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Genomic and phylogenetic characterization of a bunya-like virus from the freshwater Chinese mitten crab *Eriocheir sinensis*

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Summary. – The freshwater Chinese mitten crab (*Eriocheir sinensis*), an indigenous crustacean in China, has been cultured for more than 30 years. It was reported that the bunya-like virus from *Eriocheir sinensis* (EsBV) was associated with the tremor disease (TD), which causes high mortality and has a serious impact on production. In this study, full-length genome sequences of EsBV were pursued using next generation sequencing; the genome of EsBV was found to be composed of 6.7 kb L, 3.3 kb M, and 0.8 kb S segments, respectively. PCR detection based genomic sequences showed that the positive rate of EsBV reached 40% in crabs from farming ponds. EsBV had the highest similarity with the Wenling crustacean virus 9, an unassigned, negative sense ssRNA virus. EsBV clustered with the Wenling crustacean virus 9 firstly, and then the branch clustered with *Peribunyaviridae* clade in every phylogenetic tree – based on L, M and S encoded sequences, respectively, indicating that EsBV can be classified in the family *Peribunyaviridae*, to which the orthobunyaviruses belongs, but not belonging to any known genera in the family *Peribunyaviridae*. There were unique complimentary terminal sequences for EsBV, with only partial consensus with members from the orthobunyaviruses. We believe that the findings of this research will be vital for future research about EsBV and will also go a long way in illuminating its relationship with TD.

Keywords: Eriocheir sinensis; tremor disease; bunyavirus; EsBV; genome sequences

Introduction

The *Bunyaviridae* is a large, diverse group of viruses, more than 350 strains of *Bunyaviridae* have been isolated around the globe and classified into five genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* (Savji *et al*., 2011). In 2017, the family *Bunyaviridae* was reclassified as *Bunyvirales* by the ICTV (International Committee on Taxonomy of Viruses) (Adams *et al*., 2017). This taxonomic shift from a family of viruses to an order of viruses resulting in all five genera formerly in the family *Bunyaviridae* (*Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus* and *Tospovirus*) are now novel viral families, some of which have been combined. These new families in *Bunyvirales* include: *Hantaviridae*, *Feraviridae*, *Fimoviridae*, *Jonviridae*, *Nairoviridae*, *Peribunyaviridae*, *Phasmaviridae*, *Phenuiviridae*, and *Tospoviridae*. Bunyavirus is an enveloped spherical virus, whose size is about 100 nm. Members of the *Bunyvirales* are enveloped viruses with a segmented negative strand RNA, the genome of bunyavirus is constructed by two or three segments, respectively. The three RNAs are named as large (L), medium (M), and small (S) RNA segment. The L segment encodes the RNA Dependent RNA-polymerase, which is necessary for viral RNA replication and mRNA synthesis. The M segment encodes the viral glycoproteins, a functional molecular aid that helps the virus in attaching to and entering the host cell. The S segment encodes the nucleocapsid protein

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Abbreviations: EsBV = bunya-like virus from *Eriocheir sinensis*; L segment = large segment; M segment = medium segment; NCRs = non-coding regions; S segment = small segment; TD = tremor disease

(N) (Ariza *et al.*, 2013). The natural host of bunyavirus are arthropods, rodents and human beings, bunyaviruses from the genus *Tospovirus* were also found in plants (Elliott, 1997; Amroun *et al.*, 2017).

Eriocheir sinensis is one of the most economically important native crustaceans in Chinese freshwater aquaculture (Wang *et al.*, 2006). Since the 1980s, the freshwater Chinese mitten crabs have been cultured in many provinces such as Jiangsu, Anhui, Hubei and Liaoning in China (Shen *et al.*, 2014b). With the rapid development of Chinese mitten crab breeding industry, many diseases have emerged in recent years. One of the most serious diseases of *E. sinensis* is tremor disease (TD). The TD first appeared in Jiangsu Province, China in 1994. The incidence and mortality are very high, in some areas the incidence is up to 90% and the mortality rate is above 70%, which makes TD currently the most dangerous disease, thus resulting in huge economic losses (Shen *et al.*, 2015). Crabs with TD show symptoms, such as tremble, sluggishness, loss of appetite and curling of the legs. Although TD has been present for more than 20 years, the pathogen of TD has not been fully confirmed (Shen *et al.*, 2015). It is, however, important to note that some other viruses have been isolated from *E. sinensis* and are also candidate TD pathogens, including several strains of reoviruses, and EsBV (Bonami and Zhang, 2011; Shen *et al.*, 2015). Until now, genome sequences of bunyaviruses in crustacean were seldom known, we were only able to find a partial sequence of M segment with 1087 nt (GenBank: KM405247.2) for EsBV by searching GenBank database. To the best of our knowledge, we report here for the first time the complete genome sequences of EsBV.

