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Rickettsia raoultii and *Rickettsia sibirica* in ticks from the long-tailed ground squirrel near the China–Kazakhstan border

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Abstract
Spotted fever group (SFG) rickettsiae cause infection in humans, domestic animals and wildlife. To date, no rickettsial agents have been reported in hard ticks from the long-tailed ground squirrel (*Spermophilus undulatus*). A total of 50 adult ticks and 48 nymphs were collected from *S. undulatus* in the border region of northwestern China. Tick species (identified according to morphological and molecular characteristics) included *Dermacentor nuttalli, Dermacentor silvarum* and *Ixodes kaiseri*. Based on the cytochrome *c* oxidase subunit I (*COI*) haplotype analysis, *I. kaiseri* from *S. undulatus* belongs to an ancestral. In addition, all tick samples were analyzed for the presence of rickettsiae by PCR amplification and sequencing of six genetic markers. *Rickettsia raoultii* and *Rickettsia sibirica* subsp. *sibirica* were shown to occur in adults and nymphs of *D. nuttalli* and *D. silvarum. Rickettsia sibirica* subsp. *sibirica* was also detected in an *I. kaiseri* adult. *Dermacentor silvarum* and *I. kaiseri* were found for the first time on *S. undulatus. Rickettsia raoultii* and *R. sibirica* subsp. *sibirica* were detected in two *Dermacentor* and one *Ixodes* species, respectively, suggesting that these rickettsiae circulate in the region of the China-Kazakhstan border by hard ticks infesting *S. undulatus*.

Keyword *Spermophilus undulatus* · Ticks · *Rickettsia raoultii* · *Rickettsia sibirica* · Northwestern China

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Introduction

The long-tailed ground squirrel (*Spermophilus undulatus*) has been listed as globally vulnerable by the International Union for Conservation of Nature (IUCN) since 2008 (Ramoslara et al. 2014). This species is mainly distributed in Kazakhstan, Mongolia, Russia and northern China including Heilongjiang, Inner Mongolia and Xinjiang Uygur Autonomous Region (XUAR) (ZipcodeDev Team 2018). According to previous reports, *S. undulatus* and its ectoparasites are reservoirs of *Yersinia pestis, Francisella tularensis* and tick-borne encephalitis virus (Zhao et al. 2017; Wang and Yang 1983; Demina et al. 2017). However, only few tick species (i.e., *Dermacentor marginatus, Dermacentor nuttalli, Rhipicephalus schulzei* and *Ixodes persulcatu*s (Wang and Yang 1983; Demina et al. 2017)) are known to infest *S. undulatus*.

Members of the tick subgenus *Pholeoixodes* prefer burrowing mammals as hosts (Hornok et al. 2017), thus can be expected to occur on *S. undulatus*. Nevertheless, no previous reports confirmed this. In a study comparing three species of this subgenus (*Ixodes kaiseri, Ixodes hexagonus* and *Ixodes canisuga*) the *16S rDNA* phylogenetic tree reflected that *I. kaiseri* is divided into at least nine *COI* haplotypes in Europe (Hornok et al. 2017). However, at that time no specimens of *I. kaiseri* were available from Asia for comparison.

Spotted fever group (SFG) rickettsiae cause infection in humans, domestic animals and wildlife (Maina et al. 2014). At least nine *Rickettsia* spp. of the SFG had been detected in XUAR (LopezVelez et al. 2015; Guo 2017). According to previous studies, *Rickettsia sibirica* subsp. *sibirica*, responsible for Siberian tick typhus, is widely distributed in China, Mongolia, Kazakhstan and Russia, and was also molecularly detected in Spain (Guo 2017; Parola et al. 2013; Byambaa et al. 2008; Palomar et al. 2012). *Rickettsia raoultii*, the causative agent of tick-borne lymphadenopathy (TIBOLA) or *Dermacentor*-borne necrosis erythema lymphadenopathy (DEBONEL) (Mediannikov et al. 2008), is prevalent in Mongolia, Europe and the Russian Federation (Oteo and Portillo 2012; Boldbaatar et al. 2017). These examples justify the importance of studying rickettsiae with broad distribution range in Eurasia. Therefore, the aim of this study was to identify ticks infesting *S. undulatus*, and to molecularly identify *Rickettsia* species in these ticks.

