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The Caspian red deer, *Cervus elaphus maral* (Mammalia: Cervidae): a new host record for *Rhipicephalus* (*Boophilus*) *annulatus* (Acari: Ixodidae) in northern Iran

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The cattle tick, *Rhipicephalus* (*Boophilus*) *annulatus* (formerly *Boophilus annulatus*) Say, 1821 is a one-host ixodid tick mostly found on cattle (D'Amico *et al.* 2017). Artiodactyla (Fam.: Bovidae) are the main hosts (Guglielmone *et al.* 2014), but occasionally sheep, goats and wild ungulates can support successful completion of the life cycle (Estrada-Peña *et al.* 2004). *Rhipicephalus annulatus* is capable of transmitting both *B. bigemina* and *B. bovis* (Gray *et al.* 2019), *Anaplasma marginale* (Hosseini-Chegeni *et al.* 2020), *Coxiella burnetii* (Reye *et al.* 2012), *Ehrlichia ruminantium* (Senbill *et al*.– unpublished GenBank database) and the various pathogenic *Rickettsia* species (Reye *et al.* 2012; de Mera *et al.* 2018). The Caspian red deer (Maral or Noble deer), *Cervus elaphus maral* (Mammalia: Cervidae) inhabits mountainous regions of Africa, Asia, Eurasia, Europe (Lovari *et al.* 2018) and lives in grasslands of Hyrcanian forested areas in northern Iran (Kiabi *et al.* 2004). Lisar, protected area, Guilan province is located in the western corner of the Hyrcanian forests of Talesh county (37° 58' 36.9" N, 48° 45' 02.7" E), and is one of the potential corridors for large mammals to the Caucasus ecoregion (Soofi *et al.* 2017). Red deer is categorized as a protected species by Iran Department of Environment, and least concern (LC) by the International Union for Conservation of Nature (IUCN) red list (Lovari *et al.* 2018). Every year, a number of them are hunted and their habitats are at risk of destruction. Ticks infest every class of terrestrial vertebrates; mammals, birds, various reptiles, and even amphibians. The tick species, *Rhipicephalus* (*Boophilus*) *annulatus* was found in red deer from the Spain. We aimed to present the first tick infestation record of Caspian red deer, *C. e. maral* by this tick species in northern Iran. Thirty female tick samples were collected from a red deer. Unfed, semi- and fully-engorged adult female ticks were collected

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from parts of the body such as under the shoulders, around the ear, and on the ribs. Tick samples were examined according to morphological characters defined by Hoogstraal (1956) at subgenus level. In order to correct identification of females at species level, PCR and Sanger sequencing techniques were performed. DNA of individual ticks was extracted using a modified CTAB (Merck®, Germany)-phenol-chloroform method (Doyle and Doyle 1987; Sambrook and Russell 2001). A fragment (ca. 800-bp) of nuclear internal transcribed spacer 2 (*ITS2*) gene and a 340-bp fragment of mitochondrial large subunit ribosomal RNA (16S rRNA) gene were amplified by conventional PCR. A PCR procedure was performed under a touchdown temperature profile using the primers; TITS2F1: 5'- CTG CGA GAC TTG GTG TGA ATT G -3', RITSDer: 5'- GAA GCA CTT GGA CCG ACG -3' (*ITS2*), F16SHa: 5'- CTG TRG TAT TTT GAC TAT ACA AAG G -3', R16SOr: 5'- CAT CGA GGT CGC AAA C -3' (16S rRNA) (Nadim *et al.* 2021). Amplification program included an initial denaturation of 4 min at 95 ℃, 11 cycles of denaturation at 94 ℃ for 50 sec, annealing at 60 ℃ for 60 sec with 1 ℃ decrease per cycle until 50 ℃, extension for 60 sec at 72 ℃, followed by 25 cycles of denaturation at 94 ℃ for 60 sec, annealing at 50 ℃ for 50 sec, extension at 72 ℃ for 60 sec and a final extension of 72 ℃ for 5 min. The PCR mixture (25 µl) contained 1.5 U of *Taq* DNA polymerase enzyme, 2.5 µl PCR 10x buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 0.5 µl forward and reverse primers (10 mM), all ingredients, except primers, were from SinaClon® Co. (Iran); template DNA (50–100 ng/µl) and 14.8 µl sterile water. The PCR products were visualized on 1% agarose gel electrophoresis under UV light. The positive PCR products were submitted to a third-party service provider (Codon Genetic Group®, Iran) for direct sequencing using applied bioSyestems-ABI, 3130XL. *ITS2* and 16S rRNA electropherograms were edited manually and nucleotide sequences analyzed with BLASTn in GenBank database (Table 1). Then, two sequences of this study were submitted to the GenBank under the accession numbers MW929216 (*ITS2*) and MZ086762 (16S rRNA). A combined *ITS2*-16S rRNA phylogenetic tree was constructed using BEAST software (Ver. 2.6.3) (Bouckaert *et al.* 2019) after a significant partition homogeneity test (PHT) (*P-value* = 0.01) according to Cunningham (1997).

