Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2026.117

RESEARCH ARTICLE

First Detection of *Rickettsia canadensis* and *Rickettsia heilongjiangensis* in China-Russia-DPRK Border areas

Guolu Yin[1†], Yunxiao Li[2†], Shengwei Ji[1†], Baoquan Huang[1†], Uday Kumar Mohanta[3], Shanshan Wang[1], Jinqi Wang[1], Ying Xu[1], Zhe Liu[1], Xin Jin[1], Jixu Li[4], Zhiqiang Xu[1], Yang Wang[1], Tianlong Han[5] and Shujiang Xue[1]*

[1]Preventive Veterinary Laboratory, College of Agriculture, Yanbian University, Yanji, 133002, Jilin, China; [2]College of Life Sciences, Shandong University, Jinan, 250100, Shandong, China; [3]Department of Microbiology & Parasitology, Sher-e-Bangla Agricultural University, Dhaka, 1207, Bangladesh; [4]Department of Parasitic Diseases, Yanbian Center for Disease Control and Prevention, Yanji, 133000, Jilin, China; [5]Da'an Municipal Animal Husbandry Administration Bureau, Da'an, 131300, Jilin, China. †Guolu Yin, Yunxiao Li, Shengwei Ji and Baoquan Huang contributed equally to this work.
*Corresponding author: sjxue@ybu.edu.cn

ARTICLE HISTORY (26-180)

Received: February 26, 2026
Revised: April 13, 2026
Accepted: April 14, 2026
Published online: April 17, 2026

Key words:
China-Russia-DPRK border
Rickettsia canadensis
Rickettsia heilongjiangensis
Tick-borne pathogens

ABSTRACT

Parasites of the rickettsial pathogens borne on ticks are an important factor of the human health problem particularly on wildlife border areas including the China-Russia-DPRK region. The paper evaluated the rate and spread of *Rickettsia* spp. across this area to design effective measures of control transboundary transmission. There were 1,961 questing samples of ticks collected altogether. The collected ticks were grouped based on the tick species, sex, collection localities and Environmental Variables once they have undergone morphological identification. The genomic DNA was extracted from each of the pools and screened for *Rickettsia* spp. by targeting the *gltA*, *ompA*, and *17-kDa* genes. PCR-positive amplicons were sequenced and analyzed by phylogenies. GLM showed that tick species and geographic location were the most significant factors driving *Rickettsia* positivity rates. Phylogenetic analysis identified two *Rickettsia* species, *Rickettsia canadensis* and *Rickettsia heilongjiangensis*, among the questing ticks found in this work. This paper is the first report based on the molecular data on presence of *R. canadensis* and *R. heilongjiangensis* in this border zone. These results highlight the urgency of increasing efforts of tick vector control to reduce transboundary disease spreading.

To Cite This Article: Yin G, Li Y, Ji S, Huang B, Mohanta UK, Wang S, Wang J, Xu Y, Liu Z, Jin X, Li J, Xu Z, Wang Y, Han T and Xue S, 2026. First detection of *Rickettsia canadensis* and *rickettsia heilongjiangensis* in china-russia-dprk border areas. Pak Vet J, 46(5): 1304-1312. http://dx.doi.org/10.29261/pakvetj/2026.117

INTRODUCTION

Tick-borne diseases (TBDs) are a major threat to public health. Non-specific initial signs of rickettsiosis tend to delay diagnosis and worsen the impact of the illness (Rymaszewska and Piotrowski, 2024). *Rickettsia* spp. are obligate intracellular Gram-negative bacteria responsible for the emerging zoonotic diseases in different parts of the world (Rymaszewska and Piotrowski, 2024). The main four groups of these pathogens transmit them through ticks, the spotted fever group (SFG), ancestral group (AG), typhus group (TG), and transitional group (TRG). Untreated SFG can be an increasing problem in global public health that may lead to fatal febrile disease. Many of the AG are probably not pathogenic or only mildly pathogeneous. TRG agents induce less severe fever and rash but require urgent medical attention (Sabour *et al.*, 2022). *Rickettsia* spp. can be divided into several pathogenic species of concern in the field of zoonoses, particularly *Rickettsia heilongjiangensis*, *Rickettsia rickettsii* among others. Such infections induced by the *Rickettsia* spp. commonly manifest with fever, rash, headache, and vasculitis and often may proceed to multiorgan dysfunction and fatal outcomes in both humans (Ivan *et al.*, 2022). SFG rickettsiae comprises *R. heilongjiangensis* whereas AG rickettsiae encompass *R. canadensis* (Salje, 2021). Molecular analysis has demonstrated the occurrence of *R. canadensis* in large geographical areas such as Canada, the United States, Russia, and some provinces in china (Xue *et al.*, 2023). Conversely, *R. heilongjiangensis* has an Asiatic distribution, modern records are recorded in Russia, Japan, and Thailand (Keirans and Litwak, 1989; Kasama *et al.*, 2019; Kim, 2022).

The study area encompasses the Tumen River Basin in northeastern Jilin Province, China, which shares ecological boundaries with Russia and the Democratic People's Republic of Korea (DPRK) (Li *et al.*, 2022). Livestock farming is a major economic pillar in this region, where ticks cause substantial direct and indirect economic losses. Cross-border movement of livestock and wildlife are important factors that fuel the spread of ticks and the disease caused by them around the world (Tsao *et al.*, 2021). But the absence of research is observed in this area regarding the prevalence of *Rickettsia* spp. In this case, we analyzed whether the place of gathering, identity of tick species and other possible drivers have an influence on the prevalence of *Rickettsia* spp. in the tri-border area. To identify potentially new types of rickettsiae, thus offering an opportunity to develop early warning and precise rickettsioses control measures in this ecosystem.

MATERIALS AND METHODS

Tick Collection and Identification: During May-July 2024, dragging method was used to collect 1,961 questing ticks across four border locations (Antu, Hunchun, Helong and Longjing) in border zones of China-Russia-DPRK. Species were identified morphologically and confirmed by sequencing mitochondrial *16S rRNA* and *COI* genes (Keirans and Litwak, 1989; Black and Piesman, 1994). The ticks were grouped by species, sex, sites and environmental factors (2–15 individuals/pool) kept at -80°C. They were stored as vouchers (No. JL-Tick-2024-0001-1961) at Yanbian University.