Materials and methods

Tick sampling and identification

A total of 36 *S. undulates* were captured in Jinghe County (2023 m above sea level; 44°35′59N, 82°53′28E), near the wetlands around Ebinur Lake, in the Northwest region of XUAR in July 2017. For this purpose, Sherman traps (H.B. Sherman Traps, Tallahassee, FL, USA) were used, which were placed at the entrances of occupied burrows (Torres-Perez et al. 2010). Each survey site included 150 traps that were checked twice a day. Each trap was removed before nightfall and replaced on the survey site the following day.

Ticks were sampled from the entire body of each *S. undulatus*, and were first identified with stereomicroscope (LEICA M165 C) according to standard morphological keys (Hornok et al. 2017). Then the genomic DNA was extracted from all ticks by using the 96 flux automatic nucleic acid extraction instrument with a matching commercial kit (Cell & Tissue Kit, Bioteke, Beijing, China) according to our previous report (Liu et al. 2018).

To confirm the morphological identification of tick species, two mitochondrial markers, the *16S rDNA* and the cytochrome *c* oxidase subunit I (*COI*) genes (Hornok et al. 2017), were analyzed according to five representative ticks for each tick species. The phylogenic relationships among the representative tick specimens were inferred using MEGA 6.0 software. Twelve nucleotide sequences from our study have been deposited in the GenBank database (*16S rDNA*: MG656445, MH324406-MH324409; and *COI*: MH079424, MH279561).

Detection of rickettsial agents and sequence analyses

Six genetic markers, including 17-kilodalton antigen (*17-kDa*), surface cell antigen 4 (*sca*4), citrate synthase (*gltA*), cell surface antigen 1 (*sca*1), and outer membrane proteins A and B (*ompA* and *ompB*), were amplified from each sample to investigate the presence of SFG rickettsiae (Anstead and Chilton 2013a, b). The primers and PCR cycling conditions in this study are shown in Supplement Appendix Table 1. Each PCR assay included a negative control (distilled water instead of tick DNA template) and a positive control (with DNA from *R. raoultii* obtained from wetlands of Ebinur Lake in XUAR). Purification and sequencing of the PCR products were done as described above (Anstead and Chilton 2013a, b). Phylogenetic trees were constructed using the maximum-likelihood and neighbor-joining methods in MEGA 6.0 software (Guo et al. 2015).

Results

A total of 98 ticks (50 adult ticks and 48 nymphs) were collected from captured *S. undulatus*. According to morphological characters and the *16S rDNA* phylogenetic tree, ticks in this study were identified as *D. nuttalli, D. silvarum* and *I. kaiseri* (shown in Fig. 1). Based on analysis of *COI* haplotype, 1-5 nucleotide differences were found in comparison with *I. kaiseri* from Europe. The phylogenetic analysis indicated that *I. kaiseri* from *S. undulatus* was in an ancestral position to nine European haplotypes ("L to T") (Hornok et al. 2017) (shown in Fig. 2).

Out of 50 adult ticks (26 *D. nuttalli*, 21 *D. silvarum* and 3 *I. kaiseri*) and 48 nymphs (27 *D. nuttalli*, 21 *D. silvarum*), 25 adults and 16 nymphs were positive for six *Rickettsia* genetic markers (*17-kDa, gltA, ompA, sca*1, *sca*4 and *ompB*). Among them, *R. sibirica* subsp. *sibirica* was found in nineteen *D. nuttalli* (8 nymphs and 11 adult ticks), 13 *D. silvarum* (6 nymphs and 7 adult ticks) and an adult *I. kaiseri*. In addition, *R. raoultii* was found in five *D. nuttalli* (one nymph and four adult ticks), three *D. silvarum* (one nymph and two adult ticks) (shown in Fig. 3; Table 1). As indicated by five genetic markers (*17-kDa, gltA, sca*1, *sca*4 and *ompB*), *R. sibirica* subsp. *sibirica* in this study had sequence similarities in the range of 98.7–100% when compared to *R. sibirica* from *D. nuttalli* infesting sheep in Jimunai County, XUAR (Guo 2017). In addition, *R. raoultii* had 99.1–100% similarity compared to *R. raoultii* strain Khabarovsk (CP010969). The detailed similarities and divergences of the sequences from this study are shown in the Supplement Appendix Table 2.

