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Molecular identification of *Haemaphysalis sulcata* (Acari: Ixodidae) larval stages collected using the Berlese funnel in Northern Iran

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Ticks are among one of the most notorious and well-known arthropods in terms of bites and transmission of serious pathogens to animals and humans such as viruses, bacteria and protozoa (Marcondes and Dantas-Torres 2017). Genus *Haemaphysalis* is the second largest ixodid tick genus around the world with numerous species found in Asia and Africa (Estrada-Peña *et al.* 2017). Finding immature stages of ticks and identifying hosts provides important information about the biology of tick species (Keskin *et al.* 2013). Some ticks of domestic animals spend their immature stages on wild hosts like *Haemaphysalis sulcata* whose immature stages have been reported from reptiles (Estrada-Peña *et al.* 2004). This tick is one of the most important species infected with Spotted Fever Group (SFG) *Rickettsia* in Iran (Hosseini-Chegeni *et al.* 2020). Two earlier studies in Iran emphasized the taxonomy of adult stages using morphological and molecular characters (Hosseini-Chegeni *et al.* 2014, 2017). Molecular detection techniques based on conventional PCR and sequencing of the partial genome of living organisms is considered an important tool for identification (Reller *et al.* 2007). In this study, we report immature *H. sulcata* isolated from soil. To our knowledge, this is the first molecular systematic study on larval ticks collected using Berlese funnel and showing usefulness of molecular techniques in Iran. In the present study, two samples of tick larvae were collected in Mirafzal forest area (36° 07' 39.0" N, 53° 35' 45.0" E) located in Mazandaran Province, northern Iran. In this study, samples were randomly selected from different regions of up to 25 cm soil depth so decayed leaves were removed. For isolation of tick samples, a modified Berlese funnel was used according to Krantz and Walter (2009). The collected samples were transferred into a glass jar containing 99% ethanol. Tick samples were initially identified using a generic identification key (Bregetova *et al.* 1955) and in order to narrow identification, PCR and Sanger sequencing techniques were performed. DNA of larval ticks was extracted using a modified CTAB (Merck®, Germany)-phenol-chloroform method (Doyle and Doyle 1987; Sambrook and Russell 2001). A PCR procedure to amplify a partial (ca. 1400-bp) fragment of internal transcribed spacer 2 (*ITS2*) was performed under a touchdown temperature profile using the

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primers designed in this study (TITS2F1: 5′- CTG CGA GAC TTG GTG TGA ATT G -3′, RITSDer: 5′- GAA GCA CTT GGA CCG ACG -3′). Amplification program included an initial denaturation of 4 min at 95 °C, 11 cycles of denaturation at 94 °C for 50 sec, annealing at 60 °C for 60 sec with 1°C decrease per cycle until 50 °C, extension for 60 sec at 72 °C, followed by 25 cycles of denaturation at 94 °C for 60 sec, annealing at 50 °C for 50 sec, extension at 72 °C for 60 sec and a final extension of 72 °C for 5 min. The PCR reactions (25 µl) contained 1.5 U of *Taq* DNA polymerase enzyme, 2.5 µl PCR 10x buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 0.5µl 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. A ca. 800-bp *ITS2* amplicon was submitted to a third-party service provider (Codon Genetic Group®, Iran) for sequencing using Applied bioSyestems-ABI, 3130XL. A single *ITS2* electropherogram was edited manually and BLASTed with the sequences deposited in GenBank database. The genetic distance among sequences were calculated using maximum composite likelihood (MCL) model in MEGA7 software (Kumar *et al.* 2016), and a phylogenetic tree was constructed using Bayesian inferences method (BI) in BEAST software (Ver. 2.6.3). A sequence of this study was submitted to the GenBank under the accession numbers MW929218.

Table 1. Genetic distance (%) differences among *ITS2* sequences of *Haemaphysalis* species.

Taxa species (accession number)	***H. sulcata* (MW929218)**	*H. sulcata* (MH414959)	*H. sulcata* (MH414961)	*H. parva* (MH414958)	*H. parva* (MH414955)	*H. parva* (MH414956)	*H. erinacei* (KU364288)	*H. campanulata* (MG721032)	H. *bispinosa* (MT297637)
***Haemaphysalis sulcata* (MW929218)**									
Haemaphysalis sulcata (MH414959)	0								
Haemaphysalis sulcata (MH414961)	0	0							
Haemaphysalis parva (MH414958)	15	15	15						
Haemaphysalis parva (MH414955)	15	15	15	1					
Haemaphysalis parva (MH414956)	15	15	15	4	5				
Haemaphysalis erinacei (KU364288)	37	37	37	45	45	43			
Haemaphysalis campanulata (MG721032)	25	25	25	32	32	34	32		
Haemaphysalis bispinosa (MT297637)	39	39	39	44	45	50	44	20	

A taxon of this study is bold and eight taxa retrieved from GenBank database are un-bold.