Table 1. Genetic identity (%) among *ITS2* and 16S rRNA sequences of *Rhipicephalus* (*Boophilus*) species in this study with sequences originated from various part of world according to BLASTn in GenBank.

Host*	Country	*ITS2*		16S rRNA	
		QC	Id.	QC	Id.
Cattle	Egypt	*R.* (*B.*) *annulatus* (MF946470)		*R.* (*B.*) *annulatus* (MF946466)	
		100	99.57	96	99.35
Cattle	Iran	*R.* (*B.*) *annulatus* (KJ410770)		No items found	
		100	99.57		
Cattle	Romania	*R.* (*B.*) *annulatus* (KC503267)		*R.* (*B.*) *annulatus* (KC503256)	
		100	99.57	96	99.35
Cattle	India	*R.* (*B.*) *annulatus* (MH541049)		*R.* (*B.*) *annulatus* (MW078977)	
		100	99.13	96	99.35
Cattle	India	*R.* (*B.*) *microplus* (MK621182)		*R.* (*B.) microplus* (EU918188)	
		100	98.4	96	98.04
Unknown	Oman	*R.* (*B.*) *kohlsi* (KC503271)		*R.* (*B.*) *kohlsi* (KC503262)	
		100	88.44	96	88.56
Cattle	Kenya	*R.* (*B.*) *decoloratus* (MN266921)		*R.* (*B.*) *decoloratus* (NC_052828)	
		100	86.94	96	89.22
Unknown	Mali	*R.* (*B.*) *geigyi* (AF271273)		*R.* (*B.*) *geigyi* (KF569942)	
		91	88.24	96	90.85

Accessions in parentheses, *R.* (*B*): *Rhipicephalus* (*Boophilus*), QC: Query cover (%), Id.: Identity (%)

Morphological results showed that tick samples belonged to *Rhipicephalus* (*Boophilus*) subgenus. According to the BLASTn analysis and identity with *R.* (*B.*) *annulatus* clade of *ITS2* and 16S rRNA phylogenetic tree, it was accepted that *Rhipicephalus* (*Boophilus*) sequences from this study are related to cattle tick, *R.* (*B.*) *annulatus* (Table 2, Fig. 1). Our *R.* (*B.*) *annulatus ITS2* and 16S rRNA sequences show ca. 100% genetic identity with other *R.* (*B.*) *annulatus* originated from various parts of world. *Rhipicephalus* (*B.*) *annulatus* clade (Figure 1 that included our sequences and four GenBank sequences had close relationship with *R.* (*B.*) *microplus* (98.4% identity) according to *ITS2*/16S rRNA sequences. Three more distantly related *Rhipicephalus* (*Boophilus*) species including *R.* (*B.*) *kohlsi* , *R.* (*B.*) *decoloratus* and *R.* (*B.*) *geigyi* were included as internal outgroup showing 86–88% and 88–90% identity with *R.* (*B.*) *annulatus* clade according to *ITS2* and 16S rRNA, respectively. The molecular technique of this study is recommended to identify females tick samples of *R.* (*B.*) *annulatus* and *R.* (*B.*) *microplus* that cannot be distinguished morphologically and share many morphological features (Beati and Keirans 2001). Phylogenetic analyses of cytochrome oxidase 1 (COI), internal transcribed spacer 2 (*ITS2*) and 12S rRNA gene sequences confirmed that the *R. microplus* complex consists of at least five taxa: *R. annulatus*, *R. australis*, and various *R. microplus* clades (Roy *et al.* 2018). BLASTn and comparison of evolutionary relationships among taxa are the next analysis steps on sequences (Faghihi *et al.* 2020). The construction of a phylogenetic tree was done using genetic distance difference; nucleotide substitution models and comparison with outgroup. Molecular evidence in the present study confirm occurrence of *R.* (*B.*) *annulatus* on the Caspian red deer, *Cervus elaphus maral* in northern Iran. The results of this study will help to better understand the biology of this tick and the presence of immature stages with probable questing behaviour on the forest vegetation environment. The results suggest a possible establishment of this vector species in a wildlife environment.

