Original Article

Bats and their ectoparasites (Nycteribiidae and Spinturnicidae) carry diverse novel Bartonella genotypes, China

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Funding information
the National Natural Science Funds of China, Grant/Award Number: 81971939; the China Postdoctoral Science Foundation Funded Project, Grant/Award Number: 2019M662720; the Innovation Research of Hubei Postdoctoral Science and Technology

Abstract
Bartonella species are facultative intracellular bacteria and recognized worldwide as emerging zoonotic pathogens. Bartonella were isolated or identified by polymerase chain reaction (PCR) in bats and their ectoparasites worldwide, whereas the association between them was scarce, especially in Asia. In this study, a retrospective analysis with frozen samples was carried out to identify the genetic diversity of Bartonella in bats and their ectoparasites and to investigate the relationships of Bartonella carried by bats and their ectoparasites. Bats and their ectoparasites (bat flies and bat mites) were collected from caves in Hubei Province, Central China, from May 2018 to July 2020. Bartonella were screened by PCR amplification and sequencing of three genes (gltA, rpoB, and ftsZ). Bats, bat flies, and bat mites carried diverse novel Bartonella genotypes with a high prevalence. The sharing of some Bartonella genotypes between bats and bat flies or bat mites indicated a potential role of bat flies and bat mites as vectors of bartonelae, while the higher genetic diversity of Bartonella in bat flies than that in bats might be due to the vertical transmission of this bacterium in bat flies. Therefore, bat flies might also act as reservoirs of Bartonella. In addition, human-pathogenic B. mayotimonensis was identified in both bats and their ectoparasites, which expanded our knowledge on the geographic distribution of this bacterium and suggested a potential bat origin with bat flies and bat mites playing important roles in the maintenance and transmission of Bartonella.

Keywords
Bartonella, bat, bat fly, bat mite, Nycteribiidae, Spinturnicidae

1 | INTRODUCTION

As the second largest order of mammals, bats are unique long-lived gregarious flying mammals with a worldwide distribution, and they have been considered as the most likely sources of several viruses causing severe emerging infectious diseases in humans, including SARS-CoV, MERS-CoV, Nipah virus, Hendra virus, Ebola virus, Marburg virus, and the currently rampant SARS-CoV-2 (Han et al., 2015; Zhou et al., 2020).
In the last two decades, a large number of novel viruses have been found in bats (Chen et al., 2014), whereas the study of bacterial agents in bats has been far more neglected (Mühldorfer, 2013).

*Bartonella* species are Gram-negative, fastidious, and facultative intracellular bacteria that parasitize erythrocytes and endothelial cells of a variety of mammals, including rodents, insectivores, carnivores, ungulates, and marine mammals such as dolphins (Birtles, 2005; Dehio, 2001; Eicher & Dehio, 2012; Kosoy et al., 2010). Several *Bartonella* species are capable of causing infections in humans, including *Bartonella henselae* (cat-scratch disease), *B. quintana* (trench fever), and *B. bacilliformis* (Carrión’s disease) (Angelakis & Raoult, 2014; Jacomo et al., 2002; Rolain et al., 2004). Besides, an increasing number of novel *Bartonella* species associated with human endocarditis were reported (Edouard et al., 2015; Okaro et al., 2017).

There was evidence that bat-borne bartonellae were associated with infections in humans and other animals. *Bartonella mayotimonensis* was first isolated from the aortic valve of a patient with endocarditis in the United States (E. Y. Lin et al., 2010). Subsequently, *Bartonella* related to *B. mayotimonensis* were identified in bats from Finland (Veikkola-Lainen et al., 2014), the United States (Lilley et al., 2017), the United Kingdom (Concannon et al., 2005), France, and Spain (Stuckey, Boulouis, et al., 2017). In addition, some *Bartonella* genotypes found in bats from Georgia were genetically related to those identified in dogs from Thailand and humans from Poland (Urushadze et al., 2017). Moreover, a novel *Bartonella* species, *Bartonella rousetti*, was isolated from fruit bats in Nigeria, and serological studies revealed that infection with this bacterium was prevalent in the local population (Bai et al., 2018). These reports highlighted the zoonotic potential of bat-borne bartonellae and underscored the need for expanded surveillance and investigation of these pathogens.

