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ORIGINAL PAPER

Genetic evidence for conspecificity between *Dermacentor marginatus* and *Dermacentor niveus*

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Abstract Ticks are hematophagous arthropods transmitting several harmful human and animal pathogens like viruses, Rickettsia, bacteria, and protozoa. The identification and speciation of ticks were normally performed in Iran using identification key of Arthur (1960) and Kaiser and Hoogstraal (J Parasitol 49:130–139, 1963) or on the basis of morphological characteristic keys recommended by Walker et al. (2003). Although these identification keys are well prepared, but there are in some cases due to the strong overlapping characteristics between species like *Dermacentor marginatus* and *Dermacentor niveus* accompanied with serious problems. *D. marginatus* and *D. niveus* have been intermittently used synonymously and there is no a generally agreement with the specification of these species. To find out more about these two species, we have analyzed the complete nucleotide sequence of ITS-2 region. Interestingly, we found indeed a sequence homology of 99% between nucleotide sequence of ITS-2 region of *D. marginatus* and *D. niveus*. Since the nucleotide sequence of ITS-2 region of *D. marginatus* in Iran has 98% sequence homology to the other in GenBank registered ITS-2 sequence of *D. marginatus*, and the morphological characteristics between both examined species showed minimal differences, therefore we believe that the *D. marginatus* and *D. niveus* could belong to the same species and 1% differences in nucleotide sequence of ITS-2 region between these two species can be understand as an intra-species polymorphism. The complete sequence of ITS-2 region of rRNA gene from *D. marginatus* and *D. niveus* registered under accession no. GQ144707 and GQ144706 by GenBank, respectively.

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Introduction

Ticks are hematophagous arthropods transmitting several harmful pathogens of human and animal, like viruses, Rickettsia, bacteria, and protozoa (Shpynov et al. 2001; Whitehouse 2004; Shayan et al. 2007). Several studies dialing with the characterization of Ticks occurring in Iran were performed (Delpy 1936, 1938; Abbasian 1961; Mazlum 1971; Hoogstral and Valdez 1980; Rahbari 1995; Telmadarraiy et al. 2004; Nabian et al. 2007). The last information about the tick fauna in Iran revealed that 20 tick species were identified as *Hyalomma anatolicum anatolicum*, *Hyalomma marginatum*, *Hyalomma detritum*, *Hyalomma aegptium*, *Hyalomma schultzei*, *Hyalomma dromedarii*, *Hyalomma asiaticum asiaticum*, *Hyalomma anatolicum excavatum*, *Haemaphysalis punctata*, *Haemaphysalis Parva, Haemaphysalis concinna*, *Haemaphysalis choldokovsky*, *Ixodes ricinus*, *Rhipicephalus sanguineus*, *Rhipicephalus Bursa*, *Rhipicephalus Turanicus*, *Boophilus annulatus*, *Dermacentor niveus*, *Dermacentor marginatus*, and *Ornithodoros lahorensis* (Rahbari et al. 2007). For the identification and speciation of ticks, the identification key of Arthur (1960), Kaiser and Hoogstraal (1963), and Walker et al. (2003) were normally used in Iran. Such identification keys are based in the first line on the morphological features and characteristics of tick genera and in the second line are extended on the morphological features and characteristics of species. Although these

Table 1 The nucleotide sequence of primers, corresponding accession no of genes used for designing of primers and the PCR product size were listed in the table

Name	Nucleotide sequences	Accession no	PCR product (bp)
P1	5′TCGTCTGTCTGAGGGTCGGA3′	AF199114	708
P2	5′ATCGTCTCGTGTAGCGTCG3′	AF199114	
P3	5′CGGTCCAAGTGCTTCGCAGT3′	AF199114	482
P4	5′TCGTCTCGCCTGCATCTGAG3′	AF199114	

identification keys are well prepared, in some cases, due to the strong overlapping characteristics between species like *D. marginatus* and *D. niveus*, these accompanied with serious problems. Currently, Nabian et al. (2008) reported the occurrence of three species of *Dermacentor* (*D. marginatus*, *D. niveous*, and *Dermacentor raskamensis*) in Iran. They specified *D. marginatus* and *D. niveus* as separate species. Some other investigators reported also about *D. marginatus* and *D. niveus* as separate species (Telmadarraiy et al. 2004; Rahbari et al. 2007; Nabian et al. 2008). Interestingly, some other investigators (Estrada-Pena and Estrada-Pena 1991; Roman and Sicrat 1957) believed that *D. niveus* is synonyms of *D. marginatus*. Since *D. marginatus* and *D. niveus* have been intermittently used synonymously and there is no a generally agreement with the specification of *Dermacentor* species, we used the molecular-based techniques to answer this problem. For this aim, the nucleotide sequence of ITS-2 region of ribosomal RNA gene were determined by both species and compared with each other.

