Pathogenic *Leptospira* Species in Insectivorous Bats, China, 2015

Hui-Ju Han, Hong-Ling Wen, Jian-Wei Liu, Xiang-Rong Qin, Min Zhao, Li-Jun Wang, Li-Mei Luo, Chuan-Min Zhou, Ye-Lei Zhu, Rui Qi, Wen-Qian Li, Hao Yu, Xue-Jie Yu

PCR amplification of the *rrs2* gene indicated that 50% (62/124) of insectivorous bats from eastern China were infected with *Leptospira borgpetersenii*, *L. kirschneri*, and several potentially new *Leptospira* species. Multilocus sequence typing defined 3 novel sequence types in *L. kirschneri*, suggesting that bats are major carriers of *Leptospira*.

Leptospirosis is a zoonotic disease caused by the pathogenic spirochetes of the bacterial genus *Leptospira* (*1*). Although leptospirosis is mainly prevalent in tropical and subtropical countries (*2*), it is considered an emerging or reemerging zoonosis of global public health concern (*1*). In China, leptospirosis is listed as a category B notifiable disease (*3*). Globally, rodents are recognized as important reservoir hosts (*4*); however, a growing number of studies highlight the potential role of bats in the epidemiology of *Leptospira* (*4*). So far, knowledge on *Leptospira* in bats in China is lacking. Therefore, we screened archived bat kidney samples for *Leptospira* species to evaluate the potential role of bats in the ecology of *Leptospira* in China.

The Study

During July–October 2015, we captured 124 insectivorous bats with nets in Mengyin County, Shandong Province, China; the bats were initially intended for viral metagenomic analysis. Details regarding anesthetization of bats and tissue sampling were described previously (*5*). We collected the kidneys, storing them at –80°C until analysis. We identified bat species by using PCR amplification and DNA sequencing of the cytochrome B (*cytB*) gene as described previously (*6*). The 124 bats were classified into 4 species of the Vespertilionidae family (26 *Eptesicus serotinus* bats from 2 farmers' houses, 30 *Myotis fimbriatus* bats and 10 *M. ricketti* bats from a city sewer, and 58 *Myotis pequinius* bats from a karst cave).

We designated bat kidney samples by using the abbreviation of Shandong plus the sample identification number (e.g., SD-49). We extracted DNA from bat kidney samples by using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. To identify the species of *Leptospira* in bats, we amplified the 16S rRNA gene (*rrs2*) by using nested PCR with primers Lepto 16S-1st-F, Lepto 16S-1st-R, Lepto 16S-2nd-F, and Lepto 16S-2nd-R (*7*). We cloned the *rrs2* PCR products (642 bp) into the pMD 19-T vectors (TaKaRa, Shiga, Japan) and randomly picked 1 colony for Sanger sequencing using M13 universal primers on both DNA strands.

On the basis of *rrs2* amplification results, 62 (50.0%) of 124 bats tested were positive for *Leptospira*. All the *E. serotinus* bats (0/26, 0.0%) were negative for *Leptospira*, whereas the *M. fimbriatus* bats (19/30 [63.3%]), *M. ricketti* bats (6/10 [60.0%]), and *M. pequinius* bats (37/58 [63.7%]) showed high rates of infection.

Using ClustalW with MEGA 7.0 (http://www.megasoftware.net), we aligned the *rrs2* sequences from this study with reference *Leptospira* species downloaded from GenBank. We constructed a neighbor-joining phylogenetic tree (also by using MEGA 7.0) (*8*). On the basis of the *rrs2* phylogeny, all *Leptospira* detected in bats in Mengyin County clustered into the pathogenic group and could be divided into ≥14 clades (clades A–N). Although clade H was most likely associated with *L. borgpetersenii*, the *Leptospira* detected in bats in Mengyin County were divergent from any known *Leptospira* species (Figure 1) and any previously published *Leptospira* sequences from bats (Figure 2); therefore, these organisms might represent potentially new *Leptospira* species. We deposited the 62 *rrs2* sequences of *Leptospira* in the Mengyin County bats into GenBank (accession nos. MF498596–657).

To characterize the *Leptospira* species detected in bats from Mengyin County, we attempted multilocus sequence typing (MLST) on 7 housekeeping genes (*glmU*, *pntA*, *sucA*, *tpiA*, *pfkB, mreA*, and *caiB*), as previously described (*9*). We assigned alleles for all 7 loci by using a publicly available *Leptospira* MLST website (https://pubmlst.org/leptospira) and defined sequence types (STs) by using the allelic profiles (*glmU-pntA-sucA-tpiA-pfkB-mreA-caiB*).

Because we conducted MLST using kidney DNA rather than DNA from isolates, the results were arduous to

Author affiliations: Wuhan University, Wuhan, China (H.-J. Han, J.-W. Liu, X.-R. Qin, M. Zhao, L.-J. Wang, C.-M. Zhou, R. Qi, X.-J. Yu); Shandong University, Jinan, China (H.-L. Wen, Y.-L. Zhu, W.-Q. Li); Shandong Center for Disease Control and Prevention, Jinan (L.-M. Luo); Fudan University, Shanghai, China (H. Yu)

