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Complete Genome Analysis of the Mandarin Fish Infectious Spleen and Kidney Necrosis Iridovirus

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The nucleotide sequence of the infectious spleen and kidney necrosis virus (ISKNV) genome was determined and found to comprise 111,362 bp with a G+C content of 54.78%. It contained 124 potential open reading frames (ORFs) with coding capacities ranging from 40 to 1208 amino acids. The analysis of the amino acid sequences deduced from the individual ORFs revealed that 35 of the 124 potential gene products of ISKNV show significant homology to functionally characterized proteins of other species. Some of the putative gene products of ISKNV showed significant homologies to proteins in the GenBank/EMBL/DDBJ databases including enzymes and structural proteins involved in virus replication, transcription, protein modification, and virus–host interaction. In addition, one major repeated sequence showing significant homology to the Red Sea bream iridovirus (RSIV) genome was identified. Based on the information obtained from biological properties (including histopathology, tissue tropisms, natural host range, and geographic distribution), physiochemical and physical properties, and genome analysis, we suggest that ISKNV, RSIV, sea bass iridovirus, grouper iridovirus, and African lampeye iridovirus may belong to a new genus of the *Iridoviridae* family and are tentatively referred to as cell hypertrophy iridoviruses.

Key Words: ISKNV; genome; sequence analysis; taxonomic position; phylogeny; cell hypertrophy iridoviruses.

INTRODUCTION

Infectious spleen and kidney necrosis virus (ISKNV) from mandarin fish, *Siniperca chuatsi* (Basilewsky), a member of the *Iridoviridae* family (He *et al.,* 1998, 2000; Deng *et al.,* 2000), is the causative agent of a disease causing high mandarin fish mortalities and severe damage to mandarin fish cultures in China. Many outbreaks of ISKNV were reported between 1994 and 1999 (Wu *et al.,* 1997; He *et al.,* 1998; Zhang and Li, 1999; Fang *et al.,* 2000). The histopathology of the viral infection is characterized by cell hypertrophy in the spleen, kidney, cranial connective tissue, and endocardium. Infected cells are enlarged with large numbers of icosahedral viral particles (150 nm) present in the cytoplasm (Weng *et al.,* 1998). The virion core contains a single linear dsDNA molecule of >110 kb. Like several other vertebrate iridoviruses, such as frog virus 3 (FV3) (the type species of the *Ranavirus* genus) and lymphocystis disease virus 1 (LCDV-1) (the type species of the *Lymphocystivirus* genus) (Willis and Granoff, 1980; Wanger *et al.,* 1985), the ISKNV genome is highly methylated at cytosines in the CpG and circularly permuted. The characterizations of the ISKNV genome by molecular cloning and physical mapping have been reported (Deng *et al.,* 2001a,b).

Iridoviruses are icosahedral cytoplasm DNA viruses that have been isolated from insect invertebrate and vertebrate host species. The *Iridoviridae* family is subdivided into four genera including *Iridovirus, Chloriridovirus, Ranavirus,* and *Lymphocystivirus* (Regenmortel *et al.,* 1999). Iridoviruses have many interesting biological and molecular biological aspects; e.g., the genome of the viruses was found to be circularly permuted and terminally redundant, which is a unique genome feature among eukaryotic viruses (Delius *et al.,* 1984; Goorha and Murti, 1982; Darai *et al.,* 1983, 1985). To date, the complete genome sequence of only one vertebrate iridovirus (LCDV-1) has been determined. The genome of LCDV-1 is 102,653 bp and potentially encodes 195 proteins (Tidona and Darai, 1997). In the present paper, we describe the organization of the ISKNV genome and report the complete DNA sequence and compare it to sequence data from genes of other species. From the results of sequence analysis and information about the virus' biological properties, we also discussed the taxonomic position of ISKNV.

RESULTS AND DISCUSSION

Sequencing of the ISKNV genome

After sequencing was completed, the actual *Bam*HI and *Hin*dIII restriction enzyme maps of the ISKNV genome identified by the Omiga 2.0 program were consis-

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tent with the restriction enzyme maps predicted by enzyme digestion and hybridization. However, the two *Kpn*I restriction enzyme maps differed slightly as some ISKNV *Kpn*I short bands were lost (Deng *et al.,* 2001b). The primer walking method revealed that the ISKNV genome was end to end, indicating a circular construction. This result was consistent with the circular ISKNV physical map generated by enzyme digestion and hybridization methods (Deng *et al.,* 2001b). Like other iridoviruses, it appears that the ISKNV genome is circularly permuted.

The ISKNV genome is 111,362 bp, which is only about 9 kb larger than that of LCDV-1 (102,653 bp) (Tidona and Darai, 1997). The G+C ratio of the ISKNV genome is 54.78% and is similar to that of FV3 (53%) and *Chloriridovirus* VeroBeach (IIV3) (54%), but significantly higher than that of LCDV-1 (29.07%) and the small invertebrate iridoviruses of the *Iridovirus* genus (29–32%) (Williams, 1996).

Repeated sequences

A complex cluster of multiple tandem, overlapping, and interdigitated direct repeated sequences (496 bp) of unknown function was identified at positions 23,273 to 23,768 in the ISKNV genome. Sequence comparisons of this highly direct repetitive region between ISKNV and Red Sea bream iridovirus (RSIV) revealed that the identity is 25.6% and the similarity is 64.7%. Repetitive sequences have been identified in the genome of numerous viruses including poxviruses (Wittek and Moss, 1980), herpesviruses (Wadsworth *et al.,* 1975), adenoviruses (Arrand and Roberts, 1979), retroviruses (Shoemaker *et al.,* 1980), and baculoviruses (Hayakawa *et al.,* 1999). These sequences can be associated with important regulatory functions during viral replication. In both *Autographa californica* nucleopolyhedrovirus and *Orgyia pseudotsugata* nucleopolyhedrovirus, repetitive sequences have been shown to function as transactivators of RNA polymerase II-mediated transcription and as origins of DNA replication in transient replication assays (Hayakawa *et al.,* 1999). Extensive repeated sequences have been found in certain regions of iridoviruses FV3, LCDV-1, Chilo iridescent virus (CIV; the type species of the *Iridovirus* genus), RSIV, and several invertebrate iridoviruses (Williams, 1996). These repeated sequences are distinct from the complementary terminal sequences that arise from the terminal redundancy of iridovirus genomes. The repetitive sequences of LCDV-1 were less extensive than CIV and RSIV (Schnitzler *et al.,* 1987), while ISKNV, RSIV, and CIV (Fischer *et al.,* 1988) have been shown to be unusually complex clusters of multiple tandem, overlapping, and interdigitated repetitive elements, in which the direct repeat sequences dominated. The direct repeated sequences (GGCCTG) within the 496-bp repeat region of the ISKNV genome are identical to the counterpart of RSIV. In contrast no significant DNA sequence homologies were detected when the DNA sequences of the ISKNV were compared to those DNA sequences of a repetitive element in the genome of LCDV-1 and CIV.

