PCR amplification and sequence analysis of the major capsid protein gene of megalocytiviruses isolated in Taiwan

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Abstract

Viruses belonging to the genus Megalocytivirus in the family Iridoviridae are one of the major agents causing mass mortalities in marine and freshwater fish in Asian countries. Outbreaks of iridovirus disease have been reported among various fish species in Taiwan. However, the genotypes of these iridoviruses have not yet been determined. In this study, seven megalocytivirus isolates from four fish species: king grouper, Epinephelus lanceolatus (Bloch), barramundi perch, Lates calcarifer (Bloch), silver sea bream, Rhabdosargus sarba (Forsskal), and common ponyfish, Leioognathus equulus (Forsskal), cultured in three different regions of Taiwan were collected. The full open reading frame encoding the viral major capsid protein gene was amplified using PCR. The PCR products of approximately 1581 bp were cloned and the nucleotide sequences were phylogenetically analysed. Results showed that all seven PCR products contained a unique open reading frame with 1362 nucleotides and encoded a structural protein with 453 amino acids. Even though the nucleotide sequences were not identical, these seven megalocytiviruses were classified into one cluster and showed very high homology with red sea bream iridovirus (RSIV) with more than 97% identity. Thus, the seven iridovirus strains isolated from cultured marine fish in Taiwan were closer to the RSIV genotype than the infectious spleen and kidney necrosis virus genotype.

Keywords: iridovirus, major capsid protein gene, megalocytivirus, phylogenetic analysis, polymerase chain reaction.

Introduction

Epizootic iridoviral disease causes serious economic losses in aquaculture worldwide. Based on the Eighth Report of the International Committee on Taxonomy of Viruses (ICTV), the family Iridoviridae is divided into five genera: Iridovirus, Lymphocystivirus, Chloriridovirus, Ranavirus and Megalocytivirus (Chinchar, Essbauer, He, Hyatt, Miyazaki, Seligy & Williams 2005). Among these genera, Megalocytivirus was recently identified as a new genus to the family. Members of this genus include the infectious spleen and kidney necrosis virus (ISKNV) (He, Wang, Zeng, Huang & Chan 2000), red seabream iridovirus (RSIV) (Inouye, Yamano, Maeno, Nakajima, Matsuoka, Wada & Sorimachi 1992), sea bass iridovirus (SBIV) (Miyata, Matsuno, Jung, Danayadol & Miyazaki 1997), rock bream iridovirus (RBIV) (Jung & Oh 2000), dwarf gourami iridovirus (DGIV), African lampeye iridovirus (ALIV) (Sudthongkong, Miyata & Miyazaki 2002a), large yellow croaker iridovirus (LYCIV) (Chen, Lin & Wang 2003), orange-spotted grouper iridovirus (OSGIV) (Lu, Zhou, Chen, Weng, Chan & He 2005) and turbot iridovirus (Kim, Oh, Jung, Kim & Kitamura 2005). The characteristics of affected fish are systemic formation of enlarged cells and necrosis of virus-infected cells in the spleen and haematopoietic tissues (Wang, Shih, Ku & Chen 2003). Megalocytiviruses have caused mass mortalities in various marine and freshwater fish species throughout Asia, indicating these viruses have a broad host range and geographical distribution (Kawakami & Nakajima 2002; Wang, Lu, Weng, Huang, Chan & He 2007).
Iridoviruses are large, cytoplasmic, double-stranded DNA viruses with an icosahedral capsid approximately 120–240 nm in diameter. The viral genome of four megacloviruses, ISKNV, RSIV, RBIV and OSGIV has been completely sequenced (He, Deng, Weng, Li, Zhou, Long, Wang & Chan 2001; Kurita, Nakajima, Hirono & Aoki 2002; Do, Moon, Kim, Ko, Kim, Son, Kim, An, Kim, Lee, Han, Cha, Park, Park, Kim & Park 2004; Lu et al. 2005). Sequence analysis of these viruses revealed high nucleotide and amino acid identities in many open reading frames (ORFs) (Eaton, Metcalf, Penny, Tcherepanov, Upton & Brunetti 2007). The viral putative major capsid protein (MCP), vascular endothelial growth factor, mRNA capping enzyme, tumour necrosis factor receptor-associated protein and adenosine triphosphatase (ATPase) genes have been used to classify new megaclovirus isolates (Sudthongkong, Miyata & Miyazaki 2002b; Wang et al. 2007). Among these genes, MCP is a suitable target for phylogenetic studies because it is highly conserved in iridoviruses (Hyatt, Gould, Zupanovic, Cunningham, Hengstberger, Whittington, Kattenbelt & Coupar 2000). Moreover, it is suggested that megaclovirus isolates can be divided into three groups based on the nucleotide sequence of the viral MCP gene (Song, Kitamura, Jung, Miyadai, Tanaka, Fukuda, Kim & Oh 2008).

