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Systematic investigation of the *Borrelia miyamotoi* spirochetes in ticks, wildlife and domestic animal hosts in Yunnan province, Southwest China

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ABSTRACT

Background: Borrelia miyamotoi is a spirochete species transmitted via hard ticks. Following its discovery in Japan, this pathogen has been detected around the world, and is increasingly confirmed as a human pathogen causing febrile disease, namely relapsing fever. Its presence has been confirmed in the Northeast China. However, there is little information regarding the presence of *B. miyamotoi* and other hard-tick-borne relapsing fever spirochetes in southern China including Yunnan province, where tick and animal species are abundant and many people both inhabit and visit for recreation.

Methods: For the present study, we collected samples of ticks, wildlife, and domestic animal hosts from different counties in Yunnan province. Nucleic acids from samples were extracted, and the presence of *B. miyamotoi* and other relapsing fever spirochetes was confirmed using polymerase chain reaction (PCR) for the 16S rRNA specific target gene fragment. The positive samples were then amplified for partial genome of the *flaB* and *glpQ* genes. Statistical differences in its distribution were analyzed by SPSS 20 software. Sequence of partial 16S rRNA, *flaB* and *glpQ* genome were analyzed and phylogenetic trees were constructed.

Results: A total of 8260 samples including 2304 ticks, 4120 small mammals and 1836 blood of domestic animal hosts were collected for screening for infection of *B. miyamotoi* and other relapsing fever spirochetes. Cattle and sheep act as the main hosts and *Rhipicephalus microplus, Haemaphysalis nepalensis*, *H. kolonini and Ixodes ovatus* were identified as the important vector host with high prevalence or wide distribution. Only one *Mus caroli* (mouse) and one *Sorex alpinus* (shrew) were confirmed positive for relapsing fever spirochetes. Evidence of vertical transmission in ticks was also confirmed. Two known strains of *B. miyamotoi* and one novel relapsing fever spirochetes, *B. theileri*-like agent, were confirmed and described with their host adaptation, mutation, and potential risk of spreading and spillover for human beings.

Conclusions: Our results provide new evidence of relapsing fever spirochetes in vector and animal hosts in Yunnan province based on large sample sizes, and offer guidance on further investigation, surveillance and monitoring of this pathogen.

1. Background

Relapsing fever is a febrile disease caused by the *Borrelia* spirochetes. It can be divided into three groups based on the disease vector: soft-tick-borne relapsing fever (STBRF), hard-tick-borne relapsing fever (HTBRF), louse-borne relapsing fever (LBRF) and avian relapsing fever [1]. *Borrelia* has been identified in the Americas, Europe, Asia and Africa in reservoir hosts and vectors, and human *Borrelia* infection has been reported, with the relative disease described [2]. Most tick-borne relapsing fever pathogens are transmitted via soft tick, including *B. duttoni*,

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B. hermsii, *B. turicatae*, *B. parkeri* [3]. However, it has been noted that the vector of *B. miyamotoi* is the hard tick [4]. Moreover, in China, diverse species of hard ticks are present and multiple human pathogens are transmitted via hard ticks [5]. *B. miyamotoi* has been discovered in *Ixodes persulcatus*, *I. ovatus*, *Haemaphysalis conncina* and *H. longicornis* in China [6]. Thus, it is important to study *B. miyamotoi* and expand research regarding this pathogen.

B. miyamotoi belongs to the class Spirochaetia, order Spirochaetales, family Borreliaceae and genus *Borrelia* [7]. It can be transmitted in ticks both horizontally and transovarially (vertically) [8]. Interestingly, an experimental study found that the transovarial transmission of *B. miyamotoi* in hard ticks is much more efficient than horizontal transmission [9], indicating the primary role in the natural history of the pathogen. This spirochete was firstly discovered in *I. persulcatus* in Japan [10], and was subsequently found in ticks in North America, Asia and Europe [11]. Based on the phylogenetic tree, known *B. miyamotoi* species comprise three genotypes: Asian or Siberian, American, and European, all of which have been found to be pathogenic to humans [12]. In addition to the identification of *B. miyamotoi* within its vectors, human cases were identified in China, the United States, west Russia, and Japan [13–16]. Common symptoms of *B. miyamotoi* infection include fever, fatigue, and headache, but only some of them manifest relapsing febrile disease. Other clinical manifestations in patients include chills, myalgia, arthralgia, lymphadenopathy, and possible erythema migrans. Laboratory findings have also included leukopenia, thrombocytopenia, and elevated liver enzymes. [17] Notably, in immunocompromised patients, prolonged course of disease, vision and hearing changes, meningoencephlitis were recorded in some case reports [18–20].

