Identification and genomic characterization of a novel fish reovirus, Hubei grass carp disease reovirus, isolated in 2009 in China

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A novel fish reovirus, Hubei grass carp disease reovirus (HGDRV; formerly grass carp reovirus strain 104, GCRV104), was isolated from diseased grass carp in China in 2009 and the full genome sequence was determined. This reovirus was propagated in a grass carp kidney cell line with a typical cytopathic effect. The total size of the genome was 23 706 bp with a 51 mol% G+C content, and the 11 dsRNA segments encoded 12 proteins (two proteins encoded by segment 11). A nucleotide sequence similarity search using BLASTN found no significant matches except for segment 2, which partially matched that of the RNA-dependent RNA polymerase (RdRp) from several viruses in the genera Aquareovirus and Orthoreovirus of the family Reoviridae. At the amino acid level, seven segments (Seg-1 to Seg-6, and Seg-8) matched with species in the genera Aquareovirus (15–46 % identities) and Orthoreovirus (12–44 % identities), while for four segments (Seg-7, Seg-9, Seg-10 and Seg-11) no similarities in these genera were found. Conserved terminal sequences, 5′-GAAUU——UCAUC-3′, were found in each HGDRV segment at the 5′ and 3′ ends, and the 5′-terminal nucleotides were different from any known species in the genus Aquareovirus. Phylogenetic analysis based on RdRp amino acid sequences from members of the family Reoviridae showed that HGDRV clustered with aquareoviruses prior to joining a branch common with orthoreoviruses. Based on these observations, we propose that HGDRV is a new species in the genus Aquareovirus that is distantly related to any known species within this genus.

INTRODUCTION

The family Reoviridae contains 15 genera with 9, 10, 11 or 12 dsRNA genome segments (Mohd Jaafar et al., 2008; Attoui et al., 2012). Reoviruses that can infect aquatic species include members of the genera Aquareovirus, Mimoreovirus and Cardoreovirus. Members of the genera Aquareovirus and Mimoreovirus have 11 dsRNA segments (Attoui et al., 2006a), whereas Cardoreovirus members have 12 dsRNA segments (Mari & Bonami, 1988; Zhang et al., 2004). Recently, a reovirus isolated from the mud crab, Scylla serrata, was sequenced and proposed to be a new genus based on its sequence dissimilarity to existing viruses (Chen et al., 2011).

Aquareoviruses have been isolated from a wide variety of aquatic animals including molluscs, finfish and crustaceans (Winton et al., 1987; Lupiani et al., 1995; Seng et al., 2002; Mohd Jaafar et al., 2008). These viruses represent a great threat to the aquaculture industry in China and East Asia (Fang et al., 1989; Zhang et al., 2004; Ke et al., 2011). Although these viruses are often isolated from apparently healthy individuals, they can also cause significant clinical signs and severe diseases (Fang et al., 1989). Aquareoviruses have been divided into seven species (AQRV-A to G) by the International Committee for the Taxonomy of Viruses (ICTV). The division is based on RNA–RNA blot hybridization and/or sequence comparisons (Attoui et al., 2012). RNA sequence analysis is a reliable classification index of genera in the family Reoviridae (Noda et al., 1994; Mertens et al., 2005). Currently, there are six
aquareoviruses that have complete genome information: grass carp reovirus 873 (GCRV873, AQRV-C), grass carp reovirus HZ08 (GCRV-HZ08, unclassified), grass carp reovirus GD108 (GCRV-GD108, unclassified), golden shiner reovirus (GSRV, AQRV-C), American grass carp reovirus (AGCRV, AQRV-G) and Scophthalmus maximus reovirus (SMReV, AQRV-A) (Fang et al., 2000; Attoui et al., 2002a; Mohd Jaafar et al., 2008; Zhang et al., 2010; Ke et al., 2011; Ye et al., 2012). Furthermore, for some aquareoviruses, partial genome sequences are available. Examples include chum salmon reovirus (CHSRV, AQRV-A), coho salmon reovirus (CSR, AQRV-B), golden ide reovirus (GIRV, unclassified) and striped bass reovirus (SBRV, AQRV-A). However, analysis of the molecular relationships among aquareoviruses has been hampered by the paucity of available genetic information (Attoui et al., 2002a).