In Taiwan, outbreaks of iridovirus disease have been reported in cultured marine fish (Chao, Yang, Tsai, Chen, Lin & Huang 2002; Wang et al. 2003). However, the phylogenetic relationships between Taiwan isolates and those from different geographic areas are unclear. Recently, we have collected seven megaclovirus isolates, two from king grouper, *Epinephelus lanceolatus* (Bloch), three from barramundi perch, *Lates calcarifer* (Bloch), one from silver sea bream, *Rhodosargus sarba* (Forsskal), and one from common ponyfish, *Leiognathus equulus* (Forsskal), cultured in southern Taiwan. In this study, the complete MCP genes of these seven megaclovirus isolates were amplified by PCR. The amplified products were sequenced and compared with known megaclovirus isolates to evaluate the genetic variation of the viruses. Their phylogenetic relationships with other iridovirus isolates were also investigated.

**Materials and methods**

**Fish**

Cultured, king grouper, barramundi perch, silver sea bream and common ponyfish showing clinical signs of iridovirus infection were obtained from Kaohsiung, Pingtung and Penghu Islands in southern Taiwan (Table 1). Spleen tissue from diseased fish was collected and stored at −80 °C until use.

**Viral DNA extraction**

Samples of about 50 mg of spleen from iridovirus-affected fish were homogenized in 500 µL lysis buffer (50 mM Tris–HCl, pH 8; 20 mM NaCl, 3% SDS, 10 mM EDTA, pH 8; proteinase K 100 µg mL⁻¹). After centrifugation at 3000 g for 10 min, the supernatant fractions were extracted by phenol/chloroform as described by Sambrook, Fritsch & Maniatis (1989). The DNA pellet was dissolved in distilled water and stored at −80 °C until use.

**PCR amplification and analysis of products**

A primer pair was designed from the complete genome sequence of OSGIV (GenBank accession no. AY894343) specific to the MCP gene. Amplification products of 1581 bp were generated by PCR. The reaction was performed in 50 µL PCR buffer containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mM of each dNTP, 10 pmol of each primer.

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**Table 1 Sources of iridoviruses isolated from cultured fish in Taiwan**

