



## Original article

## A new report of adult *Hyalomma marginatum* and *Hyalomma rufipes* in the Czech Republic

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## ABSTRACT

*Hyalomma marginatum* and *Hyalomma rufipes* are important vectors of Crimean-Congo Hemorrhagic Fever Virus (CCHFV) in North Africa and Southern Europe. They are occasionally also reported from Central and Western Europe where they are likely introduced from their natural range by migratory birds. In this study, we report findings and molecular identification of adults and one nymph of *H. marginatum* and *H. rufipes*, primarily from horses from different regions of the Czech Republic. While the number of the reported ticks is small, this is likely to be an underrepresentation of the actual number. Due to their vector competence for CCHFV and potential expansion into new areas with a changing climate, surveillance programs in Europe are warranted.

## 1. Introduction

*Hyalomma marginatum* complex (*Hyalomma marginatum* sensu stricto [Koch], *Hyalomma isaaci*, *Hyalomma rufipes*, *Hyalomma turanicum*, and *Hyalomma glabrum*) are distributed across the Afrotropical, Palearctic, and Oriental regions (Estrada-Peña et al., 2017). *Hyalomma marginatum* is common in North Africa, Southern Europe, and parts of Asia, *H. rufipes* and *H. turanicum* are present in sub-Saharan Africa and around the Red Sea, and only *H. rufipes* appears to be established also in the Eastern Palearctic region (Apanaskevich and Horak, 2008).

*Hyalomma marginatum* and *H. rufipes* are two-host ticks with one generation per year. The immature stages of *Hyalomma* spp. feed on small mammals and ground-feeding birds. They were recorded on migratory birds across Central and Western Europe (Černý and Balát, 1957, 1989, Černý, 1972; Čapek et al. 2014) as far as England (Jameson et al 2012), Netherlands (Uiterwijk et al., 2021), Norway and Sweden

(Hasle, 2013; Hasle et al., 2009). The immature stages stay on the host for 12–26 days which enables them to be passively transported over long distances. Adult ticks feed primarily on domestic and wild ungulates (Chitimia-Dobler et al., 2019; Hubálek et al., 2020). Both *H. marginatum* and *H. rufipes* also feed on people (Keskin et al., 2015; Santos-Silva et al., 2011) and are the primary vector of the Crimean Congo hemorrhagic fever virus (CCHFV) (Gargili et al., 2017). Other pathogens in genera *Rickettsia*, *Babesia*, *Theileria*, and *Anaplasma* were also detected in these two tick species; however, their vector capacity has not been investigated (Kumar et al., 2020; Sajid et al., 2018).

In Central Europe, adults of *H. marginatum* were found in Slovakia (Nosek et al., 1982), Germany (Chitimia-Dobler et al., 2019), Hungary (Hornok and Horváth, 2012), Poland (Cuber, 2016), and Austria (Duscher et al., 2018). *Hyalomma rufipes* was found in Hungary (Hornok and Horváth, 2012), Germany (Chitimia-Dobler et al., 2019), and the Czech Republic (Hubálek et al., 2020). The morphological

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differentiation between *H. marginatum* and *H. rufipes* is reliable and it is typically based on spiracular plates and setae (Hornok and Horváth, 2012). However, in a case of damaged specimens, the possibility of misidentification is likely. Sands et al. (2017) studied the evolutionary history of *Hyalomma* ticks and successfully used several genes including ITS, COI, 12S rDNA, and 16S rDNA.

The aim of this study was to identify *Hyalomma* ticks collected in the Czech Republic between 2018 and 2020 to the species level using complex molecular approaches.

## 2. Materials and methods

### 2.1. Sample collection

Ticks were collected from 2018 to 2020 during the project [najdipi jaka.cz](https://www.najdipi.jaka.cz), a citizen-science project aimed at monitoring the expansion of *Dermacentor reticulatus* and other invasive tick species in the Czech Republic. Ticks were reported either by high-quality images or physically delivered to the laboratory, personally or by mail. In total, nine *Hyalomma* ticks were collected from horses ( $n = 7$ ), household ( $n = 1$ ), and one nymph was found on a ringed common nightingale (*Luscinia megarhynchos*). Sex and stage of specimens were recorded (Table 1) and ticks were stored in 70% ethanol at  $-20^{\circ}\text{C}$  for further analysis. Three additional ticks identified as *Hyalomma* spp. were reported by images only.

