Records of Three Mammal Tick Species Parasitizing an a typical Host, the Multi-ocellated Racerunner, in Arid Regions of Xinjiang, China

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Research

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Abstract

Background: To better simulate and predict zoonotic disease outbreaks, data on arthropods acting as pathogen carriers and information on host-arthropod associations should be collected. Ticks are obligate haematophagous ectoparasites of vertebrate animals, including humans, with a wide global distribution. To date, the species discrimination and phylogenetic relationships among the ticks on lizards in China remain unclear.

Methods: In this study, 31 ticks, collected from *Eremias multiocellata* lizards in four arid desert regions of the Xinjiang Uygur Autonomous Region in China, were identified by morphological observation and molecular techniques. The *12S rDNA*, *16S rDNA*, and *COI* fragments of ticks were sequenced. As reference samples, 47 Chinese ticks from hedgehogs and one tick from brushwood were also included in this study. To infer the phylogenetic relationships among them, 32 *12S* rDNA, 77 *16S* rDNA, and 66 *COI* sequences of ticks retrieved from GenBank were also included. All samples were identified by phylogenetic analyses.

Results: The Bayesian and network analysis results revealed that the 31 ticks from the lizards belong to three genera and three species: 11 were identified as *Hyalomma asiaticum*, three as *Rhipicephalus turanicus*, and 17 as *Haemaphysalis sulcata*.

Conclusions: Our study is the first attempt to investigate ticks on lizards in the arid desert regions of Xinjiang, China. Notably, two species of ticks have been identified on lizards in China for the first time. The discoveries in this study are closely related to the geographical environment in Xinjiang, and should provide important information for the control of ticks and tick-borne pathogens in northern China.

Background

Ticks are obligate haematophagous ectoparasites of vertebrate animals, including humans, with a wide global distribution [1, 2]. They are considered significant vectors of diseases in humans, livestock, and wildlife. They belong to the suborder Ixodida, which comprises three families: Argasidae, Ixodidae, and Nuttalliellidae [3]. In China, ticks have high species diversity and have been divided into two families (Argasidae and Ixodidae) and about 120 species [4, 5].

The Xinjiang Uygur Autonomous Region, located in the northwest of China, covers more than a sixth of the country's territory. Large areas of mountains, deserts, and other additional characteristics constitute the particular landscape of Xinjiang [6]. Additionally, this region is mainly occupied with animal husbandry. Both the landscape and livestock population contribute to the survival of ticks. More than 40 tick species were confirmed to be distributed in the Xinjiang Uygur Autonomous Region, about one third of the species found in China, and most of the parasitifers were livestock [7].

Generally, wild animals serve as a huge and often unknown reservoir of hosts for zoonotic disease, including tick-borne infections [8]. Many wild animals, such as lizards, wild boars, hedgehogs, and snakes have been identified as tick hosts [9–11]. However, studies on lizard-ticks are rare, especially in China. To

date, only six tick species have been reported from lizards in China: *Amblyomma javanense*, *Amblyomma cordiferum, Amblyomma varanense, Amblyomma crassupes, Ixodes nipponensis*, and *Haenaphysalis sulcate.* [12]. Hence, we know little about lizard-ticks. Lizards serve as a suitable host for many tick species [13] and commonly share their habitats with domestic animals and human beings. A recent increase in human and animal infections from tick bites has been partly caused by a change in the hosts of ticks. Because two or three hosts are involved in the life history of many tick species, tick-associated agents, including bacteria, viruses, protozoa, and helminths, carried by lizards might infect domestic animals and eventually result in human infection [14, 15]. Different tick species are suited to carrying different pathogens. Thus, it is imperative to identify tick species rapidly and accurately.

Traditionally, ticks are identified using morphological methods and criteria [16]. However, these methods are insufficient for the identification of damaged, engorged, or immature specimens because of the loss or lack of morphological characteristics [17, 18]. Because of these limitations, only a few experienced persons can accurately identify ticks. In addition, morphological and molecular analyses can also sometimes be inconsistent [19, 20]. Molecular techniques are an alternative method. The PCR amplification of molecular markers has been developed and has become an essential method in the phylogenetic analyses and species identification of ticks [21–23]. Several genetic markers, including the mitochondrial *12S* ribosomal RNA gene (*12S rRNA*) [24, 25], the mitochondrial *16S* ribosomal RNA gene (*16S rRNA*) [26, 27], the second internal transcribed spacer (*ITS-2*) of *rRNA* [28, 29], and mitochondrial *cytochrome oxidase subunit 1* (*COI*) [25, 30], have been widely used for studies on ticks.

