Molecular characterisation of hepatopancreatic parvovirus (PmergDNV) from Australian *Penaeus merguiensis*

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Abstract

Hepatopancreatic parvovirus infection is associated with reduced growth rates of prawns during the juvenile stages and overt mortalities. Hepatopancreatic parvovirus was purified from *Penaeus merguiensis* from northern Queensland and a partial consensus sequence of 5.9 kb was obtained. Nucleotide comparisons revealed that the Australian isolate of HPV has a nucleotide similarity (87%) closer to HPVchin and the full sequence of HPV *Penaeus monodon* (PmDNV) (6321 bp) than to HPVsemi (83%). Three putative open reading frames were identified. The first open reading frame encoded a nonstructural protein (NS2) and shared an amino acid similarity of 86% with PmDNV. The second ORF overlapped the first open reading frame and shared 93% and 26% amino acid similarity with PmDNV and PstDNV, respectively, and encoded NS1. The third ORF encoded the viral structural protein and shared an amino acid similarity of 73% with the capsid protein of PmDNV and HPVchin. The phylogeny suggests that the Australian HPV isolate is closely related to the Korean HPVchin isolate than to the Indian HPVsemi and Thai PmDNV isolates. HPV strains may be following the phylogenetic relationship of penaeid prawn hosts rather than their geography.

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Keywords: Hepatopancreatic parvovirus (HPV); Parvoviridae; *Penaeus merguiensis*; Australia; PCR; Molecular sequencing

Introduction

The increasing demand for seafood around the world has led to a considerable expansion in aquaculture. Penaeid prawns are one of the most valuable commercially produced species but the increase in production has been associated with an increase in disease problems, particularly those with infectious aetiologies (Lightner and Redman, 1992). Among these, viral pathogens have contributed to substantial economic losses within penaeid culture (Fraser and Owens, 1996; Flegel, 1997).

Hepatopancreatic parvovirus (HPV) is currently considered as a member of the family *Parvoviridae* (Bonami et al., 1995). Its host range encompasses both wild and cultured penaeid species worldwide (Paynter et al., 1985; Lightner, 1996; Spann et al., 1997) and the freshwater prawn *Macrobrachium rosenbergii* (Anderson et al., 1990). Mortalities during the larval stages of penaeid prawns have been associated with HPV (Spann et al., 1997). Furthermore, HPV infection is associated with reduced growth rates of juvenile prawns (Flegel et al., 1999). However, there are no specific gross signs for HPV so diagnosis may be difficult, particularly in the presence of other pathogens that may mask the effect (Manivannan et al., 2002; Chayaburakul et al., 2004).

To date, three strains of HPV have been reported suggesting that HPV isolated from different prawn species and/or different geographic regions is genetically different. Diagnosis of HPV has primarily depended on histology which cannot detect low grade infections and differentiate between strains. Conversely, sensitive methods of detection such as polymerase chain reaction (PCR) and gene probes are strain specific (Lightner et al., 1994; Phromjai et al., 2001) as small nucleotide changes lead to negative test results. Consequently, the known strains of HPV may only represent a small proportion of the existing strains as some may have escaped detection by the current diagnostic methods. Hence, there is the need to characterise HPV in different geographical areas and from different species to effectively diagnose the virus in potential carriers.

In Australian penaeid species, HPV has been reported in *Penaeus esculentus*, *Penaeus japonicus*, *Penaeus merguiensis*...
and *Penaeus monodon* (Paynter et al., 1985; Lightner, 1996; Spann et al., 1997) but it is unknown whether these are similar strains to each other or to those already described overseas. This paper describes the first sequence data obtained for HPV from any *P. merguiensis* worldwide and from any Australian host and its relationship to other known arthropod paroviruses.

**Results**

**PCR amplification**

Using PCR, 5934 bp (approximately 94%) of the genome of HPV from Australian *P. merguiensis* (*PmergDNV*) was successfully sequenced (Genbank accession number: DQ458781).

Primers from the literature, 7490F–7852R (Phromjai et al., 2001) and 1120F–1120R (Pantoja and Lightner, 2001) (Table 1), specific for the HPV*chin* genome failed to give the expected amplicon sizes of 350 bp and 592 bp, respectively but rather produced amplicons of 240 bp and approximately 1 kb, respectively.

