

A broad-range survey of *Borrelia burgdorferi sensu lato* infection in small mammals in Sino-Burmese border area, Yunnan Province, China

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Research

Keywords: Lyme disease, *Borrelia burgdorferi* sensu lato, Small mammals, China

Posted Date: March 5th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-16141/v1>

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Abstract

Background: Lyme disease is caused by *Borrelia burgdorferi sensu lato* which is usually found in wild and domestic mammals worldwide. In China, Human cases of *B. burgdorferi* infections have been identified in a wide distribution, but little direct surveillance of potential rodent reservoirs has been performed in Yunnan Province, the tropical region, Sino-Burmese border area, southwestern China. Here we report a thoroughly investigation of *B. burgdorferi sensu lato* in small mammals collected in 2011 to 2016 in the region.

Methods: A nested PCR was done for the 5S-23S rRNA intergenic spacer gene of *Borrelia burgdorferi sensu lato*. The PCR-positive amplicons were directly sequenced. Sequence analysis was carried out using a FASTA search on the Genbank database, with phylogenetic trees constructed using MEGA software, version 6.06. Statistical Analysis were conducted using SPSS.version 17.0.

Results: Totally 57 species, 3659 mammals were captured at 159 sample sites located in 23 counties in Yunnan Province. Thirty species, 146 (3.99%) mammals were tested positive for *B. burgdorferi s.l.* by nested PCR based on 5S-23S rRNA intergenic spacer gene. 20 species mammals were first reported infected with *B. burgdorferi s.l.* Sequence analysis revealed that five genotypes of *B. burgdorferi s.l.* were detected, including *B. afzelii* , *B. burgdorferi sensu stricto*, *B. japonica* , *B. garinii* and *B. valaisiana*.

Conclusions: Significant differences in prevalence rates of *B. burgdorferi s.l.* were observed at varying landscape types and altitudes. Small mammals in forested areas had higher prevalence rates than other landscape types as did small mammals found at altitudes greater than 2500 meters. The 5S-23S rRNA intergenic spacer gene revealed that there were five genotypes of *B. burgdorferi s.l.* detected, indicating high genetic diversity within this province.

Background

Lyme borreliosis (LB) is the most commonly reported vector-borne disease across Europe, North America and Asia [1–4]. The causative agents of LB fall within the species complex *B. burgdorferi sensu lato* (BBSL), and were responsible for a wide spectrum of clinical symptoms. While there have been documented reports of human cases of Lyme disease in southwestern China [4], the only information on the prevalence of BBSL in rodent reservoirs came from one study, where a majority of rodents were trapped indoors [5]. IFA was reported to detect BBSL infection in rodents and humans from Yunnan. Yunnan Province is of particular interest given its wide topographic range and high level of small mammal biodiversity, many of which may potential reservoirs for BBSL. We performed a broad-range, systematic field investigation on the prevalence of BBSL infections in rodents sampled in 23 counties in Yunnan Province, and then analyzed the distribution and genetic diversity of the pathogen, as well as the association between infections and suspected risk factors. This study aims to evaluate the role that small mammals play in the transmission of BBSL across Yunnan Province.

Methods

Collection of Small Mammal Samples

From 2011 to 2016, small mammals were captured using animal snap traps set at agricultural, forested, and residential areas at 159 sample sites from 23 counties ranging from 530 to 4300m altitude in Yunnan Province (Figure 1). Two hundred snap traps per sample site were placed for three consecutive nights and checked daily. Mammal species were identified according to external morphology, fur color, measurements and visible characters of dentition. Each animal's sex, developmental stage, and location were recorded at the time of sample processing. After identification of species, spleen tissues were removed from the animals and stored in liquid nitrogen until tested. For unidentified species in the field, the craniums were brought to the laboratory for further identification.