This study is the first attempt to investigate ticks on the lizards of the arid desert regions of Xinjiang, China. We use molecular techniques to provide data on ticks acting as pathogen carriers and information on lizards-tick associations.

Methods

Ticks collection and identification

Thirty-one ticks were collected with tweezers from the body surfaces of lizards identified as *Eremias multiocellata* by their morphological characteristics. The lizards were hand-captured alive at four sites in the arid desert regions of the Xinjiang Uygur Autonomous Region of China (Fig. 1). These sites are characteristic of arid deserts (Table 1). In addition, 47 ticks from hedgehogs and 1 tick from brushwood were collected and used as reference samples, as shown in Table S1.These ticks were collected and preserved separately in 2 mL sample tubes containing 2 mL of 95% ethanol, for later identification and DNA extraction. All 78 ticks were stored at the Chengdu Institute of Biology, Chinese Academy of Sciences. All lizards were used in other studies. The protocol was approved by the medical ethics committee of Sichuan University (No. K2018056) and carried out under the National Guidelines for Experimental Animal Welfare (MOST of the People's Republic of China, 2006).

Table 1
List of sampling localities, hosts species in this study.

Site label	Locality	Hosts
P1	Aheqi county, Kizilsu Kirghiz Autonomous Prefecture, Xinjiang	*E. multiocellata*(3)
P2	Aheqi county, Kizilsu Kirghiz Autonomous Prefecture, Xinjiang	*E. multiocellata*(1)
P3	Hejing county, Bayingol Mongolian Autonomous Prefecture, Xinjiang	*E. multiocellata*(2)
P4	Xinyuan County, Ili Kazak Autonomous Prefecture, Xinjiang	*E. multiocellata*(1)
P5	Qiemo county, Bayingol Mongolian Autonomous Prefecture, Xinjiang	
P6	Yutian county, Hotan Prefecture, Xinjiang	Hedgehog

Table 2
List of sampling localities, hosts species, ticks species and sample size in this study.

Site label	Hosts	Ticks (total)		*H.sucata*		*H. asiaticum*		*R. turanicus*	
	(infested/total)	NO.	%	NO.	%	NO.	%	NO.	%
1	*E.multiocellata* (1/3)	2	6.5	2	6.5	0	0	0	0
2	*E.multiocellata* (1/1)	12	38.7	4	12.8	6	19.4	2	6.5
3	*E.multiocellata* (1/2)	16	51.6	11	35.5	4	12.9	1	3.2
4	*E.multiocellata* (1/1)	1	3.2	0		0		1	3.2
Total		31	100	17	54.8	10	32.3	4	12.9

DNA extraction, amplification, cloning, and sequencing protocols

The ticks were washed three times with phosphate-buffered saline (PH 7.4). Then, half of the bodies of the large ticks (widths 3–8 mm) were cut into small pieces with a pair of sterile scissors. Total genomic DNA for each tick was extracted from the pieces of half body of the large ticks or the whole bodies of the small ticks (widths 1–2 mm), using the commercial TIANamp Genomic DNA Kit (TIANGEN Bio, Beijing, China) according to the manufacturer's protocols. The extracted DNA samples were stored at -20 °C for further use. PCR primers specific for the ticks were synthesised by Tsingke Biological Technology Co., Ltd (Chengdu, China). They were used to amplify *12S rRNA* [24], *16S rRNA* [31], and *COI* [25] gene fragments for each tick sample using the genomic DNA as template and PrimeSTAR Max DNA polymerase (TaKaRa Bio, Shiga, Japan), according to the manufacturer's instructions. The following cyclic conditions were

used: denaturation at 98℃ for 10 s; 15 s for annealing at a specific temperature; and 1 min 10 s for elongation at 72℃. These three steps were repeated for 34 cycles. The negative control was treated with no template DNA and was included in all amplification runs. Successful PCR products were determined by electrophoresis on 1.5% agarose gel, and were purified using a Universal DNA Purification Kit (TIANGEN Bio). The expected product sizes were 320 bp of *12S rRNA*, 455 bp of *16S rRNA*, and 760 bp of *COI*. The PCR products were purified by excision of the band from the agarose gel using the Universal DNA Purification Kit (TIANGEN Bio) and were sequenced at Tsingke Biological Technology Co., Ltd.