**Nucleotide sequence analysis**

The base composition of the sequence obtained was 35.51% A, 15.25% C, 25.55% G and 23.69% T. The total G+C content was 40.8% and A+T content was 59.2%.

**Potential open reading frames**

Potential open reading frames in the HPV sequence obtained during this study were determined by the NCBI ORF finder. The HPV genome contained three major ORFs in which there was a slight overlap between the first and second ORF (Fig. 1), but in different reading frames, namely 1 and 3.

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Table 1

The sequences of the primers used in PCR amplification of the genome of the Australian HPV isolate from *Penaeus merguiensis*

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5′–3′)</th>
<th>Consensus nucleotide sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV434F</td>
<td>TATCCGGAAACTCATTACTCTA</td>
<td>N/A</td>
</tr>
<tr>
<td>HPV434R</td>
<td>TCCATCACAAACATTTACCTT</td>
<td></td>
</tr>
<tr>
<td>HPV452F</td>
<td>TCCCTCTGTCCCTGGCCCTTC</td>
<td></td>
</tr>
<tr>
<td>HPV452R</td>
<td>CATCATCCAAAATGCTTATG</td>
<td></td>
</tr>
</tbody>
</table>
| 1120F        | GTGTTAGTGAGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
All small ORFs did not contain significant sequence homology using BLAST.

**ORF 1**

The first ORF (ORF 1) started at nucleotide 97 in reading frame 1+ and terminated with a TAA codon at position 1119. It contained 340 amino acids, corresponding to a molecular weight of 39.84 kDa. This ORF shared an amino acid similarity of 86% with HPV from *P. monodon* of Thailand (*PmDNV*). There was no significant sequence homology between ORF 1 of *PmergDNV* and other prawn, insect or indeed any other paroviruses. This protein is speculated to encode a nonstructural protein (NS2) but its function has yet to be determined.

**ORF 2**

The second ORF (ORF 2) was the second largest of the ORFs identified for *PmergDNV*. It started at nucleotide 1104 in reading frame 3+ and terminated with a TAA codon at position 2840. It contained 578 amino acids, corresponding to a molecular weight of approximately 68.3 kDa. Using the entries in the protein database via BLAST, this ORF shared 93% amino acid similarity with the nonstructural protein 1 of *PmDNV* (DQ002873) and 26% similarity with the nonstructural protein 1 of *PstDNV* (AF218266). There were no additional significant matches with other paroviruses. This sequence also contains sequence homology to the replication initiator motifs involved in parovirus rolling hairpin mechanism (Bergoin and Tijssen, 2000). These motifs were located between amino acids 86 and 399.
415 and shared similar conserved regions with IHHNV and other paroviruses (Fig. 2A) (Shike et al., 2000). The amino acid sequence also contains sequence homology to paroviruses NTP-binding and helicase domains of the NS1 polypeptide. These domains were located between amino acids 390 and 488 and shared similar conserved regions with IHHNV and other paroviruses (Fig. 2B) (Shike et al., 2000). Hence, this protein is speculated to encode a nonstructural protein (NS1).

**ORF 3**

The third ORF (ORF 3) started at nucleotide 3255 in reading frame 3+ and terminated with a TAA codon at position 5714. It contained 817 amino acids, corresponding to a molecular weight of 91.622 kDa. This ORF shares 73% amino acid similarity with the structural protein of HPV from *P. chinensis* of Korea (HPVchin), using entries in the protein database via BLAST. There were no additional significant matches with other paroviruses. Although the function of this protein is yet unknown, it is speculated that this ORF encodes the structural protein (VP1). Alignment of the four partially sequenced HPV strains shows highly conserved regions within the viral protein region across all four HPV strains (Fig. 3).

**Phylogenetic comparison**

A phylogenetic tree (Fig. 4) based on the alignment of genomic DNA of prawn and insect paroviruses gave two main clades. Clade 1 included the insect paroviruses *Bombyx mori* densovirus (*Bm*DNV), *Periplaneta fuliginosa* densovirus (*Pf*DNV), *Junonia coenia* densovirus (*Jc*DNV), *Galleria mellonella* densovirus (*Gm*DNV) *Diatraea saccharalis* densovirus (*Ds*DNV) and the prawn parovirus spawner isolated mortality virus (SMV). Clade 2 included all strains of HPV, infectious hypodermal and haematoepoietic necrosis virus (IHHNV) and the insect parovirus *Aedes aegypti* densovirus (*Aae*DNV) and *Aedes albopictus* densovirus (*Aal*DNV). The Australian HPV isolate (*Pmerg*DNV) is phylogenetically more closely related to the South Korean strain (HPVchin) than to the Indian (HPVsemi) or Thai (*Pm*DNV) isolates, respectively.