Sample Processing and DNA Extraction: Frozen ticks were removed from storage at -80°C and thawed on ice. Ticks were processed with 1% diluted commercial bleach for 30 s, followed by rinsing in three successive baths of DNA-free water for 1 min each (Binetruy *et al.*, 2019). Each tick pool was prepared by adding 800µL Minimal Eagle's Medium (MEM) and three steel beads before homogenization using Minilys Personal TGrinder H24 Homogenizer (TIANGEN® Biotech Co., Ltd., Beijing, China) (1,750 ×g, 40 s, three times). The homogenate was cleared by centrifugation at 3,000 ×g for 5 min at 4°C (Zhou *et al.*, 2021). Genomic DNA was extracted using the TIANamp Genomic DNA Kit (TIANGEN® Biotech Co., Ltd., Beijing, China), following the manufacturer's protocol. DNA purity (A260/280: 1.8–2.0) was verified by spectrophotometry.

Molecular Detection of *Rickettsia* spp: The presence of *Rickettsia* spp. was determined with the aid of PCR that targeted the citrate synthase gene (*gltA*), the outer membrane protein A gene (*ompA*), and the 17-kDa common antigen gene (*17-kDa*) (Labruna *et al.*, 2004; Kidd *et al.*, 2008; Han *et al.*, 2021). Amplification of PCR was done using the previously used methods (Paziewska *et al.*, 2011; Cheng *et al.*, 2016; Alieva *et al.*, 2020; Xue *et al.*, 2023). Each run consisted of both negative and positive controls. Positive control was derived by amplifying one *Rickettsia*-positive tick sample kept in our laboratory. Partial positive samples randomly chosen were sequenced by Sanger sequencing method. Further characterization of *Rickettsia* spp. was done by subjecting PCR-positive samples to multilocus sequence analysis (MLSA). Three protein-coding genes used were the RNA polymerase beta subunit (*rpoB*) and *gltA* and outer membrane protein B (*ompB*) for *R. canadensis*. In contrast, three protein-coding genes *gltA*, *ompA* and surface cell antigen 4 (*sca4*) were analyzed in *R. heilongjiangensis*. The obtained sequences were subjected to homology analysis using NCBI BLASTn.

Statistical Analysis: For an accurate assessment of the infection status in pooled samples. The estimation of individual level prevalence was performed through Minimum Infection rate (MIR) and Maximum Likelihood estimate (MLE) with 95% confidence interval (CI) in the R version 4.5.0 (R Core Team, 2024). To account for potential hierarchical clustering in tick populations and quantify specific ecological effects, drivers of infection were evaluated by applying Generalized Linear Models (GLMs) and Mixed Models (GLMMs) based on the glmmTMB package (Brooks *et al.*, 2017). Because highly correlated environmental variables can cause model overfitting, the measure of multi-collinearity was determined based on the Generalized Variance Inflation Factor (GVIF<3) (Fox and Monette, 1992). With the aim of identifying the most parsimonious ecological model and ensuring robust biological inferences, model selection was based on the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) and validation was done in 5-fold cross-validation, Area Under the Receiver Operating Characteristic (AUC-ROC) analysis as well as 1,000 bootstrap re-samplings (Efron and Tibshirani, 1993).

Phylogenetic Analysis: For *16S rRNA* and *COI* sequences, the best-fit model (GTR+F) was selected via ModelFinder (Kalyaanamoorthy *et al.*, 2017). The maximum likelihood phylogenies were inferred with IQ-TREE and 1,000 ultrafast bootstraps (Minh *et al.*, 2020). NCBI Refseq Database Genomic GBFF files were obtained. Five genes (*gltA*, *ompA*, *ompB*, *sca4*, and *rpoB*) were extracted from NCBI RefSeq using PhyloSuite (Zhang *et al.*, 2020), aligned with MAFFT (Katoh and Standley, 2013), and trimmed using Gblocks (Talavera and Castresana, 2007). Best-fit model (edge-linked) of each partition was selected using ModelFinder v2.2.0 based on AICc criterion: *gltA* (GTR+F+R3), *ompB* (GTR+F+G4), *rpoB* (GTR+F+R3), *ompA* (TIM3+F+R3), sca4 (GRR+F+G4). IQ-Tree maximum likelihood phylogenies were estimated using the edge-linked partition model under 1000 ultrafast bootstraps. Outgroup was *Nuttalliella namaqua* and *Candidatus Midichloria mitochondrii*.

RESULTS

Prevalence and infection rates of *Rickettsia* spp. in ticks: Five tick species have been identified within three genera among the ticks obtained in the samples of the China-Russia-DPRK border areas (Fig. 1). The initial estimate of infection had a significant variation depending on the region (P=0.004). Antu peaked at 45% (17/38) whereas Helong and Longjing reported lower values of 14% (Table 1).

Fig. 1: Map of border areas of China, Russia, and DPRK. Gray ranges represent sampling areas. Black dots represent sampling points.

Table 1: Statistical description of infection evaluation grouped by sampling site.

Characteristic	Antu N=38[1]	Helong N=49[1]	Hunchun N=77[1]	Longjing N=36[1]	P value[2]
Species					<0.001
D. silvarum	16 (42%)	0 (0%)	6 (7.8%)	5 (14%)	
H. longicornis	0 (0%)	0 (0%)	40 (52%)	0 (0%)	
H. concinna	14 (37%)	33 (67%)	10 (13%)	24 (67%)	
H. japonica	3 (7.9%)	5 (10%)	9 (12%)	6 (17%)	
I. persulcatus	5 (13%)	11 (22%)	12 (16%)	1 (2.8%)	
Sex					0.5
Female	21 (55%)	27 (55%)	38 (49%)	23 (64%)	
Male	17 (45%)	22 (45%)	39 (51%)	13 (36%)	
EnvChar					<0.001
Forest bushes	0 (0%)	16 (33%)	63 (82%)	0 (0%)	
Forest grassland	38 (100%)	33 (67%)	14 (18%)	36 (100%)	
Pool size	9.2±3.0	9.6±3.9	10.3±4.2	9.8±2.9	0.5
Height	608±132	701±57	254±148	520±129	<0.001
Temperature	20.9±3.6	13.1±0.6	20.8±2.9	23.9±1.4	<0.001
RelativeHumidity	0.66±0.07	0.54±0.01	0.66±0.03	0.67±0.02	<0.001
Preliminary evaluation of infection	17 (45%)	7 (14%)	22 (29%)	5 (14%)	0.004

[1] n (%); Mean±SD. [2] Pearson's Chi-Squared test; One-way analysis of means

Notes: N=pool count of Site; Sample count=1961; Pool count=200; Positive pool count=sum(PosPool); Preliminary evaluation of infection=Positive pool count / N.

