Ticks and Tick-borne Diseases 10 (2019) 412–420

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Virome analysis of tick-borne viruses in Heilongjiang Province, China

Fei Meng[a,b,d,1], Meiming Ding[c,1], Zhizhou Tan[b], Zihan Zhao[b], Lin Xu[b], Jianmin Wu[a,d,*], Biao He[b,e,**], Changchun Tu[b,e,**]

[a] College of Animal Science and Technology, Guangxi University, Nanning, Guangxi Province, 530000, China
[b] Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Military Veterinary Institute, Academy of Military Medical Sciences, Changchun, Jilin Province, 130000, China
[c] Da Hinggan Ling Wildlife Conservation Center, Jiagedaqi, Heilongjiang Province, 165000, China
[d] Guangxi Key Laboratory of Veterinary Biotechnology, Guangxi Veterinary Research Institute, Nanning, Guangxi Province, 530001, China
[e] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou, Jiangsu Province, 225000, China

ARTICLE INFO

Keywords:
Ticks
Tick-borne viruses
Virome analysis
Greater Khingan mountains
China

ABSTRACT

Ticks are implicated in the transmission of various human and livestock pathogens worldwide. This study aimed to understand the geographical distribution of tick species, along with tick-associated viruses, in Heilongjiang Province, northeast China. Molecular methods were used to classify tick species, with next-generation sequencing and polymerase chain reaction-based analyses used to assess the viromes of ticks from four representative sampling locations in the Greater Khingan Mountains. Five species of ixodid ticks were identified, including *Ixodes persulcatus*, *Dermacentor nuttalli*, *Dermacentor silvarum*, *Haemaphysalis longicornis*, and *Haemaphysalis concinna*. From the 1102 ticks, 3,568,561 high-quality reads were obtained by next-generation sequencing. Following trimming, 302,540 reads were obtained, of which 6577 (2.16%) reads were annotated to viruses. Phylogenetic analysis revealed that the viral sequences shared a close relationship with *Orthonairovirus*, *Phlebovirus*, deer tick *Mononegavirales*-like virus, and Jingmen tick virus sequences, but the significance of these newly-identified tick-borne viruses to human and animal health requires further investigation. The results of this study provide a basis not only for further studies on the relationship between ticks and tick-borne viruses, but also for preventing future tick-borne epidemic outbreaks by means of vector control.

1. Introduction

Ticks transmit various bacterial, viral, and protozoan pathogens (Cheng et al., 2016; Diuk-Wasser et al., 2016; Firth et al., 2014; Lee et al., 2016) and are increasingly being identified as the vectors of human and animal diseases (Torres et al., 2012). The majority of tick-borne infections occur in farmers involved in animal husbandry or among people who live or work in forested areas. The most common of these infectious diseases include tick-borne encephalitis (TBE) (Kunze, 2016), Crimean-Congo hemorrhagic fever (CCHF) (Xia et al., 2011; Yadav et al., 2013), severe fever with thrombocytopenia syndrome (SFTS) (Suh et al., 2016) and spotted fever (Keskin et al., 2016). In 2009, several farmers bitten by ticks in Hubei and Henan provinces, China, later died from an uncharacterized hemorrhagic fever-like disease. The causative agent, a novel *Orthobunyavirus*, was identified in 2010 and later designated SFTS virus (SFTSV) (Zhang and Xu, 2016). From 2010 to October 2016, SFTS cases were reported in 23 provinces of China (Zhan et al., 2017). As it stands, tick-borne pathogens are increasingly threatening human and animal health worldwide. Therefore, as part of infectious disease prevention strategies, it is important to investigate the tick vectors as well as the viral spectrum of these ectoparasites to better predict potential outbreaks of tick-borne diseases.

Next-generation sequencing (NGS) technology has been widely applied in virology research (Shi et al., 2016) and its use in viral metagenomics is increasingly common (Bzhalava and Dillner, 2013). Using

* Corresponding author at: Guangxi Key Laboratory of Veterinary Biotechnology, Guangxi Veterinary Research Institute, Nanning, Guangxi Province, 530001, China.
** Corresponding authors at: Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Military Veterinary Institute, Academy of Military Medical Sciences, Changchun, Jilin Province, 130000, China.
E-mail addresses: heather_meng@hotmail.com (F. Meng), bbhcjck@163.com (M. Ding), tanzhizhou@gmail.com (Z. Tan), 348278640@qq.com (Z. Zhao), linxu0322@163.com (L. Xu), wu-jm20@163.com (J. Wu), heb-001001@163.com (B. He), changchun_tu@hotmail.com (C. Tu).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ttbdis.2018.12.002
Received 28 April 2018; Received in revised form 5 December 2018; Accepted 7 December 2018
Available online 17 December 2018
1877-959X/

this technology, the viromes of *Amblyomma americanum*, *Dermacentor variabilis* and *Ixodes scapularis* ticks from North America were characterized, identifying Powassan virus as well as eight novel tick-borne viruses (Tokarz et al., 2014). NGS was also used to investigate the presence of viruses in *Rhipicephalus* spp. ticks from Yunnan Province in southwest China, with resulting virus-related reads distributed across 24 virus families (Xia et al., 2015). Similarly, other researchers have applied the technique to identify novel viruses in *Ixodes ricinus* from France (Moutailler et al., 2016) and in *Dermacentor occidentalis* from the United States (Bouquet et al., 2017). Together, these studies show that metagenomic analysis can lead to a better understanding of the tick virome and help in the identification of emerging pathogens.

The Greater Khingan Mountains, bordering Russia and Mongolia in the northeast of China, have abundant natural resources, with a forest coverage rate of up to 74%. Because of its unique geographical features, this area has become a popular tourist destination. However, it is a natural epidemic focus for tick-borne encephalitis (Sun et al., 2017). With the expansion of human activities, the risk of natural focal diseases has increased. To prevent outbreaks of tick-borne diseases, it is important to understand the distribution of tick species and identify their natural habitats (Tijsse-Klasen et al., 2014). This research aimed to investigate tick species distribution and the diversity and prevalence of tick-borne viruses in the Greater Khingan Mountain district of Heilongjiang Province, northeastern China.

2. Material and methods

2.1. Sample collection and identification

A flag-drag approach was used to collect ticks from four sampling locations in the Greater Khingan Mountains during 2015 and 2016 (Fig. 1). The ticks were stored in tubes at −20 °C before transport to the laboratory, where they were stored at −80 °C. Tick species were morphologically differentiated by a trained expert in the field and then confirmed by polymerase chain reaction (PCR)-based amplification and sequencing of the first (ITS-1) and second (ITS-2) internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene, as described previously (Chitimia et al., 2009).

2.2. Tick virome analysis

2.2.1. Preparation of ticks and extraction of viral RNA

The collected ticks were separated into nine groups based on their location and species, with each group consisting of pooled samples of 20 ticks per sample (e.g., group one contains 220 ticks pooled into 11 samples) (Table 1). Approximately 60% of the samples (~1102 ticks) were subjected to NGS analysis, while the remaining samples were used for virus identification. Each tick sample was homogenized using a glass pestle in 500 µL of SM buffer (50 mM Tris, 10 mM $MgSO_4$, 0.1 M NaCl, pH 7.5), after which it was centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatants were then filtered through 0.45-µm and 0.22-µm Millex filters (Millipore, USA). A 130 µL aliquot of each filtrate was mixed with 4 µL of DNase I, 15 µL of 10× DNase I buffer (TaKaRa, China), and 1 µL of RNase A (TaKaRa, China) and incubated at 37 °C for 60-min to eliminate host genomic DNA and other free nucleic acids. Total viral nucleic acids were then extracted from a 140 µL aliquot of each treated sample using a QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Total RNA was eluted in 45 µL of RNase-free H_2O (TaKaRa, China) and either used immediately or stored at −80 °C.

