

# A Novel Hepe-Like Virus from Farmed Giant Freshwater Prawn *Macrobrachium rosenbergii*

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**Abstract:** The family Hepeviridae includes several positive-stranded RNA viruses, which infect a wide range of mammalian species, chicken, and trout. However, few hepatitis E viruses (HEVs) have been characterized from invertebrates. In this study, a hepevirus, tentatively named Crustacea hepe-like virus 1 (CHEV1), from the economically important crustacean, the giant freshwater prawn *Macrobrachium rosenbergii*, was characterized. The complete genome consisted of 7750 nucleotides and had a similar structure to known hepatitis E virus genomes. Phylogenetic analyses suggested it might be a novel hepe-like virus within the family Hepeviridae. To our knowledge, this is the first hepe-like virus characterized from crustaceans.

**Keywords:** Crustacea hepe-like virus 1; Hepeviridae; *Macrobrachium rosenbergii*

## 1. Introduction

The family Hepeviridae belongs to positive-stranded RNA viruses and infects a wide range of mammalian species, chicken, and trout [1,2]. Due to frequent identifications of novel hepatitis E viruses (HEVs) or HEV-like viruses from various animal species, the HEV nomenclature system has been undergoing dynamic changes [3–6]. According to the consensus proposal from the International Committee on Taxonomy of Viruses (ICTV), the family Hepeviridae is currently divided into two genera: *Orthohepevirus*, including all mammalian and avian HEVs; and *Piscihepevirus*, including the Cutthroat trout virus [5,7]. Within *Orthohepevirus*, four species have been designated (A–D) that infect mammals and birds [4,8], including *Orthohepevirus* A from human, swine, deer, mongoose, rabbit, and camel; *Orthohepevirus* B from chicken and wild birds; *Orthohepevirus* C from rat, greater bandicoot, Asian musk shrew, ferret, and mink; and *Orthohepevirus* D from bats. In addition, different genotypes have also been classified based on the genomic sequence divergence within species. For example, within *Orthohepevirus* A, eight genotypes have been proposed [7]. However, to our knowledge, only a few HEVs from invertebrates have been reported, of which none were directly identified from crustaceans.

## 2. Materials and Methods

### 2.1. Sample Collection and Detection of Shrimp Pathogens

In 2018, a total of 18 diseased giant freshwater prawns (*Macrobrachium rosenbergii*) with growth retardation were collected from a farming pond in Jiangsu Province, China. Total RNA and DNA were extracted from each of these *M. rosenbergii* prawns, and the detection of known shrimp pathogens was performed using RT-PCR or PCR [9–11]. The shrimp pathogens included the White spot syndrome virus (WSSV), Infectious hypodermal and haematopoietic necrosis virus (IHHNV), *Enterocytozoon hepatopenaei* (EHP), Acute hepatopancreatic necrosis disease-causing *Vibrio* (AHPND-causing *Vibrio*), Taura syndrome virus (TSV), Yellow head virus genotype 1 (YHV-1), Infectious myonecrosis virus (IMNV), Decapod iridescent virus 1 (DIV1), *Macrobrachium rosenbergii* nodavirus (MrNV), and Covert mortality nodavirus (CMNV).

### 2.2. RNA Extract and Transcriptome Sequencing

Cephalothoraxes from 15 g *M. rosenbergii* were homogenized and the supernatant was centrifuged at 120,000× g for 4 h at 4 °C. Total RNA from viral crude extracts was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the ribosomal RNA was removed by the Epicentre Ribo-zero™ rRNA Removal Kit (Epicentre, Madison, WI, USA). Sequencing libraries were generated using the rRNA-depleted RNA by NEB Next®Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA), following the manufacturer's recommendations. Then, the 150 bp paired-end sequencing of the RNA libraries were conducted using the Illumina HiSeq platform. The raw sequencing reads were adaptor- and quality-trimmed using the Trimmomatic program [12] embedded in Trinity [13]. The clean reads were directly assembled de novo using Trinity (version 2.5.1) with default parameter settings. All the assembled contigs were compared using BLASTx against the non-redundant protein database (nr) downloaded from GenBank, with an *E*-value threshold set at  $1 \times 10^{-5}$ . All potential viral contigs were identified and then merged to form longer viral contigs using Geneious (version 11.1.5).

### 2.3. Viral Genome Sequencing and Sequence Analysis

To further confirm the results from next generation sequencing (NGS), RT-PCR and Sanger sequencing of viral crude extracts based on the obtained contigs were performed. Firstly, different primer pairs were designed based on the viral contigs (Table 1). Meanwhile, both the 5' and 3' rapid amplifications of the cDNA ends (RACE) (Invitrogen) were employed to determine the termini of the obtained viral genome. Then, the structure of the complete viral genome was analyzed by searching against the Conserved Domain Database (CDD). Both the reference sequences of the RNA-dependent RNA polymerase (RdRp) and the capsid protein were downloaded from GenBank and aligned using Mafft [14]. The conserved regions of the two alignments were obtained using Trimal [15]. Phylogenetic analysis was performed using RaxML [16], with the JTT amino acid substitution model and 1000 bootstrap replicates.