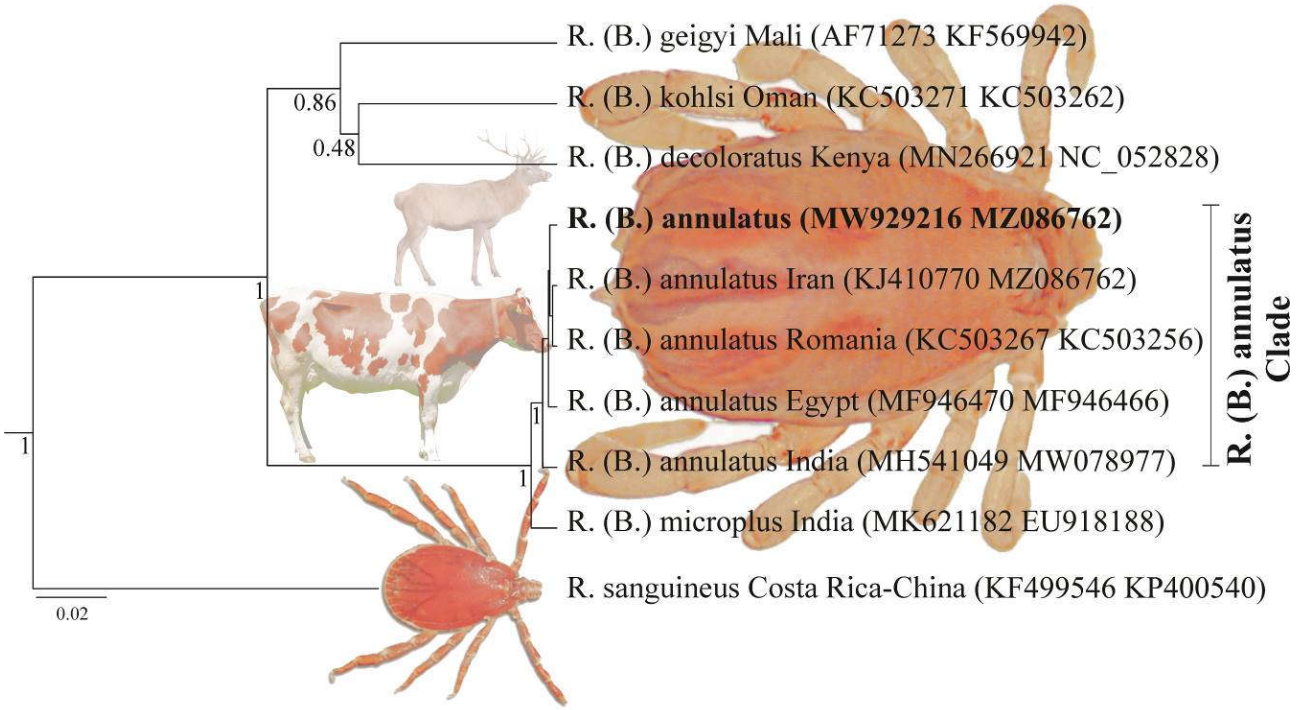

Figure 1. A combined phylogenetic tree constructed using Bayesian Inference method based on *ITS2*/16S rRNA sequence data of *Rhipicephalus* (*Boophilus*) species in this study with sequences originated from various part of world retrieved from GenBank database. The main *R.* (*B.*) *annulatus* clade separated by a vertical double headed line. The taxa were defined with a name of species, country, GenBank accession number (taxon of the present study is bold). Posterior probability values inserted in the place of nodes. Branch lengths are proportional to the evolutionary changes. *Rhipicephalus sanguineus* assigned as outgroup taxon.

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