### 2.2. DNA isolation

Each tick was washed twice with distilled water to remove ethanol, placed on a glass slide and cut in half longitudinally with a sterile blade. One half was stored in 70% ethanol at  $-20^{\circ}\text{C}$  and the other half was homogenized by a plastic pestle and placed in the 2.0 ml Eppendorf tube. The DNA was extracted by the Exgene Cell SV mini kit (GeneAll, Portugal) following manufacturer instructions. For the nymph, three legs were removed with a sterile blade and homogenized by a plastic pestle. The rest of the nymph was stored in 70 % ethanol at  $-20^{\circ}\text{C}$ . The DNA was isolated using the NucleoSpin Tissue XS kit (Macherey-Nagel, Düren, Germany) following manufacturer instructions. The isolated DNA was stored at  $-20^{\circ}\text{C}$  until further processing.

### 2.3. Tick identification

For tick identification, three genes (12S rRNA, 16S rRNA, and COI) were amplified and sequenced (Table 2). All PCRs were conducted with  $2 \times$  PCR BIO Taq Mix Red (PCR Biosystems, UK) in the total volume of 25.0  $\mu\text{l}$  with 2.0  $\mu\text{l}$  of the template DNA. PCR products were visualized on the 1.5% agarose gel with the Midori Green Advance system (Nippon Genetics Europe, Germany), products of the expected size were purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan), and sequenced at the MacroGen capillary sequencing services (MacroGen Europe, the Netherlands) using the amplification primers. Obtained sequences were edited using the Geneious Prime® 2019.2.1 software (Kearse et al., 2012) and identified by BLASTn analysis of

NCBI GenBank sequences.

### 2.4. Statistical and phylogenetic analysis

Haplotype diversity (Hd), variance of Hd, nucleotide diversity ( $\pi$ ) as well as Variable (polymorphic) sites and Parsimony informative sites were calculated using the Dna SP6 Version: 6.12.03 (Rozas et al., 2017). Median joining network (Bandelt et al., 1999) was computed for the COI gene for 9 ticks using the PopART version 1.7 (Leigh and Bryant, 2015).

The phylogenetic analysis was performed separately for each gene with the sequences from the GeneBank representing *H. marginatum* and *H. rufipes*. The sequence of *Amblyomma variegatum* was used as an out-group. The alignment was calculated using the ClustalW algorithm in the Geneious Prime® 2019.2.1 software. The phylogenetic tree was generated by the MAFFT online service, based on Neighbor-Joining method, Jukes-Cantor model; branch supports was assessed by 1000 bootstrap replicates, and the tree was visualized and edited in the Fig-Tree v1.4.1.

## 3. Results

To our knowledge, this is the first identification of *Hyalomma marginatum* and *H. rufipes* from the Czech Republic using molecular approaches. Ticks were delivered from different regions across the Czech Republic (Fig. 1). We successfully amplified and sequenced all three genes (12S rRNA, 16S rRNA, COI) from 8 out of 9 specimen and two genes (16S rDNA and COI) from the ninth tick (Table 1). All sequences were deposited to GenBank under the accession numbers: 12S rRNA–MZ662946–MZ662953; 16S rRNA–MZ662102–MZ662110; COI–MZ687111–MZ687119. For each sequence, BLAST analysis revealed 100% identity to either *H. marginatum* or *H. rufipes* (Table 1). Seven ticks were found on horses (4 *H. marginatum* and 3 *H. rufipes*). One *H. marginatum* was found in a household, and one *H. rufipes* was found feeding on the common nightingale (*Luscinia megarhynchos*) captured for ringing (Table 1). Additional three ticks were identified as adult *Hyalomma* spp. based on the high-quality images. One of these ticks was found attached to a human armpit, but this tick was unfortunately not submitted, so its identification to species level was not possible.

