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Short Communication

Rickettsia aeschlimannii and *Wolbachia endosymbiont* in *Ctenocephalides canis* from Eurasian lynx *(Lynx lynx)* Near the China-Kazakhstan Border

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Abstract

Twenty-five dog fleas *(Ctenocephalides canis)* and five ticks identified as *Hyalomma asiaticum* were collected from a Eurasian lynx *(Lynx lynx)* pup in northwestern China. Molecular analyses of four genetic markers showed the presence of *Rickettsia aeschlimannii* DNA in 5 out of 20 fleas. Only rickettsial *17-kDa* gene was detected in the blood sample of the lynx. In addition, 2 out of 20 fleas were positive to *Wolbachia endosymbiont* by targeting *16S rDNA* while there was no *Bartonella* DNA found both in 5 ticks and 20 fleas by using *gltA* and *16S-23S ITS*. Our findings suggest that i) *C. canis* parasitizing wild Eurasian lynx harbors *R. aeschlimannii* and *Wolbachia endosymbiont* in the China-Kazakhstan border, and ii) *Wolbachia endosymbiont* in present study is closer to that in *C. canis* infesting sheltered dogs in Turkey.

Keywords: *Eurasian lynx, Ctenocephalides canis, Hyalomma asiaticum, Rickettsia aeschlimannii, Wolbachia endosymbiont*

Çin-Kazakistan Sınır Bölgesinde Bir Avrasya Vaşağındaki *(Lynx lynx) Ctenocephalides canis*'te Saptanan *Rickettsia aeschlimannii* ve *Wolbachia endosymbiont*

Öz

Çin'in kuzeybatısında bir Avrasya vaşağı *(Lynx lynx)* yavrusundan yirmi beş adet köpek piresi *(Ctenocephalides canis)* ve *Hyalomma asiaticum* olarak tanımlanan beş adet kene toplandı. Dört genetik markerin moleküler analizi, 20 pirenin 5'inde *Rickettsia aeschlimannii* DNA'sının varlığını gösterdi. Vaşağın kan örneğinde ise sadece riketsiyal *17-kDa* geni tespit edildi. Buna ek olarak, 20 pire'den 2'si *Wolbachia endosymbiont*'un hedef *16S rDNA*'sı yönünden pozitifken, *gltA* ve *16S-23S ITS* kullanılarak yapılan analizde 5 kenede ve 20 pirede *Bartonella* DNA'sına rastlanmadı. Bulgularımız, i) Çin-Kazakistan sınırında vahşi Avrasya vaşaklarını enfekte eden *C. canis*'in *R. aeschlimannii* ve *Wolbachia endosymbiont*'a konakçılık yaptığını ve ii) mevcut çalışmadaki Wolbachia endosymbiont'unun, Türkiye'deki barınak köpeklerini enfekte eden *C. canis*'teki ile daha yakın olduğunu göstermektedir.

Anahtar sözcükler: *Avrasya vaşağı, Ctenocephalides canis, Hyalomma asiaticum, Rickettsia aeschlimannii, Wolbachia endosymbiont*

INTRODUCTION

The Eurasian lynx *(Lynx lynx)* is a medium-sized wild cat (Mammalia: Carnivora: Felidae), designated as a Class II protected species in China [1]. This species is widely distributed in Europe, Central- and Northern Asia, including

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Kazakhstan, China and Korea [2]. *Rhipicephalus pusillus*, *Rhipicephalus turanicus*, *Rhipicephalus sp.*, *Ixodes ricinus*, *Ixodes ventalloi*, *Ctenocephalides canis*, *Pulex irritans*, *Spilopsyllus cuniculi* and *Ctenocephalides felis* were reported from Iberian lynx *(Lynx pardinus)* [3,4]. In North America, *Amblyomma americanum* and *Dermacentor variabilis* ticks were collected from the bobcat *(Lynx rufus)* [5,6]. When ticks infesting these two lynx species were molecularly screened for tick-borne pathogens, four *Rickettsia* species belonging to the spotted fever group (SFG), i.e. *Rickettsia helvetica*, *R. massiliae, R. monacensis* and *R. rickettsii*, were detected [6,7].

Rickettsia aeschlimannii, member of spotted fever group (SFG) rickettsiae, caused human infection [8], and was molecualrly detected in the blood of camels in Israel [9]. It was firstly found in *Rhipicephalus turanicus* ticks in 2015 in northwest China [10]. Wolbachia can inhibit the transmission of certain viruses and the infectivity of the malaria-causing protozoan, Plasmodium and filarial nematodes. Furthermore, Wolbachia can cause a form of conditional sterility that can be used to suppress populations of mosquitoes and additional medically important insects [11]. In this study, the ectoparasites of Eurasian lynx *(Lynx lynx)* was reported, and *Wolbachia* and *Rickettsia* were molecularly detected in arthropod vectors.