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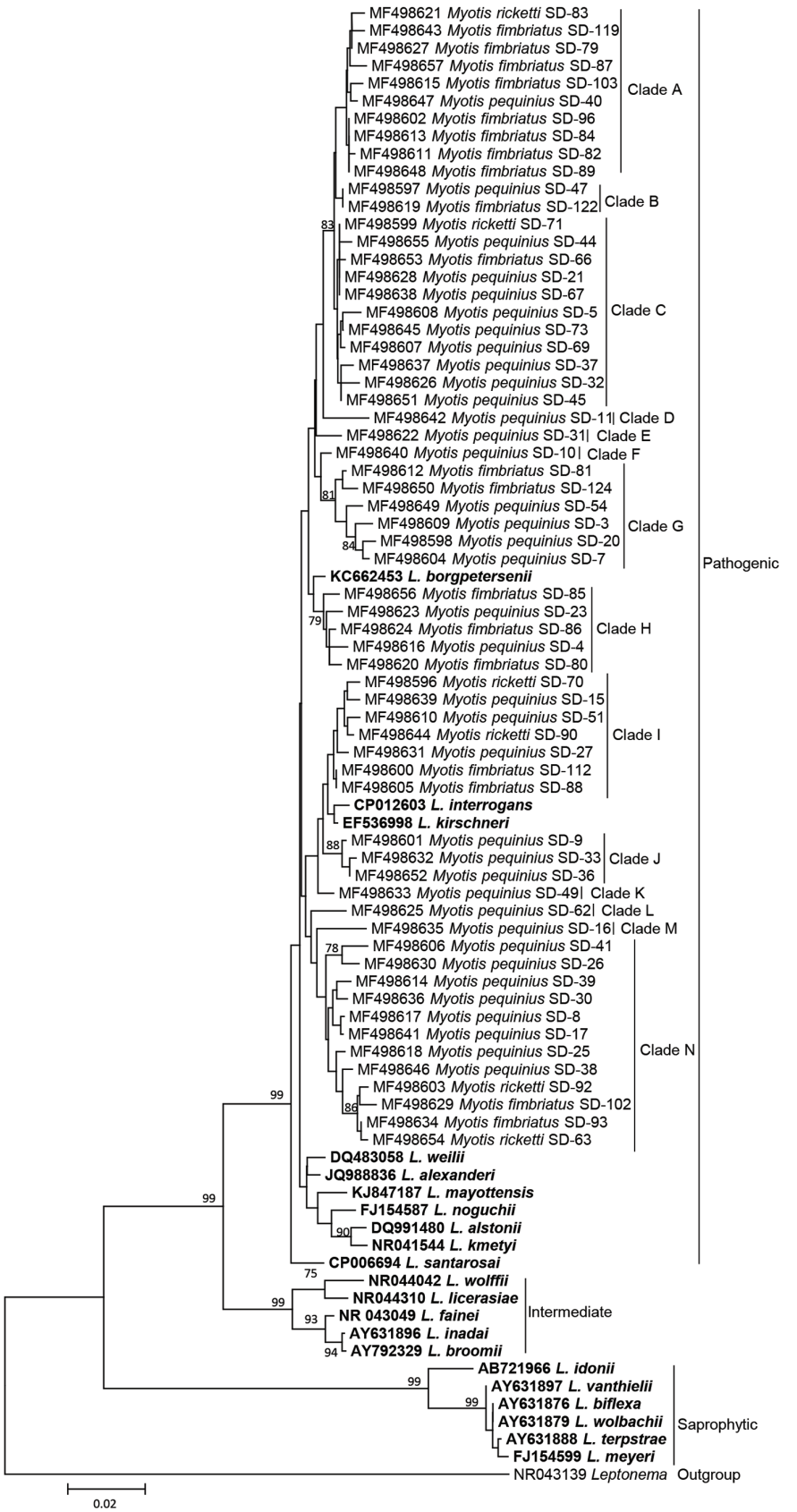

Figure 1. Neighbor-joining phylogenetic tree based on *rrs2* gene of *Leptospira* isolates from bats, Mengyin County, Shandong Province, China, and reference *Leptospira* sequences from GenBank (boldface). We constructed the tree with the *rrs2* sequences (642 bp) from this study by using the Kimura 2-parameter model with MEGA 7.0 (http://www.megasoftware.net); we calculated bootstrap values with 1,000 replicates. Sequences of *Leptospira* detected in bats in this study are shown with the GenBank accession number, the Latin name of the bat species in which *Leptospira* was detected, and the corresponding bat number. Only bootstrap values >75% are shown. Scale bar indicates nucleotide substitutions per site.

obtain. Only 35 of the 62 *rrs2*-positive bats were successfully amplified for ≥1 gene. For three samples, SD-49, SD-88, and SD-112, all 7 loci were obtained. We uploaded the allele data to the *Leptospira* MLST database and assigned a novel allele number for each gene (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/6/17-1585-Techapp1.pdf). According to the allelic profiles, we classified the organisms as *L. kirschneri*, assigning all 3 with novel STs (ST244 for SD-49, ST246 for SD-88, and ST245 for SD112).