For the COI gene, 6 different haplotypes were detected and formed two clearly separated groups. Based on the BLAST analysis, three haplotypes found in 5 samples represented *H. marginatum*, the other three from 4 samples were *H. rufipes*. The median joining network revealed the presence of 23 segregating sites between *H. marginatum* and *H. rufipes* (Fig.S1). The COI gene haplotype diversity (Hd) was found to be 0.89 and variance of Hd was 0.008 with nucleotide diversity ( $\pi$ ) 0.023. Variable (polymorphic) sites was 32 and Parsimony informative sites was 30.

For the 16S rDNA gene, two haplotypes were found, differing in 1 nucleotide, with Hd = 0.55, variance of Hd = 0.008, and  $\pi = 0.001$ . The number of variable (polymorphic) sites and Parsimony informative sites was 1.

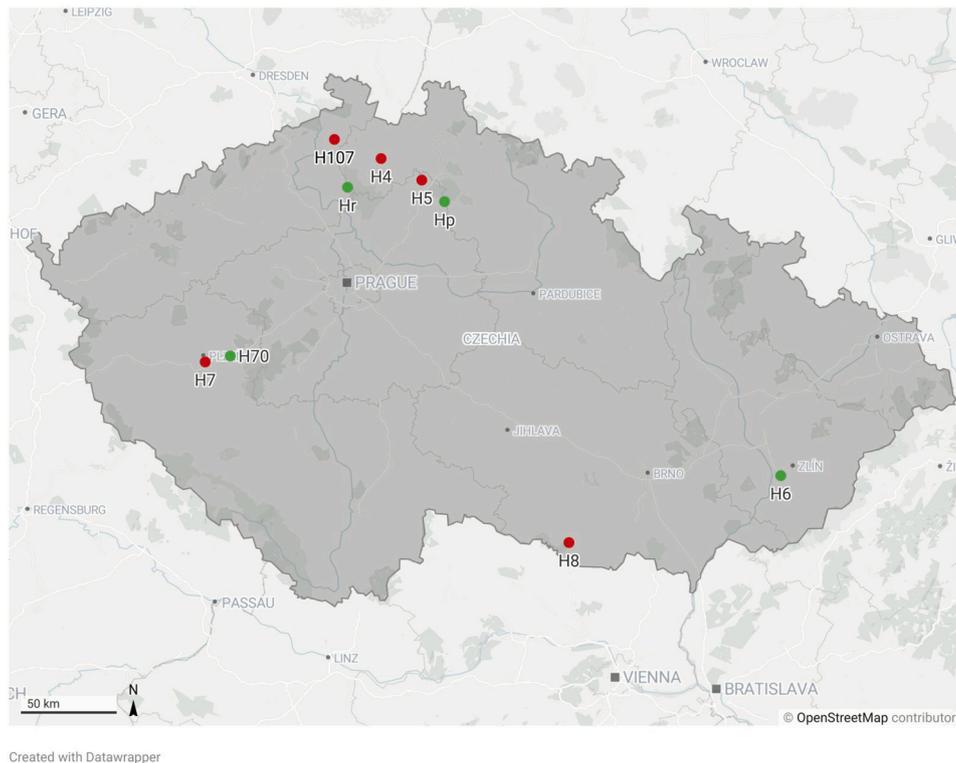
For the 12S rDNA gene, two haplotypes were detected, differing in 7

**Table 1**  
Hyalomma spp. samples information and identification.

Tick ID	Year of collection	Source	Stage	Sex	Coordinates	Region	Sequence length (bp) (12S / 16S / COI)	Identified species
H4	2018	horse	adult	F	50.6627358N,14.6734389E	Liberecký	347/404/704	<i>H. marginatum</i>
H5	2018	horse	adult	M	50.5646072N,14.9709492E	Středočeský	309/405/704	<i>H. marginatum</i>
H6	2019	horse	adult	M	49.1788133N,17.5862106E	Zlínský	352/404/714	<i>H. rufipes</i>
H7	2018	horse	adult	M	49.7188878N,13.3936817E	Plzeňský	354/383/705	<i>H. marginatum</i>
H8	2019	household	adult	F	48.8587964N,16.0435742E	Jihomoravský	353/395/706	<i>H. marginatum</i>
H70	2018	horse	adult	M	49.7435858N,13.5728467E	Plzeňský	357/406/706	<i>H. rufipes</i>
H107	2018	foal	adult	M	50.7513994N,14.3314739E	Ústecký	357/395/710	<i>H. marginatum</i>
Hr	2020	horse	adult	M	50.5322278N,14.4264403E	Ústecký	0/403/701	<i>H. rufipes</i>
Hp	2020	common nightingale	nymph	-	50.4647403N,15.1366544E	Středočeský	341/402/701	<i>H. rufipes</i>