**Discussion**

The Australian HPV isolate from *P. merguiensis* is the fourth strain of penaeid prawn HPV to be partially sequenced. We have therefore proposed to name this virus *P. merguiensis* densovirus (*Pmerg*DNV), following the convention of the International Committee for the Taxonomy of Viruses. The other three are HPVchin from *P. chinensis* of Korea (Bonami et al., 1995), *Pm*DNV from *P. monodon* of Thailand (Sukhumsirichart et al., 2006) and HPVsemi from *P. semisulcatus* of India (Manjanaik et al., 2005). An additional strain of HPV has been reported in the freshwater prawn *M. rosenbergii* (Anderson et al., 1990).

The Australian HPV isolate shared the highest nucleotide similarity (87%) with the Korean and Thai HPV isolates and the lowest similarity (83%) with the Indian HPV isolate. However, nucleotide similarity between strains is indicative since only partial sequences of HPVchin, HPVsemi and *Pmerg*DNV were analysed and the amount of known sequence from each isolate varies considerably. The phylogenetic analysis suggests that the Australian HPV isolate is more closely related to HPVchin than to *Pm*DNV and HPVsemi, respectively. This is probably because *P. merguiensis* is phylogenetically more closely related to *P. chinensis* than to *P. semisulcatus* and *P. monodon*, respectively, based on mitochondrial large subunit ribosomal RNA (16S RNA) and cytochrome c oxidase subunit I (COI) genes (Lavery et al., 2004). HPV strains may be following the phylogenetic relationship of the hosts rather than being geographically, nearest neighbour linked. Nonetheless, a complete understanding of the phylogeny of the arthropod paroviruses can only be achieved when the analyses are performed using complete genomic sequences.

The phylogenetic analysis in this study was similar to the analysis of Roekring et al. (2002), with the addition of *Pmerg*DNV, complete sequence data for *Pm*DNV and the inclusion of partial sequence data for the Australian isolate of IHHNV. The strains of HPV, IHHNV and the insect densovirus *Aae*DNV and *Aal*DNV remained in one cluster, suggesting stability. The differences were the insect densovirus *Bm*DNV, *Jc*DNV and the prawn parovirus SMV. In our phylogenetic analysis, *Bm*DNV and *Jc*DNV grouped with the insect densoviruses *Pf*DNV, *Gm*DNV and *Ds*DNV (Roekring et al., 2002). In contrast, the insect densoviruses *Aae*DNV and
Aal/DNV and the IHNV and HPV strains are now grouped together in a separate cluster. Furthermore, SMV is now more likely an outgroup than in the analysis by Roekring et al. (2002). Similar to the findings from Sukhumsirichart et al. (2006), the phylogenetic tree revealed that the HPV strains are closely related to the Brevidensoviruses (IHNV and the mosquito densovirus AaeDNV and Aal/DNV), despite differences in genome organisation (discussed below).

This is the first report of nucleotide information concerning HPV from Australia or from any *P. merguiensis*. Approximately 6 kb was successfully sequenced indicating the total genome length is larger than HPV reported from *P. chinensis* (HPVchin) and may therefore be the same size (approximately 6.3 kb) as the genome of HPV from *P. monodon* of Thailand (Sukhumsirichart et al., 2006). We report three putative ORFs, in which the two nonstructural proteins are overlapping. This is similar to the organisation structure of the PmDNV genome where the two nonstructural proteins are also overlapping but differs from the prawn parvovirus IHNV and the mosquito densovirus AaeDNV and Aal/DNV (Afanasiev et al., 1991; Boublike et al., 1994; Shike et al., 2000; Sukhumsirichart et al., 2006). The consensus sequence contains the complete sequence of putative NS1, NS2 and VP1. We were successfully able to sequence the hairpin like structure on the 5′ end of the genome. However, there have been unresolved problems sequencing the hairpin structure on the 3′ end.

