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Molecular Characterization, Sequence Analysis, and Taxonomic Position

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Within the past decade, iridoviruses have been identified as the causative agents of systemic disease in a variety of commercially and recreationally important fish. Here we examine nine iridoviruses from fish, reptiles, and amphibians and demonstrate that all isolates were more similar to frog virus 3, the type species of the genus *Ranavirus,* than to lymphocystis disease virus, the type species of the genus *Lymphocystivirus.* Comparison of viral protein synthesis profiles, restriction endonuclease digestion patterns, and the amino acid sequence of the major capsid protein indicated that iridoviruses isolated from the same geographic region were similar, if not identical, whereas viruses from different areas were distinct. Moreover, using primers complementary to the conserved major capsid protein, we found that both PCR and RT-PCR successfully amplified virus-specific nucleic acid from all nine isolates. These studies demonstrate that the piscine iridoviruses examined here were members of the genus *Ranavirus,* and suggest that surveys of pathogenic ''fish viruses'' may need to include neighboring amphibian and reptilian populations. In addition, the results indicate that PCR readily identified vertebrate iridoviruses and suggest that PCR will be useful in the diagnosis of fish disease.

INTRODUCTION

The family *Iridoviridae* consists of four genera of DNA-containing viruses infecting both invertebrates (*Iridovirus* and *Chloriridovirus*) and cold-blooded vertebrates (*Ranavirus* and *Lymphocystivirus*) (Willis, 1990). Iridoviruses are large (diameter 120–300 nm) icosahedral viruses (reviewed in Willis *et al.,* 1984a, Williams, 1996) whose formation takes place in the cytoplasm within distinct viral assembly sites (Darlington *et al.,* 1966). The viral genome is a single molecule of linear double-stranded DNA 170–200 kb in size (Goorha, 1995). In all iridoviruses, the genome is both circularly permuted and terminally redundant (Goorha and Murti, 1982). However, in contrast to invertebrate iridoviruses, vertebrate iridoviruses are characterized by a genome in which ~20% of cytosine residues are methylated (Willis and Granoff, 1980). Methylation, catalyzed by a distinct virus-coded methyltransferase, appears to be important for DNA encapsidation and may play a role in protecting viral DNA from endonucleolytic attack (Goorha *et al.,* 1984).

Although identification of a newly isolated virus as an iridovirus is relatively straightforward based on characteristic virion morphology, determining the appropriate genus or whether viruses isolated from different hosts are members of the same or different virus species is less clear-cut. Recent work with invertebrate iridoviruses indicates that some viruses infect multiple insect species (Williams and Cory, 1994). Similar observations have also been made with vertebrate iridoviruses. For example, lymphocystis disease virus type 1 (LCDV-1) infects a wide variety of marine fish (Wolf, 1988), and several newly isolated iridoviruses infect multiple fish species (Hetrick and Hedrick, 1993). Surprisingly, these latter viruses appear to be members of the genus *Ranavirus,* which originally was thought to include only viruses infecting frogs and other amphibians (Hedrick *et al.,* 1992b). Moreover, unlike LCDV, newly isolated fish viruses cause systemic disease in infected animals and are associated with high morbidity and mortality (Ahne *et al.,* 1989; Hedrick and McDowell, 1995; Moody and Owens, 1994; Pozet *et al.,* 1992; Eaton *et al.,* 1991; Inouye *et al.,* 1992; Hedrick *et al.,* 1992b; Langdon *et al.,* 1986, 1988).

To clarify the taxonomic position of newly isolated piscine iridoviruses and to develop tools and criteria for their identification and classification, we have undertaken a comparative study of 6 newly isolated fish iridoviruses along with 2 reptilian viruses, a virus isolated from *Rana aurora,* and the type species frog virus 3 (FV3). Although preliminary studies have been performed with some of these viruses, this is the first attempt at a side-by-side comparison of 10 vertebrate iridoviruses using a battery of molecular approaches. In addition to electrophoretic analysis of proteins from virus-infected cells and restriction endonuclease (REN) analysis of viral DNA, a series of PCR primers were synthesized based on the conserved major capsid protein gene and used to amplify

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TABLE 1

Iridovirus Isolates Tested in This Study

Virus[a]	Host species	Geographic region	Group[b]
ESV[1]	Sheatfish (*Siluris glanis*)	Europe	I
ECV[2]	Catfish (*Ictalurus melas*)	Europe	I
RFPV[3]	Redfin perch (*Perca fluviatilis*)	Australia	II
RTV[3]	Rainbow trout (*Oncorhynchus mykiss*)	Australia	II
DFV[4]	Doctor fish (*Labroides dimidatus*)	S.E. Asia	III
GV6[4]	Guppy (*Poecilia reticulata*)	S.E. Asia	III
TV3[5]	Box turtle (*Terrapene c. carolina*)	N. America	IV
TV5[5]	Tortoise (*Testudo horsfieldi*)	N. America	IV
276[5]	Tadpole (*Rana aurora*)	N. America	IV
FV3[6]	Frog (*Rana pipiens*)	N. America	IV

[a] The viruses used in this study are identified by trivial name (e.g. European sheatfish virus [ESV]), isolate number, and a key reference. The viruses designated RFPV and RTV are strains (isolated from different fish species) of epizootic hematopoietic necrosis virus (EHNV).

[1] Isolate 62.90; Ahne *et al.,* 1989.

[2] Isolate 59.90; Pozet *et al.,* 1992.