*Bats harbour numerous ectoparasites, including bat flies (Diptera: Nycteribiidae and Streblidae), bat bugs (Hemiptera: Cimicidae and Polyctenidae), bat fleas (Siphonaptera: Ischnopsyllidae), bat ticks (Ixodidae: Ixodidae and Argasidae), and bat mites (Mesostigmata: Spinturnicidae and Macronyssidae) (Szentiványi et al., 2019). In the last decade, diverse novel Bartonella strains/genotypes were identified in bats and their ectoparasites worldwide (Figure 1, Table S1). Besides, a recent study inferred that bats had a great influence on both the origin and spread of *Bartonella* among other mammals and geographic regions (Mckee et al., 2021). However, there was a lack of understanding on the maintenance and transmission of *Bartonella* in bat populations. Bat flies and bat mites are obligate hematophagous ectoparasites of bats, and they may play important roles in the transmission and maintenance of bat pathogens. Bat flies were the most studied ectoparasites of bats in terms of bartonellae. The presence of identical genotypes of *Bartonella* in bat flies and their bat hosts was frequently reported (Brook et al., 2015; Dietrich et al., 2016; Judson et al., 2015; Kamani et al., 2014; Qiu et al., 2020); therefore, bat flies were generally considered as vectors for transmitting bartonellae among bats. Few studies reported the occurrence of *Bartonella* in bat mites (Hornok et al., 2012; Ikeda et al., 2016; Reeves et al., 2016), and a recent study in Poland found that identical genotypes of *Bartonella* were shared among bats and their *Spinturnix myoti* mites, indicating the possible role of bat mites in the acquisition and transmission of *Bartonella* (Szubert-Kruszyńska et al., 2019).

Studies on the genetic diversity of *Bartonella* in bats and their ectoparasites in Asia were quite limited (Figure 1), and there was a lack of research on the relationship between them. In China, *Bartonella* were previously identified in bats from Shandong Province by our team (Han et al., 2017) and from Taiwan (J.-W. Lin et al., 2012). However, there were hardly any reports of *Bartonella* in bat ectoparasites other than

**FIGURE 1** Map showing our current understanding of *Bartonella* in bats and their ectoparasites worldwide. Countries or regions with reports of *Bartonella* in bats or their ectoparasites are highlighted in gold. The map was created based on data from Table S1.
one bat fly in China (Morse et al., 2012). Considering the highly diverse novel Bartonella genotypes identified in bats from China in our previous work (Han et al., 2017), there was a need for further investigation on the relationship of Bartonella in bats and their ectoparasites.

In this study, a retrospective analysis with frozen samples was carried out to identify the genetic diversity of Bartonella in bats and their ectoparasites and to investigate the relationships of Bartonella carried by bats and their ectoparasites. Our study will provide insight into the evolution and ecology of Bartonella in bat populations.

## MATERIALS AND METHODS

### 2.1 Ethics statement

Collection of bats for microbiological studies was approved by the Ethics Committee of the Medical School, Wuhan University (WHU2020-YF0023), and every effort was made to minimize the discomfort of bats.

### 2.2 Sampling and species identification of bats and their ectoparasites

Frozen bats, bat flies, and bat mites stored in our laboratory, which were sampled for an ongoing programme aiming at identifying pathogens in bats, were used for the analysis of Bartonella. These bats were collected with mist nets, which were settled near the entrance of karst caves at sunset when bats left roosts for night feeding, and bats were collected in the next early morning. Captured bats were put in a bag, sacrificed by inhaling of ethyl ether in the field, and then transported back to the laboratory on ice as soon as possible. Once arrived at the laboratory, bats were checked for ectoparasites with for- ceps, with bat flies in the fur and bat mites on the membrane wings. After collection of ectoparasites, thoracic and abdominal organs of bats were collected. All the specimen were stored at -80°C until use. Bat species were preliminary identified by morphology, and then confirmed by polymerase chain reaction (PCR) amplification and sequencing of the cytochrome B (cytB) gene as described previously (Li et al., 2021). Bat flies and bat mites were molecularly identified by the cytochrome oxidase subunit I (COI) and mitochondrial 16S rRNA genes (Bruyndonckx et al., 2009) (Table S2; Castro et al., 2002; Folmer et al., 1994).

