

Nucleotide sequence analysis of the Second Internal Transcribed Spacer (ITS2) in *Hyalomma anatolicum anatolicum* in Iran

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Abstract

Ticks are important acarina that infest animals. They are obligatory blood sucker arthropods which economically impact cattle industry by reducing weight gain and production. Moreover, they are important vectors of viral, bacterial, rickettsial and parasitic pathogens infecting humans and animals. In view of the importance of *Hyalomma anatolicum anatolicum* in pathogen transmission, including *Theileria lestoquardi* in Iran, the accurate identification of this tick is critical. Although many keys are available as aids, morphological identification of tick species such as *Hyalomma anatolicum anatolicum* (Koch, 1844; Hoogstral and Kaiser, 1959) is difficult and expert knowledge is required. False morphological identification at the level of species and subspecies is common, particularly for *Hyalomma excavatum* complex members which are prevalent in Iran. For example, the high similarity between *Hyalomma anatolicum anatolicum* and *Hyalomma anatolicum excavatum* is the cause of confusion in the identification of these species. In this study, polymerase chain reaction (PCR) techniques were used for identification of *Hyalomma anatolicum anatolicum* based on analysis of the gene sequence of the Second Internal Transcribed Spacer (ITS2) of this tick. The ITS2 nucleotide sequence of *Hyalomma anatolicum anatolicum* was 963 base pairs (bp) in length and exhibited 93% homology with other GenBank registered ITS2 sequences of this subspecies (accession no: FJ593700.1). The complete ITS2 region sequence was identified in this study and registered in GenBank under accession number HQ123320.

Introduction

Ticks are important acarina that infest humans and animals. The subspecies of *Hyalomma* which are geographically distributed in Africa and Asia infect both wild and domestic animals. They are of significant veterinary and medical importance as a result of their role in transmission of viral, bacterial, rickettsial and parasitic pathogens.

Hyalomma anatolicum anatolicum, *Hyalomma anatolicum excavatum*, *Hyalomma asiaticum asiaticum*, *Hyalomma detrium*, *Hyalomma aegyptium*, *Hyalomma dromedarii* are commonly distributed in Iran (Rahbari *et al.*, 2007). Several studies have investigated the distribution of tick fauna in Iran (Delpy, 1936; Abbasian, 1961; Mazlum, 1971; Hoogstral and Valdez, 1980; Rahbari, 1995; Telmadarraiy *et al.*, 2004; Nabian *et al.*, 2007; Razmi *et al.*, 2007). Nabian *et al.* (2009) reported the presence of *Hyalomma anatolicum*, *Hyalomma marginatum*, *Hyalomma detrium* and *Hyalomm asiaticum* species in

all four zoogeographical zones of Iran.

The identification of tick species is frequently based on morphological keys (Koch, 1844; Hoogstral and Kaiser, 1959). Apanaskevich *et al.* (2005) reported the taxonomic status of *Hyalomma anatolicum anatolicum* and *Hyalomma anatolicum excavatum* (Acari: Ixodidae) with redescription of all stages.

Despite the availability of numerous keys, morphological identification of tick species including *Hyalomma anatolicum anatolicum* (Koch, 1844; Hoogstral and Kaiser, 1959) is difficult and expert knowledge is required. False identification at the level of species and subspecies is common, particularly for *Hyalomma excavatum* complex members, which are prevalent in Iran.

This study aimed to circumvent the problems associated with identification based on taxonomy and morphological characteristics by development of an identification protocol based on polymerase chain reaction (PCR) techniques for the detection of species specific polymorphisms in the ITS2 gene.

Material and Methods

Sample collection and tick identification

Fifty male ticks, previously identified as *Hyalomma anatolicum anatolicum*, were stored in 70% ethanol and were received from the Iranian National Museum of Parasitology, Faculty of Veterinary Medicine, University of Tehran. Following examination under a stereomicroscope, 10 of these ticks were re-identified as *Hyalomma anatolicum anatolicum* using available taxonomic keys (Walker, 2007). The dorsal integument of the male ticks was removed carefully under a dissecting microscope using a scalpel. The salivary glands and midgut were separated and homogenized in PBS (pH 7.2) as described by Purnell and Jouyner (1968). The resulting suspensions were centrifuged at 5,000 rpm for 5 min at 4°C and stored at -20°C prior to analysis.