Over the past three years, an increasing number of *B. miyamotoi* in ixodid ticks has been identified in many countries including the Czech Republic, Egypt, and Austria [21–23]. There has also been an increase in human infections in France, Austria, and Inner Mongolia Autonomous Region of China [24–26]. Therefore, it is highly likely that the distribution of this pathogen covers a wider range, resulting in a public health burden that is more serious than initially believed. While human cases have only been discovered in Northeast China, *B. miyamotoi* has been detected in other parts of the country, indicating that more studies should be conducted in other Chinese provinces.

Yunnan is a southwestern province in China that is famous for its diversity in species. Moreover, with 47 ticks species and 187 species of small mammals in Yunnan [27,28], it is a hot spot for zoonotic disease. A recent study found that the prevalence of *Borrelia* relapsing fever is highest in ticks in southwestern China, reaching 5.58% [29]. This highlights the need for a detailed study on its prevalence in different counties to uncover the risks of disease spillover.

This study focuses on describing the prevalence of *B. miyamotoi* and other potential hard-tick-borne relapsing fever spirochetes in ticks, wildlife (small mammals) and domestic animal hosts (cattle, sheep, dogs, horses, donkeys). It also looks at the molecular relationship of relapsing fever spirochetes with different origins to reveal the transmission route and provide evidence of potential vertical transmission. This information can be applied to the targeted prevention of relapsing fever caused by relapsing fever spirochetes.

2. Method

2.1. Study sites and sample collection

Geographical characteristics were considered, and samples were collected from ticks, wildlife, and domestic animal hosts in 12, 29 and 15 counties in Yunnan province, respectively (Fig. 1(a)). Domestic animal host samples were collected using EDTA anticoagulant vacuum blood collection tubes. Small mammals were trapped in different habitats using traps laid out overnight. After morphological identification, liver and spleen tissue samples were acquired in the field and stored in a liquid nitrogen tank. Then samples were brought back to the laboratory and stored in a freezer set at −80 °C for later testing. The unfed ticks in their natural habitat were collected using the flag method and parasitic ticks on the skin of the domestic hosts were collected with sterilized tweezers [30]. When collecting samples, basic information including the collection date, location, habitat, altitude, and parasitic status was recorded for data analysis. The habitats of unfed and parasitic ticks were classified as broad-leaved forest, mixed forest, artificial area (farms and roadside), bush, grassland, and arid valleys. In addition, adult, engorged and lymph ticks collected from domestic animal hosts in 6 counties in Yunnan province were used for the tick-borne spirochete culture.

2.2. Pathogen isolation

The collected ticks were soaked in 75% ethanol for 10 min and then washed three times with sterile phosphate buffer saline (PBS). Engorged ticks were kept in an artificial climate cabinet under the condition of 26 °C, 85% humidity and 16 h daylight. Once the eggs were laid and hatched, larvae ticks were acquired. Tick samples at different life stages were grouped, with a group comprising either 1–15 ticks or approximately 100 eggs. Afterwards, samples were ground and homogenized. They were then inoculated onto a BSK-II medium for culturing and isolation of the spirochete at 31 °C. Each week, a small amount of supernatant from each sample was observed under dark-field microscopy. DNA was also extracted, and subsequent sequencing was conducted for the *B. miyamotoi* 16S rRNA gene.

2.3. Nucleic acid extraction

Nucleic acid was extracted from the domestic host blood samples and small mammal tissue samples in accordance with the instructions of the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China, Cat. No. 4992199). The nucleic acid of tick samples and the supernatant of cultured cells were extracted in accordance with the instructions of the DNeasy Blood & Tissue Kit (Qiagen, USA, Cat. No. 69504), and the extracted DNA was stored in the freezer set at −80 °C for later use.

2.4. Gene amplification

The common primers targeting the 16S rRNA specific target gene fragment in relapsing fever spirochetes were used for screening by PCR. The positive samples were then tested for *flaB* gene and *glpQ* gene. The used primers, amplification conditions and references are shown in Table A.1.

2.5. Sequencing and data analysis

The positive products were directly sent to Sangon Bioetech (Shanghai) Co., Ltd. for sequencing, and the sequences were successfully assembled using the CLC genomics workbench. Sequence comparisons were performed using the Basic Local Alignment Search Tool (BLAST) in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). MEGA 11 software was used to construct a phylogenetic tree for analysis. After the experimental data and basic data were prepared, statistical analysis was carried out using SPSS 20.0.