[3] Isolate 13.91 (RFPV) and 12.91 (RTV); Eaton *et al.,* 1991.

[4] Isolate F93-20#16 (DFV) and F93-20#6 (GV6); Hedrick and McDowell, 1995.

[5] Isolates CP4-4562 (TV5), CP4-4398B (276), and CP4-4234 (TV3); F. M. Hetrick and D. E. Green, Maryland Department of Agriculture.

[6] Granoff *et al.,* 1966.

[b] Group designations are based on polypeptide profiles, REN patterns, and nucleotide/amino acid sequence data.

viral genomic DNA. Subsequently, the amplified fragments were cloned, the nucleotide sequence of the 5′-most 300 nucleotides of the major capsid gene was determined, and this information was used to construct a phylogenetic tree. The results indicate that all 9 isolates were more closely related to the ranavirus FV3 than to the lymphocystivirus LCDV-1. Furthermore, they confirmed and extended earlier serological and biochemical studies (Hedrick *et al.,* 1992b; Eaton *et al.,* 1991) suggesting that some isolates from fish were similar to FV3. Finally, the ability to amplify viral DNA from all 9 isolates suggests that primers corresponding to conserved regions within the major capsid protein may be the key to developing PCR assays for iridovirus identification.

MATERIALS AND METHODS

Cells and virus

Iridoviruses were grown and assayed in fathead minnow (FHM) cells at 28° in Eagle's minimum essential medium supplemented with 5% fetal calf serum. FHM cells were chosen over CCO (channel catfish ovary) cells because the latter, although permissive for piscine iridovirus infection, could not be used to determine virus yields by plaque assay. Iridovirus isolates from sheatfish (ESV), catfish (ECV), redfin perch (RFPV), rainbow trout (RTV), doctor fish (DFV), and guppy (GV6) were provided by R. P. Hedrick and T. S. McDowell; isolates from tortoise (TV5), turtle (TV3), and tadpole (276) were provided by F. M. Hetrick and D. E. Green (Maryland Department of Agriculture, College Park, MD). Information concerning the isolates used in this study is summarized in Table 1.

Protein synthesis in virus-infected cells

Monolayers of FHM cells grown on 35-mm tissue culture dishes were infected with iridoviruses at multiplicities of infection (m.o.i.) ranging from 8 to 60 PFU/cell. Replicate cultures were labeled with methionine-free MEM containing 25 μCi/ml [^{35}S]methionine (Amersham) from 24 to 25 hr postinfection. Radiolabeled cells were lysed in 300 μl of sample buffer (125 m*M* Tris [pH 6.8], 2% SDS, 10% glycerol, 0.02% 2-mercaptoethanol, and 0.01% bromophenol blue) and samples boiled for 3–5 min. Radiolabeled proteins were separated on 10% SDS–polyacrylamide gels (Laemmli, 1970) and visualized by autoradiography (Chinchar and Granoff, 1984).

Restriction endonuclease analysis

Concentrated virus stocks or lysates from infected cells were digested with proteinase K in the presence of 0.5% SDS for 4 hr at 37° (Goorha *et al.,* 1984). Purified virions were digested with 2 mg/ml proteinase K, whereas virus-infected cell lysates were incubated with 150 μg/ml proteinase K. In the latter case, viral DNA was radiolabeled by incubating iridovirus-infected FHM cells in media containing 20 μCi/ml [*methyl*-^{3}H]thymidine from 4 to 6 hr postinfection. Following protease treatment, viral DNA was extracted with phenol/chloroform and digested with the restriction enzymes *Hin*dIII, *Xba*I, *Hpa*II, and *Msp*I according to the manufacturer's directions. Restricted DNA was analyzed by electrophoresis on 1% agarose gels. Unlabeled DNA was visualized by ethidium bromide staining, whereas radiolabeled fragments were detected by fluorography.

TABLE 2

Primers Used for PCR and RT-PCR Analysis

Primer	Gene product	Nucleotide sequence[a] [5′ to 3′]	Amino acid position[b]
1	MCP[c]	ATGTCTTCTGTAGCAGGTTCA	1–7
2	MCP	TTANAAGATNGGRAANCCCAT	463–458
3	MCP	CCACCTRATNGTNCCRTT	105–100
4	MCP	GACTTGGCCACTTATGAC	15–20
5	MCP	GTCTCTGGAGAAGAAGAA	191–186
6	MCP	GGTGCATGAGATTTTGGTCC	111–106
7	18K[d]	GAATTCCGGGACAATCGCCTTCACTT	NA[e]
8	18K	ATCGATCTCTTTATATTACCGAGAGG	NA

[a] Nucleotide sequence of primers used in this study. The underlined nucleotides in primers 7 and 8 are the recognition sites for *Eco*RI and *Cla*I, respectively.

[b] The position of the indicated FV3 protein to which the cognate oligonucleotide primer was generated.

[c] The FV3 MCP (Mao *et al.,* 1996).

[d] The FV3 18-kDa early protein (Willis *et al.,* 1984).

[e] NA, not applicable. The primer binds within the 5′ (7) and 3′ (8) flanking regions of the 18K gene.