Sequence alignment

Geneious Prime 2019.1.3 was used to study and edit the chromatograms. First, the obtained sequence data were preliminarily identified by GenBank searches using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All the nucleotide sequences obtained in this study have been submitted to the GenBank database (Tables S1–S2). Subsequently, all the sequences of each gene locus were multiple-aligned with a set of tick sequences for that locus. These sequences were retrieved from GenBank (Tables S3) using the default options of ClustalW in MEGA [32] (Molecular Evolutionary Genetic Analysis v7.0.26) and refined manually. All ambiguous alignment segments were manually removed. Finally, the alignments were concatenated into a single matrix (1229-bp in size) by Seaview to infer the phylogenetic relationships between the ticks. Then, the aligned sequences were used for the following analyses.

Phylogenetic analysis

The programme PartitionFinder v2.1.1 was used to choose the most appropriate models of nucleotide substitution for phylogenetic analyses using Bayesian information criterion. the phylogenetic relationships between the ticks were generated from a dataset concatenated from *12S rRNA, 16S rRNA*, and *COI*, using a commonly applied phylogenetic method: heuristic searches using Bayesian inference in MrBayes v.3.2 [33]. In the Bayesian inference analyses, gaps were treated as missing data. Four Markov chains were run for 20 million generations. To avoid local optima, we used two independent runs, and to improve the swapping of states between the heated and cold chains, the heating parameter was decreased to 0.02. Trees were sampled every 1000 generations, and the first 5000 trees were discarded as burn-in. The sampled trees were used to construct one Bayesian consensus tree and to calculate the posterior probabilities of the clades. The consensus tree was rooted at its midpoint and visualised using FigTree v.1.4.2 (available at http://tree.bio.ed.ac.uk/software/figtree/).

Network reconstructions

To better present the relationships among haplotypes within species, the median joining (MJ) network reconstruction method was implemented using the programme NETWORK v5.0.0.3 (available at http://www.fluxus-engineering.com/sharenet.htm).

Results

Ticks samples collection from lizards.

In total, 31 ticks parasitising lizards (identified as *E. multiocellata*) were collected from four sampling sites in Xinjiang. The majority of ticks were found in the lizards' armpits and crotch areas, and a few ticks were found on the head, the sides of the chest, or in the pericloacal region. Based on morphological keys and descriptions in previous reports [34], preliminary examination identified these ticks to three species of three genera: *Hyalomma asiaticum*, *Rhipicephalus turanicus*, and *H. sulcata*.

Sequence characteristics

The PCR amplification of each locus resulted in amplicons of the expected lengths (approximately 320 bp for *12S rRNA*, 455 bp for *16S rRNA*, and 760 bp for *COI*). The estimated mean frequencies of the GC were as follows: 23.5% for *12S rRNA*, 21.31% for *16S rRNA*, and 32.7% for *COI.* Thus, both the lengths and GC content were within the range of tick species [23].

Phylogenetic relationships

Bayesian information criterion analysis conducted with the aid of jModeltest identified GTR + I + G as the most appropriate model. The Bayesian majority rule consensus tree is shown in Fig. 2. The haplotypes obtained from the lizards are shown in red.

As shown in the Bayesian majority rule consensus tree (see Fig. 2), three clades were recovered, corresponding to the three genera *Haemaphysalis, Hyalomma*, and *Rhipicephalus.* As expected, within the first phylogroup, all the ticks obtained from the desert *E. multiocellata* lizards clustered together and had a closer relationship with *H. sulcata* than with any other species. In the *Hy. asiaticum* clade, the two *Hy. asiaticum* sequences obtained from GenBank were nested within 22 haplotypes (four obtained from lizards) with high support. Furthermore, haplotype H15 shared identical sequences to eight *Hy. asiaticum* isolates obtained from the lizards and 10 from hedgehogs. In the *Rhipicephalus* clade, two haplotypes from the lizards formed a strongly supported cluster with other *R. turanicus* haplotypes (posterior probability = 0.99).

