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Molecular characterization of '*Candidatus* Rickettsia vini' in *Ixodes arboricola* from the Czech Republic and Slovakia

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ABSTRACT

The aim of this study was to analyze the prevalence of rickettsiae in the tree-hole tick *Ixodes arboricola* in the Czech Republic and Slovakia. During May to September of 2009 and 2013, bird boxes belonging to three different areas were screened for ticks. In total, 454 nestlings and 109 nests of 10 hole-breeding bird species were examined. Ticks were found on *Ficedula albicollis*, *Parus major*, *Cyanistes caeruleus* and *Sitta europaea* and/or in their nests. In total, 166 ticks (17 nymphs, 10 males and 139 females) were found at 3 areas (arithmetic mean ± standard error: 55.3 ± 45.9). All ticks were tested for the presence of *Rickettsia* species by polymerase chain reaction targeting the rickettsial genes *gltA*, *ompA*, *ompB* and *htrA* and amplicon sequencing. All individuals except 3 nymphs were infected with '*Candidatus* Rickettsia vini'. Multilocus sequence typing showed closest proximity to *Rickettsia japonica* and *Rickettsia heilongjiangensis* cluster. The presence of '*Ca.* R. vini' is reported for the first time in Slovakia.

Introduction

Ixodes arboricola Schulze and Schlottke, also known as the tree-hole tick, is a nidicolous ectoparasite that preferably feeds on hole-breeding birds. The whole life cycle of the tick is restricted to different types of cavities, such as tree holes or nest boxes. Birds play a significant role as reservoirs of various tick-borne pathogens (Hubálek, 2004; Palomar et al., 2012a). Tick-borne rickettsioses belong to emerging and reemerging infections. These zoonoses are among the longest-known vector-borne diseases and several rickettsial species have been associated with human infections (Weinert et al., 2009; Parola et al., 2013). Still, new *Rickettsia* species are discovered, including the recently described '*Candidatus* Rickettsia vini' found in *I. arboricola* individuals from Spain, the Czech Republic and Turkey (Keskin et al., 2014; Palomar et al., 2012a; Spitalska et al., 2011). In this study, we screened *I. arboricola* ticks for *Rickettsia* spp., and compared the genetic sequences from positive samples with those from known species in GenBank. Ticks were collected from nestlings and nest-boxes from the Czech Republic and Slovakia.

Material and methods

During the nestling period (May–June), nest boxes installed in the forests were surveyed at 3 locations: Breclav, 48°43′ N, 16°54′ E, 150 m above sea level (a.s.l.), an oak-ash flood-plain forest, 2009 and 2013; Velky Kosir, 49°32′ N, 17°2′ E, 350 m a.s.l., an oak hill forest, the nestling period and September (the after-breeding period) 2013 – both in the Czech Republic; and Ziar nad Hronom, Slovakia, 48°34′ N, 18°52′ E, 450 m a.s.l., a beech-oak mountain forest, 2013.

Individual bird nestlings were identified using Svensson et al. (2010), ringed, examined for the presence of ticks and put back into their nests. Ticks were collected by tweezers and stored in 96% ethyl alcohol. If there were no nestlings because fledged or predated, nest

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Table 1
Nest boxes and nestlings surveyed for the presence of *Ixodes arboricola*.

Birds		Study area/year				Total
Family	Species	Breclav 2009 No. of nest boxes (no. of nestlings)	Breclav 2013	Velky Kosir 2013	Ziar nad Hronom 2013	
Certhiidae	*Certhia* sp.	1(0)				1(0)
Muscicapidae	*Ficedula albicollis*	87(147)	12(51)	55(102)	17(27)	171(327)
Paridae	*Cyanistes caeruleus*	10(17)				10(17)
	Parus major	27(47)	1(10)		4(11)	32(68)
	Parus palustris	1(0)				1(0)
	Periparus ater				1(11)	1(11)
Passeridae	*Passer montanus*	3(5)				3(5)
Sittidae	*Sitta europaea*	1(0)			2(4)	3(4)
Sturnidae	*Sturnus vulgaris*	1(6)				1(6)
Picidae	*Jynx torquilla*	5(16)				5(16)
Total		136(238)	13(61)	55(102)	24(53)	228(454)

was placed into zip-lock plastic bag. In the laboratory, nests were examined for the presence of ticks and identified to species according to characteristic appearance of the nest. Ticks were classified to species, stage and blood meal volume according to Nosek and Sixl (1972) and sequencing of the tick mitochondrial 16S rDNA gene (Mangold et al., 1998).