PCR primers and amplification of viral DNA

Oligonucleotides (DNA International) corresponding to different regions of the FV3 major capsid protein gene (Table 2) were used as primers in polymerase chain reactions (Mao *et al.,* 1996). PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

DNA sequence analysis

Following excision from agarose gels, PCR products were purified and cloned into the vector pCRII using the TA cloning method (InVitrogen) and both DNA strands were sequenced (Sanger *et al.,* 1977). A multiple alignment of the deduced amino acid sequences of the nine novel isolates along with FV3, LCDV, and two invertebrate iridoviruses was generated using the program MEGALIGN (DNASTAR, Madison, WI). The parameters used to generate the multiple alignment and the subsequent phylogenetic tree were gap penalty 10; gap length penalty 10, K-tuple 1, window 5, and PAM 250. Based on the alignment generated by MEGALIGN, a phylogenetic tree was constructed using the CLUSTAL V algorithm within DNASTAR. The final phylogeny was generated by applying the neighbor-joining method of Saitou and Nei (1987) to the distance and alignment data.

RT-PCR amplification from iridovirus-infected cells

FHM cells were mock and virus infected (FV3, DFV, or ESV) and 24 hr later total RNA was isolated (Chomcyznski and Sacchi, 1987). cDNA synthesis was performed using primers complementary to conserved regions within the FV3 major capsid protein (MCP) transcript (Table 2: primers 5 and 6). The reverse transcription reaction (25 μl total) contained 3–5 μg of RNA, 3 m*M* $MgCl_2$, 3.5 mmol of each dNTP, 50 m*M* Tris–HCl, pH 8.3, 75 m*M* KCl, 10 m*M* DTT, 1 μmol primer, 40 units of RNasin (Promega), and 200 units of Moloney murine leukemia virus reverse transcriptase (Warr *et al.,* 1992). Samples were incubated for 1 hr at 37° after which 1 μl was used directly as template in a PCR reaction.

RESULTS

Polypeptide synthesis in virus-infected FHM cells

To determine whether the proteins synthesized by newly isolated iridoviruses differed from each other and from FV3, replicate FHM cultures were infected with each isolate and virus-specific protein synthesis was analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). As with FV3, infection with the novel iridoviruses resulted in a marked inhibition of host-cell protein synthesis making it possible to detect virus-specific (or virus-induced) proteins without labeling cellular polypeptides. As shown in Fig. 1, all isolates possessed a prominent band at about 50 kDa which is presumed to be the MCP. Although the polypeptide profiles of newly isolated viruses were similar, they could be ordered into four groups based on the mobility of several signature proteins (Table 1)—ESV and ECV (group I), RTV and RFPV (group II), GV6 and DFV (group III), and FV3, TV3, TV5, and 276 (group IV). Taken together, our results confirmed earlier observations that novel iridoviruses resembled FV3 in their overall viral protein profile (Hedrick *et al.,* 1992b) and indicated that these viruses could be differentiated based on the mobility of specific proteins.

REN digestion patterns

To determine the DNA sequence relatedness of amphibian, piscine, and reptilian iridoviruses, REN analysis was performed using *Hin*dIII and *Xba*I. A representative REN profile is shown in Fig. 2a and depicts three distinct patterns defined by ECV/ESV, DFV/GV6, and FV3. It should be noted that the ECV and ESV profiles, although similar, differed in the mobility of one or more high-molecular-weight fragments. Additional work showed that the profiles obtained with RTV and RFPV were identical to each other and distinct from the other three patterns (data not shown). Likewise, REN profiles for TV3, TV5, and 276 were identical to each other, but differed slightly from FV3 and markedly from the other iridoviruses. As shown in Fig. 2b, *Hin*dIII digests of TV3, TV5, and 276 were identical to FV3 except for a single low-molecular-weight fragment, whereas *Xba*I digests showed marked differences between these 3 isolates and FV3. Taken together REN analysis showed that the 10 viruses could be ordered into four groups which were the same as

