rDNA-ITS2 Identification of *Hyalomma*,
*Rhipicephalus, Dermacentor and Boophilus* spp.
*(Acari: Ixodidae)* Collected from Different Geographical Regions of Iran

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Abstract

Hard ticks (Acari: Ixodidae) are important obligatory blood feeding external parasites of human and animals. There have been a lot of studies on taxonomy of Iranian ixodidae. On the basis of morphological features found basically in adult male. There are problems in tick identification due to their intraspecific variations and morphological changes of the surface body of ticks after blood feeding. Molecular taxonomic tools have been regarded and internal transcribed spacer2 has been selected to amplify the specific part of the genome of ticks. Then using sequence variations of this marker to drive a molecular phylogeny for collected ticks from Iran. Ticks have been collected during favorable seasons in 2007. They have been diagnosed and DNA was extracted. After PCR amplification of the specific segment, fifty two specific bands have shown related to fifty two individuals. Thirty clear bands have been purified and prepared for sequencing. The sequences have been aligned and released in PubMed and their accession numbers are listed. The levels of intraspecific and interspecific variations have been analyzed using suitable soft wares. These variations yielded different phylogenetic arrangement of ITS2 sequences. The level of intraspecific variability of sequences within *H. anatolicum anatolicum* was high but for *D. marginatus* and *R. turanicus* appeared to be low. All *D. marginatus* sequences were at the same branch, *H. dromedarii* was most resembled to *H. anatolicum anatolicum*, and the sequence of *R. bursa* differed only by 16 fixed bp from the sequence of *R. turanicus*. These results indicate the advantage of ITS2-rDNA analysis in phylogeny of hard ticks and additional trials to collect more ticks from different places of Iran is going to be done to promote the results.

Keywords: ITS2; *Hyalomma* spp., *Rhipicephalus* spp., *Dermacentor marginatus* and *Boophilus annulatus*

1. Introduction

Hard ticks (Acari: Ixodidae) are important obligatory blood feeding ectoparasites transmitting different viral, bacterial and protozoan agents to humans and animals. There are six different reported genera from the ixodidae family in Iran: *Hyalomma* Koch, 1844, *Rhipicephalus* Koch, 1844, *Dermacentor* Koch, 1844, *Boophilus* Curtice, 1891, *Haemaphysalis* Koch, 1844 and *Ixodes* Latreille, 1795. *Hyalomma* and *Rhipicephalus* are the most prevalent genera and related species, *Hyalomma* spp. and *Rhipicephalus* spp., can be found nearly in all provinces of Iran (Mazlum 1971; Abdigoudarzi 2004). These species are vectors of *Theileria annulata* Dschunkowsky and Luhs, 1904 and *Babesia* spp. in cattle and sheep. Although there is not a documented study on direct loss due to tick borne diseases in Iran, but it could be estimated very high when economical cost just for vaccination against *Theileria*
annulata in cattle is estimated more than 2 million dollars per year in Iran (Fesharki, Personal communication).

There have been a lot of studies on taxonomy of Iranian Ixodidae on the basis of morphological characters found in adult male ixodidae ticks (Delpy, 1936, 1946 and 1949; Abdigoudarzi, 2004). The identification of these ticks, particularly Hyalomma and Rhipicephalus species, is difficult even when using nomenclatures (Adler and Feldman-Muehsam, 1948; Hoogstraal, and Kaiser, 1959) Apanaskevich and Horak, 2006 tried to discriminate Hyalomma anatolicum anatolicum Koch and Hyalomma anatolicum excavatum Koch based on morphological characters which were very weak such as size and colour of the scutum of the different stages of tick. Concerning Rhipicephalus species three were described in Iran: R. sanguineus Latreille, R. bursa Can. and Fanz. and R. turanicus Pomerantzev. These tick species are very similar and it should be clarified if they are subspecies or distinct species. The problem is the same for the Dermacentor rare genus in Iran where two very similar species were reported (Mazlum, 1971). In fact, using morphological characters for species identification could be very misleading because of intraspecific variations and morphological changes of the surface body of ticks after feeding process.