DNA Extraction and PCR Analysis

DNA was extracted from spleen tissue using the DNA blood and tissue kit (Tiangen Biotechnology, Beijing, China) according to the manufacturer's instruction. A nested PCR for the 5S-23S rRNA intergenic spacer gene of BBSL was done as previously described [6]. The PCR-positive amplicons were directly sequenced with an automated DNA sequencer (ABI PRISM 373; Perkin-Elmer, Norwalk, CT). Sequence analysis was carried out using a FASTA search on the Genbank database, with phylogenetic trees constructed using MEGA software, version 6.06 [7]. The 5S-23S rRNA intergenic spacer gene of BBSL obtained in this study was deposited in Genbank under accession numbers MK333406-MK33427 and KP677523.1 respectively.

Statistical Analysis

Univariate analysis was used to assess the association between gender, developmental stage of rodents, environmental landscape, altitude, and testing positive for *B. burgdorferi* s.l. using a chi-square test. All variables with a *P*-value of <0.05 from univariate analysis were entered into a multivariate forward stepwise logistic regression analysis. All analyses were conducted using SPSS (version 17.0, SPSS Inc. Chicago, IL).

Results

A total of 3659 small mammals belonging to 57 species were collected (Table 1). The *Apodemus draco* was the most common species (15.82%, 579/3659), followed by *Rattus tanezumi* (15.66%, 573/3659). A total of 146 (3.99%) rodents were tested positive for BBSL, with *Ochotona gloveri* (33.33%, 1/3), *Sorex cylindricauda* (14.28%, 7/49), *Soriculus leucops* (14.94%, 13/87), and *Rattus tueckestanicus* (14.28%, 1/7) actively infected with BBSL. The positive mammals originated from 14 out of 23 sample counties (Figure 1), including Deqin, Weixi, Yulong, Gongshan, Fugong, Jinggu, Tengchong, Yongde, Menghai, Yunxian, Shiping, Mile, Yiliang and Yunlong, with Gongshan (S1) having the highest prevalence (8.58%), followed by Deqin (S2, 7.85%), and Yiliang (S16, 6.38%). The prevalence of BBSL in small mammals in forested landscapes, agricultural landscapes and residential landscapes were 5.19%, 3.14% and 0.63%,

respectively ($\chi^2=14.945$, $p=0.001$) (Table 2). There was significant difference in prevalence of BBSL in small mammals at the altitude classes of <1500 meters, 1500-2500 meters, >2500 meters with 0.80%, 2.92% and 5.86%, respectively ($\chi^2=43.089$, $p=0.001$). The multivariate logistic regression analysis also revealed that samples found at altitudes greater than 1500 meters and in agricultural landscapes were more likely to be infected with BBSL, OR (95%CI) 4.524, $p < 0.01$.

Sequencing was successful for all 146 positives amplicons samples. The comparative analysis with the BLAST program revealed that 83 samples were *B. afzelii*, 27 were *B. burgdorferi sensu stricto*, 30 were *B. garinii*, 4 were *B. valaisiana* and 2 were *B. japonica*. Deqin County had a distribution of four *Borrelia* spp. except *B. japonica* which was only found in Yunlong County. Additionally, four *Borrelia* spp. except for *B. valaisiana* were detected in *Apodemus draco* (Table 1). The nucleotide sequences of the *B. afzelii* sequences were closely related to the sequence from a patient in China (JX888444.1). All *B. burgdorferi sensu stricto* sequence were identical to the sequence from strain BRE-13 sequenced from a patient's CSF in France (KY594010.1). *B. garinii* sequences in this study showed 99% identity with the strain YN12/2012 from *Canis familiaris* in Yunnan Province. *Borrelia japonica* sequences showed 99% identity with strain Cow611C from a tick in Japan (L30125.1). The *B. valaisiana* sequences were 98% similar to the strain KM2 from *Ixodes granulatus* ticks in Taiwan, China (HM100110.1) and 98% similar to the strain CKA2a from *Apodemus agrarius* in Zhejiang province, China (AB022124.1). Phylogenetic analyses based on different representative sequences in this study revealed that all detected *Borrelia* fell within five separate clades belonging to five different types of BBSL including *B. afzelii*, *B. burgdorferi sensu stricto*, *B. garinii*, *B. japonica* and *B. valaisiana* (Figure 2).