Materials and Methods

Ethical statement. This study was approved by the Animal Care and Use Committee of the Freshwater Fisheries Research Center at the Chinese Academy of Fishery Sciences.

Animal collection. Chinese mitten crabs manifesting signs of TD were collected from a farm located in the Yandu District, Yancheng City, Jiangsu Province, China; these crabs were selected to perform high-throughput sequencing. Samples were also randomly collected from crab ponds for PCR detection.

RNA isolation, illumina sequencing and data analysis. Total RNA from various tissues of the Chinese mitten crabs was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The genomic DNA was removed using RNase-free DNase I (Qiagen). The RNA integrity was evaluated using 1.5% agarose gel electrophoresis, and RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA-seq library preparation, sequencing and data analysis was performed as per our published method (Shen *et al.*, 2014a, 2015).

Sequence phylogenetic analysis and bioinformatics analysis. The Test Maximum Likelihood tree was constructed from multiple sequence alignments with a total of 23 L segments encoding RNA Dependent RNA-polymerases sequences derived from the GenBank database using molecular evolutionary genetics analysis (MEGA) software, version 6.0. Similarly, a total of 23 M segments encoding viral glycoproteins, 24 S segments encoding nucleocapsid proteins were also collected to construct the Test Maximum Likelihood trees, respectively. Conserved protein domain was analyzed by searching SMART database (http://smart.embl-heidelberg.de/) and using MOTIF Search tool (http://www.genome.jp/tools/motif/)

PCR amplification. PCR was used to validate the genome sequences and detect the infection rate of EsBV in the crabs. The primers based on L segment were designed (Forward: 5'-ACACCT GTGAGAGAGGTCTTGC-3', Reverse: 5'-GTCTGCTGAGCAAC TAGCCATTCT-3'), and the expected product was 161 bp. Total RNA was isolated from the muscle of crabs using Trizol reagent and then reverse transcribed using M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's protocol. The synthesized cDNA was used as a template for PCR. The following PCR program was used: denaturation at 94°C for 3 min; 30 amplification cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; a final extension at 72°C for 7 min. The PCR products were analyzed on a 1.5% agarose gel using DNA markers.

Results

Complete genomic sequences of EsBV and genome organization of EsBV

Based our previous high-throughput sequencing data (Shen *et al.*, 2015), we identified three sequences related to bunya-like virus of *Eriocheir sinensis*. In all three sequences of EsBV, L segment has 6691 nt, (GenBank Acc. No. MH717874), M segment has 3266 nt, (NCBI GenBank Acc. No. MH717875), S segment has 836 nt, (NCBI GenBank Acc. No. MH717876). Conserved protein domain was analyzed by searching SMART database and using MOTIF Search tool. The L segment encodes a RNA-dependent RNA polymerase with 2173 amino acids, in which 2 motifs were recognized; an L_protein_N domain (PF15518) (aa No. 11–121, 8.3e-05) and a Bunya_RdRp domain (PF04196) (aa No. 617–1332, 4.3e-66) (Fig. 1); the estimated molecular weight is 248.1 kDa. The M segment encodes a glycoprotein with 914 aa length with an estimated molecular weight of 101.8 kDa. Bunyavirus glycoprotein G1 domain (PF03557) was identified (2.8e-24) at amino acids 406–785 (Fig. 1). The S segment is 836 bp long (Fig. 1) and encodes a 212 aa nucleocapsid protein with an estimated molecular weight of 23.2 kDa. No conserved domain was found. The non-coding regions (NCRs) varied among different segments: for the S segment, the 3' NCR (genome sense) was 77 nt

and the 5' NCR was 122 nt; for the M segment, the 3' NCR (genome sense) was 46 nt and the 5' NCR was 477 nt; for the L segment, the 3' NCR (genome sense) was 65 nt and the 5' NCR was 107 nt.