We obtained 32 incomplete allelic profiles in all. After searching the *Leptospira* MLST database for each gene, we found that the loci of this study could not match with any known alleles and that they represented novel

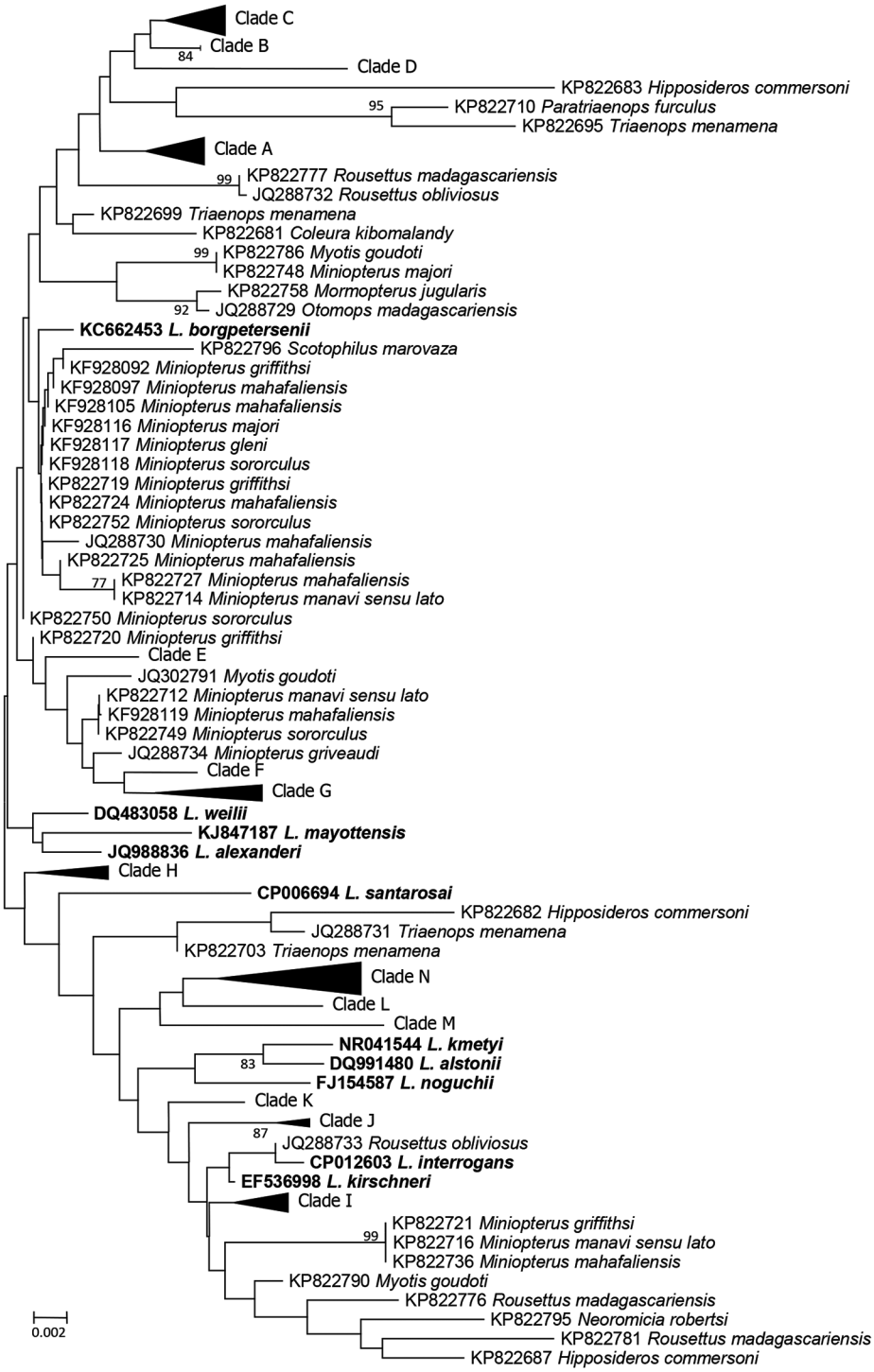

Figure 2. Neighbor-joining phylogenetic tree based on *rrs2* gene of pathogenic *Leptospira* isolates from bats, Mengyin County, Shandong Province, China, and reference *Leptospira* sequences from GenBank that had been previously isolated from bats (boldface). We constructed the tree with bat *Leptospira rrs2* sequences (446 bp) from this study and previous studies by using Kimura 2-parameter model with MEGA 7.0 (http://www.megasoftware.net); we calculated bootstrap values with 1,000 replicates. *Leptospira* detected in this study are shown with compressed clades, and bat *Leptospira* sequences from previous studies are shown with the GenBank accession number and the Latin name of the bat species in which *Leptospira* was detected. Only bootstrap values >75% are shown. Scale bar indicates nucleotied substitutions per site.

alleles; however, novel alleles could not be assigned for incomplete allelic profiles. Phylogenies based on each of the 7 genes showed inconsistent topologies for individual bats, indicating co-infection with different *Leptospira* species in the Mengyin County bats (online Technical Appendix Table, online Technical Appendix Figures 1–7). For SD-103, for example, we amplified 5 of the 7 genes. On the basis of *glmU*, *tpiA*, *pfkB*, and *mreA*, SD-103 clustered with *L. borgpetersenii*; however, on the basis of *sucA*, SD-103 fell into the group of the potentially new *Leptospira* sp. 2.

Because we did not conduct culture isolation with the archived bat kidney samples, further isolation with fresh bat kidney or urine samples will be needed to enable a thorough genotyping analysis of *Leptospira* species in the Mengyin County bats. Altogether, our study suggested that bats in Mengyin County carried a wide diversity of *Leptospira*, and the actual genetic diversity is likely even higher.

