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Molecular Identification of *Rickettsia* Species in *Haemaphysalis* Ticks Collected from Southwest China

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Abstract

Rickettsia species are obligate intracellular Gram-negative bacteria that can infect a wide range of vertebrate hosts, including humans, through arthropod vectors such as Ixodid ticks. These ticks are a threat to humans and animals because they are the primary vectors or reservoirs for rickettsiae, which is of public health importance. In this study, we report the identification and percent of positive of *Rickettsia* spp. in ticks collected from Cangxi County, Southwest China. *Haemaphysalis longicornis* comprised 48.4% of the 188 ticks collected followed by *Haemaphysalis flava* (29.3%), *H. doenitzi* (12.2%), and *Haemaphysalis hystricis* (10.1%). A total of 63 (33.5%) ticks were positive with *Rickettsia* spp., with 48 (57%) of those being *H. longicornis* and 15 (27.3%) being *H. flava*. The other two tick species, however, did not have any ticks positive for rickettsial DNA. In addition, two different *Rickettsia* spp. were identified using *glt*A and *omp*A as molecular markers. The sequence of *Rickettsia* sp. infecting *H. longicornis* ticks was found to be identical to the *Rickettsia* sequences from Northeastern China and Japan (KF728367, AB516964). Phylogenetic analyses using these molecular markers support the notion that *Rickettsia* species from *H. flava* is the most close to a member of the *Candidatus* Rickettsia gannanii subgroup. The high percentage of *Rickettsia* positive in this Southwest China region suggests potential public health threat in the future and warrants to be monitored.

Keywords: *Haemaphysalis* ticks, *Rickettsia* spp., *glt*A, *omp*A, Cangxi County

Introduction

Rickettsia species are obligate intracellular Gram-negative bacteria that can infect a variety of vertebrate hosts, including humans. These pathogens are transmitted by arthropods, such as ticks, mites, fleas, and lice (Parola et al. 2005). They have been classified based on the morphological, antigenic, and metabolic characteristics, and genetic traits (Noh et al. 2017). Approximately 60% (19/30) of the documented *Rickettsia* species are human pathogens. For example, *Rickettsia conorii*, *Rickettsia rickettsii,* and *Rickettsia japonica* cause Mediterranean, Rocky Mountain, and Japanese spotted fever, respectively (Merhej and Raoult 2011, Anstead and Chilton 2013). Recently, a number of other putative *Rickettsia* species have been reported based on phylogenetic analyses of different gene loci (*e.g.*, citrate synthase A [*glt*A], outer membrane protein A [*omp*A], and outer membrane protein B [*omp*B]) (Yu et al. 1993, Lupo et al. 2012, Speck et al. 2012, Tian et al. 2012, Anstead and Chilton 2013, Igolkina et al. 2016, Yang et al. 2016). Rickettsioses could present diverse clinical symptoms ranging from asymptomatic to severe symptoms in humans. Recently, the disease was considered one of the important emerging or reemerging diseases with a worldwide distribution. (Merhej et al. 2014, Sun et al. 2015, Lu et al. 2017).

Rickettsia sp. are transmitted by hematophagous arthropods that need repeated blood feedings for their reproductive and metabolic needs, thus allowing them to be vectors of numerous devastating infectious diseases (Attardo et al. 2005, Hou et al. 2015, Wang et al. 2017). Ticks are obligate ectoparasites of vertebrates and are distributed worldwide in various natural environments. The Ixodid ticks continue to be

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a threat to human and animal health because they are the primary vectors or reservoirs of an array of rickettsiae species. In Asia, *Haemaphysalis longicornis*, *Haemaphysalis flava*, *Haemaphysalis concinna*, *Ixodes persulcatus*, *Ixodes nipponensis*, and *Dermacentor silvarum* have been reported to be infected with *R. japonica*, *R. conorii*, *Rickettsia honei*, *Rickettsia sibirica*, *Rickettsia monacensis*, *Rickettsia heilongjiangensis*, and several unclassified *Rickettsia* spp. (Chung et al. 2006, Speck et al. 2012, Tian et al. 2012, Shin et al. 2013, Cheng et al. 2016, Igolkina et al. 2016, Yang et al. 2016, Noh et al. 2017). Recently, more *Rickettsia* species causing Spotted fever group (SFG) rickettsioses have been detected in China (Wei et al. 2015, Guo et al. 2016). However, research on the species associated with tick-borne rickettsial diseases is still limited in less developed regions of China.