Table 1
Distribution of tick species across the four sampling locations.

Location	Species of ticks	Groups	Numbers	Samples	Date
A'muer Town	*I. persulcatus*	1	220	11	2016
Beiji Village	*I. persulcatus*	2	547	28	2015
Chuonahe preservation zone	*H. longicornis*	3	377	19	2015
	H. concinna	4	495	25	2015
	D. nuttalli	5	40	2	2015
	I. persulcatus	6	137	7	2015
Shuanghe preservation zone	*D. nuttalli*	7	26	2	2015
	D. silvarum	8	23	2	2015
	I. persulcatus	9	16	1	2015
Total			**1881**		

2.2.2. cDNA library preparation for NGS

Viral RNA was reverse transcribed into cDNA using M-MLV RTase (TaKaRa, China). Briefly, 38 µL of each RNA extract were separately mixed with 100 pmol/µL anchored random primers, heated at 75 °C for 5 min, and then cooled on ice for 2 min before the addition of 3 µL of dNTPs (10 mM, TaKaRa, China), 1 µL of recombinant RNase inhibitor (TaKaRa, China), 1 µL of M-MLV RTase, 10 µL of 5× M-MLV buffer (TaKaRa, China), and RNase-free H_2O to a final volume of 50 µL. The mixtures were incubated at 42 °C for 60 min, followed by another 10 min at 85 °C to inactivate the RTase. For double-stranded cDNA (dscDNA) synthesis, mixtures containing 15.8 µL of the cDNA synthesis reaction and 100 pmol/µL random primers were incubated at 75 °C for 2 min, followed by cooling on ice for 5 min. The remaining reagents, including 1 µL of dNTPs (10 mM, TaKaRa, China), 1 µL of Klenow fragment (TaKaRa, China), and 2 µL of 10× Klenow buffer, were then added along with ddH_2O to a final volume of 20 µL. Reactions were incubated at 37 °C for 60 min, followed by inactivation at 75 °C for 10 min. To obtain sufficient viral nucleic acid for NGS, dscDNA was further amplified via sequence-independent single-primer amplification. Briefly, 50 µL reaction mixtures containing 4 µL of dscDNA mixture, 2 µL of barcode primer (20 µM), 2× Taq PCR MasterMix (Tiangen, China), and ddH_2O were subjected to thermal cycling at 94 °C for 2 min,

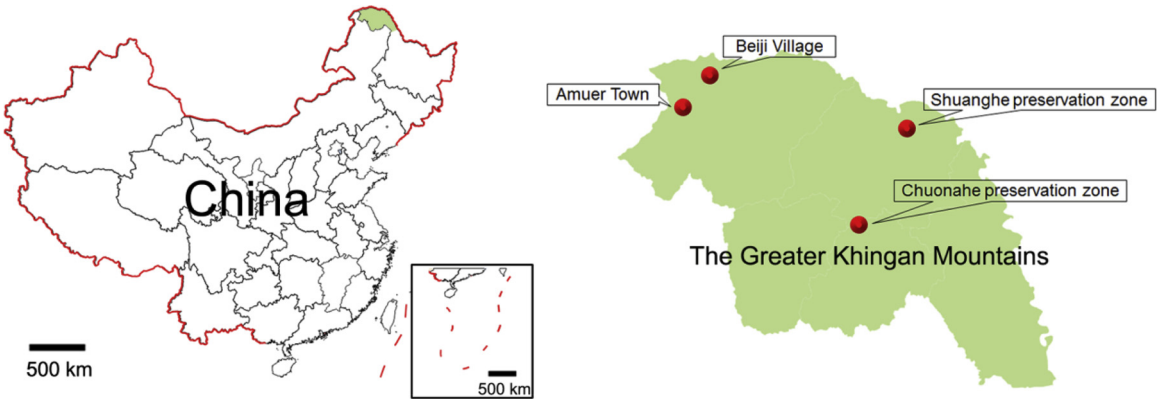

Fig. 1. Map of tick collection sites.

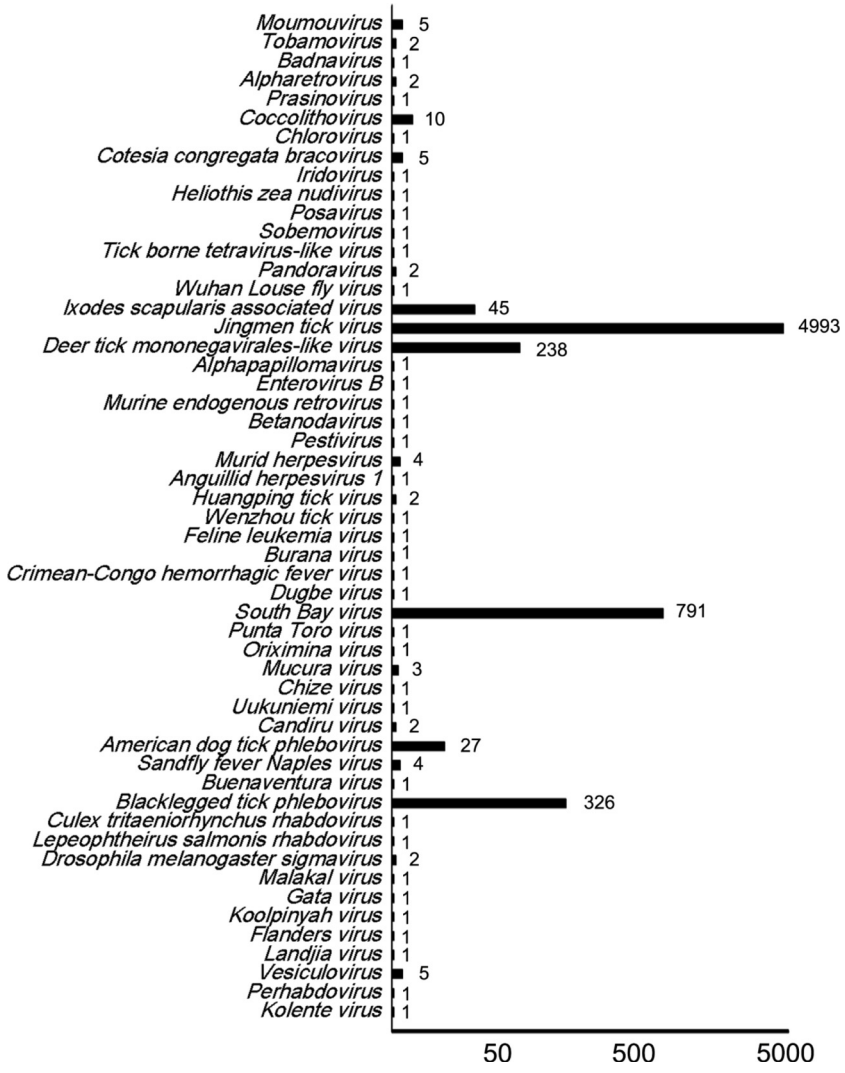

Fig. 2. Overview of viral reads obtained from the samples.

followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 8 min. The amplicons generated from each sample were then purified using a QIAquick PCR Purification Kit (Qiagen, Germany) and dissolved in 50 μL of TE buffer (100 mM Tris-HCl,10 mM EDTA, pH 8.0) (He et al., 2013).

