Short Communication

Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family *Herpesviridae*

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Outbreaks of a new disease responsible for mass mortality in common carp (*Cyprinus carpio*) and coloured carp or koi (*Cyprinus carpio koi*) may have occurred as early as 1996 (Haenen et al., 2004). The first formal descriptions of the disease were from Israel and the USA in 1998 (Hedrick et al., 1999) and from Germany in 1997–1998 (Bretzinger et al., 1999). Based on preliminary transmission electron microscopy of gill tissues from moribund koi in these studies, a herpesvirus was the suspected agent responsible for the disease. Furthermore, Hedrick et al. (1999, 2000) were able to isolate the virus observed by electron microscopy by using a newly developed cell line (KF-1) originating from koi. The isolated virus reproduced the disease in experimentally infected koi and was reisolated from the same fish, confirming that this was the aetiological agent of the new disease. Developmental and morphological features supported inclusion of this new virus in the family *Herpesviridae* (Hedrick et al., 2000).

The new virus was therefore designated koi herpesvirus (KHV) after the host from which the virus was isolated originally (Hedrick et al., 2000). More recently, a very similar or identical virus was isolated by Neukirch & Kunz (2001) and Ronen et al. (2003). The latter group preferred the designation carp nephritis and gill necrosis virus (CNGV) and have now confirmed by PCR experiments that CNGV is the same as KHV (Hutoran et al., 2005).

Current placement of viruses in the family *Herpesviridae* is based upon the presence of a linear, double-stranded DNA...
genome packaged within an icosahedral capsid that is surrounded by a proteinaceous tegument layer and finally a host-derived envelope (Minson et al., 2000). Capsid architecture is highly conserved among the herpesviruses and consists of 162 capsomers arranged as a $T=16$ icosadeltahedral lattice. In herpes simplex virus type 1 (HSV-1), the 150 hexameric and 12 pentameric capsomers consist of the major capsid protein (VP5) plus a small protein (VP26) associated with the exterior of each subunit of the hexamers. These are joined by intercapsomeric triplexes, each consisting of two copies of one protein (VP23) and one copy of another (VP19C) (Zhou et al., 2000). Production of herpesvirions involves capsid formation, capsid maturation, DNA replication, DNA packaging, addition of tegument, envelopment of capsids and release of mature particles. Each of these processes requires a suite of virally encoded proteins. DNA replication is dependent on seven viral proteins, which include a two-subunit DNA polymerase, a three-subunit helicase–primase complex, a single-stranded DNA-binding protein and a helicase that recognizes the origins of DNA replication (Roizman & Pellett, 2001; Wu et al., 1988). DNA packaging also requires seven proteins, including a terminase that is unique to double-stranded DNA bacteriophages and herpesviruses (Brown et al., 2002; Serwer et al., 2004). Subsequent processes in viral morphogenesis and maturation are at least as complex and relatively poorly understood.

The existence of three fundamental clades within the family Herpesviridae is evident from the almost complete lack of sequence similarity between herpesviruses that infect mammals, birds and reptiles, herpesviruses that infect fish and frogs, including two previously classified cyprinid herpesviruses (CyHV-1 and CyHV-2), remain as members of the family Herpesviridae (Minson et al., 2000).

In order to investigate the genetic relationships between KHV, CyHV-1 and CyHV-2, sequences were determined and compared for two genes presumed to be involved in DNA replication and two genes encoding putative capsid proteins. The sequences were also compared to the most extensively characterized fish herpesvirus, channel catfish virus (Ictalurid herpesvirus 1, IcHV-1). In addition, genetic variation in a region of the DNA polymerase gene was assessed for 12 KHV isolates from koi and common carp in the USA, Israel, Malaysia and Taiwan.