**Table 1.** Primers used for amplification and sequencing of the Crustacea hepe-like virus 1 genome.

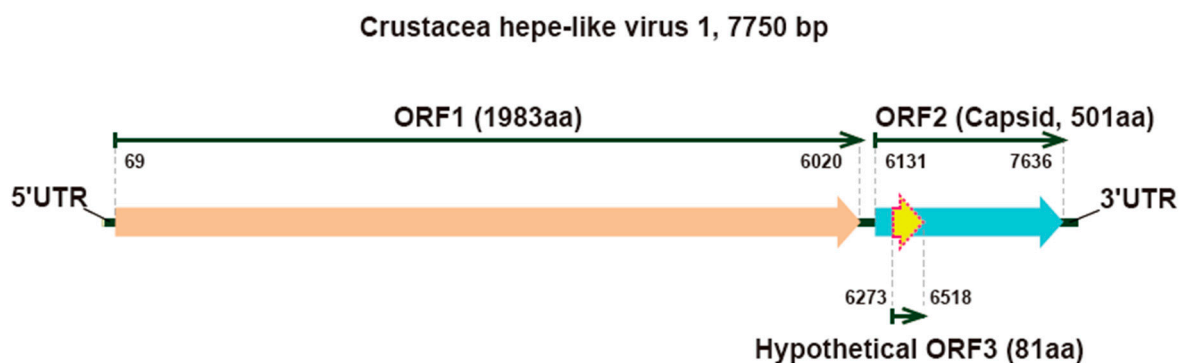
| Primer     | Sequence 5'-3'                                         |
|------------|--------------------------------------------------------|
| HE-1F      | TGATAACGATGGATATTAATCCACAT                             |
| HE-1R      | AGAAGTTGAAAATGCCGCTGAT                                 |
| HE-2F      | TCGTTTCAAGAAAGGCAACAA                                  |
| HE-2R      | TAGAGGGAGCATGACTGGTTTGT                                |
| HE-3F      | TTCACGTATGTCCAACACAATAACTA                             |
| HE-3R      | GTGGGGTTGGTCTTATAGCGTA                                 |
| HE-4F      | ACTTCTTGGACATCCATTCCACA                                |
| HE-4R      | GTGTAAAGATGTCTTACTTGCTCTGTT                            |
| HE-5F      | GAAAACATCCACGCTCAAAATC                                 |
| HE-5R      | GGTTCATACCATTCTTTCTCCAGTT                              |
| HE-6F      | CAAGCTAAGAAGCAATGCCGT                                  |
| HE-6R      | TTGTTCCAAGTCCTCAGGTGTGT                                |
| HE-7F      | ATACATCTCCAACATTGTCACACCA                              |
| HE-7R      | CTTTGAGTGCCTTGGCAATGT                                  |
| HE-8F      | GGAAAGCCTGGGACAGAGATA                                  |
| HE-8R      | ATTGCGATACTATGTAGGCCCA                                 |
| 5' adaptor | GCTGTCAACGATACGCTACGTAACGGCATGACAGTGCCCCCCCCCCCCCCCC   |
| 3' adaptor | GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTT |
| 5.3' outer | GCTGTCAACGATACGCTACGTAAC                               |
| 5.3' inner | GCTACGTAACGGCATGACAGTG                                 |
| CHEV-H-F1  | CGTCCAGGAAAGGGCCAGTTAACAC                              |
| CHEV-H-F2  | CGATCAAAATCGATGGGGCCTAAAGT                             |
| CHEV-H-R2  | GCTGCAGATATTGATCCCTGTGTCGTT                            |
| CHEV-H-R1  | ATTGGTCGACGATAAGTGGATCATTGTTTATA                       |
| CHEV-H-RT2 | TTGACTGCGTAAGCGTAA                                     |
| CHEV-H-RT1 | GAGGTATTAGGTTGATGTCTG                                  |

