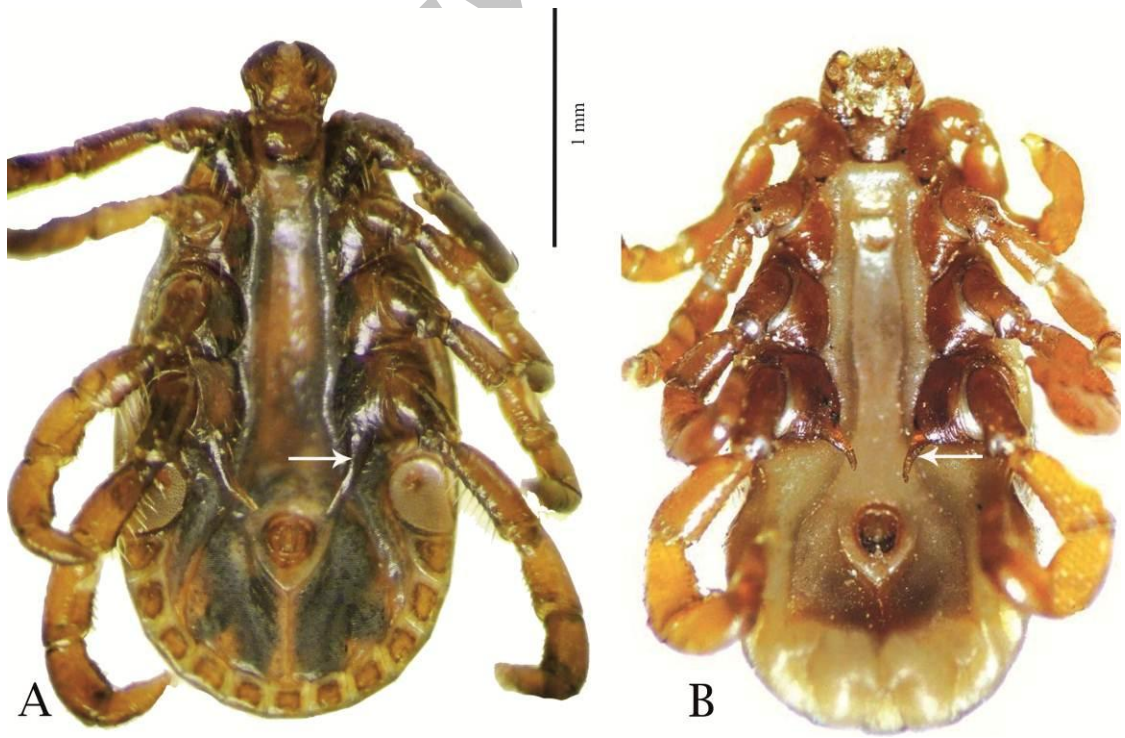

Figure 2. The ventral morphological aspect of representative male specimens of *Haemaphysalis punctata* (A) collected from Mazandaran province and *Ha. sulcata* (B) collected from Lorestan province; white arrows showing position of character spur of coxa IV (size of specimens was not considered).