Materials and methods

Samples and species determination

D. marginatus and *D. niveus* were collected from different area of Iran and preserved in 70% ethanol until used (50 male ticks from each species). The specification was carried out on the basis of morphological characteristic keys recommended by Arthur (1960) and Estrada-Pena and Estrada-Pena (1991) and the male ticks were used for genetical analysis. The preparation of salivary glands was performed according to Brown and Askenase (1986).

Extraction of DNA from salivary gland of ticks

Deoxyribonucleic acid (DNA) was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. Briefly, salivary gland was first lysed in 180 µl lysis buffer, and the proteins were degraded with 20 µl proteinase K 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 to the solution, and after vortexing, the complete volume was transferred to the MBST column. The MBST column was first centrifuged and then washed twice with 500 µl washing buffer. Finally, DNA was eluted from the carrier with 50 µl elution buffer.

PCR amplification

Approximately 100–500 ng DNA was used for the polymerase chain reaction (PCR) analysis. The PCR was performed on 100 µl total volume including one-time PCR buffer; 2.5 U Taq Polymerase (Cina gene, Iran); 2 µl of each primer (P1/P2, P3/P4, 20 mM, Cina gene); 200 µM of each deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate (Fermenta); and 1.5 mM MgCl2 in automated Thermocycler (Eppendorf, Germany) with the following program: 5 min incubation at 95°C to denature double-strand DNA, 36 cycles of 45 s at 58°C (annealing step), 45 s at 72°C (extension step), and 45 s at 94°C (denaturing step). Finally, PCR was completed with the additional extension step for 10 min. The PCR products were analyzed on 1.8% agarose gel in 0.5× TBE buffer (5× TBE buffer, 54 g Tris base, 27.5 g acide boric, and 20 ml 0.5 M EDTA (pH 8.0) in 1 l H_2O) and visualized using ethidium bromide and an UV illuminator (Table 1).

Table 2 The main differentiation characters between *D. marginatus* and *D. niveus* are given

Dermacentor spp.	Basis capituli	Dorsal scutum	Second segment of palp
D. marginatus	Rectangular Dorsally one and	Ornamented Multishape	Small spur or strong postero-median spur
	Half times as broad as long (including conua)	Enamel white	
D. niveus	Rectangular Dorsally one and	Ornamented Base color brown	Strong pronounced spur at the dorsal side
	Half times as broad as long (including cornua)	Enamel yellow-white	

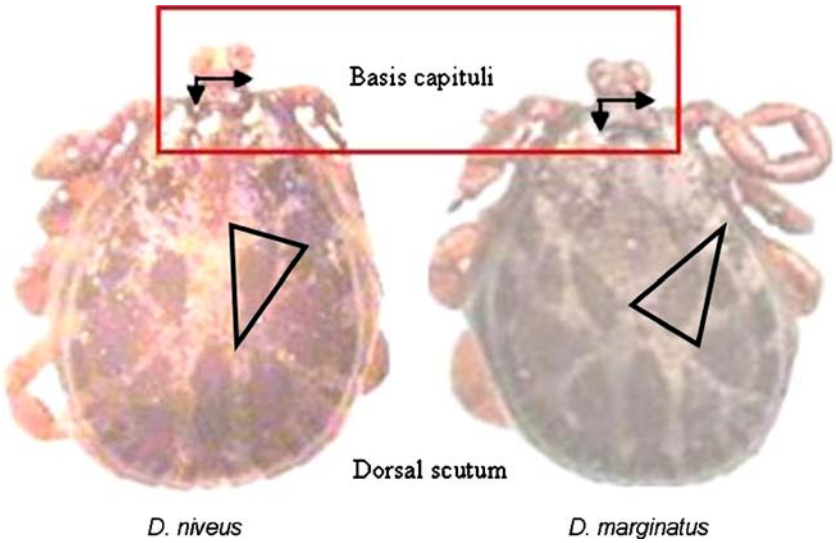

Fig. 1 Basis capituli of *D. marginatus* is like *D. niveus* rectangular and is dorsally one and half times as broad as long in both species. The central dorsal spots are organized approximately triangular with the apical angle directed antrolateral in *D. marginatus* whereas these two spots are vice versa in *D. niveus*