In the family Reoviridae, the genus Orthoreovirus is considered to be most closely related to the genus Aquareovirus. Aquareovirus particles physically resemble those of the mammalian orthoreoviruses (MRVs) when viewed by cryo-electron microscopy. They areicosahedral in shape and are composed of a turreted central ‘core’ (containing the genome) surrounded by a double-layered capsid (Shaw et al., 1996). Aquareovirus structure displays a marked similarity to the infectious subviral particles of mammalian orthoreoviruses, suggesting a close evolutionary relationship (Mohd Jaafar et al., 2008). Additionally, phylogenetic analysis based on the viral RNA-dependent RNA polymerase (RdRp) also indicated that aquareovirus and orthoreoviruses may have originated from a common ancestor much more recently than other members of the family Reoviridae (Attoui et al., 2002a). However, a number of typical differences exist between these two genera including their distinct G+C content, different numbers of genome segments, absence of an antigenic relationship, different cytopathic effects (CPEs), and specific eco-niches. They also lack the ability to reassort (Attoui et al., 2002a).

Grass carp is an important freshwater aquaculture fish widely cultured in Asian countries. In China, the annual production is estimated to exceed 4 Mt. GCRV causes serious disease in this fish, characterized by severe haemorrhage and up to 80% mortality in fingerlings and yearlings (Fang et al., 1989). In 1983, GCRV was the first fish virus isolated in China, and has been recognized by the ICTV as a species belonging to the genus Aquareovirus, AQRV-C (Samal et al., 2005; Attoui et al., 2012). Although many GCRV strains have been isolated in China, complete genome sequence information is available for only three, GCRV873, GCRV-HZ08 and GCRV-GD108.

In this study, we report a novel reovirus strain, Hubei grass carp disease reovirus (HGDRV), which was first isolated from diseased grass carp with severe haemorrhages on the muscle, intestine, fins and gills in Hubei province, China, in 2009. Its complete genome was sequenced and analysed. Comparison of its genome sequence with others has placed this virus in the aquareovirus group, although the genome of HGDRV shows very limited similarities at both nucleotide and amino acid levels to aquareoviruses or orthoreoviruses.

**RESULTS**

**Pathology and morphology analyses**

HGDRV was grown in the CIK cell line. Typical CPE of aquareovirus infection was observed as early as 24 h post-infection (Fig. 1): the cells shrank, their boundaries became invisible and a degree of cell fusion appeared. The cell monolayer was destroyed on day 4. After three consecutive passages, the CPE became more consistent. Healthy grass carp fingerlings were intraperitoneally injected with purified virus derived from cell culture and exhibited haemorrhagic symptoms that were similar to those found in naturally diseased fish at approximately 5 days post-injection. The mortality was approximately 80% after 1 week.

HGDRV showed a level of resistance to freeze–thaw cycles, acidity and higher temperatures than normal (28°C). Treatment with ether or chloroform did not appear to affect the viral infectivity. Electron microscopy revealed typical aquareovirus morphology including an inner nucleocapsid surrounded by double-layered capsids. The diameter was about 70 nm (Fig. 2).

**HGDRV genome**

The genomes of HGDRV and GCRV were analysed by SDS-PAGE. Comparisons with the GCRV standard strain showed that HGDRV had a unique electropherotype (Fig. 3). HGDRV genome segments separated into nine distinct bands. Seven of the genome segments (Seg-1, Seg-4 to Seg-8, and Seg-11) migrated individually, while bands 2 and 8 contained two co-migrating segments each (Seg-2 and Seg-3, and Seg-9 and Seg-10, respectively).

Complete sequences of HGDRV genome segments 1–11 were determined. The length of individual genome segments and the proteins they encode are listed in Table 1. HGDRV genome segments ranged from 876 (Seg-11) to 3943 (Seg-1) bp, with a total size of 23706 bp. The G+C content was determined to be 51 mol%, which was at the lower end for sequenced aquareoviruses (52–60%) but higher than sequenced orthoreoviruses (44–48%). Each genome segment encoded one protein, except for Seg-11 which encoded two proteins (Table 1). Nucleotide sequence similarity searches using BLASTN found no significant matches except for segment 2, which partially matched with the gene encoding the RNA-dependent RNA polymerase (RdRp) of several viral species in the genera Aquareovirus and Orthoreovirus and other unclassified reoviruses. The highest nucleotide sequence identity was 66% over a length of 16% of total size of segment 2 (full-length is 3877 bp) from GCRV (AQRV-C). At the amino acid level, seven segments (Seg-1 to Seg-6 and Seg-8) matched with aquareoviruses and orthoreoviruses as...
revealed by the BLASTX, while four (Seg-7 and Seg-9 to Seg-11) found no similarity in these two genera (Table 2).