Subsequently, it is important to find more relevant identification methods to discriminate the different species and subspecies of ticks. Molecular methods have then been used (Zahler et al. 1995) to clarify the problem of species identification. Zahler et al. (1997) used DNA amplification of partial sequence of ITS2 region when studying Rhipicephalus spp. and Dermacentor spp. Murrell et al., 2001, studied the phylogenetic relationships of the subfamilies of hard ticks. There has been noticed that ITS2 could provide resolution of intrageneric and relationships among genera. The length of the ITS2 in their studies on ticks was approximately 1Kb, but the regions that could be aligned reliably were almost 422 bp. They also noticed that no sequences were available for ITS2 for species from the genus Hyalomma on that time.

Different studies also reported partial sequences of ITS2 region for Hyalomma spp. and Boophilus spp. (Rees et al. 2003; Murrell et al. 2001; Abdigoudarzi unpublished data) Finally, Fukunaga (2000) studied the phylogenetic relation of Ixodidae ticks using ITS2 region and considered it as a good marker for such analyses. Some populations of ticks are efficient vectors of pathogens but others are not. For example, Theileria lestoquardi is the causative agent of malignant theileriosis of sheep and goats, causing morbidity and mortality in these animals in Iran, but the spread of the disease is not compatible with the vector's distribution and there are local small endemic areas for disease. There may be geographical strains of tick which has the potential of protozoan transmission. Regarding molecular systematics of ticks, Hutcheson, et al. (2000) proposed nine questions. Three important of these questions are the position of the Rhipicephalinae, the position of Rhipicephalus and the position of Hyalomma that should be clarified. Accordingly, the purpose of this study was to show if ITS2 molecular marker could be helpful discriminating different genera and species of Ixodidae members after amplification of the gene by
pcr and using sequence variation of ITS2 (ribosomal DNA) to drive a molecular phylogeny for Iranian hard ticks. Then Intraspecific variations and interspecific variations for selected individuals will be denoted.

2. Materials and Methods

2.1. Tick collection

Hyalomma anatolicum anatolicum, Hyalomma marginatum Koch, Rhipicephalus turanicus, Rhipicephalus bursa, Dermacentor marginatus Sulzer and Boophilus annulatus Say were collected during field campaigns in spring and summer 2007. Information regarding the geographical origins and number of individuals collected for each species are summarized in tables 1, 2. Host preferences of the collected ticks were generally sheep and goats, otherwise it is commented.
Table 1: List of taxa, their geographical origins and the GenBank accession numbers