Discussion

Human cases of LB have been confirmed in almost every province found on mainland China including Yunnan Province. However, most of patients only had serological evidence and were not confirmed for specific genotypes. BBSL has been reported in small mammals trapped in the provinces Qinghai, Hunan, Shanxi, Liaoning, Sichuan, Fujian, Zhejiang, Gansu, Guangdong, Jilin and Yunnan [8–16], suggesting that small mammals are likely the main reservoir hosts in China. This study presents a large sample size extending over a wide geographic area, which provides insight into the prevalence, spatial distribution and genetic diversity of BBSL in small mammals collected in Sino-Burmese border area, Yunnan Province.

We documented BBSL infection in 30 species of small mammal, among which, 20 species had not been previously documented. These species may be infected occasionally, whether they serve as reservoir hosts need a further study. The *Rattus tanezumi* (573/3659, 15.66%) was the predominant species trapped in residential area in Yunnan. *Apodemus draco* (579/3659, 15.82%) and *Apodemus chevrieri* (402/3659, 10.99%) were the predominant hosts species in Yunnan, which was consistent with results from Europe where *Apodemus* are considered a major reservoir of *Borrelia* [17]. BBSL was detected in *Apodemus draco* and in *Apodemus chevrieri* in Yunnan, with *Apodemus draco* capable of carrying four *Borrelia* spp. The *Ochotona gloveri*, *Soriculus leucops* and *Rattus tuekkestanicus* also had a much higher

prevalence (> 14%) with much larger sample sizes in this study than in other provinces in China [12; 18; 19; 20; 21; 22]. *Rattus norvegicus* is the prominent household species in Yunnan, which had a high prevalence (12.50%) and was detected positive for pathogenic genotypes (*B. afzelii* and *B. burgdorferi sensu stricto*). We also found that the uncommon species *Sorex cylindricauda* in this study tested positive for *B. burgdorferi* s.l DNA, requiring further investigation to fully understand their role in maintaining or amplifying infections in nature.

Our findings indicated that prevalence rates in rodents are ranked highest to lowest by landscape type as follows: forest landscape > agricultural landscape > residential landscape (Table 2), which is likely related to tick vector density and preferred habitat. This reiterates the need for individuals traveling into potential tick habitats, like the forest, to take proper protective measures to limit tick bite exposure. Sampling locations in this survey contained a broad range of altitudes from 500 meters to 4500 meters. Among the three altitude classes, small mammals with the highest prevalence of BBSL were found above 2500 m. It was reported that *Ixodes ricinus* distribution in Sumava National Park extended toward higher altitudes, probably in relation to warming climates [23]. The roles temperature and humidity play in tick reproduction and reservoir preferences requires further investigation within these altitude ranges. Additionally, there are no reported human cases at these heights, which might reflect lower populations living in these areas.