Coding capacity of the ISKNV genome

Computer-assisted analysis of the complete DNA sequence of ISKNV identified 124 potential open reading frames (ORFs) with coding capacities for polypeptides ranging from 40 to 1208 amino acids (Table 1 and Fig. 1). The codon usage of ISKNV genes differed significantly from that of LCDV-1 (Tidona and Darai, 1997). For ISKNV, CGC (Arg), AAC (Asn), GAC (Asp), CAG (Gln), GGC (Gly), CAC (His), CTG (Leu), AAG (Lys), AGC (Ser), TAC (Tyr), and GTG (Val) are used more frequently, while for LCDV-1, AGA (Arg), AAT (Asn), GAT (Asp), CAA (Gln), GGT (Gly), CAT (His), TTA (Leu), AAA (Lys), TCT (Ser), TAT (Tyr), and GTT (Val) are used more frequently. The classical or slightly modified canonical promoter and termination signal were found upstream and downstream of the start and termination codons of the majority of the individual ORFs (Table 1).

Sequence similarities to proteins in databases

Analysis of putative proteins from the individual ORFs by GenBank/EMBL/DDBJ database search revealed that 35 of the 124 potential gene products of ISKNV show significant similarity or homology to functionally characterized proteins of other species. These include enzymes and structural proteins involved in virus replication, transcription, protein modification, and virus–host interaction.

Several putative gene products of ISKNV contain highly conserved domains and active site motifs of enzymes involved in protein processing and modification. For example, ORF7L contains an asparaginase/glutaminase active site signature 1, ORF13R contains a serine/threonine protein kinase domain, and ORF64L contains a tyrosine-specific protein phosphatase active site.

Proteins involved in DNA replication, modification, and processing

The deduced amino acid sequence of ISKNV ORF19R shows significant homology to DNA polymerases of cellular and viral origin, belonging to the family B DNA polymerases. On the basis of structural, mutational, and biochemical studies, it has been proposed that DNA polymerases have separate $3' \rightarrow 5'$ exonuclease and polymerization motifs located at the N-terminal and C-terminal portions of the protein (Joyce, 1991; Rodriguez *et al.,* 1993). ISKNV ORF19R codes for a protein of 948 amino acids which contains all the $3' \rightarrow 5'$ exonuclease and polymerization regions which are conserved in DNA-dependent DNA polymerases, in the same spatial arrangement on the polypeptide. The amino acid sequence of the conserved regions in the ISKNV protein confirms

TABLE 1

Potential Open Reading Frames of the ISKNV Genome

ORF	Position (length, aa)	M_r (kDa)	pI	Promoter	Conserved domain or signature[a]	Prosite/CD accession no.[b]	Best match: BlastP score[c]	Best match: % identity[d]	Best match: Accession no.[e]	Best match: Species	Predicted function/similarity[f]	Reference(s)
001L	1,270–137 (378)	40.66	7.23									
002R	1,394–2,041 (216)	24.62	9.72	CCAAT								
003L	2,634–2,080 (185)	20.39	5.40									
004L	2,890–2,684 (69)	7.61	8.87									
005L	3,648–2,896 (251)	27.55	7.68				93.2	35	NP_078678.1	LCDV-1	ORF64	
006L	5,155–3,797 (453)	49.61	6.25	CCAAT			444	47	L63545	LCDV-1	Major capsid protein	Schnitzler and Darai (1993)
007L	6,631–5,177 (485)	51.51	8.50		Asparaginase/glutaminase active site signature 1	PS00144	172	30 (394)	AF368229	Regina ranavirus	LCDV ORF 20-like protein	
008R	6,669–8,243 (525)	58.05	6.06	TATA								
009R	8,342–8,500 (53)	5.90	9.67									
010L	9,054–8,665 (130)	14.58	9.03									
011L	9,311–9,054 (86)	9.50	7.98									
012R	9,330–9,659 (110)	12.94	8.55		Zinc finger, C3HC4 type (RING finger), signature	PS00518	76.6	32	AE003519	*Drosophila melanogaster*	CG12477 gene product (zinc finger protein)	Tidona and Darai (1997); Schnitzler *et al.* (1994b)
013R	9,669–11,051 (461)	50.87	6.88	TATA	Serine/threonine protein kinases	smart00220						
014R	11,309–12,265 (319)	34.81	5.66									
015R	12,278–13,066 (263)	29.89	9.58	TTGACA								
016L	13,716–13,132 (195)	21.12	4.03									
017L	14,095–13,721 (125)	14.11	9.69	TATAAT								
018L	14,513–14,325 (63)	7.42	12.83									
019R	14,579–17,422 (948)	107.07	8.16	TATA	DNA polymerase family B signature	PS00116	1872	96	AB007366	RSIV	DNA polymerase	Kurita *et al.* (1998); Rodriguez *et al.* (1993)
020L	17,642–17,457 (62)	6.24	4.26	CCAAT								
021L	17,900–17,781 (40)	4.70	8.83	AGGAGG								
022L	19,489–17,993 (499)	54.37	6.85	TATA	EF-hand calcium-binding domain	PS00018						
023R	19,562–22,129 (856)	91.95	4.50	TATA	EGF-like domain signature 1, 2; Laminin-type EGF-like domain signature	PS00022 PS01186 PS01248	215	42 (309)	J03202	*Homo sapiens*	Laminin B2 precursor	
024R	22,300–23,235 (312)	35.60	5.01	TATA	Ribonucleotide reductase small subunit signature	PS00368	596	96	AB018418	RSIV	Ribonucleotide reductase small subunit	Oshima *et al.* (1996); Li *et al.* (1997)
025R	23,354–23,776 (141)	14.97	12.73									
026L	24,145–23,825 (107)	12.35	5.92	TATA								
027L	25,063–24,170 (298)	33.45	8.44		XPG I-region, XPG N-region, 5′-3′ exonuclease	smart00484 smart00485 smart00475	554	91	AB018418	RSIV	DNA repair protein RAD2	Lee and Wilson (1999); Tidona and Darai (1997)
028L	28,559–25,083 (1159)	128.50	7.31		RNA polymerase α subunit, RNA polymerase I subunit A N-terminus	pfam00623 smart00663	2264	95	AB018418	RSIV	Largest subunit of the DNA-dependent RNA polymerase	Moss (1990); Yanez *et al.* (1993a); Schnitzler *et al.* (1994a)
029L	28,814–28,596 (73)	8.34	8.50	TATA	C2C2 zinc finger; nucleic-acid-binding motif in transcriptional elongation factor TFIIS and RNA polymerases	smart00440	43.1 43.1	30 30	A55263 L27650	*Thermococcus celer*	Transcription-associated protein, DNA-directed RNA polymerase subunit M	Moss (1990); Yanez *et al.* (1993a); Schnitzler *et al.* (1994a)
030R	28,886–29,044 (53)	5.97	8.83	TATA								
031R	29,110–29,349 (80)	8.70	8.36	TATA								
032R	29,447–30,058 (204)	23.23	6.83	CCAAT	ATP/GTP-binding site motif A (P-loop)	PS00017	67.4	30 (165)	AF198100	Fowlpox virus	Deoxycytidine kinase	
033L	31,079–30,141 (313)	36.52	9.60									
034R	31,144–34,275 (1044)	116.67	8.44		RNA polymerase β subunit	pfam00562	761	42	NP_078633.1	LCDV-1	DNA-directed RNA polymerase II second largest chain	Moss (1990); Yanez *et al.* (1993a); Schnitzler *et al.* (1994a)