### 2.3 Molecular detection of Bartonella in bats, bat flies, and bat mites

DNA was extracted from bat flies, bat mites, and bat liver tissue samples using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, Spain). Bartonella were screened by PCR amplification and sequencing of gltA, rpoB, and ftsZ genes as described previously (Bai et al., 2015; Gonçalves-Oliveira et al., 2020) (Table S2).

The PCR reaction was performed in a 50 μl mixture containing 0.25 μl 5 U/μl TaKaRa Ex Taq (TaKaRa, Shiga, Japan), 5 μl 10x Ex Taq buffer, 4 μl 25 mM MgCl2, 4 μl 2.5 mM dNTP mixture, 5 μl 10 μM each forward and reverse primer (Sangon Biotech, Shanghai, China), 27.75 μl nuclease-free water, and 5 μl sample DNA. PCR was performed with one denaturation cycle at 95°C for 5 min followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s, and an additional final cycle at 72°C for 10 min. Each PCR assay included nuclease-free water as a negative control. PCR products were analyzed by 1.2% agarose gel electrophoresis. Bands of expected size were excised from the gels, and purified using a Gel Extraction Kit (TSINGKE Biological Technology, Wuhan, China). The purified amplicons were cloned into the pMD19-T vector (TaKaRa, Shiga, Japan), and at least three positive clones were selected for sequencing with the universal primers M13-47/ M13-48. Chromatograms were checked with Chromas 2.5.1 (Technelysium, Tewantin, QLD) to ensure sequencing accuracy. The sequences were compared with those in public databases using the Nucleotide BasicLocal Alignment Search Tool (BLASTn) on the website of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

### 2.4 Phylogenetic analysis

Sequences of interest were imported into MEGA 7.0, and primers were removed after alignment with ClustalW. Phylogenetic trees were constructed based on the neighbour-joining method using the Kimura 2-parameter model in MEGA 7.0, and bootstrap values were calculated using 1000 replicates.

### 2.5 Statistical analyses

Since information on the age, sex, reproduction status, or ectoparasite intensity of bats was not collected in this study, statistical analyses were not performed for these factors. Chi-squared test and Fisher’s exact test were used to evaluate the Bartonella prevalence by bat species, and differences were statistically significant if $p$-values < .05.

## RESULTS

### 3.1 Sampling and species identification of bats and their ectoparasites

Frozen bat tissue samples and bat ectoparasites used in this study were collected from bats sampled from three cities (Songzi, Jingmen, and Xianning) in Hubei Province, Central China, during May 2018 to July 2020. In each of the three cities, bats were sampled once from a single karst cave (Table 1).

In September 2019, bat flies were collected from 16 bats sampled from a karst cave in Jingzhou, Hubei Province, China. Initially, these...
bat flies were divided into 16 pools (bat flies from several bats were mixed as a pool). Based on the COI and 16S rRNA genes, 16 bat fly pools consisted of three bat fly species belonging to three genera (Nycteribia, Penicillidia, and Phthiridium) in the family Nycteribiidae. Based on the COI gene, the three bat fly species shared 98.2%, 98.3%, and 96.4% nucleotide identity with Penicillidia monoceros (GenBank accession number: MW590972). Nycteribia sp. (Korea) (GenBank accession number: MT362944), and Phthiridium hindlei (GenBank accession number: AB632568), respectively. Based on the 16S rRNA gene, the three bat fly species shared 98.2%, 98.5%, and 96.3% nucleotide identity with P. monoceros (GenBank accession number: AB632585), N. pleuralis (GenBank accession number: AB632581), and P. hindlei (GenBank accession number: AB632587), respectively. Therefore, the three bat fly species found in this study were identified as P. monoceros, Nycteribia sp. (Korea), and a potentially novel Phthiridium sp. (Figures 2 and 3, Table 1). The 16 bat hosts were identified as Myotis davidii (13) and Miniopterus schreibersii (3) based on the cytB gene.

Due to inability to identify bat flies and bat mites using morphological keys, molecular identification was performed to confirm their species. To meet the requirement of DNA extraction, for ectoparasites collected from Songzi in 2018, and Xianning in 2020, big bat flies were individually processed, while small bat flies and bat mites were randomly pooled for Bartonella analysis.