The complete ITS-2 nucleotide sequence was amplified in two steps. In the first step the upstream region of the ITS-2 was amplified using forward primer derived from the nucleotide sequence 1 to 20 of 5.8 S rRNA gene (P1, accession no. AF199114) and the reverse primer derived from nucleotide sequence 690 to 708 of ITS-2 region (P2). In the second step the downstream region of ITS-2 was amplified using forward primer derived from upstream of the first reverse primer from nucleotide 628 to 647 (P3, accession no. AF199114) and the reverse primer derived from nucleotide 1,091 to 1,110 of 5` region of 28 S rRNA (P4, accession no. AF199114) to have the PCR product with the overlapping region with the first PCR product. The primers are listed in Table 2.

Sequencing of PCR products

The PCR products were sequenced after they were extracted from agarose gel or after purification of PCR products. Twenty microliters of PCR product was run on a 1.5% agarose in 0.5% TBE buffer. After visualization of the positive band using ethidium bromide under UV, the PCR product was extracted from the gel using DNA extraction kit from agarose gel (MBST, Iran) according to the manufacturer's instructions. Briefly, the PCR product was cut from the gel under UV control and dissolved in 340 μl binding buffer for 5 min at 60°C. After the addition of 255 μl ethanol (96%) to the sample, the mixture was applied to the spin column and centrifuged for 1 min at 8,000×*g*. The column was washed twice with washing buffer, and the adsorbed DNA was eluted from the column using 20 μl elution buffer.

PCR product was purified from the salts and proteins using PCR purification kit (MBST, Iran). Briefly, 200 μl binding buffer was added to 100 μl PCR product solution. After adding 150 μl ethanol (96%) to the sample, the mixture was applied into the column. The column was washed twice with washing buffer, and PCR product was eluted from the column using 100 μl elution buffer. The sequencing was performed from both sites of each PCR products by Kawsar Biotech Company in Iran basis on Sanger method (1977).

Results and discussion

D. marginatus and *D. niveus* were specified according to the main specific characters such as dimensions of basis capituli, the basis color of scutum, and the second segment of palp. Basis capituli of *D. marginatus* is like *D. niveus* which is rectangular. This was dorsally one and a half times as broad and as long in *D. marginatus* as well as in *D. niveus*. These parameters were consistent in 50 ticks from each *D. marginatus* and *D. niveus* species. In contrast Estrada-Pena and Estrada-Pena (1991) have measured the dorsal basic capituli for *D. marginatus* 0.5–0.9 times as broad and as long (excluding cornua) and they have characterized *D. niveus* provided by Arthur as *Dermacentor* species conspecific with *D. marginatus* lacking the produced dorsal palpal spur and having a darker dorsal pattern than those of the paratypic series of *niveus* collected at Iran. They have also analyzed three males collected from Morocco displaying a palpal shape typical for *D. niveus*, with pronounced spur at the dorsal side of the second palpal

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CLUSTAL format alignment by MAFFT (v6.704b)


D. n Iran     1 atcacatatcaagagagccttcggcgcacagggaacgtgcgtccgtcgactcgttttgac 60
D. m Iran       atcacatatcaagagagccttcggcgcacagggaacgtgcgtccgtcgactcgttttgac 60
D. m            atcacatatcaagagagccttcggcgcacagggaacgtgcgtccgtcgactcgttttgac 60
D. r            atcacatatcaagagagccttcggcgcacagggaacgtgcgtccgtcgactcgttttgac 60
D. a            atcacatatcaagagagacttcggcgcacagggaacgtgcgtccgtcgactcgttttgac 60
                ***************** ****************************************