In our study, all the *E. serotinus* bats were negative for *Leptospira*, whereas *M. fimbriatus*, *M. ricketti*, and *M. pequinius* bats showed a high rate of infection. This finding might be explained by a widely accepted belief that *Leptospira* mainly circulate in humid environments (*10*). *E. serotinus* bats were captured from the eaves of 2 farmers' houses, where the habitats were dry, whereas the *M. fimbriatus*, *M. ricketti*, and *M. pequinius* bats were sampled from the humid city sewer and karst cave.

So far, *Leptospira* has been detected in ≈50 bat species belonging to 8 bat families from tropical and subtropical regions as well as part of Europe (*4*). Although the role of bats as carriers of *Leptospira* associated with human leptospirosis remains uncertain, intrusion into bat habitats and increasing urbanization that results in the cohabitation of bats and humans are likely to increase the opportunity for batborne *Leptospira* spillover (*11*). Moreover, bats might play an important role in the epidemiology of *Leptospira* through transmission between bats and rodents, with rodents being a major source of human infection (*12*).

Conclusions

Myotis spp. bats in Mengyin County in Shandong Province of China showed a high rate of infection with *Leptospira borgpetersenii*, *L. kirschneri*, and several potentially new *Leptospira* species, suggesting that bats are important carriers of *Leptospira* in Mengyin County. To date, knowledge of batborne leptospirosis is lacking, and the monitoring of the potential spillover of batborne *Leptospira* to humans is needed.

The collection of bats for microbiologic studies was approved by the Ethics Committee of Prevention Medicine of Shandong University (approval no. 20150501). Bats were captured with the help of the Mengyin County Center for Disease Control and Prevention. This study was supported by a grant from the National Natural Science Funds of China (grant no. 31570167).

About the Author

Ms. Han is a PhD candidate in the School of Health Sciences, Wuhan University. Her research interests include emerging infectious diseases and vectorborne diseases, especially batborne diseases.

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Address for correspondence: Xue-Jie Yu, School of Health Sciences, Wuhan University, Wuhan 430071, China; email: yuxuejie@whu.edu.cn

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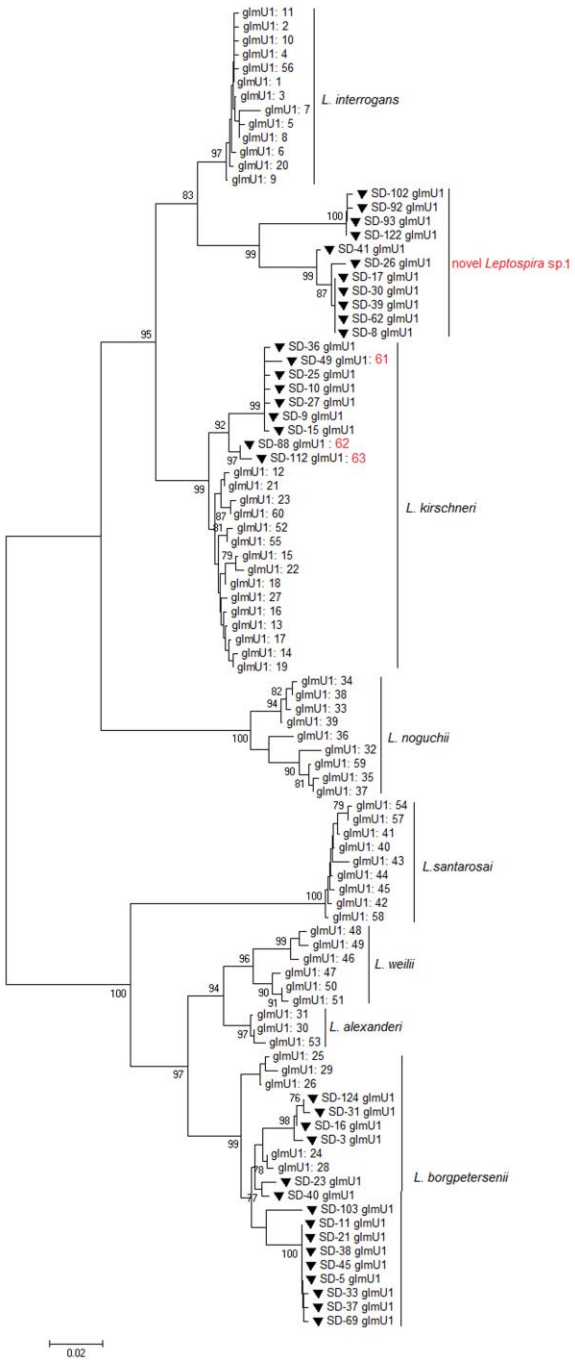
Technical Appendix