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample code number</th>
<th>Geographical origin</th>
<th>Life Stage</th>
<th>Lg. bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. turanicus</td>
<td>1rt</td>
<td>Iran-Fars- Arjan- Benroud- 2/3/1386</td>
<td>M/F</td>
<td>376</td>
</tr>
<tr>
<td>R. turanicus</td>
<td>2rt</td>
<td>Iran-Fars- Arjan- Benroud- 2/3/1386</td>
<td>F_1</td>
<td>202</td>
</tr>
<tr>
<td>R. turanicus</td>
<td>3rt</td>
<td>Iran-Fars- Arjan- Benroud- 2/3/1386</td>
<td>M/F</td>
<td>621</td>
</tr>
<tr>
<td>R. turanicus</td>
<td>4rtf</td>
<td>Iran-Fars- Arjan- Benroud- 2/3/1386</td>
<td>F</td>
<td>504</td>
</tr>
<tr>
<td>R. turanicus</td>
<td>Rtf(t2)</td>
<td>Iran-Fars- Arjan- Benroud- 2/3/1386</td>
<td>F</td>
<td>916</td>
</tr>
<tr>
<td>R. bursa</td>
<td>5rb</td>
<td>Iran-Az.sharghi-Marand-Kashksaray-23/5/86</td>
<td>F</td>
<td>898</td>
</tr>
<tr>
<td>R. bursa</td>
<td>7rb</td>
<td>Iran-Az.sharghi-Marand-Kashksaray-23/5/86</td>
<td>F</td>
<td>579</td>
</tr>
<tr>
<td>R. bursa</td>
<td>9rb</td>
<td>Iran-Az.sharghi-Marand-Kashksaray-23/5/86</td>
<td>F</td>
<td>485</td>
</tr>
<tr>
<td>R. bursa-cattle</td>
<td>12rb</td>
<td>Iran-Az.sharghi-Marand-Kashksaray-23/5/86</td>
<td>F</td>
<td>677</td>
</tr>
<tr>
<td>R. bursa-cattle</td>
<td>99rb</td>
<td>Iran-Az.sharghi-Marand-Kashksaray-23/5/86</td>
<td>F</td>
<td>818</td>
</tr>
<tr>
<td>R. turanicus</td>
<td>28rs</td>
<td>Iran-Fars- Ghir va Karzin-Baghe nou-Karzin14/6/86</td>
<td>F</td>
<td>212</td>
</tr>
<tr>
<td>R. turanicus</td>
<td>29rs</td>
<td>Iran-Fars- Ghir va Karzin-Baghe nou-Karzin14/6/86</td>
<td>N_3</td>
<td>723</td>
</tr>
<tr>
<td>D. marginatus</td>
<td>6dm</td>
<td>Iran-Az.sharghi-Zonouz-Zonouzagh-23/5/86</td>
<td>M_2</td>
<td>457</td>
</tr>
<tr>
<td>D. marginatus</td>
<td>8dm</td>
<td>Iran-Az.sharghi-Jolfà-Galin ghiyeh-23/5/86</td>
<td>M/F</td>
<td>376</td>
</tr>
<tr>
<td>D. marginatus</td>
<td>10dm</td>
<td>Iran-Az.sharghi-Zonouz-Zonouzagh-23/5/86</td>
<td>F</td>
<td>661</td>
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<tr>
<td>D. marginatus</td>
<td>11dm</td>
<td>Iran-Az.sharghi-Zonouz-Varagh-23/5/86</td>
<td>M</td>
<td>772</td>
</tr>
<tr>
<td>D. marginatus</td>
<td>13dm</td>
<td>Iran-Az.sharghi-Jolfà-Siahroud-sheep-23/5/86</td>
<td>F</td>
<td>627</td>
</tr>
<tr>
<td>H. marginatum</td>
<td>15hm</td>
<td>Iran-Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>M</td>
<td>201</td>
</tr>
<tr>
<td>H. marginatum</td>
<td>30hs</td>
<td>Iran- Fars- Ghir va Karzin-Baghe nou-Karzin14/6/86 Iran-</td>
<td>F</td>
<td>740</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>14har</td>
<td>Iran- Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>M</td>
<td>481</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>Ha1</td>
<td>Iran- Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>F</td>
<td>892</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>Ha3al</td>
<td>Iran- Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>M</td>
<td>289</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>Ha4al</td>
<td>Iran- Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>F</td>
<td>274</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>Ha5al</td>
<td>Iran- Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>M</td>
<td>273</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>Ha6al</td>
<td>Iran- Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>M</td>
<td>287</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>Ha7al</td>
<td>Iran- Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>M</td>
<td>949</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>Ha8al</td>
<td>Iran- Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>F</td>
<td>960</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>Ha9al</td>
<td>Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>F</td>
<td>960</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>Ha10al</td>
<td>Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>M</td>
<td>285</td>
</tr>
<tr>
<td>H. anatolicum anatolicum</td>
<td>Ha10ffal</td>
<td>Fars- Ghir va Karzin-ashayere dashte bilaki-14/6/86</td>
<td>M</td>
<td>960</td>
</tr>
</tbody>
</table>

1=F= Female  
2=M= Male  
3=N=Nymph  
4= Mixed(Male+Female)  
5=in these cases the host is cattle
Table 2 –Geographical origins and number of the sampled ticks’ species with positive amplified bands for ITS2 from spring and summer 2007 tick collection.