In May 2018, 15 big bat flies and one small bat fly pool (about five bat flies) were collected from six bats from a karst cave in Songzi City, Hubei Province, China. The six bats were identified as M. davidii (2) and Myotis adversus (4) based on the cytB gene. Based on the COI and 16S rRNA genes, the 15 big bat flies were identified as P. monoceros, and the one pool of small bat flies was identified as Nycteribia sp. (Korea) (Figures 2 and 3, Table 1).

In July 2020, 203 bats were sampled from a karst cave in Xianning City, Hubei Province, China. Based on the cytB gene, the 203 bats were identified as M. davidii (105), M. adversus (29), and Rhinolophus pusillus (69). Bat flies collected from these bats included 119 big bat flies and six small bat fly pools (approximately 20 bat flies per pool). Based on the COI and 16S rRNA genes, the 119 big bat flies were all identified as P. monoceros, and the small bat fly pools consisted of Nycteribia sp. (Korea) and Phthiridium sp. (Figures 2 and 3, Table 1).

Bat mites collected from bats sampled in 2020 were randomly divided into 11 pools (approximately 20 bat mites per pool). Phylogenetic analysis based on the COI and 16S rRNA genes showed that bat mites in this study included two species belonging to the genus Spinturnix and the genus Eyndhovenia in the family Spinturnicidae, respectively. Based on the COI gene, Spinturnix sp. of this study shared 90.1% nucleotide identity with S. myotis (GenBank accession number: FJ225911), and Eyndhovenia sp. of this study shared 90.3% nucleotide identity with Eyndhovenia euryalis (GenBank accession number: EU784906). Based on the 16S rRNA gene, Spinturnix sp. of this study shared 96.7% nucleotide identity with S. myotis (GenBank accession number: FJ225969), and Eyndhovenia sp. of this study shared 94.3% nucleotide identity with E. euryalis (GenBank accession number: EU784846). Therefore, Spinturnix sp. and Eyndhovenia sp.

### Table 1
**Sampling information and prevalence of Bartonella in bats, bat flies, and bat mites from Hubei Province, China**

| Sampling date | Sampling area | Sample type | Family | Species | Number of samples | Number of positive samples (%) | p-Value |
|---------------|---------------|-------------|--------|---------|------------------|-------------------------------|---------|
| May 2018      | Songzi, Hubei, China (30°17’N, 111°77’E) | Bat fly     | Nycteribiidae | Penicillidia monoceros | 15 | 10 (66.7) | – |
|               |               | Bat fly     | Vespertilionidae | Nycteribia sp. | 1’ | 1’ (100) | – |
|               |               | Bat fly     | Vespertilionidae | Myotis adversus | 4 | 2 (50.0) | – |
|               |               | Bat fly     | Vespertilionidae | Myotis davidii | 6 | 0 (0) | – |
| September 2019| Jingzhou, Hubei, China (30°35’N, 112°19’E) | Bat fly     | Nycteribiidae | Penicillidia monoceros | 16’ | 16’ (100) | – |
|               |               | Bat fly     | Nycteribiidae | Nycteribia sp. | 16’ | 16’ (100) | – |
|               |               | Miniopteridae | Miniopterus schreibersii | 3 | 0 (0) | – |
|               |               | Miniopteridae | Miniopterus schreibersii | 3 | 0 (0) | – |
| July 2020     | Xianning, Hubei, China (29°85’N, 114°30’E) | Bat fly     | Nycteribiidae | Penicillidia monoceros | 119 | 60 (50.4) | – |
|               |               | Bat fly     | Nycteribiidae | Nycteribia sp. | 119 | 6’ (100) | – |
|               |               | Bat fly     | Nycteribiidae | Phthiridium sp. | 119 | 6’ (100) | – |
|               |               | Miniopteridae | Miniopterus schreibersii | 3 | 0 (0) | – |
|               |               | Miniopteridae | Miniopterus schreibersii | 3 | 0 (0) | – |
|               |               | Bat mite    | Spinturnicidae | Spinturnix sp. | 11’ | 11’ (100) | – |
|               |               | Bat mite    | Spinturnicidae | Eyndhovenia sp. | 11’ | 11’ (100) | – |
|               |               | Rhinolophidae | Rhinolophus pusillus | 69 | 3 (4.3) | – |
| Total         |               |             |             |             | 397 | 130 (32.7) | – |

Note: Asterisk (*) represents pooled samples, and en-dash (-) indicates that no statistical analysis was performed.
identified in this study represented two novel species (Figures 2 and 4, Table 1).

