Jiao *et al. Zoonoses* (2022) 2:24
DOI 10.15212/ZOONOSES-2022-0026

ORIGINAL ARTICLE

First detection of *Rickettsia aeschlimannii* in *Hyalomma marginatum* in Tibet, China

Jun Jiao[1,#], Yonghui Yu[1,#], Peisheng He[1], Weiqiang Wan[1], Xuan OuYang[1], Bohai Wen[1], Yi Sun[1,*] and Xiaolu Xiong[1,*]

Abstract

Objective: *Hyalomma marginatum* is an important arthropod vector in the transmission of various zoonoses. The aim of this study was to identify the tick-borne pathogens (TBPs) maintained in *Hy. marginatum* in Tibet and to estimate the risk of human tick-borne diseases.

Methods: Adult *Hy. marginatum* ticks (n = 14) feeding on yaks were collected. The individual DNA samples of these ticks were sequenced with metagenomic next-generation sequencing to survey the presence of TBPs. TBPs in individual ticks were identified with nested polymerase chain reaction (PCR) combined with DNA sequencing.

Results: The presence of *Rickettsia*, *Anaplasma*, and *Ehrlichia* in individual ticks was indicated by the taxonomic profiles at the genus level, but only *Rickettsia aeschlimannii* (100%, 13/13) was further detected in the ticks by nested PCR.

Conclusion: This study provides information on the microbial communities of *Hy. marginatum* in Tibet, China, and provides the first report of *R. aeschlimannii* found in *Hy. marginatum* in Tibet. The results of this study indicated that yaks in Tibet are exposed to *R. aeschlimannii*.

Keywords: *Hyalomma marginatum, Rickettsia aeschlimannii,* metagenomic next-generation sequencing, Tibet

Edited by:
Jianwei Shao, School of Life Science and Engineering, Foshan University

Reviewed by:
Reviewer 1, Giorgi, Chakhunashvili, National Center for Disease Control and Public Health Tbilisi, Communicable Disease, Georgia
The other two reviewers chose to be anonymous.

[#]These authors contributed equally to this work.
***Corresponding authors:**
E-mail: xiongxiaolu624@sohu.com (XX); sunyi7310@sina.com (YS)

[1]State Key Laboratory of Pathogen and Biosecurity; Beijing Institute of Microbiology and Epidemiology, Beijing, PR China

Received: June 20 2022
Revised: July 19 2022
Accepted: July 27 2022
Published Online: August 09 2022

INTRODUCTION

Hard ticks (Acari: Ixodidae) are obligate blood-sucking parasitic arthropods that are vectors for a wide range of zoonoses, such as tick-borne encephalitis; Lyme borreliosis; and *Anaplasma*, *Coxiella*, *Ehrlichia* and *Rickettsia* and *Babesia* infections [1-3]. Tick-transmitted infections often occur in people working in forested areas or farmers engaged in animal husbandry [4]. Therefore, hard ticks are considered a relatively greater threat to animal and human health. *Hyalomma marginatum*, a species of hard ticks, is a known arthropod vector of several viruses, such as Thogoto, Dhori, Crimean-Congo hemorrhagic fever, and West Nile viruses [5], as well as bacteria, such as *Rickettsia aeschlimannii*, which is associated with spotted fever infection in humans [6].

To date, 119 tick species in ten genera have been reported in China, including 100 species of hard ticks [7,8]. The increasing cases of tick-borne diseases in humans, including Q fever [9], spotted fever [10], ehrlichiosis [11], anaplasmosis [12], brucellosis [8], tick-borne encephalitis [13], and babesiosis [14], have been reported in ticks in this region. Tibet, located in the southwestern of Qinghai-Tibet Plateau in China, supports the survival of several microorganisms that are adapted to the harsh environment of the plateau. Because of the harsh natural environment, animal husbandry and livestock

are cornerstone industries in Tibet. *Haemaphysalis tibetensis* [15], *Boophilus microplus* [16], and *Dermacentor* spp. [17,18] have been found in Tibet, and several tick-borne pathogens (TBPs), such as *Anaplasma marginale*, *Anaplasma ovis*, GRD spirochetes, and *Ehrlichia* spp. have been reported in ticks in this region [12]. However, limited knowledge exists regarding the distribution of *Hy. marginatum* and TBPs in *Hy. marginatum* in Tibet.

In this study, TBPs in *Hy. marginatum* feeding on yaks in Tibet were analyzed through metagenomic next-generation sequencing (mNGS) followed by genus/group-specific nested polymerase chain reaction (PCR). The results of this study provide broader information on the microorganisms maintained by *Hy. marginatum* in the region.

METHODS

Study design

A two-stage protocol was used in the present study: tick species were first identified through morphological characterization and molecular biology methods; mNGS was then used for TBP identification in individual ticks and was followed by genus/group-specific nested PCR (Fig 1).