MATERIAL and METHODS

Twenty-five fleas and five ticks were collected from a road-killed Eurasian lynx pup, in Toli County (805 m above sea level; 82°41'30E 45°19'04N) in northwestern China in August, 2019. A blood sample was also collected from the Eurasian lynx pup. The sampled fleas and ticks were carefully surface-sterilized, washed in 3% hydrogen peroxide (H_2O_2) followed by 70% ethanol (EtOH) [12]. Genomic DNA was extracted individually from 20 fleas (the other five fleas were used as morphological identification by making slides [13]), 5 ticks and the pup's blood sample by using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). The molecular identification was carried out by amplifying the mitochondrial *18S rRNA* and the cytochrome c oxidase subunit I *(cox1)* genes for fleas and the *16S rRNA* gene for ticks. PCR primers were shown in *Table 1*. The presence of rickettsial DNA was investigated by PCR amplification and sequencing of parts of the following four genetic markers: 17 kilodalton antigen *(17-kDa)*, citrate synthase *(gltA)*, cell surface antigen 1 *(sca1)*, and outer membrane proteins A *(ompA)* as described previously [14]. Phylogenetic relationships of the detected rickettsial agent were investigated by the Maximum Likelihood and Neighbor-Joining analyses. In addition,

Table 1. *Characteristics of amplified fragments and corresponding primer sequences*

Species	Gene		Primer	Fragment	Ref.
Tick	*16S rRNA*		F-CTGCTCAATGATTTTTTAAATTGCTGTGG	460	[14] [16]
			R-CCGGTCTGAACTCAGATCAAGT		
Flea	*18S rRNA*		F-CCTGAGAAACGGCTACCACATC	1150	
			R-GCATCACAGACCTGTTATTGC		
	cox1		F-GGTCAACAAATCATAAAGATATTGG	710	
			R-TAAACTTCAGGGTGACCAAAAAATCA		
Rickettsia	*17-kDa*	out	F-GCTTTACAAAATTCTAAAAACCATATA	434	[14]
			R-TGTCTATCAATTCACAACTTGCCGTT		
		in	F-GCTCTTGCAACTTCTATGTT		
			R-CATTGTTCGTCAGGTTGGCG		
	gltA	out	F-ATGACCAATGAAAATAATAAT	1178	[14]
			R-ATTGCAAAAAGTACAGTGAACA		
		in	F-GGAATCTTGCGGCATCGAGGATATG	931	
			R-CCATAGCTTTATAGATAATACCCG		
	sca1	out	F-GGTGATGAAGAAGAGTCTC	657	[14]
			R-CTCTTTAAAATTATGTTCTAC		
		in	F-GAGGTTTGTGGATGCGTGGT	553	
			R-ACTGTGACTTTAGTACCGACA		
	ompA	out	F-ATGGCGAATATTTCTCCAAAA	532	[14]
			R-AGTGCAGCATTCGCTCCCCCT		
		in	F-CTTAAAGCCGCTTTATTCACCACCTC	433	
			R-CCTGTATAATTATCGGCAGGAGC		
Wolbachia	*16S rDNA*		F-TTGTAGCCTGCTATGGTATAACT	936	[17]
			R-GAATAGGTATGATTTTCATGT		

Wolbachia and *Bartonella* were also molecularly detected by targeting *16S rDNA*, *gltA* and *16S-23S rRNA* intergenic spacer region, respectively [15].

RESULTS

The fleas were identified morphologically as *C. canis* according to the following key features *(Fig.1)*. The first spine is half as long as second spine of the genal comb. The pattern of metatibial formula of chaetotaxy is 2-2-2-2-2-1-1-3. The shape of the manubrium of the clasper expanded apically [18]. The sampled five ticks (3 male and 2 female) were identified as *Hyalomma asiaticum* by morphological observation *(Fig. 2)*. Briefly, Coxa I deeply was incised with two contiguous and unequal spurs. Legs harbor areas of enamelling. Cervical grooves were deep and long while lateral grooves short [19]. Then the morphological results were confirmed by amplifying *18S rRNA* (GenBank: MN565695), *cox1* (MN565696) and *16S rRNA* (MN625446) genes. Rickettsial DNA was detected in 5 out of 20 (25%) *C. canis* fleas, but not positive in any of the 5 *H. asiaticum* ticks *(Fig. 3-A)*. Interestingly, only the *17-kDa* gene was detected in lynx blood sample.