In addition to mortality, HPV reduces the growth rate of infected individuals (Flegel et al., 1999), resulting in production losses for farmers because they continue to spend money on feed and maintenance of ponds where prawns will not grow. To date, HPV has been of little concern and has been overlooked. However, as investigations continue, there is now an impression that HPV is an emerging disease and that there are different strains of HPV associated with different species and/or geographical areas. Consequently, HPV is probably much more widespread than previously thought and may therefore have a much wider host range than reported. This study was the first to obtain molecular data for HPV in Australia. Its biology, prevalence and pathogenesis in other species await investigation and should be focuses of further research.

**Materials and methods**

**Strain of hepatopancreatic parovirus**

*P. merguiensis* (40) stored frozen at James Cook University, Queensland were used as the source of HPV. Infected specimens were originally obtained from three commercial prawn farms in northern Queensland as early as June 2003. Infected specimens originated from the same HPV outbreak and were classified as being HPV-positive by an experimental PCR.

**Viral purification**

The method for isolating HPV was modified from the procedure of Bonami et al. (1995) and Rutpratanporn et al. (2005). Hepatopancreata were removed from the gnatathoraxies of *P. merguiensis* and homogenised in TN buffer (0.02 M Tris–HCl, 0.4 M NaCl, pH 7.4). The homogenate was centrifuged at 7000 and 13,000×g respectively, for 15 min each at 4 °C using Suprafuge 22 12.50 rotor. Liquid supernatant was vacuum filtered through Whatman GF/B, Whatman GF/F filter and Millipore Nitrocellulose 0.45 μm membrane filter, respectively.

The filtered supernatant was subsequently centrifuged at 4 °C for 1 h at 142,459×g using Beckman Coulter Optima L-90K Ultracentrifuge (Beckman Coulter, USA) Type 70 Ti rotor. The pellet was resuspended in 500 μl of TN buffer, layered onto the top of a 20–40% sucrose gradient and re-centrifuged at 113,652×g in a SW 40 Ti rotor for 3 h at 4 °C. The pellet was resuspended in 200 μl of TN buffer and stored at −80 °C until required.

**Nucleic acid extraction**

Total DNA was extracted from the viral suspension using the High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer’s instructions.

**PCR amplification**

Ten sets of primers were used to amplify segments of the HPV genome (Table 1). The PCR program and primer set (1120F and 1120R) described from Pantoja and Lightner (2001) were expected to yield a 592 bp amplicon from the HPV template. The PCR program and primer set (7490F and 7852R) described from Phromjai et al. (2001) were expected to yield a 350 bp amplicon from the HPV DNA template.

Additional primers were subsequently designed from the genome of *PmDNV* (DQ002873), the HPVchin genome (AY008257) and from new sequences obtained from amplifications of the genome of the Australian HPV isolate. All primers were designed using Oligo 6.60 Software (Molecular Biology Insights, USA) and synthesised by Sigma-Genosys, Australia. The PCR reaction mixture contained 1 μl (20–50 ng) of HPV template, 1× *Taq* buffer (750 mM Tris–HCl pH 8.8, 200 mM (NH₄)₂SO₄, 0.1% Tween20), 2.5 mM MgCl₂, 0.75 U *Taq* polymerase (MBI Fermentas), 200 μM each dNTP and 50 pmol of each primer. The PCR reaction volume was adjusted with sterile distilled water to a final volume of 25 μl. Amplification was performed in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Germany) with a PCR profile consisting of an initial 94 °C for 7 min, 35 cycles at 94 °C for 45 s denaturation, 55 °C annealing for 45 s and polymerisation at 72 °C for 1 min and an additional 5 min at 72 °C following the last cycle. PCR products were visualised by running all of the 25 μl PCR reaction mixture on 1% agarose gel containing ethidium bromide at a concentration of 0.5 μg ml⁻¹.