D. n Iran       cgcgtcggcatcacggacagtacgttgagtgctgaagccacgcgccagcggcctcacgtg 120
D. m Iran       cgcgtcggcatcacggacagtacgttgagtgctgaagccacgcgccagcggcctcacgtg 120
D. m            cgcgtcggcatcacggacagtacgttgagtgctgaagccacgcgccagcggcctcacgtg 120
D. r            cgcgtcggcatcacggacagtacgttgagtgcagaagccacgtgccaacggcctcacgag 120
D. a            cgcgtcggcatcatggacagtacgttgagcgctaaagccacgcgccagcggcctcacgag 120
                ************* *************** **  ******** **** ********** *


D. n Iran       aggaagacggtggcgaactga-actgttgtgccaaaacttcgcagagactgaaacgaggc 179
D. m Iran       aggaagacggtggcgaactga-acggttgtgccaaaacttcgcacagacggaaacgaggc 179
D. m            aggaagacggtggcgaactga-actgttgtgccaaaacttcgcagagacggaaacgaggc 179
D. r            aaggagagyggtggcgagctgacactgttgtgcctaaacttcgaagagacggaaacgaggc 180
D. a            agggagacggtggcaa------accgttgtgccaattcttcgaaaagacggaaacgaggc 174
                * * *** ****** *      ** ******** *  ***** * **** **********


D. n Iran       attgtactactgcagcacgacgagcgcgcgcctccgaagagaaccgccgcaggatggagt 239
D. m Iran       attgtactactgcagcacgacgagcgcgcgcctccgaagagaaccgccgcaggatggagt 239
D. m            attgtactactgcagcacgacgagcgcgcgcctccgaagagaaccgccgcaggatggagt 239
D. r            a--acactactgcagcacgacgaatgcgtgcctccgaagag-accgccgcaggatggagt 237
D. a            a--atactactgcagcgtgacgagtgcgcgcctctagcaag-accgccgcaggatggagt 231
                *    ***********  *****  *** *****     ** ******************


D. n Iran       cggacacctgcagggaaagtgcggtctgagcgcgaggcgcgaacgtctgttgcaacagca 299
D. m Iran       cggacacctgcagggaaagtgcggtctgagcgcgaggcgcgaacgtctgttgcaacagca 299
D. m            cggacacctgcagggaaagtgcggtctgagcgcgaggcgcgaacgtctgttgcaacagca 299
D. r            cggacacctgcagggaaagagcggtccaagtgtgaggcgcgaacgtctgttgcgac---- 293
D. a            cggatacctgcagggaaagagcggtccaagcacgaggcgcgaacgtctgttgccat---- 287
                **** ************** ******  **   ******************** *


D. n Iran       gtagcgcgcacgtttgcgagagagtcggaagtgcccgcttgcgtgcacggacaacgcggg 359
D. m Iran       gtagcgcgcacgtttgcgagagagtcggaagcgcccgcttgcgtgcacggacaacgcggg 359
D. m            gtagcgcgcacgtttgcgagagagtcggaagcgcccgcttgcgtgcacggacaacgcggg 359
D. r            gtagcgcgcacgtttgcgagagagtcggaagcgcacgcttgcgtgcacggaaaarcgcgg 353
D. a            gtagcgcgcacgtttgcgagagagtcggaagcgcacgcttgcgtgcacggaaaacgtggg 347
                ******************************** ** ***************** **    **


D. n Iran       aagcaaacgccggccgattcccgcgccgtgcgcgaagcaagcgagcgcgatcgcacgttg 419
D. m Iran       aagcaaacgccggccgattcccgcgccgtgcgcgaagcaagcgagcgcgatcgcacgttg 419
D. m            aagcaaacgccggccgattcccgcgccgtgcgcgaagcaagcgagcgcgatcgcacgttg 419
D. r            aa---------aaccctttccgctccgtgcgca----aagccagcgcgatcgcagtttg 400
D. a            aatgaaacgccggccgattcccgcgccgtgcgca----aagccagcgcgatcgcaatttg 403
                **           **  ** **** ********     **** ************  ***
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Fig. 2 Alignment of complete nucleotide sequence of ITS-2 regions of rRNA genes from Iranian *D. niveus* (D. n Iran, GenBank accession no. GQ144706), Iranian *D. marginatus* (D. m Iran, GenBank accession no. GQ144707), *D. marginatus* (D. m GenBank accession no. S83081.1), *D. reticulatus* (D. r GenBank accession no. S83080.1), and *D. andersoni* (D. a GenBank accession no. EU520395.1) using CLUSTAL format alignment by MAFFT

segment and a dark color of scutum. In accordance with our results Arthur (1960) described the basis capituli of *D. niveus* one and a half times as broad and as long (including cornua).