Median joining network

To further evaluate the relationships among the intraspecific or interspecific genes of lizard-ticks MJ networks were constructed using the MJ algorithm network method. Ultimately, two haplotypes were obtained for 16S rDNA and eight for COI (Table S4). To gain additional insight into the relationship between lizard-ticks and other species of the genus *Haemaphysalis* (Fig. 3), all species of *Haemaphysalis* which have the sequences of 16S rDNA were included. The network effectively portrayed the relationship between the haplotypes in this study and other species of the genus *Haemaphysalis*. Q1 and Q2, collected from lizards in the northwest of China, were closely related to each other and were fewer mutation steps away from *H. sulcata* than the other sequences.

The network based on the *COI* haplotype of *R. turanicus* is shown in Fig. 4. It shows that the *R. turanicus* from China cluster together. Z1 and the other seven sequences from China share the same haplotype as one sequence from Iran (KT313117) and two from Kazakhstan (MN907846 and MN853166). Furthermore, at the intraspecific level, the haplotype network is superior to the bifurcating tree in detail, which could directly reflect the small genetic distances between the Z1 haplotype obtained in this study and other adjacent haplotypes.

Figure 5 present the MJ network based on the *COI* haplotype of *Hy. asiaticum*. Apparently, The haplotype network of *Hy. asiaticum* is centred around haplotype Z3. Meanwhile, the haplotypes from Xinjiang cluster together. The same haplotype was shared by Z3 (11 sequences), two ticks from Gansu Province (MK292000 and JQ737072) and another three from Kazakhstan (MN892553, MN961479, and MN907845). In addition, the haplotype network of *Hy. asiaticum* could intuitively reflect the distances between the three obtained haplotypes (one mutational step).

Discussion

This study was the first to investigate ticks on lizards from the arid desert regions of Xinjiang, China, using molecular techniques. The results show that the 31 ticks from *E. multiocellata* lizards at four sampling sites in this study belonged to three genera and three species (*H. sulcata*, *Hy. asiaticum*, and *R. turanicus*). Among them, *H. sulcata* was the most abundant species and *R. turanicus* the rarest, with only four ticks. *Hy. asiaticum* and *R. turanicus* were reported in lizards in China for the first time.

The tick species *H. sulcata* is a three-host tick species. The adults have been reported to usually feed on domestic and wild ungulates, while the immature stages have a special preference for reptiles [35]. In Turkey, *H. sulcata* was documented on *A.cappadocica*, *L.stellio*, and *O. elegans* [36]. Recently, six lizard species have been found to be reptilian hosts of *H. sulcata.* Consistent with previous research [12], we found *H. sulcata* in lizards. In this study, the *H. sulcata* haplotypes obtained from the lizards were clustered together with the *H. sulcata* sequences derived from GenBank. Meanwhile, the MJ network (Fig. 3) showed that *H. sulcata* has a shorter distance to these haplotypes than to any others. Therefore, we can conservatively infer that 17 of the ticks collected from the lizards have been identified to the species *H. sulcata*

Interestingly, we found not only *H. sulcata* feeding on the lizards, but also found two local tick species that are dominant in Xinjiang, *Hy. asiaticum* and *R. turanicus.* A previous survey of ticks on livestock also reported that *H. sulcata*, *Hy. asiaticum* and *R. turanicus* were found in the Tarim Basin, in Xinjiang. As the prevalent species in Xinjiang [37, 38], *Hy. asiaticum* has a broad host range, reaching more than 50 species, mainly artiodactyls and small mammals. Furthermore, all hosts can be accidentally infested at all stages of *Hy. asiaticum* [39]. This is the first report of *Hy. asiaticum* infestation on lizards in China. *Hy. asiaticum* mostly occurs in desert type habitats in Asia, including China, Kazakhstan, and Mongolia [39]. The MJ network based on the *COI* haplotype of *Hy. asiaticum* showed that Z3 shared the same haplotype

as two ticks from Gansu and three from Kazakhstan (Fig. 5). The reason could be their geographical origins, as Gansu and Kazakhstan both border Xinjiang. However, there are still unique lizard-tick haplotypes (Z2 and Z4) in Xinjiang.