The Minimum Infection Rate (MIR) and the Maximum Likelihood Estimation (MLE) estimates were used to determine individual prevalence by compensating pooling bias. MIR was 26.01/1,000 ticks whereas MLE was used to attain a single percentage of 2.95% (95% CI: 2.22 –3.83%).

Pool size was not significantly associated with infection positivity (P=0.790), indicating that the pooled sampling approach did not introduce detectable bias.

Preliminary assessment of *Rickettsia* spp. infection in ticks using generalized linear mixed models (GLMM)

Variable screening via variance inflation factor (VIF); Multicollinearity was assessed using variance inflation factors (VIFs; threshold=5). Temperature and relative humidity showed severe collinearity (VIF=10.27 and 11.28, respectively), and were strongly correlated (Pearson's r=0.96, Fig. 2a). The resulting combination of these variables as a composite Temp-RH index was then used in further models. With this change, all predictors exhibited satisfactory collinearity (VIF less than 3), and the final fixed effects design was determined as: ~ Sex+EnvChar+Pool size_z+Height_z+Temp RH index.

Optimization of GLMMs: Three models that are candidate GLMMs were used to compare and identify the best random-effect structure (Table 2). To select the best one, Model 3, having random intercepts of Site and Site:Species, was chosen because it had the lowest AIC (233.19) and non-singularity. Cross-validation was used to assess predictive performance. Mean AUC of the respective Model 1, Model 2, and Model 3 values were 0.615 (SD=0.127), 0.620 (SD=0.109), and 0.620 (SD=0.106). Even though Models 2 and 3 had equal mean AUC values, Model 3 was preferred as it had a lower value of AIC (233.19) and better stability. The residual diagnostics revealed that the model was well fitted (Fig. 2b).

Table 2: Comparison of candidate GLMM structures and predictive performance.

Model	Fixed Effects	Random Effects	AIC	Singular
Model 1	Sex+EnvChar+Pool size (z)+ Height (z)+Temp-RH index	(1 | Site)	234.27	FALSE
Model 2	Sex+EnvChar+Pool size (z)+Height (z)+ Temp-RH index	(1 | Site)+ (1 | Species)	234.33	FALSE
Model 3	Sex+EnvChar+Pool size (z)+Height (z)+Temp-RH index	(1 | Site)+(1 | Site: Species)	233.19	FALSE

Table footnote. AIC, Akaike information criterion; EnvChar, environmental characteristics; (z), variables were z-transformed before analysis; Temp-RH index, Temperature-Humidity Index; Site:Species, interaction between site and species.

Model-based analysis, interpretation and visualization of variables: The total McFadden R^2 for the model was 0.044. This value indicates a relatively low level of fit, reflecting limited explanatory power of the fixed effects. Based on the forest plot of the optimal model (Fig. 2c), the environmental variables (EnvChar) had an unmistakable negative correlation with *Rickettsia* positivity (Table 3). In contrast, Temp-RH index had a positive relationship, but this effect was not statistically significant (P>0.05). Sex, size of the pool and altitude were among some other factors that failed to have a statistically significant effect on the infection level. Such results indicate that the main determinants are environmental; however, the possible role of temperature and humidity should be explored through further modeling.

Table 3: Summary of regression model results for factors influencing *Rickettsia* positivity rates.

Term	Estimate	Std. Error	z value	P value	95% CI
EnvChar	-0.55	0.57	-0.96	0.336	[-1.67, 0.57]
Height (z)	-0.22	0.29	-0.76	0.447	[-0.78, 0.34]
Pool size (z)	-0.11	0.21	-0.54	0.590	[-0.53, 0.30]
Sex	0.02	0.36	0.06	0.950	[-0.68, 0.72]
Temp-RH index	0.17	0.87	0.19	0.847	[-1.54, 1.88]

Table footnote. Std. Error, standard error; CI, confidence interval; EnvChar, environmental characteristics; (z), variables were z-transformed before analysis; Temp-RH index, Temperature-Humidity Index.