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Species</th>
<th>Size</th>
<th>Region</th>
<th>Year</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KGIV-Ph-05</td>
<td><em>Epinephelus lanceolatus</em></td>
<td>2 kg</td>
<td>Penghu</td>
<td>2005</td>
<td>EU847414</td>
</tr>
<tr>
<td>KGIV-Kh-07</td>
<td><em>Epinephelus lanceolatus</em></td>
<td>600–1000 g</td>
<td>Kaohsiung</td>
<td>2007</td>
<td>EU847415</td>
</tr>
<tr>
<td>BPIV-Kh-05</td>
<td><em>Lates calcarifer</em></td>
<td>200–300 g</td>
<td>Kaohsiung</td>
<td>2005</td>
<td>EU847416</td>
</tr>
<tr>
<td>BPIV-Kh-07</td>
<td><em>Lates calcarifer</em></td>
<td>300–600 g</td>
<td>Kaohsiung</td>
<td>2007</td>
<td>EU847417</td>
</tr>
<tr>
<td>BPIV-Kh-08</td>
<td><em>Lates calcarifer</em></td>
<td>50–100 g</td>
<td>Kaohsiung</td>
<td>2008</td>
<td>EU847418</td>
</tr>
<tr>
<td>SSIV-Ph-05</td>
<td><em>Rhodosargus sarba</em></td>
<td>10–15 g</td>
<td>Penghu</td>
<td>2006</td>
<td>EU847419</td>
</tr>
<tr>
<td>CPIV-Pt-05</td>
<td><em>Leiognathus equulus</em></td>
<td>20–30 g</td>
<td>Pingtung</td>
<td>2005</td>
<td>EU847420</td>
</tr>
</tbody>
</table>
(MCP-OS-F 5′-GTGGCTCTGCTTGTGATT-3′, MCP-OS-R 5′-GTCGGACTGTTGGTCTTG-3′), 1 unit of Taq DNA polymerase and 1 µg DNA template, using the following steps: denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 1 min and elongation at 72 °C for 2 min, ending with an additional elongation step of 10 min at 72 °C. The amplified products were analysed by electrophoresis on a 1.5% agarose gel.

**Cloning**

To determine the homology of nucleotide sequences of iridovirus isolates from Taiwan with other megalocystivirus isolates, the PCR products from diseased fish species were purified from agarose gels using a PCR clean-up system (Promega Corporation). The DNA fragments were individually ligated into pGEM-T Easy Vector (Promega Corporation) and transformed into *Escherichia coli* DH-5α. The recombinant plasmid DNA from the different clones was extracted separately, and automatic sequencing was performed commercially (Genomics BioSic & Tech Co., Ltd). Three clones from each viral MCP gene isolated from different fish species were sequenced to verify replication error.

**Phylogenetic analysis**

Nucleotide sequence analyses of the MCP gene from the different megalocytivirus isolates were compared with other known isolates retrieved from the GenBank databases. These isolates included ALIV (AY285745), DGIV (AY989901), grouper sleepy disease iridovirus (GSDIV) (AY285746), ISKNV (AF371960), Korean flounder iridovirus (KFIV) (AY633987), LYCIV (AY779031), Murray cod iridovirus (MCIV) (AY936203), olive flounder iridovirus (OFIV) (DQ198145), orange spotted grouper iridovirus (OSGIV) (AY894343), RBIV (AY532613), red sea bream iridovirus (RSIV) (AY310918), SBIV (AY310917) and turbot red body iridovirus (TRBIV) (AY590687) using BLAST (Altschul, Madden, Schäffer, Zhang, Zhang, Miller & Lipman 1997) and Clustal W (Thompson, Higgins & Gibson 1994). The phylogenetic analysis was performed using the neighbour-joining method (Saitou & Nei 1987) as implemented in the phylogeny inference package (PHYLIP) version 3.76. Bootstrap analysis was performed using 1000 data resamplings.

**Results**

Fish samples were collected during outbreaks of mortality since 2005. Affected fish displayed abnormal swimming and lethargy and although the sizes of affected fish were different, the cumulative mortality in all outbreaks reached 30–50% within 1 month. Using the primers corresponding to the MCP gene approximately 1581-bp PCR products were obtained from all seven isolates (Fig. 1). These iridovirus isolates were designated as KGIV-Ph-05, KGIV-Kh-07, BPIV-Kh-05, BPIV-Kh-07, BPIV-Kh-08, SSIV-Ph-05 and CPIV-Pt-05, respectively (Table 1). The PCR amplified products were separately cloned and sequenced. The nucleotide sequences of the complete viral MCP genes have been deposited in GenBank (Table 1). All seven PCR products contained a unique open reading frame with 1362 nucleotides and encoded a structural protein with 453 amino acids (Fig. 2). The nucleotide sequences of the viral MCP gene showed a 97% identity between all seven PCR products. Comparison of the nucleotide sequences of the seven megalocytiviruses isolated from Taiwan showed 94%, 97% and 97% identity with the ISKNV, RSIV and OSGIV genotypes, respectively (Table 2). Among the Taiwan megalocytivirus