<table>
<thead>
<tr>
<th>Species</th>
<th>Hyalomma anatolicum</th>
<th>Hyalomma marginatum</th>
<th>Rhipicephalus turanicus</th>
<th>Rhipicephalus bursa</th>
<th>Dermacentor marginatus</th>
<th>Boophilus annulatus</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iran, Fars, Shiraz, Arjan</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Iran, Fars, Ghir Karzin 1</td>
<td>16</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>Iran, Fars, Ghir Karzin 2</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Iran, Az. Sh. Marand</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Iran, Ghom</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>28</strong></td>
<td><strong>3</strong></td>
<td><strong>11</strong></td>
<td><strong>4</strong></td>
<td><strong>5</strong></td>
<td><strong>1</strong></td>
<td><strong>52</strong></td>
</tr>
</tbody>
</table>

2.2. Identification of Ticks

Ticks were transported in small cotton sealed tubes under suitable temperature and relative humidity to keep their vitality. They have been diagnosed morphologically using different illustrated identification keys (Delpy 1936, 1946, 1949; Pomerantsev 1946, 1950; Hoogstraal 1959; Kaiser et al. 1964; Mazlum 1971). They have been additionally confirmed after their comparison to paratypes kept in Reference Laboratory for ticks and tick-Borne diseases at Razi Institute (denominated by Iranian Ministry of Science, Research and Technology).

2.3. DNA extraction

Ticks were first frozen in liquid nitrogen and crushed individually in microtubes by using small sterile cylindrical glass. Total nucleic acid was extracted from each tick by using DNA isolation kits (Biospin Tissue Genomic DNA Extraction Kit(Bioflux))
rDNA-ITS2 identification

Bioer Technology Co., Ltd. (China) and Dneasy Blood & Tissue Kit from Qiagen (Germany)) or by using a phenol-chloroform-isoamyl extraction method. The concentration of DNA was photometrically measured and quality of PCR products reflects good quality of extracted DNA.

2.4. PCR amplification and DNA sequencing

A pair of degenerative primers was designed for ITS2 amplification and the sequences was kindly sent by Dr. H. J. Hutcheson from Colorado State University, (forward 5´- YTGCGARACTTGGTGTGAAT-3´ and reverse 5´- TATGCTTAARTTYAGSGGGT-3´).

PCR reaction was performed with 2 µl of the extracted DNA template, 2 µl of each primer, 44 µl of dNTPs and PCR buffer, 0.2 µl of Taq DNA polymerase (5U/µl), in a total volume of 50 µl. The reactions mixture were subjected to 2.5 min DNA denaturation at 94°C, 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min and elongation at 72°C for 1 min. The reaction was completed by a further 30 min step at 72°C. Electrophoresis of 1% agarose gels were carried out in 1x TBE buffer at 80 volts for 1 hour. Gels were then examined under the UV light by a gel documentation system from Biorad Company.

PCR products were purified using QIAquick PCR Purification Kit from Qiagen, Germany. The purified PCR products were air dried in a vacuum chamber and have been sent for sequencing to MWG Biotech. Company (Germany). Sequencing of the samples was performed in both directions (forward and reverse).

2.5. Sequence analyses and phylogenetic tree

Sequence alignment was accomplished with MAAFT (Multiple Alignment using Fast Fourier Transform), (Katoh et al. 2002). E-INS-i strategy was used when setting the parameters. Scoring matrix for nucleotide sequences was 200PAM/K=2 and the gap opening penalty was 1.53. DNA sequence analyses for nucleotide and haplotype diversity were conducted using DnaSP version 4.10.9 (Rozas et al. 2003). Gaps and missing data were excluded from analyses. Phylogenetic analyses using the criteria of Neighbour-Joining model and UPGMA model were performed with CLC sequence viewer. Branch supports were calculated by bootstrap analyses (100 replicates). Branches with bootstrap values $\geq 70\%$ were considered resolved (Hillis and Bull 1993).