### 3. Results and Discussion

For all of the diseased giant freshwater prawns (*M. rosenbergii*), our molecular tests showed that they were negative for all known shrimp pathogens, including WSSV, IHNV, EHP, AHPND-causing *Vibrio*, TSV, YHV-1, IMNV, DIV1, MrNV, and CMNV. However, the NGS of the viral crude extracts from the pooled samples identified one contig (size = 7696 bases) associated with Hepeviridae, with 3608 non-repetitive reads and mean depth of  $60.94 \pm 18.52$ . The first hit in the BLASTx output for this contig was AFR11848.1 (506 aa) with a sequence identity of 55%, which was a protein encoded by the opening reading frame (ORF) 2 of a *Hepelivirus* from sewage from Nepal in 2009 [17]. The second hit was ASM94024.1 (1797 aa), the polyprotein encoded by a Barns Ness breadcrumb sponge hepe-like virus 2 from *Halichondria panacea* from Scotland in 2014, with only 26% sequence identity in the helicase and RdRp regions from positions 920 to 1771. Because both *Hepelivirus* and Barns Ness breadcrumb sponge hepe-like virus 2 are currently proposed to be unclassified viruses in the Hepeviridae family, we propose that the novel viral isolate may belong to the Hepeviridae family and was tentatively named as Crustacea hepe-like virus 1 (CHEV1).

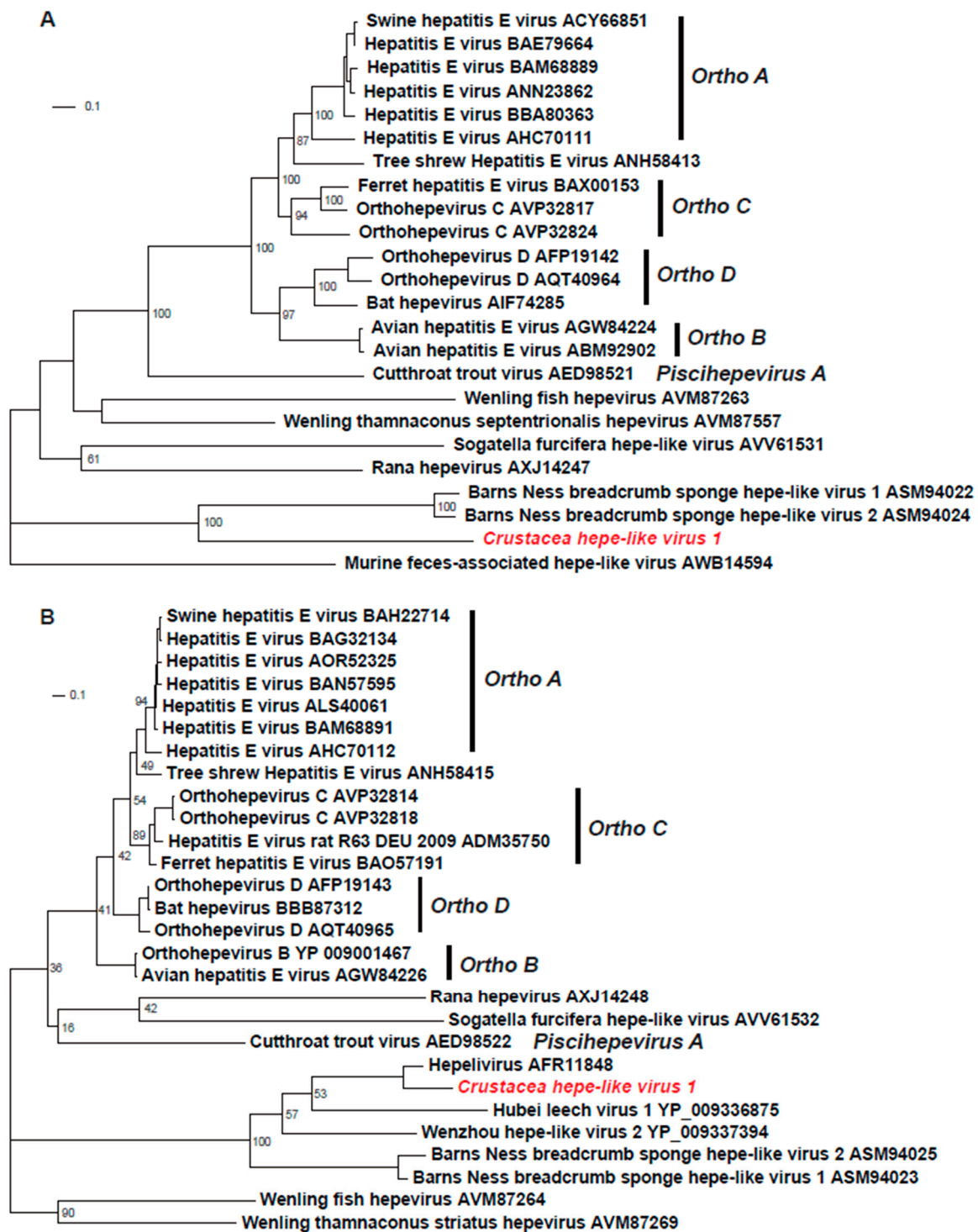
To further confirm the assembly results, RT-PCR and Sanger sequencing based on this contig were performed using primers designed based on the NGS results (Table 1). In addition, both 5' and 3' RACE were employed to determine the termini of the CHEV1 genome. The full genome of CHEV1 with 7750 nucleotides (nt) in length was successfully obtained and deposited in GenBank under accession number MK580123. Similar to other recognized HEVs, the newly sequenced genome also included a 5' untranslated region (UTR), followed by two major ORFs and one hypothetical ORF, and a 3' UTR (Figure 1). The first ORF encodes a polyprotein of 1983 amino acids (aa) from genome positions 69 to 6020 nt (Figure 1). As mentioned above, only the 3' terminus of the polyprotein showed certain sequence identity with hypothetical helicase and RdRp of some other unclassified HEVs. The second ORF from genome positions 6131 to 7636 encoded a protein of 501 aa, which may encode the capsid