DNA was extracted from all individuals. Success of DNA extraction was confirmed by polymerase chain reaction (PCR) using primers 16S+1 and 16S–1 amplifying a ≈460-bp fragment of the tick mitochondrial 16S rDNA gene. PCR with primers CS-78 and CS-323, CS-239 and CS-1069 was used to amplify a ≈1090-bp fragment of the *gltA* gene that occurs in all *Rickettsia* species (Labruna et al., 2004). Detection of *Rickettsia* belonging to the spotted fever group (SFG) was performed by PCR using primers Rr190.70 and Rr190.602 targeting a 532-bp fragment, a part of the *ompA* gene that occurs in the majority of SFG rickettsiae (Regnery et al., 1991). This gene is usually highly polymorphic and is considered the most valuable for the identification of *Rickettsia* species (Santibáñez et al., 2013). To enhance the identification, nested PCR with primers rompB OF, rompB OR, rompB SFG IF and rompB SFG/TG was used to amplify a 425-bp fragment of the *ompB* gene and primers 17kD1 and 17kD2 to amplify a 433-bp fragment of *htrA* gene (Choi et al., 2005; Webb et al., 1990).

PCR products were purified by ExoSAP-IT® and DNA-sequenced by Sanger dideoxy sequencing. Sequences were subjected to National Center for Biotechnology Information nucleotide BLAST analysis to compare to published sequences of species. Alignment of sequences was conducted using MEGA 6 (Tamura et al., 2013). Phylogenetic analysis was performed by PAUP* 4.0 (Swofford, 2003).

Statistical analyses were performed within the R environment (R Development Core Team, 2011). Infestation prevalence in two infested bird species was compared by Two-sample test for equality of proportions. The difference in infestation rate on studied localities was tested using Pearson's Chi-squared test for count data. We compared localities only for year 2013, because in 2009 the only locality studied was Breclav.

Results

In total, 228 nest boxes were surveyed, in which 454 pulli were found (Table 1). One hundred nine nests were taken for observation in the laboratory. In total, 166 (17 nymphs, 10 males and 139 females) *I. arboricola* ticks were collected: 119 (13 nymphs and 106 females) nestling-derived ticks, 47 (4 nymphs, 10 males and 33 females) individuals found in nests; arithmetic mean ± standard error: 55.3 ± 45.9 (Table 2). Ticks were derived from nests and/or nestlings of four out of ten hole-breeding songbird species (Table 2). By BLAST analysis, the 16S rDNA partial sequence was 100% (410/410) identical to the corresponding sequence of *I. arboricola* from Spain (JF791812). GenBank nucleotide sequence accession numbers are KP713675 (a nymph from the Czech Republic) and KP713676 (a nymph from Slovakia).

Ticks feeding on nestlings were observed on *F. albicollis* (mean infestation prevalence 11.5%) and *P. major* (mean infestation prevalence 7%). The difference in the infestation prevalence was not

Table 2
Ticks *Ixodes arboricola* collected on nestlings or from nests in Breclav (BR), Velky Kosir (VK) and Ziar nad Hronom (ZH) in 2009 and 2013. Nymph (N), male (M), female (F).

Bird species	Study area/year	No. infested/no. captured pulli (% prevalence)	Mean infestation intensity	No. of ticks on pulli (N/M/F)	No. of nests	No. of ticks in nests (N/M/F)
Certhia sp.	BR 13				1	
Ficedula albicollis	BR 09	16/51 (31)	2.2	1/0/34		
	BR 13	21/147 (14)	3.9	10/0/70	43	0/6/21
	VK 13	1/102 (1)	1.0	0/0/1	21	3/1/9
	ZH 13	0/27 (0)			9	0/0/1
Cyanistes caeruleus	BR 13	0/17 (0)			6	0/1/1
Parus major	BR 09	1/47 (2)	1.0	0/0/1	21	
	BR 13	1/10 (10)	1.0	1/0/0		
	ZH 13	1/11 (9)	1.0	1/0/0	3	1/1/1
Parus palustris	BR 13				1	
Periparus ater	ZH 13	0/11 (0)				
Passer montanus	BR 13	0/5 (0)			2	
Sitta europaea	BR 13				1	0/1/0
	ZH 13	0/4 (0)			1	
Sturnus vulgaris	BR 13	0/6 (0)				
Jynx torquilla	BR 13	0/16 (0)				
Total		41/454 (9)		13/0/106	109	4/10/33