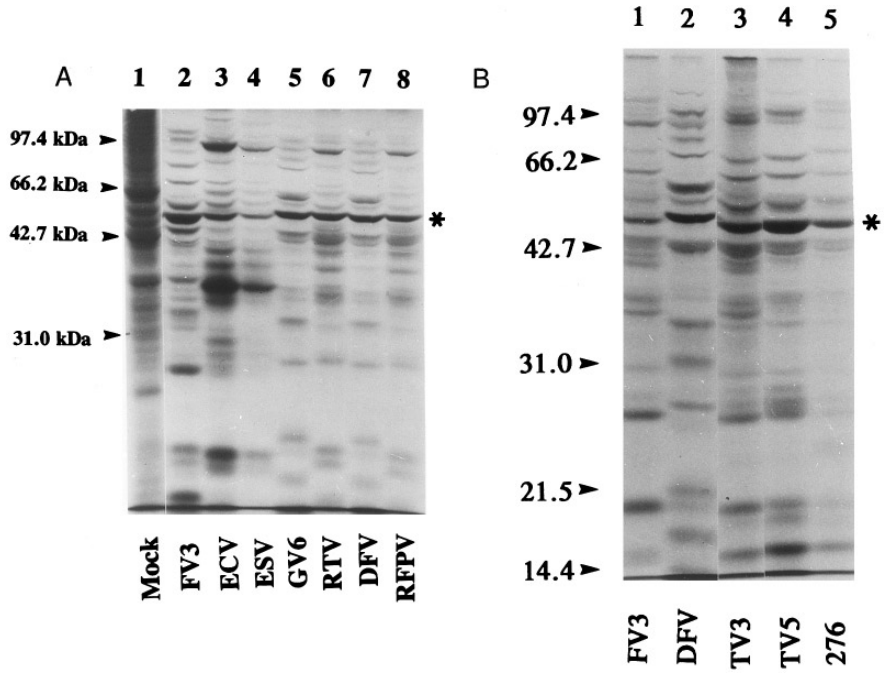

FIG. 1. Protein synthesis in iridovirus-infected cells. FHM cells were mock infected or infected with the indicated iridoviruses at multiplicities of infection ranging from 8 to 20 PFU/cell for the six piscine, two reptilian, and amphibian isolate 276, and at a m.o.i. of 60 PFU/cell for FV3. Replicate cultures were incubated at 28° and radiolabeled in methionine-deficient growth medium containing 25 μCi/ml [^{35}S]methionine from 24 to 25 hr postinfection. Radiolabeled proteins were separated by electrophoresis on 10% SDS–polyacrylamide gels and visualized by autoradiography. (A) Protein synthesis in cells infected with FV3 and six piscine iridovirus isolates. (B) Protein synthesis in cells infected with FV3, DFV, TV3, TV5, and 276. The MWs of marker proteins are indicated to the left. The asterisk indicates the position of the major capsid protein.

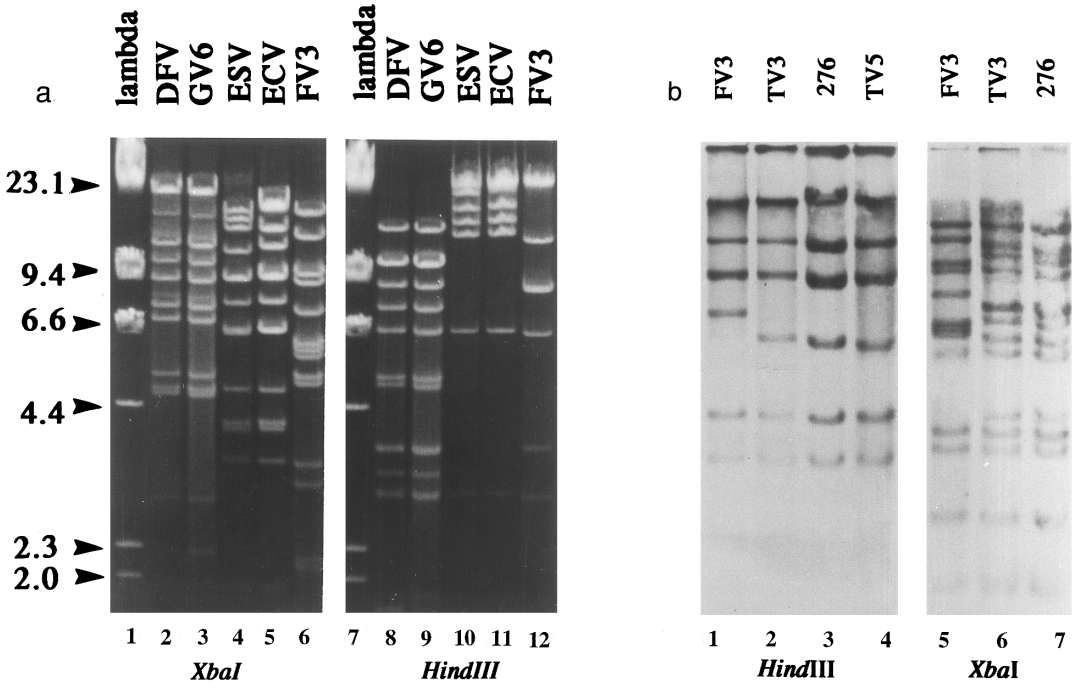

FIG. 2. Restriction endonuclease digestion of iridovirus genomic DNA. (a) Genomic DNA was isolated from purified stocks of the indicated iridoviruses and digested with either *Xba*I (lanes 2–6) or *Hin*dIII (lanes 8–12). Digested DNA was separated on a 1% agarose gel and visualized by staining with ethidium bromide. A *Hin*dIII digest of λ DNA served as a size marker and is shown in lanes 1 and 7; sizes (in kb) of the marker fragments are shown to the left. (b) Viral DNA, radiolabeled *in vivo* from 4 to 6 hr postinfection in medium containing 20 μCi/ml [*methyl*-^{3}H]thymidine, was isolated as described under Materials and Methods and digested with *Hin*dIII (lanes 1–4) or *Xba*I (lanes 5–7). Restriction fragments were separated by electrophoresis on 1% agarose gels and viral DNA was visualized by fluorography.