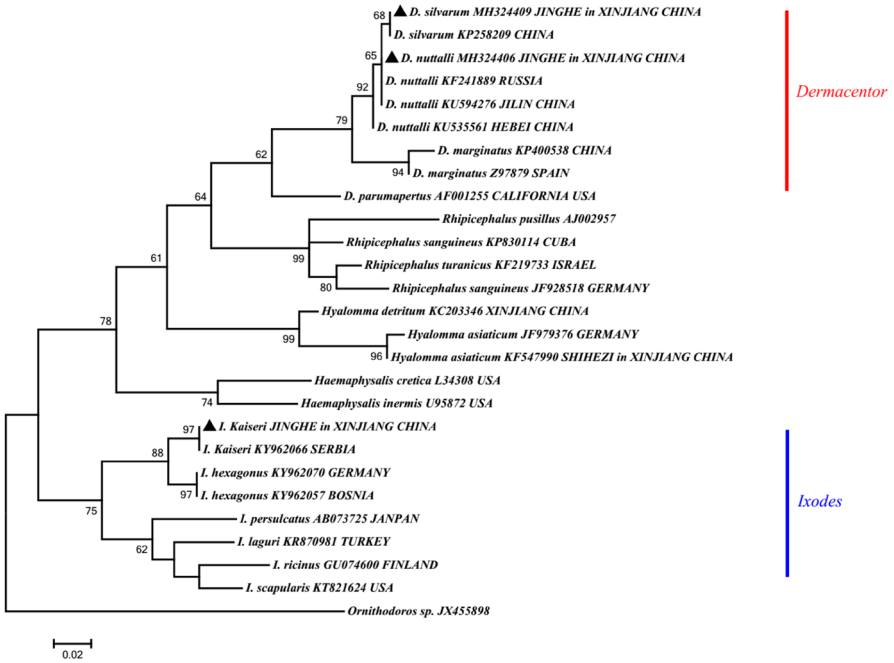

Fig. 1 The phylogenetic tree inferred from the *16S rDNA* sequences of representative tick specimens. The evolutionary history was inferred using the maximum-likelihood method. The new sequences provided by the present study are indicated by a black triangle (containing the accession number). The phylogenetic analyses were conducted using MEGA 6.0 software

Discussion

Spermophilus undulatus is a burrowing mammalian species, inhabiting mountain areas 1600–3000 m above sea level (a.s.l), with habitats along wetter front hills, forest margins and river valleys (Wang and Yang 1983). Previously, *D. marginatus, D. nuttalli, Rh. schulzei* and *Ixodes persucatus* were sampled from *S. undulatus* (Wang and Yang 1983).

Dermacentor silvarum, a three-host tick species, is widely distributed in North China, Russia and Mongolia (Deng and Jiang 1991; Kulik and Vinokurova 1983). The adult ticks parasitize cattle, horses, sheep, goats, hares and hedgehogs, and the larvae and nymphs mainly infest voles, squirrels and birds (Wang and Yang 1983). On the other hand, *I. kaiseri* occurs in the western Palearctic, including Germany, Hungary, southern Moldavia, Ukraine, Crimea, Romania, Egypt, Israel, Azerbaijan and Georgia, where it typically infests badgers, foxes, steppe polecats, raccoon dogs, common hedgehogs and domestic dogs (ZipcodeDev Team 2018; Hornok et al. 2017). In the present study, adult ticks (*D. nuttalli, D. silvarum* and *I. kaiseri*) and nymphs (*D. nuttalli* and *D. silvarum*) were found on *S. undulatus* in northwestern China. Our results extend the host range of *D. silvarum* and *I. kaiseri*. The ancestral phylogenetic position of its *COI* haplotype from China supports that the genetic diversity of *I. kaiseri* might be related to various geographic locations (Hornok et al. 2017).

To date, *R. sibirica*, including *R. sibirica* subsp. *sibirica* and *R. sibirica* subsp. *mongolotimonae*, have been reported from *Hyalomma anatolicum, Hyalomma truncatum, Hy.*