3. Results

3.1. Prevalence of relapsing fever spirochetes in ticks, wildlife, and domestic animal hosts

A total of 8260 samples including 2304 ticks, 4120 small mammals and 1836 blood of domestic animal hosts were collected for screening of *B. miyamotoi* and other relapsing fever spirochetes. The distribution of samples collected and the positive samples by species are shown in Fig. 1 (b). A total of 1354 individual ticks were homogenized and screened one by one. 6 out of 12 (50.0%) sample collection counties were detected

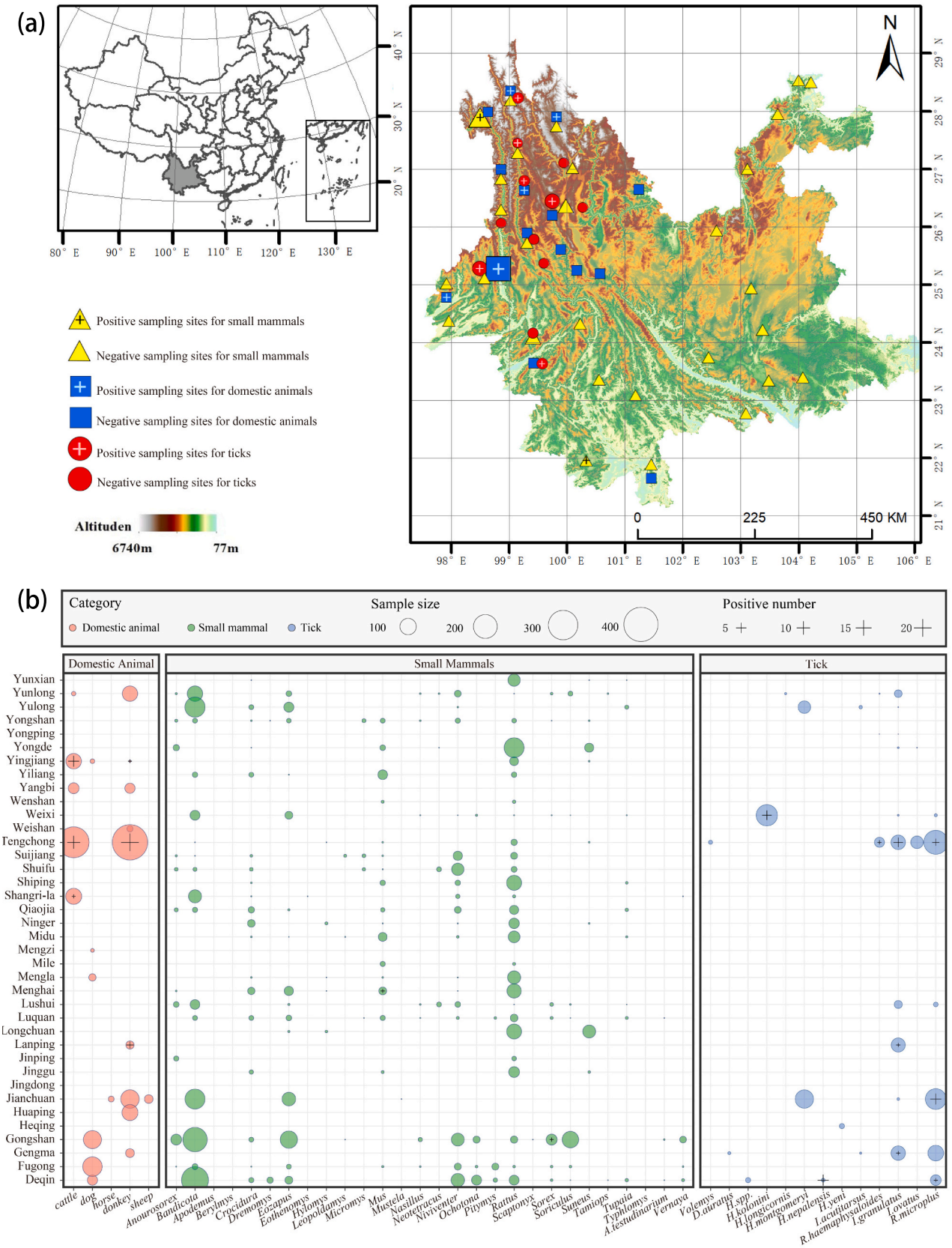

Fig. 1. Distribution of tick, wildlife and domestic animal samples collected.

(a) Distribution of different types of samples and positive samples in different counties in Yunnan province. The bigger the size of symbols, the larger the number of samples. (b) Distribution of samples in different species in different counties in Yunnan province.

with relapsing fever spirochetes, with the highest positivity rates being 10% (7/70) in Deqin County (Table 1). The ticks belong to 13 species, of which 26 ticks (1.92%) tested positive, including 5 species of *H. nepalensis*, *H. kolonini*, *Rhipicephalus microplus*, *I. ovatus* and *I. granulatus* with a significant prevalence difference of 66.67% (6/9), 2.45% (4/163), 1.74% (9/518), 1.96% (6/306) and 2.22% (1/45), respectively ($P < 0.001$) (Table 2).

As for the epidemiological information of ticks, there were significant differences in the positivity rates of ticks carrying relapsing fever spirochetes in different counties ($P < 0.001$) (Table 1), tick species (Table 2), life stage ($P < 0.05$) and habitat ($P < 0.001$) (Table 3).

Small mammals, belonging to 59 species of 28 genera, 11 families and 5 orders were captured in 29 counties (Table A.2). One *Mus caroli* (mouse) in Menghai County (0.57%) and one *Sorex alpinus* (shrew) in Gongshan County (0.15%) were confirmed positive for relapsing fever spirochetes (Table 1).