In Cangxi County, Sichuan Province, Southwest China, a *H. concinna* tick outbreak was reported in 2005 (Sun et al. 2006). This species was found in 97.4% of the county, consistent with previous reports, and no new *Rickettsia* species were reported in the region. Following the availability of molecular techniques, new *Rickettsia* species have been identified in places where no rickettsioses were reported (Dobler and Wolfel 2009). In this study, a preliminary investigation was conducted to identify the presence and prevalence of *Rickettsia* spp. in ticks collected from Cangxi County, Sichuan Province, Southwest China.

Materials and Methods

Tick collection

Cangxi County is located in northern Sichuan province and belongs to Guangyuan City. This county is spread from N31°37′ to 32°10′ and from E105°43′ to 106°28′ and is a low mountain area. During May 2014, ticks were collected from three sites around this county (Table 1). All ticks were collected using drag cloth or removed from animals. Then, all collected ticks were identified based on morphological characteristics visualized through a light microscope (Lu and Wu 2003). Morphological identification was confirmed by sequencing the mitochondrial 16S ribosomal RNA (16S rRNA) gene as described previously (Rydkina et al. 1999). The ticks were stored at −80°C until DNA extraction.

DNA extraction

Genomic DNA from individual adult or nymph tick was isolated using the MasterPure DNA Purification kit (Epicentre Biotechnologies) with a modified protocol. Briefly, whole body of the arthropod was mixed with 5-mm stainless steel beads and 50 μL of Tissue and Cell Lysis Solution. Samples were disrupted using a Tissuelyser-24 (Jingxin, Shanghai, China) and then centrifuged for 1 min. To each sample, 250 μL of Tissue and Cell Lysis Solution containing 1 μL of Proteinase K (50 μg/μL) was added. Samples were then incubated at 65°C for 60 min, and placed on ice for 5 min. Then, 150 μL of MPC Protein Precipitation Reagent was added and vortexed. After centrifugation, supernatants were transferred to clean tubes and DNA was recovered using ice-cold isopropanol and resuspended in DNase-free water.

Molecular detection

Extracted DNA was screened for the presence of *Rickettsia* spp. using two-step PCR assays. The first step was a PCR screen for genus *Rickettsia* by amplifying a 381 bp fragment of the rickettsial citrate synthase (*glt*A) gene (Roux et al. 1997) by using RpCS.877p (*5′-GGGGGCCTGCTCACGGCGG-3′*) and RpCS.1258n (*5′-ATTGCAAAAAGTACAGTGAACA-3′*) primers. Then, all samples were screened by a nested PCR assay, which amplified a fragment of rickettsial outer membrane protein A (*omp*A) gene (Regnery et al. 1991, Blair et al. 2004) by using SFG *Rickettsia*-specific outer primers, Rr190.70 (*5′-ATGGCGAATATTTCTCCAAAA-3′*) and Rr190.701 (*5′-GTTCCGTTAATGGCAGCATCT-3′*), and inner primers Rr190.FN (*5′-AAGCAATACAACAAGGTC-3′*) and Rr190.RN (*5′-TGACAGTTA TTATACCTC-3′*). To avoid cross-contamination, a dedicated clean room was used for PCR assays and another room for DNA extraction.

Sequencing and phylogenetic analysis

The PCR products were purified using the QIAquick (Qiagen) kit and directly sequenced. Sequences of *omp*A, and *glt*A (Blair et al. 2004) genes retrieved from GenBank (National Center for Biotechnology Information, Bethesda, MD) were used to align and identify those from unknown SFG *Rickettsia* species. Sequence alignment and neighbor-joining phylogenetic analyses were conducted using MEGA version 7 (Kumar et al. 2016). Tree support was evaluated by bootstrap with 1000 replications.

GenBank accession numbers

The *glt*A and *omp*A sequences of *Rickettsia* from *Haemaphysalis* ticks are deposited in GenBank with the accession numbers MF590724 to MF590727, respectively.

Results

In total, we collected 188 ticks: 92 from hosts and 96 from vegetation (Table 1). The majority of those collected were *H. longicornis* 89 (48.4%), followed by *H. flava* 55 (29.3%), *H. doenitzi* 23 (12.2%), and *Haemaphysalis hystricis* 19 (10.1%). Notably, only *H. longicornis* was collected from all three sites.