Characterization of terminal nucleotides of EsBV

The terminal nucleotide sequences of EsBV genome were conserved. Here, for all three segments of EsBV, the consensus extends to 13 nt that are complementary apart from an exception at position 8 (Fig. 2). These 13 terminal nt are followed by 4 (S segment) or 3 (M and L segments) nt that are complementary and conserved on a segment-specific basis (Fig. 2). The exceptional nucleotide at position 8 is A-G pairing in L segment and U-U pairing in M and S segments, exhibiting neither complementarity nor conservation. The complementary terminal sequences were found in all three segments of EsBV, which also suggests that we obtained the full-length genome sequences of EsBV.

Phylogenic trees of members of the family Bunyaviridae

The L-segment was selected to search for homology using BLASTX, the top 122 hits were with Ortobunyavirus (Peribunyaviridae) (E < 4e-71). The best hit was the Wenling crustacean virus 9 (E = 0.0), whose identity reached 96% for RNA-dependent RNA polymerase, the second hit was RNA-dependent RNA polymerase from Bimiti virus (E = 3e-92) with 23% identity. The Wenling crustacean virus 9 was an unassigned, negative sense ssRNA virus (Shi *et al.*, 2016). The genome sequences of EsBV and the Wenling crustacean virus 9 were compared; the S segment sequence of Wenling crustacean virus 9 is partial, it lacks about 37 nt sequence of 3' terminus. The identity of the L segment between EsBV and the Wenling crustacean virus 9 reached 87%, for the M segment the identity reached 86%, while for the S segment the identity reached 88%. Because of its sequence conservation, viral RdRp is the most common protein used for the phylogenetic analysis of RNA viruses, and it has been widely applied to confirm the evolutionary relationship of various species (Attoui *et al.*, 2000; Mohd Jaafar *et al.*, 2008; Deng *et al.*, 2012). Here, 23 RNA-dependent RNA polymerasees from five families of *Bunyvirales*, formerly known as five genera in the *Bunyaviridae* family, were used to construct a phylogenetic tree (Fig. 3a). These studies showed that members from the same family were clustered in the same clade. EsBV clustered with the Wenling crustacean virus 9 firstly, and then the branch clustered with peribunyaviridae clade, which was consistent with the results based on sequence similarity.

Similarly, 23 glycoproteins coded by the M segment, 24 nucleocapsid proteins coded by the S segment from five family of *Bunyvirales* were used to construct the phylogenetic tree, respectively (Fig. 3b and 3c). Both phylogenetic trees

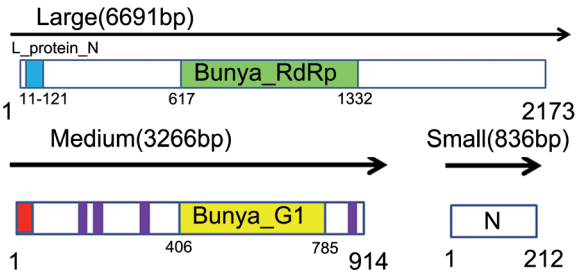

Fig. 1

Genome organization of EsBV

The RNA-dependent RNA polymerase is shown in long rectangle box, in which motif L_protein_N is highlighted in blue, and Bunya_RdRp in green. The glycoprotein precursor is shown in the middle rectangle box, in which motif Bunya_G1 is highlighted in yellow, a signal peptide is highlighted in red in its N-terminus, four transmembrane regions in the glycoprotein precursor are highlighted in purple. The nucleoprotein encoded by the small segment is shown in the short rectangle box. The respective amino acid positions of the elements in the genome are shown at the bottom of the figures (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Fig. 2

Consensus 3' and 5' terminal nucleotide sequences of EsBV genome RNA segments

were similar with the phylogenetic tree based on RNA-dependent RNA polymerase amino acid sequences. EsBV clustered with the Wenling crustacean virus 9 firstly, and then the branch clustered with peribunyaviridae clade, which was consistent with the result from phylogenetic tree based on RNA-dependent RNA polymerase sequences. The results from three phylogenetic trees indicate that EsBV and the Wenling crustacean virus 9 can be classified in the family *Peribunyaviridae*, to which the orthobunyaviruses belong.