2.2.3. Next-generation sequencing

The tagged and purified PCR products for each of the nine groups were pooled and submitted to the Beijing Genome Institute (BGI, Shenzhen, China) for Solexa sequencing. The resulting sequence data were aligned against the non-redundant and viral reference GenBank databases using BLASTx and BLASTn analyses. An E-value cutoff of $\leq 10e^{-5}$ was used to identify homologous sequences. Contigs containing bacterial or eukaryotic sequence were eliminated, and virus-like sequences were subjected to further analysis.

2.2.4. Virus screening

To validate the results of Solexa sequencing, Primer 5 (Premier Biosoft International, USA) was used to design primers which target on RNA-dependent RNA polymerase gene for verification of the identified viral sequences. Primer sequences are provided in the Supplementary material. Viral RNA was extracted using RNeasy mini kit (Qiagen, Germany) and was synthesized cDNA used PrimeScript™ II 1st Strand cDNA synthesis kit (TaKaRa, China), the amplification of cDNA was conducted with PCR Master Mix (Tiangen, China) by nested-PCR according to the manufacturer's protocol. The products were visualized on 1.0% agarose gels stained with ethidium bromide (Tiangen, China) and the positive PCR products were sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems, USA). In addition, primers which targeted on nucleocapsid protein (Supplementary material) were designed to screen the samples for CCHF virus (CCHFV). To detect CCHFV, the cDNA was performed by touchdown PCR in 50 μL reaction volumes containing 1 μL of cDNA, 2 μL of each primer (10 μM), 25 μL of 2× Taq PCR Master Mix (Tiangen, China) and ddH_2O. Thermal cycler parameters consisted of 94 °C for 3 min, followed by 20 cycles of 94 °C for 30 s, 65 °C for 30 s (decreasing in 0.5 °C increments/cycle), and 72 °C for 60 s, a further 20 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 60 s, and a final extension at 72 °C for 8 min. The products were visualized on 1.0% agarose gels stained with ethidium bromide (Tiangen, China).

2.2.5. Phylogenetic analysis

Nucleotide sequence alignment was conducted using Clustal W and phylogenetic trees were generated using the neighbor-joining method in MEGA 6 with 1000 bootstrap replicates (Tamura et al., 2013).

3. Results

3.1. Tick collection, classification, and distribution

A total of 1881 ticks were collected from the Greater Khingan Mountains and were found to belong to five different species: *I. persulcatus*, *H. longicornis*, *H. concinna*, *D. nuttalli* and *D. silvarum*. Tick collection sites are shown in Fig. 1, and the distribution of ticks across the four sites is summarized in Table 1. Analysis showed that *I. persulcatus* was most prevalent (48.9%) and was distributed across all four sampling sites. *D. nuttalli* (3.5%) was identified in both the Shuanghe and Chuonahe preservation zones, *H. longicornis* (20.1%) and *H. concinna* (26.3%) were only found in the Chuonahe Preservation Zone, and *D. silvarum* (1.2%) was only found in the Shuanghe Preservation zone.

3.2. Tick virome profiling

A total of 2030 Mb of raw reads were obtained by Solexa sequencing. After the removal of tick sequences following comparison against tick genomes available from the NCBI databases, a total of 3,568,561 reads remained. Deletion of non-overlapping reads (7.85%), resulting in a total of 3,288,385 overlapped reads with an average length of 134 bp. Initial analyses of the clean data against the NCBI bacterial, fungal, and viral genome databases identified 395 (0.011%), 19,165 (0.537%), and 287,839 (8.066%) reads with identity to known viral, fungal and bacterial sequences, respectively. Gene prediction using MetaGeneMark revealed that 304,540 reads showed identity to annotated microbial ORFs, with 6577 (2.16%, 6577/304,540) reads showing identity to viral ORFs based on functional annotations under the defined cutoff value. Based on the most significant BLASTn similarities (E-value $\leq 10e^{-5}$), the viral sequences were classified into 18 virus families (Fig. 2). Approximately 98.42% (6473/6577) of the reads were annotated to vertebrate viruses spanning 12 virus families, including *Phenuiviridae*, *Nairoviridae*, *Rhabdoviridae*, *Retroviridae*, *Peribunyaviridae*, *Alloherpesviridae*, *Herpesviridae*, *Flaviviridae*, *Nodaviridae*, *Picornaviridae*, *Papillomaviridae* and an unclassified virus family that includes Jingmen tick virus (JMTV), deer tick *Mononegavirales*-like virus (DTMV), *Ixodes scapularis*-associated virus, *Pandoravirus*, Wuhan louse fly virus, *Sobemovirus*, *Posavirus*, and tick-borne tetravirus-like virus. Most of the reads were attributed to JMTV (75.92%, 4993/6577), followed by South Bay virus (SBV; 12.03%, 791/6577), blacklegged tick phlebovirus (BTPV; 4.96%, 326/6577), and DTMV (3.62%, 238/6577). All other reads showed only low sequence identity to known viral nucleic acid/amino acid sequences. Insect virus families comprised 0.11% of the reads, while plant viruses accounted for 0.26% of the annotated reads.

The 6577 virome reads were assembled into 91 contigs. *Orthobunyavirus*-like sequences made up more than 60% of all contigs, with 30 contigs annotated to SBV, 28 to BTPV, 14 to DTMV, and 19 to JMTV. The Solexa sequencing results were also verified by nested PCR using primers specific for these vertebrate viruses (Table 2). The results indicated that ticks belonging to the same species collected from different locations displayed different PCR-positive virus profiles, with ticks belonging to different species but from the same location also demonstrating differing virome profiles. Interestingly, samples belonging to the same tick species from the same location also showed variation in PCR-positive virus profiles.

3.3. Screening for Orthonairovirus sequences

The family *Nairoviridae*, belonging to the order *Bunyavirales*, is comprised of 12 species, many of which are tick-borne viruses. Virome studies conducted on ticks from North America in 2014 identified SBV and classified it as a novel virus belonging to the family *Nairoviridae* (Tokarz et al., 2014). SBV is highly divergent compared with previously identified tick-borne *Orthonairovirus* species. In the current study, 12708-bp and 1092-bp fragments of the L and S segments, respectively, were amplified from several SBV-positive samples and sequenced. Phylogenetic analysis of the resulting sequences with sequences from representatives of all 12 species of *Orthonairovirus* showed that one sequence from a sample collected in Beiji, and therefore designated Beiji nairovirus, did not cluster with any of the described *Orthonairovirus* species (Fig. 3A&B). The sequence from the L segment of Beiji nairovirus shared the highest nucleotide identity (81%) with Pustyn virus from Russia and 68% nucleotide identity with SBV from United States, and similarly the S segment sequence showed the highest identity (79%) with Pustyn virus and 67% identity with SBV. These results suggested that the Beiji nairovirus in this study may belong to a new *Orthonairovirus* species.

NGS only produced one read annotated to CCHFV, which showed low nucleotide sequence identity. PCR analysis of the tick samples using primers targeting the CCHFV N gene also did not support the presence of CCHFV in ticks from this region of China.