Blood samples were collected from 5 species of domestic animal hosts, including cattle, sheep, dogs, horses, and donkeys in 15 counties in Yunnan. As shown in Table 4, the total positivity rate was 2.12% (39/1836), with 2.66% (23/866) for sheep, 2.78% (16/575) for cattle based on 16S rRNA specific target gene fragment. There were significant differences among the infection rates of different species of domestic hosts ($P < 0.05$). The samples from 4 out of 15 counties tested positive, with significantly different positivity rates ($P < 0.001$) in Yingjiang (6.54%), Tengchong (3.83%), Lanping (6.90%), and Shangri-La (1.04%) (Table 1).

3.2. Isolation and culture of tick-borne spirochetes

Of all the 2304 collected ticks, the 950 samples, including 902 unfed ticks and 48 engorged ticks, were used for culture of relapsing fever spirochetes. The unfed tick samples, including 855 adult ticks and 47 nymphs of 5 species, were subsequently divided into 204 and 8 pools, respectively, which were collected from 6 counties. From the engorged ticks, 41 egg pools and 7 hatched larvae tick pools were acquired and used for inoculation after homogenization (Table 5). A total of 260 pools of tick samples were inoculated into BSK-II cultures. After culturing and observing for three months, the growth of spirochete was not observed morphologically. However, by screening for 16S rRNA specific target gene fragment, a total of 24 (9.23%) cultured tick samples were positive for relapsing fever spirochetes, including 22 pools of adult ticks, one pool of larvae ticks and one pool of tick eggs, with positivity rates of 10.78% (22/182), 12.5% (1/7), and 2.44% (1/40), respectively. All the positive samples were *R. microplus* and had a positivity rate of 13.41% (24/179).

Table 1
Prevalence of relapsing fever spirochetes in different counties.*

County	Positive/Total number (Positiviry rate (%))				
	Domestic animals	Small mammals	Ticks detected	Pool ticks cultred	Total
Deqin	0/43 (0)	0/509 (0)	7/70 (10.00)	0/7 (0)	7/629 (1.11)
Fugong	0/138 (0)	0/119 (0)	–	–	0/257 (0)
Gengma	0/33 (0)	0	1/184 (0.54)	0/22 (0)	1/239 (0.42)
Gongshan	0/120 (0)	1/686 (0.15)	–	–	1/806 (0.12)
Heqing	–	0	0/14 (0)	–	0/14 (0)
Huaping	0/96 (0)	0	–	–	0/96 (0)
Jingdong	–	0	–	0/7 (0)	0/7 (0)
Jianchuan	0/174 (0)	0/221 (0)	6/284 (2.11)	0/18 (0)	6/697 (0.86)
Jinggu	–	0/76 (0)	–	–	0/76 (0)
Jinping	–	0/27 (0)	–	–	0/27 (0)
Lanping	2/29 (6.90)	0	1/76 (1.32)	–	3/105 (2.86)
Longchuan	–	0/167 (0)	–	–	0/167 (0)
Luquan	–	0/120 (0)	–	–	0/120 (0)
Lushui	–	0/110 (0)	0/40 (0)	–	0/150 (0)
Menghai	–	1/175 (0.57)	–	–	1/175 (0.57)
Mengla	0/24 (0)	0/81 (0)	–	–	0/105 (0)
Mengzi	–	0/22 (0)	–	–	0/22 (0)
Midu	0/9 (0)	0	–	–	0/9 (0)
Mile	–	0/110 (0)	–	–	0/110 (0)
Ninger	–	0/88 (0)	–	–	0/88 (0)
Qiaojia	–	0/127 (0)	–	–	0/127 (0)
Shangri-la	1/96 (1.04)	0/94 (0)	–	–	1/190 (0.53)
Shiping	–	0/127 (0)	–	–	0/127 (0)
Shuifu	–	0/129 (0)	–	–	0/129 (0)
Suijiang	–	0/93 (0)	–	–	0/93 (0)
Tengchong	29/758 (3.83)	0/35 (0)	7/398 (1.76)	24/173 (13.87)	60/1364 (4.39)
Weishan	0/18 (0)	–	–	–	0/18 (0)
Weixi	–	0/92 (0)	4/174 (2.30)	–	4/266 (1.50)
Wenshan	–	0/17 (0)	–	–	0/17 (0)
Yangbi	0/91 (0)	–	–	–	0/91 (0)
Yiliang	–	0/86 (0)	–	–	0/86 (0)
Yingjiang	7/107 (6.54)	0/37 (0)	–	–	7/144 (4.87)
Yongde	–	0/221 (0)	0/6 (0)	–	0/227 (0)
Yongping	–	–	0/2 (0)	–	0/2 (0)
Yongshan	–	0/97 (0)	–	–	0/97 (0)
Yulong	–	0/220 (0)	0/72 (0)	0/33 (0)	0/325 (0)
Yunlong	0/100 (0)	0/166 (0)	0/34 (0)	–	0/300 (0)
Yunxian	–	0/68 (0)	–	–	0/68 (0)
Total	39/1836 (2.12)	2/4120 (0.05)	26/1354 (1.92)	24/260 (9.23)	91/7570 (1.20)

* Fisher's exact method result of positivity rates of ticks at different counties: $P < 0.001$.

