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Pathogenicity and complete genome sequence analysis of the mud crab dicistrovirus-1

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ABSTRACT

A virus with a particle diameter of approximately 30 nm and no envelope was purified from diseased mud crab, *Scylla paramamosain* and it was demonstrated to be pathogenic to mud crab. The complete nucleotide sequence analysis indicated that its genome was a single molecule of linear positive-sense ssRNA with a length of 10,415 nucleotides, excluding the 3′poly (A) tail. It consisted of two open reading frames (ORF) separated by an intergenic region (IGR) and flanked by a 5′untranslated region (5′-UTR) and a 3′untranslated region (3′-UTR). The 5′-ORF encode five putative non-structural proteins, including BIR (Baculovirus Inhibitor of Apoptosis Protein Repeat), helicase, VPg (the genome-linked viral protein), 3C-like protease and RdRP (RNA-dependent RNA polymerase), while the 3′-ORF encode the structural protein precursors. This genome organization was consistent with the typical organization of dicistrovirus and the virus was designated as mud crab dicistrovirus-1 (MCDV-1). The results of the phylogenetic analysis of the putative structural protein precursor suggest that MCDV-1 has a closer genetic relationship with Taura syndrome virus (TSV) than do other dicistroviruses and that MCDV-1 is a new member of the family *Dicistroviridae* and assigned into the genus *Aparavirus*.

1. Introduction

The mud crab, *Scylla paramamosain*, previously identified as *S. Serrata* (Lin et al., 2007), is an economically important species cultured in China for the past 100 years, with a production in excess of 115 thousand metric tons in 2009 (China Fishery Statistical Yearbook, 2010). However, with the development of intensive culture, various diseases have frequently begun to appear that have severely affected production. From May to November 2004, mass mortalities, in excess of 70% in some cases, occurred in the cultured mud crab population and resulted in significant economic losses. We previously described that the mud crab reovirus infection is the main cause of mortality (Weng et al., 2007). In our subsequent investigations, we found that an additional virus with a diameter of about 30 nm accompanied the mud crab reovirus. Our further studies including the complete genome nucleotide analysis revealed this accompanied virus to be a dicistrovirus.

Dicistrovirus in the family *Dicistroviridae* belongs to the order *Picornavirales* (Gall et al., 2008), which includes two genera, *Cripavirus* and *Aparavirus*. Its members can be distinguished from the members of the *Iflaviridae*, *Marnaviridae*, *Picornaviridae* and *Secoviridae* in the order *Picornavirales* by their dicistronic RNA genome with two non-overlapping ORFs that are separated by an intergenic region (IGR) (Chen et al., 2012). Dicistroviruses are a group of pathogenic agents that infect some insects, including the aphid (D'Arcy et al., 1981; Williamson et al., 1988), bee (Allen and Ball, 1996; Maori et al., 2007), cricket (Reinganum et al., 1970), *Drosophila* (Jousset et al., 1977), and *Triatoma* (Ronderos and Schnack, 1987), as well as the pacific white shrimp *Litopenaeus vannamei* (Crustacean) (Hasson et al., 1995). At present, the complete genomes of 14 species of dicistrovirus have been described, including the Acute bee paralysis virus (ABPV) (Govan et al., 2000), Aphid lethal paralysis virus (ALPV) (Munster et al., 2002), Black queen cell virus (BQCV) (Leat et al., 2000), Cricket paralysis virus (CrPV) (Wilson et al., 2000), Drosophila C virus (DCV) (Koonin and Dolja, 1993), Himetobi P virus (HiPV) (Nakashima et al., 1999), Plautia stali intestine virus (PSIV) (Sasaki et al., 1998), *Rhopalosiphum padi* virus (RhPV) (Moon et al., 1998), Triatoma virus (TrV) (Czibener et al., 2000), Taura syndrome virus (TSV) (Mari et al., 2002), Kashmir bee virus (KBV) (Miranda et al., 2004), Solenopsis invicta virus-1 (SINV-1) (Valles et al., 2004), Homalodisca coagulata virus-1 (HoCV-1) (Hunnicutt et al., 2006), and Israel acute paralysis virus of bees (IAPV) (Maori et al., 2007). In this paper, the pathogenicity of the virus particles isolated from the mud crab was demonstrated by

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an infection test. The complete genome of this virus was described and compared with that of other reported dicistroviruses, revealing the presence of two ORFs separated by an intergenic region. The genome organization, multiple sequence alignments, and phylogenetic analysis of the genome sequence all help define this virus as a new member of the family *Dicistroviridae*, named *mud crab virus*.

2. Materials and methods

2.1. Virus purification

A 20 g sample of gills from the diseased mud crabs, which were collected in the farm of Zhuhai city (Guangdong Province, China) in 2006, was homogenized in PBS (2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) in an ice bath with a glass tissue blender. After centrifugation for 30 min at 1000 × *g* in a Beckman centrifuge (Avanti™ J-20XP, JA-14 rotor) at 4 °C, the supernatant was centrifuged again for 1 h at 10,000 × *g* at 4 °C. The virions in the supernatant were then pelleted for 2 h at 200,000 × *g* in a Beckman centrifuge (Optima™ LE-80K, 70Ti rotor) at 4 °C and resuspended in PBS buffer before being layered onto a 15–45% (w/w) CsCl gradient and centrifuged for 8 h at 200,000 × *g* (Optima™ LE-80K, SW41Ti rotor) at 15 °C. Viral fractions were collected and washed in PBS by centrifugation at 200,000 × *g* at 4 °C for 2 h. The virus was finally resuspended in 0.3 ml of PBS buffer and examined under Philips CM10 transmission electron microscopy after negative staining with 2% phosphotungstic acid (pH 6.8).