Tick collection

All ticks collected were feeding on yaks from Chagyab County in Tibet in October of 2019 (Fig 2). The ears, groin, tail, abdomen, and neck regions of yaks of different ages and sexes were examined for the presence of ticks, and all visible

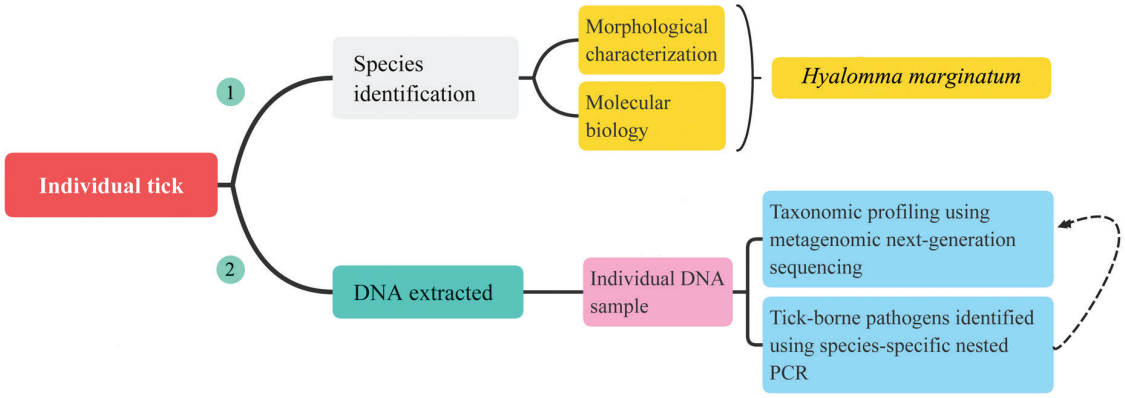

FIGURE 1 | Study workflow. Morphological characterization and molecular biology methods were performed for identification of tick species, and metagenomic next-generation sequencing followed by genus/group-specific nested PCR was subsequently used for tick-borne pathogen identification in individual ticks.

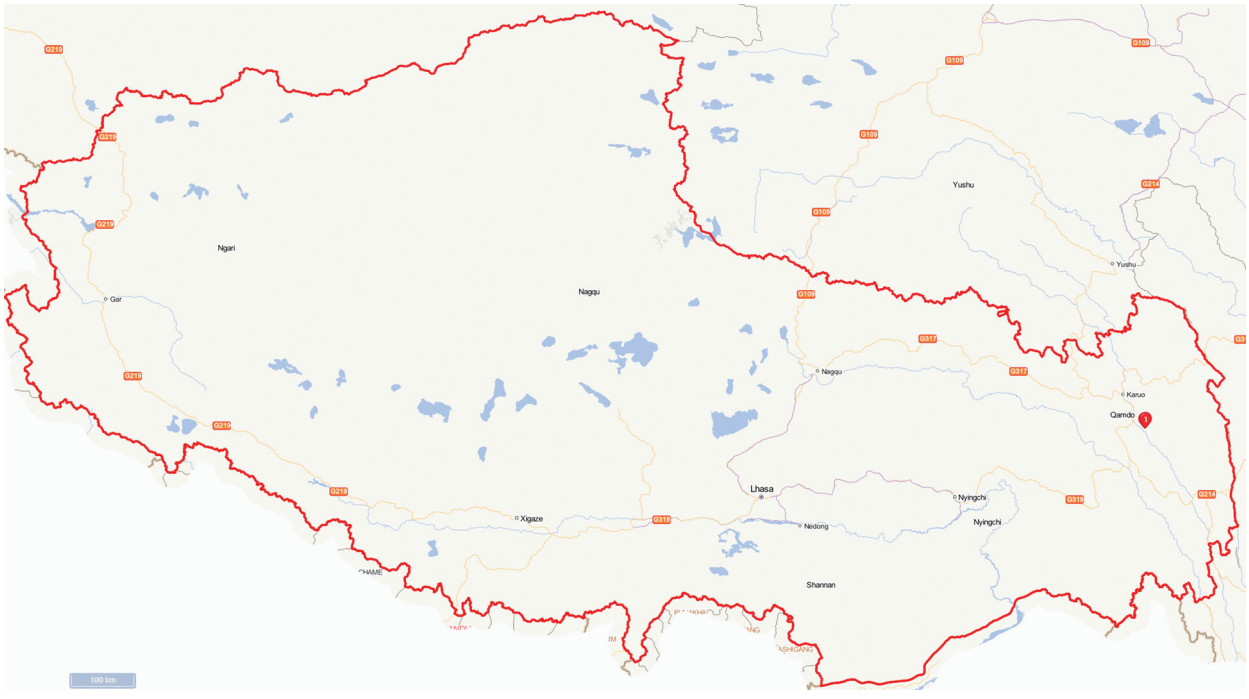

FIGURE 2 | Map of the sampling sites in Tibet, China. The red dot indicates the sampling region in the present study.

ticks were collected with forceps. Tick species were identified on the basis of morphological characterization and molecular biology methods, according to the sequence of the mitochondrial cytochrome c oxidase I (*COI*) gene, as described in previously [19].

DNA extraction

First, ticks were surface sterilized with 75% ethanol twice, followed by phosphate-buffered saline twice to remove environmental contaminants. Then individual ticks were homogenized in phosphate-buffered saline with MagNA Lyser Green Beads (Roche, Mannheim, Germany), and DNA was extracted with a QIAamp® Fast DNA Tissue Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. Deionized water was added as an extraction control. Finally, each extracted DNA sample was eluted in 100 μL deionized water and stored at -20°C until further analysis.

Metagenomic sequencing and taxonomy prediction

Individual DNA samples were sequenced on the Illumina HiSeq platform with paired-end 150-bp reads by Novogene (Beijing, China). Reads with low quality bases (quality threshold value ≤ 38) above a certain length (more than 40 bp) and/or with more than 10 bp of "N" bases were removed. Reads with more than 15 bp overlap with the adapter were also removed. Reads of host origin were finally filtered. Consequently, clean data were obtained.

The clean reads were assembled and analyzed with SOAPdenovo [20]. The scaffolds were broken at N into the scaftigs, and the scaftigs (<500 bp) were filtered [21]. The scaftigs (≥500 bp) were predicted according to the open reading frames by MetaGeneMark [22,23], and CD-HIT was used to remove redundancy and obtain the initial unique gene catalog [24,25]. For determination of gene abundance, the reads were realigned with the gene catalog with Bowtie 2. Only genes with two or more mapped reads were deemed to be present in a sample [26]. The relative abundance of each gene in each sample was calculated according to the number of mapped reads and the length of the gene [27-29].