Currently, seven species A to G are included in the genus *Aquareovirus*, mainly based on VP7 protein (Seg-10 gene) variation level (Attoui et al., 2012). A different species normally shows >45% nucleotide sequence variation from any other. Such nucleotide dissimilarity should also be reflected in the amino acid sequence variation (>64%) of the VP7 proteins. As Seg-10 of HGDRV was not found to have significant similarity to any of species A to G, this new isolate should be placed as a tentative species in this genus.

### Sequence analysis of the individual HGDRV segments

Segment 1 was predicted to encode the core protein VP1. BLASTP searches showed that VP1 possessed putative conserved domains, which belonged to the reovirus L2 superfamily, consisting of several reovirus core-spike protein L2 sequences (Marchler-Bauer et al., 2011). VP1 was predicted to encode the guanylyltransferase protein, which functions as an mRNA capping enzyme. Four conserved amino acids, two lysines and two histidines, were found in the HGDRV VP1 N terminus (Lys173 and Lys193, and His226 and His235). The VP1 protein shared
Table 1. Characteristics of genome segments and predicted protein functions in HGDRV

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>GenBank accession no.</th>
<th>Length (bp)</th>
<th>GC%</th>
<th>5’NCR (bp)</th>
<th>3’NCR (bp)</th>
<th>Position of ORF (nt)</th>
<th>Coding potential</th>
<th>Length (aa)</th>
<th>MM (kDa)</th>
<th>Isoelectric point (pI)</th>
<th>Predicted function</th>
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<tbody>
<tr>
<td>Seg-1</td>
<td>JN967629</td>
<td>3943</td>
<td>50.87</td>
<td>16</td>
<td>42</td>
<td>17–3901</td>
<td>VP1</td>
<td>1294</td>
<td>142.51</td>
<td>5.25</td>
<td>Core protein, guanylyltransferase</td>
</tr>
<tr>
<td>Seg-2</td>
<td>JN967630</td>
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<td>49.92</td>
<td>15</td>
<td>24</td>
<td>16–3840</td>
<td>VP2</td>
<td>1274</td>
<td>142.59</td>
<td>8.09</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
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<td>15</td>
<td>39</td>
<td>16–3690</td>
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<td>17</td>
<td>45</td>
<td>18–2165</td>
<td>VP5</td>
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<td>79.73</td>
<td>7.60</td>
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</tr>
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<td>35</td>
<td>54</td>
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<td>VP11</td>
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<td>85.57</td>
<td>3.73 6.31</td>
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</table>

Table 2. Conserved terminal nucleotide sequences and percentage sequence identities of proteins between HGDRV and other aquareovirus and orthoreovirus species

GenBank accession numbers are presented in Table S1 (available in JGV Online). CHSRV, chum salmon reovirus; SMReV, Scophthalmus maximus reovirus; GCRV-873, grass carp reovirus 873; GSRV, golden shiner reovirus; AGCRV, American grass carp reovirus; GCRV-HZ08, grass carp reovirus HZ08; GCRV-GD108, grass carp reovirus GD108; MRV-1/2/3, mammalian orthoreovirus 1/2/3; ARV-SSRV, avian orthoreovirus-Steller sea lion reovirus; ‘–’, complete sequence not available; NH, no homologous protein.

<table>
<thead>
<tr>
<th>Compared virus</th>
<th>HGDRV segment: (Seg-1)</th>
<th>VP1 (Seg-2)</th>
<th>VP2 (Seg-3)</th>
<th>VP3 (Seg-4)</th>
<th>VP4 (Seg-5)</th>
<th>NS66 (Seg-6)</th>
<th>VP5 (Seg-7)</th>
<th>VP6 (Seg-8)</th>
<th>VP39 (Seg-9)</th>
<th>VP38 (Seg-10)</th>
<th>VP8/VP15 (Seg-11)</th>
<th>Conserved terminal nucleotide sequences (5’-GAAUU.....UCAUC-3’)</th>
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<tr>
<td>AQRV–A CHSRV</td>
<td>30</td>
<td>40</td>
<td>30</td>
<td>20</td>
<td>26</td>
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<td>20</td>
<td>75</td>
<td>5’-GUUUAU.....UCAUC-3’</td>
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<td>26</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>20</td>
<td>75</td>
<td>5’-GUUUAU.....UCAUC-3’</td>
</tr>
<tr>
<td>AQRV–C GCRV873</td>
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<td>44</td>
<td>35</td>
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<td>22</td>
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<td>32</td>
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<td>35</td>
<td>23</td>
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<td>27</td>
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<tr>
<td>AQRV Not proposed GCRV-HZ08</td>
<td>29</td>
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<td>36</td>
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<td>26</td>
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<td></td>
<td>MRV-2</td>
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<td>17</td>
<td>75</td>
<td>5’-GUUUAU.....UCAUC-3’</td>
</tr>
</tbody>
</table>
identities of between 29 and 32 % with the core turret VP1 protein of related aquareoviruses, including CHSRV (AQRV-A), SMReV (AQRV-A), GCRV873 (AQRV-C), GSRV (AQRV-C), AGCRV (AQRV-G), GCRV-HZ08 (unclassified) and GCRV-GD108 (unclassified). The protein also displayed higher sequence identities (25–26 %) to four species of the genus Orthoreovirus, \( \lambda 2 \) of MRV1–3 and \( \lambda C \) of avian orthoreovirus (ARV) (Table 2).