035L	35,508–34,363 (382)	43.31	6.12									
036R	35,546–36,598 (351)	38.66	6.96									
037L	37,950–36,601 (450)	50.08	6.13	TATA								
038L	39,395–37,962 (478)	53.27	7.60	TATA								
039R	39,439–40,308 (290)	32.15	6.69									
040L	41,443–40,307 (379)	41.53	4.65	TATA								
041L	42,788–41,448 (447)	49.26	7.07									
042R	42,803–43,393 (197)	21.48	6.46									
043L	43,842–43,483 (120)	13.33	10.51				75.9	33 (103)	NP_078699.1	LCDV-1	ORF79	
044L	44,645–43,848 (266)	29.69	5.88	CCAAT								
045L	45,564–44,653 (304)	34.01	6.32									
046L	46,241–45,561 (227)	25.86	9.26		C-5 cytosine-specific DNA methylase active site	PS00094	179	43	U15575	FV3	Cytosine DNA methyltransferase	Kaur *et al.* (1995); Tidona and Darai (1997)
047R	46,401–46,661 (87)	10.04	9.81									
048R	46,661–47,002 (114)	12.43	9.11	TATA	Platelet-derived and vascular endothelial growth factors (PDGF, VEGF) family	smart00141 pfam00341	52.0 52.0	34 (85) 32 (85)	AJ401391 AF022952	*D. melanogaster* *Rattus norvegicus*	PDGF/VEGF-like protein VEGF-B	
049R	47,021–47,188 (56)	6.71	9.41									
050L	47,678–47,253 (142)	16.10	5.94	TATA								
051R	47,733–47,861 (43)	4.84	8.36									
052L	48,403–47,954 (150)	16.78	7.70	CCAAT								
053R	48,405–48,617 (71)	7.61	7.35	CCAAT								
054L	49,559–48,636 (308)	34.48	5.68									
055L	50,508–49,585 (308)	34.15	10.28	TATAAT								
056L	51,166–50,522 (215)	23.43	4.60				70.9	28 (146)	AF003534	CIV	Hypothetical protein 009R	
057L	51,433–51,176 (86)	9.53	4.60	TTGATA								
058L	51,759–51,463 (99)	10.65	8.72									
059R	51,854–52,000 (49)	5.49	7.29	TTGACA								
060L	52,200–52,039 (54)	5.96	10.73	TATA								
061L	53,162–52,362 (267)	31.01	8.56		Myb DNA-binding domain repeat signature 1	PS00037						
062L	56,785–53,162 (1208)	138.94	5.58	CCAAT	Rabphilin-3A effector domain							
063L	59,875–57,230 (882)	100.70	9.16		DEAH-box subfamily ATP-dependent helicase signature	PS00690	435	31	NP_078720.1	LCDV-1	ORF4 (nucleoside triphosphatase I putative helicase)	Tidona and Darai (1997); Yanez *et al.* (1993b)
064L	61,393–59,921 (491)	54.65	7.83	TATA	ATP/GTP-binding site motif A (P-loop); tyrosine-specific protein phosphatase active site	PS00017 PS00383	221	32	AF034568 AF025653	*Mus musculus*	RNA guanylyltransferase and 5′-phosphatase, mRNA capping enzyme	Yue *et al.* (1997); Pena *et al.* (1993)
065L	61,900–61,442 (153)	16.95	8.08		RING finger	smart00184						
066L	63,025–61,985 (347)	38.95	6.80	TTGACA	Zinc finger, C3HC4 type (RING finger), signature	PS00518						
067R	63,055–63,651 (199)	23.68	10.06									
068L	65,329–63,899 (477)	53.47	4.90									
069L	66,001–65,339 (221)	23.99	8.05	TATAAT								
070L	66,331–66,104 (76)	8.66	9.78	CCAAT								
071L	68,042–66,435 (536)	61.01	6.38									
072R	68,173–69,174 (334)	38.47	6.10	TTGAGA								
073R	69,203–69,619 (139)	15.53	6.20									
074R	69,669–70,679 (337)	37.99	6.92	TTGAAA								
075L	71,043–70,780 (88)	10.20	11.18									
076L	74,017–71,048 (990)	111.95	6.52	CCAAT			135	22 (723)	NP_078748.1	LCDV-1	ORF2	
077R	74,035–75,366 (444)	47.84	4.85		Ankyrin repeats	pfam00023 smart00248	70.9	35 (139)	AY026253	*M. musculus*	Gene trap ankyrin repeat containing protein	White *et al.* (1992)
078R	75,366–75,827 (154)	17.20	7.57									
079L	76,053–75,835 (73)	8.70	8.23	TATA								
080L	76,368–76,168 (67)	7.53	8.14	CCAAT								
081R	76,367–76,861 (165)	18.56	6.18									
082L	78,007–76,904 (368)	40.66	7.37	TTGACA								
083L	78,288–78,139 (50)	5.14	6.02	CCAAT								
084L	79,881–78,529 (451)	50.64	7.80	TATA								
085R	79,884–80,483 (200)	22.65	8.88									
086L	80,483–80,944 (154)	17.78	9.25	TTGAGA			110 103	49 (122) 50 (114)	AJ279814 AF367980	Ascovirus DpAV4 Regina ranavirus	Iridoviral protein homologue P8.141C	