Unigenes were aligned to the NR database (https://www.ncbi.nlm.nih.gov/) of NCBI with DIAMOND [30]. The aligned results of each gene with e value ≤ the smallest e value × 10 were retained [26] and then processed with the Lowest Common Ancestor-based algorithm implemented in MEGAN to ensure the species annotation information of sequences [31]. The final results contained the number of genes and the abundance information for each sample, and the relative abundance of each taxonomic group was calculated by addition of the relative abundance of genes annotated to the same feature [21,23,32].

Polymerase chain reaction (PCR)

Genus/group-specific nested PCR was performed to confirm the presence of TBPs in individual ticks. The PCR primers for spotted fever group rickettsia (SFGR) [33,34], *Anaplasma* spp. and *Ehrlichia* spp. [35] are presented in Table 1. Briefly, 2 μL DNA of sample was subjected to the initial PCR run, and 2 μL of PCR product from the first round was subjected to a second round of PCR. All PCR amplifications were performed with PrimeSTAR® HS (Premix) (TaKaRa, Beijing, China) and a PCR System 9700 (Applied Biosystems, GeneAmp®, USA). The nested PCR products were separated electrophoretically in 1.5% agarose gel, and positive amplicons were sequenced.

Phylogenetic analysis

The obtained DNA sequences were compared with the reference sequences in GenBank with the NCBI-BLAST server (http://blast.ncbi.nlm.nih.gov/blast.cgi), and multiple sequences were aligned with ClustalW with default parameters in MEGA 7.0. The phylogenetic tree of outer membrane protein A (*ompA*), citrate synthase (*gltA*), *ompB*, *gene D*, and *17kDa* for SFGR was constructed with the maximum likelihood method on the basis of the T92+G model, T92 model, T92 model, T92 model, and k2 model, with 1000 bootstrap replicates in MEGA 7.0 [36,37].

RESULTS

Taxonomic classification

All 14 adult ticks were identified as *Hy. marginatum*, according to morphological identification and species-specific PCR targeting the *COI* gene. Thirteen individual DNA samples were successfully analyzed with metagenomic sequencing. Sequencing yielded between 52597 and 58706 million reads per sample library, all of which had high quality (Clean_Q20 > 95%) (Table 2). The construction of the metagenomic library of sample 1.9 failed.

Metagenomic sequencing was performed to analyze the microbial community. A total of 276419 core genes were predicted to be common to all 13 tick samples, thus indicating their similar microbial community (Fig 3). The presence of *Rickettsia*, *Anaplasma*, and *Ehrlichia* at the genus level in individual tick samples was identified according to the taxonomic profiles. *Rickettsia* spp. was most abundant in all samples, followed by *Ehrlichia* spp. In addition, *Anaplasma* spp. was detected in samples CYP1.3, CYP1.4, and CYP1.10, but with lower abundance than that of *Ehrlichia* spp. In addition, *Staphylococcus* spp. were the most abundant microorganisms in all samples, followed by *Escherichia* spp. (Fig 4A).

Prevalence of *Rickettsia* in individual ticks

The presence of *Rickettsia*, *Anaplasma*, and *Ehrlichia* was identified in individual ticks with mNGS, and the identification of TBPs in individual ticks was confirmed by genus/group-specific PCR combined with sequencing.

Consequently, detection of *Anaplasma* spp. and *Ehrlichia* spp. yielded no positive results with nested PCR targeting the *16S rRNA* gene. Only *Rickettsia* spp. detection was positive with nested PCR targeting the *ompA*, *gltA*, *ompB*, *gene D*, and *17kDa* genes, and positive amplification of these

TABLE 1 | Target genes and primer sequences used for PCR

Pathogen	Target gene	Primer name	Sequence (5′-3′)
SFGR	*gltA*	CS2d	ATGACCAATGAAAATAATAAT
		CSEndr	CTTATACTCTCTATGTACA
		RpCS.877p	GGGGACCTGCTCACGGCGG
		RpCS.1258n	ATTGCAAAAAGTACAGTGAACA
	ompA	Rr190.70p	ATGGCGAATATTTCTCCAAAA
		Rr190.602n	AGTGCAGCATTCGCTCCCCCT
		190.70-38s1	AAAACCGCTTTATTCACC
		190.602-384r1	GGCAACAAGTTACCTCCT
	17 kDa	R17122	CAGAGTGCTATGGAACAAACAAGG
		R17500	CTTGCCATTGCCCATCAGGTTG
		TZ15	TTCTCAATTCGGTAAGGGC
		TZ16	ATATTGACCAGTGCTATTTC
	ompB	BG1-21	GGCAATTAATATCGCTGACGG
		BG2-20	GCATCTGCACTAGCACTTTC
	Gene D	D1F	ATGAGTAAAGACGGTAACCT
		D928R	AAGCTATTGCGTCATCTCCG
Anaplasma spp.	*16S rRNA*	Eh-out1	TTGAGAGTTTGATCCTGGCTCAGAACG
Ehrlichia spp.		Eh-out2	CACCTCTACACTAGGAATTCCGCTATC
		Eh-gs1	GTAATAACTGTATAATCCCTG
		Eh-gs2	GTACCGTCATTATCTTCCCTA