3. Results

Fifty two individuals from five different geographical places were positive after ITS2 amplification and have got prominent bands on Agarose gel 1% and electrophoresis.
(Figure 1 and 2). Different genera could be discriminated even on gel electrophoresis and using a ladder marker, PeqGOLD DNA-Leiter Mix (Peqlab) (Figure 1 and 2). According to the size marker the sizes for *Hyalomma* spp. and *Rhipicephalus* spp. were estimated 1200bp and 1100 bp respectively. Thirty out of fifty two of samples were selected on the basis of the quality of the bands on gel electrophoresis. They have been purified and sent for sequencing as mentioned in part 2-4 of materials and methods in this manuscript. Results are summarised in table 1.

3.1. Data sequencing and alignment
Accession numbers of the analysed sequences are listed in table 1. The alignments were obtained with MAFFT and obviously misaligned characters were detected and realigned by eye. Estimated mean frequencies of the 4 nucleotides were as follow: adenine 18.5%, cytosine 30.2%, thymidine 17.6% and guanine 33.7%. The transition/transversion rate ratios were estimated \( k_1 = 2.402 \) (purines) and \( k_2 = 2.812 \) (pyrimidines). The overall transition/transversion bias is \( R = 1.626 \), where \( R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)] \) (Tamura et al., 2004). All positions containing gaps and missing data were eliminated from the data set.

3.2. Intraspecific variation
The level of intraspecific variability of sequences within *D. marginatus* (Az. Sh. Marand, Iran), and *R.turanicus* appeared to be extremely low (\( \pi \) values were respectively 0.0019 and 0.0267). No sequence variations were observed for *R. bursa* (Table 3).

Table 3 – Different sizes of ITS2 aligned sequences and calculated \( \pi \) values used for confirmation of sequence variations (Intraspecific variation and Interspecific Variation).

<table>
<thead>
<tr>
<th>Species</th>
<th>Lg (bp)</th>
<th>Nb sequences</th>
<th>Nb haplotypes</th>
<th>S</th>
<th>Hd</th>
<th>( \pi )</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. anatolicum</em> anatolicum</td>
<td>273 - 960</td>
<td>11</td>
<td>7</td>
<td>83</td>
<td>0.818</td>
<td>0.2398</td>
</tr>
<tr>
<td><em>H. marginatum</em></td>
<td>201 - 740</td>
<td>2</td>
<td>2</td>
<td>62</td>
<td>1</td>
<td>0.5345</td>
</tr>
<tr>
<td><em>H. dromedarii</em></td>
<td>297</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0.00673</td>
<td>0</td>
</tr>
<tr>
<td><em>R. bursa</em></td>
<td>485 - 898</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>R. turanicus</em></td>
<td>202 - 916</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>0.4</td>
<td>0.0267</td>
</tr>
<tr>
<td><em>D.marginatus</em></td>
<td>376 - 772</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0.4</td>
<td>0.0019</td>
</tr>
</tbody>
</table>
The 5 *D. marginatus* sequences of 376-772 bp differed by 4 base pair where 3 base pair were gaps and a single base pair corresponded to a segregating site (substitution of G by A). *R. turanicus* 5 sequences differed by 7 segregating sites and 3 gaps. The 273-960 bp long sequences of *H.anatolicum anatolicum* from Fars-Ghir Va Karzin were characterized by higher levels of variability with 83 segregating sites and 108 insertion/deletion sites. Similarly the two sequences of *H. marginatum* showed high level of DNA polymorphism with 63 segregating sites and 67 gaps. On the opposite, the two sequences of *H. dromedarii* Koch showed extremely low levels of nucleotidic diversity (\( \pi = 0.00673 \)) where only 2 sites were polymorphes.