**Table 2**  
Primers used in this study

Gene	Primer name	Primer sequence (5'- 3')	Length (bp)	Annealing temperature (°C)	Source
COI	AR-U-COIa	AAACTRTKTRCCTTCAAAG	664	45°C	Cangi et al. (2013)
	AR-L-COIa	GTRITAAARTTTCGATCSGTTA			
12S rDNA	T1B	AAACTAGGATTAGATACCCT	320	50°C	Beati and Keirans (2001)
	T2A	AATGAGAGCGACGGGGCGATGT			
16S rDNA	16S-F	TTAAATTGCTGRGTATT	455	45°C	Lv et al. (2014)
	16S-R1	CCGGTCTGAACTCASAWC			



**Fig. 1.** Localities of *Hyalomma* spp. found in the Czech Republic 2018-2020. *H. marginatum* – red dots; *H. rufipes* – green dots.

nucleotides with  $H_d = 0.54$ , variance of  $H_d = 0.01$ , and  $\pi = 0.01$ . The number of variable (polymorphic) sites and Parsimony informative sites was 7.

The phylogeny was generated for each gene separately. In the preliminary analysis, all sequences available in the GenBank for *H. marginatum* and *H. rufipes* for each targeted gene were included (data not shown). For the final analysis, only the representative sequences for each species and country were included. Based on all three genes, *H. marginatum* and *H. rufipes* form a clearly separate and highly supported clades. For 12S rDNA and 16S rDNA, the intraspecific variability was close to zero, the COI gene formed subclades on the species level but with no clear correlation to the tick geographical origin (Fig. 2).

#### 4. Discussion

In Europe, the *H. marginatum* tick complex (Apanaskevich and Horak, 2008) is endemic to southern and eastern regions, including several Mediterranean countries (Jameson et al., 2012) where the CCHFV was detected (Portillo et al., 2021). Over the past several years, *H. marginatum* and *H. rufipes* were occasionally found in Europe outside of their natural range (Hansford et al., 2019). Findings of immature stages of these ticks on the migrating birds in Europe are common, while reports of adult ticks are limited to single ticks from Slovakia, Poland, Austria, Hungary, and the Czech Republic (reviewed in Hubálek et al.,

2020). Series of recent findings from Germany (2007, 2016, and 2017) extend the data set to thirty-five specimens. Of these, eighteen individuals were identified to the species level by morphological characteristics (10 *H. marginatum* and 8 *H. rufipes*); identification of the remaining 17 ticks was based on images and to the genus level only (Chitimia-Dobler et al., 2019).

Here, we report the finding of 11 adults and one nymph of *Hyalomma* spp. from the Czech Republic. Nine physically delivered ticks were identified to the species level (five *H. marginatum* and four *H. rufipes*) using molecular analysis. Remaining three ticks were identified to the genus level based on morphology from tick images.

It is very likely that these adult ticks were brought to the Czech Republic as nymphs feeding on migratory birds and moulted to adults after full engorgement. It appears that the conditions (temperature and humidity) in Central Europe provide the suitable environment for *Hyalomma* spp. to complete their development and to find appropriate hosts for subsequent blood feeding. It has been suggested that *H. rufipes* overwinters in Central Europe (Rudolf et al., 2021); however, this needs to be investigated further. Long distance dispersal on migratory birds combined with climate changes (Malhi et al., 2020) may result in an expansion of the *Hyalomma marginatum* complex natural habitat range (Domşa et al., 2016) and can also impact the epidemiology of tick-borne pathogens such as the CCHFV.