TABLE 2 | Pooling strategies for metagenomic next-generation sequencing

Sample ID	InsertSize (bp)	SeqStrategy	RawData (MB)	CleanData (MB)	Clean_Q20	Clean_GC (%)
CYP1.1	350	(150:150)	57,988.81	56,715.91	96.95	47.63
CYP1.2	350	(150:150)	55,630.90	54,744.23	96.59	47.42
CYP1.3	350	(150:150)	55,016.44	53,340.28	96.61	48.19
CYP1.4	350	(150:150)	56,170.53	54,486.79	96.91	47.62
CYP1.5	350	(150:150)	56,013.06	54,700.57	97.19	47.41
CYP1.6	350	(150:150)	54,318.37	52,685.77	96.93	48
CYP1.7	350	(150:150)	52,597.53	51,261.33	96.22	47.61
CYP1.8	350	(150:150)	53,026.99	51,275.89	96.31	47.28
CYP1.10	350	(150:150)	55,921.43	54,575.12	96.36	46.72
CYP1.11	350	(150:150)	54,589.54	52,202.90	96.94	47.31
CYP1.12	350	(150:150)	57,403.60	55,920.88	96.31	47.4
CYP1.13	350	(150:150)	57,792.73	56,846.56	96.34	47.75
CYP1.14	350	(150:150)	58,706.93	56,559.08	97.37	46.97

two genes was found in all 13 tick samples (13/13, 100%) (Fig 4B). The obtained sequences for each gene for SFGR from all 13 tick samples were 99.61% and 100% identical to *R. aeschlimannii*, respectively.

Phylogenetic analysis

According to phylogenetic analysis based on the partial sequences of *ompA*, *gltA*, *ompB*, *gene D*, and *17kDa* genes, *R. aeschlimannii*, identified in *Hy. marginatum* in the present

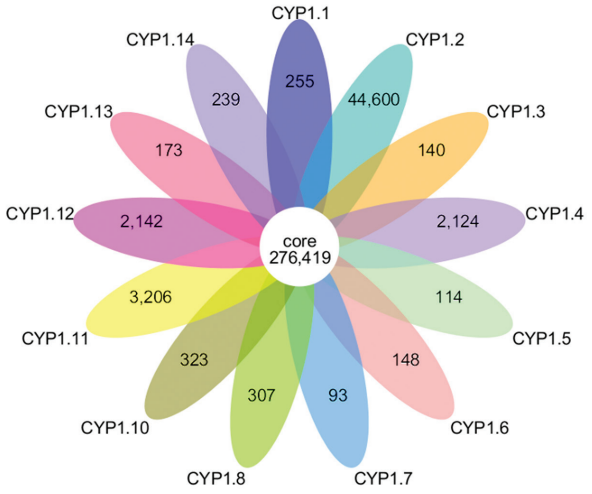

FIGURE 3 | Total numbers of shared and unique core genes of individual ticks. CYP1.1 to CYP1.14, individual DNA samples of *Hy. marginatum* for metagenomic next-generation sequencing.

study, clustered with other *R. aeschlimannii* subspecies and was most closely associated with *R. aeschlimannii* stavropol (DQ235777), which had been isolated from *Hy. marginatum* in Russia (Figs 5 and 6).

DISCUSSION

Recently, increasing attention has been paid to the distribution of ticks and TBPs worldwide, and this knowledge can aid in the prevention and control of tick-borne diseases. Although a variety of TBPs have been identified in ticks in China, knowledge regarding the distribution of *Hy. marginatum* and TBPs in Tibet remains limited. In the present study, mNGS combined with nested PCR was applied to survey TBPs in *Hy. marginatum* feeding on yaks in Tibet, China.

mNGS has been successfully used to identify known and/or unknown microorganisms in arthropods [38,39], thus enabling analysis of microorganism diversity. By mNGS, the most abundant bacteria identified in ticks include *Rickettsia* spp., *Coxiella* spp., *Francisella* spp., and "*Candidatus* Midichloria mitochondrii" [40]. Ravi et al. have applied mNGS to analyze the microbiomes in hard ticks collected in Palestine and detected high levels of important TBPs, including *Coxiella* spp., *Rickettsia* spp., and *A. ovis*, and generated a genome sequence of a canine parvovirus [41]. Beyond these bacteria, environmental and skin-associated bacteria, such as *Pseudomonas*, *Acinetobacter*, *Enterobacter*, and *Stenotrophomonas*, are common in hard ticks [42,43]. Bacteria such as *Staphylococcus* and *Escherichia* might have been predominant in the present study.

The results of mNGS in the present study revealed the presence of *Rickettsia*, *Anaplasma*, and *Ehrlichia* in *Hy. marginatum*. However, only *Rickettsia* spp. was positively detected in genus/group-specific nested PCR. After sequence comparison, *R. aeschlimannii* was found in all 13 *Hy. marginatum*. The results of genus/group-specific nested PCRs were partially consistent with those of mNGS, possibly because less abundant species might not have been sensitively amplified with PCR when highly abundant sequences were over-represented [40]. In addition, *Coxiella* spp., *Babesia* spp., and *Borrelia* spp. were often detected in ticks. Thus each tick was detected by genus/group-specific nested PCR in the present study, and no positive amplicons were observed (data not shown).