To further explore the Taxonomic status of EsBV in orthobunyaviruses, EsBV, the Wenling crustacean virus 9 and 66 bunyaviruses from 15 different serogroup were selected

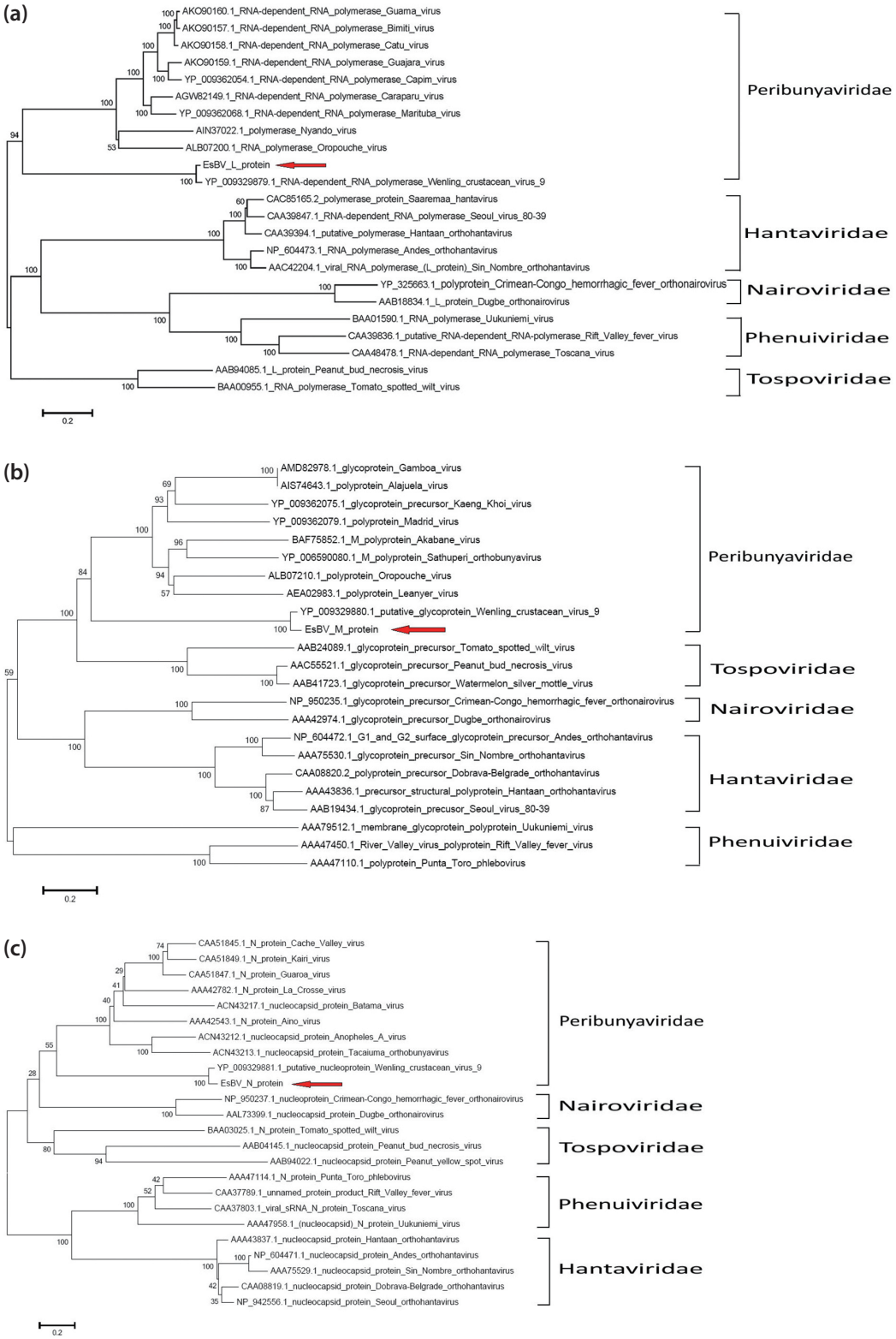

Fig. 3

Molecular phylogenetic analysis by the maximum-likelihood method showing representative viruses from different viruses in the *Bunyvirales* order using (a) L-, (b) M-, (c) S- segment ORF amino acid sequences

GenBank Acc. Nos. are indicated before to virus names.

to construct a phylogenetic tree, the results showed that EsBV and the Wenling crustacean virus 9 were clustered in a unique clade and belonged to no known serology (Supplementary Fig. S1).