Among the 188 ticks collected, 63 (33.5%) were positive for *Rickettsia* spp. in PCR screening assays. A large proportion of those positive for *Rickettsia* spp. were *H. longicornis* (48, 52.7%), followed by *H. flava* (15, 27.3%). However, other two tick species were not positive in the *Rickettsia* PCR screening assays. In Tongan Village, 16 out of 26 (61.5%) *H. longicornis* ticks collected from goats and 31 out of 55 (56.4%) ticks collected from plants were positive for *Rickettsia* spp. In Longquan Village, 13 out of 44 (29.5%) *H. flava* ticks collected from dogs and 2 out of 11 (18.2%) ticks collected from plants amplified *Rickettsia* spp. DNA.

Two different *Rickettsia* spp. were identified by sequencing *glt*A and *omp*A genes. The *glt*A sequence (MF590724) from *H. longicornis* was 100% identical to those from Northeastern China and Japan (KF728367 and AB516964). The *omp*A sequence (MF590726) from *H. longicornis* tick was 99.8–100% identical to those obtained from Southern China and Korea (KC888951, AF169629).

Table 1. Tick Collection Information

Collection sites	*Tick species*	*Hosts*	*Stage*	*No. ticks*	*No. positive*	*% positive*
Longquan village	*Haemaphysalis longicornis*	Dog	Female	0	0	0
			Male	0	0	0
			Nymph	3	0	0
		Plants	Female	0	0	0
			Male	0	0	0
			Nymph	5	1	20.0%
			Subtotal	8	1	12.5%
	Haemaphysalis flava	Dog	Female	26	10	38.5%
			Male	2	0	0
			nymph	16	3	18.8%
		Plants	Female	1	0	0
			Male	4	2	50.0%
			Nymph	6	0	0
			Subtotal	55	15	27.3%
	Haemaphysalis hystricis	Dog	Female	11	0	0
			Male	8	0	0
			Nymph	0	0	0
		Plants	Female	0	0	0
			Male	0	0	0
			Nymph	0	0	0
			Subtotal	19	0	0
Tongan village	*H. longicornis*	Goat	Female	9	8	88.9%
			Male	13	6	46.2%
			Nymph	4	2	50.0%
		Plants	Female	22	10	45.5%
			Male	17	12	70.6%
			Nymph	16	9	56.3%
			Subtotal	81	47	58.0%
Lingjiang village	*H. longicornis*	Plants	Female	0	0	0
			Male	0	0	0
			Nymph	2	0	0
			Subtotal	2	0	0
	Haemaphysalis doenitzi	Plants	Female	13	0	0
			Male	9	0	0
			Nymph	1	0	0
			Subtotal	23	0	0
			Totals	188	63	33.5%

The *glt*A (MF590725) and *omp*A (MF590727) sequences were 98.98% identical to uncultured *Rickettsia* sp. clone Y27-1 (KT921891) and 98.1% identical to uncultured *Rickettsia* sp. clone Y27-1 (KT921894), respectively. Uncultured *Rickettsia* sp. clone Y27-1 was isolated from *Haemaphysalis qinghaiensis* ticks from Gannan, China.

Topology of the phylogenetic tree produced from the *glt*A gene sequence is similar to the *omp*A tree (Figs. 1 and 2). The *Rickettsia* spp. in *H. longicornis* from Cangxi County clustered together with other *Rickettsia* isolated from *H. longicornis* collected from several different locations in East Asia. The *Rickettsia* spp. in *H. flava* from Cangxi County clustered with the clade containing *Rickettsia* from *H. qinghaiensis*, and this clade was separate from other known SFG *Rickettsia* spp. (Yang et al. 2016).

Discussion

In this study, we collected four different tick species belonging to the genus *Haemaphysalis* from Cangxi County. The results showed a highly diverse tick species in this region, although *H. concinna*, which were reported to be the cause for the 2005 outbreak, was not found in our collection. It is likely that in the past 10 years, this species may have moved from this area, or simply due to the different collection time warranting a more intensive survey. Currently, *H. longicornis* is the most abundant tick species in this region from this study. Three tick species were collected from the Longquan Village, likely due to its well-conserved ecological environment. Tourists visiting this area should be alerted about tick bites as this village is a popular tourist area.