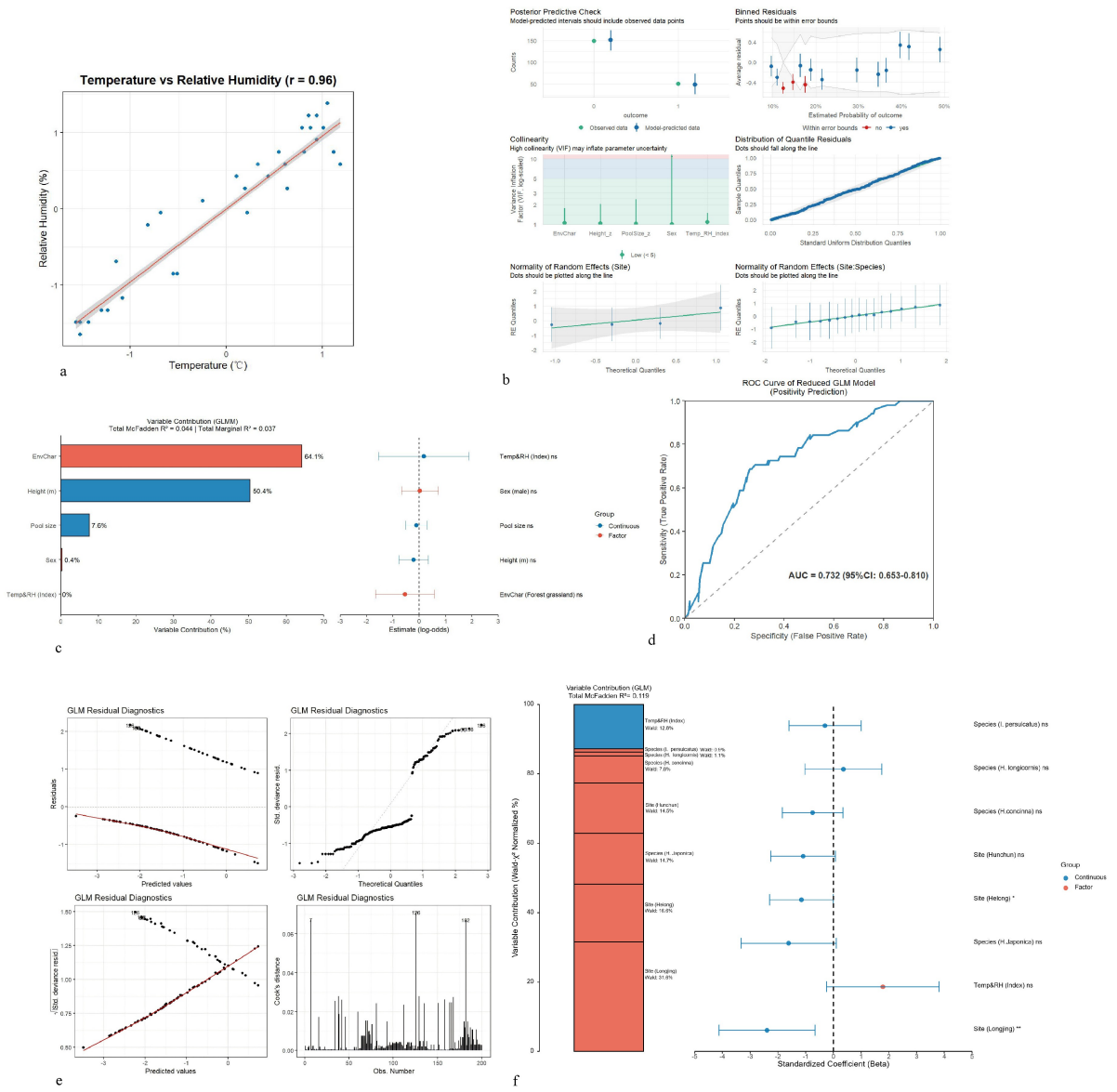

Fig. 2: Model construction, optimization, and diagnostic evaluation. (a): Correlation between standardized temperature and relative humidity. (b): Evaluation of the optimal model. (c): Variable importance and forest plot analysis of the optimal Generalized Linear Mixed Model (GLMM). (d): ROC curve and AUC value of the simplified model. (e): The residual evaluation for the optimal model. (f): Variable contribution and forest plot of the GLM model.

Marginal R^2 value across all grouped fixed effects was 0.037 with their contribution approaching zero (<0.01%). This result is similar to the overall McFadden R^2 of 0.044. In this framework, Site and Species have been identified as random effects and the rest are fixed effects. The negligible marginal R^2 values signify low explanatory power of the present fixed predictors. The random effects are the major components of the model, accounting for spatial and interspecific heterogeneity. Thus, random effects are largely responsible for the variability of *Rickettsia* infection rates.

GLM-based assessment of *Rickettsia* spp. infection status in ticks

Model definition and variable selection: To account for the interaction between environmental factors, a Temp-RH index was incorporated into the Generalized Linear Mixed Model (GLMM). This model structure was specified as: Formula: "*PosPool ~ Temp-RH index+Height+Pool size+Site+Species+Sex+EnvChar*".

The values of VIF were computed to determine the possible existence of multicollinearity between the predictors. The threshold of VIF<5 was used to help stabilize the estimates of the model and as a means of selecting variables.

The Generalized Variance Inflation Factor (GVIF) was used to test multicollinearity. The GVIF of the variable Site was 39.601, which is a high GVIF value compared to the traditional threshold of 10. It had an adjusted value of 1.846 and this indicated that there may be some level of collinearity between the various levels of the variable or its interaction with other predictors. On the contrary, the rest of variables were within acceptable limits. Their VIF values were always less than five, indicating no severe multicollinearity problems.

Validation of GLM after handling multicollinearity

Significance test of model coefficients: The statistical analysis showed that Longjing and *H. japonica* were core significant variables (P<0.05) (Table 4). In particular, the Longjing location was significantly associated with a decrease in the risk of infection by ticks (coefficient value=-2.372). Equally, *H. japonica* demonstrated significantly less infection risk compared to the reference species (coefficient value=-1.837). The Temp-RH index had no significant result (P=0.246) but it had a positive coefficient (1.483). Therefore, the variable kept capturing possible weak effects and this was compatible with the conclusion of GLMM.

Table 4: Results of the regression analysis for factors influencing *Rickettsia* positivity rates

Variable	Estimate	95% CI	P value
Intercept	1.248	(-0.415, 2.925)	0.139
Temp-RH index	1.483	(-0.974, 4.071)	0.246
Height	-0.401	(-1.172, 0.342)	0.297
Pool size	-0.228	(-0.655, 0.186)	0.283
Site			
Helong	-1.092	(-2.469, 0.185)	0.103
Hunchun	-1.875	(-4.099, 0.305)	0.092
Longjing	-2.372	(-4.402, -0.484)	0.017*
Species			
H. longicornis	0.416	(-0.965, 1.864)	0.560
H. concinna	-0.746	(-1.881, 0.390)	0.195
H. japonica	-1.837	(-3.880, -0.223)	0.040*
I. persulcatus	-0.826	(-2.408, 0.736)	0.300
Sex (Male)	-0.032	(-0.748, 0.678)	0.929
Environment (Forest grassland)	-0.370	(-1.825, 1.142)	0.619

Table footnote. CI, confidence interval; * P<0.05.

Model selection and validation: To determine the most parsimonious ecological model, two candidate Generalized Linear Models (GLMs) were constructed and compared: the full model (*PosPool ~ Temp-RH index+Height+Pool size+Site+Species+Sex+EnvChar*) and the simplified model (*PosPool ~ Temp-RH index+Site+Species*). Based on the evaluation, the simplified GLM was selected over the full model. The simplified model provided a better fit (AIC=218.01 vs. 223.65) and demonstrated superior calibration in the Hosmer-Lemeshow goodness-of-fit (GOF) test (P=0.555 vs. 0.194). Additionally, it maintained comparable discriminative ability, yielding an AUC of 0.732 (95% CI: 0.653-0.810) compared to the full model's AUC of 0.741 (95% CI: 0.661-0.821). The strong discriminative ability of the simplified model was also validated by the ROC curve (Fig. 2d). To ensure the robustness of this optimal model and reduce the likelihood of overfitting to a specific location, site-stratified 5-fold cross-validation was conducted, demonstrating consistent predictive performance and generalization across different geographic folds (Table 5). Additionally, internal validation using 1,000 bootstrap resamplings confirmed that the parameter estimates maintained a strong level of fitting consistency without overfitting the specific sample data (Table 6). Consequently, this rigorously validated optimal model was used to evaluate the specific ecological drivers of *Rickettsia* infection. The residual evaluation for the optimal model is detailed in the Fig. 2e.