Our study found five genospecies of BBSL in small mammals in Yunnan Province, four of them except for *B. japonica*, have previously been associated with LB [24–25]. *B. afzelii* was the prominent genotype (83/146, 56.84%) in this study, which detected in 24 species. There exists a wide distribution and genetic diversity of BBSL in Yunnan, compared to only 1–2 genospecies of BBSL in most provinces in China, such as Qinghai, Zhejiang, Guizhou and Guangxi. According to the sequence analysis carried out in this study, most of the *B. afzelii* sequences shared 99% identity with clinical isolates from patients in northeastern China [26]. Most of the *B. burgdorferi sensu stricto* sequences were identical to the sequence from a human case reported in France (KY594010.1). At this time, there have been no confirmed patients with registered sequence of Lyme disease spirochetes in Yunnan province, requiring further investigation in the near future. The sequence of *B. valaisiana* obtained from small mammals cluster into two clades, one cluster within the sequence from Guizhou and Zhejiang province, the other three cluster fell within close proximity to sequences from Europe. Birds are major reservoirs for *B. valaisiana* in Europe, however the transmission cycle maintaining *B. valaisiana* in Yunan may be different from other areas. *B. japonica* have only been found in Yunlong county, with this representing the first report documenting *B. japonica* in *Apodemus draco* and *Niviventer excelsior* in China. *B. garinii* is the most common genospecies in China, followed by *B. afzelii* [27]. However, *B. garinii* was detected from different species of small mammals only in Deqin County. We found that *B. afzelii* was the main genospecies detected in Yunnan, which is consistent with previous reports [4]. *B. burgdorferi sensu stricto* has been detected in Sika deer from Jilin province and in *Caprolagu ssinensis* from Hunan province, and detected in small mammals in Yunnan province within the more populated counties of Gongshan, Deqin, and Weixi (S1, S2, S5) found in northwestern Yunnan (Fig. 1). These findings reflect that Yunnan Province is of particular interest given

its diverse topographic range and high level of biodiversity in small mammals that are potential reservoirs for BBSL.

In conclusion, Yunnan Province is an important natural foci of *Borrelia burgdorferi* sensu lato in China, and given the absence of reported human cases within this region, efforts to expand clinical surveillance are urgently needed.

Abbreviations

BBSL: *Borrelia burgdorferi* sensu lato; LB: Lyme borreliosis; IFA: [Indirect Immunofluorescent Assay](#); PCR: polymerase chain reaction; OR: Odds ratio; 95% CI: 95% confidence interval

Declarations

Acknowledgements

The authors wish to thank all the people who had collected rodent specimens in the field.

Availability of data and materials

The dataset used and analyzed by the authors during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JJ, WC and CD conceived and designed the experiments. ZH, BJ, YZ, LT and MY performed the experiments. JJ, ZH and BJ analyzed the data. NJ and MF provided very constructive suggestions for revisions. Sample collections were implemented by ZG, ZS, ZL, YL and EP. All authors read and approved the final manuscript.

Ethics Statement

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, 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

All authors approved the final submitted version of the manuscript and consented for publication.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Prevalence of *Borrelia burgdorferi* sensu lato in small mammals of different species.