protein of CHEV1 (Figure 1). The hypothetical ORF3 was predicted to be located from positions 6273 to 6518 and was fully overlapped with ORF2. It encoded a protein of 81 aa with the start codon CTG rather than ATG, which was used by the first two ORFs. It also should be noted that the hypothetical ORF3 was smaller than those of other recognized HEVs. We further compared the protein sequences of the predicted ORF3 of CHEV1 and representative HEVs [18] (Figure S1). However, these proteins were so divergent that only a few conserved amino acids could be observed. Therefore, the existence of ORF3 of CHEV1 warranted further verification. However, CHEV1 exhibited a relatively low amino acid identity to the Orthohepevirus species A Genotypes 1 (M73218, D11092, X98292, AY23020, AY204877, and JF443721) and 2 (M74506) that can infect humans [19]: The predicted ORF1, 17.3% to 17.6% for Genotype 1 and 17.5% for Genotype 2; the predicted ORF2, 14.4% to 14.7% for Genotype 1 and 13.8% for Genotype 2; and the predicted ORF3, 12.9% for Genotype 1 and 10.6% for Genotype 2.



**Figure 1.** The predicted genome structure of the novel Crustacea hepe-like virus 1.

In order to study the phylogenetic position of CHEV1, the RdRp and capsid protein sequences were extracted and phylogenetically analyzed with those of representative HEVs. In both trees, the HEVs clustered into two major branches, which basically represented HEVs infecting vertebrates and invertebrates, respectively. The branch of vertebrate hepeviruses included the two previously classified *Orthohepevirus* and *Piscihepevirus* genera, as well as several unclassified species from fish, whereas CHEV1 was located in the branch of invertebrate hepeviruses (Figure 2). Consistent with the previous BLASTx search, phylogenetic analysis of the RdRp protein sequences showed that CHEV1 clustered with Barns Ness breadcrumb sponge hepe-like viruses 1 and 2, despite with a very long branch separating them (Figure 2A). In the tree constructed using the capsid proteins, CHEV1 formed a separate cluster with *Hepelivirus* (Figure 2B). Remarkably, CHEV1 did not fall within the two classified genera, and hence may represent a novel hepe-like virus in the family Hepeviridae (Figure 2).



**Figure 2.** Phylogenetic analyses of the RNA-dependent RNA polymerase (RdRp) and capsid protein sequences of representative hepeviruses. (A) Phylogenetic analyses of RdRp sequences of representative hepeviruses. (B) Phylogenetic analyses of capsid protein sequences of representative hepeviruses. In both panels, *Ortho* represents *Orthohepevirus* and the novel virus is highlighted in red. The reference sequences were downloaded from GenBank and aligned using Mafft. Phylogenetic analysis was performed using RaxML, with the JTT amino acid substitution model and 1000 bootstrap replicates.

#### 4. Conclusions

In summary, we described a novel hepe-like virus from diseased *M. rosenbergii*, which possessed two major ORFs and a hypothetical ORF3 and might represent a novel hepe-like virus in the family Hepeviridae. To our knowledge, it is the first hepe-like virus identified from crustaceans. Although we could not determine the novel virus as the causative agent for the outbreak based on current evidence, our study highlighted the expanding host range and the increasing species diversity of the family Hepeviridae.

**Supplementary Materials:** Supplementary materials can be found at <http://www.mdpi.com/1999-4915/12/3/323/s1>.

**Author Contributions:** X.D., W.S., Q.Z. and J.H. designed this study; X.D., T.H., Q.L., C.L. and Y.S. performed experiments; T.H. and Y.W. analyzed the data; X.D., W.S., Q.Z. and J.H. wrote the manuscript. All of the authors reviewed the manuscript and approved the final version.

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**Conflicts of Interest:** The authors declare no conflict of interest. All authors have seen and approved the manuscript and have contributed significantly to the work.

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