### 3.3. Interspecific Variation

The low degree of sequence variation observed within species (excepting *H. anatolicum anatolicum*) contrasted with moderate to high levels of interspecific variation. This variation yielded different phylogenetic arrangement of ITS2 sequences and different relationships when using UPGMA and neighbour-joining analyses (Figs. 3, 4). Thus several major features of the phylogeny were well supported. First, all *D. marginatus* sequences clustered on the same branch. Second, the *H. dromedarii* sequences also clustered together and most ressembled the *H. anatolicum anatolicum* sequences. Interestingly, no fixed differentiation was observed between these two species. Third, both *R. turanicus* and *R. bursa* sequences clustered and shared a recent ancestor with each other. The sequences of *R. bursa* differed only by 16 fixed bp from the sequences of *R.turanicus*. In both UPGMA and neighbour-joining analyses, *H. anatolicum anatolicum* appeared not to be a monophyletic group. This is probably due to the lenght polymorphism of the available sequences of ITS2 that were mostly partial sequences.
Figure 3- Phylogenetic tree using UPGMA method (CLC Sequence Viewer)
4. Discussion

Murrell et al (2001) studied totally information into the evolution of ticks, and they noted in their studies that the size of ITS2 in ticks should be near 1kb but the aligned region for *Haemaphysalis* spp. was 422 bp. This problem was noted in our study too. They also noticed that no sequences were available for ITS2 for species from the genus *Hyalomma* on that time. The problem is more prominent for *Hyalomma* spp. (figure 2, 3 and table 3) but we could finally amplify the ITS2 segment and complete sequence of 829bp for *B. annulatus* and it was successfully aligned. There are discriminative bands for *Rhipicephalus* and *Boophilus* genera on agarose gel (1%) after electrophoresis and they could be identified just with respect to a ladder marker (figure2).

![Sample agarose gel electrophoresis of PCR products related to ITS2 amplification for Iranian Ixodidae.](image)

1- *Hyalomma marginatum*
2- *Boophilus annulatus* (Accession no. AY702974) (829bp)
3- *Hyalomma marginatum*
4- *Hyalomma anatolicum anatolicum*
5- *Hyalomma anatolicum anatolicum*
6- *Rhipicephalus sanguineus* (not amplified)
7- *Rhipicephalus sanguineus*
Figure 2- Sample agarose gel electrophoresis of PCR products related to ITS2 amplification for Iranian Ixodidae.

1- *Hyalomma sp. female* (≈1200 bp)
2- *Rhipicephalus sp. female* (≈1100 bp)
3- *Hyalomma anatolicum anatolicum* male
4- *Hyalomma sp. female*
5- *Hyalomma anatolicum anatolicum* male
6- *Hyalomma anatolicum anatolicum* male
7- *Hyalomma anatolicum anatolicum* male

M) - PeqGOLD DNA-Leiter Mix (Peqlab)

(Additional time was needed for expansion of the ladder)
There was a code in this study (28rs) and it was a dead female tick, but with soft tissue. It was selected for DNA extraction and regarded as *Rhipicephalus* sp.. After PCR was done and the sequences were ready and aligned, the results shown that it should be *R. turanicus* (table 1). There was another code, 29rs and it was a dried dead nymph. It was selected for DNA extraction and regarded as *Rhipicephalus* sp.. After PCR was done and the sequences were ready and aligned, the results shown that it should be *R. turanicus* too (table 1). There was a 30hs code and it was primarily identified as a female tick from the genus *Hyalomma*. Then after PCR study it was confirmed to be *H. marginatum* (FJ416322).

So, as it can be seen in these three cases, using the ITS2 sequence to identify a species is crucial when studying larval or nymphal stages and even females.