3.4. Screening Phlebovirus sequences

The genus *Phlebovirus* also belongs to the order *Bunyavirales*, with viruses belonging to this genus showing genome identity to viruses of the genus *Orthonairovirus*. Based on NGS data, the specific primers were designed to examine the prevalence of BTPV in the samples via RT-PCR-based amplification. Nested RT-PCR of all tick samples targeting a 540-bp fragment of the L segment confirmed the results of Solexa sequencing. As shown in Table 2, the prevalence of BTPV was high among the tested tick samples, with prevalence rates ranging from 55% in *I. persulcatus* to 100% in *D. nuttalli* and *D. silvarum*. A 2003-bp fragment from the L segment of phlebovirus sequences was amplified from the PCR-positive samples and sequenced. Sequence comparison showed that the resulting sequences shared 81%, 78%, 76%, and 70% nucleotide sequence identity with Norway phlebovirus 1, BTPV-1, BTPV-2, and BTPV-3, respectively. Phylogenetic analysis based on the L fragment sequences showed that the Beiji phlebovirus sequence identified in the current study belonged to the tick-borne phlebovirus cluster, but formed a monophyletic clade away from the Uukuniemi group and SFTS group clades (Fig. 4)

3.5. Screening for deer tick mononegavirales-like virus sequences

The order *Mononegavirales* contains eight families and includes several important zoonotic viruses, including Marburg, Ebola, and rabies. DTMV was first reported in North America in 2014 (Tokarz et al.,

Table 2
Prevalence of selected viruses in various tick species from each of the sampling locations.

Viruses	A'muer	Beiji	Chuonahe				Shuanghe		
	I. persulcatus	*I. persulcatus*	*H. longicornis*	*H. concinna*	*D. nuttalli*	*I. persulcatus*	*D. nuttalli*	*D. silvarum*	*I. persulcatus*
SBV	100%	96%	16%	20%	100%	0	0	0	0
BTPV	55%	96%	95%	60%	100%	100%	100%	100%	100%
DTMV	36%	100%	0	28%	100%	86%	50%	100%	100%
JMTV	27%	7%	11%	4%	100%	43%	50%	0	0

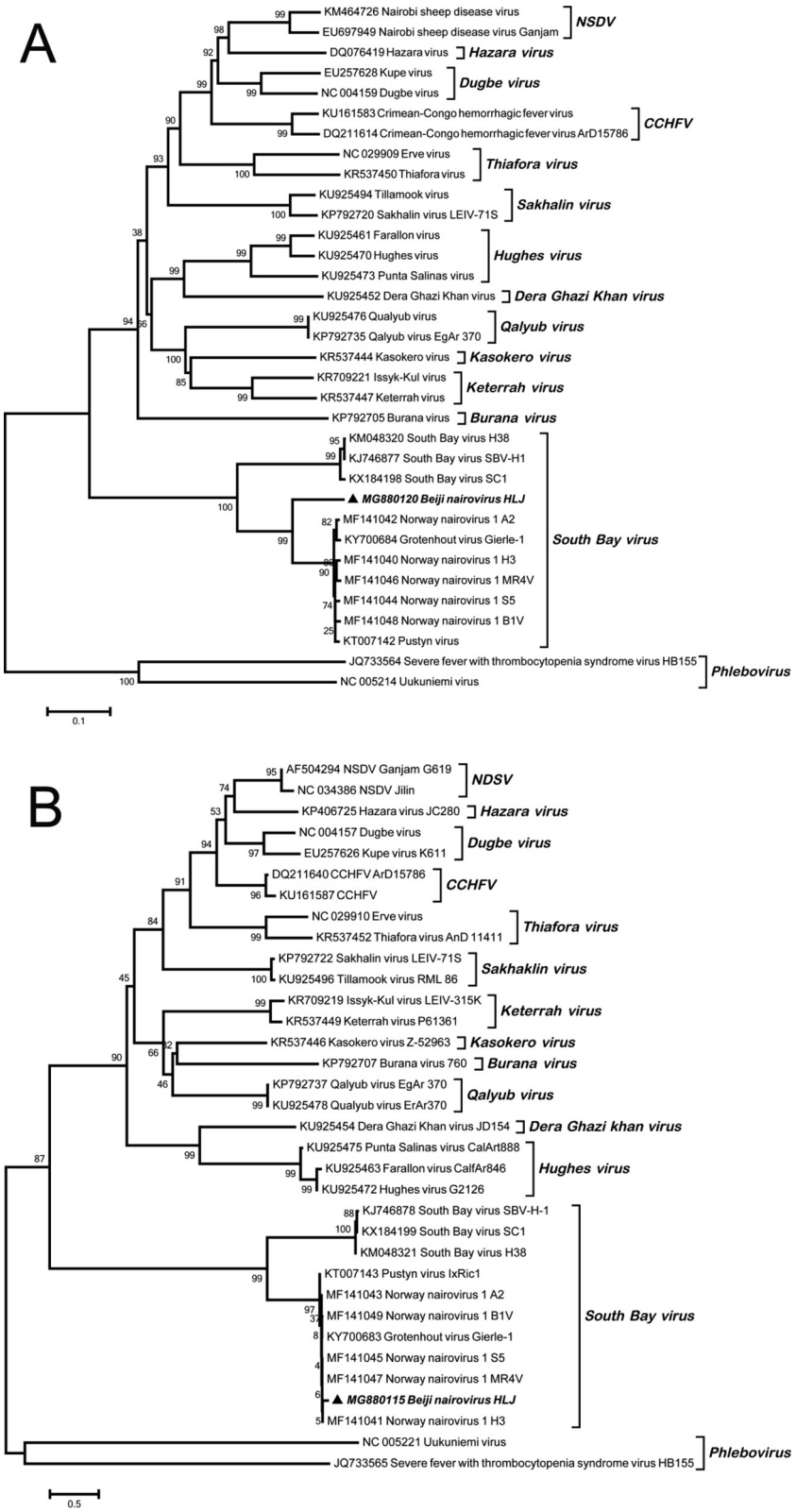

Fig. 3. (A) Phylogenetic analysis based on a 12,708-bp region of the RNA-dependent RNA polymerase gene (L segment) from Beiji nairovirus and other representative tick-borne viruses. **(B)** Phylogenetic analysis based on a 1092-bp region of the nucleocapsid protein gene (S segment) of Beiji nairovirus with other representative tick-borne viruses. The trees were generated using the neighbor-joining (NJ) method with a Maximum Composite Likelihood model. Analysis included 1000 bootstrap replicates. Numbers above the branches indicate NJ bootstrap values. Bold triangles indicate the Beiji nairovirus sequence obtained in the current study.