TABLE 1—*Continued*

ORF	Position (length, aa)	M_r (kDa)	p*I*	Promoter	Conserved domain or signature[a]	Prosite/CD accession no.[b]	Best match: BlastP score[c]	Best match: % identity[d]	Best match: Accession no.[e]	Best match: Species	Predicted function/similarity[f]	Reference(s)
087R	80,940–81,707 (256)	28.95	8.13	TATA	Ribonuclease III family	smart00535 pfam00636	102	28	NP_078726.1	LCDV-1	ORF44 (ribonuclease homologue)	Tidona and Darai (1997)
088R	81,717–83,717 (667)	74.23	9.15	TATA								
089R	83,729–83,857 (43)	3.75	3.75	CCAAT								
090L	84,025–83,879 (49)	5.97	11.64									
091L	84,553–84,317 (79)	9.65	11.76									
092R	84,588–84,860 (91)	10.37	5.07									
093L	85,786–84,863 (308)	32.70	10.53									
094L	86,296–85,799 (166)	17.91	6.38	TATA								
095L	87,481–86,324 (386)	42.43	5.44									
096L	88,298–87,492 (269)	29.48	6.42									
097R	88,317–88,439 (41)	4.30	6.28									
098R	88,467–88,715 (83)	9.11	8.71									
099L	89,097–88,777 (107)	11.82	4.80	TATA	Zinc finger, C3HC4 type (RING finger), signature	PS00518						
100L	89,689–89,147 (181)	19.83	6.34	TATA								
101L	90,251–89,739 (171)	19.02	5.25									
102R	90,311–91,750 (480)	53.68	8.19									
103R	91,760–92,158 (133)	15.04	8.47		Src homology 2 domains	smart00252 pfam00017	105 105	42 (116) 42 (116)	AJ243123 U88325	*R. norvegicus* *M. musculus*	Suppressor of cytokine signaling 1	
104R	92,215–92,988 (258)	29.32	8.90	CCAAT								
105R	92,993–93,355 (121)	14.00	6.14		Prenyl group-binding site (CAAX box)	PS00294						
106L	94,501–93,485 (339)	37.01	7.67	CCAAT								
107L	94,812–94,600 (71)	7.42	6.93	CCAAT								
108R	94,934–95,080 (49)	5.75	10.69									
109L	97,950–95,188 (921)	103.41	6.18	TTGACA	ATP/GTP-binding site motif A (P-loop)	PS00017	501	34	NP_078717.1	LCDV-1	ORF6	
110R	97,997–98,149 (51)	5.85	8.57	TTGATA								
111L	99,039–98,152 (296)	32.51	6.57	TTGAGA	Zinc finger, C3HC4 type (RING finger), signature	PS00518	116	35 (167)	S56163	*H. sapiens*	Tumor necrosis factor Receptor-associated protein	Song and Donner (1995)
112R	99,059–99,799 (247)	26.97	7.20				105	31	NP_078615.1	LCDV-1	ORF45	
113R	99,937–100,287 (117)	12.34	9.93									
114L	103,159–100,337 (941)	106.70	7.44									
115R	103,203–104,210 (336)	38.27	6.82									
116R	104,219–105,664 (482)	52.19	4.66									
117L	106,395–105,724 (224)	25.29	7.61	TATA								
118L	108,093–106,726 (456)	50.16	6.34									
119R	108,105–108,389 (95)	10.73	8.15									
120R	108,424–108,930 (169)	19.49	9.25									
121L	109,584–108,937 (216)	24.93	8.77									
122R	109,594–110,310 (239)	27.35	7.69	CCAAT	ATP/GTP-binding site motif A (P-loop)	PS00017	487 487 487	99 99 99	AB007367 AB043977 AB043978	RSIV SBIV GIV	Adenosine triphosphatase (ATPase)	Kurita *et al.* (1998)
123R	110,391–110,573 (61)	6.58	8.58									
124L	111,351–110,668 (228)	24.95	6.31									

[a] Conserved domain or signature was constructed using the program Prosite or CD-Search within BlastP.

[b] Accession numbers starting with PS are Prosite-derived numbers, while those starting with smart and pfam are CD-Search within BlastP-derived numbers.

[c] BlastP scores represent bits of information.

[d] The figure within the parentheses in the "Percentage identity" column represents the ORF length of the match or it refers to the entire ORF.

[e] Accession numbers starting with NP_xx are NCBI-derived protein numbers.

[f] The NCBI-derived ORF numbers in the "Predicted function/Similarity" column do not correspond to the published LCDV-1 ORF numbers.

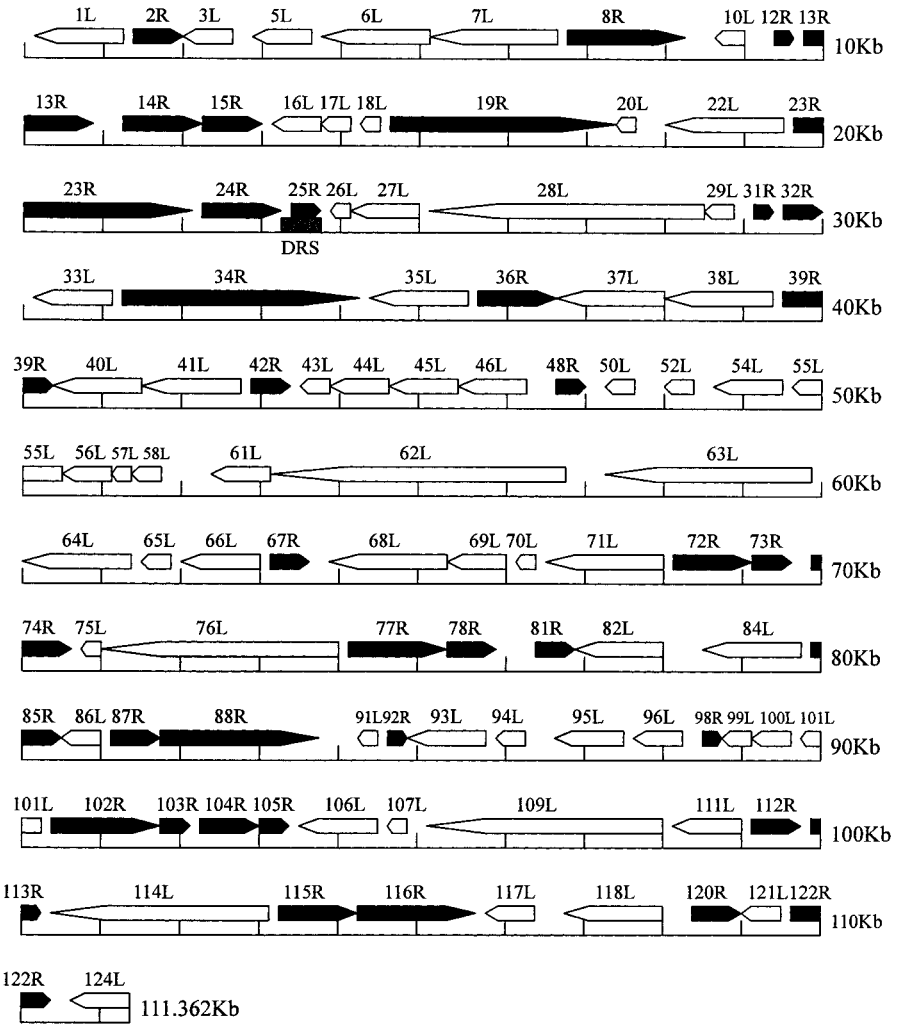

FIG. 1. Organization of the ISKNV genome. The arrows represent largely nonoverlapping open reading frames with respect to their size, position, and orientation. The scale is in kilobase pairs. DRS, direct repeated sequence.

that the protein belongs to the family B DNA polymerases.