Segment 2 was predicted to encode the core protein VP2, which is an RdRp. The catalytic domain of RdRp was identified between aa 558 and 794 in HGDRV VP2 by ScanProsite (ExPASy proteomics server). Previous studies had identified five signature motifs, motifs A–E, in RdRp (O’Reilly & Kao, 1998). Motif A (DXXXD, aa 588–593), motif B (SGXXXT, aa 684–689), and motif C (GDD, aa 735–737) were found in the predicted catalytic domain of HGDRV VP2. HGDRV VP2 shared high identities (42–46 %) with VP2 homologues from related aquareoviruses and also showed high identities (43–44 %) to \( \lambda 5 \) from MRV and \( \lambda B \) from ARV (Table 2).

Segment 3 has been predicted to encode the VP3 core protein, which functions as a helicase and NTPase, involved in the transcription and capping of viral RNA (Ke et al., 2011). A zinc finger motif (Cys-His-Cys-His) was identified in the HGDRV VP3 at aa 128–149, which are known to bind RNA. As revealed by Cryo-EM analysis, the amino acids Glu502, Ser503, Thr504 and Thr505 are known to bind RNA. As revealed by Cryo-EM analysis, the amino acids Glu502, Ser503, Thr504 and Thr505 are involved in GCRV RNA transcription (Cheng et al., 2010). Alignments showed that the four amino acids were conserved in MRV, ARV, GCRV, GSRV and AGCRV. However, the corresponding amino acids in HGDRV were Glu512, Glu513, Ser514 and Thr515. HGDRV VP3 shares 30–36 % identities with the homologue of aquareoviruses, and it also showed 33 % identity to \( \lambda 1/\lambda A \) of MRV and ARV (Table 2).

Segment 4 has been predicted to encode the minor core protein VP5. BLASTp searches showed that VP5 possesses a putative conserved domain belonging to the Reovirus \( \mu 2 \) superfamily. Two conserved lysine residues (aa 399 and 403) in VP5 amino acid sequence alignments were detected for HGDRV, which were also conserved in MRV, ARV and aquareovirus species other than CHSRV. This alignment result is consistent with previous research in SMReV (Ke et al., 2011). HGDRV VP5 showed 19–23 % identities to aquareovirus homologues and also shared 18–20 % identities with \( \mu 2/\mu A \) of MRV and ARV, respectively.

Segment 5 was predicted to encode the outer capsid protein VP4. BLASTp analysis of the VP4 showed that it possesses a conserved domain belonging to the Reovirus_M2 superfamily. This family consists of several major reovirus virion structural proteins and \( \mu 1/\mu C \) (M2) sequences that affect host cell membrane penetration (Marchler-Bauer et al., 2011). An autolytic cleavage site has been predicted to be between HGDRV VP4 amino acids Asn42 and Pro43, which are also conserved in orthoreoviruses and other aquareoviruses. Three possible N-linked glycosylation sites (\(^{10} \text{NVT}, \quad ^{23} \text{NSTG} \) and \(^{54} \text{NETR} \) were observed in VP4. In addition, the first three amino acid residues ‘MGN’ at the N-terminal sequence of VP4 in aquareoviruses and orthoreoviruses were highly conserved. Sequence alignment shows that HGDRV VP4 had 26–29 % identities to the aquareovirus homologue, and 25 % identity to \( \mu 1/\mu B \) of MRV and ARV.

Segment 6 may encode the non-structural protein designated NS66 based on its theoretical molecular mass. NS66 shares a low similarity with non-structural aquareovirus proteins (15–21 %), and it also shared a low identity of 12 % with \( \mu NS \) proteins of orthoreoviruses. One coil was found at aa 451–515 via the Coils program. The analysis also revealed that the homologous \( \mu NS \) proteins of orthoreoviruses all contained two coils in the corresponding regions.