Details of the KHV isolates are listed in Table 1. The CyHV-1 (Sano et al., 1985) and CyHV-2 isolates were provided by one of the authors (H. Fukuda). The KHV and CyHV-1 isolates were grown in the KF-1 cell line, and the CyHV-2 isolate was grown in a goldfish fin cell line (GF-1) provided by H. Fukuda. Stocks of each virus (1 ml), previously frozen at $-80^\circ$C, were thawed and used to inoculate flasks containing monolayers of the appropriate cell line. After CPE was complete, cells and culture media were collected. All centrifugation steps were carried out at 10°C. Cellular debris was separated from the culture medium by centrifugation at 3500 $g$ for 20 min. The pellet was then Dounce-homogenized with 10 ml minimal essential medium containing 2% (v/v) fetal bovine serum. The supernatant obtained by centrifugation at 3500 $g$ for 20 min was combined with the culture medium and centrifuged at 95 300 $g$ for 90 min. The virus pellet was resuspended by Dounce homogenizing in 1 ml TNE (50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5) and layered onto a 10–60% (w/v) linear sucrose gradient in TNE. After centrifugation at 77 000 $g$ for 18 h, two visible bands near the bottom of the tube were collected together, diluted in

Table 1. List of KHV isolates used in this study, including the reference in which the isolate was first reported, year of isolation, fish species infected and country of origin

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Reference</th>
<th>Year of isolation</th>
<th>Species</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>KHV-U</td>
<td>Hedrick et al. (2000)</td>
<td>1998</td>
<td>Koi</td>
<td>USA</td>
</tr>
<tr>
<td>KHV-1</td>
<td>Hedrick et al. (2000)</td>
<td>1999</td>
<td>Koi</td>
<td>Israel</td>
</tr>
<tr>
<td>SAM</td>
<td>This paper</td>
<td>1999</td>
<td>Koi</td>
<td>USA</td>
</tr>
<tr>
<td>R00-16-2</td>
<td>This paper</td>
<td>2000</td>
<td>Koi</td>
<td>Israel</td>
</tr>
<tr>
<td>F01-18</td>
<td>This paper</td>
<td>2001</td>
<td>Koi</td>
<td>USA</td>
</tr>
<tr>
<td>C01-52-3</td>
<td>This paper</td>
<td>2001</td>
<td>Koi</td>
<td>USA</td>
</tr>
<tr>
<td>D-132</td>
<td>Gilad et al. (2003)</td>
<td>2001</td>
<td>Koi</td>
<td>USA</td>
</tr>
<tr>
<td>D-164</td>
<td>Gilad et al. (2003)</td>
<td>2001</td>
<td>Koi</td>
<td>Malaysia</td>
</tr>
<tr>
<td>F04-10-1</td>
<td>This paper</td>
<td>2004</td>
<td>Koi</td>
<td>USA</td>
</tr>
<tr>
<td>KHV-Taiwan</td>
<td>This paper</td>
<td>2004*</td>
<td>Koi</td>
<td>Taiwan</td>
</tr>
<tr>
<td>SC04-06</td>
<td>This paper</td>
<td>2004*</td>
<td>Common carp</td>
<td>USA</td>
</tr>
<tr>
<td>SC04-04</td>
<td>This paper</td>
<td>2004*</td>
<td>Common carp</td>
<td>USA</td>
</tr>
</tbody>
</table>

*Tissue samples from which DNA was extracted for PCR, but virus was not isolated in cell culture.*
Fig. 1. Amino acid sequence alignments for four genes conserved between KHV and lower-vertebrate herpesviruses. The alignments were computed by using CLUSTAL W (Thompson et al., 1994). Conserved amino acid residues are shown as black blocks. (a) The complete helicase for IcHV-1 (GenBank accession no. NC_001493), KHV D-132 (AY939857) and CyHV-1 (AY939868) and a partial sequence for CyHV-2 (AY939863). (b) The complete intercapsomeric triplex protein for IcHV-1 (NC_001493), KHV-U (AY939862) and CyHV-1 (AY939865) and a partial sequence for CyHV-2 (AY939864).
Fig. 1. cont.

(c) DNA polymerase

RaHV-1
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fresh TNE and centrifuged at 151 000 g for 1 h. The resulting virus pellet was eluted in 1 ml TNE and stored at −80 °C. Difficulties in growing significant quantities prevented CyHV-2 from being purified by using this protocol. Instead, the infected cell pellet was collected after CPE was complete, eluted into 1 ml TNE and stored at −80 °C. DNA was isolated from each preparation by treatment with proteinase K in lysis buffer [50 mM Tris/HCl, 100 mM NaCl, 100 mM EDTA, 1% (w/v) SDS, pH 8–0], extraction with phenol/chloroform/isoamyl alcohol and ethanol precipitation (Sambrook et al., 1989). The DNA was eluted in TE (50 mM Tris/HCl, 1 mM EDTA, pH 7–5) and the concentration was determined by spectrophotometry. DNA preparations were stored at −20 °C.