Table 2
Prevalence of Relapsing Fever Spirochetes in individual ticks of different species.

Tick species*	Positive number	Negative number	Total	Positivity rate (%)
Haemaphysalis nepalensis	6	3	9	66.67
Haemaphysalis kolonini	4	159	163	2.45
Haemaphysalis longicornis	0	5	5	0
Haemaphysalis yeni	0	14	14	0
Haemaphysalis montgomeryi	0	185	185	0
Haemaphysalis spp.	0	14	14	0
Rhipicephalus microplus	9	509	518	1.74
Rhipicephalus haemaphysaloides	0	64	64	0
Ixodes ovatus	6	300	306	1.96
Ixodes acutitarsus	0	14	14	0
Ixodes granulatus	1	44	45	2.22
Amblyomma testudinarium	0	10	10	0
Dermacentor auratus	0	7	7	0
Total	26	1328	1354	1.92

* Fisher's exact method result of positivity rates of different tick species: $P < 0.001$.

Table 3
The Positivity rate of ticks of different sex, parasitic status, hosts and altitudes.

Variable	Positive	Negative	Positivity Rate (%)	Chi-square Value	*P*
Life Stage					
Larva	0	1	0		0.002[a]
Lymph	7	75	8.54		
Adult	22	1249	1.73		
Sex					
Male	5	462	1.08	1.885	0.170
Female	17	788	2.11		
Habitats					
Broad-leaved Forest	13	584	2.18	85.807	<0.001
Mixed Forest	7	503	1.37		
Artificial Area	1	91	1.09		
Bush	1	75	1.32		
Grassland	6	12	33.33		
Arid Valleys	1	60	1.64		
Parasitic Status					
Unfed	6	368	1.60	0.712	0.399
Parasitic	23	957	2.35		
Hosts					
Domestic Animal	22	904	2.38	0.61	1.000
Small Mammal	1	53	1.85		
Altitudes					
<1500	1	87	1.14	4.315	0.109
1500–2500	8	582	1.36		
>2500	20	656	2.96		

[a] Fisher's exact method result.

Table 4
Prevalence of Relapsing Fever Spirochetes in domestic animals.

Source*	Location#	Sample size	Positive number	Positivity rate(%)
Cattle	Yingjiang	90	6	6.67
	Tengchong	330	9	2.73
	Shangri-La	96	1	1.04
	Yunlong	11	0	0
	Yangbi	48	0	0
	Total	575	16	2.78
Sheep	Yingjiang	5	1	20
	Lanping	29	2	6.90
	Weishan	18	0	0
	Tengchong	428	20	4.67
	Yunlong	89	0	0
	Jianchuan	125	0	0
	Huangping	96	0	0
	Yangbi	43	0	0
	Gengma	33	0	0
	Total	866	23	2.66
Dog	Deqing	43	0	0
	Yingjiang	12	0	0
	Fugong	138	0	0
	Gongshan	120	0	0
	Mengla	24	0	0
	Midu	9	0	0
	Total	346	0	0
Horse	Jianchuan	32	0	0
Donkey	Jianchuan	17	0	0
Total		1836	39	2.12

* Chi-square test result of positivity rates of different sources: $\chi^2 = 10.949$, $P = 0.038$.

Fisher's exact method result of positivity rates of different locations: $P < 0.001$.

3.3. Sequence and phylogenetic analysis

3.3.1. 16S rRNA specific target gene fragment for screening

91 sequences (353 bp) of 16S rRNA specific target gene fragment were obtained, with identities of 95.80%–100%. The positive samples were mainly distributed in Tengchong and Yingjiang in western Yunnan province. The main vector host was *R. microplus*, while *Ixodes* and *Haemaphysalis* ticks also harbor relapsing fever spirochetes. The domestic animal hosts were cattle and sheep. However, dogs and donkeys were not found to carry relapsing fever spirochetes. Representative sequences were selected for BLAST, and the sequences represented by LP-T-18 from tick sources were most similar to *B. miyamotoi* from the strain detected in human blood in Russia (CP036726.1) and *I. ovatus* in Japan (LC164122.1), which shows 100% identity. Those from domestic animals, represented by DQ-B-45, have 100% identity to *B. theileri* from a sheep in Egypt (MN621894.1). The sequence from a small mammal (GS-653) shares 98.30% identity with *Borrelia.* sp. A126 (MW889882.1) from a parasitic tick on a pangolin in China, while that of another small mammal (MH-60) is 100% identical with *B. miyamotoi* (CP021872.1) from *I. pacificus* in the United States.