Segment 7 is likely to encode a protein of 511 aa, designated VP55. BLASTp searches indicate that VP55 had no homology to any other reovirus protein. One coil was predicted between aa 28 and 106 at the N-terminal region of HGDRV VP55. Interestingly, the coil structures were also predicted in \( \alpha 1/\sigma C \) of orthoreoviruses and segment 7 encoded proteins of GCRV-HZ08 and GCRV-GD108.

Segment 8 was predicted to encode the core protein VP6. BLASTp analysis showed that VP6 possesses conserved domains belonging to the reoviral \( \sigma 1/\sigma 2 \) superfamily. VP6 shared only 20–22 % identities with the homologues of aquareoviruses, and 17–19 % to \( \sigma 2 \) of MRV and \( \sigma A \) of ARV (Table 2). Secondary structure predictions for VP6 revealed a large N-terminal domain (spanning approximately three-quarters of the protein) made up predominantly of \( \beta \)-sheets and turns and a smaller C-terminal domain rich in \( \alpha \)-helices, which are characteristics of \( \sigma 2/\sigma A \) MRV and ARV proteins (Dermody et al., 1991; Yin et al., 2000; Thalmann et al., 2010). In addition, most of the amino acid residues in the C-terminal regions of HGDRV VP6 and the \( \sigma 2/\sigma A \) proteins of MRV and ARV are hydrophilic, while the amino acid residues in SMReV VP6 were hydrophobic (Ke et al., 2011).

Segment 9 was predicted to encode a protein of 354 aa, designated VP39. No homologous reovirus protein was found by BLASTp. However, BLASTp searches showed that VP39 possessed putative conserved domains, which belonged to the poly(C)-dependent poly(G) polymerase (PolyG_pol) superfamily. In addition, the same putative conserved domains were also found in \( \sigma NS \) of orthoreoviruses and segment 9 encoded proteins of aquareoviruses, including GCRV873, CHSRV, GSRV and AGCRV.

Segment 10 was shown to encode a protein of 346 aa, VP38. No homologous reovirus protein was found. A hydrophathy plot of the proteins encoded by Seg-10 from MRV, ARV and HGDRV showed similar hydrophathy profiles in the N-terminal part of the protein, with four domains in the order hydrophobic–hydrophilic–hydrophobic–hydrophilic (Fig. 4). Similar hydrophathy profiles
Among the 12 proteins, seven of them were found to have motif comparisons or roles of the individual viral proteins (Mohd Jaafar et al., 2008). In our study, the HGDRV viral proteins shared a number of conserved domains and structural motifs with those of MRV and ARV in the genus Orthoreovirus, suggesting a close evolutionary relationship between HGDRV and orthoreoviruses. Similar results were also found between GSRV (AQRV-C) and MRV (Attoui et al., 2002a).

**Non-coding regions of the HGDRV genome**

Short non-coding regions (NCRs) at the 5’ and 3’ terminal regions were found in all of the HGDRV genome segments (Table 1), which are characteristic features of aquareovirus genomes. The length of HGDRV NCRs ranged from 13 to 35 nt at the 5’ end and 24 to 70 nt at the 3’ end. All of the segments shared five conserved nucleotides at the 5’ and 3’ ends (5’-GAAAU—UCAUC-3’). The conserved motifs at the 5’ end differed from those of any known aquareoviruses or orthoreoviruses (Table 2). Moreover, the first and last two nucleotides for each segment of HGDRV were inverted complements, which are not seen in other aquareoviruses.

**Phylogenetic analysis**

A phylogenetic tree was constructed based on RdRp amino acid sequences among members of 14 genera in the family Reoviridae (Fig. 6). All known species of aquareoviruses clustered in a branch and the genus *Aquareovirus* was clustered closely with *Orthoreovirus*. HGDRV was closer to the newly isolated strains GCRV-GD108 and GCRV-HZ08, and tended to cluster with aquareoviruses prior to joining a branch common with orthoreoviruses. This suggests that HGDRV belongs to the genus *Aquareovirus*, although it was distinct from the known species of this genus. In addition, there are two subfamilies in the Reoviridae, *Spinareovirinae* and *Sedoreovirinae*. Members of *Spinareovirinae* are turreted reoviruses, which have turrets situated on the virus core structure. Members of the *Sedoreovirinae* are non-turreted reoviruses (Attoui et al., 2012). Two large groups of genera also existed in the present phylogenetic analysis. On the right side of Fig. 6 (divided by a skewed dashed line) are members of the *Spinareovirinae*. Members of the *Sedoreovirinae* are clustered on the left side of Fig. 6.