Hyalomma spp. (e.g., *Hy. marginatum*) are mainly endemic to southern and eastern Europe [44], and these ticks also have been reported in birds in countries where these ticks are not autochthonous [45]. One possible explanation is that the larvae and nymphs of *Hyalomma* spp. may be transported passively by migratory birds from southern Europe to their breeding areas in the northern hemisphere [44,45]. *Hy. marginatum*, a species of *Hyalomma* spp., is distributed primarily in northern Africa, southern and eastern Europe, the Middle East and several parts of Asia [5,46]. To date, *Hy. marginatum* has been found in Inner Mongolia, Gansu, and Xinjiang in China [10,47,48], thus indicating that this

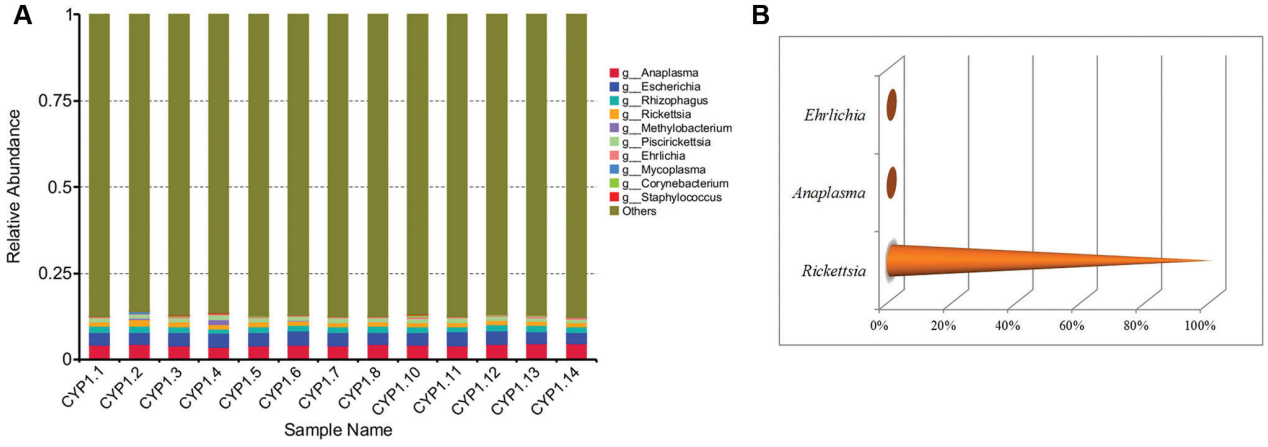

FIGURE 4 | Analysis of the microbial community in *Hyalomma marginatum*. The relative abundance of the potential top ten bacteria at the genus level was analyzed with metagenomic next-generation sequencing (A), and then the tick-borne pathogens maintained in individual ticks were confirmed by genus/group-specific nested PCR (B). CYP1.1 to CYP1.14, individual DNA samples of *Hy. marginatum*.

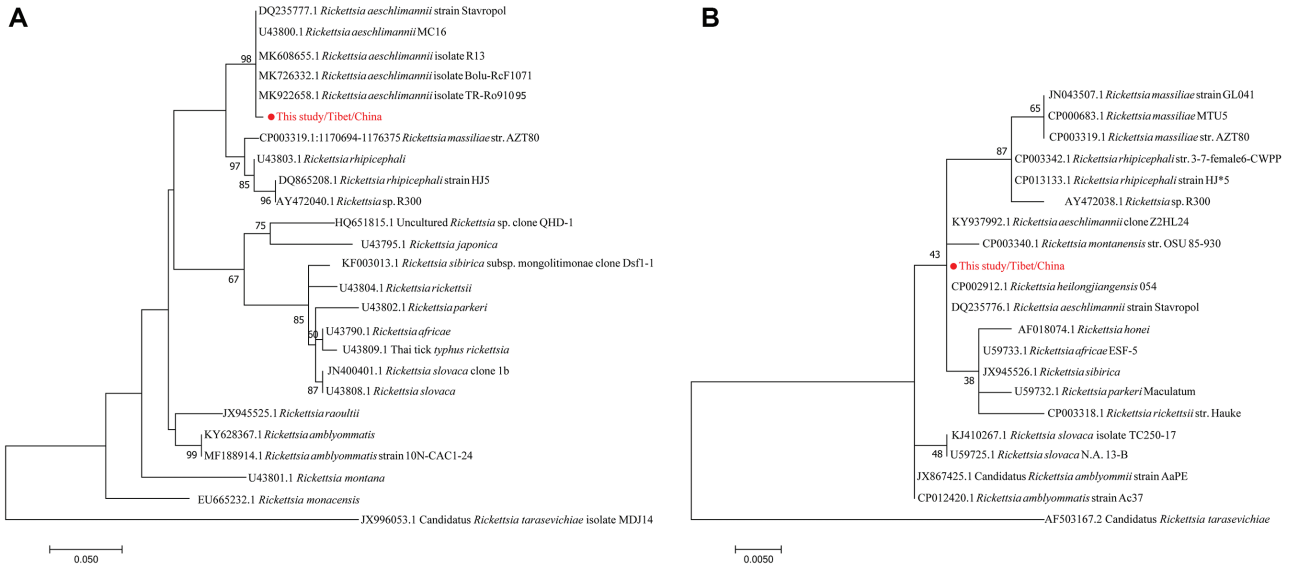

FIGURE 5 | Phylogenetic analysis based on the sequences of the *ompA* and *gltA* genes of *Rickettsia aeschlimannii*. The obtained sequences in the present study are indicated with red dots. Multiple **s**equences were aligned with the ClustalW tool in MEGA 7.0. Phylogenetic analysis of *ompA* (258 bp) (A) and *gltA* (285 bp) (B) of *R. aeschlimannii* was performed with the maximum likelihood method based on the T92+G model and T92 model with 1000 bootstrap replicates in MEGA 7.0.