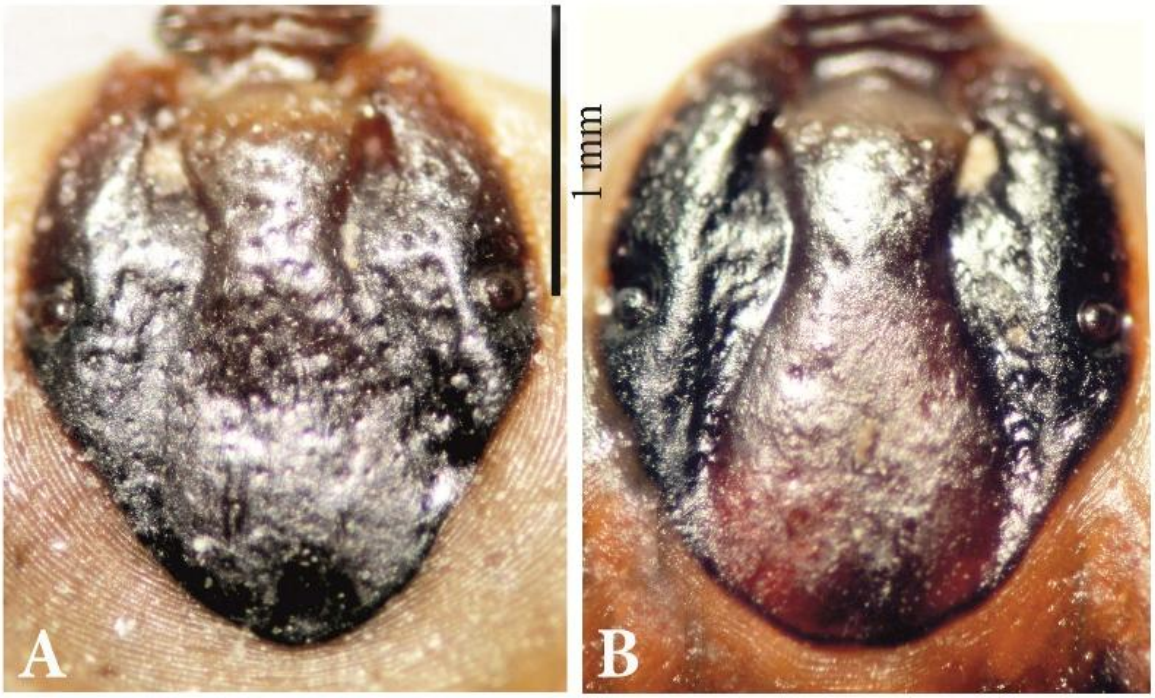

Figure 3. General schema of representative female dorsal scutum of *Hyalomma anatolicum* (A) and *Hy. asiaticum* (B), both collected from Lorestan province (size of specimens was not considered).

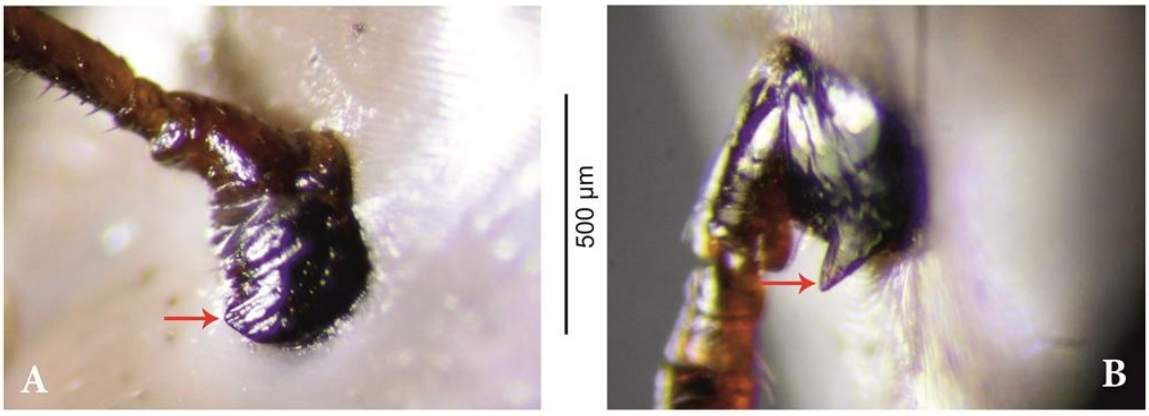

Figure 4. Fourth female coxal spur of *Haemaphysalis sulcata* (A) and *Ha. punctata* (B), both collected from Mazandaran province, Iran.

Multiplex primers

The designed primers could accurately amplify target gene fragments in all examined tick specimens collected from different populations of each species. All primers produced amplicons with the expected length specific for each species. In *Hyalomma*, the length of ITS2 amplicons for primer pairs Fanas/Ran and Fanas/Ras was 749 and 408 base pair, respectively (Fig. 5A). In *Haemaphysalis* the length of COI amplicons for primer pairs Fsul/Rpun and Fsul/Rsul was 524 and 614 base pair, respectively (Fig. 5B).