There are different articles reporting partial sequences for ITS2 regions for *Rhipicephalus* and *Dermacentor* spp. (Zahler et al., 1997), report of partial sequence for ITS2 region for *Hyalomma marginatum* (accession number, AY228234) (Abdigoudarzi, unpublished data), reports of different partial sequences for *Hyalomma marginatum* (Rees et al., 2003), reports of complete sequence of ITS2 for *Boophilus annulatus* (accession number, AF271272) (Murrell,A., et al, 2001) and *Boophilus annulatus* (accession number, AY702974) (Abdigoudarzi, unpublished data). Amplification of the entire ITS2 spacer according to different studies produced unsatisfactory results (Zahler et al., 1995, 1997). It may be due to the specific nature of ITS2 in ticks and the repeated fragments in it (Barker, 1998 and Murrell, A. et al., 2001a).

W. C. Black (1994) concluded members of the subfamilies *Rhipicephalinae* and *Hyalomminae* form a monophyletic group with 85-100% support. Their data suggest that *Hyalomma* species share a common ancestor with the *Rhipicephalinae* and should not be placed in a separate subfamily. In our study in both UPGMA and neighbour-joining analyses, *H. anatolicum anatolicum* appeared not to be a monophyletic group too. Or, this is probably due to the length polymorphism of the available sequences of ITS2 that were mostly partial sequences. This problem will be resolved if complete sequence of the ITS2 could be available in future studies.

In our study Since there were little information about molecular study of *Hyalomma anatolicum anatolicum* and *H. marginatum* two partial ITS2 sequences for *H. dromedarii* were selected from Pubmed (GenBank accession no: AJ437375, AJ437376) and data analysis was set according to these and other data from this study totally. The *H. dromedarii* sequences also clustered together and most resembled the *H. anatolicum anatolicum* sequences. Interestingly, no fixed differentiation was observed between these two species. Since, there is no complete hybrid sterility in ticks (Sonenshine, 1991 ) It means that the hybrids could lay viable eggs. This may occur in very relevant species of ixodidae (for example, *H. anatolicum ana.* and *H. dromedarii* where they live at the same ecological niche and select the same host in Bousher province (Southern part of Iran) (Zarif-fard, M and Abdigoudarzi, M, 2000 ).
This could resulted to more complexity of molecular markers (for example ITS2). Murrell et al., 2001b in their study pointed out that a wide taxonomic sample was achieved for 12S, COI and ITS2, but no sequences were available for ITS2 for species from the genera *Hyalomma* and *Nosomma* on that time. Murrell, A. et al., (2001a) studied ITS2 segment in 22 species of ticks in Rhipicephalinae subfamily. There was a specific region and it was repeated in tick's genome. *Rhipicephalus bursa* was not included in this study and the list of taxa in their manuscript does not contain *Rhipicephalus bursa*. There are sequences of *R. bursa* in our study and they could be regarded in future study of the members of Rhipicephaline subfamily. Both *R. turanicus* and *R. bursa* sequences clustered and shared a recent ancestor with each other. The sequences of *R. bursa* differed only by 16 fixed bp from the sequences of *R. turanicus*. This data are consistent with the proposal from Zahler et al. (1997) on conspecific species of (*R. sanguineus, R. turanicus, R. pumilio* and *R. camicasi*). In general these results indicate that examination of the ITS2-rDNA will be useful in examining the phylogenetics of hard tick taxa at genus and species level. Moshavernia et al (2009) in a recent study on *D. marginatus* and *D. niveus* proposed these two species are conspecific. Since the identity and origin of *D. niveus* was neglected and the specimens was not been confirmed by a reference center or expert, the molecular data of this study was not regarded in our study. All *D. marginatus* sequences clustered on the same branch in our study. They have been collected from Iran, Az. Sh., Marand. Additional collection of specimens from other parts of Iran is in progress to promote this study.

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Figure 4-Phylogenetic tree using Neighbour-Joining method (CLC Sequence Viewer)