Molecular characterization of Ribosomal DNA (ITS2) of hard ticks of Iran, in understanding the conspecificity of Dermacentor marginatus and D. niveus

Parisa Soltan-Alinejad
Urmia University of Medical Sciences
Zahra Ramezani
Urmia University of Medical Sciences
Hamideh Edalat
Tehran University of Medical Sciences
Zakkyeh Telmadarraiy
Tehran University of Medical Sciences
Farrokh Dabiri
Urmia University of Medical Sciences
Hassan Vatandoost
Tehran University of Medical Sciences
Mohammad Ali Oshaghi
Tehran University of Medical Sciences
Mehdi Mohebali
Tehran University of Medical Sciences
Seyyed Javad Seyyed-Zadeh
Urmia University of Medical Sciences
Zabihollah Zarei
Tehran University of Medical Sciences
Haleh Hanifian
Tehran University of Medical Sciences
faham faghihi
Urmia University of Medical Sciences
Mandan Abolhasani
Tehran University of Medical Sciences
Mulood Mohammadi Bavani
Urmia University of Medical Sciences
Jalil Musavi
Urmia University of Medical Sciences

Olle Terenius
SLU
Ali Reza Chavshin (chavshin@gmail.com)
Urmia University of Medical Sciences https://orcid.org/0000-0002-2359-8610

Research note

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Abstract

Objectives: Hard ticks (Acari: Ixodidae) are ectoparasites of human and animals and transmit a wide range of pathogens. The species identification of ticks is normally based on morphological keys. However, considering the importance of proper identification of the species and their bio-ecological characteristics, relying on morphological characteristics alone can be questionable. In this study, ITS2 and COI were selected for phylogenetic evaluation of Iranian species belonging to three genera: *Dermacentor*, *Hyalomma* and *Rhipicephalus.*

Results: The ticks were from 10 populations from three different climatic and zoogeographical zones of Iran.

The study of important morphological characteristics revealed notable differences in these characteristics between different populations of *D. marginatus* species.

Contrary to the results of ITS2 sequence analysis which giving additional support for the view that *D. niveus* and *D. marginatus* should be viewed as one species only, the sequence analysis of COI and phylogeny favored the separation of the two species of *D. niveus* and *D. marginatus*. Given the greater importance of COI in identifying and distinguishing species, the Being two species should be considered.

Introduction

Hard ticks are obligate hematophagous ectoparasites of terrestrial vertebrates that play important roles in the transmission of a wide range of diseases such as ehrlichiosis, Lyme disease, Crimean Congo hemorrhagic Fever (CCHF), tularemia and anaplasmosis [1]. The importance of proper identification of the species and their bio-ecological characteristics emphasizes the need for research on the taxonomic and phylogenetic relationships among hard-tick taxa. Morphological methods have been widely used for these purposes, but in the case of some species solely relying on morphological methods may be questionable [2]. Molecular methods, which focus on DNA-sequence differences, seems to be a better way to assess variation within and between species [3, 4]. Because of the rapid evolution of the ITS regions, in comparison to coding regions, they are useful for distinguishing between closely related taxa [5] and have been used in characterization of different taxa of ticks [6–10].The hyper-variability of the second internal transcribed spacer (ITS 2) can also reflect intra-specific differences allowing the separation even between different populations of the same species [11, 12].

One disagreement among researchers is the status of the species *D. niveus* [13]. Filippova and Plaksina (2005) discussed the difficulties involved in differentiating species of the *D. marginatus* complex, which includes *D. marginatus*, *D. niveus*, *D. nuttalli*, *D. silvarum* and *D. ushakovae*. Some authors treat *D. niveus* as a synonym of *D. daghestanicus* while other authors consider *D. niveus* a valid species and *D. daghestanicus* as its junior synonym [14]. In some cases both *D. daghestanicus* and *D. niveus* are listed as valid species [15], but this opinion has not generally been accepted [2].Also, the separation of *D. marginatus* and *D.niveus*was evaluated by molecular tools using ITS2 [16], in which the conspecificity of

these two species were suggested, but since other authors continue to treat them as separate species (e.g. [2]) the validation of the suggestion of conspecificity awaits results from further studies.

Because of the importance of molecular characterization of Ixodidae ticks and evaluation of the utility of the ITS2 fragment for tick phylogeny, in current study, 10 populations of four species belonging to three genera of Iranian hard ticks were characterized based on ITS2 fragment. Additionally the possibility of the conspecificity of *D.niveus* and *D. marginatus* was examined using proper phylogenetic analysis using both ITS2 and COI fragments.

Materials And Methods

Study area

Tick samples were collected from 5 provinces across Iran: Ardabil, Kerman, Tehran, Isfahan, and the Kurdistan Province (Figure S1).