The dorsal scutum of *D. marginatus* is like *D. niveus* which is ornamented and multishape, but its basic color in *D. marginatus* is enamel white, whereas the corresponding areas in *D. niveus* is enamel yellow-white. Arthur (1960) described the base color of dorsal scutum of *D. niveus* as brown, enamel yellow-white such as we observed in our samples. According to the scutum pattern, our observation confirmed the description of Estrada-Pena

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D. n Iran       cgttgtttgccttcggagtacgtcgagctctagcagaaggtcgctcgtccgcgtcaccgc 479
D. m Iran       cgttgtttgccttcggagtacgtcgagctctagcagaaggtcgctcgtccgcgtcaccgc 479
D. m            cgttgtttgccttcggagtacgtcgagctctagcagaaggtcgctcgtccgcgtcaccgc 479
D. r            cgttgtttgccttcgtagtacgtcgagctctagcaaaaggtcgctcgtccgcgtcagcgc 460
D. a            cgctgtttgccttcgaagtacgtcgagctccag-agctggtcgctcgttcacgtcaccgc 462
                ** ************ ************** ** *   ********** * ***** ***

D. n Iran       acgggtgcaggcgccctagtccgggtttcgtcgcaggaataagagtcggaaaagattctt 539
D. m Iran       acgggtgcaggcgccctagtctgggtttcgtcgcaggaataagagtcggaaaagattctt 539
D. m            acgggtgcaggcgccctagtccgggtttcgtcgcaggaataagagtcggaaaagattctt 539
D. r            acgggtgtcggcgctct--tccgggcttcgtcgcaggaataggagtcggaaaagattcct 518
D. a            agctgtgtgggcgccct--tccgggcttcgtcgcaggaatgggaatcgg--cagattctt 518
                *   ***  ***** **  ** *** **************  ** ****   ****** *

D. n Iran       gcgcggagcggggaggacaagggtgcgccccggagcggtagtgacgcggttacgcgagtg 599
D. m Iran       gcgcggagcggggaggacaagggtgcgccccggagcggtagtgacgcggttatgcgagtg 599
D. m            gcgcggagcggggaggacaagggtgcgccccggagcggtagtgacgcggttacgcgagtg 599
D. r            gcgc-------ggagggaaagggtgcgccccgaagcggtagtgacgcggcaacacgagtg 571
D. a            gcgaggagcggggaggagaagggtggccccccgaa--------agcggttcgacgcgacag 570
                ***        *****  *******  ***** *          **     *  ***  *
D. n Iran       cgccgtctgcgagaagcgaagaaaacggcacgactgaatagtcgccgcgaagcggaaaat 659
D. m Iran       cgccgtctgcgagaagcgaagaaaaccgcacgactgaatagtcgccgcgaagcggaaaat 659
D. m            cgccgtctgcgagaagcgaagaaaacggcacgactgaatagtcg--gcgaagcggaaaat 657
D. r            tgccgtccgcgag-cgcgaagaaaacggcacggcggagcagtcgcggcgactcggaaaat 630
D. a            cgccgtctgcgagcgagaagagtcacggcacggcggag-aattgccgcgaaacggaaaat 629
                 ****** *****     *     ** ***** * **  * * *  ****  ********

D. n Iran       gtctccctcgaaagcgtgagtttgcccgttggcggagctgaagcgttccgtcgtagtccg 719
D. m Iran       gtctccctcgaaagcgtgagtttgcccgttggcggagctgaagcgttccgtcgtagtccg 719
D. m            gtctccctcgagagcgtgagtttgcccgttggcggagctgaagcgttccgtcgtagtccg 717
D. r            gtctccctcgagag------------agttggcygagctgaagcattccgtcgtagtccg 678
D. a            gtctccttcgagagcgtgggtgc-cccgttggcggagctgaagcgttccgtcgtagtccg 688
                ****** **** **             ****** ********** ***************