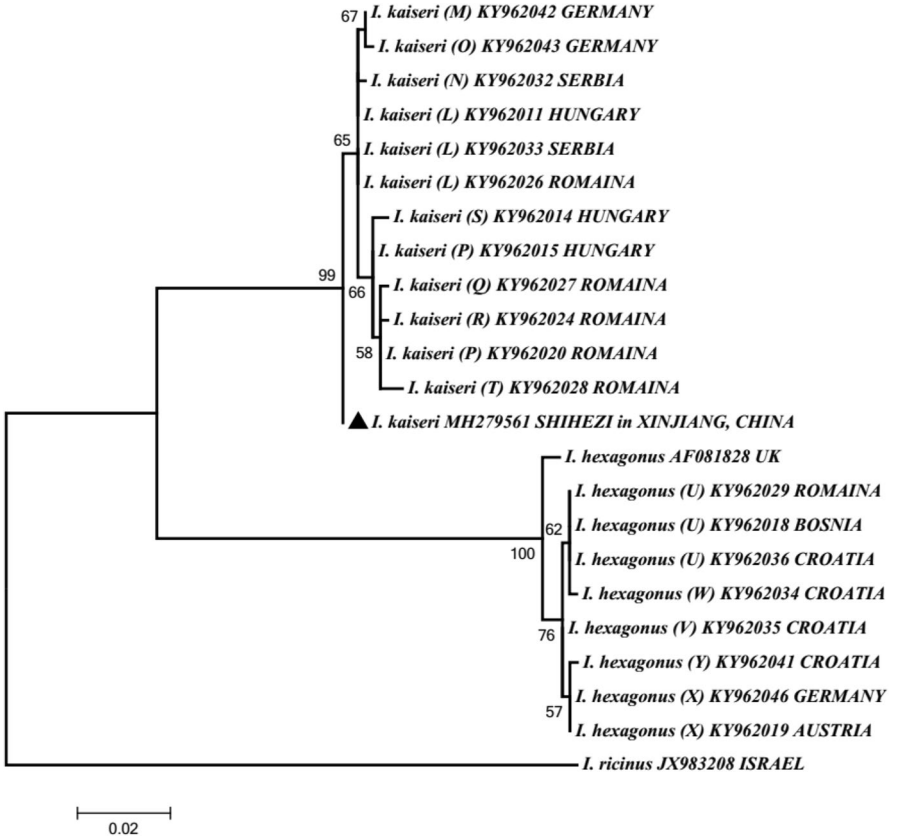

Fig. 2 Phylogenetic tree based on the *COI* gene, including sequences obtained in this study and representative sequences of other *Ixodes* spp.

asiaticum, Rh. pusillus, Dermacentor sinicus, D. nuttalli, D. marginatus, D. reticulatus, D. silvarum, Haemaphysalis yeni, Haemaphysalis concinna and *I. persulcatus* (Parola et al. 2013). In XUAR, *R. sibrica* subsp. *sibirica* has already been isolated from *D. nuttalli* in Jinghe County in 1974 (Kong et al. 1982). However, our finding is the first molecular evidence on the presence of *R. sibirica* subsp. *sibirica* in *I. kaiseri* from *S. undulatus*.

Another *Rickettsia* species, *R. raoultii*, is highly prevalent in XUAR and its neighboring countries. Among the others, it was detected in *Haemaphysalis erinacei* from marbled polecats in the Ebinur Lake wetlands (189 m above sea level; 82°48′51E 45°04′22N) in northwest China in 2014 (Anstead and Chilton 2013a, b). Jinghe County, neighboring Ebinur Lake wetlands, has a similar geographic habitat. In the present study, *R. raoultii* was detected in *D. nuttalli* and *D. silvarum* from *S. undulatus*. These findings suggest that *D. nuttalli, D. silvarum* and *I. kaiseri* parasitizing *S. undulatus* may serve as reservoirs and carriers for *R. raoultii* and *R. sibirica*.

Jinghe County, with the largest density of *S. undulatus* in China (10–50/hectare), was listed as a main *Marmot baibacina–S. undulatus* plague focus since 1967 (Wang and Yang 1983). *Yersinia pestis* was isolated from 3.22% (66/2051) of *S. undulatus* (Zhang and Sheng 1991). In our current study, the rickettsial *17-kDa* gene was detected from two