The phylogenetic tree shows that the sequences of 16S rRNA specific target gene fragments falls into three clades (Fig. 2(a)), among which Clade 1, represented by WX-T-58, was *B. miyamotoi* strain, which could be further divided into the American strain that was closest to the one found in the United States and Siberian strains closest to those found in Northeast China and Inner Mongolia of China, and Japan. Clade 2 is close to *B. theileri*, which can be further divided into three branches: *B. theileri*-like strain, *Borrelia.* sp. *HM*-like strain and *Borrelia* sp. strain. Clade 3 consists of a sample collected from a shrew in Gongshan, which falls in the same clade with *B. javanense* previously found from a pangolin in China.

3.4. *flaB* and *glpQ* gene

A total number of 28 partial *flaB* genome (506 bp) sequences were obtained from 12 domestic animals, 1 small mammal, 3 ticks and 12 tick cultures, which share 92.43%–100% identity. The BLAST result shows the acquired sequences were most identical with *B. theileri* (ON113497.1) from cattle blood in Brazil with 100% identity. The results of the phylogenetic tree showed that the samples from domestic animals, ticks and tick cultures fall into one clade, and divided into two subclades (Fig. 2(b)). The YJ-B-34 and other sequences in the same subclade with *B. theileri*, but TC-B-291 shows 1 base difference from them. The other subclade represented by C-TC-T-53 is in an isolated branch with no known sequence. It is worth noting that the *flaB* sequence of MH-60 from a small mammal in Menghai was in the same clade with *B. miyamotoi* European strain, and 99.59% identical with *B. miyamotoi* isolated from *I. ricinus* in Czech Republic, with 2 bases different.

26 partial *glpQ* genome sequences (461 bp) were obtained from domestic animals, ticks, and tick cultures, which were 5, 3, and 17, respectively. The identity among sequences was 88.44%–100%, and the samples were from ticks, tick cultures or domestic animals in Tengchong, Jianchuan and Yingjiang. It was found by BLAST that those sequences were closest to *B. theileri* (MG601738.1). The highest identity was 99.78%, with 1 base difference. Phylogenetic analysis showed that all the *glpQ* gene sequences from ticks and domestic animals were in one single clade with a *B. theileri* in Brazil (MG601738.1) (Fig. 2(c)).

3.5. Vertical transmission of Borrelia in ticks

The partial 16S rRNA genome sequences of an engorged tick (TC-T-53) and its eggs (TC-T-83) were 100% identical, and the partial functional *glpQ* gene sequences were also 100% identical, suggesting the vertical transmission of this relapsing fever spirochete.

4. Discussion

The present study described the wide distribution of a variety species of relapsing fever spirochetes in Yunnan province. We also identified their main hosts, vectors, and vertical transmission among ticks, as well as potential risk of spreading and spillover, which offer guidance on

Table 5
Pools of ticks from different counties used for culturing.

County	*Rhipicephalus microplus*				*Ixodes ovatus*		*Ixodes granulatus*	*Haemaphysalis montgomeryi*	*Ixodes acutitarsus*	Total
	Adult	Nymph	Larva	Egg	Adult	Egg	Adult	Adult	Adult	
Tengchong	105[a]	2[a]	0	35[a]	25	6	0	0	0	173
Yulong	0	0	0	0	0	0	0	28	5	33
Gengma	5	2	0	0	13	0	2	0	0	22
Jianchuan	13	4	0	0	0	0	0	1	0	18
Jingdong	6	0	0	0	0	0	1	0	0	7
Deqing	0	0	7	0	0	0	0	0	0	7
Total	129	8	7	35	38	6	3	29	5	260

[a] Among which 22 adult ticks, 1 larva tick and one tick egg pools were positive.