We report a relatively high percent of *Rickettsia* spp.-positive *H. longicornis* in the Tongan Village. The 58% in our study is higher than 5.5% reported in *H. longicornis* ticks from Zhejiang province (Sun et al. 2015) and 6.9% from Hebei province (Zou et al. 2011). Such high prevalence could be due to our tick collection from a goat farm, where *Rickettsia* spp. could be transferred between ticks in goats and ticks in the Tongan Village. The *Rickettsia* species detected in *H. longicornis* ticks remains unclassified and is not associated with any known rickettsiosis yet. However, visitors to this area should be cautioned due to the high percent of *Rickettsia* spp. that may cause a future public health concern.

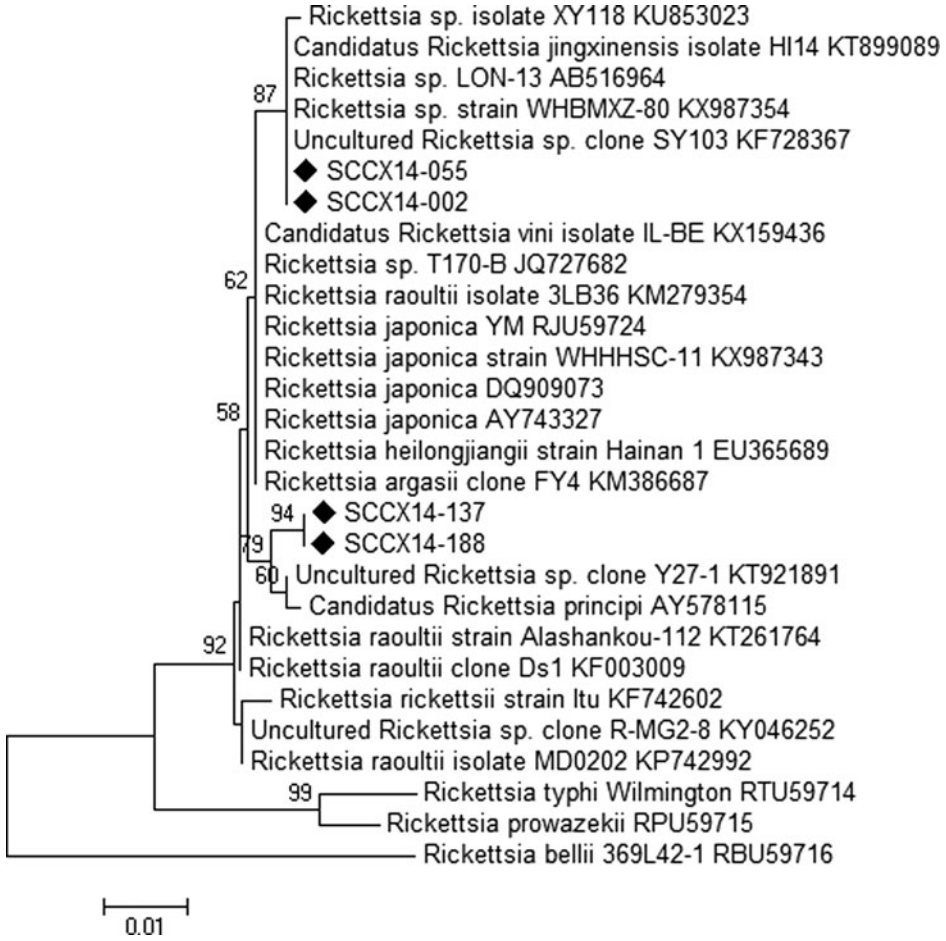

FIG. 1. Phylogenetic analysis of *Rickettsia* spp. in *Haemaphysalis* ticks. The tree was constructed by NJ method based on *glt*A gene sequences. Bootstraps analysis was performed with 1000 replicates. *Rickettsia* spp. MF590724 (SCCX14-002 and SCCX14-055 from *Haemaphysalis longicornis*) and MF590725 (SCCX14-137 and SCCX14-188 from *Haemaphysalis flava*) obtained in this study are denoted with "◆." The sequence uncultured *Rickettsia* sp. clone Y27-1 KT921891 is from *Haemaphysalis qinghaiensis*.