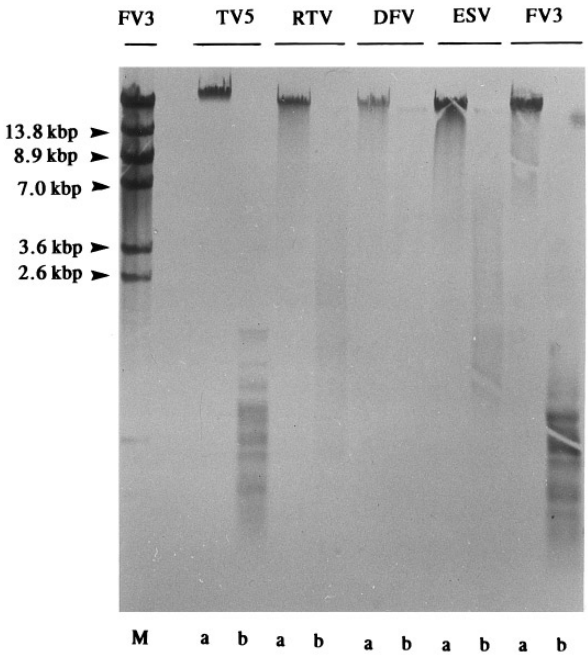

FIG. 3. Digestion of iridovirus DNA with methylation-sensitive endonucleases. FHM cells were infected with the indicated iridoviruses and at 4 hr postinfection viral DNA was selectively labeled with [*methyl*-^{3}H]thymidine for 2 hr. Radiolabeled viral DNA was isolated from infected cultures and digested with the isoschizomers *Hpa*II (which is unable to cleave methylated DNA) and *Msp*I (which cleaves methylated DNA). Digested DNA was separated by electrophoresis on 1% agarose gels and viral DNA fragments were visualized by fluorography. Lane M, *Hin*dIII digest of FV3 DNA as a size marker; lane a, DNA digested with *Hpa*II; lane b, DNA digested with *Msp*I.

those identified by SDS–PAGE analysis of viral protein synthesis.

Methylation of viral DNA

A characteristic feature of the genomic DNA of vertebrate iridoviruses such as FV3 and LCDV is the high level of methylated cytosine (Willis and Granoff, 1980). Among vertebrate iridoviruses methylation is sequence specific and each cytosine within the sequence CpG is methylated. To determine whether novel fish iridoviruses also possess a methylated genome, we chose one representative virus from each group and digested purified viral DNA with the isoschizomers *Hpa*II and *Msp*I. Although both enzymes recognize the same target sequence (i.e., CC/GG), only *Msp*I can cleave the target sequence when the internal cytosine is methylated (Willis and Granoff, 1980). Following digestion and agarose gel electrophoresis we found that all viral DNAs tested were resistant to *Hpa*II cleavage but were extensively digested by *Msp*I, indicating that methylated cytosine was present in genomic DNA (Fig. 3). In contrast, unmethylated plasmid DNA was readily digested by *Hpa*II (data not shown).

PCR amplification and sequence analysis

Based on marked amino acid sequence identity within the iridovirus MCP gene (Mao *et al.,* 1996), we sought to determine whether primers complementary to the FV3 MCP gene, or regions conserved within the MCP gene of different iridoviruses, could be used to amplify genomic DNA from novel iridoviruses. Viral genomic DNA was isolated from concentrated virus stocks and used as template in a series of PCR reactions. Using primers 1 and 2 (Table 2), we were able to amplify full-length MCP cDNA from all six piscine iridovirus isolates, whereas primers 1 and 3 amplified a ~300 nucleotide fragment corresponding to the 5′ coding region from ECV, ESV, RTV, and RFPV (Fig. 4 and data not shown). Furthermore, another set of primers (4 and 5, Table 2), corresponding to two highly conserved regions within the MCP gene, were capable of amplifying a PCR product of about 0.5 kb from all isolates (data not shown).

To confirm that DNA amplified from all nine viruses was the authentic product of the MCP gene, ~300-bp fragments corresponding to the 5′ coding region were cloned and the deduced amino acid sequences compared by multiple alignment (Fig. 5). As shown in Fig. 5, there was marked sequence similarity within the amino-terminal region of the iridovirus MCP. Match ratios (Table 3) showed high levels of identity and similarity (i.e., conservative amino acid substitution) among all isolates tested with the exception of LCDV-1. This result indicated that newly isolated fish, amphibian, and reptilian viruses were more closely related to the ranavirus FV3 than to the lymphocystivirus LCDV-1. Although it is possible that some of the observed amino acid changes were the re-

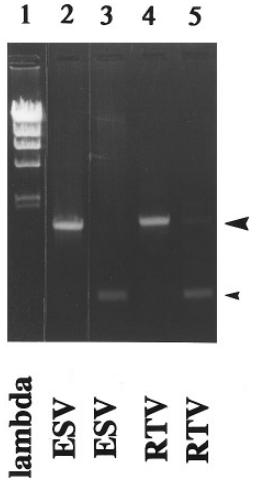

FIG. 4. PCR analysis of iridovirus DNA. Purified viral genomic DNA was used as template in standard PCR reactions using oligonucleotide primers capable of amplifying the complete coding region of the major capsid protein (primers 1 and 2, lanes 2 and 4) or a sequence of ~300 bp at the 5′ end of the gene (primers 1 and 3, lanes 3 and 5). PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining. *Hin*dIII-digested λ DNA was used a size marker (lane 1). PCR products generated using DNA isolated from ESV (lanes 2 and 3) and RTV (lanes 4 and 5) as template are shown. The band identified by the large arrowhead is 1392 bp in length, whereas that identified by the small arrowhead is 315 nucleotides long.