![Figure 1](image_url)
Figure 2 Multiple deduced amino acid sequence alignment of the major capsid protein gene of different Megalocytivirus isolates from Taiwan with red sea bream iridovirus (RSIV) (AY310918), infectious spleen and kidney necrosis virus (ISKNV) (AF371960), rock bream iridovirus (RBIV) (AY532613) and orange spotted grouper iridovirus (OSGIV) (AY894343). The sequences have been shaded to indicate variation within different isolates.

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isolates, KGIV-Kh-07 and BPIV-Kh-05 contain 0–67 nucleotide replacements compared with OSGIV and ISKNV, and SSIV-Ph-05 is most variable with 29–79 nucleotide replacements. Furthermore, a comparison of the deduced amino acid sequences of seven Taiwan isolates with four other megalocytivirus isolates revealed strong homology with more than 98% identity (Table 3). These amino acid substitutions, occurred at position 225 (L → V) and 232 (A → T) in SSIV-Ph-05, at position 280 (N → D) in BPIV-Kh-07 and at position 396 (L → P) in BPIV-Kh-08 (Fig. 2). The nucleotide sequence of the MCP gene from 20 isolates was used to construct a phylogenetic tree to examine the relationship between the seven megalocytivirus isolates identified in this study and 13 previously identified iridovirus isolates. All Taiwan megalocytivirus isolates from four species of fish had more affinity with the RSIV genotype than the ISKNV genotype.

**Discussion**

Iridoviruses are one of the major pathogenic agents in mariculture in Taiwan. In a previous study, iridovirus infection has been confirmed in the hybrid grouper, giant seaperch and largemouth bass using nested PCR (Chao et al. 2002). Furthermore, the ATPase gene and CY15 fragment of Taiwan grouper iridovirus (TGIV) has been cloned and sequenced. Comparison of deduced amino acid sequences suggested TGIV is closer to ISKNV, but has over 90% similarity to the RSIV group (Chao, Chen, Lai, Lin & Huang 2004). In this study, seven iridovirus isolates were collected from four species of fish in southern Taiwan, and the viral MCP genes were separately cloned and sequenced. It is concluded that all the Taiwanese megalocytiviruses have more affinity with the RSIV genotype than the ISKNV genotype.

**Megalocytivirus** was identified as a new genus within the *Iridoviridae* family (Chinchar et al. 2005). Recently, megalocytiviruses have been divided into three groups based on the MCP gene, which is one of the most important genes for analysis of phylogenetic relationships among this family (Do, Cha, Kim, An, Park, Kim, Park & Park 2005). Song et al. (2008) evaluated the relationships between genetic variation and geographic distribution of the genus *Megalocytivirus* isolated from Korea and Japan. They suggested that viruses belonging to the genotype I were widely distributed among various fish species in many Asian countries. Conversely, the epidemic viruses belonging to genotypes II and III may be constrained in their prevalence and geographic distribution because of their restricted host fish species range (Song et al. 2008). Our results confirm that the seven Taiwanese isolates in this study belong to genotype I of the genus

### Table 2 Percentage similarities of the nucleotide sequence of the MCP gene between the megalocytiviruses isolated in Taiwan with reference fish iridoviruses (ISKNV, RSIV, RBIV and OSGIV). The numbers of substitute nucleotides are given in brackets