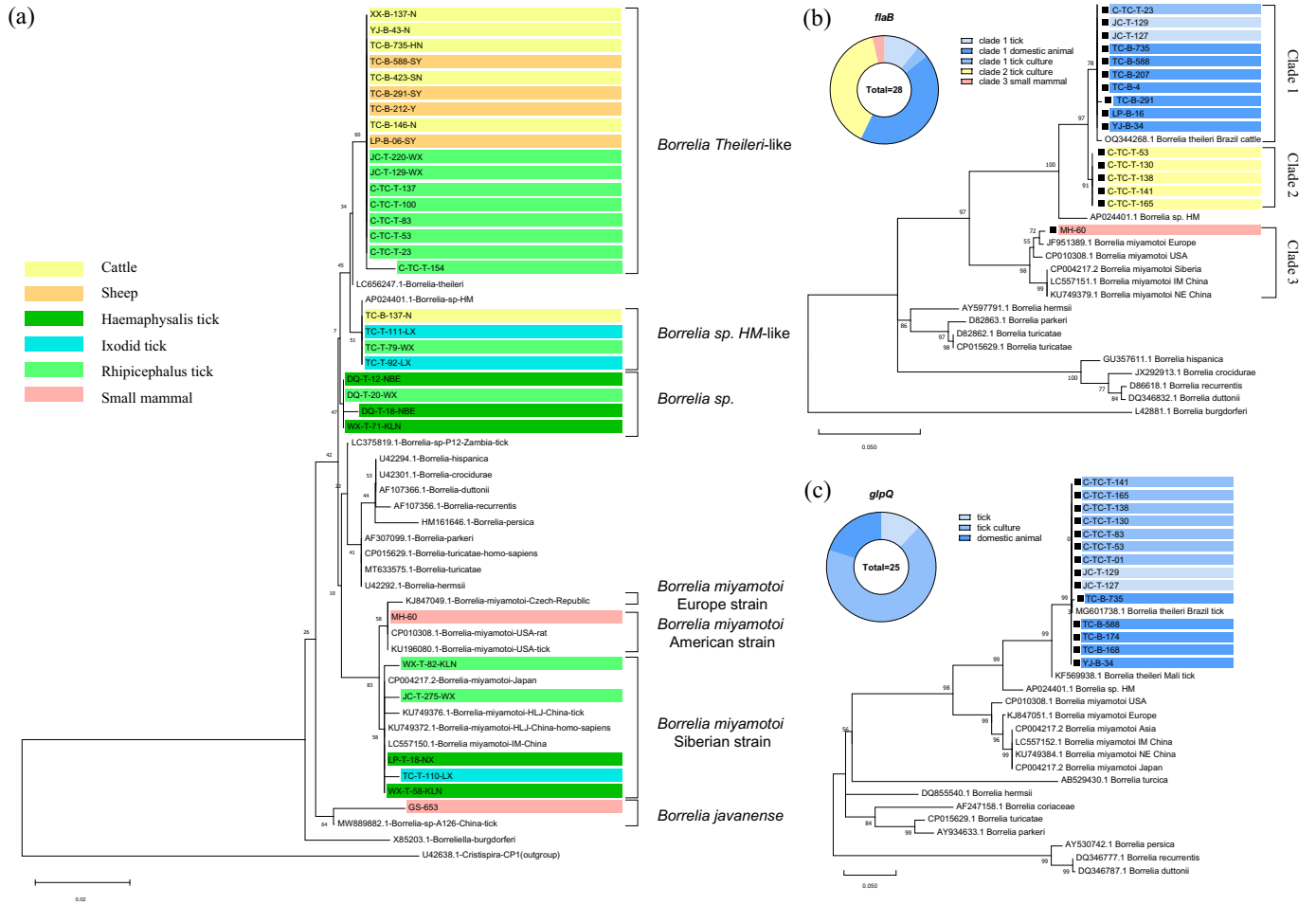

Fig. 2. Phylogenetic trees of 16S rRNA specific target screening fragment, partial *flaB* and partial *glpQ* genome.

(b) Phylogenetic tree of 16S rRNA specific target screening fragment. Different colors represent different types of samples. (b) Pie chart of *flaB* genome from different orignins and phylogenetic tree of partial *flaB* genome. Names start with black squares represent samples acquired in this study. (c) Pie chart of *glpQ* genome from different orignins and phylogenetic tree of partial *glpQ* genome. Names start with black squares represent samples acquired in this study.

further investigation and prevention of these pathogens.

Since its first discovery of *B. miyamotoi* in Japan in 1995 [10], this pathogen and other relapsing fever spirochetes have been widely studied, demonstrating ticks as the primary vector for this group of pathogens. The main vectors of *B. miyamotoi* differ in different countries, such as *I. scapularis* and *I. pacificus* in the United States, *I. ricinus* in Europe, *I. persulcatus*, *I. ovatus, H. concinna* and *H. longicornis* in China, *I. persulcatus* and *I. pavlovski* in Japan, *I. ricinus* and *I. persulcatus* in Russia [6]. An increasing number of *B. miyamotoi* strains in ixodid ticks have been identified in many countries including the Czech Republic, Egypt, and Austria [21–23]. Here, our study showed that *R. microplus*, *I. ovatus*, *H. nepalensis* and *H. kolonini* may be novel vectors of relapsing fever spirochetes in Yunnan province. In addition, a higher positivity rate was observed in cultures inoculated with homogenized *R. microplus*. Interestingly, *B. theileri*-like was detected positive in cultures of samples from an adult *R. microplus* tick and its eggs, showing the vertical transmission of relapsing fever spirochetes, which is consistent with the previous finding by Geller et al. [31]. However, we failed to isolate the spirochetes with traditional BSK-II medium [10], which is now considered not optimal for culturing relapsing fever spirochetes. A modified BSK-R should be a better choice according to a recent study [32]. Regardless, *R. microplus* could be considered as the main and important vector host of relapsing fever spirochetes in Yunnan province and *Ixodes* and *Haemaphysalis* ticks should not be ignored. As relapsing fever

spirochetes can be transmitted to humans through tick bites, the risk of tick-borne relapsing fever in humans should be highly concerned.