3.2 Prevalence of Bartonella in bats, bat flies, and bat mites

*Bartonella* positive rates for bats collected in 2018, 2019, and 2020 were 20.0% (2/10), 18.8% (3/16), and 10.3% (21/203), respectively. For individual big bat flies (*P. monoceros*) collected in 2018 and 2020, *Bartonella* infection rates were 66.7% (10/15) and 50.4% (60/119), respectively. One small bat fly pool of 2018 (*Nycteribia* sp.), 16 bat fly pools of 2019 (*P. monoceros*, *Nycteribia* sp., and *Phthiridium* sp.), six small bat fly pools (*Nycteribia* sp. and *Phthiridium* sp.), and 11 bat mite pools (*Spinturnix* sp. and *Eyndhovenia* sp.) of 2020 were all positive for *Bartonella* (Figure 2, Table 1, and Table S3).

3.3 Molecular characterization of Bartonella in bats, bat flies, and bat mites

For pooled samples (bat flies and bat mites) and bats, all the samples were screened for *Bartonella* with the three genes (gltA, rpoB, and ftsZ). For individual big bat fly samples, they were firstly screened with the gltA gene. Based on the gltA gene, *Bartonella* identified in individual big bat flies clustered into only two groups. Therefore, representative samples were further selected for *Bartonella* characterization by the rpoB and ftsZ genes to reduce workload (Table S3). Phylogenetic analysis showed that bats, bat flies, and bat mites carried diverse novel genotypes of *Bartonella*, clustered with previously reported bat/bat fly-borne bartonellae. Based on the gltA gene, some *Bartonella* genotypes identified in this study were closely related to those identified in bats from China, as well as in bat flies from South Korea. Based on the rpoB gene, some *Bartonella* genotypes identified in this study were clustered with those identified in bats from other places of China as well as from other countries such as Georgia and Japan. Based on the ftsZ gene, some *Bartonella* genotypes identified in this study were clustered with bat-borne *Bartonella* from China, Georgia, Japan, and Kenya (Figures 5–7).

In 2003, La Scola et al. (2003) proposed a gene-sequence-based criterion for species identification of *Bartonella*: Bartonella isolates should be considered as a new species if the gltA fragment (327 bp) and the rpoB fragment (825 bp) share less than 96.0% and 95.4% sequence similarity, respectively, to those of validated species. According to this criterion, bartonellae identified in bats and their ectoparasites (bat flies, and bat mites) in this study could be divided into seven and 13 *Bartonella* genotypes by gltA and rpoB, respectively, among which three *Bartonella* genotypes (II, V, and VII) defined by gltA, and 7 *Bartonella* genotypes (II, IV, V, VII, VIII, XII, and XIII) defined by rpoB represented novel genotypes of *Bartonella*. Based on the gltA gene, three, six, and three *Bartonella* genotypes were identified in bats, bat flies, and bat mites, respectively, and based on the rpoB gene, there were 4, 10, and 5 genotypes, respectively. Genotypes of *Bartonella* identified in bats (II, IV, and VIII by gltA; III, V, XI, and XIII by rpoB) were shared by their ectoparasites (bat flies, and bat mites). Some genotypes of *Bartonella* were identified in bat flies and bat mites, but not in bats in this study.
**FIGURE 3** Molecular identification and photos of bat flies. Phylogenetic trees based on the (a) COI and (b) 16S rRNA genes of bat flies, and photos of the dorsal and ventral sides of (c and d) *Penicillidia monoceros* and (e and f) *Nycteribia* sp. under an optical microscope.

(II, III, V by gltA; I, IV, VI, VII, VIII, IX, X, and XII by rpoB) (Figures 5 and 6).

Based on the 825 bp rpoB fragment, *Bartonella* genotype (XI) identified in this study in both bats and their ectoparasites (bat flies and bat mites) shared 95.1%–95.8% nucleotide identity with *B. mayotimonensis* (GenBank accession number: FJ376736), the causative agent of human endocarditis. It was close to the cutoff value (95.4%) by the gene-sequence-based criteria for *Bartonella* species definition for rpoB; therefore, *Bartonella* genotype (XI) could be identified as *B. mayotimonensis* (Figure 6).

