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Short Communication

Isolation and characterization of a novel invertebrate iridovirus from adult *Anopheles minimus* (AMIV) in China

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ABSTRACT

An invertebrate iridovirus (designated AMIV) was isolated from adult wild-captured *Anopheles minimus* mosquitoes in China. AMIV was pathologically and morphologically characterized and sequenced using the Ion Torrent™ sequencing platform. Phylogenetic analysis based on both the major capsid protein and core genes revealed that AMIV differs from all the members of the family *Iridoviridae*. The AMIV negatively strained virion has a diameter of about 130 nm. AMIV contains a linear DNA molecule of 163,023 bp, with 39% G+C content and 148 coding sequences. The genome analysis revealed that AMIV genome encodes a high content of replication associated genes including BRO-like genes. This is the ninth complete genome of IIV reported.

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1. Introduction

The family *Iridoviridae* consists of five genera: *Chloriridovirus*, *Iridovirus*, *Lymphocystivirus*, *Megalocytivirus*, and *Ranavirus* (King et al., *2012*). Members of the last three genera infect only poikilothermic vertebrates, which are economically important, especially in aquaculture (Do et al., 2004; Zhang et al., 2004). In contrast, viruses of the genera *Iridovirus* and *Chloriridovirus* are known as invertebrate iridescent viruses (IIVs), infecting predominantly insects (Webby and Kalmakoff, 1998; Williams et al., 2005). The dsDNA genomes of IIVs are circularly permuted, with terminal redundancy, and range from 100 to 210 kb in length (Fauquet et al., 2005). IIVs are icosahedral particles with a diameter in the range of 110–300 nm (Fauquet et al., 2005). The important effects of IIVs on insects, including sublethal effects, debilitation, and the induction of apoptosis, have been examined (Marina et al., 2003a, 1999). Unfortunately, many functional activities associated with the viruses and the interactions between the viruses and host cells remain to be clarified. Furthermore, there is little complete sequence information on IIVs, leading to considerable confusion, especially regarding their taxonomic definition (Williams, 1994; Williams and Cory, 1994), and few proteomic analyses (Eaton et al., 2007). Eight complete IIV genomes have been reported to date: for IIV-3 (mosquito iridovirus) (Delhon et al., 2006), IIV-6 (Jakob et al., 2001), IIV-9 (*Wiseana* iridovirus; (Wong et al., 2011), IIV-22 (Piegu et al., 2013) and the recently reported four genomes (IIV-25 (Piegu et al., 2014b), IIV-22A (Piegu et al., 2014d), IIV-30 (Piegu et al., 2014a) and IIV-31 (Piegu et al., 2014c)). Of those viruses, Only IIV-3 was originally isolated from mosquitoes. Several IIVs have been reported that infect mosquitoes, the most important viral vector for human diseases (Fukuda, 1971a; Marina et al., 2003b). However, most of the previously reported mosquito isolates were found in *Aedes*, *Ochlerotatus*, and *Psorophora*, with no record of them in *Anopheles* or *Uranotaenia* (Williams, 2008). The literature suggests that IIVs have strong replicative capacities in a wide range of insect cell lines (Constantino et al., 2001; Williams et al., 2005) and broad host range, displayed in laboratory tests (Henderson et al., 2001; Jakob et al., 2002). Furthermore, almost all IIVs have been isolated in the larval but not adult stages.

Here we describe a novel IIV isolated from wild captured adult *Anopheles minimus* in China, designated AMIV. The viral isolate was observed under electron microscopy and sequenced on the Ion Torrent™ sequencing platform. The complete genome of AMIV was characterized and submitted to GenBank under accession number KF938901.

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2. Materials and methods

2.1. Biological material

Mosquitoes were sampled during the active mosquito season (from June to September) in Yunnan Province, southwest China from 2007 to 2010 using light traps baited with CO_2. The collected mosquitoes were frozen at −20 °C for 30 min and the blood-fed mosquitoes were removed. They were then grouped on the basis of collection site and mosquito species. A group of approximately 50–100 mosquitoes was considered as one mosquito pool. A total of 20,256 mosquitoes were trapped and sorted into 247 pools according to species, location, and date of collection for viral isolation. Each mosquito pool was homogenized with RPMI-1640 medium containing 2% fetal calf serum and then centrifuged at 4000 rpm for 5 min. The supernatants were filtered through a 0.22 μm Millipore filter and were cultured with *Aedes albopictus* C6/36 cells. The cells were examined daily for evidence of cytopathic effect (CPE). A specimen was considered a positive isolate if it caused CPE in three successive passages. Two cell lines BHK21 and Vero-E6 were also used to explore the host range of AMIV: 500 μl virus supernatant from infected C6/36 cells was added to the new cultured cell lines (C6/36, BHK21 and Vero-E6) on 5 ML cell culture plate. As negative control, 500 μl cell culture was also added to the 3 cell lines. The virus particles were concentrated from positive isolate supernatant samples, and subsequently placed onto a formvar-coated copper grid, negatively stained with 1% uranyl acetate and viewed with a transmission electron microscope (TecnaiG2F20 S-TWIN) to examine the morphological characteristics of AMIV particles.