D. n Iran       ccgtcggtccaagtgcttcgcagtctctgcccttaaaaaagactgggccactccagttgg 779
D. m Iran       ccgtcggtccaagtgcttcgcagtctctgccctt-aaaaagactgggccactccagttgg 778
D. m            ccgtcggtccaagtgcttcgcagtctctgccctt-aaaaagactgggccactccagttgg 776
D. r            ccgtcggtccaagtgcttcgcagtctctgtccc--gaawagactgggccactccagtt-g 735
D. a            ccgtcggtccaagtgcttcgcagtctctgtccc--ctaaagactgggccactccagtt-g 745
                **************************** **     * ******************* *

D. n Iran       gggcaggggcgacgctacactagacgatgccctgtgccaggctagagtcg-tcctgtggc 838
D. m Iran       gggcaggggcgacgctacactagacgatgccctgtgccaggctagagtcg-tcctgtggc 837
D. m            gggcaggggcgacgctacactagacgatgccctctgccaggctagagtcg-tcctgtggc 835
D. r            gggcgggggcgacgctacactagacgatgcctactgccaggctagagtcg-tcctgcggt 794
D. a            gggcaggggcgacgctacacgagacgatgcctcccgccaggttttagtcgcccctgcggt 805
                **** *************** **********    ****** *  *****  **** **

D. n Iran       gcccgctgaagcggtgcgctgcgagggtgg--catgcctcggcggtgtttgggcttcaga 896
D. m Iran       gcccgctgaagcggtgcgctgcgagggtgg--catgcctcggcggtgtttgggcttcaga 895
D. m            gcccgctgaagc---gcgctgcgagggtgg--catgcctcggcggtgtttgggcttcaga 890
D. r            gcccgctaaagc-gcgcgctgcgagggtgg--catgcctcggcggtgtttgggcttcaga 851
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Fig. 2 (continued)

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D. a            gcccgctgaagcggcgcgctgctaaggcgatctttgcctcggtggtgtttgggcttcaga 865
                ******* ****   ******* * ** *     ******** *****************

D. n Iran       cacggtcgtttgccacgcaactgctcgtgcgacgcacgcgcgctaaggcggttcaagaaa 956
D. m Iran       cacggtcgtttgccacgcaactgctcgtgcgacgcacgcgcgctaaggcggttcaagaaa 955
D. m            cacggtcgtttgccacgcaactgctcgtgcgacgca--cgcgctaaggcggttcaagaaa 948
D. r            cacggtcgtttaccacgcaactgctcgtgcgccgcacgcgtgt---ggcggttt-----a 903
D. a            cacggtcgcttaccacgcaactgctcgtgcgccgcacgcgcgc--aggcggttc-----a 918
                ******** ** ****************** ****  ** *    *******      *

D. n Iran       ccacctgccagcctcgtctataagtagctccgtgttgggcgaaggacgtggtagggcgtc 1016
D. m Iran       ccacctgccagcctcgtctataagtagctccgtgttgggcgaaggacgtggtagggcgtc 1015
D. m            ccacctgccagcctcgtctataagtagctccgtgttgggcgaaggacgtggtagggcgtc 1008
D. r            ccgcctgccagcctcgtctataagtagctccgtgttgggcgaatgacgtggtagggcgtc 963
D. a            ccgcctgccagcctcgtctataagtagctccgtgttgggcgaaggacgtggtagggcgtc 978
                ** ************************************** ****************

D. n Iran       gtactcggtttgcgctgggctttcgaacgtgtcgagtcctttc--cagcac--------- 1065
D. m Iran       gtactcggtttgcgctgggctttcgaacgtgtcgagtcctttc--cagcac--------- 1064
D. m            gtactcggtttgcgctgggctttcgaacgtgtcgagtccttt---cagcac--------- 1056
D. r            gtactcggttcgcgctgggttttcgaacgtgtcgagtcctttcagctgcac--------- 1014
D. a            gcactcggttggcgctgggttttcgaacgtgtcgagtcctttt--cggcataccgctcgt 1036
                * ******** ******** **********************   * ***

D. n Iran       ------------------------------------------------------------ 1065
D. m Iran       ------------------------------------------------------------ 1064
D. m            ------------------------------------------------------------ 1056
D. r            ------------------------------------------------------------ 1014
D. a            atgacgcacgcgcgcaggcgttgtgccgcctgccagcctcgtccgtaaggacgtggcagg 1096