<table>
<thead>
<tr>
<th></th>
<th>KGIV-Ph-05 (%)</th>
<th>KGIV-Kh-07 (%)</th>
<th>BPIV-Kh-05 (%)</th>
<th>BPIV-Kh-07 (%)</th>
<th>BPIV-Kh-08 (%)</th>
<th>SSIV-Ph-05 (%)</th>
<th>CPIV-Pt-05 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISKNV</td>
<td>95 (67)</td>
<td>95 (67)</td>
<td>95 (67)</td>
<td>95 (68)</td>
<td>95 (68)</td>
<td>94 (79)</td>
<td>95 (68)</td>
</tr>
<tr>
<td>RSIV</td>
<td>99 (5)</td>
<td>99 (4)</td>
<td>99 (4)</td>
<td>99 (5)</td>
<td>99 (5)</td>
<td>97 (29)</td>
<td>99 (7)</td>
</tr>
<tr>
<td>RBIV</td>
<td>99 (2)</td>
<td>99 (1)</td>
<td>99 (1)</td>
<td>99 (2)</td>
<td>99 (2)</td>
<td>97 (30)</td>
<td>99 (4)</td>
</tr>
<tr>
<td>OSGIV</td>
<td>99 (1)</td>
<td>100 (0)</td>
<td>100 (0)</td>
<td>99 (1)</td>
<td>99 (1)</td>
<td>97 (29)</td>
<td>99 (3)</td>
</tr>
</tbody>
</table>

MCP, major capsid protein; ISKNV, infectious spleen and kidney necrosis virus; RSIV, red sea bream iridovirus; RBIV, rock bream iridovirus; OSGIV, orange-spotted grouper iridovirus.

### Table 3 Percentage similarities of the deduced amino acid sequence of the MCP gene between the megalocytiviruses isolated in Taiwan with reference fish iridoviruses (ISKNV, RSIV, RBIV and OSGIV). The numbers of substitute amino acids are given in brackets

<table>
<thead>
<tr>
<th></th>
<th>KGIV-Ph-05 (%)</th>
<th>KGIV-Kh-07 (%)</th>
<th>BPIV-Kh-05 (%)</th>
<th>BPIV-Kh-07 (%)</th>
<th>BPIV-Kh-08 (%)</th>
<th>SSIV-Ph-05 (%)</th>
<th>CPIV-Pt-05 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISKNV</td>
<td>98 (6)</td>
<td>98 (6)</td>
<td>98 (6)</td>
<td>98 (7)</td>
<td>98 (7)</td>
<td>98 (8)</td>
<td>98 (6)</td>
</tr>
<tr>
<td>RSIV</td>
<td>99 (1)</td>
<td>99 (1)</td>
<td>99 (1)</td>
<td>99 (2)</td>
<td>99 (2)</td>
<td>99 (3)</td>
<td>99 (1)</td>
</tr>
<tr>
<td>RBIV</td>
<td>99 (1)</td>
<td>99 (1)</td>
<td>99 (1)</td>
<td>99 (2)</td>
<td>99 (2)</td>
<td>99 (3)</td>
<td>99 (1)</td>
</tr>
<tr>
<td>OSGIV</td>
<td>100 (0)</td>
<td>100 (0)</td>
<td>100 (0)</td>
<td>99 (1)</td>
<td>99 (1)</td>
<td>99 (2)</td>
<td>100 (0)</td>
</tr>
</tbody>
</table>

MCP, major capsid protein; ISKNV, infectious spleen and kidney necrosis virus; RSIV, red sea bream iridovirus; RBIV, rock bream iridovirus; OSGIV, orange-spotted grouper iridovirus.
Megalocytivirus. It is interesting that barramundi perch larvae are usually reared in sea water, and juveniles are gradually acclimatized to fresh water for grow-out in Taiwan. Although the BPIV-Kh-05 and BPIV-Kh-08 isolates were collected from infected fish in freshwater ponds, they were closer to genotype I than genotype II, which is mainly distributed in freshwater fish in Southeast Asian countries. In these cases, the rearing sites of the affected barramundi perch were distant from other fish farms. It is speculated that the iridovirus infection occurred in the larval stage and the virus further adapted to saline conditions in grow-out ponds.