Mammal Species	Positive/Tested (%)	<i>B. afzelii</i>	<i>B. burgdorferi</i>	<i>B. garrinii</i>	<i>B. valaisiana</i>	<i>B. japonica</i>
<i>Rattus tanezumi</i>	4/573 (0.70)	3	0	0	1	0
<i>Rattus tuekkestanicus</i>	1/7 (14.29)	1	0	0	0	0
<i>Rattus norvegicus</i>	2/16 (12.50)	1	1	0	0	0
<i>Rattus brunneusculus</i>	2/94 (2.13)	1	0	0	1	0
<i>Apodemus latronum</i>	9/166 (5.42)	1	1	7	0	0
<i>Apodemus chevrieri</i>	20/402 (4.98)	16	1	3	0	0
<i>Apodemus draco</i>	19/579 (3.28)	10	5	3	0	1
<i>Mus caroli</i>	3/75 (4.00)	3	0	0	0	0
<i>Mus pahari</i>	6/91 (6.59)	6	0	0	0	0
<i>Niviventer andersoni</i>	3/57 (5.26)	0	0	2	1	0
<i>Niviventer eha</i>	2/32 (6.25)	0	2	0	0	0
<i>Niviventer confucianus</i>	14/144 (9.72)	6	1	7	0	0
<i>Niviventer excelsior</i>	1/29 (3.45)	0	0	0	0	1
<i>Eothenomys eleusis</i>	12/160 (7.50)	8	4	0	0	0
<i>Eothenomys cachinus</i>	4/38 (10.53)	3	1	0	0	0
<i>Eothenomys custos</i>	2/95 (2.11)	1	1	0	0	0
<i>Pitymys leucurus</i>	1/42 (2.38)	0	0	1	0	0
<i>Volemys clarkei</i>	2/35 (5.71)	1	0	1	0	0
<i>Dremomys pernyi</i>	3/26 (11.54)	0	0	3	0	0
<i>Crocidura attenuata</i>	2/41 (4.88)	1	0	0	1	0
<i>Crocidura dracula</i>	1/62 (1.61)	1	0	0	0	0
<i>Soriculus caudatus</i>	1/46 (2.17)	1	0	0	0	0
<i>Soriculus leucops</i>	13/87 (14.94)	6	7	0	0	0
<i>Sorex alpinus</i>	1/25 (4.00)	1	0	0	0	0
<i>Sorex cylindricauda</i>	7/49 (14.28)	6	1	0	0	0
<i>Anourosorex squamipes</i>	2/114 (1.75)	0	2	0	0	0
<i>Suneus murinus</i>	1/59 (1.69)	1	0	0	0	0
<i>Nasillus gracilis</i>	1/31 (3.23)	1	0	0	0	0
<i>Ochotona thibetana</i>	6/92 (6.52)	3	0	3	0	0
<i>Ochotona gloveri</i>	1/3 (33.33)	1	0	0	0	0
Others	0/389 (0)	0	0	0	0	0
Total	146/3659 (3.99)	83	27	30	4	2

Table 2. Risk factors related to *Borrelia burgdorferi* sensu lato based on univariate analyses.

variable	simple size	BBSL Infection		
	constituent ratio (%)	positive rate (%)	χ^2	<i>P</i>
altitude (m)				
~1500	868/3659 (23.72%)	7/868 (0.81%)		
1500~2500	823/3659 (22.49%)	24/823 (2.92%)	43.089	0.001
2500~	1968/3659 (53.79%)	115/1968 (5.84%)		
gender				
male	1753/3659 (47.91%)	81/1753 (4.62%)	3.492	0.062
female	1906/3659 (52.09%)	65/1906 (3.41%)		
age				
adult	3337/3659 (91.20%)	133/3337 (3.99%)	0.002	0.964
pubertal	322/3659 (8.80%)	13/322 (4.04%)		
landscape				
residential	158/3659 (4.32%)	1/158 (0.63%)		
agricultural	1786/3659 (48.81%)	56/1786 (3.14%)	14.945	0.001
forest	1715/3659 (46.87%)	89/1715 (5.19%)		
Total		146/3659 (3.99%)		