ISKNV ORF46L encodes a homologue of cytosine DNA methyltransferase (MTase) present in other vertebrate iridoviruses (Kaur *et al.,* 1995; Tidona and Darai, 1997). Like the FV3 MTase gene, the predicated ISKNV ORF46L amino acid sequence contains a C-5 cytosine-specific DNA methylase active site and shows strong homologies to the first four motifs, but it does not seem to possess the long variable region or the fifth motif (Fig. 2). Methylation of DNA is a ubiquitous phenomenon in prokaryotic as well as eukaryotic cells (Bird, 1992). In prokaryotes, DNA methylation is the basis for the restriction/modification phenomenon whereby certain strains of bacteria recognize and degrade foreign DNA (Wilson and Murray, 1991). In vertebrates, methylation is responsible for transcriptional silencing of genes and control of gene expression (Maegawa *et al.,* 2001).

The predicted ORF27L codes for a protein of 298 amino acids in size. It showed significant homology to the DNA repair protein RAD2 Class I of RSIV and LCDV-1 (Tidona and Darai, 1997). It contains a xeroderma pigmentosum G (XPG) internal region (I-region), an XPG N-terminal region (N-region), and a 5′ → 3′ exonuclease domain, which are conserved domains in the RAD2 family of nucleases. The XPG-like proteins operate in nucleotide excision repair to incise the target strand to the 3′-side of the bubble-like, damage-containing structure formed as an intermediate during the repair event (van Steeg and Kraemer, 1999). The RAD2 family of nucleases exhibits a blend of substrate-specific exo- and endonuclease activities and contributes to DNA repair, replication, and recombination (Lee and Wilson, 1999). Interestingly, the putative protein also shows significant homology to an endonuclease of *Homo sapiens* and *Xenopus laevis.* Consequently, the putative protein derived from ORF27L is a suitable candidate for representing the counterpart of the DNA MTase in a viral restriction modification system. This system is able to degrade the host genomic DNA while the viral genome is protected from

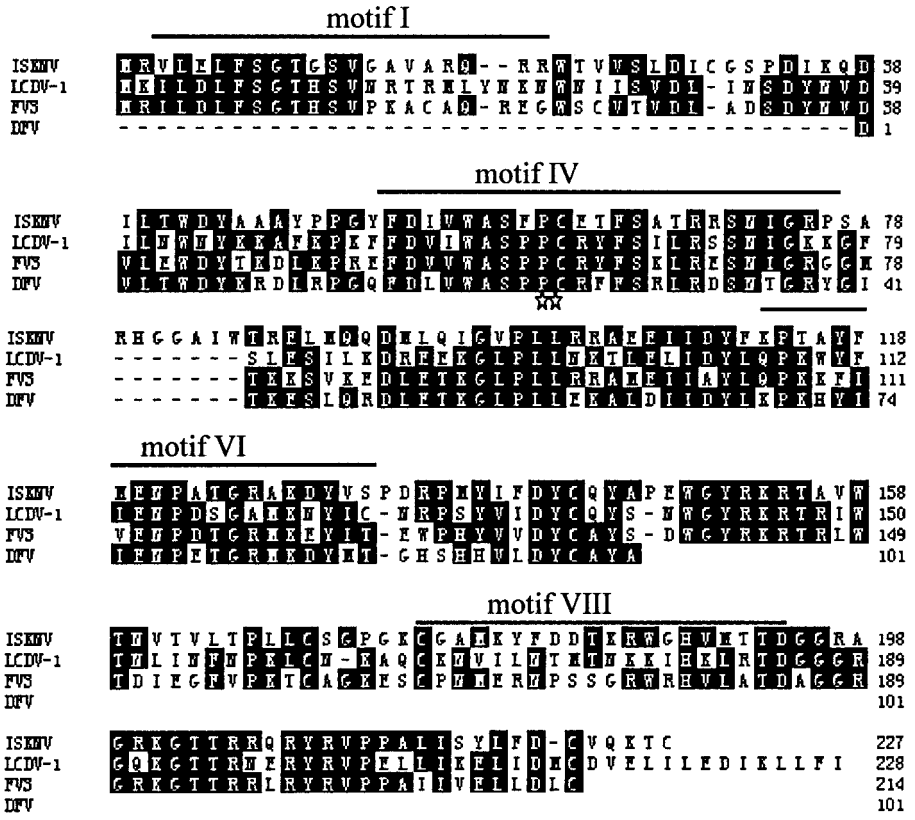

FIG. 2. Multiple amino acid sequence alignment of protein encoded by ISKNV ORF46L with cytosine DNA methyltransferase of FV3 (AAA86959.1), LCDV-1 (AAB50571.1), and DFV (AAC79867.1). Identical amino acids are indicated by shading. Gaps are indicated by a dash. The predicted ISKNV ORF46L amino acid sequence contains the first four motifs (I, IV, VI, and VIII) of DNA methyltransferase. The Pro-Cys motif is indicated by an asterisk.

endonucleolytic cleavage during viral DNA replication and allows the production of mature progeny DNA (Goorha *et al.,* 1984).

Database searches with the sequence of the predicted protein of ORF63L showed that this protein is similar to a group of helicases of superfamily II. This group includes D11L and D6R proteins of vaccinia virus (VV) and other poxviruses and the D1133L and Q706L protein of African swine fever virus (ASFV) (Gorbalenya and Koonin, 1989; Yanez *et al.,* 1993b). The five most conserved motifs in the helicases of superfamily II are present in ISKNV ORF63L, and it was shown that ISKNV ORF63L encodes a protein that can be classified as a member of this group of helicases of superfamily II (Matson and Kaiser-Rogers, 1990).

Proteins involved in transcription and nucleotide metabolism

ISKNV encodes homologues of proteins involved in transcription of DNA such as the two largest subunits of the DNA-dependent RNA polymerase (ORF28L and 34R), a ribonucleotide reductase small subunit (ORF24R), ribonuclease (ORF87R), and mRNA guanylyltransferase and mRNA capping enzyme (ORF64L).