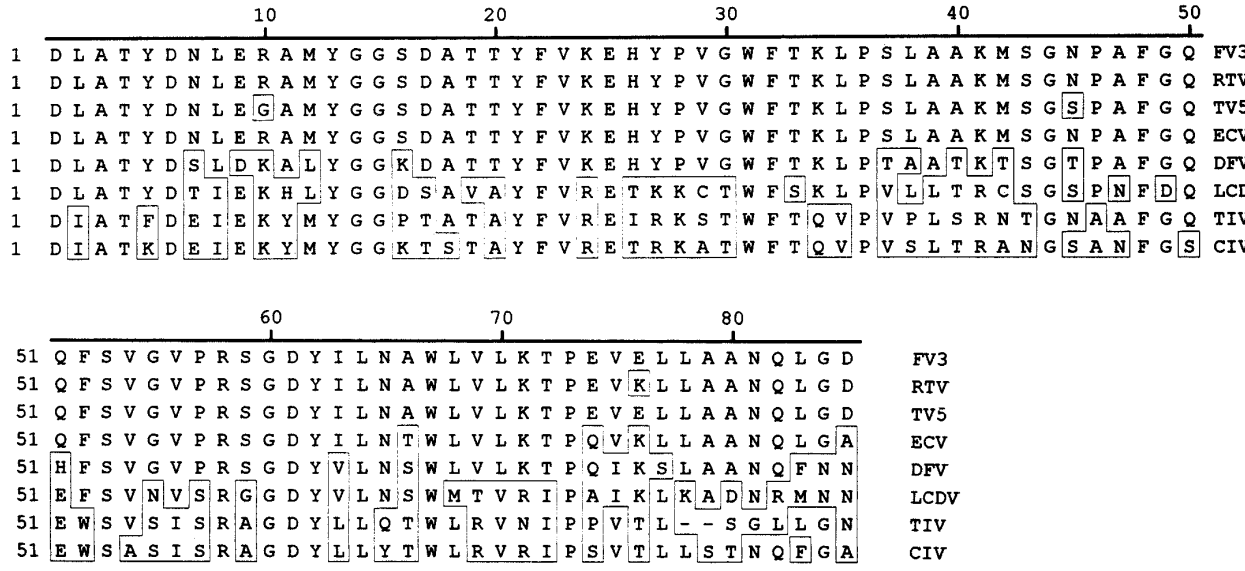

FIG. 5. Multiple alignment of iridovirus capsid sequences. DNA sequences from the nine novel isolates tested in this study were amplified by PCR and cloned into the vector pCRII. The nucleotide sequence of the 5′ end of the iridovirus major capsid protein gene was determined by the method of Sanger *et al.* (1977) and the amino acid sequence deduced. Amino acid sequences for the corresponding regions in FV3, LCDV, Chilo iridescent virus (CIV), and Tipula iridescent virus (TIV) were obtained from published work (cited in Mao *et al.,* 1996). Multiple alignment was performed using the program MEGALIGN (DNASTAR). Within the region depicted, the amino acid sequences for FV3, TV3, and 276 were identical, as were those for RTV and RFPV, ECV and ESV, and DFV and GV6. Amino acids that differed from the FV3 sequence are boxed. The sequence shown here begins at the 15th amino acid from the amino-terminus of the FV3 MCP and extends to amino acid 99. Nucleotide and amino acid sequences have been submitted to GenBank and assigned the following Accession Nos.: DFV, U82550; ECV, U82551; RTV, U82552; TV3, U82553; and TV5, U82554.

sult of nucleotide misincorporation by *Taq* polymerase, the low error rate observed in an earlier study (no misincorporation within the 1452-nucleotide MCP gene) suggests that the sequence determined here was authentic (Mao *et al.,* 1996).

TABLE 3

Amino Acid Identity and Similarity within the Major Capsid and 18K Proteins of Selected Iridoviruses

Target Gene	Virus(es)	% identity[a]	% similarity[b]
Major capsid	FV3, TV3, 276	100	100
Major capsid	RTV, RFPV	99	99
Major capsid	TV5	98	98
Major capsid	ECV, ESV	95	96
Major capsid	DFV, GV6	77	86
Major capsid	LCDV	48	62
18K	FV3	100	100
18K	ECV	92	94
18K	RTV	90	92

[a] % identity is the number of identical amino acids between FV3 and the test virus divided by the total number of amino acids in the region of interest times 100. The length of the compared sequence was 85 amino acids (i.e., amino acids 15–99 from the amino-terminus of the FV3 major capsid protein).

[b] % similarity is equal to the number of identical and conserved amino acids between FV3 and the test virus divided by the total number of amino acids in the region of interest times 100. For determination of conservative amino acid substitutions, equivalences were based on the PAM 250 matrix of Schwarz and Dayhoff (1978); i.e., R = K; I = L; I = V; M = L; F = Y; E = D; Q = E; Q = H; S = T.

To demonstrate that sequence identity among iridoviruses was not an artifact of the gene chosen for study, we used a similar approach to clone and sequence the 18-kDa early protein gene (18K) of ESV and RTV. Oligonucleotide primers complementary to the 5′ and 3′ flanking regions of the FV3 18K mRNA (Willis *et al.,* 1984b) were used to amplify cDNA from RTV and ESV genomic DNA. The resulting PCR products were cloned into pCRII and the sequence of the 18K gene was determined. Multiple alignment of the inferred amino acid sequence of the 18K proteins of FV3, ESV, and RTV showed them to be ~90% identical (Fig. 6 and Table 3). This result suggests that the identity detected by analysis of the MCP gene was most likely not an artifact of the gene chosen, but reflects the overall relatedness of the viral genomes. Taken together, these results clearly indicate that novel iridovirus isolates were more similar to FV3 than to LCDV.

To determine the evolutionary relationship of newly isolated iridoviruses to FV3 and LCDV, a phylogenetic tree was constructed based on the multiple alignment shown in Fig. 5. To generate a rooted tree, the NH-terminal region (amino acids 15–99) of the major capsid protein of the phycodnavirus PBCV-1 was used as an outgroup. The PBCV-1 MCP was chosen because it has limited sequence identity to the iridovirus capsid protein within its central and COOH-terminal regions (Mao *et al.,*

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FV3  (   1)  MRMIQAYLCDSVSGEPYTCKGDLCEIPFNRNFTIDLVNLSVSTEFQVKIT
ECV  (   1)  .S.........................S.................R..
RTV  (   1)  .S.........................S.................R..