**DISCUSSION**

**Putative functions of HGDRV proteins revealed by motif comparisons**

As several genes of HGDRV are relatively closely related to cognate genes of other members of the genus *Aquareovirus*, RNA segment reassortment may play an important role in the evolution of segmented RNA viruses. Although the levels of sequence identity that could be detected between homologous proteins of different reoviruses are often low by the standards of other genetic groups, they are very strongly supported by similar functional and structural motifs, or roles of the individual viral proteins (Mohd Jaafar et al., 2008). In our study, the HGDRV viral proteins shared a number of conserved domains and structural motifs with those of MRV and ARV in the genus *Orthoreovirus*, suggesting a close evolutionary relationship and similar protein functions between HGDRV and related orthoreoviruses.

Four conserved amino acids (two lysines and two histidines) were detected in HGDRV VP1. It has been reported previously that the four conserved residues are essential amino acids for the guanylyltransferase activity of the homologous proteins in MRV, ARV and GCRV (Luongo et al., 2000; Hsiao et al., 2002; Qiu & Luongo, 2012).
amino acids were identified in SMReV VP1 (Ke et al., 2003). Thus, HGDRV VP1 is also likely to possess guanylyltransferase activity. The same four conserved amino acids were identified in SMReV VP1 (Ke et al., 2011), while only two conserved amino acids (lysines) were found in the GCRV-HZ08 homologue (Zhang et al., 2010).

Three signature motifs (A, B and C) for the reovirus RdRp were detected in HGDRV VP2. Amino acid alignments revealed that these three motifs were also conserved in the RdRPs of aquareovirus and orthoreovirus species, which is consistent with the results of a previous study in SMReV (Ke et al., 2011). However, there was also a distinct difference at the motif B location in the RdRp sequence when compared with previous research. We located motif B (SGXXXT) in HGDRV at aa 684–689 (the equivalent position in SMReV is aa 688–693), while the position in the previous study was aa 648–649 in SMReV (Ke et al., 2011). Moreover, our study also showed that motif B is conserved in 11 genera among the compared 14 genera of the family Reoviridae, the three exceptions being the genera Cardoreovirus, Mimoreovirus and Seadornavirus.

Four conserved consecutive residues and the zinc-finger C2H2 domain were found in HGDRV VP3. Four consecutive residues at aa 502–505 of VP3A are conserved between GCRV and orthoreoviruses. These residues are situated in the RNA molecule and are located near the putative channel responsible for the nascent mRNA pathway and RNA transcription (Reinish et al., 2000; Cheng et al., 2010). The zinc-finger motif has been observed in other aquareoviruses, including GCRV873 (aa 119–140), GCRV-HZ08 (aa 137–158), GCRV-GD108 (aa 137–158) and SMReV (aa 113–136) (Cheng et al., 2010; Zhang et al., 2010; Ke et al., 2011; Ye et al., 2012).

The conserved domain of the Reovirus_μ2 superfamily was found in HGDRV VP5. Previous studies showed that this conserved domain is a microtubule-associated protein that plays a key role in the formation and structural organization of reovirus inclusion bodies (Zou & Brown, 1992; Parker et al., 2002). Previous studies also showed that this conserved domain is an RNA-binding protein involved in MRV core particle NTPase activities and viral mRNA synthesis (Noble & Nibert, 1997; Brentano et al., 1998; Kim et al., 2004). The two conserved lysine residues found in HGDRV VP5 are essential for ATPase activity in the ARV_μA homologue (Su et al., 2007). Based on the results of BLASTP and amino acid sequence comparisons with MRV and ARV, HGDRV VP5 is considered/proposed to be a putative NTPase.

The autolytic cleavage site, Asn42 and Pro43, is required for cell membrane penetration during the early stages of MRV infection (Odegard et al., 2004). A similar site was identified in HGDRV VP4. A highly sensitive cleavage site was also found in the same amino acid positions in GCRV873, GCRV-GD108, SMReV and other aquareoviruses (Qiu et al., 2001; Ke et al., 2011, Ye et al., 2012). Previous research reported that VP4 and VP7 formed heterodimers of the outer capsid in GCRV (Cheng et al., 2008). In the early stage of infection, the outer capsid is proteolytically cleaved and disassembled to form infectious subviral particles which have enhanced infectivity (Fang et al., 2008).