The predicted amino acid sequence of ISKNV ORF28L showed significant homology to the largest subunit of the DNA-dependent RNA polymerase of RSIV and LCDV-1, and ISKNV ORF34R, which is 2585 bp away from ORF28L, encoded a protein that showed significant homology to the DNA-directed RNA polymerase II second largest chain of eukaryotic cells. ISKNV ORF29L contains a C2C2 zinc finger and nucleic acid-binding motif in a transcriptional elongation factor, transcription factor S-II (TFIIS), and RNA polymerases. ISKNV ORF29L may be a small subunit of DNA-directed RNA polymerases. The DNA-dependent RNA polymerase (DdRP) is a complex multi-subunit enzyme consisting of two large subunits with a M_r of >100K and several small polypeptides that are all present in stoichiometric amounts. Viruses that replicate in the cytoplasm of eukaryotic cells have no access to the host transcriptional machinery. Thus, DdRPs are essential enzymes for cytoplasmic DNA viruses, such as VV, ASFV, and iridoviruses (Moss, 1990; Yanez *et al.,* 1993a; Schnitzler *et al.,* 1994a). The presence of two ORFs in the ISKNV genome that encoded proteins similar to the two largest subunits of the DNA-dependent RNA polymerase from eukaryotic organisms and viruses suggest that viral transcription is carried out by DdRPs.

The ISKNV ORF24R translation product contains the

ribonucleotide reductase small subunit (RNRS) signature and it has over 50% amino acid identity to the RNRS from many organisms. Alignment of the RNRS amino acid sequences of viruses and cells revealed that the RNRS gene is highly conserved. Ribonucleotide reductase is a central enzyme in DNA metabolism, providing the only route for *de novo* synthesis of deoxyribonucleotides. This enzyme, which consists of two nonidentical subunits, carries out direct reduction of all four ribonucleotides to the corresponding deoxyribonucleotides (Eriksson *et al.,* 1984). Many large DNA-containing viruses, such as the poxviruses, ASFV, herpesvirus, and baculoviruses, encode both ribonucleotide reductase subunits (Oshima *et al.,* 1996; Li *et al.,* 1997). But in the present study, we found only the small subunit and not the large subunit of this enzyme in the ISKNV genome. The reason for this discrepancy requires further investigation.

ISKNV ORF64L encodes a protein that shares significant similarity with the RNA guanylyltransferase and mRNA capping enzyme of *Mus musculus* and *Paramecium bursaria Chlorella* virus 1 (PBCV-1). It contains a motif around a lysine residue ("LysXAspGly") that is common in the RNA guanylyltransferase of ASFV, poxviruses, and yeast guanylyltransferases (Pena *et al.,* 1993; Tomkinson *et al.,* 1991). The mRNAs of the iridoviruses have methylated cap structures, suggesting the presence in the virus genome of a guanylyltransferase and methyltransferase gene. To our knowledge, this is the first RNA guanylyltransferase and mRNA capping enzyme gene found in the iridovirus genome. The capping reaction should be extensively studied in the iridovirus system.

Proteins involved in host-related functions

ISKNV ORF111L encodes a protein that has some similarity to the tumor necrosis factor receptor-associated protein (TRAP) of *H. sapiens* and *M. musculus.* In human and mouse, TRAP specifically binds to the type-2 tumor necrosis factor receptor and plays a role in promoting or regulating cellular proliferation (Song and Donner, 1995). Like the human and mouse proteins, the ISKNV protein also contains the C3HC4-type zinc finger (RING finger) domain often found in DNA-binding proteins including transcription factors. This result indicated that the ISKNV ORF111L protein might be a DNA-binding protein and might therefore contribute to inhibition of apoptosis in the virus-infected host cells, but more extensive study is necessary to confirm this assumption.

ISKNV ORF48R contains a platelet-derived and vascular endothelial growth factor (PDGF, VEGF) family domain and has similarities to VEGF-B of *D. melanogaster,* mouse, and human. PDGF is a potent activator for cells of mesenchymal origin. Members of the VEGF family are homologues of PDGF. VEGF is a homodimeric glycoprotein that is mitogenic for endothelial cells and is an angiogenic factor that acts via the endothelial-specific receptor tyrosine kinases (VEGF receptors, VEGFRs) (Thomas, 1996). VEGF is also a potent inducer of vascular permeability (Senger *et al.,* 1983). VEGF-B, which is approximately 43% identical in amino acid sequence to VEGF, can form heterodimers with VEGF and may be involved in angiogenesis in muscle and heart. The function of the ISKNV ORF48R protein is currently unknown and needs to be further investigated.

ISKNV ORF77R contains ankyrin repeat motifs and encodes a protein that displays some similarity to the gene trap ankyrin repeat-containing protein of *M. musculus* (White *et al.,* 1992). However, these resemblances disappear if the ankyrin repeat sequences are removed from the analyses. To our knowledge, this is the first record of ankyrin repeat genes found in the iridovirus genome. Many proteins contain ankyrin repeats motifs, which are involved in mediating protein–protein interactions (Lin *et al.,* 1999).

Structural proteins

Significant homologies were found between the amino acid sequence deduced from ISKNV ORF6L and the major capsid proteins (MCPs) of other iridoviruses (i.e., FV3, LCDV-1, and CIV), viruses in the *Phycodnaviridae* family (i.e., PBCV-1), and ASFV (Mao *et al.,* 1996; Schnitzler and Darai, 1993; Stohwasser *et al.,* 1993; Graves and Meints, 1992; Lopez-Otin *et al.,* 1990). The ISKNV MCP gene (ORF6L) encodes a protein that contains 453 amino acids and it is the shortest of the known MCP amino acid sequences of the iridoviruses. MCP is the predominant structural component of the virus particles, comprising 40–50% of the total particle polypeptide. The iridovirus MCP is a late gene product whose expression appears to be translationally regulated (Williams, 1996). Tidona *et al.* (1998) used the amino acid sequences of the known MCPs in comparative analyses including three insect iridoviruses, seven vertebrate iridoviruses isolated from fish, amphibians, or reptiles, PBCV-1, and ASFV. The analyses revealed that the amino acid sequence of the MCP is a suitable target for the study of viral evolution since it contains highly conserved domains. Furthermore, their results suggested that a substantial revision of the taxonomy of iridoviruses based on molecular phylogeny is required.