FV3  (  51)  MTPHHDLGTFVVEPKNVFSIKRAVKGDAAFKVERAAGWLPDTPQVLTLFV
ECV  (  51)  ..............K.......A........A......L......S...
RTV  (  51)  ..............K.......A........A.............S...

FV3  ( 101)  YERLNPVEWHSECMYENLETDGGTVIVPGEATGQRFGTATEVPTMFLFKR
ECV  ( 101)  .K..H......G.L.D.......................A..AR.....
RTV  ( 101)  .K..H......G.L.........................A...R.....

FV3  ( 151)  MFVVKGV
ECV  ( 151)  .......
RTV  ( 151)  .......
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FIG. 6. Multiple alignment of the iridovirus 18-kDa early protein. The coding region of the 18-kDa early gene of ECV and RTV was amplified by PCR using primers 7 and 8 (Table 2) and cloned into pCRII. The nucleotide sequence was determined by the method of Sanger *et al.* (1977), and the deduced amino acid sequence is shown. Multiple alignment was performed using Align Plus (Scientific and Educational Software). Amino acids identical to the FV3 sequence are indicated by a dot (.). Nucleotide and amino acid sequence data have been submitted to GenBank and assigned the following Accession Nos.: RTV, U82631; ECV, U82632.

1996; Kutish *et al.,* 1996). The resulting tree (Fig. 7) shows that the 6 fish iridoviruses, the 2 reptilian viruses, and the virus isolated from *R. aurora* were more closely linked to FV3 than to LCDV. Furthermore, the data indicate that the 10 viruses that make up the ranavirus cluster are about as distantly related to LCDV-1 as they are to the invertebrate iridoviruses CIV and TIV.

RT-PCR analysis

Based on the sequence relatedness detected above, we set out to determine whether RT-PCR could detect iridovirus-specific RNA within infected cells. FHM cells were mock infected or infected with three representative viruses (FV3, DFV, and ESV) and RT-PCR was performed. A typical agarose gel of the amplified products is shown in Fig. 8. The presence of a unique PCR product in virus-infected, but not mock-infected cells, indicates that RT-PCR can be a rapid and reliable diagnostic substitute for electron microscopy and should facilitate the detection of novel iridoviruses from infected tissues.

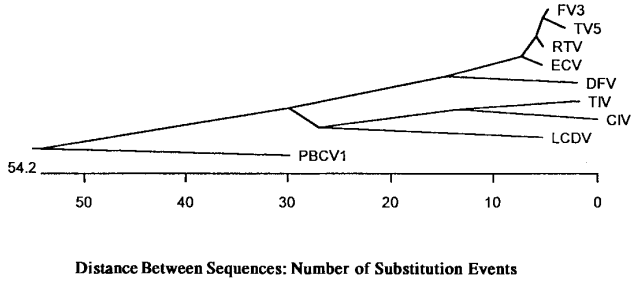

FIG. 7. Phylogenetic tree: iridovirus major capsid protein. A phylogenetic tree was constructed based on the multiple alignment shown in Fig. 5 using the CLUSTAL V algorithm within MEGALIGN (DNASTAR). The NH-terminal portion (amino acids 15–99) of the major capsid protein of the phycodnavirus PBCV-1, a protein with limited sequence identity to the FV3 MCP (Kutish *et al.,* 1996), was used as an outgroup. For clarity, only one virus is indicated at a terminal node even though two or more viruses may share sequence identity. Within this region, FV3, TV3, and 276 were identical, as were RTV and RFPV, ECV and ESV, and DFV and GV6. Branch length is proportional to the number of amino acid substitutions, which is indicated by the scale beneath the tree.

DISCUSSION

The identification of iridoviruses responsible for serious disease in commercially and recreationally important

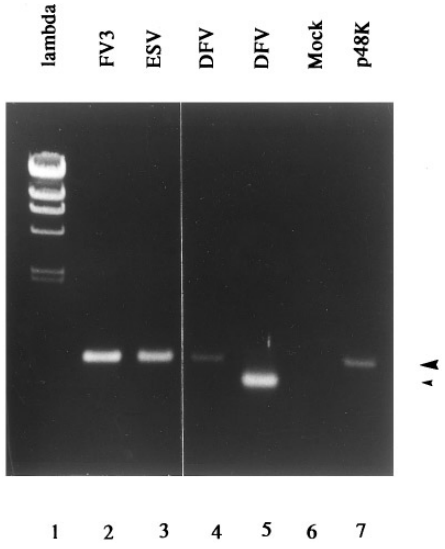

FIG. 8. RT-PCR analysis of iridovirus-infected cells. Total RNA from mock- and iridovirus-infected cells was isolated by the method of Chomcyznski and Sacchi (1987) and used as template in a reverse transcription-PCR reaction. Primer 5 was used in the reverse transcription reactions shown in lanes 2–4 and lane 6, whereas primer 6 was used in the reaction shown in lane 5. *Hin*dIII-digested λ DNA was used as a size marker (lane 1), and a plasmid bearing the complete FV3 MCP gene (p48K) was used as a positive PCR control (lane 7). The large arrowhead indicates a product of 531 bp; the small arrowhead a product of 271 bp.