D. n Iran       -----------------gctggaaggggcgaaagcgatgtatccgtt----------gtc 1098
D. m Iran       -----------------gctggaaggggcgaaagcgatgtatccgtt----------gtc 1097
D. m            -----------------gctggaaggggcgaaagcgatgtatccgtt----------g-- 1087
D. r            -----------------gctggaaggggcgaaagcgatgtatccgtt----------g-- 1045
D. a            gcgtcgtactcggtcgtgctggaatgggcgaaagcgatgtatccgttcgactcagtccgc 1156
                                 ******* **********************


D. n Iran       ga 1100
D. m Iran       ga 1099
D. m            -- 1087
D. r            -- 1045
D. a            ca 1158
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Fig. 2 (continued)

and Estrada-Pena (1991). They described the scutal pattern of *D. marginatus* as very variable, with spots in the scutum generally with the inner surface of each spot mostly studded with base color pattern. Furthermore, we observed that the two central dorsal spots are organized approximately triangular with the apical angle directed antrolateral in *D. marginatus* whereas these two spots are vice versa in *D. niveus* (Fig. 1). The second segment of palp in *D. marginatus* had small or strong postero-median spur. In contrast, the second segment of palp in *D. niveus* had strong pronounced spur at the dorsal side (Fig. 1).

After speciation of these two species, DNA was extracted from salivary gland of each tick separately. DNA was then amplified using specific primers to amplify the complete ITS-2 region in two steps. In the first step of PCR, PCR product of approximately 708 bp in length was amplified using primers P1/P2 and in the second step PCR

product of approximately 482 bp in length was amplified using primers P3/P4. The PCR products were purified or extracted from the gel and sequenced from both sites of the DNA. The nucleotide sequence of both PCR products had overlapping region and could be adapted (Fig. 2). The complete nucleotide sequence of *D. marginatus* and *D. niveus* consist of 1,099 bp and 1,100 bp respectively. The alignment of these two sequences with each other was performed by BLAST software and showed 99% nucleotide homology. The both nucleotide sequences distinguished only in 5 bp scattered in the whole sequences. The alignment of both sequences with the nucleotide sequence registered in the DataBank showed 98% homology to the nucleotide sequence of *D. marginatus* (Accession no S83081.1), 87% with *Dermacentor reticulates* (accession no. S83080.1). Nucleotide sequence of ITS-2 region of *D. marginatus* had 85% homology to the *D. andersoni*, whereas *D. niveus* had 86% homology to this sequence (accession no. EU520395.1). Zahler et al. (1995) showed that the nucleotide sequence of ITS-2 region of *D. reticulates* had 87.8% homology to *D. marginatus* and *D. andersoni* was 79.4% and 79.5% identical to *D. reticulates* and *D. marginatus*, respectively. They analyzed the nucleotide sequence of ITS-2 region of *D. reticulates* individuals from different geographic origins and determined a 99.6% homology between the individuals and described it as intra-specific differences. In their study the differences in nucleotide sequence of more than 10% was determined as interspecific identity, which can be understood as not suggestive for common gene pool. ITS-2 sequence was already used for determination of validity of species status of *Ixodes dammini* (Wesson et al. 1993), or for differentiation of *Rhipicepalus* spp. as well (Barker 1998). Since the nucleotide sequence of ITS-2 region of *D. marginatus* in Iran has 98% sequence homology to the other in GenBank registered ITS-2 sequences of *D. marginatus*, and the morphological characteristics between both examined species showed minimal differences, therefore we believe that the *D. marginatus* and *D. niveus* could belong to the same species and 1% differences in nucleotide sequence of ITS-2 region between these two species can be understood as an intra-species polymorphism. It seems that the ITS-2 nucleotide sequence can be used as a sufficient molecular marker for phylogenetical analysis of *Dermacentor* spp.

Rahbari et al. (2007) and Nabian et al. (2008) determined the tick fauna in Iran and could find *D. marginatus* and *D. niveus* mostly in the mountainous areas. Interestingly, they could find *D. niveus* in North-East of Iran (Khorassan province) whereas *D. marginatus* was more collected in North-West of Iran. Taken these results with our results together, it could be speculated that ecological environments could have direct influence on some morphological characters of ticks, which must be studied in future.

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