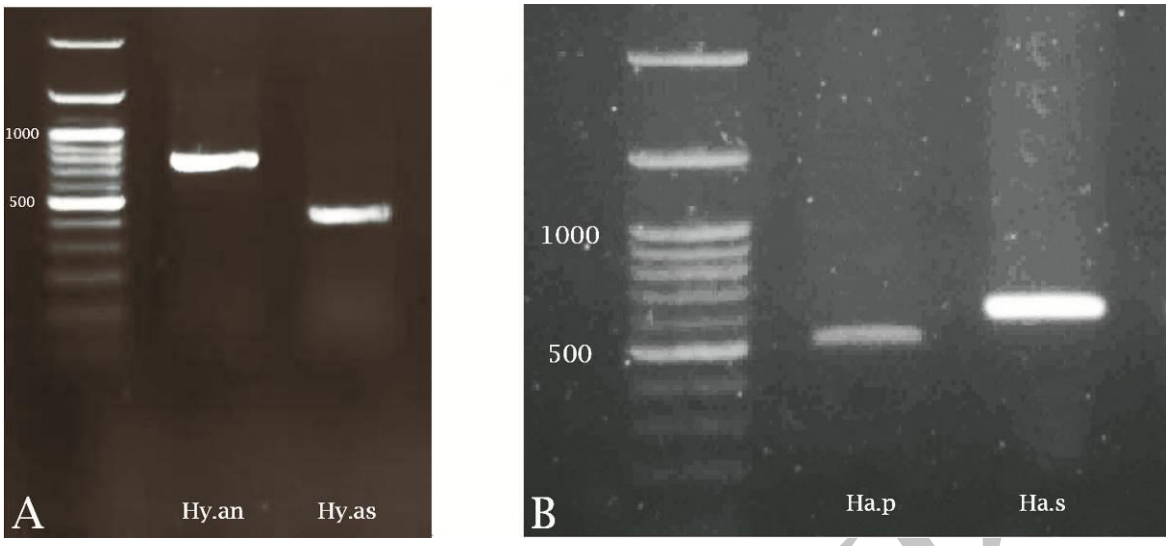

Figure 5. One percent agarose gel electrophoresis stained with Cyber Safe® showing ITS2 fragments amplified using primer pairs Fanas/Ran for *Hyalomma anatolicum* (amplicon size 749 bp) and Fanas/Ras for *Hy. asiaticum* (amplicon size 408 bp) (A), COI fragments amplified using primer pairs Fsul/Rpun and Fsul/Rsul for *Haemaphysalis punctata* (amplicon size 524 bp) and *Ha. sulcata* (amplicon size 614 bp) (B). (100 bp DNA ladder).

DISCUSSION

The molecular taxonomy should be used for the identification of organisms since for certain species or species groups in each of the taxa, traditional identification can be a daunting task, requiring extensive time, training, and experience (Black and Munstermann 2005). In terms of molecular identification genera *Hyalomma* and *Haemaphysalis*, the number of mitochondrial and nuclear genes including ITS2, COI, 12S and 16S were examined (Rees *et al.* 2003; Anderson *et al.* 2004; Chitimia *et al.* 2009; Lv *et al.* 2014). The ticks identification system based on electrophoretic banding patterns were designed for some ixodid and argasid tick species e.g. *Argas*, *Ixodes*, *Hyalomma* (Poucher *et al.* 1999; El-Fiky *et al.* 2003; Abdigoudarzi *et al.* 2004; El-Kammah and El-Fiky 2005). However, a few studies were published on multiplex PCR identification of ixodid and argasid ticks. Only a single study of Anderson *et al.* (2004) developed a multiplex PCR procedure combined with RFLP analysis for differentiation of the tick genera *Amblyomma*, *Dermacentor*, *Rhipicephalus*, and *Haemaphysalis* based on the length of the PCR amplicon and subsequent restriction digestion profile. The present study similar to Anderson *et al.* (2004) was designed to differentiate two pairs of *Hy. anatolicum-Hy. asiaticum* and *Ha. punctata-Ha-sulcata* usually with the problem of detecting them (Hosseini-Chegeni *et al.* 2013, 2014). The molecular diagnostic described here helps to easily, inexpensively, rapidly and correctly determine closely related tick species, the pairs *Hy. anatolicum-Hy. asiaticum* and *Ha. punctata-Ha. sulcata* either to confirm morphological identification or verify material processed in the laboratory assays for faunal works, vector genetics or pathogen surveillance, anti-tick vaccine experiments, etc. All tick species used in the present study are predominant ticks widely distributed in their geographical area including Iran, except *Ha. punctata* which seems to be occupying more limited habitats in northern regions of Iran (Hosseini-Chegeni *et al.* 2013, 2014). All species are medically and veterinary important. The species *Hy. anatolicum* and *Hy. asiaticum* were found to be highly infected with Crimean Congo Hemorrhagic Fever Virus (CCHFV), as well as *Ha. punctata* but to a lesser degree compared to the *Hyalomma* species (Kayedi *et al.* 2015; Telmadarraiy *et al.* 2015). Currently, the taxonomic status of the two closely related *Hy. anatolicum* and *Hy. asiaticum* will be confirmed as distinct species based on COI and ITS2 genes (Hosseini-Chegeni 2015). However, many intermediate forms can complicate the correct identification of male and female *Hy. anatolicum* and *Hy. asiaticum*