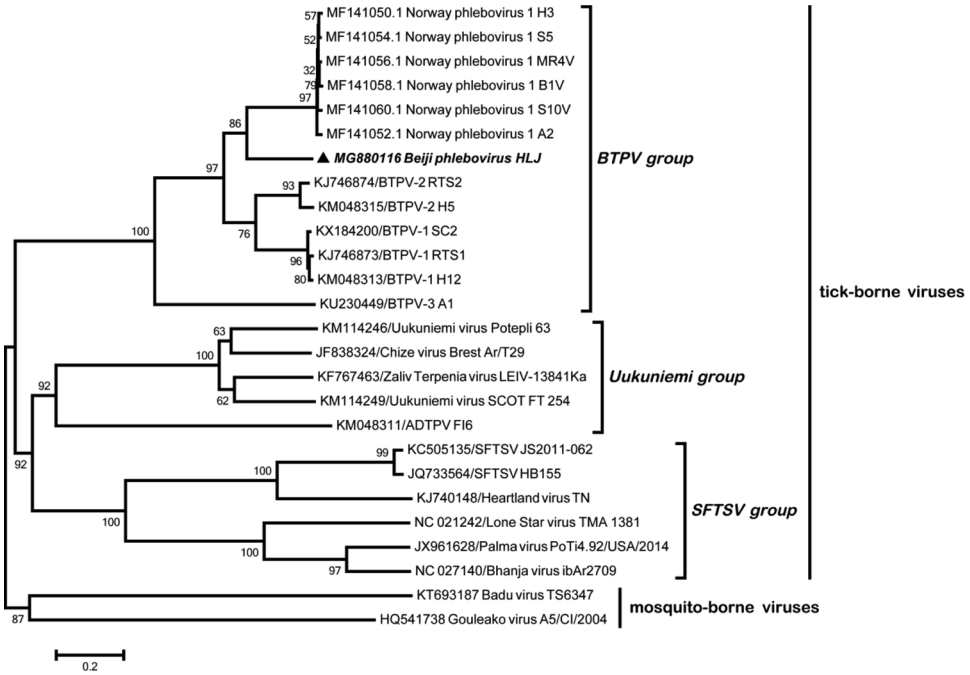

Fig. 4. Phylogenetic analysis of Beiji phlebovirus and other representative tick-borne viruses based on a 2003-bp region of the RNA-dependent RNA polymerase gene. The tree was generated using the neighbor-joining (NJ) method with a Maximum Composite Likelihood model. Numbers above the branches indicate NJ bootstrap values. Bold triangles indicate the Beiji phlebovirus sequence obtained in the current study.

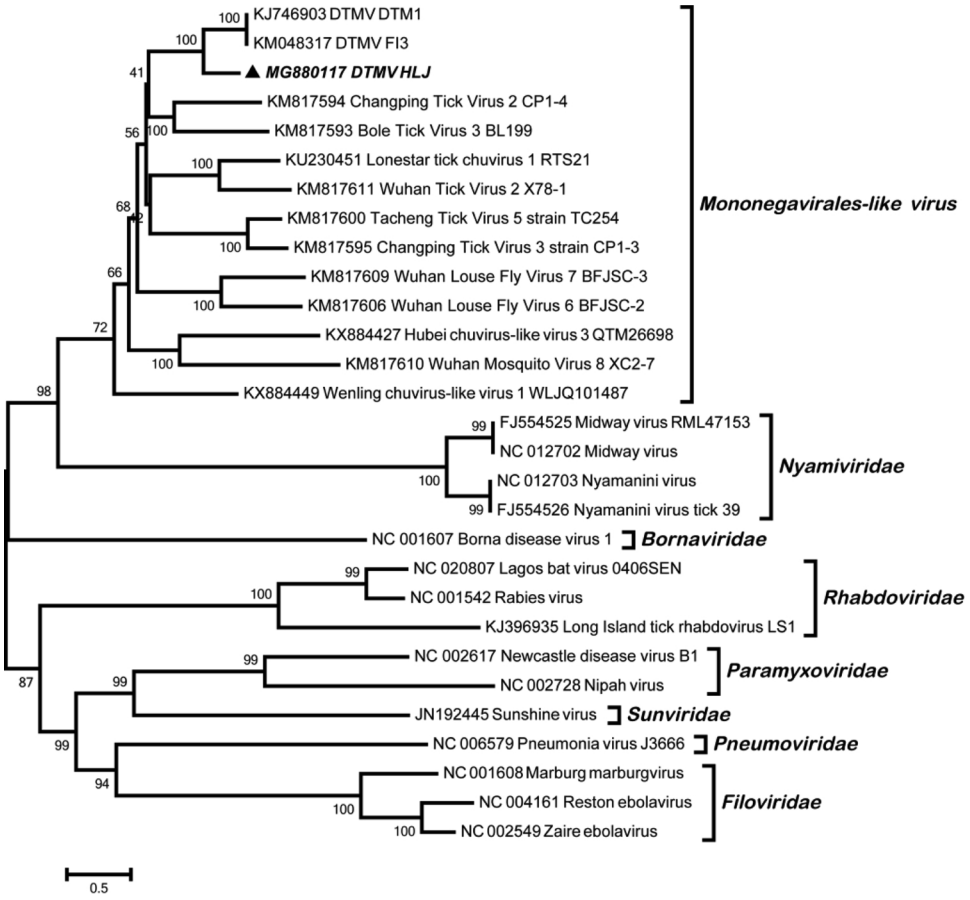

Fig. 5. Phylogenetic analysis of deer tick *Mononegavirales*-like virus (DTMV) strain HLJ and other representative viruses based on a 3985-bp fragment of the RNA-dependent RNA polymerase gene. The tree was generated using the neighbor-joining (NJ) method with a Maximum Composite Likelihood model. Numbers above the branches indicate NJ bootstrap values. Bold triangles indicate DTMV strain HLJ identified in the current study.

2014). In the current study, the prevalence of DTMV was detected by NGS and confirmed by nested RT-PCR targeting a 320-bp fragment of the viral L segment. The prevalence of DTMV was 36–100% in *I. persulcatus* samples, 0% in *H. longicornis*, 28% in *H. concinna*, 50–100% in *D. nuttalli* samples, and 100% in *D. silvarum* (Table 2). A 3985-bp fragment from the L segment of DTMV (designated DTMV HLJ) was obtained by PCR from the NGS-positive samples. This sequence shared 72% nucleotide sequence identity with DTMV strains DTM1 and F13. Phylogenetic analysis showed that the DTMV HLJ sequence formed a separate monophyletic clade within the order *Mononegavirals* (Fig. 5).