Table 1: The major characteristics of male *Hyalomma anatolicum anatolicum* (Walker, 2007).

<i>Hyalomma anatolicum anatolicum</i>
Dorsal view:
Cervical field depression is apparent
Conscutum is pale in color
Lateral grooves are short
Caudal depression is present
Central festoon is dark in color
Paracervical festoons are separate anteriorly
Posteromedial groove is present (long and narrow)
Paracervical grooves are small
Leg coloration in pale rings
Punctuation sizes are small
Ventral view:
Subanal plates in alignment with adanal plates
Subanal plates indistinct, small and may be absent
Adanal plates shape has rounded end
Spiracle areas have sparse setae

DNA extraction

Genomic DNA was extracted from suspensions of salivary gland and midgut tissue from each tick using a DNA Isolation kit (MBST, Iran) according to the instructions provided by the manufacturer. Briefly, salivary glands and midgut were lysed in 180 µl of lysis buffer. Proteins were degraded by the addition of 20 µl proteinase K and incubation for 10 min at 55°C. After addition of 360 µl binding buffer and incubation for 10 min at 70°C, 270 µl ethanol (100%) was added and the solution was vortexed. The MBST column was centrifuged and then washed twice with 500 µl washing buffer before the complete volume for extraction of DNA was transferred to the MBST column. Finally DNA was eluted from the carrier with 100 µl elution buffer.

PCR amplification of ITS2

Total genomic DNA extracted from ticks was used as the template for PCR. The complete ITS2 nucleotide sequence was amplified in two steps. In the first step the upstream region of ITS2 was amplified using a forward primer (P1) derived from the nucleotide sequence 1 to 20 of the ITS2 region (accession no: FJ593703) and a

reverse primer (P2) derived from the nucleotide sequence 517 to 536 of the ITS2 region (Accession N°. FJ593703). In the second step the downstream region of ITS2 was amplified using a forward primer (P3) derived from a sequence upstream of the first reverse primer (nucleotide 460 to 480) and a reverse primer (P4) derived from nucleotides 943 to 963 of the ITS2 region (accession no: FJ593703), resulting in a PCR product with a region overlapping the first PCR product. Primer sequences are listed in Table 2. The PCR was performed in a total volume of 100 µl containing 1xPCR buffer; 2.5 U Taq polymerase (Cina Gene, Iran), 2 µl of each primer (P1/P2, P3/P4, 20 mM Cina Gene), 2 µl dNTPs (20 mM, Fermentase), 1.5 mM MgCl₂, 2 µl DNA (100 ng) and made up to 100 µl with sterile distilled water. The PCR was carried out using an automated thermocycler programmed as follows: 5 min denaturation at 95°C followed by 35 cycles of 45 s at 94°C (denaturing step), 58-62°C (annealing step), 1 min at 72°C (extension step) and Finally PCR was completed with an additional extension step for 10 min. Simultaneous negative (no template) controls were included in all PCR experiments.

Table 2: Details of PCR primers used for amplification of the ITS2 region.

Name	Nucleotide sequence	GenBank Accession Number	PCR product (bp)
P1 (forward)	5'ggcttcccttggtctgtc3'	FJ593703	536
P2 (reverse)	5'cgacacogagacggctgcac3'	FJ593703	
P3 (forward)	5'catgttgctatcggagtc3'	Sequence data from this study	500
P4 (reverse)	5'gagcagttgcgtgataagcga3'	FJ593703	

Gel electrophoresis

PCR products were analyzed by 1.5% agarose gel electrophoresis in 0.5xTBE buffer (5 x TBE buffer, 54 g Tris base, 27.5 g boric acid and 20 ml 0.5 M EDTA (pH 8.0) in 1 (H₂O)) containing ethidium bromide for visualization under UV illumination. The molecular weight standard used was the 100-bp DNA Marker (Fermentase). Migration distances of ITS2 amplicons were measured for molecular weight calculation.