An understanding of host range of a virus is important for the control of disease outbreaks. Kawakami & Nakajima (2002) reported that the disease caused by RSIV occurred in 31 cultured fish species in Japan. Recently, ISKNV-like viruses were detected from 13 cultured fish species and 39 wild fish species collected in the South China Sea (Wang et al. 2007). This was the first report of iridovirus infection in king grouper and common ponyfish in the grow-out stage. Because fish are transported worldwide for commercial, recreational and ornamental use, they may be the vehicle by which virus is introduced into a new host or environment (Go, Lancaster, Deece, Dhungyel & Whittington 2006). Thus, BPIV-kh-07 and 08 were collected from diseased barramudi imported as larvae from Thailand. It is not clear whether the megalocytiviruses were present in the imported larvae or whether the virus was acquired in the grow-out ponds in Taiwan.

The MCP gene of iridovirus is a suitable target for phylogenetic studies as it is highly conserved. However, differences in nucleotide sequences of the MCP gene are not apparent between megalocytiviruses isolated from different locations, years or host species based on the nucleotide sequences of the various genomic regions, such as the ATPase gene, the DNA polymerase gene and the ribonucleotide reductase small subunit gene (Sudthongkong et al. 2002b; Jeong, Kim, Kim, Chung, Komisar & Jeong 2006). In this study, the MCP nucleotide sequences of KGIV-Kh-07 and BPIV-Kh-05 were 100% identical to OSGIV, and KGIV-Kh-07, BPIV-Kh-07 and BPIV-Kh-08 have only one replacement nucleotide. The viral genome of OSGIV has been completely sequenced (Lu et al. 2005) and the relationship between these isolates from Taiwan and OSGIV merits further study to confirm they are the same virus strain.

The emergence of variants of iridoviruses is unexpected because intraspecific variation is a hallmark of RNA viruses, but is not generally observed in large, complex DNA viruses. Our results showed that viruses isolated from king grouper and barramundi perch from different years had less than one substitute nucleotide, suggested that intraspecies variation of iridovirus does not occur. This present study revealed that iridoviruses have spread over a wide area and transmitted to various fish species in Taiwan.

Figure 3 Phylogenetic neighbour-joining tree deduced from analysis nucleotide sequences of major capsid protein gene of seven Taiwan Megalocytivirus isolates were compared with 13 different geographic isolates. GenBank accession numbers for nucleotide sequences of MCP genes are as follows: African lampeye iridovirus (ALIV) (AY285745), dwarf gourami iridovirus (DGIV) (AY989901), grouper sleep disease iridovirus (GSDIV) (AY285746), infectious spleen and kidney necrosis virus (ISKNV) (AF371960), Korean flounder iridovirus (KFIV) (AY633987), large yellow croaker iridovirus (LYCIV) (AY779031), Murray cod iridovirus (MCIV) (AY936203), olive flounder iridovirus (OFIV) (DQ198145), orange spotted grouper iridovirus (OSGIV) (AY894343), rock bream iridovirus (RBIV) (AY532613), red sea bream iridovirus (RSIV) (AY310918), sea bass iridovirus (SBIV) (AY310917) and turbot reddish body iridovirus (TRBIV) (AY590687). Taiwan isolates abbreviated as in Table 1.
Analysis of the complete MCP gene demonstrated more than 97% identity of the nucleotide sequence between all the amplicons. It is concluded that the seven iridovirus strains could be classified into one cluster with more affinity to the RSIV genotype than the ISKNV genotype. Phylogenetic analysis suggested that these isolates from four different fish species at three geographically different locations have a single origin. The phylogenetic relationships between these strain variants were related neither to host-dependent evolution nor to geography. In the future, more powerful and extensive analyses using different viral genes should be performed for the discrimination of various megalocytiviruses isolated in Taiwan.

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