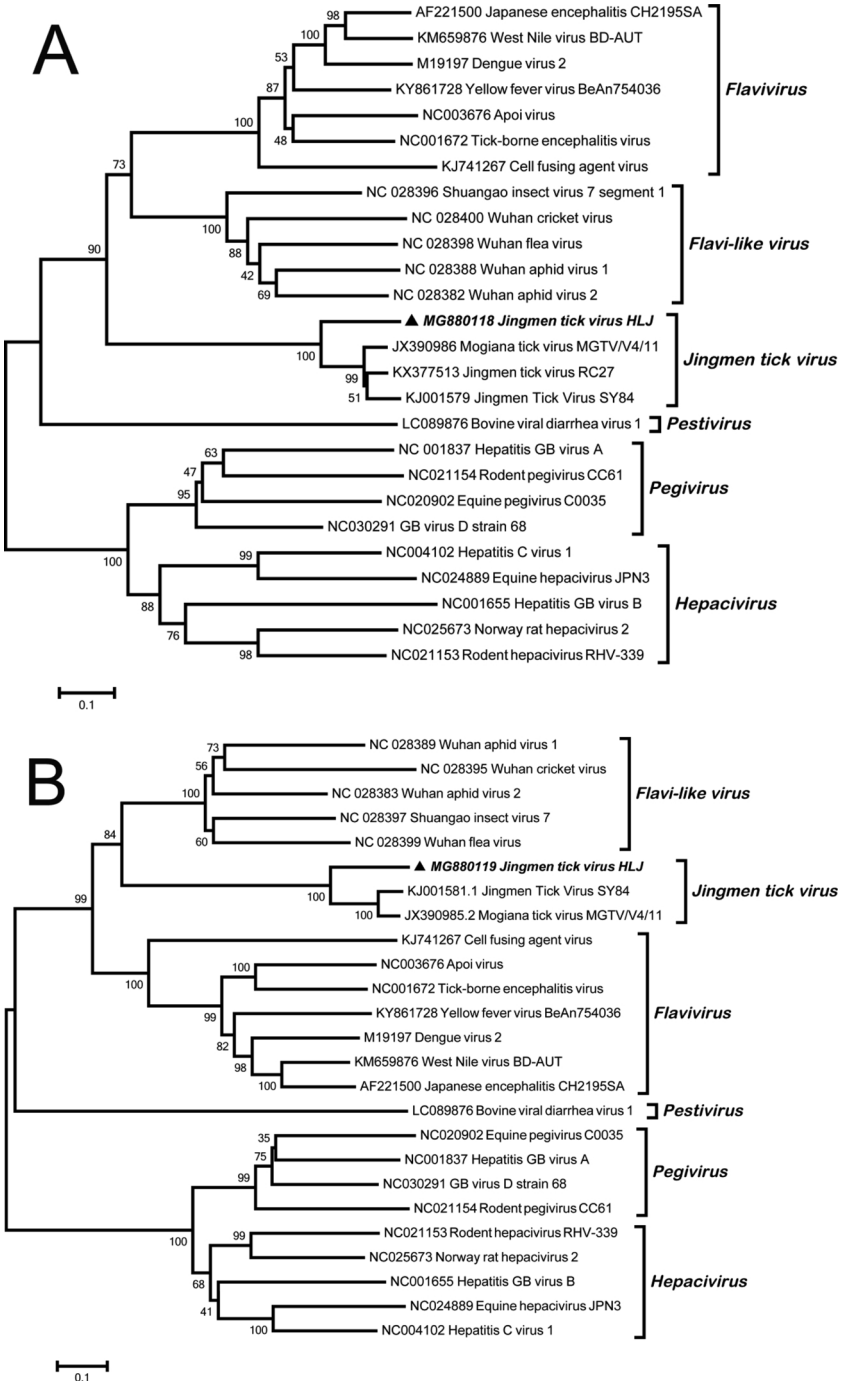

Fig. 6. Phylogenetic analysis of novel Jingmen tick virus (JMTV) strain HLJ identified from ticks in the current study with representative viruses of the genus *Flavivirus* (family *Flaviviridae*). The phylogenetic trees were constructed based on a 1298-bp fragment of the NS5 (segment 1) gene **(A)** and a 1865-bp fragment of the NS3 (segment 3) gene **(B)**. Trees were generated using a neighbor-joining (NJ) method with a Maximum Composite Likelihood model. Numbers above the branches indicate NJ bootstrap values. Bold triangles indicate JMTV strain HLJ identified in the current study.

3.6. Screening for Jingmen tick virus sequences

JMTV has a segmented RNA genome derived from an unsegmented *Flaviviridae* virus and is widely distributed amongst tick populations across China (Qin et al., 2014). RT-PCR-based screening of all samples using specific primers targeting segment 1 revealed JMTV prevalence rates of 0–43% in *I. persulcatus* samples, 10% in *H. longicornis*, 4% in *H. concinna*, and up to 100% in *D. nuttalli* (Table 2). However, JMTV was not detected in *D. silvarum* samples (Table 2). A 1298-bp fragment of segment 1 and 1865-bp fragment of segment 3 were obtained by PCR from some of the PCR-positive samples and subsequently sequenced. Phylogenetic analyses showed that the JMTV sequences clustered with *Flavivirius* and flavi-like virus sequences in both phylogenetic trees (Fig. 6A&B)..

4. Discussion

In this study, a total of 1881 ticks were collected from four representative sampling sites in the Greater Khingan Mountains area of China and classified into five species. Prevalence analysis suggests that *I. persulcatus* is the major tick species in this area, and that the distribution of different tick species differs across the region. A previous

study identified 11 tick species in Heilongjiang Province, China (Chen et al., 2010), a significantly greater number than the five species identified in the current study. It is not clear if the difference in tick species diversity between the two studies is because of differences in the timing of sample collection, or is the result of differences in climate, ecological environment, and human activity, as suggested by others (Esteve-Gassent et al., 2016).

NGS resulted in only 6577 reads with similarity to viral ORFs, which were subsequently assembled into 91 contigs. This is significantly less than the 735,413 contigs and 230,000 reads annotated to virus sequences in two similar studies (Bouquet et al., 2017; Pettersson et al., 2017). We speculate that the higher sample conservation temperature (−20 °C), different specimen preparation procedures, and different sequencing platforms may be behind the discrepancies. The tick samples were initially stored at −20 °C before transportation and then transferred to −80 °C in the laboratory prior to analysis, the higher initial temperature may have resulted in degradation of the viruses. In addition, the kit used for cDNA library preparation and the chosen sequencing platform may significantly impact viral detection rates.

Despite lower detection rates, several viruses were identified via NGS, which were confirmed by PCR and sequencing. The prevalence of these viruses in ticks was also determined. It is worth noting that the prevalence data for viruses in different zones may have been skewed by the sample sizes and the timing of sample collection. Nevertheless, BLASTx and BLASTn analyses revealed that many of the sequences obtained by NGS showed a high level of similarity to sequences from *Nairoviridae, Phenuiviridae* (*Bunyavirales*), *Mononegavirales*, and *Flaviviridae* viruses. The order *Bunyavirales* contains nine families, including *Nairoviridae*, and includes many tick-borne viruses responsible for serious diseases in humans and animals such as CCHF and Nairobi sheep disease (NSD). While recognized vectors of CCHFV and NSD virus (NSDV) are present in China (Gong et al., 2015; Sun et al., 2009), these viruses were not detected in the current study, although a number of sequences related to *Orthonairovirus* species were identified by NGS and confirmed by RT-PCR analyses. The *Orthonairovirus* identified in a sample from Beiji, designated Beiji nairovirus shared 68% nucleotide sequence identity with SBV, while phylogenetic analysis of L and S segment sequences showed that it belonged to the family *Nairoviridae*. SBV was first detected in *I. scapularis* ticks in North America, and our results show that *I. persulcatus*, *H. longicornis*, *H. concinna* and *D. nutalli* ticks may also host SBV. Beiji nairovirus showed relatively high amino acid identity (86%) to Pustyn virus from Russia and Norway nairovirus 1 from Norway, indicating that this group of viruses is present in diverse geographic regions.

The genus *Phlebovirus* belongs to the family *Phenuiviridae* and is classified into the mosquito/sandfly-borne and tick-borne viral groups (Alkan et al., 2016). Phlebovirus SFTSV, which causes serious diseases in humans and livestock, is transmitted by *Haemaphysalis* and *Rhipicephalus* ticks and was first identified in China (Yu et al., 2011). However, Solexa sequencing of tick viromes did not detect the presence of SFTSV in any of the samples from the current study. In contrast, another phlebovirus, BTPV, was highly prevalent in all tick species identified in the current study and was widespread across the sampling locations. Phylogenetic analysis based on the L segment of this virus, designated Beiji phlebovirus, showed that it forms a cluster with the tick-borne phlebovirus group, but is separate from the Uukuniemi virus and SFTSV groups. Attempts to sequence the M segment of the SBV and BTPV viruses identified in this study were unsuccessful. Previous studies have shown that the secondary structure of the M segment of SBV and BTPV viruses may prevent the synthesis and/or amplification of cDNA (Tokarz et al., 2014; Xia et al., 2015). However, the high genetic divergence of Beiji phlebovirus from known *Phlebovirus* species may indicate that it is a novel virus within the genus *Phlebovirus*.