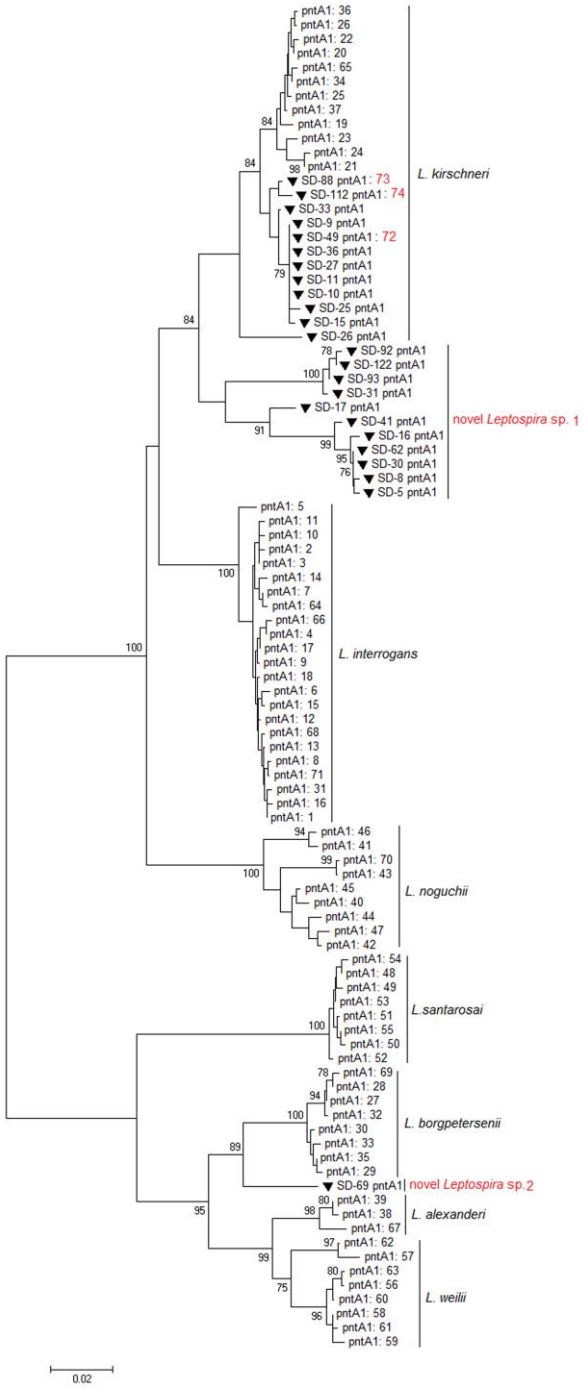
Technical Appendix Table. Multilocus sequence typing (MLST) of *Leptospira* in bats, Mengyin County, Shandong Province, China

Bat species	Bat no.	*glmU*	*pntA*	*sucA*	*tpiA*	*pfkB*	*mreA*	*caiB*	ST	Co-infection
M. fimbriatus	SD-88	*Lk* (62)	*Lk* (73)	*Lk* (68)	*Lk* (63)	*Lk* (88)	*Lk* (65)	*Lk* (60)	ST246	1
M. fimbriatus	SD-112	*Lk* (63)	*Lk* (74)	*Lk* (69)	*Lk* (64)	*Lk* (89)	*Lk* (66)	*Lk* (60)	ST245	1
M. fimbriatus	SD-124	*Lb*	—	*Lb*	—	—	—	—	—	1
M. fimbriatus	SD-122	N1	N1	N1	—	—	N1	—	—	1
M. fimbriatus	SD-93	N1	N1	N1	N1	N1	—	—	—	1
M. fimbriatus	SD-103	*Lb*	—	N2	*Lb*	*Lb*	*Lb*	—	—	2
M. fimbriatus	SD-102	N1	—	N1/N2	N1	N1/*Lb*	N1	—	—	3
M. ricketti	SD-92	N1	N1	N1/*Lb*	N1	N1	N1	—	—	2
M. pequinius	SD-49	*Lk* (61)	*Lk* (72)	*Lk* (67)	*Lk* (62)	*Lk* (87)	*Lk* (64)	*Lk* (59)	ST244	1
M. pequinius	SD-10	*Lk*	*Lk*	*Lk*	—	*Lk*	*Lk*	*Lk*	—	1
M. pequinius	SD-15	*Lk*	*Lk*	*Lk*	—	*Lk*	*Lk*	*Lk*	—	1
M. pequinius	SD-36	*Lk*	*Lk*	*Lk*	—	*Lk*	—	—	—	1
M. pequinius	SD-21	*Lb*	—	—	—	—	—	—	—	1
M. pequinius	SD-23	*Lb*	—	*Lb*	*Lb*	*Lb*	—	*Lb*	—	1
M. pequinius	SD-38	*Lb*	—	*Lb*	*Lb*	*Lb*	—	—	—	1
M. pequinius	SD-8	N1	N1	N1	—	—	N1	—	—	1
M. pequinius	SD-30	N1	N1	N1	—	N1	N1	—	—	1
M. pequinius	SD-62	N1	N1	N1	—	N1	N1	—	—	1
M. pequinius	SD-41	N1	N1	N1	N1	N1	—	—	—	1
M. pequinius	SD-39	N1	—	N1	—	N1	N1	—	—	1
M. pequinius	SD-25	*Lk*	*Lk*	*Lk*	—	*Lb*	—	*Lk*	—	2
M. pequinius	SD-9	*Lk*	*Lk*	*Lk*	*Lb*	*Lk*	*Lk*	*Lk*	—	2
M. pequinius	SD-27	*Lk*	*Lk*	*Lk*	*Lb*	*Lk*	*Lk*	*Lk*	—	2
M. pequinius	SD-3	*Lb*	—	*Lb*	—	N2	*Lb*	—	—	2
M. pequinius	SD-40	*Lb*	—	*Lb*	—	N2	*Lb*	—	—	2
M. pequinius	SD-5	*Lb*	N1	N1	—	*Lb*	—	—	—	2
M. pequinius	SD-69	*Lb*	N2	N2	—	*Lb*	*Lb*	—	—	2
M. pequinius	SD-33	*Lb*	*Lk*	N2	—	*Lb*	*Lb*	—	—	2
M. pequinius	SD-37	*Lb*	—	N2	—	*Lb*	*Lb*	—	—	2
M. pequinius	SD-45	*Lb*	—	N2	—	*Lb*	*Lb*	—	—	2
M. pequinius	SD-26	N1	*Lk*	N1	N1	N1	N1	*Lk*	—	2
M. pequinius	SD-31	*Lb*	N1	*Lb*	*Lb*	N2	N1	—	—	3
M. pequinius	SD-16	*Lb*	N1	*Lb*	—	N2	*Lb*	—	—	3
M. pequinius	SD-11	*Lb*	*Lk*	N4	—	—	—	*Lk*	—	3
M. pequinius	SD-17	N1	N1	N3	N1	N1	N1	*Lk*	—	3