In a previous study, *R. turanicus* was a widely distributed species in desert and semi-desert areas in the southern region of Xinjiang [40, 41]. However, the abundance of this species seems to be partly reduced owing to the limited sample size in the present study. Although this species has been reported to feed on lizards [20], the record of *R. turanicus* collected from lizards in China was blank until we found the three lizard-ticks in this study. As shown in Fig. 4, in the *R. turanicus* clade, one haplotype (Z1, three sequences from lizards and three from hedgehogs) shared an identical sequence with one Chinese *R. turanicus* and was clustered with two sequences derived from GenBank. To extend the knowledge of tick species infesting lizards and their potential to cause tick-borne diseases, more investigations are needed. Reptile-ticks could perhaps be believed to be less widely distributed in China only because of the lack of research on them.

Thousands of lizards were captured by our lab in the summers of 2015 to 2019. They belonged to the genera *Eremias* and *Phrynocephalus* and were caught at various sampling sites in arid desert regions, covering 68 counties in Xinjiang [12, 42]. No ticks were found on the lizards of other species belonging to the genus *Eremias* or on any species of *Phrynocephalus*. In addition, no ticks were found on *E. multiocellata* lizards from sampling sites other than the four sites used in this study (all in the north of the Tarim Basin). Several factors may affect the distribution of tick species, such as the climate, human land-use patterns, geographical habitats, and hosts. The Tarim Basin, an endorheic basin in southern Xinjiang, is located between the Kunlun Mountains, the Tianshan, and the Altun Mountains. Various landscapes in the Tarim Basin are composed of desertified grassland, salinised desert, and human and animal inhabited oases [5]. This was consistent with the adapted living environment of the three tick species found in the lizards [20, 34, 38]. In conclusion, we suggest that the characteristics found in this study of ticks in Xinjiang is closely related to the geographical environment.

Xinjiang, adjacent to eight countries (Russia, Kazakhstan, Kyrgyzstan, Tajikistan, Pakistan, Mongolia, India, and Afghanistan), is an important transportation hub. The economic system of Xinjiang is mainly agriculture and animal husbandry; thus, farmers and herders are in close contact with livestock, reptiles, and ticks. Therefore, the spread and epidemic risk of tick-borne disease in this area is grim, and the potential for the early warning, prevention, and control of tick-borne diseases should be improved. Further studies on the storage and transmission of pathogens in ticks are needed to help us control ticks and tick-borne diseases efficiently.

Conclusions

Based on molecular techniques, ticks parasitic on lizards have been identified for the first time in the arid desert regions of Xinjiang in China. The ticks belong to three species and three genera, including *Hy. asiaticum*, *R. turanicus*, and *H. sulcata*. Furthermore, some of these ticks share the same genotype as

their counterparts in neighbouring countries. The characteristics of ticks found in our study in Xinjiang are closely related to the geographical environment. Our findings could extend our knowledge about tick species infesting wild animals and help us to understand the relationship between lizards and ticks in Xinjiang.

Abbreviations

Hy.asiaticum: *Hyalomma asiaticum*; *H.sulcata*: *Haemaphysalis sulcate*; *R. turanicus*: *Rhipicephalus turanicus.*

Declarations

Acknowledgements

Not applicable.

Ethics approval and consent to participate

The protocol was approved by medical ethics committee of Sichuan University (No. K2018056) and carried out under the National Guidelines for Experimental Animal Welfare (MOST of People's Republic of China, 2006).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JPC, QZ, JL and DLC designed and supervised the study. HC, JHZ, JLH and ZWZ undertook the laboratory work. XGG, JLL, QS, XG and DLC collected the samples. QZ and JL wrote the manuscript. DLC, XGG and JPC revised the manuscript and polished the language. All authors read and approved the final manuscript.

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