Residual evaluation of the optimal model: Fitted models were compared using the goodness-of-fit test. The best model that included the Temp - RH index was characterized by the AIC value of 218.011 and BIC value of 247.695 and the model that did not contain the Temp - RH index had an AIC value of 219.305 and BIC value of 245.692. It means that the use of the Temp-RH index improves the fitness of the model and ensures the parsimony in the model statistics. Thus, the chosen optimal model is verified to be the most suitable.

Table 5: AUC metrics of simplified and full models evaluated via site-stratified 5-fold cross-validation

Model	Mean Train AUC	Mean Test AUC	SD Test AUC	Min Test AUC	Max Test AUC	Valid Folds
Simplified Model	0.7421	0.6617	0.0930	0.5344	0.7596	5
Full Model	0.7522	0.6431	0.0974	0.5063	0.7380	5

Table footnote. AUC, area under the receiver operating characteristic curve; SD, standard deviation.

Table 6: Internal validation of the final GLM coefficients using 1,000 bootstrap resamples

Statistic	Original Value	Bias	Std. Error
Intercept	0.7106	0.0336	0.6071
Temp-RH index	1.7828	0.2166	1.0744
Site			
Helong	-1.1511	-0.0438	0.6900
Hunchun	-1.0909	-0.0985	0.7629
Longjing	-2.3929	-0.2895	1.3893
Species			
H. longicornis	0.3623	0.0639	0.9447
H. concinna	-0.7487	0.0109	0.6851
H. Japonica	-1.6146	-2.0322	5.3731
I. persulcatus	-0.3037	0.0037	0.7782

Table footnote. Std. Error, standard error; Temp-RH index, Temperature-Humidity Index.

Ecological and environmental drivers of *Rickettsia* infection in ticks: The model examined the relationships between the infection of ticks by *Rickettsia* and the environmental variable (Temp-RH index), the sampling site, and the tick species. Site was found as the major predictor of infection risk (Table 7; Table 8). Even though Temp-RH index and Species did not reach statistical significance their observed trends were consistent with ecological predictions. Each predictor was displayed in a forest plot (Fig. 2f) representing its relative impact.

Table 7: Summary of GLM parameter estimates for factors associated with *Rickettsia* positivity rate

Term	Estimate	SE	Statistic	P value	Wald chi2	Wald %	Group
Temp-RH index	1.780	1.030	1.73	0.084	2.98	12.80	Continuous
Site							
Helong	-1.150	0.585	-1.97	0.049*	3.87	16.60	Factor
Hunchun	-1.090	0.595	-1.83	0.067	3.36	14.50	Factor
Longjing	-2.390	0.883	-2.71	0.007**	7.34	31.60	Factor
Species							
H. longicornis	0.362	0.707	0.513	0.608	0.263	1.13	Factor
H. concinna	-0.749	0.557	-1.34	0.179	1.80	7.77	Factor
H. japonica	-1.610	0.875	-1.85	0.065	3.41	14.70	Factor
I. persulcatus	-0.304	0.662	-0.459	0.647	0.210	0.906	Factor

Table footnote. SE, standard error; CI, confidence interval; Temp-RH index, Temperature-Humidity Index; * P<0.05, ** P<0.01.

Precisely, the infection risks in Longjing were much smaller in comparison to the reference site (OR=0.091, P<0.01). The same was also true with Helong, which had a significant decrease in infection risk (OR=0.316, P<0.05). Hunchun had a stable negative trend (OR = 0.336, P>0.05) it failed to achieve a statistically significant level. The observed infection odds of *H.*

japonica were less than that of the reference group (OR=0.199, P>0.05). The others, including *H. longicornis*, *H. concinna*, *I. persulcatus* showed odds ratios close to unity and large P values (0.179-0.647) indicating infection rates similar to the baseline. The effect of the Temp-RH index on *Rickettsia* infection did not reach statistical significance (OR=5.95, P>0.05).

In summary, sampling locations, types of ticks, and the Temp-RH indicator were the main sources of the model. Particularly, the risk of infection in the Longjing area was much less compared to the risk of infection in the reference area, whereas similar downward tendencies were detected in Helong and Hunchun. Although a positive coefficient was observed for the Temp-RH index, it lacked statistical significance (P>0.05).

Table 8: GLM Optimal Model Coefficient Table.

Variable	Estimate	SE	OR	95% CI	P value
Temp-RH index	1.780	1.030	5.950	0.873–52.780	0.084
Site					
Helong	-1.150	0.585	0.316	0.097–0.981	0.049*
Hunchun	-1.090	0.595	0.336	0.099–1.048	0.067
Longjing	-2.390	0.883	0.091	0.014–0.478	0.007**
Species					
H. longicornis	0.362	0.707	1.440	0.368–6.049	0.608
H. concinna	-0.749	0.557	0.473	0.158–1.424	0.179
H. japonica	-1.610	0.875	0.199	0.027–0.961	0.065
I. persulcatus	-0.304	0.662	0.738	0.198–2.722	0.647

Table footnote. SE, standard error; OR, odds ratio; CI, confidence interval; Temp-RH index, Temperature-Humidity Index; * P<0.05, ** P<0.01.