(Tavakoli *et al.* 2012). Sometimes the central/lateral direction of coxal spur IV (as the main taxonomic character) in males and the presence/absence of coxal spur II–IV in *Ha. punctata* and *Ha. sulcata* may be subject to debates. Therefore, the Multiplex PCR method presented here should discriminate two species. No taxonomic molecular work has been found on discrimination of *Ha. punctata* from *Ha. sulcata* to date. To determine specificity and sensitivity of a marker (here primer), not only laboratory colonies or single field collections of each species should be examined but also sampling of taxa in the entire dispersal range is needed (Black and Munstermann 2005). According to this, in the present study we successfully adapted the designed primers for different populations of both *Hyalomma* species scattered within Iran.

CONCLUSION

The molecular taxonomy may be better than only morphological identification especially for differentiation of closely related tick species. The rapid, accurate and simple Multiplex-PCR method can be used in the epidemiologic surveillance of tick transmitted diseases, faunal works, laboratory assays, anti-tick vaccine experiments, etc. In the present study, results of Multiplex-PCR revealed that the pairs *Hy. anatolicum-Hy. asiaticum* and *Ha. punctata-Ha. sulcata* could be appropriately differentiated on gel electrophoresis following PCR. Consequently, the morphological misidentification of the pairs *Hy. anatolicum-H. asiaticum* and *Ha. punctata-Ha. sulcata* as well as, correct identification of female specimens even immature stages should be reduced significantly after using Multiplex-PCR.

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افتراق دو گونه از *Hyalomma* و دو گونه از *Haemaphysalis* (Acari: Ixodidae) با روش Multiplex-PCR

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چکیده

Hyalomma و *Haemaphysalis* دو جنس از مهم‌ترین کنه‌های حیوانی (Acari: Ixodidae) هستند. تشخیص سریع و دقیق نمونه‌های جمع‌آوری شده از صحرا بخشی ضروری در مطالعات فون، بررسی‌های آزمایشگاهی، تحقیقات واکسن ضدکنه و غیره است. مطالعهٔ حاضر برای معرفی Multiplex-PCR به عنوان روشی سریع و خیلی حساس برای افتراق جفت گونه‌های نزدیک به هم *Hy. anatolicum-Hy. asiaticum* و *Ha. punctata-Ha. sulcata* به عنوان ناقلین اصلی عوامل بیماری‌زای دامی و انسانی طراحی شده است. نمونه‌ها از استان‌های اردبیل، هرمزگان، خوزستان، کردستان، لرستان و مازندران جمع‌آوری و با کلیدهای تاکسونومیک تا سطح گونه شناسایی شدند. DNA هر نمونه با روش فنل کلروفرم استخراج شد. سپس PCR برای هر نمونهٔ مجزا با استفاده از سه زوج آغازگر طراحی شده (یک رفت و دو برگشت) منطبق بر ژن‌های به ترتیب ITS2 و COI برای کنه‌های *Hyalomma* و *Haemaphysalis* انجام شد. نتیجه نشان داد که گونه‌های *Hy. anatolicum-Hy. asiaticum* و *Ha. punctata-Ha. sulcata* را می‌توان روی ژل الکتروفورز افتراق داد. اشتباه در تشخیص این گونه‌ها با استفاده از روش Multiplex-PCR می‌تواند تا حد زیادی کاهش پیدا کند.

واژگان کلیدی: گونه‌های نزدیک به هم؛ COI؛ *Hyalomma*؛ *Haemaphysalis*؛ ITS2؛ شناسایی.

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