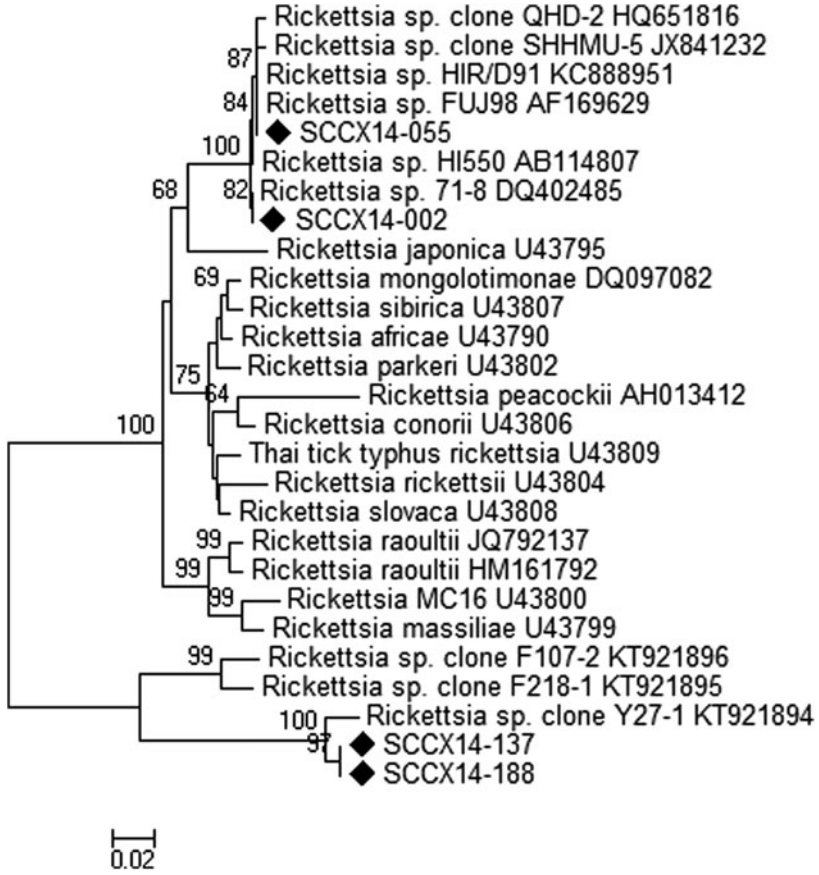

FIG. 2. Phylogenetic analysis of Spotted Fever Group *Rickettsia* spp. in *Haemaphysalis* ticks. This tree was constructed based on ompA gene sequences by NJ method. Bootstraps analysis was performed with 1000 replicates. *Rickettsia* spp. MF590726 (SCCX14-002 and SCCX14-055 from *H. longicornis*) and MF590727 (SCCX14-137 and SCCX14-188 from *Haemaphysalis flava*) obtained in this study are denoted with "◆." The sequences uncultured *Rickettsia* sp. clone Y27-1 KT921894, clone F107-2 KT921 895, and clone F218-1 KT921896 are from *H. qinghaiensis*.

The percent of *Rickettsia* species DNA in *H. flava* was also relatively high. The 27% of positive rate is higher than 1% in *H. flava* reported in Korea (Noh et al. 2017), but similar to *H. qinghaiensis* (18.5%) (Yang et al. 2016).

Based on our results, the *Rickettsia* species from *H. flava* is the most close to a member of the *Candidatus* Rickettsia gannanii subgroup. Both *glt*A and *omp*A phylogenetic trees show this *Rickettsia* species clustered in a clade with ''*Candidatus* Rickettsia gannanii.'' Yang et al. (2016) reported that the novel SFG *Rickettsia* species from *H. qinghaiensis* has three clusters (Fig. 1, *e.g.*, clone Y27-1, F107-2, and F218-1), and that they formed a distinct new subgroup out of SFG, TG, and *R. bellii* in genus *Rickettsia*. The *Rickettsia* species in *H. flava* from Cangxi County is likely a new member of the *Candidatus* Rickettsia gannanii subgroup. The Cangxi County, Sichuan Province, and Gannan District, Gansu Province, are separated by $\sim$500 km. The relationship between the two *Rickettsia* species needs additional investigation.

Conclusions

We detected a high percent of *H. longicornis* and *H. flava* ticks collected from Cangxi County, Sichuan Province, with *Rickettsia* DNA. We have also shown a *Rickettsia* species from *H. flava* and its close relationship with *Candidatus* Rickettsia gannanii subgroup from Gannan, China.

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Author Disclosure Statement

No competing financial interests exist.

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