Sequence and phylogenetic analysis: Five tick species were found to have identities of 97.7-99.8% per cent to reference sequences in GenBank. The *H. concinna*, *H. longicornis*, *H. japonica*, *D. silvarum*, and *I. persulcatus* nucleotide identities to their respective sequences in GenBank were 98.7-99.4, 99.1-99.8, 97.7-99.6, 99.0-99.8 and 99.3-99.7%. Two distinct species of *Rickettsia* were detected in ticks through multi-locus sequence alignment in GenBank. *R. canadensis* exhibited 99.2-99.9% nucleotide identity, while *R. heilongjiangensis* exhibited 99.5-99.8% nucleotide identity to the respective sequences deposited in GenBank. Notably, both species were simultaneously detected in *H. concinna*. Phylogenetic analysis showed that *Haemaphysalis japonica* clustered with isolates from China (MG253031, NC_037246) and Japan (LC567919, LC567918). Additionally, *H. concinna* formed a distinct clade with Chinese isolates (OM368287, KY364906, NC_034785). *H. longicornis* grouped with isolates from Korea (PQ380094). *D. silvarum* was closely related to other Chinese isolates (OM368309, OM368310, NC_026552) and *I. persulcatus* clustered with Chinese isolates (OM368271, OM368270, KU935457, NC_004370). *Nuttalliella namaqua* from Africa was used as the outgroup (Fig. 3a). *R. canadensis* detected in this study formed a strongly supported clade along with Canadian (NC_009879) isolate (Fig. 3b). However, *R. heilongjiangensis* isolate in this study formed a monophyletic cluster together with a Japanese isolate (NZ_AP019864) (Fig. 3c).

All obtained sequences were deposited in GenBank under accession numbers (Table 9).

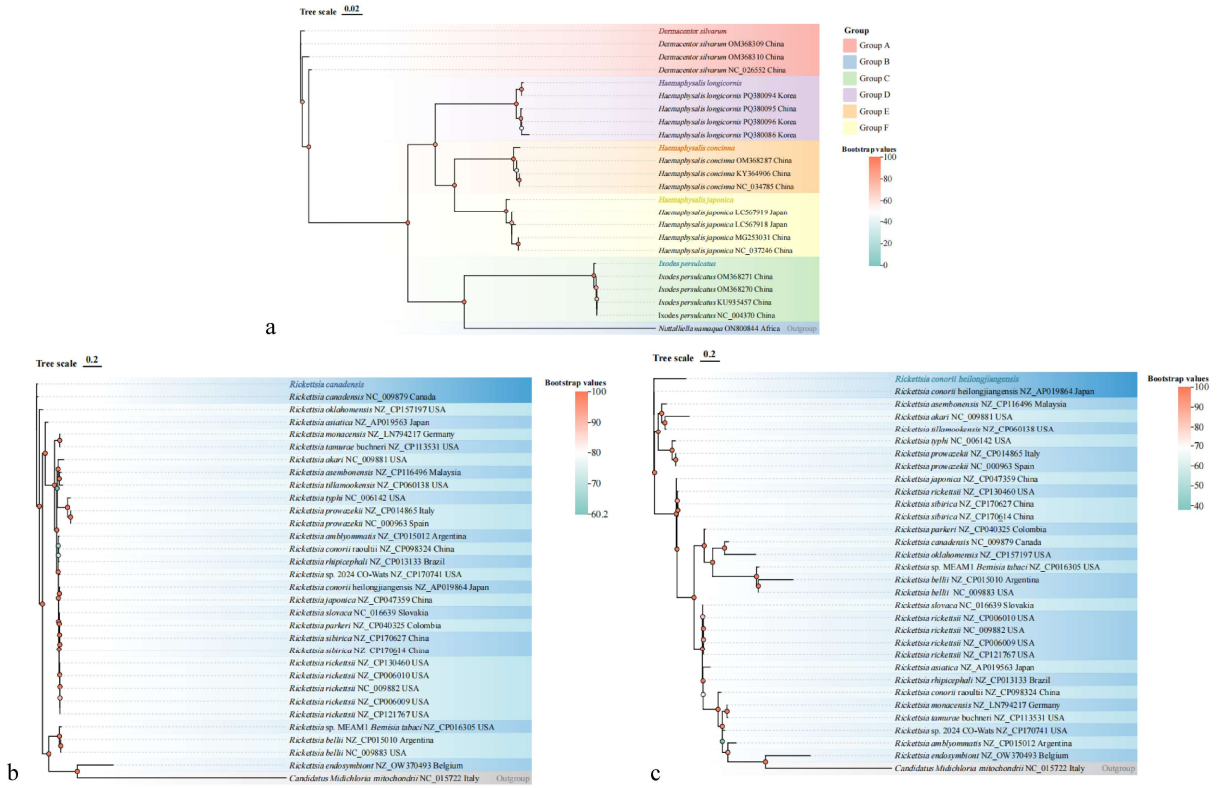

Fig. 3: Phylogenetic tree of samples included in this study. Use colored dots to visually indicate SH-aLRT / UFBoot support values directly on the phylogenetic-tree nodes. (a): Phylogenetic tree based on *16S rRNA* and *COI* sequences of ticks, *Nuttalliella namaqua* was used as the outgroup. (b): Phylogenetic tree based on concatenated *gltA*, *ompB*, *rpoB* sequences of *R. canadensis*. *Candidatus Midichloria mitochondrii* was used as the outgroup. (c): Phylogenetic tree based on concatenated *gltA*, *ompA*, *sca4* sequences of *R. heilongjiangensis*.

Table 9: GenBank accession numbers of mitochondrial and rickettsial sequences generated in this study

Species	Target gene	Accession No.
H. concinna	*COI*	PX435395
H. concinna	*16S rRNA*	PX287155
H. japonica	*COI*	PX435430
H. japonica	*16S rRNA*	PX287153
H. longicornis	*COI*	PX239504
H. longicornis	*16S rRNA*	PX285352
D. silvarum	*COI*	PX239602
D. silvarum	*16S rRNA*	PX287154
I. persulcatus	*COI*	PX239656
I. persulcatus	*16S rRNA*	PX435390
R. heilongjiangensis	*gltA*	PX454776
R. heilongjiangensis	*ompA*	PX454777
R. heilongjiangensis	*sca4*	PX454778
R. canadensis	*gltA*	PX454773
R. canadensis	*ompB*	PX454774
R. canadensis	*rpoB*	PX454775

DISCUSSION

Ticks infests a wide range of endothermic hosts, including humans, livestock, and wildlife, serving as a competent vector for multiple zoonotic pathogens (Peňazziová *et al.*, 2024; Shu *et al.*, 2024). Its broad host range and vector capacity pose significant threats to both human and animal health. The detection of *R. canadensis* and *R. heilongjiangensis* in ticks collected from the China-Russia-DPRK border region represents a significant expansion of their known geographical distribution.