Cloning and Sequencing of ITS2

PCR products (10 µl) were separated by 1.5% agarose gel electrophoresis in 0.5xTBE buffer. Positive bands were visualized using ethidium bromide under UV illumination. PCR products were sequenced after extraction from agarose gel (agarose gel extraction kit, MBST, Iran) or after purification of PCR products (PCR purification kit, MBST, Iran). In this case, direct sequencing of the PCR products was not possible. Therefore, fresh (less than 1-day-old) purified PCR products were first cloned into pTZ57R/T using a pTZ57R/T cloning kit (Fermentase) according to the instructions provided by the manufacturer. Cloned PCR products were transformed into *E. coli* strain DH5α. Positively transformed colonies were identified by blue/white colony screening using IPTG in conjunction

with X-Gal. If X-gal and an inducer of β -galactosidase (usually IPTG) is contained within an agar medium on a culture plate, colonies which have a functional *lacZ* gene can easily be distinguished. After the transformation process, the bacteria is spread on nutrient agar plates, which mostly contain antibiotics as well. Successfully transformed bacteria has a truncated β -galactosidase gene, causing white colonies on the plate. The identity of purified clones was confirmed by PCR, diagnostic restriction endonuclease (*Bam*HI and *Eco*RI) digestion and nucleotide sequencing. Sequences were obtained from clones purified using a High Pure plasmid isolation kit (MBST, Iran). Sequencing was performed from both sides (Faza Biotech and Kawsar Biotech Co., Iran) using the Sanger method (1977). Nucleotide sequences were compared using BLAST (Basic Local Alignment Search Tool) software.

Results

Hyalomma anatolicum anatolicum were identified using available taxonomic keys (Walker, 2007) based on the features presented in Table 1. DNA was extracted from suspensions of midgut and salivary gland tissue obtained from individual ticks. The complete ITS2 region was amplified in two steps using two pairs of specific primers. In the first step a PCR

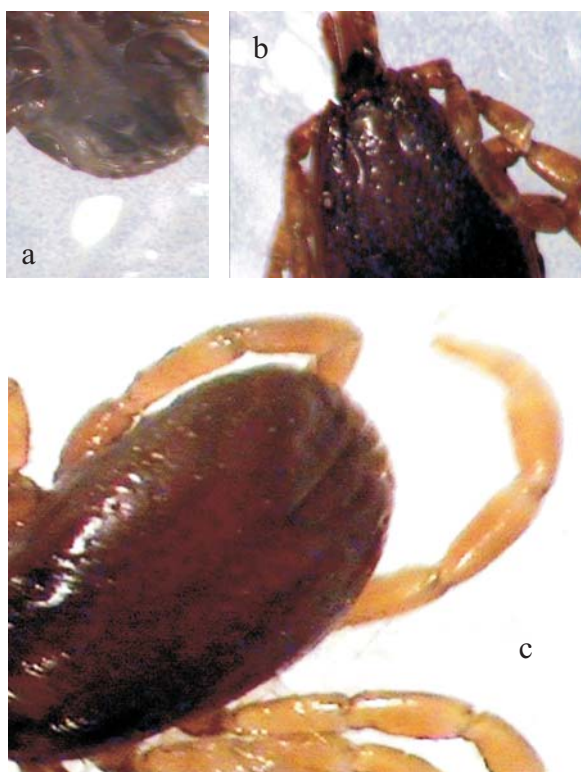


Figure 1: *Hyalomm anatolicum anatolicum* male, ventral (a): subanal plates alignment is with the adanal plates dorsal (b): cervical field depression is apparent, dorsal (c): lateral grooves are short and central festoon is dark colored.

product of approximately 536-bp was amplified using the P1/P2 primer pair and cloned into the pTZ57R/T plasmid. PCR was used for direct analysis of positive transformants and successful amplification was confirmed. The identity of clones was further confirmed by diagnostic double digestion of the resultant plasmids with *Bam*HI and *Eco*RI (Figure 2a). These results demonstrated that a 536 bp fragment of the ITS2 gene sequence was successfully cloned into the pTZ57R/T plasmid. In the second step, a PCR product of approximately 500 bp in length was amplified using the P3/P4 primer pair (Figure 2b). The full length of the ITS2 gene of *Hyalomma anatolicum anatolicum* was identified as 963 bp the sequence was registered with GenBank (accession no: HQ123320).