2.2. Genome sequencing and assembly

The viral particles were obtained from the positive isolate supernatant. The DNA of the virus was extracted with the Qiagen Viral RNA Mini Kit (Qiagen, Valencia, CA), and used to construct a short-insert sequencing library (with 400 bp inserts) and a mate-paired sequencing library (with 4 kbp inserts), sequenced with the Ion Torrent™ sequencing platform. The raw data were assembled into two large scaffolds using Newbler (version 2.8). The interscaffold and intrascaffold gaps were closed with a combined strategy involving GapFiller (version 1.1) (Boetzer and Pirovano, 2012) and Sanger sequencing.

2.3. Genome analysis

A phylogenetic tree based on the amino acid sequence of the major capsid protein (MCP) and one based on core genes of the family *Iridoviridae* were generated to explore the evolutionary relationships between members of the family *Iridoviridae* (Fig. 2). Sixteen reported viruses belonging to the genera *Chloriridovirus* and *Iridovirus*, together with four representative species from three other genera, were selected. The phylogenetic tree was inferred with the maximum likelihood method, with a 1000-replicate bootstrap test, using MEGA version 5 (Tamura et al., 2011) (Fig. 2A). Similarly, eleven complete genomes were selected to generate another phylogenetic tree based on core genes, using Pan-based software PGAP (Zhao et al., 2012) (Fig. 2B). The intermediate stage results show that six genes of AMIV (AMIV_039, AMIV_011, AMIV_067, AMIV_094, AMIV_069 and AMIV_096) have homology with that of other IIVs.

3. Results

Three cell lines (BHK21, Vero-E6, and C6/36) were used to culture AMIV to explore its host cell range and pathogenic effects. The cells were regularly observed under light microscopy at different times after AMIV inoculation. A CPE was only observed in C6/36 cells. The C6/36 cells showed a significant CPE, starting about 9 h after infection with AMIV. Within 36 h, almost all the cells in the plate were lysed, indicating the pathogenic infection of C6/36 cells by AMIV.

The electron microscope results showed that the AMIV virion has a diameter of about 130 nm (Fig. 1A). The diameter of the particle was close to that of the members in the genus *Iridovirus* (Williams, 1998). The proposed defining differences between the *Chloriridovirus* and *Iridovirus* genera are particle sizes (King et al., 2012), AMIV should be classified into genus *Iridovirus*.

The complete genome of AMIV is a linear DNA molecule of 163,023 bp, with a G+C content of 39%. The genome was annotated using the RAST server (Aziz et al., 2008) and verified with BLAST against the non-redundant database of NCBI. In total, 148 open reading frames (ORFs) were annotated, 94 of which encode hypothetical proteins, and 54 were functionally annotated. The coding ORFs cover 83.3% of the entire genome. The characteristics of AMIV and the other eight published complete IIV genomes are listed in Supplementary Table 2. Nine AMIV ORFs (003, 004, 032, 059, 073, 102, 135, 146, and 148) have no orthologs in the other IIV genomes. Repeated sequences were identified within the genome, known as BRO-like genes. In total, three BRO-like genes were found in AMIV with the same number in IIV-6. Previous researches have shown that BRO-like gene may enhance replication of invertebrate virus (Bideshi et al., 2003). Interestingly, replication associated genes (17 genes) were widely distributed on the complete genome (Fig. 1B). This high content of replication associated genes, together with the 3 BRO-like genes, may explain why the AMIV has a potent CPE on cultured cell lines.

As shown in both trees of Fig. 2, AMIV clustered in the group of IIVs but formed a single clade that was distant from the other IIVs. The phylogenetic analysis also showed that AMIV is more closely related to members in the genus *Iridovirus* than to IIV-3 in the genus *Chloriridovirus*, suggesting that AMIV is quite different from the previously reported mosquito-derived IIV-3. Interestingly, the genome size, G+C content and ORF number of AMIV are intermediate between those of IIV-3 and members of the genus *Iridovirus*, which is consistent with the phylogenetic trees in Fig. 2. The discovery of AMIV provides one more record of a natural infection of mosquitoes by an IIV, revealing the genetic diversity of these viruses. AMIV can be defined as a novel species isolated from a natural infection of *Anopheles minimus*. The full sequence extends our knowledge of the *Iridovirus* genomes and may have great reference value for virologists and entomologists in defining interspecies variations in the IIVs.

4. Discussion

High-throughput sequencing (HTS), a frequently used tool, makes the identification of unknown isolates easy. With the paucity of complete genomic data available and the strong demand for genomic analyses, HTS is undoubtedly an efficient approach to the large-scale and economically feasible determination of the complete genomes of the iridescent viruses.

Insect vector diversity in the Yunnan region may be conducive to the spread of arthropod-borne viruses. The origin of the AMIV host and information about its geographic distribution extends the known distribution of the IIVs. This is the first natural IIV infection recorded in *Anopheles* mosquitoes, except for the IIV-6 infections established in the laboratory (Fukuda, 1971b).

Infection of cell lines with AMIV in this study revealed the very rapid onset of CPE. Although there is insufficient evidence to show

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