In addition to the *Orthobunyavirus*-like viruses, the L segment of a *Mononegavirales*-like virus, designated DTMV HLJ, was also detected in the current study. DTMV HLJ was highly prevalent in *I. persulcatus* ticks. Phylogenetic analysis based on the partial L segment sequence of DTMV HLJ placed it within a clade comprising other *Mononegavirales*-like viruses, including louse fly virus and mosquito virus, within the family *Nyamiviridae* (order, *Mononegavirales*). We therefore hypothesize that DTMV HLJ belongs to a novel genus within *Nyamiviridae*.

The family *Flaviviridae* comprises small enveloped viruses with RNA genomes (Simmonds et al., 2017). The majority of known members of the genus *Flavivirus* are arthropod-borne, and many are important human and veterinary pathogens. Tick-borne encephalitis, also known as forest encephalitis in China, has been reported in Heilongjiang, Jilin, Inner Mongolia, and Xinjiang provinces (Fang et al., 2015). Serological analysis of tick-borne encephalitis virus (TBEV) confirmed human cases of TBEV infection in Tibet and Yunnan, two provinces located in southwest China (Xing et al., 2017). There are three TBEV subtypes, designated Far Eastern (FE), Siberian (Sib), and European, with the Far Eastern subtype currently the predominant subtype in China. However, the Siberian subtype has recently also been detected in Xinjiang Province (Liu et al., 2016). The primary vector of the European subtype is *I. ricinus*, while *I. persulcatus* is the primary vector of the Far Eastern and Siberian subtypes. However, neither FE-TBEV nor Sib-TBEV sequences were detected in the current study.

JMTV is a segmented RNA virus whose genome comprises four segments (S1-S4), two of which are highly divergent segments of unknown origin, while the remaining two segments share common ancestry with the NS3 and NS5 regions of *Flavivirus* genomes (Qin et al., 2014). In the current study, the average prevalence of JMTV across all samples was 26.89%, which was much lower than that of the other three viruses. Phylogenetic analysis indicated that S1 and S3 sequences from JMTV cluster with *Flavivirus* sequences, which is consistent with previous findings (Shi et al., 2015).

Of note, attempts to isolate viruses in the current study by inoculation of PCR-positive specimens onto Vero and baby hamster kidney fibroblast (BHK-21) cell cultures were unsuccessful.

5. Conclusions

This study identified five species of ticks in samples from the Greater Khingan Mountains, while virome analysis suggested the presence of several likely novel tick-borne viruses. Viruses on the natural epidemic focus list of northeast China, including TBEV, STSFV, CCHFV, and NSD virus, were not detected in the current study, possibly as a result of geographical isolation, tick sample sizes, and sampling time. It is unclear if the newly identified viruses can be transmitted to vertebrate hosts via blood feeding; thus, their significance to human and animal health will require further study. In addition, studies using samples from a larger geographical area need to be conducted to obtain a better understanding of tick distribution and the prevalence of tick-borne viruses, which would aid in the prevention and control of tick-borne diseases.

Declarations of interest

The authors have no conflict of interest.

Data availability

All sequence reads generated in this project are available from the NCBI Short Read Archive (SRA) under accession number SRR6659068. All consensus virus genome sequences have been deposited in GenBank (accession numbers MG880115-MG880120).

Funding

This work was supported by the National Key Basic Research and Development Program of China [grant number 2017YFD0500100] and the Youth Talent Supporting Project [grant number 17-JCJQ-QT-035].

The sponsors had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the article for publication.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2018.12.002.

References

Alkan, C., Kasap, E.O., Alten, B., Lamballerie, D.X., Charrel, R.N., 2016. Sandfly-borne phlebovirus isolations from Turkey: new insight into the Sandfly fever Sicilian and Sandfly fever Naples species. PLoS Negl. Trop. Dis. 10, e0004519.

Bouquet, J., Melgar, M., Swei, A., Delwart, E., Lane, R.S., Chiu, C.Y., 2017. Metagenomic-based surveillance of Pacific Coast tick *Dermacentor occidentalis* identifies two novel Bunyaviruses and an emerging human Ricksettsial pathogen. Sci. Rep. 7, 12234.

Bzhalava, D., Dillner, J., 2013. Bioinformatics for viral metagenomics. J. Data Mining Genomics Proteomics 04, 1000134.

Chen, Z., Yang, X.J., Bu, F.J., Yang, X.H., Yang, X.L., Liu, J.Z., 2010. Ticks (Acari ixodoidea argasidae, Ixodidae) of China. Exp. Appl. Acarol. 51, 393–404.

Cheng, C., Fu, W., Ju, W., Yang, L., Xu, N., Wang, Y.M., Li, H., Wang, Y.L., Hu, M.X., Wen, J., Jiao, D., Geng, C., Sun, Y., 2016. Diversity of spotted fever group *Rickettsia* infection in hard ticks from Suifenhe, Chinese-Russian border. Ticks Tick. Dis. 7, 715–719.

Chitimia, L., Lin, R.Q., Cosoroaba, I., Braila, P., Song, H.Q., Zhu, X.Q., 2009. Molercular characterization of hard and soft ticks from Romania by sequences of the internal transcribed spacers of ribosomal DNA. Parasitol. Res. 105, 907–911.

Diuk-Wasser, M.A., Vannier, E., Krause, P.J., 2016. Coinfection by *ixodes* tick-borne pathogens: ecological, epidemiological, and clinical consequences. Trends Parasitol. 32, 30–42.

Esteve-Gassent, M.D., Castro-Arellano, I., Feria-Arroyo, T.P., Patino, R., Li, A.Y., Medina, R.F., Perez de Leon, A.A., Rodriguez-Vivas, R.I., 2016. Translating ecology, physiology, biochemistry, and population genetics research to meet the challenge of tick and tick-borne diseases in North America. Arch. Insect Biochem. Physiol. 92, 38–64.

Fang, L., Liu, K., Li, X., Liang, S., Yang, Y., Yao, H., Sun, R., Sun, Y., Chen, W., Zuo, S., Ma, M., Li, H., Jiang, J., Liu, W., Yang, X.F., Gray, G.C., Krause, P.J., Cao, W., 2015. Emerging tick-borne infections in mainland China: an increasing public health threat. Lancet Infect. Dis. 15, 1467–1479.

Firth, C., Bhat, M., Firth, M.A., Williams, S.H., Frye, M.J., Simmonds, P., Conte, J.M., Ng, J., Garcia, J., Bhuva, N.P., Lee, B., Che, X., Quan, P.L., Lipkin, W.I., 2014. Detection of zoonotic pathogens and characterization of novel viruses carried by commensal *Rattus norvegicus* in New York City. mBio. 5, e01933–01914.

Gong, S., He, B., Wang, Z., Shang, L., Wei, F., Liu, Q., Tu, C., 2015. Nairobi sheep disease virus RNA in Ixodid ticks, China, 2013. Emerg. Infect. Dis. 21, 1697–1703.

He, B., Li, Z., Yang, F., Zheng, J., Feng, Y., Guo, H., Li, Y., Wang, Y., Su, N., Zhang, F., Fan, Q., Tu, C., 2013. Virome profiling of bats from Myanmar by metagenomic analysis of tissue samples reveals more novel Mammalian viruses. PLoS One 8, e61950.

Keskin, A., Bursali, A., Keskin, A., Tekin, S., 2016. Molecular detection of spotted fever group rickettsiae in ticks removed from humans in Turkey. Ticks Tick. Dis. 7, 951–953.

Kunze, U., 2016. The International Scientific Working Group on tick-borne encephalitis (ISW TBE): review of 17 years of activity and commitment. Ticks Tick. Dis. 7, 399–404.