A comparison of the sequence identified in this study with previously registered *Hyalomma anatolicum anatolicum* nucleotide sequences in GenBank showed 93% homology (Accession N° FJ593700.1). Furthermore, the nucleotide sequence of *Hyalomma anatolicum anatolicum* described here showed less homology with the sequences of other tick species, for example 29% to *Hyalomma marginatum*

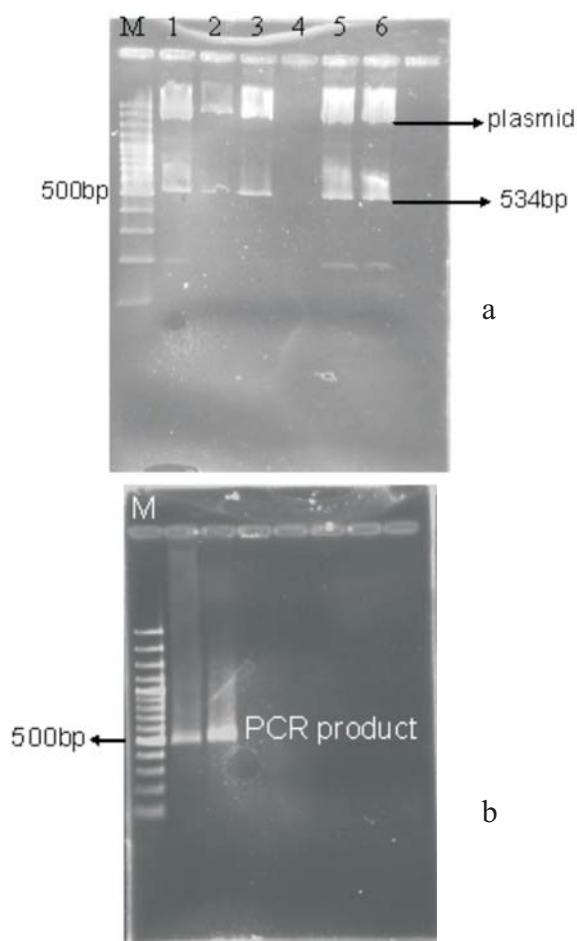


Figure 2: Agarose gel electrophoresis of upstream (a) and downstream (b) regions of ITS2, 100-bp DNA marker.

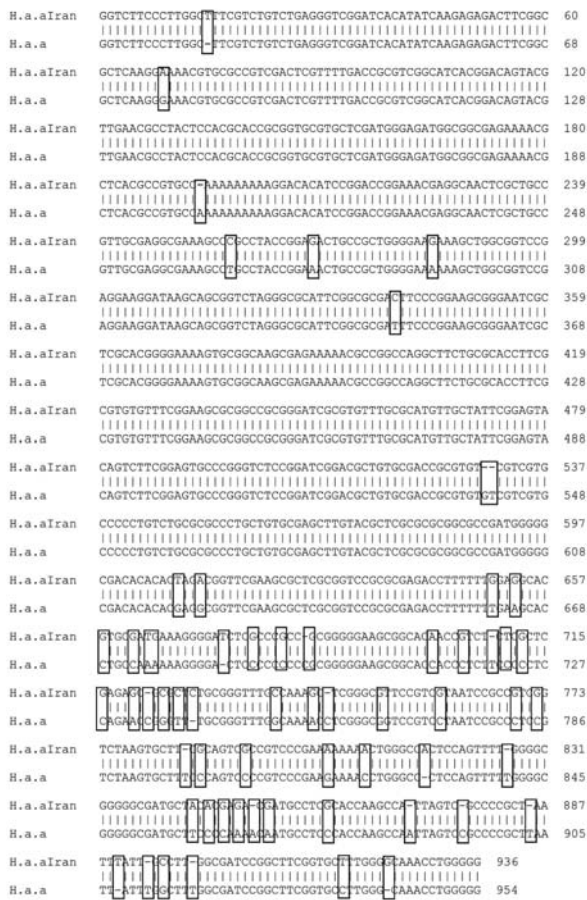


Figure 3: Alignment of complete nucleotide sequence of ITS2 region of rRNA gene from Iranian *Hyalomma anatolicum anatolicum*.