### 3.5 Nucleotide sequence accession numbers

The representative sequences of this study were deposited in the GenBank database with the accession numbers: MH888178 and MW085077-MW085079 (bat cytB), MZ350230-MZ350231, MZ483869, and MZ483871-MZ483872 (bat fly COI), MZ502972-MZ502976 (bat fly 16S rRNA), MZ483874-MZ483876 (bat mite COI), MZ238086-MZ238089 (bat mite 16S rRNA), MZ208681-MZ208702 (*Bartonella* gltA) MZ208739-MZ208766 (*Bartonella* rpoB), and MZ208703-MZ208735 (*Bartonella* ftsZ).

### 3.4 Statistical analyses

Statistical analyses of *Bartonella* prevalence by bat species were performed for bats collected from a cave in Xiaining in 2020, and the *Bartonella* prevalence among the three bat species (*M. adversus*, *M. davidii*, and *R. pusillus*) was statistically significant ($\chi^2 = 8.031$, $p = .018 < .05$). Exactly, significant difference of the *Bartonella* prevalence was found between *M. davidii* and *R. pusillus* ($\chi^2 = 5.740$, $p = .017 < .05$).

### 4 DISCUSSION

Bats, bat flies, and bat mites were collected from Hubei Province in Central China for the detection of bartonellae. The positive rate of *Bartonella* in bats in this study was 3.9% (8/203; gltA) and 12.8% (26/203; gltA, rpoB, and ftsZ). Our previous study showed that the infection rate of *Bartonella* in bat blood from Shandong Province in East China was 25.2% (27/107) by gltA amplification (Han et al., 2017), and previous
FIGURE 4 Molecular identification and photos of bat mites. Phylogenetic trees based on the (a) COI and (b) 16S rRNA genes of bat mites (Spinturnicidae), and photos of the dorsal and ventral sides of (c and d) Spinturnix sp. under an optical microscope.

reports of Bartonella prevalence in bats were 83.2% (94/113) in Peru and 45% (36/80) in Belize by gltA amplification (Becker et al., 2018); 33.3% (21/63) in Costa Rica (Judson et al., 2015), 33.1% (39/118) in Guatemala (Bai et al., 2011), 24.1% (27/112) in Peru (Bai et al., 2012), and 30.2% (106/331) in Kenya (Kosoy et al., 2010) by blood culture. The prevalence of Bartonella in bats in this study was much lower than that reported in previous studies, which may be due to the use of liver tissue rather than blood samples, as Bartonella generally parasitize erythrocytes and endothelial cells (Dehio, 2001), blood samples should be the first choice for PCR screening (Kosoy et al., 2018).