Sample collection and identification

The animal dwellings were visited and Ixodidae ticks were collected from hosts (sheep, cows, goats and dogs) seasonally [17]. Samples were identified to species level according to the standard morphological keys available [18,19]. In the case of morphological differentiation of *D. marginatus* and *D.niveus*, the keys of [13,20] were used.

Genomic DNA extraction and PCR reactions

Samples were frozen in liquid nitrogen and homogenized. Genomic DNA was extracted from tick samples individually using AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea) according to the manufacturer manual High yield extracted DNA obtained successfully from each species was subjected to PCR reactions using the primers:
DITS2-F, 5´-GTGCGTCCGTCGACTCGTTTTGA-3´ and
DITS2-R, 5´-ACGGCGGACTACGACGGAATGC-3´[12], in order to amplify the ITS2 region. The amplification condition for theITS2 region was 95°C for 5 min followed by 30 cycles of [95°C for 30 s, 49.5°C for 30 s, and 72°C for 30 s] and a final incubation of 72°C for 5 min [11].

Also the COI fragment was amplified using the universal primers Forward: 5'-GGAGGATTTGGAAATTGATTAGTTCC -3' and Reverse: 5'- CCCGGTAAAATTAAAATATAAACTTC -3' [21] and PCR conditions were set as follows: initial denaturation at 94 °C for 5 min; 30 cycles of [94 °C for 30 s, 48 °C for 30 s, 72 °C for 30 s] and a final extension at 72 °C for 7 min.

All the amplicons were sequenced (Bioneer Co., South Korea) and the results analyzed using BLAST search (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis

The evolutionary history of the ticks in the current study was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [22]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter =2.0513 for the three genera of ixodide ticks and +G, parameter =0.0500 for the *Dermacentor* comparative analysis)). Evolutionary analyses were conducted in MEGA6 [23].

Results

In this study, four species belonging to *Dermacentor*, *Hyalomma* and *Rhipicephalus* from 10 populations in three different climatic and zoogeographical zones of Iranwere investigated (Table 1). Phylogenetic analysis of the amplified fragments showed that the ITS2 sequences obtained successfully could differentiate the three genera studied (Fig. 1).

Table 1
The species, collection sites, Climatic zone and the accession numbers of their ITS2 fragment

Species	Location	Climatic zone	Accession No.
Dermacentor marginatus	Kerman	tropical/ Desert	KJ004032
Rhipicephalus sanguineus	Tehran	subtropical/plain	KJ004033
Hyalomma anatolicum	Tehran	subtropical/plain	KJ004034
Rhipicephalus bursa	Meshkin-Shahr	mediterranean/ mountainous	KJ004035
Rhipicephalus sanguineus	Kurdistan	mediterranean/ mountainous	KJ004036
Dermacentor marginatus	Meshkin-Shahr	mediterranean/ mountainous	KJ004037
Rhipicephalus sanguineus	Meshkin-Shahr	mediterranean/ mountainous	KJ004038
Dermacentor marginatus	Meshkin-Shahr	mediterranean/ mountainous	KJ004039
Hyalomma anatolicum	Isfahan	subtropical/plain	KJ004040
Dermacentor niveus	Meshkin-Shahr	mediterranean/ mountainous	KJ004041
Dermacentor niveus	Kerman	Tropical/ Desert	KJ004042

Similarly, the two populations of *H.anatolicum*, were well separated. Also the members of *Dermacentor* were well segregated from the rest of the genera, but the studied species of this genus (*D. marginatus* and *D. niveus*) were not distinguished clearly at the species level (Fig. 1).

For further analysis of the phylogenetic relationship between the different populations of *D. marginatus* and *D. niveus*, the acquired sequences of these species in this study and from other related studies were analyzed (Fig. 2). Based on the phylogenetic analysis of different populations of both *D. marginatus* and *D. niveus*, although it was clearly shown that the ITS2 sequences clustered according to location rather than to species. Thus, *D. marginatus* and *D. niveus* from Kerman formed their own clade outside the group of *D. marginatus* and *D. niveus* from Ardabil, but analysis of the COI fragments also revealed that the mentioned species can be considered as two distinct species (Fig. 2).

Discussion

This study is one of the few studies that have focused on the molecular characterization of Ixodidae ticks in Iran. Previously, the ITS2 fragment of different populations from two species of hard ticks of Iran (*R. sanguineus* and *D. niveus)* has been characterized [11, 16].

Based on the results obtained in our study, which confirm earlier studies, it can be safely said that ITS2 is a suitable molecular marker at the genus level for distinguishing the examined genera (*Dermacentor, Hyalomma* and *Rhipicephalus*). In addition to the genus levels, the results of this study also demonstrated that this fragment could successfully separate between different species. These results also reinforce the suggestion of ITS2 as a standard DNA barcode in the case of malfunction of Cytochrome Oxidase I (COI).