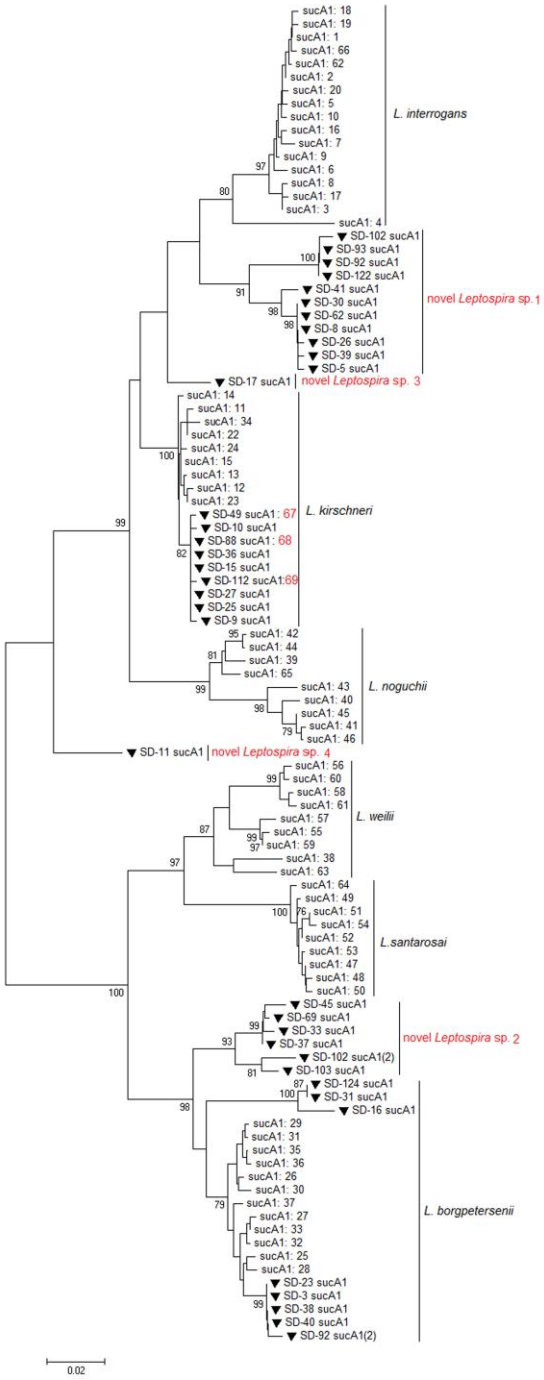
*For SD-49, SD-88, and SD-112, novel alleles assigned are shown in the parentheses. *Lb*, *L. borgpetersenii*; *Lk*, *L. kirschneri*; N1, potential new *Leptospira* sp. 1; N2, potential new *Leptospira* sp. 2; N3, potential new *Leptospira* sp. 3; N4, potential new *Leptospira* sp.



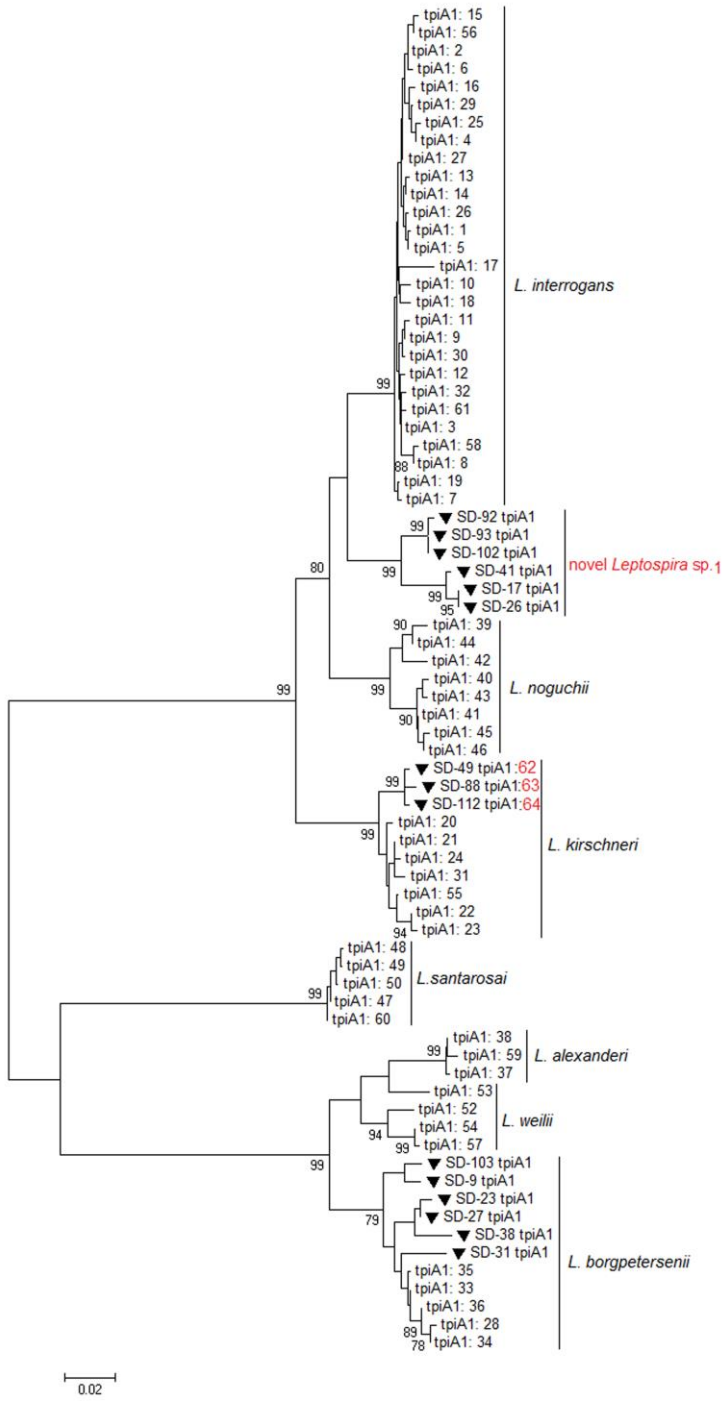
Technical Appendix Figure 1. Neighbor-joining phylogenetic tree for *glmU* gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura's 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.