Morphological results showed that tick samples belonged to *Haemaphysalis* genus because they had a relatively short capitulum, no eyes with festoons (Fig. 1).

DNA extraction, PCR amplification and sequencing of an ITS2 fragment of tick sample were done successfully. The results of BLASTn showed that the larvae belonged to species *Haemaphysalis sulcata*. Table 1 summarizes genetic distance among sequences including a single DNA sequence obtained in this study and eight ITS2 sequences of *Haemaphysalis* from the GenBank database. Our *H. sulcata* ITS2 sequence shows 15–39% genetic difference with other *Haemaphysalis* species. *Haemaphysalis sulcata* clade (Fig. 2) that included our sequence and two GenBank ITS2 sequences had 0% genetic difference. Three more distantly related *Haemaphysalis* species including *H. campanulata*, *H. erinacei* and *H. bispinosa* were included as outgroup showing 25%, 37% and 39% distance difference with *H. sulcata* clade, respectively. The molecular

technique of this study is recommended to identify immature and dead tick samples that cannot be reared until the adult stage. BLASTn and comparison of evolutionary relationships between taxa are the next analysis steps on sequences (Faghihi *et al.* 2020). The construction of a phylogenetic tree is done using genetic distance difference; nucleotide substitution models and comparison with outgroup. The present study was the first report on the identification of *H. sulcata* larvae isolated from Berlese funnel. The result of this study will help to better understand the biology of this tick and the presence of immature larval stages with questing behaviour on the soil environment.

Figure 1. Dorsal aspect of a tick larvae sample collected in this study.

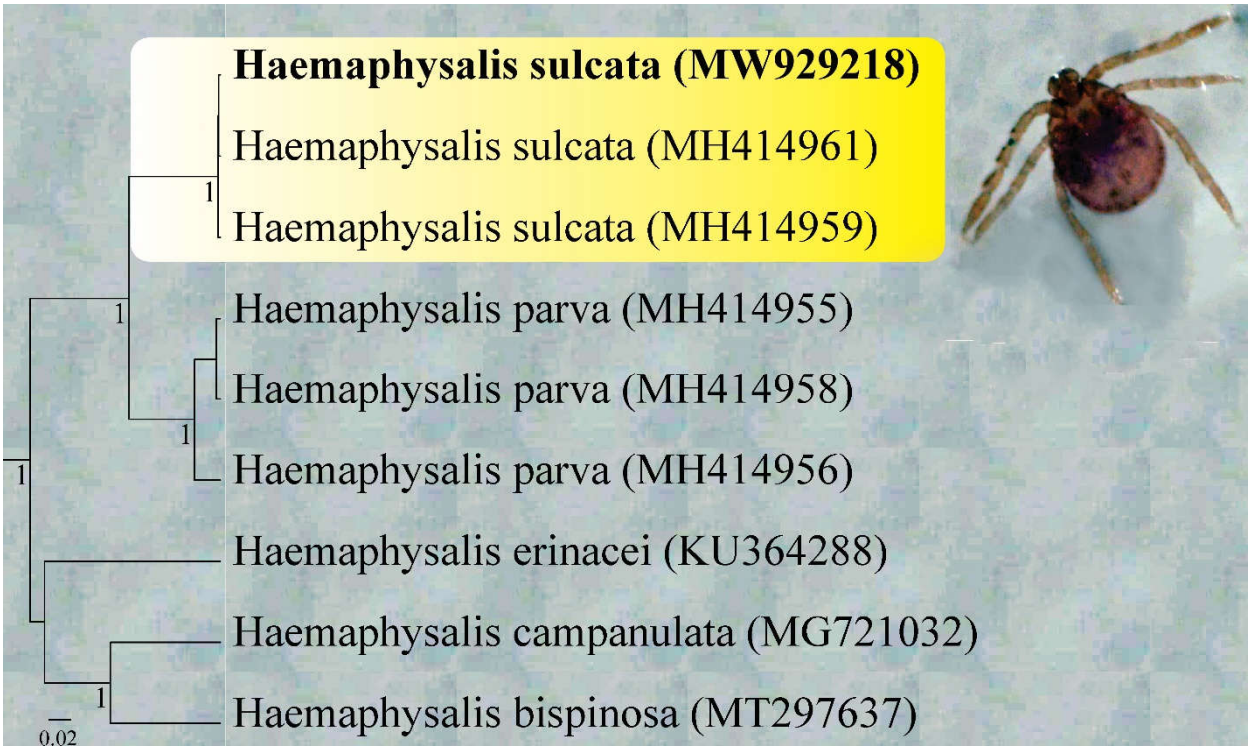

Figure 2. Phylogenetic tree generated based on *ITS2* sequence data of the *Haemaphysalis* species of this study and sequences retrieved from GenBank database constructed using Bayesian Inference method. The main clade of tree separated by a yellow rectangular box. The taxa were defined with a name and GenBank accession number and taxa of the present study is bold. Posterior probability values inserted in the place of nodes. Branch lengths are proportional to the evolutionary changes.

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