BLAST search results showed that sequences of three genes *(17-kDa*, *ompA*, and *sca1)* from the fleas were 100% identical to that of *R. aeschlimannii* isolate VGD7 from *C. felis* infesting domestic animals in Peru, while the obtained *gltA* sequence showed only 97.52% (908/931 bp) identity in the same context. All new sequences were deposited in GenBank *(17-kDa*: MN557354; *sca1*: MN557355; *gltA*: MN603746; *ompA*: MN557357). The phylogenetic analysis of concatenated sequences confirmed that the rickettsial agent in this study clustered closest to *R. aeschlimannii (Fig. 4)*.

Wolbachia DNA was also detected in 2 out of 20 (10%) *C. canis* fleas but negative in 5 ticks while there was no *Bartonella* DNA both in ticks and fleas *(Fig. 3-B)*. The phylogenetic analysis of *Wolbachia 16S rDNA* indicated that *Wolbachia endosymbiont* in present study was clustered into that in *C. canis* collected from sheltered dogs in Turkey *(Fig. 5)* [20].

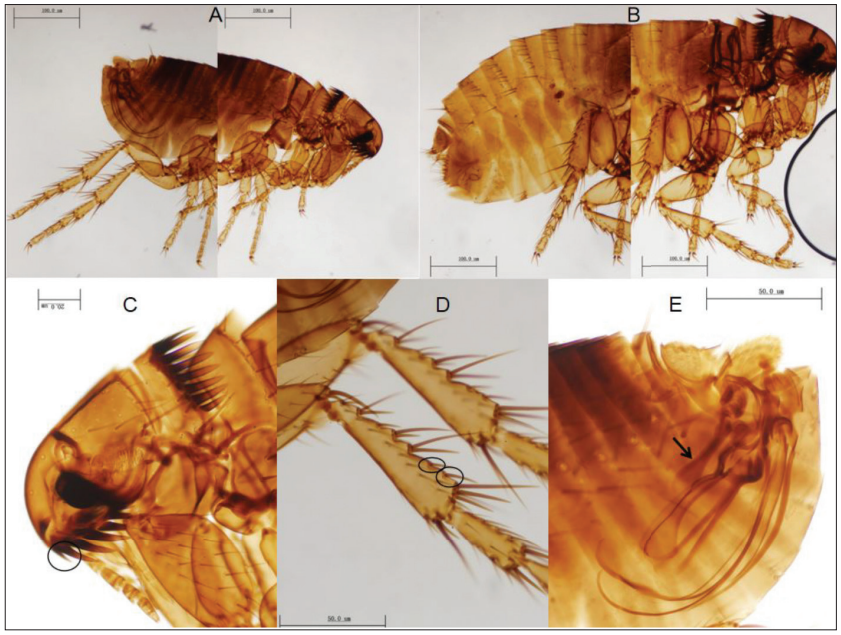

Fig 1. The key features of *C. canis* fleas. A: The male. B: the Female. C: The first and second spines of the genal comb. D: The metatibial formula of chaetotaxy. E: The shape of the manubrium of the clasper

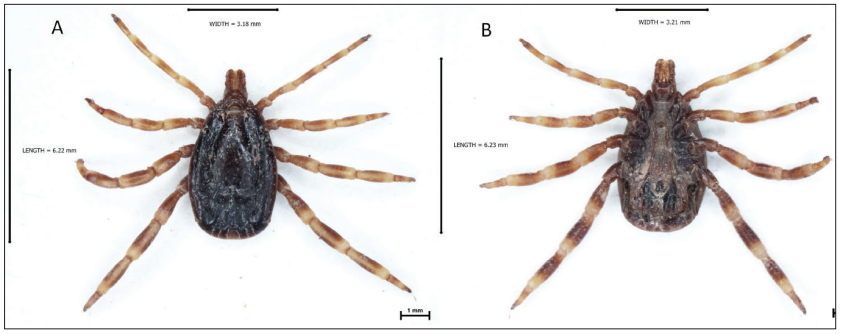

Fig 2. *Hyalomma asiaticum* collected from Eurasian lynx pup. A: Dorsal view. B: Ventral view

DISCUSSION

Hyalomma asiaticum is a highly abundant tick species in Xinjiang Uygur Autonomous Region (XUAR), Northwestern China. The main habitat of this tick species includes desert or semi-desert areas, where it is typically found on camel, sheep, cattle, horses and other livestock, but can also infest humans and wild animals [21]. In addition, *C. canis* (Siphonaptera: Pulicidae) is known as an ectoparasite of dogs and cats [13], Here *H. asiaticum* and *C. canis* are reported from Eurasian lynx.