Other proteins

ISKNV ORF12R, 66L, 99L, and 111L encoded proteins with the C3HC4-type zinc finger (RING finger) domain, as found in the LCDV-1 and CIV genomes (Tidona and Darai, 1997; Schnitzler *et al.,* 1994b). A number of eukaryotic and viral proteins contain the C3HC4-type zinc finger domain and are probably involved in mediating protein–protein interactions (Borden and Freemont, 1996). The majority of the members of the RING family are involved

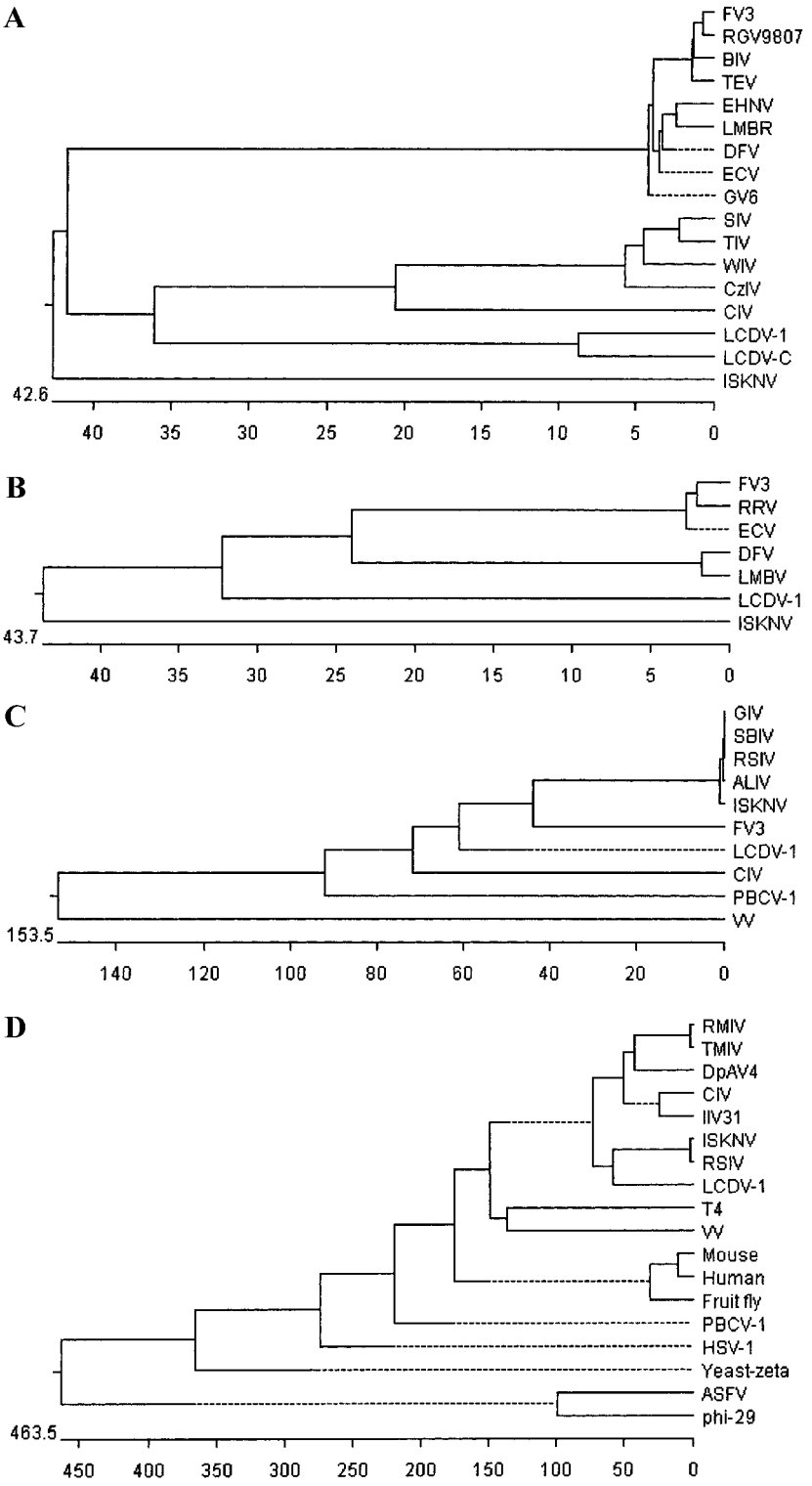

FIG. 3. Using the CLUSTAL V algorithm within MEGALIGN (DNASTAR), phylogenetic trees were constructed based on the multiple alignment of the amino acid sequence of a 163-aa region of the major protein of iridoviruses (A), the amino acid sequence of a 68-aa region of the cytosine DNA methyltransferase of vertebrate iridoviruses (B), the complete amino acid sequences of the ATPase of viruses (C) and the DNA polymerase of organisms (D). Branch length is proportional to the number of amino acid substitutions, which is indicated by the scale beneath the tree (Distance between sequences = Number of substitution events). Accession numbers, given in parentheses, were retrieved from the EMBL data library or as otherwise indicated: (A) FV3, frog virus 3 (U36913); RGV9807, iridovirus RGV9807 (AF192508); BIV, bohle iridovirus (AF157651); TEV, tadpole edema virus (AF157681); EHNV, epizootic hematopoietic necrosis virus (AF157667); LMBR, largemouth bass ranavirus (AF080250); DFV, doctor fish virus (AF157665); ECV, catfish iridovirus (AF157659); GV6, guppyfish iridovirus 6 (AF157671); SIV, simulium iridescent virus (M32799); TIV, tipula iridescent virus (M33542); WIV, *Wiseana* iridescent virus (AF025774); CzIV, *Costelytra zealandica* iridescent virus (AF025775); CIV, chilo iridescent virus

in the regulation of transcription and in the repair of damaged DNA and DNA binding (Schnitzler *et al.,* 1994b). In ISKNV, there are four ORFs encoding proteins containing the RING finger domain, suggesting that they may be involved in viral gene expression or protein–protein interaction. However, an inhibitor of apoptosis gene with a zinc finger motif in baculoviruses, which has a homologue in CIV (No. M81387) (Noeman *et al.,* 1993), was not found in the ISKNV genome.

Additional putative gene products of ISKNV homologous to other DNA viruses include adenosine triphosphatase (ATPase) (ORF122R) and the hypothetical proteins of CIV, LCDV-1, and Regina ranavirus (RRV) (ORF5L, 7L, 43L, 56L, 76L, 86L, 87R, 109L, and 112R).

Taxonomic position of ISKNV

The predicted gene products of ISKNV show the highest homology to the corresponding viral proteins of RSIV, sea bass iridovirus (SBIV), grouper iridovirus (GIV), and African lampeye iridovirus (ALIV), and they were listed in the unassigned species in the *Iridoviridae* family. There are five predicted amino acid sequences of RSIV in the GenBank/EMBL/DDBJ databases, including DNA polymerase, ribonucleotide reductase small subunit, DNA repair protein RAD2, the largest subunit of the DNA-dependent RNA polymerase, and ATPase (Nos. AB006954, AB007366, AB007367, and AB018418). When the sequences of the ISKNV genome and RSIV were compared, five of the predicted ISKNV polypeptides (ORF19R, ORF24R, ORF27L, ORF28L, and ORF122R) showed a high degree of sequence identity to the RSIV homologues (with identities of 96, 96, 91, 95, and 99%, respectively). In addition, the identities of the amino acid sequence of ISKNV ORF123R and SBIV (AB043977) (99%), GIV (AB043978) (99%), and ALIV (AB043979) (98%) are also higher than those of the other iridoviruses. These sequence analysis results indicated that ISKNV was much more closely related to RSIV, SBIV, GIV, and ALIV than to other iridoviruses.