Spatial variations suggest that infection risk is tightly linked to local ecological characteristics. For instance, the markedly reduced infection risk in Longjing may be attributed to a lower prevalence of *Rickettsia* in ticks or reduced tick population densities.

It is important to note that *I. persulcatus* is well-known as an important vector of *Rickettsia* spp. (Igolkina *et al.*, 2016). In contrast, our model was associated with no significant association (OR=0.738). This mismatch could be due to the local ecological diversity or the fact that the region under study has a low incidence of pathogen. Moreover, the small number of individuals in some taxa might have limited the statistical ability to identify interspecific differences.

The acquisition and passage of *Rickettsia* in tick could be conditioned by ideal hygrothermal parameters. As an example, high humidity reduces tick desiccation and promotes the growth of pathogens (Gray *et al.*, 2009). The findings of Temp-RH index are consistent with eco-physiological drivers of tick biology but must be tested at a bigger scale (Lindgren *et al.*, 2000; Dong *et al.*, 2020).

Major weakness of this research is that several variables, including Hunchun, *H. japonica*, and the Temp-RH index, exhibited marginal significance. To add more statistical power and reliability, especially in the underrepresented sites and taxa, future studies will further increase the sample size. Additionally, although the pooling strategy limits direct observation of exact individual prevalence, we addressed this by calculating a true prevalence of 2.95% using MLE. All pools were strictly monospecific, and GLM analysis confirmed that pool size did not significantly affect pool positivity (P=0.79), ensuring our ecological conclusions remain robust and unconfounded.

In comparison with Helong and Longjing, Antu showed an exceptionally high likelihood of infection indicating that it should become a key area of prioritization in case of surveillance against *Rickettsia*. Although the Temp-RH index showed a positive trend, it was not statistically significant. Its potential influence on infection risk should be interpreted with caution and requires further investigation. If the low-risk pattern of *H. japonica* is confirmed, it can provide some hints on the possibilities of reducing risks through interspecific replacement of dominant species. While our model identified significant ecological drivers, its explanatory power is relatively low (McFadden R^2=0.044; marginal R^2=0.037). In fact, this aligns with general ecological studies, where the mean variance explained typically ranges from only 0.025 to 0.054 (Møller and Jennions, 2002). Our model validation confirms that the identified predictors remain robust and biologically meaningful risk indicators.

The phylogenetic analysis indicated that the *R. canadensis* identified in the present study was highly related to the isolate found in Canada. This genetic similarity suggests potential bird-mediated dispersal (Silatsa *et al.*, 2020), though it remains speculative without direct host tracking data. Also, the DNA sequences of *R. heilongjiangensis* were grouped into clusters of sequences of Japan, implying possible epidemiologic relationships between such areas. Theoretically, the ecological connectivity of tri-national borders could facilitate cross-border pathogen spillage, but the specific role of avian and wildlife movement requires future empirical validation (Silatsa *et al.*, 2020; Wu *et al.*, 2021 and Kuno, 2024).

The PCR-based approach used in this work aiming at a variety of genetic loci has proven to be an efficient detection and species identification mechanism used in *Rickettsia*, laying a solid basis on which later examinations can be based on (Kuno, 2024). The fact that pathogenic *Rickettsia* has been confirmed as being present within border areas underlines the necessity of long-term surveillance to track tick infections in various environments and reservoir hosts.

Conclusions: The current research offers the initial molecular proof of *R. canadensis* and *R. heilongjiangensis* in ticks of the China-Russia-DPRK border zone, specifically, Jilin Province. The most significant contributors were the type of tick species and geographic location which altogether accounted to much of the variability of *Rickettsia* positivity rates. As we have found, there are considerable spatial differences. The incidence rate of infection in Longjing was much less than in the control area, whereas in the other two areas (Helong and Hunchun) it was also relatively low. Phylogenetic similarities suggest potential transcontinental and cross-border dispersal, the specific role of migratory birds remains a hypothesis requiring future empirical validation. These results indicate the necessity to strengthen the inter-country monitoring of tri-border illnesses caused by rickettsiosis in this ecologically vulnerable tri-country area.

Authors contribution: GY, YL, SJ, BH, SX planned, executed the research; All statistical analyses were

conducted by YL. UKM, SW, JW, YX, ZL, XJ, JL, ZX, YW, TH provided help to execute this project through materials and finances and also in the editing of the draft; All the members were actively involved in write up and editing of the manuscript.

Funding: This study was supported by grants from the Jilin Province Science and Technology Development Plan Item (20250601056RC), the Natural Science Foundation of Jilin Province (YDZJ202201ZYT616), the National Natural Science Foundation of China (32360886), and the Department of Education of Jilin Province (JJKH20250418).

Institutional review board statement: This research did not involve human subjects, human material, or vertebrate animals; therefore, no Institutional Review Board (IRB) or Institutional Animal Care and Use Committee (IACUC) approval was required.

Data availability statement: All relevant data are within the paper and its Supporting Information files. Assembled mitogenomes, alignments and phylogenetic trees are archived in Zenodo (https://doi.org/10.5281/zenodo. 17522980).

Biosafety: All sample processing was performed in a Biosafety Level 2 (BSL-2) laboratory in accordance with institutional biosafety guidelines.

Acknowledgments: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

REFERENCES

Alieva EE, Bondarenko EI, Maliy KD, *et al.*, 2020. The role of *Rhipicephalus sanguineus* ticks parasitizing dogs in the spread of tick-borne rickettsial pathogens in the city of Sevastopol. New Microbes New Infection 36:100704.

Black WC IV and Piesman J, 1994. Phylogeny of hard- and soft-tick taxa (*Acari: Ixodida*) based on mitochondrial *16S rDNA* sequences. Proceedings of National Academy of Science 91:0034-8.

Binetruy F, Dupraz M, Buysse M, *et al.*, 2019. Surface sterilization methods impact measures of internal microbial diversity in ticks. Parasites and Vectors 12:268.

Brooks ME, Kristensen K, van Benthem KJ, *et al.*, 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. The R Journal 9:378-400.

Cheng C, Fu W, Ju W, *et al.*, 2016. Diversity of spotted fever group *Rickettsia* infection in hard ticks from Suifenhe, Chinese-Russian border. Ticks Tick Borne Diseases 7:715-9.