Infection rate of EsBV in the crabs

Forty-two samples were randomly collected from crab farming ponds, and analyzed by RT-PCR for the presence of the L segment using specific primers. The results showed that seventeen samples had strong bands, showing a positive rate of 40% (Supplementary Fig. S2). These results indicate a high infection rate of EsBV in crabs from farming ponds included in this study.

Discussion

In this report, the genomic and phylogenetic characterization of EsBV was analyzed. EsBV had the highest sequence identity with the Wenling crustacean virus 9, genome sequences of two viruses were compared, the identity of three segments of genome for the two viruses reached 86%–88%. The Wenling crustacean virus 9 was found in tissues from 13 species of crustaceans (Shi *et al*., 2016) with the exception of *Eriocheir sinensis*. Among the 13 species, the closest species with *Eriocheir sinensis* are *Ovalipes punctatus*, a crab from the family *Portunidae*, the order *Decapoda*, and two species from the family *Latreilliidae*, the order *Decapoda*, so we can conclude that the host of the Wenling crustacean virus 9 is not *Eriocheir sinensis*. Phylogenetic trees based on protein sequences encoded by three RNA segments showed EsBV and the Wenling crustacean virus 9 formed a unique novel clade under *Orthobunyvirus* (*Peribunyaviridae*). Phylogenetic analyses of the S segments of EsBV also showed that it does not belong to any serogroup of orthobunyvirus. All these results indicate that it is taxonomically classified under *Orthobunyvirus* (*Peribunyaviridae*), but does not belong to any reported genera in the family *Peribunyaviridae*.

Bunyaviruses exhibite complementary terminal sequences; terminal nucleotide complementarity between their 3' and 5' non-translated regions (NTRs) was reported to be associated with transcription and RNA replication (Guu *et al*., 2012; Ferron *et al*., 2017). Normally, these complementary terminal nucleotide sequences are conserved among members of same family of bunyaviruses, the consensus terminal sequences of orthobunyavirus of the L, M and S genome segments are UCAUCACAUG… at the 3' end and AGUAGUGUGC… at the 5' end. However, EsBV's complementary terminal sequences of the L, M and S genome segments are UCAUCAUAUGUCC… at the 3' end and AGUAGUAGACAGG… at the 5' end. The terminal six nucleotide sequences are UCAUCA at the 3' terminus and AGUAGU at the 5' terminus in all three segments of EsBV, which is in line with other reported orthobunyaviruses. The other part of the terminal sequences of EsBV is different than in other orthobunyaviruses and exhibits a unique feature. In addition, the non-complementary nucleotide of the terminal sequences of the three segments is at the position 8, unlike at the position 9 in other reported members of orthobunyavirus. However, there is a conserved U at the position 9 of the 3' UTR in all three segments of EsBV. The nucleotide U is strictly necessary at the position 9 of the 3' UTR, the corresponding nucleotide at the 5' UTR may vary without affecting transcription initiation from the vRNA template (Barr and Wertz, 2005).

Several crabs were reported infected by Bunyavirus-like particles, including *Carcinus maenas* (Bang, 1971, 1974), *Macropipus depurator* (Decapoda) (Bonami *et al*., 1975), the edible crab, *Cancer pagurus* (L.) (Corbel *et al*., 2003); Athtabvirus, a Bunya-like virus, was also reported in crayfish (*Cherax quadricarinatus*) from farms in northern Australia (Sakuna *et al*., 2018). It has to be noted that in all reported bunyaviruses in crustacean, except Athtabvirus, were obtained the L and M sequences, the genome sequences of all other reported bunyaviruses in crustacean were not yet obtained. Here, the genome sequences of EsBV are the first example of whole genome sequences of bunyavirus in crustacean. The causative agents of TD are controversial, with several related viruses being suggested, including several strains of reoviruses, and EsBV (Bonami and Zhang, 2011; Shen *et al*., 2015). Here, the whole genome sequences of EsBV were obtained, which will act as a foundation block for future research on EsBv and go a long way in illuminating its relationship with TD.