Although the ITS2 marker has been shown to have acceptable results in identifying and examining mosquitoes of different populations within a species, it has also shown acceptable function at the identification of ticks' species level.

Among the published lists of ticks, the position of some species and the validity of their names are questionable [24, 2]. The species *D. marginatus* and *D. niveus* were previously distinguished solely based on morphological evaluations, but the limitation of the morphological characters was expressed [16]. In addition, based on the comparison of morphological characteristics and examination of the syntype series of *D. niveus*, it was concluded that *D. niveus* is conspecific with *D. marginatus* [13]. Also the molecular results obtained from the current study revealed a high similarity (> 98%) between *D. marginatus* and *D. niveus* based on the different populations of both species across this study and other sequences obtained from Iran [16] or elsewhere [25] (Fig. 2).

On the other hand, analysis of the COI-barcode as a reliable marker for species identification, mark the two species separately and indicate them as two separate species. This adds to the complexity of identifying the actual status of the taxonomy of these two species.

The results of the analysis of COI fragment in the present study can be used as a support to the recently published data suggest that *D. marginatus* and *D. niveus* are different species [2].

Limitations

Few specimens of *D. niveus* have been caught and studied in the present study. The existence of spatial and temporal variability of these samples were the results of this study.

Abbreviations

ITS2
Internal transcribed spacer 2
COI
Cytochrome Oxidase 1

Declarations

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Author contribution statements

ARC and OT designed and supervised the study, PSA, HE, ZT, FD, HV, MAO and ZR did the field and laboratory activities. MM, SJS, ZZ, HH, FF and MA analyzed the DATA. Also ARC, PSA, OT, MMB and SJM wrote the draft of the manuscript and ARC and OT finalized the Draft. All authors reviewed and approved tha final version of manuscript for publication.

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Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This project was approved by the Ethics Committee of Urmia University of Medical Sciences.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