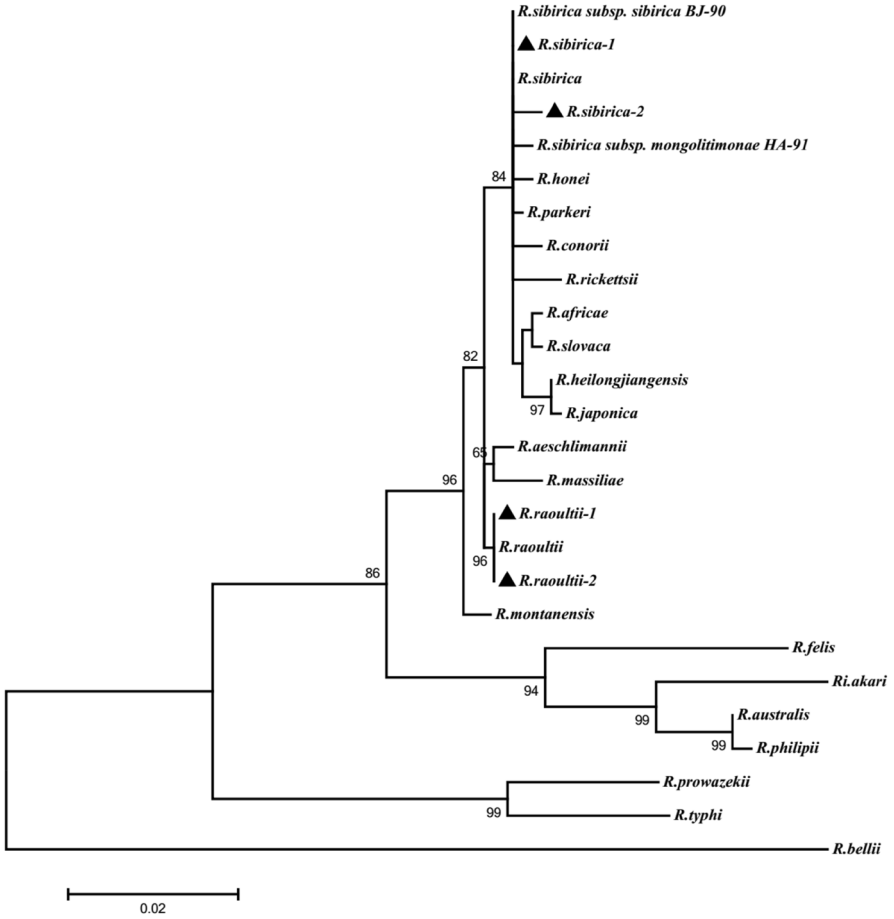

Fig. 3 Phylogenetic tree of the *17-kDa-ompA-gltA-sca*4-*sca*1-*ompB* concatenated sequence of rickettsial agents in adult ticks and nymphs

Table 1 The prevalence of *Rickettsia sibirica* and *R.raoultii* in *Dermacentor nuttalli, D. silvarum* and *Ixodes kaiseri* from *Spermophilus undulatus*

Tick species	*Rickettsia* species	Prevalence (%) in nymphs	Prevalence (%) in adults
D. nuttalli	*R. sibirica*	16.7 (8/48)	22.0 (11/50)
	R. raoultii	2.1 (1/48)	8.0 (4/50)
D. silvarum	*R. sibirica*	12.5 (6/48)	14.0 (7/50)
	R. raoultii	2.1 (1/48)	4.0 (2/50)
I. kaiseri	*R. sibirica*	0	6.0 (3/50)
	R. raoultii	0	0

naturally killed *S. undulatus* (data not shown). These findings indicate that several vector-borne pathogens co-circulate in Jinghe County, and imply potential risks associated with

populations of *S. undulatus*. In the future, it is necessary to confirm whether *D. nuttalli, D. silvarum* and *I. kaiseri* infesting *S. undulatus* could serve as vectors, transmitting rickettsial agents to *S. undulatus*.

Conclusions

Dermacentor nuttalli, D. silvarum and *I. kaiseri* were sampled from *S. undulatus* near the China-Kazakhstan border, and *I. kaiseri* in this study had an ancestral *COI* haplotype when compared to conspecific European sequences. *Rickettsia raoultii* was detected in the adult and nymph stages of *D. nuttalli* and *D. silvarum*, and *R. sibirica* also in one *I. kaiseri* adult. These findings extend our knowledge on the range of tick species infesting *S. undulatus* and harboring rickettsial agents.

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Author contributions SZ, MY, MJ and YW conceived and designed the study. BY, SZ, WY, BW and WH processed the samples and performed molecular and phylogenetic analyses. SZ and SH contributed to writing the manuscript. All authors read and approved the final version of the manuscript.

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Data Availability The datasets supporting the conclusions of this article are available in GenBank (National Center for Biotechnology Information) [unique persistent identifier and hyperlink to datasets in http://www.ncbi.nlm.nih.gov/genbank/].

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and informed consent This study was approved by the Animal Ethics Committee of Shihezi University (Approval No. AECSU2017-22).

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