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Supplementary information is available in the online version of the paper.

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Supplementary information

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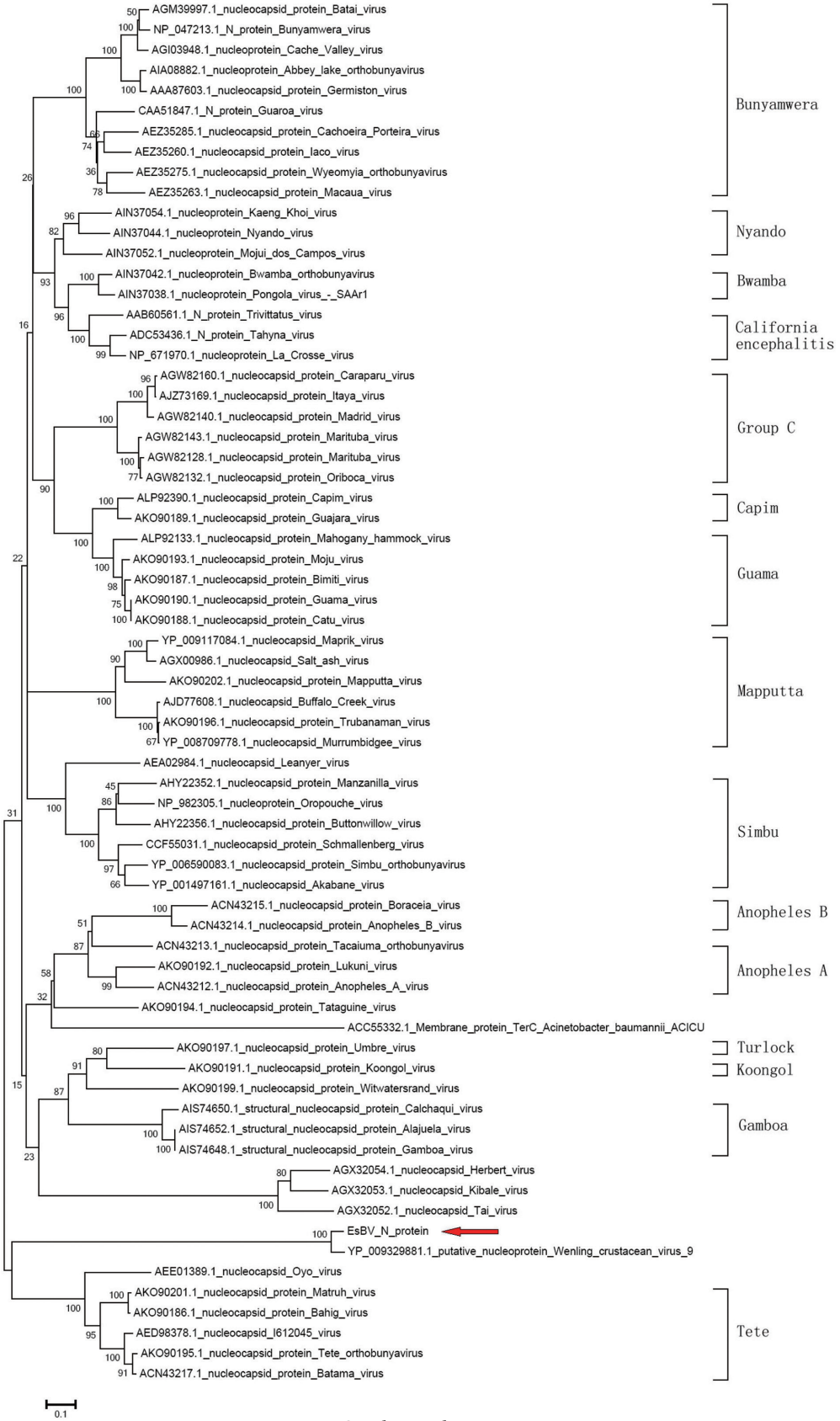
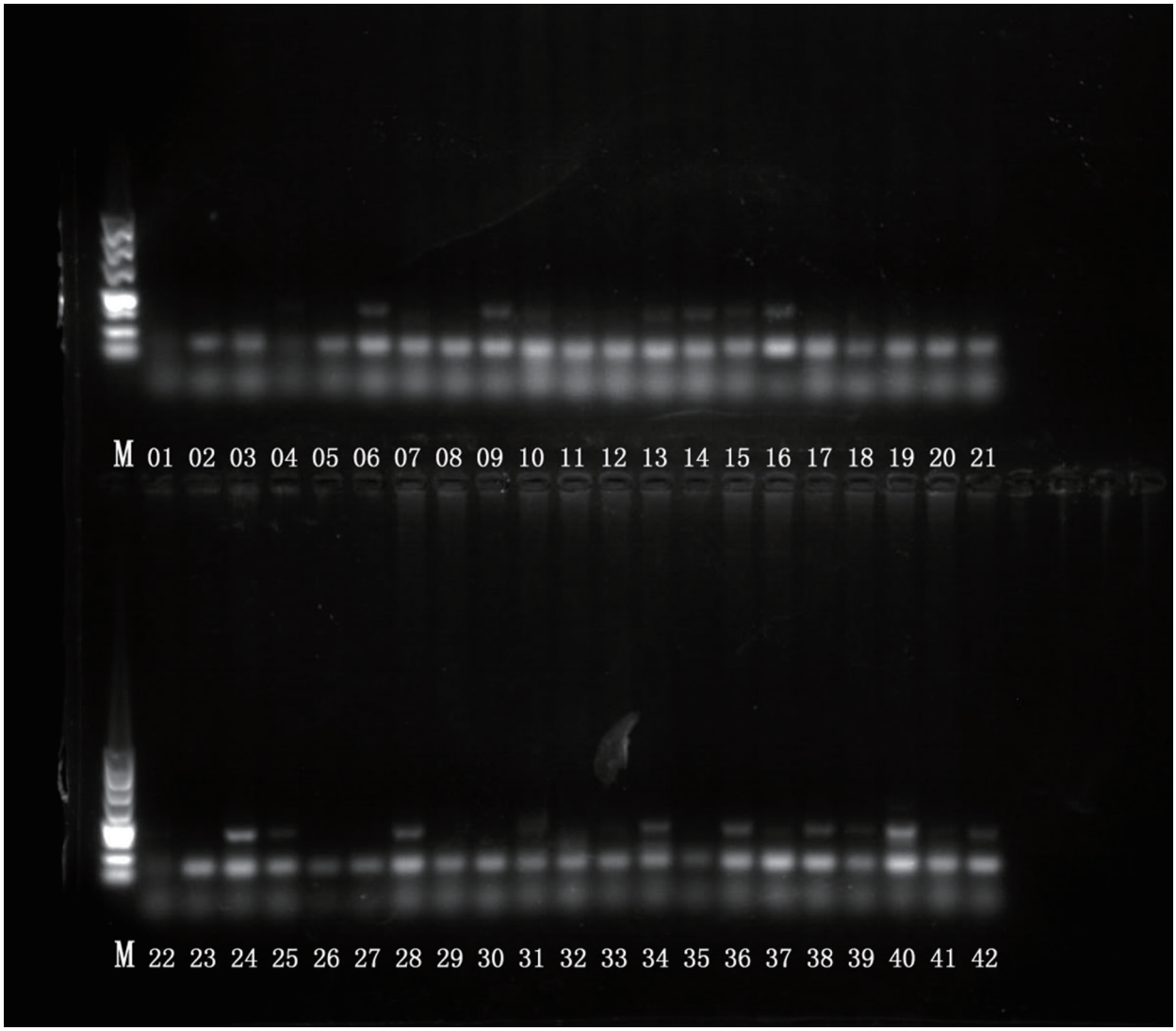


Supplemental Fig. 1

Molecular phylogenetic analysis by the maximum-likelihood method showing representative viruses from different EsBV, the Wenling crustacean virus 9 and 66 members from orthobunyavirus using S- segment ORF amino acid sequences

GenBank Acc. Nos. are indicated before the virus names.



Supplemental Fig. 2

Example of agarose gel showing bands of PCR amplicons obtained using Chinese mitten crab samples
01–42, crab samples; M, 500 bp DNA Ladder.