Figures

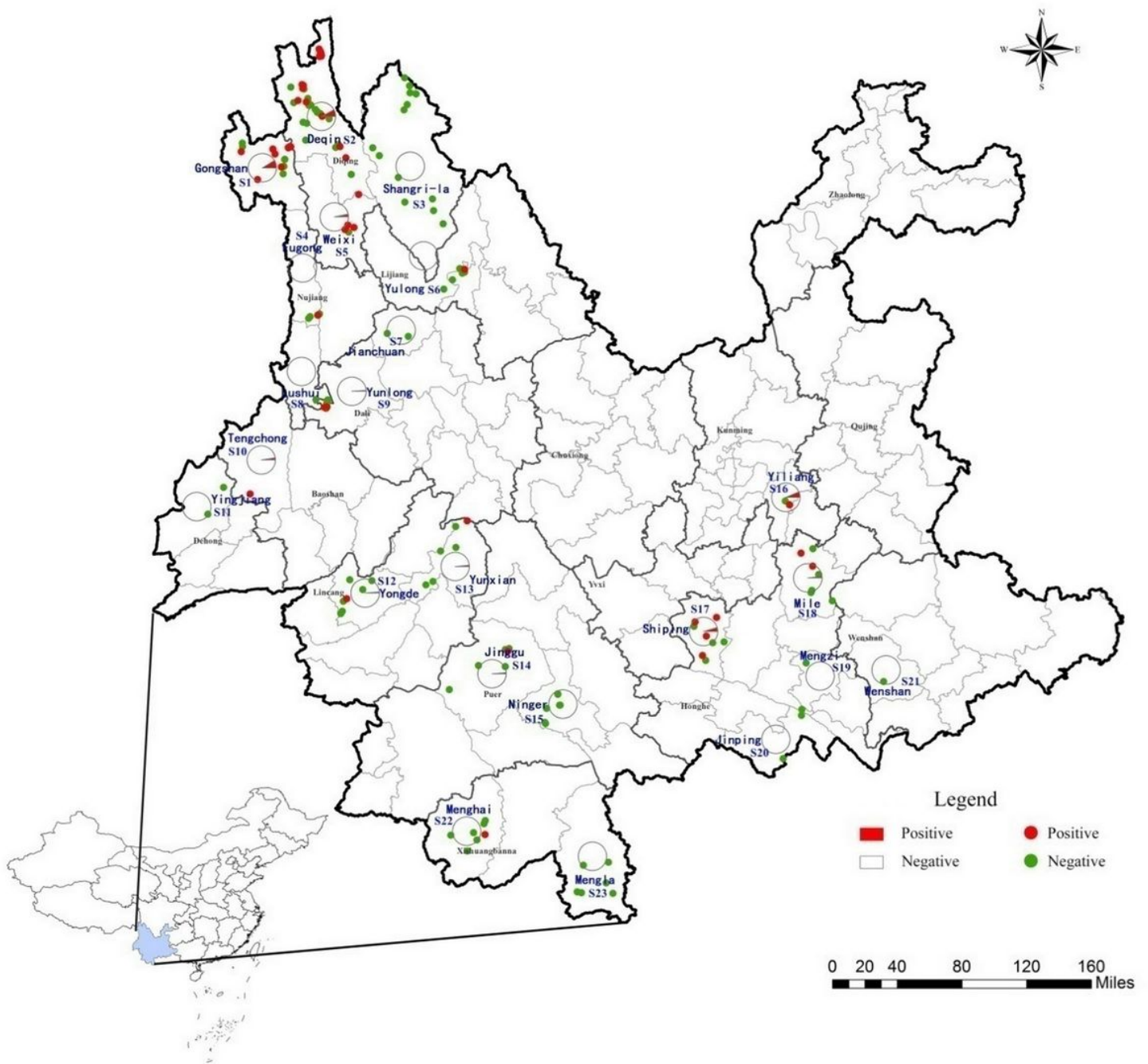


Figure 1

The distribution of sampling sites and prevalence of *Borrelia burgdorferi sensu lato* in Yunnan Province. A total of 3659 small mammals were collected from 23 sample counties, including 14 positive sample counties, Deqin, Weixi, Yulong, Gongshan, Fugong, Jinggu, Tengchong, Yongde, Menghai, Yunxian, Shiping, Mile, Yiliang, Yunlong, and 9 negative sample counties, Shangri-la, Jianchuan, Lushui, Yingjiang, Ninger, Mengzi, Jinping, Wenshan, Mengla. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



Figure 2

Phylogenetic tree of 5S-23S rRNA intergenic spacer gene of *Borrelia burgdorferi* sensu lato. Maximum Likelihood phylogenetic tree based on a comparison of *Borrelia burgdorferi* sensu lato 5S-23S rRNA intergenic spacer gene sequences obtained from Yunnan small mammals with *Borrelia burgdorferi* sensu lato reference strains. The number on each branch shows the percent occurrence in 1000 bootstrap replicates. Black circles stood for novel sequences identified in this study.

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