Lee, S., Lee, S.H., VanBik, D., Kim, N.H., Kim, K.T., Goo, Y.K., Rhee, M.H., Kwon, O.D., Kwak, D., 2016. First molecular detection and phylogenetic analysis of Anaplasma phagocytophilum in shelter dogs in Seoul. Korea. Ticks Tick Borne Dis. 7, 945–950.

Liu, R., Zhang, G.L., Liu, X.M., Li, Y.C., Zheng, Z., Sun, X., Yang, Y.H., 2016. Detection of the Siberian tick-borne encephalitis virus in the Xinjiang Uygur Autonomous Region, northwestern China. Bing Du Xue Bao 32, 26–31.

Moutailler, S., Popovici, I., Devillers, E., Vayssier-Taussat, M., Eloit, M., 2016. Diversity of viruses in *Ixodes ricinus*, and characterization of a neurotropic strain of Eyach virus. New Microbes New Infect. 11, 71–81.

Pettersson, J.H., Shi, M., Bohlin, J., Eldholm, V., Brynildsrud, O.B., Paulsen, K.M., Andreassen, A., Holmes, E.C., 2017. Characterizing the virome of Ixodes ricinus ticks from northern Europe. Sci. Rep. 7, 10870.

Qin, X.C., Shi, M., Tian, J.H., Lin, X.D., Gao, D.Y., He, J.R., Wang, J.B., Li, C.X., Kang, Y.J., Yu, B., Zhou, D.J., Xu, J.G., Plyusnin, A., Holmes, E.C., Zhang, Y.Z., 2014. A tick-borne segmented RNA virus contains genome segments derived from unsegmented viral ancestors. Proc. Natl. Acad. Sci. U. S. A. 111, 6744–6794.

Shi, M., Lin, X.D., Vasilakis, N., Tian, J.H., Li, C.X., Chen, L.J., Eastwood, G., Diao, X.N., Chen, M.H., Chen, X., Qin, X.C., Widen, S.G., Wood, T.G., Tesh, R.B., Xu, J., Holmes, E.C., Zhang, Y.Z., 2015. Divergent viruses discovered in arthropods and vertebrates revise the evolutionary history of the *Flaviviridae* and related viruses. J. Virol. 90, 659–669.

Shi, M., Lin, X.D., Tian, J.H., Chen, L.J., Chen, X., Li, C.X., Qin, X.C., Li, J., Cao, J.P., Eden, J.S., Buchmann, J., Wang, W., Xu, J., Holmes, E.C., Zhang, Y.Z., 2016. Redefining the invertebrate RNA virosphere. Nature 540, 539–543.

Simmonds, P., Becher, P., Bukh, J., Gould, E.A., Meyers, G., Monath, T., Muerhoff, S., Pletnev, A., Rico-Hesse, R., Smith, D.B., Stapleton, J.T., Ictv Report, C., 2017. ICTV virus taxonomy profile: flaviviridae. J. Gen. Virol. 98, 2–3.

Suh, J.H., Kim, H.C., Yun, S.M., Lim, J.W., Kim, J.H., Chong, S.T., Kim, D.H., Kim, H.T., Kim, H., Klein, T.A., Johnson, J.L., Lee, W.J., 2016. Detection of SFTS virus in *Ixodes nipponensis* and *Amblyomma testudinarium* (*Ixodida: Ixodidae*) collected from reptiles in the Republic of Korea. J. Med. Entomol. 3, 1–7.

Sun, S.R., Dai, X., Aishan, M., Wang, X.H., Meng, W.W., Feng, C.H., Zhang, F.H., Hang, C.S., Hu, Z.H., Zhang, Y.J., 2009. Epidemiology and phylogenetic analysis of Crimean-Congo hemorrhagic fever viruses in Xinjiang. China. J. Clin. Microbiol. 47, 2536–2543.

Sun, R.X., Lai, S.J., Yang, Y., Li, X.L., Liu, K., Yao, H.W., Zhou, H., Li, Y., Wang, L.P., Mu, D., Yin, W.W., Fang, L.Q., Yu, H.J., Cao, W.C., 2017. Mapping the distribution of tick-borne encephalitis in mainland China. Ticks Tick. Dis. 8, 631–639.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Tijsse-Klasen, E., Koopmans, M.P., Sprong, H., 2014. Tick-borne pathogen - reversed and conventional discovery of disease. Front. Public Health 2, 73.

Tokarz, R., Williams, S.H., Sameroff, S., Sanchez Leon, M., Jain, K., Lipkin, W.I., 2014. Virome analysis of *Amblyomma americanum, Dermacentor variabilis*, and *Ixodes scapularis* ticks reveals novel highly divergent vertebrate and invertebrate viruses. J. Virol. 88, 11480–11492.

Torres, F.D., Chomel, B.B., Otranto, D., 2012. Ticks and tick-borne diseases: a one health perspective. Trends Parasitol. 28, 437–447.

Xia, H., Li, P., Yang, J., Pan, L., Zhao, J., Wang, Z., Li, Y., Zhou, H., Dong, Y., Guo, S., Tang, S., Zhang, Z., Fan, Z., Hu, Z., Kou, Z., Li, T., 2011. Epidemiological survey of Crimean-Congo hemorrhagic fever virus in Yunnan, China, 2008. Int. J. Infect. Dis. 15, e459–463.

Xia, H., Hu, C., Zhang, D., Tang, S., Zhang, Z., Kou, Z., Fan, Z., Bente, D., Zeng, C., Li, T., 2015. Metagenomic profile of the viral communities in Rhipicephalus spp. ticks from Yunnan. China. PloS One 10, e0121609.

Xing, Y., Schmitt, H.J., Arguedas, A., Yang, J., 2017. Tick-borne encephalitis in China: a review of epidemiology and vaccines. Vaccine 35, 1227–1237.

Yadav, P.D., Cherian, S.S., Zawar, D., Kokate, P., Gunjikar, R., Jadhav, S., Mishra, A.C., Mourya, D.T., 2013. Genetic characterization and molecular clock analyses of the Crimean-Congo hemorrhagic fever virus from human and ticks in India, 2010–2011. Infect. Genet. Evol. 14, 223–231.

Yu, X.J., Liang, M.F., Zhang, S.Y., Liu, Y., Li, J.D., Sun, Y.L., Zhang, L.H., Zhang, Q.F., Popov, V.L., Li, C., Qu, J., Li, Q., Zhang, Y.P., Hai, R., Wu, W., Wang, Q., Zhan, F.X., Wang, X.J., Kan, B., Wang, S.W., Wan, K.L., Jing, H.Q., Lu, J.X., Yin, W.W., Zhou, H., Guan, X.H., Liu, J.F., Bi, Z.Q., Liu, G.H., Ren, J., Wang, H., Zhao, Z., Song, J.D., He, J.R., Wan, T., Zhang, J.S., Fu, X.P., Sun, L.N., Dong, X.P., Feng, Z.J., Yang, W.Z., Hong, T., Zhang, Y., Walker, D.H., Wang, Y., Li, D.X., 2011. Fever with thrombocytopenia associated with a novel *Bunyavirus* in China. N. Engl. J. Med. 364, 1523–1532.

Zhan, J., Wang, Q., Cheng, J., Hu, B., Li, J., Zhan, F., Song, Y., Guo, D., 2017. Current status of severe fever with thrombocytopenia syndrome in China. Virol. Sin. 32, 51–62.

Zhang, Y.Z., Xu, J.G., 2016. The emergence and cross species transmission of newly discovered tick-borne Bunyavirus in China. Curr. Opin. Virol. 16, 126–131.