4.1 The public health significance of bat-associated Bartonella

Based on the 825 bp rpoB fragment, the Bartonella genotype (XI) identified in this study in bats, bat flies, and bat mites shared 95.2%–95.7% nucleotide identity with B. mayotimonensis (GenBank accession number: FJ376736), which was close to the cutoff value (95.4%) of the gene sequence-based criteria for Bartonella species definition proposed by La Scola et al. (2003). While based on the 327 bp gltA fragment, Bartonella genotypes identified in this study (I and II) shared 91.2%–92.6% nucleotide identity with B. mayotimonensis (GenBank accession number: FJ376732), which was well below the cutoff value (96.0% by gltA). The inconsistency of the gltA and rpoB gene might be explained by recombination events as observed in rodent-borne bartonellae (Paziewska et al., 2011). Several studies reported the discovery of Bartonella species related to B. mayotimonensis in bats collected in the Northern hemisphere (Finland, the United States, the United Kingdom, France, and Spain) (Concannon et al., 2005; Lilley et al., 2017; Stuckey, Boulouis, et al., 2017; Veikkolainen et al., 2014). These bat-borne bartonellae related to B. mayotimonensis were characterized using a short gltA sequence (~327 bp). With the exception of Bartonella identified in bats from the United States that shared 99.7% nucleotide identity with
**Figure 5** Phylogenetic analysis of bat-borne *Bartonella* based on the *gltA* gene (327 bp). Phylogenetic tree was constructed using the neighbour-joining method with the Kimura 2-parameter model in MEGA 7.0. Bootstrap values more than 50% were indicated at nodes. Representative *Bartonella* sequences with hosts (bat, bat fly, and bat mite) from each sampling area (green: Songzi, Blue: Jingzhou and red: Xianning) were included in the analysis. Genotypes of *Bartonella* defined in this study are highlighted with grey box. Reference *Bartonella* sequences downloaded from GenBank were included in the analysis, and bat-derived *Bartonella* sequences are shown in bold.
**Figure 6**  Phylogenetic analysis of bat-borne *Bartonella* based on the *rpoB* gene (825 bp). Phylogenetic tree was constructed using the neighbour-joining method with the Kimura 2-parameter model in MEGA 7.0. Bootstrap values more than 50% were indicated at the nodes. Representative *Bartonella* sequences with hosts (bat, bat fly, and bat mite) from each sampling area (green: Songzi, blue: Jingzhou and red: Xianning) were included in the analysis. Genotypes of *Bartonella* defined in this study were highlighted with grey box. Reference *Bartonella* sequences downloaded from GenBank were included in the analysis, and bat-derived *Bartonella* sequences are shown in bold.
FIGURE 7 Phylogenetic analysis of bat-borne *Bartonella* based on the *ftsZ* gene (885 bp). Phylogenetic tree was constructed using the neighbour-joining method with the Kimura 2-parameter model in MEGA 7.0. Bootstrap values more than 50% were indicated at the nodes. Representative *Bartonella* sequences with hosts (bat, bat fly, and bat mite) from each sampling area (green: Songzi, Blue: Jingzhou and red: Xianning) were included in the analysis. Reference *Bartonella* sequences downloaded from GenBank were included in the analysis, and bat-derived *Bartonella* sequences are shown in bold.
Novel bat fly and bat mite species

Bat flies and bat mites as potential vectors and reservoirs of Bartonella

As described in previous reports (Judson et al., 2015; Sándor et al., 2018; Szubert-Kruszyńska et al., 2019), some Bartonella genotypes were shared by bats and their ectoparasites in this study. Bartonella genotypes identified in bats included I, IV, and VII by gltA, and III, V, XI, and XIII by rpoB, and these genotypes were also detected in bat flies and bat mites. Although the sharing of Bartonella among bats and their ectoparasites could be a result of bloodsucking, more and more evidence suggested that bat flies and bat mites were probably not simple carriers of Bartonella. A recent study showed that the ectoparasites intensity of bats was positively correlated with Bartonella infection, indicating the possible role of these ectoparasites as vectors of Bartonella (Stuckey, Chomel, et al., 2017). In addition, previous studies reported the isolation of Bartonella from bat flies (Billetter et al., 2012; Nabeshima et al., 2020), confirming the viability of Bartonella in bat flies, and highlighting the possibility of bat flies as vectors of Bartonella.

The genetic diversity of bartonellae identified in bat ectoparasites was higher than that in bats in this study, which was also reported in a previous study (Judson et al., 2015). Bat fly/bat mite-unique Bartonella genotypes of this study clustered with bartonellae found in bats in previous studies, it was unlikely that they were symbiotic bacteria of bat flies and bat mites. The absence of some Bartonella genotypes in bats might be partially explained by the low detection rate of Bartonella in bats in this study due to the use of liver tissue rather than blood samples for Bartonella detection, or due to the immune response of bats to clear certain Bartonella species. Moreover, bat flies have evolved a unique reproductive strategy, viviparous pupation: The larval stage develops within a female bat fly, and when internal development is complete, a larva is laid, and it immediately forms a puparium, from which an unfed adult fly emerges after a pupal stage (Figure S1) (Dick & Dittmar, 2014). During the internal developmental stages, the larva feeds on the ‘milks’ glands, which may facilitate vertical transmission of some bacteria, including Bartonella (Szentiványi et al., 2019). A previous study reported the identification of Bartonella in female bat flies and their pupae, indicating vertical transmission of Bartonella across developmental stages (Morse et al., 2012), and suggesting the potential roles of bat flies as reservoirs of bartonellae.

Studies on the role of bat mites in the maintenance and transmission of bartonellae were scarce, and a study in Poland reported the presence of identical Bartonella genotypes in bats and their mites (Szubert-Kruszyńska et al., 2019). The role of bat flies and bat mites for Bartonella will be better elucidated with epidemiological investigation of Bartonella across all life stages of these ectoparasites and laboratory infection experiments.