Rickettsia aeschlimannii was previously detected in *Hyalomma aegyptium*, *H. turanicum*, *H. excavatum*, *H. impeltatum*, *H. dromedarii*, *Hyalomma marginatum* and *Hyalomma rufipes* ticks in Algeria, Israel, Southern Algeria, Morocco, Croatia, Spain, southern France, Portugal, Italy, Russia, Cyprus, Germany, Turkey, Hungary, and the Greek island of Cephalonia [9,16,22-24].

In 2015, *R. aeschlimannii* was detected in *Rhipicephalus turanicus* ticks from sheep in Yining County, XUAR [25]. Additional SFG rickettsiae shown to be present in other arthropods, such as *R. raoultii* and *R. slovaca* in the sheep ked, *Melophagus ovinus* (Diptera: Hippoboscidae) [16], as well

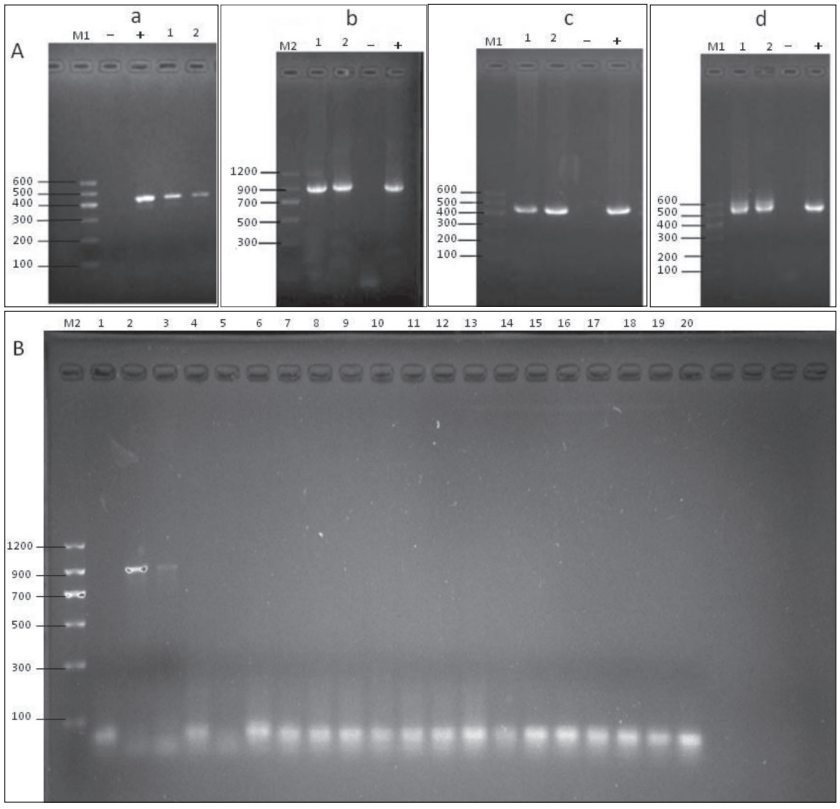

Fig 3. The PCR products of the Rickettsiae *17-KDa*, *gltA*, *ompA*, *sca1* genes and *Wolbachia 16S rDNA* genes. A: a, *17-KDa*. b, *gltA*. c, *ompA*. d, *sca1*. B: *16S rDNA*

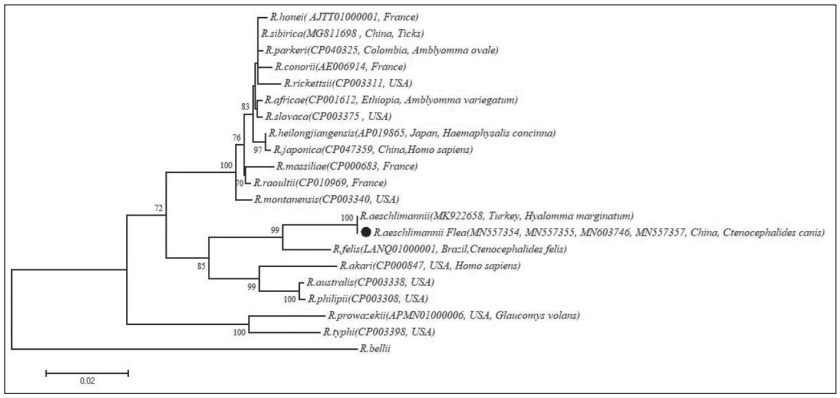

Fig 4. Phylogenetic tree of the *17-kDa-ompA-gltA-sca1* concatenated sequence of rickettsial agents in Eurasian lynx and infesting fleas. The new sequences provided by the present study are indicated by a black circle (containing the accession number). The phylogenic analyses were conducted using MEGA 6.0 software