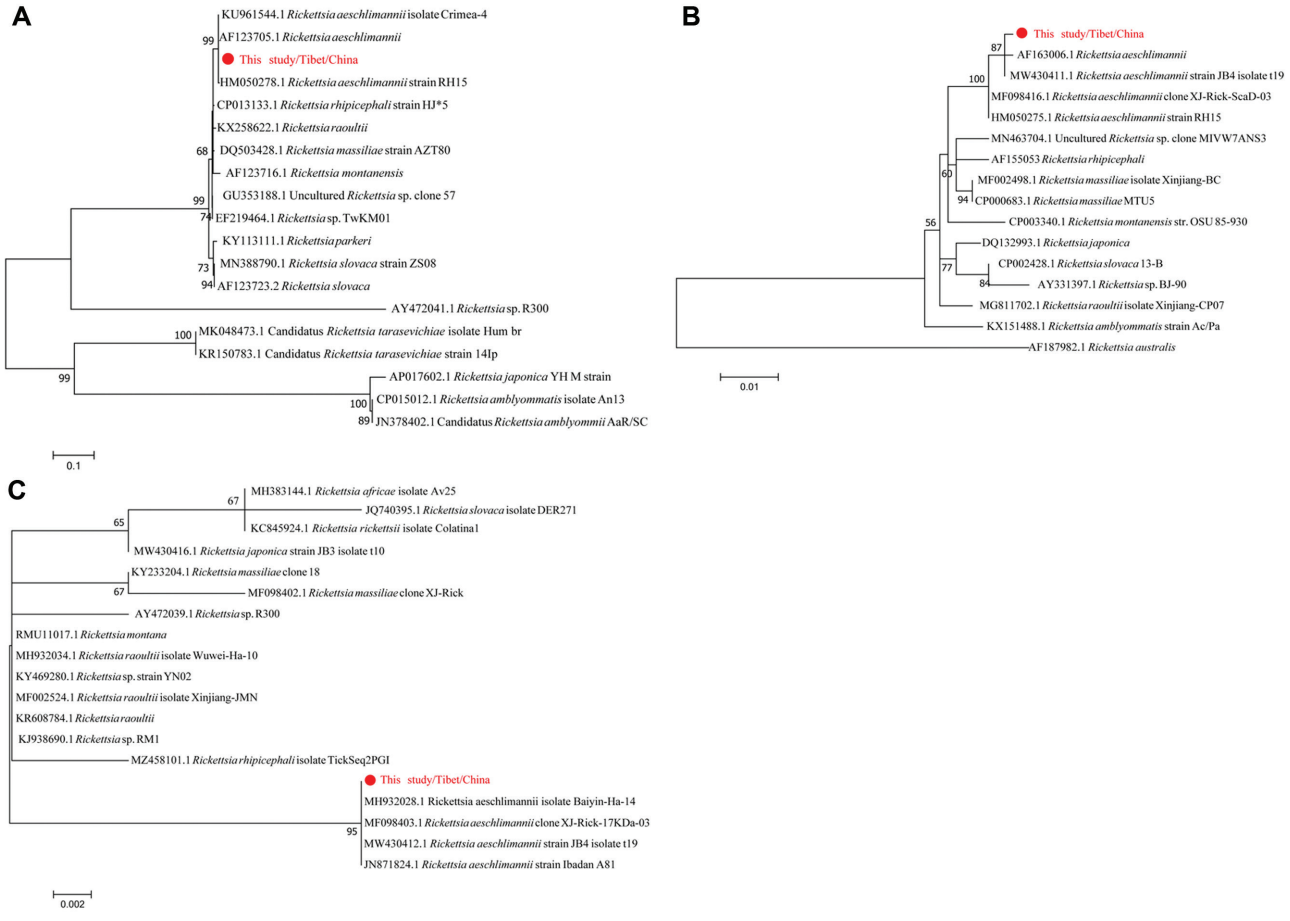

FIGURE 6 | Phylogenetic analysis based on the sequences of the *ompB, gene D,* and *17 kDa* genes of *Rickettsia aeschlimannii*. The obtained sequences in the present study are indicated with red dots. Multiple **s**equences were aligned with the ClustalW tool in MEGA 7.0. Phylogenetic analysis of *ompB* (564 bp) (A), *gene D* (729 bp) (B), and *17 kDa* (163 bp) (C) of *R. aeschlimannii* was performed with the maximum likelihood method based on the T92 model, T92 model, and k2 model with 1000 bootstrap replicates in MEGA 7.0.

tick is not autochthonous in China. To our knowledge, this is the first report of *Hy. marginatum* in Tibet, thus indicating its increasing geographical distribution in China.

R. aeschlimannii belongs to SFGR and was first detected in *Hy. marginatum* in Morocco in 1997 [49]. A clinical case of infection caused by this pathogen was first reported in 2002 [6]. Symptoms of *R. aeschlimannii* infection in humans are highly similar to those of Mediterranean spotted fever caused by *R. conorii* and may be associated with liver dysfunction [50]. In China, *R. aeschlimannii* has been detected in *Rhipicephalus turanicus* [51], *Hy. asiaticum*, [10,47], *Hy. marginatum* [10], and *Haemaphysalis punctata* [52]. Yang et al. have reported a case of *R. aeschlimannii* infection in a woman from Xingjiang, and this pathogen was also detected in *Hy. asiaticum* around the patient's residence [53]. In the present study, *R. aeschlimannii* was detected in all *Hy. marginatum*, thereby indicating its dominant prevalence in these ticks; this finding may be relevant to the health of humans who may have spotted fever after being bitten by these ticks.

This study has several limitations. Although the researchers went to Zogang County, Chamdo County, and Chagyab County, and verified nearly 100 yaks for tick collection, the high altitude (>4000 meters) and low environmental temperature (<20°C) of the sampling sites might have made this region unsuitable for tick adaptation extending the development phase (120 days) at temperatures of 18°C [54]. In addition, a small number of tick samples was collected from one location in the present study. Another limitation is that no livestock sera were collected because of the local cultural customs.