Viral DNA isolated from KHV D-132 was digested with EcoRI and fragments of 4 kb or larger were purified from an agarose gel and cloned into pGEM (Promega). The sizes of plasmid inserts were determined by digestion with EcoRI. DNA sequence data were obtained from one insert (a 7.3 kb EcoRI fragment) and similarities between putative encoded amino acid sequences and proteins in GenBank were screened by using BLASTP (Altschul et al., 1990). Significant similarities to IcHV-1 were detected for two adjacent, tandem-oriented open reading frames (ORFs) in this fragment. These ORFs were significantly similar to IcHV-1 ORF25 (encoding a putative helicase; Davison, 1992) and ORF27 (encoding a putative capsid triplex protein that corresponds, although unrelated in sequence, to HSV-1 VP23; Davison, 1992; Davison & Davison, 1995). Sequence alignments then facilitated the design of primers to amplify partial sequences from the CyHV-1 and CyHV-2 helicase genes (5′-CTGATCATCGACGAGTACGG-3′ and 5′-CACACGCGTGCACACNACRTA-3′; 867 bp product) and triplex protein genes (5′-CACTCTGGCGACGCTTATG-3′ and 5′-CATCACAGAGTTCTTGACNGC-3′; 259 bp product). BLASTP searches showed that the sequences of the products were related closely to those of KHV and more distantly to those of IcHV-1.

In addition, a region of the DNA polymerase gene was amplified by PCR from KHV-U, CyHV-1 and CyHV-2 by using primers whose 3′ ends were based on two regions conserved among the DNA polymerases of herpesviruses,
Fig. 1. cont.

(d) Major capsid protein

<table>
<thead>
<tr>
<th>IC</th>
<th>KHV</th>
<th>CyHV-1</th>
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<tbody>
<tr>
<td>5'-CGGAATTCTAGAYTTYGCNWSNYTNTAYCC-3'</td>
<td>497 bp product.</td>
<td>BLASTP searches of the sequences again revealed close relationships between the three cyprinid viruses, with the next closest neighbours being the corresponding portions of the DNA polymerases of ICHV-1, Anguillid (eel) herpesvirus 1 and Ranid (frog) herpesvirus 1 (RaHV-1). The sequence data generated by using the degenerate primers facilitated the construction of KHV-specific primers (5'-GACTTT-GCCAGGCCTGTACCAGC-3' and 5'-CGGTGTGCGGTACAGCAGCGT-3' product). The 448 bp product.</td>
</tr>
</tbody>
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sequence (i.e. the 496 bp product lacking the primer-binding sites) generated for the 12 KHV isolates in Table 1 was identical to the sequence determined previously by us for KHV-U (GenBank accession no. AY572853).

The data obtained thus far indicated that KHV is related most closely to CyHV-1 and CyHV-2 and more distantly to herpesviruses of non-cyprinid fish and of frog. Subsequently, data accumulating during KHV-U and CyHV-1 genome-sequencing projects being carried out in Tokyo, Japan, and Davis, CA, USA, respectively, permitted extension of the partial information obtained by PCR to the entire helicase, triplex protein and DNA polymerase genes (Fig. 1a–c). KHV-U and KHV D-132 were found to be identical in the helicase gene and a single, synonymous nucleotide difference was noted in the triplex protein gene. In interpreting the results, it should be registered that the capsid proteins, including those constituting the intercapsomeric triplex, are specific to herpesviruses and that their conservation constitutes strong evidence for evolution from a common ancestral herpesvirus. Moreover, capsid proteins of fish and frog herpesviruses exhibit no detectable sequence similarity to their counterparts in higher-vertebrate herpesviruses (Davison & Davison, 1995). In contrast, helicases and DNA polymerase genes are ubiquitous in organisms and acquisition by horizontal transfer rather than vertical inheritance is a significant possibility. Therefore, further data were extracted from the genome-sequencing projects for another herpesvirus-specific gene, encoding the major capsid protein (ORF39 in IcHV-1). The pattern of relationships for this gene was similar to that observed for the others (Fig. 1d). In summary, KHV, CyHV-1 and CyHV-2 are related closely in each of the four genes analysed and are related more distantly to IcHV-1 and, where data are available, to other lower-vertebrate herpesviruses. Development of a detailed phylogenetic scheme is anticipated when more sequence data for a larger set of viruses become available.