Little information on the natural hosts of relapsing fever spirochetes is available, with limited studies confirming that the main natural reservoir is rodents, but rarely found in other mammals [33]. During our investigation of a large number ($n = 4120$) of small mammals including rodents, insectivores, rabbits, climbing shrews and carnivores, we detected only two small mammals infected with relapsing fever spirochetes, which is inconsistent with previous the report that *B. miyamotoi* may use small rodents as reservoirs [33]. It is also different from our previous reports that small mammals are the main reservoir hosts of Lyme disease spirochetes in Yunnan province [34]. Moreover, our study demonstrates that sheep and cattle may be the main natural host of relapsing fever spirochetes with high prevalence. Our findings reflect different spirochete positivity rates of hosts in different regions. As positive domestic animals are widely distributed in certain human-populated areas and tourist destinations in Yunnan province, there is an increased chance of contact with them, increasing risk of pathogen spillover.

Among our sampling counties, Tengchong shows the highest positive number (Table 1). Among the relapsing fever spirochetes detected, *B. theileri*-like was the dominant species (66/91, 72.53%), also mostly distributed in Tengchong (50/66, 75.76%) (Table A.3), indicating that the *Borrelia theileri*-like relapsing fever spirochetes show regional clustering in Tengchong in western Yunnan province. On the contrary, there were only 7 *B. miyamotoi*, a pathogen of human victims, out of 91 relapsing fever spirochetes detected (Table A.3), scattered in Jianchuan, Lanping, Menghai, Tengchong and Weixi, mainly in northwestern Yunnan province. Therefore, the distribution patterns of *B. theileri* and *B. miyamotoi* are different, suggesting different risks of transmission of relapsing fever spirochetes among hosts and vectors.

Two different strains of *B. miyamotoi* were discovered in this study based on 16S rRNA. One was the Siberian strain, which was all from ticks, and the other was the American strain, which was all from small mammals. No domestic animal was found to be infected with *B. miyamotoi*. One small mammal sample (MH-60) was confirmed to carry *B. miyamotoi* of American strain based on 16S rRNA. For *B. theileri*, based on *flaB* gene, they were divided into two subclades, clade 1 was originated from domestic animals, clade 2 was all from ticks (Fig. 2). These results indicates the host or vector tropism of different relapsing fever spirochetes.

There has also been an increase in human infections of *B. miyamotoi* in France, Austria, and China [24–26]. A recent study has mapped human relapsing fever spirochetes in the world [35]. However, besides northeastern China, no human relapsing fever spirochetes case has been reported in other provinces including Yunnan province. *B. theileri* was not found to be a human pathogen, but our study shows its wide distribution in vectors and hosts in Yunnan province and *B. miyamotoi*, the most common relapsing fever pathogen, is found among ticks, and domestic animals in Yunnan province, consolidating the importance of consistent active surveillance of relapsing fever spirochetes.

5. Conclusions

In conclusion, our results provide new evidence on genetic diversity, wide distribution, and potential risk of spillover of relapsing fever spirochetes in vector, wild and domestic animal hosts in Yunnan province based on large sample sizes. It offers guidance on further investigation, surveillance and monitoring of these pathogens.

Author contributions

CD and JJ conceived and designed the experiments. MY, YS and BJ performed the experiments. JY, YZ, LH, RX and CL analyzed the data. HZ, SZ, PE, WF, BS, LZ, DC, LM implemented sample collections. JJ and JY drafted and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Ethics approval and consent to participate

The research protocol for trapping wild small animals and collecting samples was approved by the Animal Subjects Research Review Boards at the Yunnan Institute of Endemic Diseases Control and Prevention (2017–001), in accordance with the medical research regulations of China and the Regulation of the People's Republic of China for the Implementation of the Protection of Terrestrial Wildlife.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Chun-Hong Du: Investigation, Resources, Methodology, Project administration, Supervision. **Ji-Hu Yang:** Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Validation. **Ming-Guo Yao:** Data curation, Investigation. **Bao-Gui Jiang:** Data curation, Investigation. **Yun Zhang:** Data curation, Formal analysis. **Zhi-Hai He:** Resources. **Rong Xiang:** Data curation, Formal analysis. **Zong-Ti Shao:** Resources. **Chun-Feng Luo:** Conceptualization, Project administration, Supervision, Methodology, Resources. **En-Nian Pu:** Resources. **Lin Huang:** Data curation, Formal analysis. **Yu-Qiong Li:** Resources. **Fan Wang:** Resources. **Shuang-Shuang Bie:** Resources. **Zhi Luo:** Resources. **Chao-Bo Du:** Resources. **Jie Zhao:** Resources. **Miao Li:** Resources. **Yi Sun:** Methodology, Data curation, Investigation. **Jia-Fu Jiang:** Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2024.100735.

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