2.2. Infection test

The mud crabs with the size of 30–40 g/crab used for the challenge test were collected from the coastal area of Zhuhai, Guangdong Province, China. The mud crabs had been maintained in the tanks covered with sand for more than 2 weeks and feed with low-valuable molluscus. During the period of acclimatization and experiment, the flowing water and aeration were adopted, the water temperature was 28–30 °C, the salinity is 28‰, no crabs with clinical symptom were observed.

The purified virus solution in what no mud crab reovirus was observed under electronic microscope was diluted 1000 times in PBS buffer and passed through 0.22 μm filter membrane (Millipore, USA). Twenty mud crabs were injected at the base of forth appendage with 0.1 ml virus filtrate per crab and cultured in two tanks. Twenty crabs were injected with 0.1 ml PBS (pH 7.4) per crab as control. The number of the dead crab was recorded daily. 5 crabs were sampled randomly in the control group and 5 moribund crabs in the test group for MCDV-1 detection in their gill, gut, stomach, intestine, heart, hepatopancreas, gonads, muscles, hemolymph and horacic ganglion tissues.

2.3. Viral RNA extraction

The RNA was extracted with Trizol (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 10 volumes of Trizol were added to the viral suspension and vortexed a few minutes, then mixed with 0.2 volume of chloroform and left at room temperature for 3 min before centrifugation at 10,000 × *g* for 10 min at 4 °C. The upper aqueous layer containing the RNA was transferred to a fresh tube, recovered by precipitation with isopropyl alcohol, and washed with 75% ethanol. The RNA was resuspended in DEPC-treated water and kept at −70 °C until use, after quality assessment with agarose gel electrophoresis.

2.4. RT-PCR detection

One pair of primers were designed with PrimerPrimer 5 software (Primier, Canada) based on the MCDV-1 genome sequence, which are:

DF (sense): 5-AGGAACCTTAGTGGGTAGGAATG-3, DR (antisense): 5-TCCTTTGATGTAAATCGTGCTCT-3, the expected product size is 439 bp.

The same tissue of 5 crabs were pooled together with 50 mg for each crab to extract total RNA with Trizol (Invitrogen, USA). cDNA was synthesized by the RT reaction following the instruction of M-MLV reverse transcriptase system manufacture modified slightly(Promega, USA). The RNA extracted from purified virus, healthy and diseased mud crab gill tissue with Trizol (Invitrogen, USA) respectively in 16 μl DEPC-treated water was heated at 65 °C for 5 min with 4 μl random primer (N6) and 1 μl RNAsin ribonuclease inhibitor (Promega, USA), then, quickly cooled in ice for 5 min. Then, the following agent was added into the mixture and vibrated: 8 μl first-strand buffer (5×), 1 μl RNAsin ribonuclease inhibitor, 4 μl dNTP mix (10 mM each), 4 μl DTT (0.1 M), 2 μl M-MLV reverse transcriptase. After that, the mixture was incubated for 1 h at 37 °C, then, heated to 90 °C for 5 min to inactivate reverse-transcriptase activity before the mixture was cooled in ice.

PCR amplification was carried out in a 20 μl reaction mixture containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5% Tween-20, 0.2 mM each dNTP, 0.25 mM of primer, 1 unit of Taq DNA polymerase and cDNA. The mixture was incubated in a PTC-200 DNA thermal cycler (MJ Research Inc., USA) at 95 °C for 3 min, and then for 30 cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min) plus a final 10 min extension at 72 °C. The PCR products were analyzed in 0.8% agarose gels containing ethidium bromide at a final concentration of 0.5 μg/ml and visualized under a UV transilluminator.

2.5. Sequencing of the MCDV-1 genome

To sequence the RNA of the MCDV-1, double-stranded cDNA with blunt ends was synthesized with M-MLV RTase cDNA synthesis kit (TAKARA, Japan) with a random primer and cloned with the Mighty DNA cloning Kit (Blunt END) (TAKARA, Japan). The positive clones were screened by the method described by Sambrook and Russell (2001) and sequenced by the BigDye Terminator Kit (Applied Biosystems, Inc.) using the ABI PRISM377 DNA sequencer.

The sequences obtained from the cDNA clones were assembled by computer, and then primers were designed based on the obtained sequences to bridge the gap between the known sequence fragments. The sequences of both ends were amplified with 3′ and 5′-RACE respectively. Finally, the assembled sequence was confirmed twice by PCR with overlapped products.

2.6. Computer analysis of the sequence

All computational sequence analysis was done using the software in the DNAstar package (DNASTAR®, Inc., USA) and the BLAST suite at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). EditSeq was used for the sequence editing, ORF finding and deduction of amino acid sequences based on the nucleotide acid sequences. Multiple sequence alignments were performed with the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). An unrooted phylogenetic tree was constructed by the neighbor-joining method with MEGA 3.1 software using the amino acid alignment of the putative structural polyptrotein. Confidence was carried out by bootstrapping 1000 replications.

Besides the MCDV-1 sequence, all other dicistrovirus sequences used for analysis in this paper were obtained from the NCBI