CONCLUSION

This study provides information on the microbial communities of *Hy. marginatum* in Tibet, China, and provides the first report of *R. aeschlimannii* in *Hy. marginatum* in Tibet. The results of this study also indicated that yaks in Tibet are exposed to *R. aeschlimannii*. Molecular and serology methods are important for investigation of *R. aeschlimannii* in livestock and farmers with close contact with *Hy. marginatum* in Tibet.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No 32000139) and the National Key Research and Development Program of China (No. 2019YFC1200500).

REFERENCES

1. Liu XY, Bonnet SI. Hard tick factors implicated in pathogen transmission. PLoS Negl Trop Dis. 2014;8(1):e2566.
2. Li LH, Zhang Y, Wang JZ, Li XS, Yin SQ, Zhu D, et al. High genetic diversity in hard ticks from a China-Myanmar border county. Parasit Vectors. 2018;11(1):469.
3. Dong X, Soong L. Emerging and re-emerging zoonoses are major and global challenges for public health. Zoonoses. 2021;1(1):1-2.
4. Yang X, Gao Z, Wang L, Xiao L, Dong N, Wu H, et al. Projecting the potential distribution of ticks in China under climate and land use change. Int J Parasitol. 2021;51(9):749-759.
5. Duscher GG, Hodzic A, Hufnagl P, Wille-Piazzai W, Schotta AM, Markowicz MA, et al. Adult *Hyalomma marginatum* tick positive for *Rickettsia aeschlimannii* in Austria, October 2018. Euro Surveill. 2018;23(48):1800595.
6. Raoult D, Fournier PE, Abboud P, Caron F. First documented human *Rickettsia aeschlimannii* infection. Emerg Infect Dis. 2002;8(7):748-749.
7. Sheng J, Jiang M, Yang M, Bo X, Zhao S, Zhang Y, et al. Tick distribution in border regions of Northwestern China. Ticks Tick Borne Dis. 2019;10(3):665-669.
8. Jia W, Chen S, Chi S, He Y, Ren L, Wang X. Recent progress on tick-borne animal diseases of veterinary and public health significance in China. Viruses. 2022;14(2):355.
9. Jiao J, Zhang J, He P, OuYang X, Yu Y, Wen B, et al. Identification of Tick-Borne Pathogens and Genotyping of *Coxiella burnetii* in *Rhipicephalus microplus* in Yunnan Province, China. Front Microbiol. 2021;12:736484.
10. Guo WP, Wang YH, Lu Q, Xu G, Luo Y, Ni X, et al. Molecular detection of spotted fever group rickettsiae in hard ticks, northern China. Transbound Emerg Dis. 2019;66(4):1587-1596.
11. Zhang L, Liu Y, Ni D, Li Q, Yu Y, Yu XJ, et al. Nosocomial transmission of human granulocytic anaplasmosis in China. JAMA. 2008;300(19):2263-2270.
12. Yu Z, Wang H, Wang T, Sun W, Yang X, Liu J. Tick-borne pathogens and the vector potential of ticks in China. Parasit Vectors. 2015;8:24.
13. Lu Z, Broker M, Liang G. Tick-borne encephalitis in mainland China. Vector Borne Zoonotic Dis. 2008;8(5):713-720.
14. Qi C, Zhou D, Liu J, Cheng Z, Zhang L, Wang L, et al. Detection of *Babesia* divergens using molecular methods in anemic patients in Shandong Province, China. Parasitol Res. 2011;109(1):241-245.
15. Liu M, Li T, Yu ZJ, Qiu ZX, Yan P, Li Y, et al. Seasonal dynamics of the tick *Haemaphysalis tibetensis* in the Tibetan Plateau, China. Med Vet Entomol. 2017;31(4):449-456.
16. Wen B, Jian R, Zhang Y, Chen R. Simultaneous detection of *Anaplasma marginale* and a new *Ehrlichia* species closely related to *Ehrlichia chaffeensis* by sequence analyses of 16S ribosomal DNA in *Boophilus microplus* ticks from Tibet. J Clin Microbiol. 2002;40(9):3286-3290.
17. Wang Y, Liu Z, Yang J, Chen Z, Liu J, Li Y, et al. *Rickettsia raoultii*-like bacteria in *Dermacentor* spp. ticks, Tibet, China. Emerg Infect Dis. 2012;18(9):1532-1534.
18. Li T, Liu M, Zhang TT, Li Y, Wang WY, Li MM, et al. The life cycle and development characteristics of *Dermacentor everestianus* (Acari: *Ixodidae*) under field conditions in Qinghai-Tibet Plateau. Exp Appl Acarol. 2018;76(4):513-522.
19. Chitimia L, Lin RQ, Cosoroaba I, Wu XY, Song HQ, Yuan ZG, et al. Genetic characterization of ticks from southwestern Romania by sequences of mitochondrial *cox1* and *nad5* genes. Exp Appl Acarol. 2010;52(3):305-311.
20. Luo R, Liu B, Xie Y, Li Z, Huang W, Yuan J, et al. SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. Gigascience. 2012;1(1):18.
21. Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, et al. An integrated catalog of reference genes in the human gut microbiome. Nat Biotechnol. 2014;32(8):834-841.
22. Qin N, Yang F, Li A, Prifti E, Chen Y, Shao L, et al. Alterations of the human gut microbiome in liver cirrhosis. Nature. 2014;513(7516):59-64.
23. Karlsson FH, Fak F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, et al. Symptomatic atherosclerosis is associated with an altered gut metagenome. Nat Commun. 2012;3:1245.
24. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 2006;22(13):1658-1659.

25. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics. 2012;28(23):3150-3152.
26. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.
27. Villar E, Farrant GK, Follows M, Garczarek L, Speich S, Audic S, et al. Ocean plankton. Environmental characteristics of *Agulhas rings* affect interocean plankton transport. Science. 2015;348(6237):1261447.
28. Cotillard A, Kennedy SP, Kong LC, Prifti E, Pons N, Le Chatelier E, et al. Dietary intervention impact on gut microbial gene richness. Nature. 2013;500(7464):585-588.
29. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature. 2013;500(7464):541-546.
30. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 2015;12(1):59-60.
31. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. Genome Res. 2007;17(3):377-386.
32. Feng Q, Liang S, Jia H, Stadlmayr A, Tang L, Lan Z, et al. Gut microbiome development along the colorectal adenoma-carcinoma sequence. Nat Commun. 2015;6:6528.
33. Cheng C, Fu W, Ju W, Yang L, Xu N, Wang YM, et al. Diversity of spotted fever group Rickettsia infection in hard ticks from Suifenhe, Chinese-Russian border. Ticks Tick Borne Dis. 2016;7(5):715-719.
34. Tadepalli M, Vincent G, Hii SF, Watharow S, Graves S, Stenos J. Molecular evidence of novel spotted fever group Rickettsia species in *Amblyomma albolimbatum* ticks from the Shingleback Skink (*Tiliqua rugosa*) in Southern Western Australia. Pathogens. 2021;10(1):35.
35. Qin XR, Han FJ, Luo LM, Zhao FM, Han HJ, Zhang ZT, et al. *Anaplasma* species detected in *Haemaphysalis longicornis* tick from China. Ticks Tick Borne Dis. 2018;9(4):840-843.
36. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33(7):1870-1874.
37. Hall BG. Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol. 2013;30(5):1229-1235.
38. Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. Annu Rev Pathol. 2019;14:319-338.
39. Kim JY, Yi MH, Mahdi AAS, Yong TS. iSeq 100 for metagenomic pathogen screening in ticks. Parasit Vectors. 2021;14(1):346.
40. Greay TL, Gofton AW, Paparini A, Ryan UM, Oskam CL, Irwin PJ. Recent insights into the tick microbiome gained through next-generation sequencing. Parasit Vectors. 2018;11(1):12.
41. Ravi A, Ereqat S, Al-Jawabreh A, Abdeen Z, Abu Shamma O, Hall H, et al. Metagenomic profiling of ticks: Identification of novel rickettsial genomes and detection of tick-borne canine parvovirus. PLoS Negl Trop Dis. 2019;13(1):e0006805.
42. Narasimhan S, Fikrig E. Tick microbiome: the force within. Trends Parasitol. 2015;31(7):315-323.
43. Carpi G, Cagnacci F, Wittekindt NE, Zhao F, Qi J, Tomsho LP, et al. Metagenomic profile of the bacterial communities associated with *Ixodes ricinus* ticks. PLoS One. 2011;6(10):e25604.
44. Jameson LJ, Morgan PJ, Medlock JM, Watola G, Vaux AG. Importation of *Hyalomma marginatum*, vector of Crimean-Congo haemorrhagic fever virus, into the United Kingdom by migratory birds. Ticks Tick Borne Dis. 2012;3(2):95-99.
45. Hasle G. Transport of ixodid ticks and tick-borne pathogens by migratory birds. Front Cell Infect Microbiol. 2013;3:48.
46. Grandi G, Chitimia-Dobler L, Choklikitumnuey P, Strube C, Springer A, Albihn A, et al. First records of adult *Hyalomma marginatum* and *H. rufipes* ticks (Acari: *Ixodidae*) in Sweden. Ticks Tick Borne Dis. 2020;11(3):101403.
47. Yin X, Guo S, Ding C, Cao M, Kawabata H, Sato K, et al. Spotted fever group Rickettsiae in Inner Mongolia, China, 2015-2016. Emerg Infect Dis. 2018;24(11):2105-2107.
48. Yang Y, Tong J, Ruan H, Yang M, Sang C, Liu G, et al. Genetic diversity of hard ticks (Acari: *Ixodidae*) in the South and East regions of Kazakhstan and Northwestern China. Korean J Parasitol. 2021;59(1):103-108.
49. Roux V, Rydkina E, Eremeeva M, Raoult D. Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the *rickettsiae*. Int J Syst Bacteriol. 1997;47(2):252-261.
50. Tosoni A, Mirijello A, Ciervo A, Mancini F, Rezza G, Damiano F, et al. Human *Rickettsia aeschlimannii* infection: first case with acute hepatitis and review of the literature. Eur Rev Med Pharmacol Sci. 2016;20(12):2630-2633.
51. Wei QQ, Guo LP, Wang AD, Mu LM, Zhang K, Chen CF, et al. The first detection of Rickettsia aeschlimannii and *Rickettsia massiliae* in *Rhipicephalus turanicus* ticks, in northwest China. Parasit Vectors. 2015;8:631.
52. Song S, Chen C, Yang M, Zhao S, Wang B, Hornok S, et al. Diversity of *Rickettsia* species in border regions of northwestern China. Parasit Vectors. 2018;11(1):634.
53. Yang M, Jia Y, Dong Z, Zhang Y, Xie S, Liu Q, et al. *Rickettsia aeschlimannii* Infection in a Woman from Xingjiang, Northwestern China. Vector Borne Zoonotic Dis. 2022;22(1):55-57.
54. Chavez-Larrea MA, Cholota-Iza C, Medina-Naranjo V, Yugcha-Diaz M, Ron-Roman J, Martin-Solano S, et al. Detection of *Babesia* spp. in high altitude cattle in Ecuador, possible evidence of the adaptation of vectors and diseases to new climatic conditions. Pathogens. 2021;10(12):1593.