Furthermore, primers HPV4657F, HPV5836F and HPV5765 (Table 1) were designed at the ends of the *PmergDNV* genome to primer walk the ends of the genome incorporating the inverted terminal repeats. The PCR reaction mixture contained
Table 2

Source and origin of arthropod sequences used for the phylogenetic comparison with the sequence of the Australian HPV isolate

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
<th>Host species</th>
<th>Genbank accession number and/or source</th>
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<tr>
<td>HPVchinensis (HPVchin)</td>
<td>Hepatopancreatic parovirus</td>
<td>Peneaus chinensis</td>
<td>AY008257</td>
</tr>
<tr>
<td>HPYmonodon (PmDNV)</td>
<td>Hepatopancreatic parovirus</td>
<td>Peneaus monodon</td>
<td>DQ002873</td>
</tr>
<tr>
<td>HPVsemisulcatus (HPVsemi)</td>
<td>Hepatopancreatic parovirus</td>
<td>Peneaus semisulcatus</td>
<td>Manjanaik et al., 2005</td>
</tr>
<tr>
<td>IHHNV (Australian strain)</td>
<td>Infectious hypodermal and haematopoietic necrosis virus</td>
<td>Peneaus monodon</td>
<td>AYS90120, K. Krabetswe unpubl. data, pers. comm.</td>
</tr>
<tr>
<td>IHHNV (PstDNV)</td>
<td>Infectious hypodermal and haematopoietic necrosis virus</td>
<td>Peneaus stilyorstris</td>
<td>AF218266</td>
</tr>
<tr>
<td>SMV</td>
<td>Spawner isolated mortality virus</td>
<td>Peneaus monodon</td>
<td>AF499102</td>
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<td>Diatraea saccharalis densovirus</td>
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<td>GmDNV</td>
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<td>JeDNV</td>
<td>Junonia coenia densovirus</td>
<td>Junonia coenia</td>
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<td>PfDNV</td>
<td>Periplaneta fuliginosa densovirus</td>
<td>Periplaneta fuliginosa</td>
<td>NC009936</td>
</tr>
</tbody>
</table>

1 µl (50–100 ng) of HPV template, 1 × Taq buffer (100 mM Tris–HCl (pH 8.8 at 25 °C), 500 mM KCl, 0.8% Nonidet P40), 5.0 mM MgCl2, 5 U Taq polymerase (MBI Fermentas), 10 mM dNTPs and 0.6 µM of each primer. The PCR reaction volume was adjusted with sterile distilled water to a final volume of 50 µl. Amplification was performed in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Germany) with a PCR profile consisting of 10 cycles at 95 °C for 30 s denaturation, 40 °C for 1 min annealing and polymerisation at 72 °C for 4 min and 30 s, followed by 30 cycles at 95 °C for 30 s denaturation, annealing at 60 °C for 1 min and polymerisation at 72 °C for 4 min and 30 s and an additional 5 min at 72 °C following the last cycle. PCR products were visualised by running all of the 50 µl PCR reaction mixture on 1% agarose gel containing ethidium bromide at a concentration of 0.5 µg ml⁻¹.

Cloning and sequencing

DNA fragments were purified from agarose gels using WizardSV Gel and PCR Clean-Up System (Promega, USA) and cloned into Escherichia coli JM 109 cells, using pGEM-T Easy Vector System (Promega, USA), according to the manufacturer’s instructions. At least three white colonies were selected for isolation of the recombinant plasmids according to the protocol of the Eppendorf Fast Plasmid Mini Prep Kit (Eppendorf, Germany). Recombinant plasmids were digested with SpeI followed by electrophoresis on 1% agarose gel to screen for DNA inserts. Recombinant plasmids containing DNA inserts were sequenced using the Amersham Chemistry DYEnamic ET terminator sequencing kit (Amersham Biosciences) and M13 universal primers. Three forward and three reverse reactions were performed for each clone. Samples used either a MegaBACE Sequence Analyser at the Advanced Analytical Centre at James Cook University, Townsville (Amersham Biosciences) or a Macrogen Inc (Korea) Sequencher software (Gene Codes Corporation) to analyse and align overlapping sequences for each clone.

Nucleotide sequence analysis

Sequence information derived from HPV in this study was compared with sequence information from other strains of HPV using BLAST. Putative open reading frames (ORFs) in the nucleotide sequence were determined by computer analysis using NCBI ORF finder.

Phylogenetic comparisons

Alignment and phylogenetic analysis of sequences was performed using ClustalX Multiple Sequence Alignment Program version 1.8 (National Centre for Biotechnology Information) and GeneDoc Multiple Sequence Alignment Editor and Shading Utility Version 2.6.002 (Pittsburgh Supercomputing Centre) using sequence information from known arthropod paroviruses (Table 2). Alignments were visualised using NJplot (http://pbil.univ-lyon1.fr/software/njplot.html) (Perriere and Guoy, 1996). Bootstrap values for the phylogenetic analysis were calculated from 1000 replications.

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