SMReV NS88 was shown to contain a region of two coils, with conserved histidine and cysteine (Ke et al., 2011).
Fig. 6. Phylogenetic analysis based on the amino acid sequences of RdRp from the family Reoviridae. The filled arrow indicates the position of HGDRV. The scale bar indicates the average number of amino acid substitutions per site. The two subfamilies, Spinareovirinae and Sedoreovirinae, are divided by a skewed dashed line. The abbreviations and RdRp GenBank accession numbers are presented in Table S2.
However, only one coil region was predicted in HGDRV NS66 and no conserved histidines and cysteines were found in corresponding regions. It has been shown that NS proteins play significant roles in viral replication and assembly processes within the infected target cell (Broering et al., 2004; Jayaram et al., 2004; Owens et al., 2004). The C-terminal conserved region of GCRV NS80 has been suggested to be involved in the formation of viral inclusion structures (Fan et al., 2010), while the SMReV NS88 was hypothesized to be necessary to form viral inclusion bodies during virus infection (Ke et al., 2011). Whether the HGDRV NS66 protein possesses a similar function is currently unknown.

The conserved domains of the reovirus σ1 and σ2 superfamily were found in HGDRV VP6. Previous studies showed that σ1 and σ2 consist of two concentric icosahedral layers, the inner core and the outer capsid layer (Marchler-Bauer et al., 2011). σ2 plays a role in stabilizing the σ1 shell and in binding the base of μ1 for outer capsid assembly (Reinsch et al., 2000; Kim et al., 2002; Liemann et al., 2002). Similarly, GCRV VP6 functions in the formation of a continuous capsid shell via clamping to VP3 (Cheng et al., 2008).

The putative conserved domains PolyG_pol were found in HGDRV VP39 and in the σNS of MRV/ARV. It has previously been reported that reovirus sigma NS, which has a PolyG_pol activity, may act during infection as condensing agents to bring together 10 viral single-stranded RNA templates in preparation for dsRNA synthesis (Gomatos et al., 1981). Therefore, VP39 may possess a similar function.

Conserved terminal regions of HGDRV genome segments

All members of the family Reoviridae exhibited conserved terminal sequences in genomic dsRNAs. This feature may be considered to be useful in reovirus classification (Attoui et al., 2002a; Mohd Jaafar et al., 2008). Each segment of HGDRV has the same conserved terminal sequences and the first and last two nucleotides are inverted complements. Notably, the 5′-terminal nucleotides (GAAUU) of HGDRV were different from any viruses reported thus far within the genera Aquareovirus and Orthoreovirus, an indication that HGDRV does not belong to any previously described species. Genome segments of most member viruses of the family Reoviridae have their first and last mono-, di-, or tri-nucleotides as inverted complements (Attoui et al., 2000a, b, 2002a, b, 2005, 2006a, b; Mohd Jaafar et al., 2008). It has been suggested that the complementary nature of sequences at the 5′- and 3′ NCRs could hold each RNA transcript in a circular form, either by itself or via interactions with protein components, particularly the replication complex (Anzola et al., 1987; Chen & Patton, 1998).

Phylogenetic relationship analysis based on RdRp

RdRp was used to elucidate the evolutionary relationships of the viruses among genera in the family Reoviridae. This is because the polymerase protein is thought to be the most conserved among viral proteins (Zhang et al., 2010). Members within an Aquareovirus species show >95% amino acid sequence identity of their RdRps, while viruses belonging to different genera have low amino acid sequence identity (<20%) in the polymerase genes (Attoui et al., 2002a). Previous studies also revealed that most reoviruses from a single genus will have amino acid identity >30% in the reovirus polymerase (Attoui et al., 2002a, b, 2005). Overall identities of 42–46% of RdRp were found between HGDRV and other aquareoviruses, indicating that HGDRV may belong to the genus Aquareovirus but not to any of the currently known species. The highest level of amino acid sequence identity that was detected between the polymerase of HGDRV and a member of a distinct genus of reovirus MRV was 44%, supporting the conclusion that aquareoviruses and orthoreoviruses originated from a common ancestor (Attoui et al., 2002a).