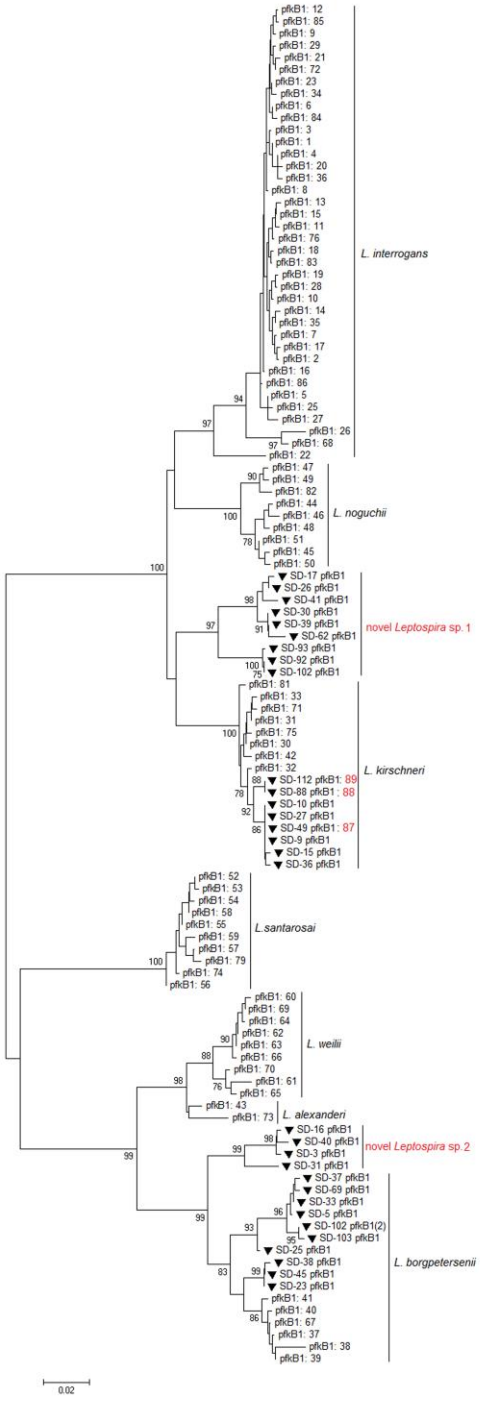
Technical Appendix Figure 2. Neighbor-joining phylogenetic tree for *pntA* gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura's 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.