as Candidatus *R. barbariae* in the flea *Vermipsylla alakurt* [26]. Concerning the flea species, *C. canis*, it is known to carry *R. felis*, as reported in Uruguay [27]. Here *R. aeschlimannii* and *Wolbachia endosymbiont* were detected in *C. canis* from Eurasian lynx. Interestingly, the rickettsial *17-kDa* gene was also detected here from lynx blood (MN603747). These findings suggest that *C. canis* parasitizing wild Eurasian lynx harbors *R. aeschlimannii* and *Wolbachia endosymbiont* in the China-Kazakhstan border.

In previous studies, *C. canis* was collected from feral cat *(Felis catus)*, feral dog *(Canis lupus)*, red fox *(Vulpes vulpes)* and chilla foxes *(Pseudalopex griseus Gray)* [10,28]. In this study, *C. canis* was sampled from Eurasian lynx and *Wolbachia* DNA was also detected in *C. canis*.

In conclusion, the flea species *C. canis* and the tick species *H. asiaticum* were reported in Eurasian lynx *(Lynx lynx)*. Furthermore, *R. aeschlimannii*, an emerging member of the spotted fever group (SFG) [25], was molecularly detected in *C. canis* fleas. These findings extend our knowledge on the geographical distribution of *R. aeschlimannii* and the range of its carriers, which now includes not only ticks but also fleas. In addition, the presence of *Wolbachia endosymbiont* in *C. canis* might be more related to flea species rather than infesting hosts.

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Author's Contributions

Gang Liu, Shuo Zhao, Xinli Gu and Yuanzhi Wang conceived and designed the study, and wrote the manuscript. Gang Liu, Shuo Zhao, Meihua Yang and Wurelihazi Hazihan performed the experiments, and analyzed the data. Sándor Hornok critically revised the manuscript. All authors read and approved the final manuscript.

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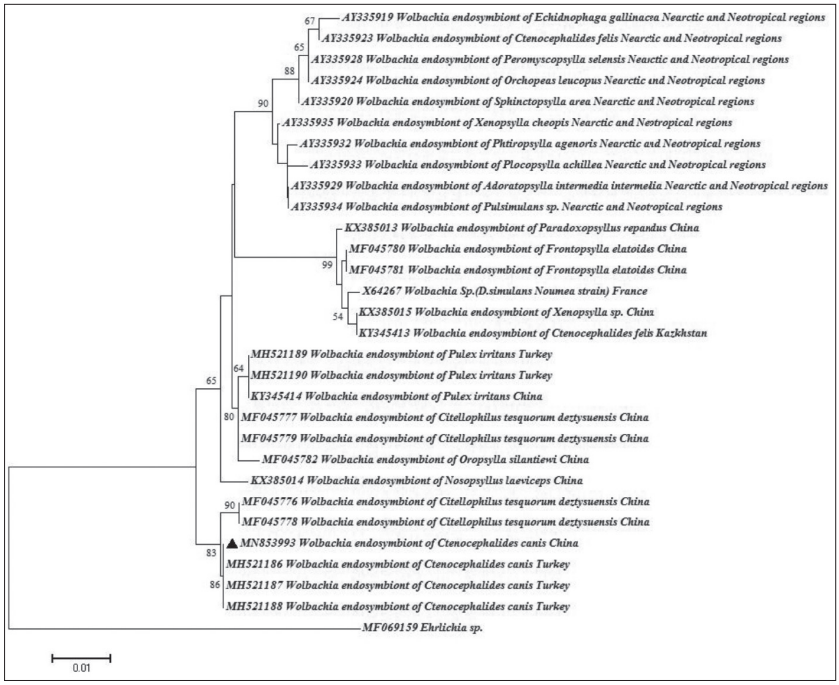

Fig 5. Phylogenetic tree of the *Wolbachia 16S rDNA* in *Ctenocephalides canis* from Eurasian lynx. The new sequences provided by the present study are indicated by a black triangle (containing the accession number). The phylogenic analyses were conducted using MEGA 6.0 software

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