(Accession N° AY228234. 1), 21% to *Hyalomma dromedarii* (Accession N° AJ437371.1), 21% to *Hyalomma truncatum* (Accession N° AJ437394. 1), 13% to *Rhipicephalus turanicus* (Accession no DQ849268.1) and 13% *Rhipicephalus bursa* (Accession N° FJ416323.1).

Discussion

This is the first report of the use of nucleotide sequence analysis of ITS2 for identification of *Hyalomma anatolicum anatolicum* and prior to this report, only a partial sequence of *Hyalomma anatolicum anatolicum* was registered in GenBank. Abdigoudarzi *et al.* (2004) used raid amplification of polymorphic DNA PCR (RAPD-PCR) for the differential analysis of *Hyalomma anatolicum anatolicum* and *Hyalomma marginatum* (Acari: Ixodidae) in Iran. ITS2 nucleotide sequence is currently used for determination of other closely related species of hard ticks. Dergousoff *et al.* (2007) differentiated three species of Ixodid tick *Dermacentor andersoni*, *Dermacentor variabilis* and *Dermacentor albipictus* using a PCR-based approach for specific

amplification of a ribosomal DNA (rDNA) marker. Furthermore, numerous ITS2 rDNA sequencing studies in closely related tick species have been reported (Zahler *et al.*, 1995; Zahler and Gothe 1995; Barker 1998; Fukunaga *et al.*, 2000; Murrell *et al.*, 2001; Show *et al.*, 2002; Kawther *et al.*, 2005). The nucleotide sequence of the ITS2 region of *Hyalomma anatolicum anatolicum* in Iran exhibited 93% homology with other GenBank registered ITS2 sequences of this subspecies (FJ593700.1). Similar comparisons of the complete nucleotide sequence of ITS2 in *Hyalomma anatolicum anatolicum* determined in this study with other GenBank sequences revealed a nucleotide diversity of 7%. Sequence analysis indicated the occurrence of greater diversity in the downstream region of the gene (6%, from nucleotide 608 to 936) than in the upstream region (3%, from nucleotide 1 to nucleotide 607). Rees *et al.* (2002) identified less homology in the COI gene sequences of two species of *Hyalomma* (*H. dromedarii* or *H. truncatum*). Sequence analysis revealed 0.3% diversity of the COI gene between normal individuals of both *H. dromedarii* and *H. truncatum*, whereas differences of 6% to 2.7% were identified in COI nucleotide sequences analyzed from putative hybrids of these ticks, respectively. Analysis of the ITS2 nucleotide sequence of normal and putative hybrids of *H. dromedarii* indicated the possible occurrence of gene flow among the *Hyalomma* species. However, it was concluded that the observed gene flow had no influence on the morphology of individuals. It can be speculated that the diversity in nucleotide sequence of ITS2 of *Hyalomma anatolicum anatolicum* identified in this study and those previously registered with GenBank are the result of gene flow in *Hyalomma* spp. in Iran. The common part of ITS2 (upstream region) represents a target region for identification of *Hyalomma anatolicum anatolicum*, which is essential for the study of the ecology and transmission of tick-borne disease.

Conclusion

A polymorphism in the nucleotide sequence of ITS2 was detected in *Hyalomma anatolicum anatolicum*. Further investigation is required in a greater number of samples of *Hyalomma anatolicum anatolicum* and other members of *Hyalomma excavatum* complex from different geographical parts of Iran in order to validate the use of ITS2 polymorphisms as a basis for identification of these groups of ticks.

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