Although almost all Bartonella genotypes identified in bats in this study were found in bat flies and bat mites, there were still some Bartonella genotypes unique to bats (Figure 7). The role of other ectoparasites such as hard ticks, soft ticks, fleas, and bugs in the transmission of Bartonella in the bat population should be investigated. Interestingly, although bartonellae are generally considered as vector-borne bacteria, they were also identified in the saliva and faeces of bats, indicating that biting and faecal exposure might also contribute to the transmission and maintenance of bartonellae among bat population (Becker et al., 2018; Veikkolainen et al., 2014). Future studies on the infection dynamics of bartonellae will shed light on how these bacteria circulated among bats.

Novel bat fly and bat mite species

Bat flies are obligate bloodsucking ectoparasites that commonly parasitize in the fur and wing membranes of bats. Bat flies are divided into two families: the wingless, spider-like Nycteribiidae, and the more traditionally fly-like Streblidae (Dick & Patterson, 2006). Currently, the family Nycteribiidae consists of 275 species in 21 genera, and the family Streblidae consists of 227 species in 31 genera. The family Nycteribiidae has a worldwide distribution, while the family Streblidae is mainly found in Western Hemisphere (Szentiványi et al., 2019). Based on the COI and 16S rRNA genes, three bat fly species belonging to three genera (Nycteribia, Penicillidia, and Phthiridium) in the family Nycteribiidae were identified in this study, with Phthiridium sp. as a potentially novel bat fly species.

Mites of the family Spinisciidae are highly specialized bat ectoparasites. The taxonomy of Spinisciidae was mainly based on morphology, and molecular identification of this family was explored by Bryndonckx et al. (2009), making species identification of this family possible even for non-experts in morphology. The family Spinisciidae includes 12 genera. The genus Spinturnix is the most diverse of this family and includes more than 50 named species, most of which are associated with the Old World bats (Orlova et al., 2020). Currently, only two species are recognized in the genus Eynhovenia, E. euryalis, and E. brachypus, with the latter only morphology described from Rhinolophus rouxi bats in China (Luong et al., 2021, Yu-Mei Sun, 1986). Eight bat mite species of three genera (Spinturnix, Eynhovenia, and Paraperiglischrus) in the family Spinisciidae were morphologically described in China, with four species in the genus Spinturnix (Spinturnix psi, S. myoti,
S. sinicus, and S. kolenatoidei) (Rui-Yu Ye, 1996; Tian, 2009; Yu-Mei Sun, 1986). Bat mites of this study were molecularly identified as two species in the family Spinturnicidae, with one species belonging to the genus Spinturnix, and the other to the genus Eyndhovenia. Whether the Spinturnix sp. identified in this study is S. sinicus or S. kolenatoidei, or just another novel species, and whether the Eyndhovenia sp. identified in this study is the E. brachypus or another novel species need further study. The combination of morphological characteristics and molecular data will get these bat mites better defined in the future.

Due to the lack of knowledge on the morphology of bat flies and bat mites at the beginning of the study, they were not pooled by species for Bartonella analysis, which left a gap in understanding the host-vector specificity of Bartonella. However, differences in the Bartonella genotypes carried by different bat fly species were observed in this study. Bartonella genotypes I and VII by gltA; VII and XI by rpoB) were identified in the big bat fly (P. monocrates), while for the small bat fly pools (Nyciterbia sp., and Phthiridium sp.), Bartonella genotypes II, III, VI, and VII by gltA; X and VI by rpoB) were identified (Table S3). Further studies will be needed to reveal the roles of each bat fly or mite species in the transmission and maintenance of Bartonella.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Funds of China (grant number: 81971939), the China Postdoctoral Science Foundation Funded Project (grant number: 2019M662720), and the Innovation Research of Hubei Postdoctoral Science and Technology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/

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How to cite this article: Han, H.-J., Li, Z.-M., Li, X., Liu, J.-X., Peng, Q.-M., Wang, R., Gu, X.-L., Jiang, Y., Zhou, C.-M., Li, D., Xiao, X., & Yu, X.-J. (2022). Bats and their ectoparasites (Nycteribiidae and Spinturnicidae) carry diverse novel Bartonella genotypes, China. Transboundary and Emerging Diseases, 69, e845–e858. https://doi.org/10.1111/tbed.14357