fish has awakened interest in this little-studied virus family (Ahne *et al.,* 1989; Hedrick and McDowell, 1995; Moody and Owens, 1994; Pozet *et al.,* 1992; Eaton *et al.,* 1991; Hedrick *et al.,* 1992a; Langdon *et al.,* 1986, 1988; Inouye *et al.,* 1993). Until about 10 years ago only one iridovirus, LCDV, had ever been linked to fish disease (Wolf, 1988). LCDV infection induces hypertrophy of connective tissue cells and results in neoplastic-like growths on the skin, mesenteries, and peritoneum. LCDV-induced lesions are unsightly, but seldom cause death and eventually regress. In contrast to LCDV, recently isolated fish iridoviruses have been associated with serious disease among both native fish and those raised in aquaculture facilities. For example, in one aquaculture facility in Germany mortality of sheatfish following iridovirus infection reached 100% (Ahne *et al.,* 1989). Similar infections characterized by high morbidity and mortality were also seen in France (Pozet *et al.,* 1992), Australia (Langdon *et al.,* 1986, 1988; Eaton *et al.,* 1991), Japan (Tamai *et al.,* 1997; Oda *et al.,* 1993), and the United States (Hedrick *et al.,* 1992a).

To determine how these newly isolated fish iridoviruses are related to previously described iridovirus species, we compared six of these isolates, along with two novel reptilian isolates and one from *R. aurora,* to FV3, the type species of the genus *Ranavirus.* The results described above demonstrate that all nine isolates were more closely related to FV3 than to LCDV and confirmed earlier serological and biochemical data (Hedrick *et al.,* 1992b) suggesting that some fish isolates were similar to FV3. For example, protein profiles (Fig. 1), REN analysis, and amino acid sequence determinations (Fig. 5) suggested that viruses isolated from redfin perch (RFPV) and rainbow trout (RTV) were identical and represent different isolates of epizootic hematopoietic necrosis (EHNV). However, whether viruses isolated from catfish, sheatfish, guppy, and doctor fish are strains of EHNV, or distinct, but related, viral species remains to be determined. Although the ability of these last four isolates to cross-react in fluorescent antibody and immunoblot assays (Hedrick *et al.,* 1992b; Hedrick and McDowell, 1995) indicates that they share common antigens, the observation that they show unique protein (Fig. 1) and REN (Fig. 2) profiles and possess different amino acid sequences (Fig. 5 and 6) indicates that they are not identical. Interestingly, viruses isolated from four geographically distinct regions — i.e., North America, southeast Asia, Australia, and Europe — were distinguishable from each other, whereas viruses isolated from the same geographic region were similar if not identical.

The work described in this report is important to both fisheries management and virus taxonomy. The analyses described herein strengthen the observation that the genus *Ranavirus* contains viruses capable of infecting three different classes of cold-blooded vertebrates (fish, amphibians, and reptiles). However, it leaves open the question whether viruses infecting one taxonomic class (e.g., fish) infect members of different classes (e.g., amphibians or reptiles). Preliminary studies suggest that although some ranaviruses are limited to infection of a single class (Wolf *et al.,* 1968), others can infect members of different classes (Moody and Owens, 1994; Hedrick and McDowell, 1995). If the latter finding is common, it will have important implications for fish biologists and suggest that surveillance of aquatic environments for "fish viruses" should be extended to include amphibians and reptiles.

Although LCDV and FV3 are currently placed within separate genera within the family *Iridoviridae* (Goorha, 1995), amino acid sequence analysis (Fig. 5) suggests that vertebrate iridoviruses may be more appropriately classified as members of a single genus rather than as members of two distinct genera. It remains to be determined whether possession of a single key feature — a methylated genome — is sufficient grounds to join LCDV, FV3, and related vertebrate viruses into a single genus, or whether differences in virion size, GC content, host range, amino acid sequence, and spectrum of clinical illness warrant separation into distinct genera. Perhaps possession of a common gene set along with moderate (35–85%) sequence identity is more important in defining members of a genus than differences in biological properties (Ward, 1993). Additional sequencing studies involving other viral genes (e.g., the methyltransferase and viral DNA polymerase) will be necessary to determine the relatedness of vertebrate iridoviruses.

From a diagnostic view, the results described above suggest that PCR can be a powerful tool to identify iridovirus infections. Williams (1993) showed that covert infection of blackfly larvae by insect iridovirus 22 was readily detected by PCR using primers directed at the major capsid protein. Recently PCR analysis has been used to detect several vertebrate iridoviruses. Gould *et al.* (1995) demonstrated that primers targeted to an unknown open reading frame within the EHNV genome amplified DNA fragments from EHNV strains isolated from redfin perch and rainbow trout, as well as from the amphibian virus, Bohle iridovirus. Significantly, no PCR products were obtained when DNA from LCDV or diamond python erythrocytic iridovirus was used as template. Using the same primers as Gould *et al.,* Hedrick and McDowell (1995) reported that viral DNA from iridoviruses isolated from the guppy and the doctor fish could also be amplified. More recently, Tamai *et al.* (1997) and Oda *et al.* (1993) used PCR primers specific for the FV3 18-kDa early protein gene and the gene encoding FV3 ICR-489 to detect iridoviruses from red seabream, striped jack, and yellow tail. These studies suggest that PCR approaches can be successful in identifying closely related iridovirus isolates. As reported here, the use of primers specific for consensus regions within the highly conserved major

capsid protein gene may provide the best chance of identifying members of the vertebrate iridovirus family.

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