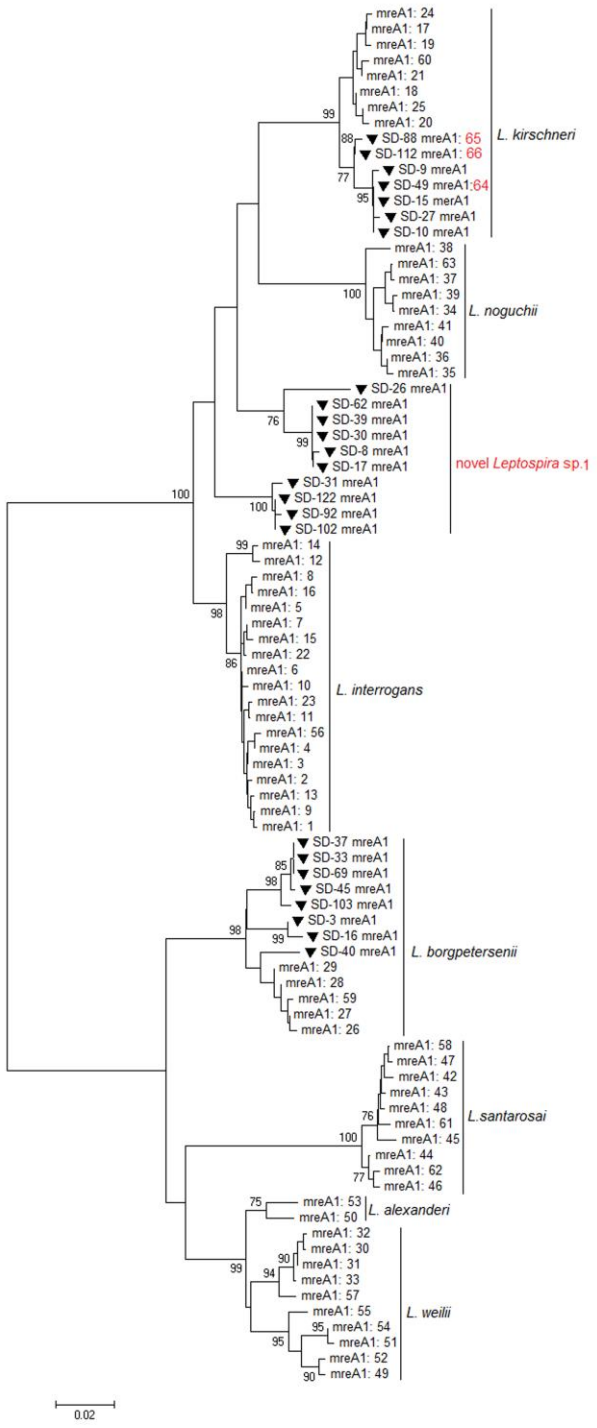
Technical Appendix Figure 3. Neighbor-joining phylogenetic tree for *sucA* gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura's 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.



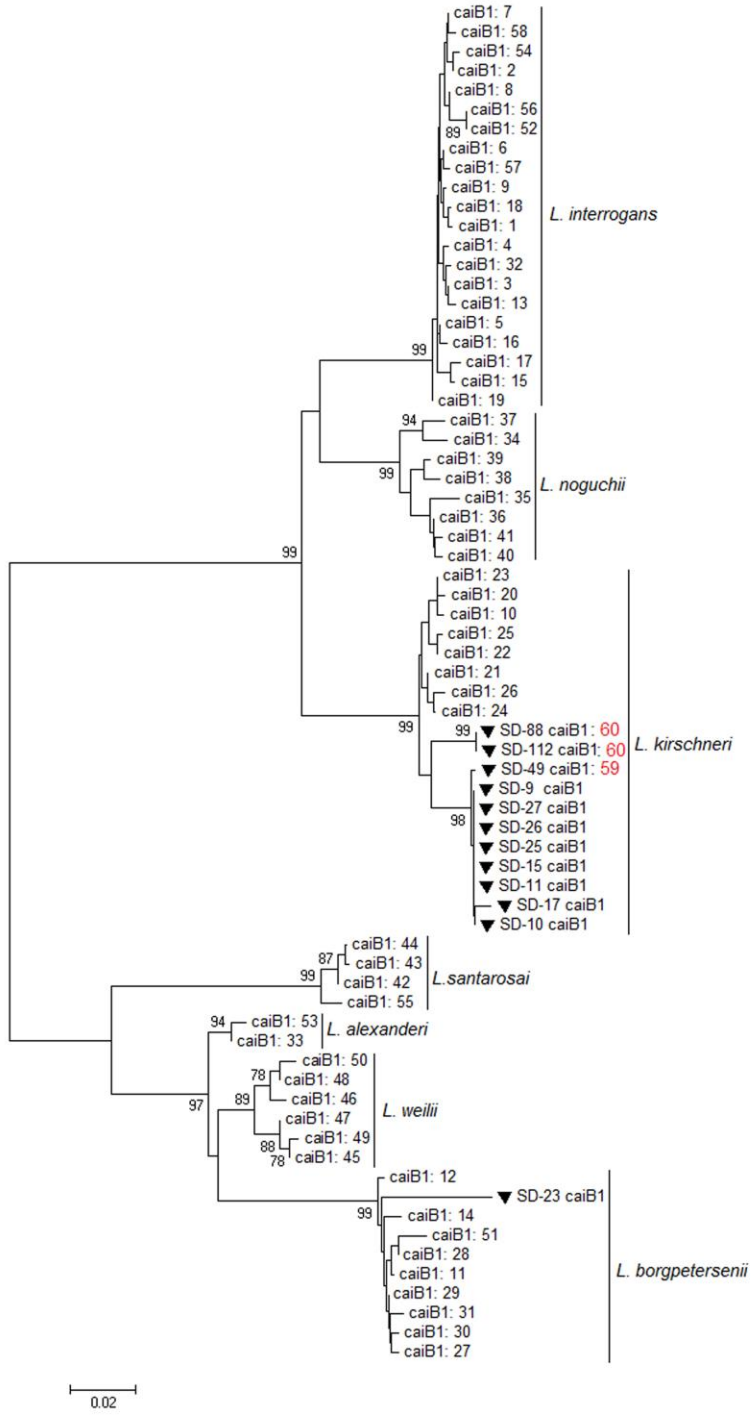
Technical Appendix Figure 4. Neighbor-joining phylogenetic tree for *tpiA* gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura's 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.



Technical Appendix Figure 5. Neighbor-joining phylogenetic tree for *pfkB* gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura's 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.



Technical Appendix Figure 6. Neighbor-joining phylogenetic tree for *mreA* gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura's 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.



Technical Appendix Figure 7. Neighbor-joining phylogenetic tree for *caiB* gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura's 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.