Adult *Hyalomma* prefer feeding on large mammals, particularly

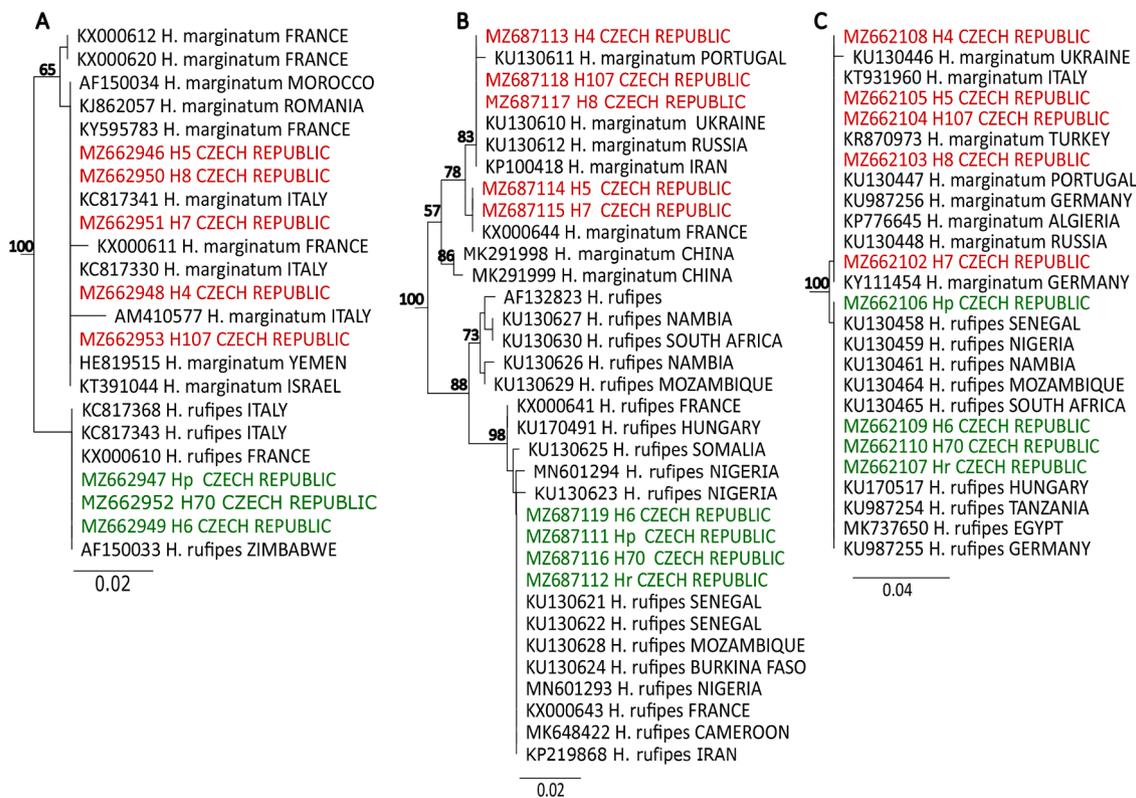


Fig. 2. The phylogenetic analysis of *Hyalomma* ticks using the Neighbour-joining method, Jukes-Cantor model with 1000 bootstrap replicates. A. 12S rDNA, B. COI, C. 16S rDNA. *H. marginatum* is marked in red colour, *H. rufipes* is in green colour

ungulates grazing in open areas (Grandi et al., 2020). The number of *Hyalomma* specimens found on horses in this study probably reflects the fact that horses are generally commonly handled and examined for ectoparasites. However, *Hyalomma* ticks likely feed also on free-ranging ungulates and cattle where their presence remains overlooked.

It is too early to speculate whether *H. marginatum* and *H. rufipes* have established in the Czech Republic and other parts of Central and Western Europe. However, a general pro-active strategy for the country-wide surveillance of exotic ticks is warranted. It is also important to implement educational programs for general public to raise an awareness on exotic ticks and associated pathogens.

**Fig. S1.** The median joining network computed for the COI gene revealed the presence of 23 segregating sites between five *H. marginatum* and four *H. rufipes*

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