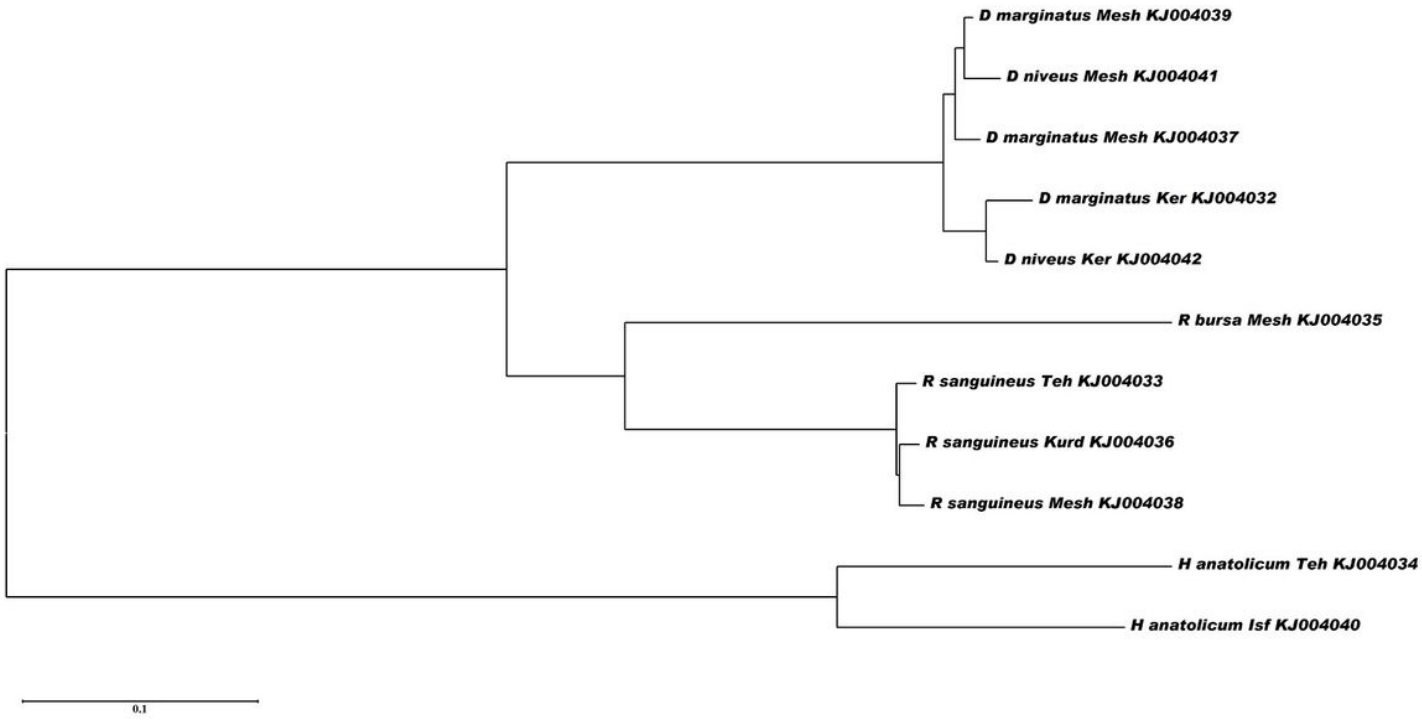

Figure 1

Phylogenetic relationships based on DNA sequences of ITS2 rDNA region for 11 populations of 5 species belong to 3 genera of hard ticks. (for the species names and abbreviations: D: Dermacentor, R: Rhipicephalus, H: Hyalomma, and Mesh (sample from Ardabil Province, Meshkin-Shahr, The: Tehran Province, Ker: Kerman Province, Kurd: Kurdistan Province and Isf: Isfahan Province)

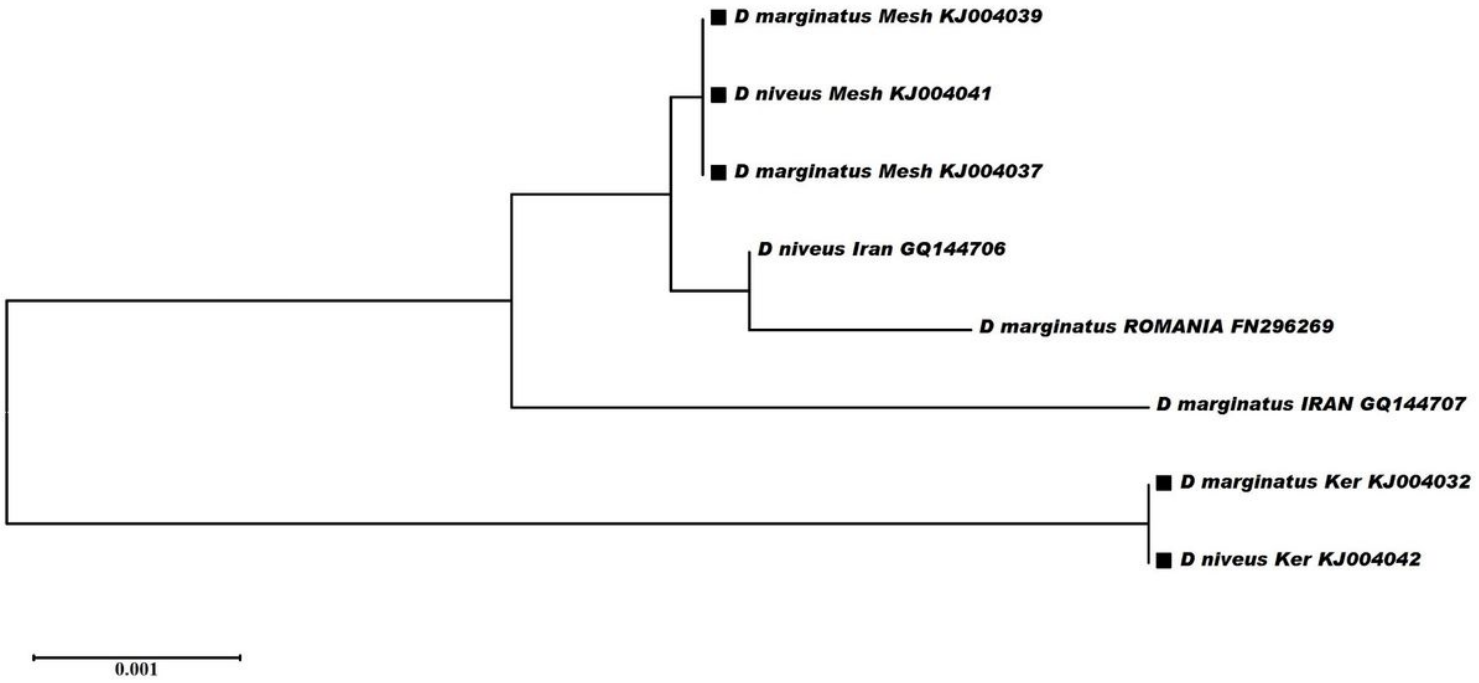

Figure 2

Phylogenetic relationships for DNA sequences of ITS2 rDNA region for different populations of D. marginatus and D. niveus. (indicates the sequences acquired in this study, GQ144706, 7 from other Iranian study [16] and FN296269 from Romania [25]).

Supplementary Files

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