Dong Y, Huang Z, Zhang Y, *et al.*, 2020. Comparing the climatic and landscape risk factors for Lyme disease cases in the Upper Midwest and Northeast United States. International Journal of Environmental Research and Public Health 17:1548.

Efron B and Tibshirani RJ, 1993. An introduction to the bootstrap. Chapman & Hall, New York, xvi+436 pp., ISBN 0-412-0423-2.

Fox J and Monette G, 1992. Generalized collinearity diagnostics. Journal of American State Association 87:178-83.

Gray JS, Dautel H, Estrada-Peña A, *et al.*, 2009. Effects of climate change on ticks and tick-borne diseases in Europe. Interdisciplinary Perspect of Infectious Diseases 2009:593232.

Han SW, Chae JB, Jo YS, *et al.*, 2021. First detection of *Borrelia* and *Rickettsia* species from *Ornithodoros* ticks in the Republic of Korea. Ticks and Tick Borne Diseases 12:101689.

Igolkina Y, Bondarenko E, Rar V, *et al.*, 2016. Genetic variability of *Rickettsia* spp. in *Ixodes persulcatus* ticks from continental and island areas of the Russian Far East. Ticks and Tick Borne Diseases 7:1284-9.

Ivan T, Matei IA, Novac CŞ, *et al.*, 2022. Spotted fever group *Rickettsia* spp. diversity in ticks and the first report of *Rickettsia hoogstraalii* in Romania. Veterinary Science 9:343.

Kalyaanamoorthy S, Minh BQ, Wong TKF, *et al.*, 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nature Methods 14:587-9.

Kasama K, Fujita H, Yamamoto S, *et al.*, 2019. Genomic features of *Rickettsia heilongjiangensis* revealed by intraspecies comparison and detailed comparison with *Rickettsia japonica*. Frontiers in Microbiology 10:2787.

Katoh K and Standley DM, 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30:772-80.

Keirans JE and Litwak TR, 1989. Pictorial key to the adults of hard ticks, family *Ixodidae* (*Ixodida*: *Ixodoidea*), east of the Mississippi River. Journal of Medical Entomology 26:435-48.

Kidd L, Maggi R, Diniz PP, *et al.*, 2008. Evaluation of conventional and real-time PCR assays for detection and differentiation of spotted fever group *Rickettsia* in dog blood. Veterinary Microbiology 129:294-303.

Kim HK, 2022. *Rickettsia*-host-tick interactions: knowledge advances and gaps. Infection and Immunity 90:e00621-21.

Kuno G, 2024. Mechanisms of yellow fever transmission: gleaning the overlooked records of importance and identifying problems, puzzles, serious issues, surprises and research questions. Viruses 16:84.

Labruna MB, Whitworth T, Horta MC, *et al.*, 2004. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of São Paulo, Brazil, where Brazilian spotted fever is endemic. Journal of Clinical Microbiology 42:90-8.

Li Y, Powell J, Jin A, *et al.*, 2022. Community attitudes towards Amur tigers (*Panthera tigris altaica*) and their prey species in Yanbian, Jilin province, a region of northeast China where tigers are returning. PLoS ONE 17:e0276554.

Lindgren E, Tälleklint L and Polfeldt T, 2000. Impact of climatic change on the northern latitude limit and population density of the disease-transmitting European tick *Ixodes ricinus*. Environment and Health Perspectives 108:119-23.

Minh BQ, Schmidt HA, Chernomor O, *et al.*, 2020. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. Molecular Biology and Evolution 37:1530-4.

Møller AP and Jennions MD, 2002. How much variance can be explained by ecologists and evolutionary biologists? Oecologia 132:492-500.

Paziewska A, Harris PD, Zwolińska L, *et al.*, 2011. Recombination within and between species of the alpha proteobacterium *Bartonella* infecting rodents. Microbial Ecology 61:134-45.

Peňazziová KL, Chitimia-Dobler L, Csank T, *et al.*, 2024. First detection and a new avian host of the tick *Ixodes ventalloi* Gil Collado, 1936, in Slovakia. Parasitology Research 123:268.

R Core Team, 2024. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rymaszewska A and Piotrowski M, 2024. *Rickettsia* species: genetic variability, vectors, and rickettsiosis-a review. Pathogens 13:661.

Sabour S, Azimi T, Nasser A, *et al.*, 2022. A global overview of the most important zoonotic bacteria pathogens transmitted from *Rattus norvegicus* to humans in urban environments. Infectious Medicine 1:192-207.

Salje J, 2021. Cells within cells: *Rickettsiales* and the obligate intracellular bacterial lifestyle. Nature Reviews in Microbiology 19:375-90.

Shu C, Intirach J, Zhou Y, *et al.*, 2024. Microbial community characteristics and pathogens detection in *Rhipicephalus sanguineus* and *Haemaphysalis hystricis* from Hainan Island, China. Frontiers in Microbiology 15:1450219.

Silatsa BA, Simo G, Githaka N, *et al.*, 2020. First detection of *Theileria parva* in cattle from Cameroon in the absence of the main tick vector *Rhipicephalus appendiculatus*. Transboundary Emerging Diseases 67:68-78.

Talavera G and Castresana J, 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Systemic Biology 56:564-77.

Tsao JI, Hamer SA, Han S, *et al.*, 2021. The contribution of wildlife hosts to the rise of ticks and tick-borne diseases in North America. Journal of Medical Entomology 58:1565-87.

Wu DL, Shih HC, Wang JK, *et al.*, 2021. Commensal rodent habitat expansion enhances arthropod disease vectors on a tropical volcanic island. Frontiers in Veterinary Science 8:736216.

Xue J, Chen SS, Jian R, *et al.*, 2023. Molecular evidence of *Rickettsia canadensis* in ticks, Hebei, China. Infection Genetics and Evolution 115:105506.

Zhang D, Gao F, Jakovlić I, *et al.*, 2020. PhyloSuite: An integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. Molecular Ecology Resources 20:348-55.

Zhou Q, Li J, Guo X, *et al.*, 2021. Records of three mammal tick species parasitizing an atypical host, the multi-ocellated racerunner lizard, in arid regions of Xinjiang, China. Parasites and Vectors 14:135.