The observations that KHV has a large genome (estimated at 277 kbp from PFGE) and lacks convincing nucleotide sequence similarity to herpesviruses (Ronen et al., 2003; Hutoran et al., 2005) have been cited as reasons for not including this virus in the family Herpesviridae, despite its morphological characteristics. The large size (295 kbp) has been confirmed during the KHV genome-sequencing project (T. Aoki, unpublished data). Indeed, this may be characteristic of cyprinid herpesviruses, as PFGE has revealed a value of 295 kbp for CyHV-1 (J. Jeung, personal communication). Genomes vary greatly in size among mammalian and avian herpesviruses (125–245 kbp) and among herpesviruses from frogs and fish (134–217 kbp) (Davison, 1992, 1998, 2002; Davison et al., 1999). However, genome size is not a criterion for exclusion from the family Herpesviridae (Minson et al., 2000). The hesitation in classifying KHV may be explained by a combination of the scarcity of sequence data for lower-vertebrate herpesviruses (including KHV) and the substantial phylogenetic distance between KHV and IcHV-1 (evident qualitatively in Fig. 1). From an analysis of a few small genome fragments based on relatively insensitive nucleic acid sequence comparisons, Ronen et al. (2003) detected little convincing evidence for a relationship between KHV and herpesviruses. Recent work by this group again underscored the degree of divergence of KHV from other viruses, but nonetheless cited several examples of genetic similarity between KHV and IcHV-1 revealed by amino acid sequence comparisons (Hutoran et al., 2005). For example, a 715 bp KHV fragment, reported initially as being unrelated to herpesviruses (Ronen et al., 2003), was shown subsequently to be related to IcHV-1 ORF56 (Hutoran et al., 2005). The significance of this particular finding is strengthened by the observation that ORF56 is conserved not only in KHV and IcHV-1, but also in CyHV-1 and RaHV-1 (Fig. 2). We also note that a substantial portion of a recently deposited GenBank sequence (accession no. Y787402) from this group is identical to part of our sequence of the KHV major capsid protein gene.

As well as possessing several genes whose closest relatives are found in lower-vertebrate herpesviruses, KHV resembles herpesviruses and other large DNA viruses in apparently having acquired certain genes by horizontal transfer. They include genes encoding thymidine kinase (H. Bercovier, R. Hedrick, Y. Fishman and R. Nahary, unpublished data; GenBank accession no. AJ535112) and the large subunit of ribonucleotide reductase (Hutoran et al., 2005). Both of these are related closely to cellular genes and thus to the

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**Fig. 2.** Amino acid sequence alignment for part of a previously unidentified ORF in a KHV sequence (denoted here as CNGV; nt 218–631 in GenBank accession no. AY208988; Ronen et al., 2003), homologues in CyHV-1 (AY939866) and RaHV-1 (residues 503–635; AAD12270; Davison et al., 1999) and IcHV-1 ORF56 (residues 455–587; AAA88159). The alignment was computed by using CLUSTAL W (Thompson et al., 1994). Conserved amino acid residues are shown as black blocks.
same genes captured by other viruses. Marginal similarities indicate that KHV also possesses a distant relative of an ancillary membrane-protein gene found in some members of the family Poxviridae (Hutoran et al., 2005). Horizontal gene transfer between viruses is well-documented, even for IchV-1, which possesses a gene derived from a bacteriophage (Davison, 2002).

Our study has shown that four complete KHV genes of substantial size are conserved in lower-vertebrate herpesviruses. Two of these encode structural proteins that contribute to the capsid architecture conserved among all herpesviruses, and are detectably similar in sequence only among lower-vertebrate herpesviruses. The other two encode proteins involved in DNA replication and, even though they have relatives in many other organisms, are related most closely to counterparts in fish and frog herpesviruses. The comparisons demonstrate that KHV is related closely to, but distinguishable from, CyHV-1 and CyHV-2 at each locus examined. The three cyprinid viruses are also different from each other in their biological properties. These factors substantiate the case for continued inclusion of CyHV-1 and CyHV-2 in the family Herpesviridae and prompt us to propose that KHV should join them in this family as Cyprinid herpesvirus 3 (CyHV-3).

Acknowledgements

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References