METHODS

Virus isolation and culture. Diseased grass carp were sampled from a fish farm in the Hubei province of China on 4 October 2009, and the liver, kidney and spleen were collected and homogenized in Dulbecco’s PBS. After being frozen at −80°C and thawed three times, the homogenate was centrifuged at 2880g for 30 min at 4°C. The supernatant fluid was then filtered through a 0.22μm membrane and stored at −80°C as the original viral isolate for cell culture. A Ctenopharyngodon idellus kidney (CIK) cell line, maintained in our laboratory (Zuo et al., 1986), was used for virus isolation. CIK cells grew into confluent monolayers in T-25 cm² cell culture flasks in minimum essential medium (MEM) supplemented with 10% FBS (Sigma) at 28°C. After the culture medium had been removed from the monolayer, 1 ml filtered homogenate was added into the flask and incubated for 1 h allowing viral adsorption onto the cells. The suspension materials were then removed and 5 ml MEM with 2% fetal bovine serum was added. Cells were incubated at 28°C for 7–8 days. CPE was checked daily. Non-infected cells were used as control. The virus cultures were used in a challenge test.

Artificial infection of fish. Healthy grass carp fingerlings with body length 6–12 cm were used for artificial infection experiments. Fish were cultured in two tanks (50 fish in each) with cycling water at 28°C. Fish in the control group were infected by intraperitoneal injection with 0.3 ml of the fourth passage of HGDRV, and fish in the control group were injected intraperitoneally with 0.3 ml Dulbecco’s PBS. The virus was re-isolated from experimentally infected fish and the propagated virus was used to infect the healthy fish again, using the method described above.

Physical and chemical properties detection. CIK cells were harvested at 3 or 4 days post-infection when a typical CPE appeared. Harvested virally infected cell cultures were frozen at −80°C and thawed at room temperature for three cycles, followed by centrifugation at 2880g for 30 min to remove cell debris. The supernatant was collected as a viral suspension and stored at −80°C. Whether the HGDRV NS66 nucleotides as inverted complements (Attoui et al., 2000a, 2001, 2005). Overall identities of 42–46% of RdRp were found between HGDRV and other aquareoviruses, indicating that HGDRV may belong to the genus Aquareovirus but not to any of the currently known species. The highest level of amino acid sequence identity that was detected between the polymerase of HGDRV and a member of a distinct genus of reovirus MRV was 44%, supporting the conclusion that aquareoviruses and orthoreoviruses originated from a common ancestor (Attoui et al., 2002a).

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**Electron microscopy.** The viral suspension was ultracentrifuged at 112700g (Beckman rotor SW28) for 2 h at 4 °C. The pellet was resuspended in 1 ml twice distilled H2O. Drops of the purified virus were negatively stained with 2 % phosphotungstic acid (pH 7.0) for 30 s on Butvar-coated grids and then examined by transmission electron microscopy (Hitachi-7650).

**Viral dsRNA full-length amplification.** The purified virus was used for RNA extraction using Trizol (Invitrogen). Extracted dsRNAs were stored at −80 °C. Viral genomic dsRNAs were separated by SDSPAGE on vertical slab gels (9 % polyacrylamide gel) in Laemmli’s buffer (Laemmli, 1970) and visualized by silver staining. The GCRV standard (JX09-01 strain) used as a control in SDS-PAGE was derived from the Pearl River Fisheries Research Institute of China by material exchange, and was propagated in the CIR cell line and under growth conditions appropriate for HGDVR.

Amplification of the entire HGDRV genome was carried out using the full-length amplification of cDNA (FLAC) technique as described previously (Rao, 2002; Shapiro et al., 2005; Maan et al., 2007). Briefly, a 35 nt oligonucleotide ‘anchor-primer’ was synthesized: 5′-p-GACCTCTGAGGATTCTAAAC/iSp9/TCCAGTTAGAATCC-OH-3′, which has a C9 (phosphoramidite) spacer between two complementary halves and a phosphorylated 5′ terminus (Integrated DNA Technology). The anchor-primer was ligated to both of the 3′-ends of the dsRNA segments using T4 RNA ligase (New England Biolab). After the ligation reaction, dsRNA with the anchor-primer was extracted using an RNA clean up kit (Omega), followed by first-strand cDNA synthesis using an AMV Reverse Transcription System (Promega) and PCR amplification using a complementary primer, 5′-15-1 (5′-GAGGGATCCAGTTAGAATCCGCAGTCTGC-3′).

**Cloning, sequencing and analysis.** PCR products were separated on a 1 % agarose gel and visible bands were purified using a silica bead DNA Gel Extraction kit (Fermentas) and cloned into a pMD19-T simple vector (Takara). Positive clones were sequenced as described by Maan et al. (2007).

Aquareovirus and other reovirus sequences used in this study for comparison were obtained from GenBank and accession numbers are presented in Tables S1 and S2 (available in JGV Online). Nucleic acid sequences in wound tumor virus genome and defective interfering RNAs. Proc Natl Acad Sci U S A 84, 8301–8305.

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**REFERENCES**