To determine the evolutionary relationship of ISKNV to the other iridoviruses, such as CIV, FV3, and LCDV-1, phylogenetic trees based on the multiple alignments of the known amino acid sequences involving several viral proteins (including MCP, ATPase, MTase, and DNA polymerase) were constructed (Fig. 3). The resulting trees subdivided the viruses into four groups: (1) invertebrate iridoviruses (including CIV, *Costelytra zealandica* iridescent virus, simulium iridescent virus, tipula iridescent virus, iridovirus RMIV, iridovirus TMIV, iridovirus IV31, and wiseana iridescent virus; (2) lymphocystiviruses (including LCDV-1 and Chinese flounder lymphocystis disease virus; (3) ranaviruses (including FV3, catfish iridovirus (ECV), sheatfish iridovirus (ESV), epizootic hematopoietic necrosis virus (EHNV), tadpole edema virus (TEV), bohle iridovirus, largemouth bass ranavirus (LMBR), RRV, doctor fish virus (DFV), guppyfish iridovirus 6 (GV6) and iridovirus RGV9807); and (4) unassigned viruses (including ISKNV, RSIV, SBIV, GIV, and ALIV).

There are two genera (*Lymphocystivirus* and *Ranavirus*) in the *Iridoviridae* family that infect vertebrates such as fish, amphibians, and reptiles. During the past two decades, iridoviruses have gained significant economic importance, as there are a growing number of unclassified iridovirus-like isolates from different species of insects and vertebrates, especially from commercially important aquatic animals, such as rainbow trout, sheatfish, and catfish (Rodger *et al.,* 1987; Langdon *et al.,* 1988; Chua *et al.,* 1994; Miyata *et al.,* 1997; Nakajima *et al.,* 1998; Chou *et al.,* 1998). Mao *et al.* (1997) and Hyatt *et al.* (2000) compared the newly isolated vertebrate iridoviruses with FV3 and LCDV-1 using electron microscopy, SDS–PAGE, restriction endonuclease digestion, DNA hybridization, and DNA sequencing. The seventh report of ICTV described many iridoviruses newly isolated from fish, reptile, and amphibian iridoviruses, such as FV3, EHNV, ESV, ECV, LMBR, DFV, GV6, TEV, box turtle virus 3, and tortoise virus 5, which were members of the *Ranavirus* genus (Regenmortel *et al.,* 1999). Hyatt *et al.* (2000) also suggested that the taxonomic position of at least two other vertebrate iridovirus groups requires further investigation. These are the erythrocytic iridoviruses (identified in blood cells from reptiles, fish, and amphibians) and the second group of viruses that produce hypertrophic cells in the lamina propia, in the epidermis, and within organs such as kidney and spleen, which include RSIV, SBIV, GIV, and ALIV. The biochemical and pathological character of ISKNV (He *et al.,* 1998, 2000, in press; Weng *et al.,* 1998; Wu *et al.,* 1997) suggested that ISKNV might be related to the second group (RSIV, SBIV, GIV, and ALIV), which is characterized by cell hypertrophy throughout the spleen, kidney, cranial connective tissue, and endocardium (Table 2). Furthermore, analysis

(AF303741); LCDV-1, lymphocystis disease virus 1 (L63545); LCDV-C, Chinese flounder lymphocystis disease virus (AF126405); ISKNV, infectious spleen and kidney necrosis virus (AF371960). (B) FV3 (U15575); RRV, Regina ranavirus (AF100200); ECV (AF100201); DFV (AF100202); LMBR (AF100199); LCDV-1 (L49526). (C) GIV, grouper iridovirus (AB043978); SBIV, sea bass iridovirus (AB043977); RSIV, Red Sea bream iridovirus (AB007367); ALIV, African lampeye iridovirus (AB043979); FV3 (M80551); LCDV-1 (GenBank NP_078656.1); CIV (AF003534); PBCV-1, *Paramecium bursaria Chlorella* virus 1 (U42580); VV, vaccinia virus (M32854). (D) RMIV, iridovirus RMIV (AJ279822); TMIV, iridovirus TMIV (AJ279825); DpAV4, ascovirus DpAV4 (AJ279812); CIV (AF083915); IIV31, iridovirus IV31 (AJ279821); RSIV (AB007366); LCDV-1 (GenBank NP_078724.1); T4, bacteriophage T4 (M10160); VV (M36339); mouse, *Mus musculus* (Z21848); human, *Homo sapiens* (M81735); fruit fly, *Drosophila melanogaster* (AE003529); PBCV-1 (U42580); HSV-1, herpes simplex virus 1 (X04495); yeast-zeta, *Saccharomyces cerevisiae* (M60416); ASFV, African swine fever virus (U18466); ϕ29, bacteriophage ϕ29 (V01155).

TABLE 2

Comparative Analysis of Properties of Vertebrate Iridoviruses

	Lymphocystivirus	Ranavirus	Cell hypertrophy iridovirus	Erythrocytic iridovirus
Morphology	Icosahedral	Icosahedral	Icosahedral	Icosahedral
G+C ratio	30.7 (LCDV-1)	53 (FV3)	54.8 (ISKNV)	
Natural host range	Fish	Fish, amphibians, and reptiles	Fish	Fish
Histopathology	Chronic, benign, and papilloma-like lesions which consist of enormous hypertrophy of dermal connective tissue cells	Necrosis of the renal hematopoietic tissue, liver, spleen, and pancreas; lethal	Hypertrophy of cells in the lamina propia, in the epidermis, and within organs such as kidney and spleen; lethal	Occlusion bodies in erythrocytic cytoplasm
Main tissue tropisms	Dermal connective tissue	Liver	Spleen and kidney	Erythrocytes
Geographic distribution	All over the world	Europe, Australia, USA, Asia	East and Southeast Asia (including China, Japan, Thailand, Indonesia)	
Sensitivity of culture cell	No	Yes	No	
Known gene amino acid, sequence identity	MCP gene identity between LCDV-1 and LCDV-China is 84.4%	MCP gene identity between FV3 and LMBV, DFV, GV6 is about 75%; MCP gene identity between FV3 and the others is over 90%	The known gene identity between cell hypertrophy iridoviruses is over 90%	

of the ISKNV genome confirmed that members of this group of iridoviruses have higher levels of homology with one another than with the other vertebrate iridoviruses, suggesting that they may belong to a new genus of the *Iridoviridae* family, which is tentatively referred to as the cell hypertrophy iridoviruses.

MATERIALS AND METHODS

Infected fish

Spleen and kidney of moribund mandarin fish, *S. chuatsi* (Basilewsky), that showed symptoms of ISKNV infection were collected and kept at −80°C from fish farms in Nanhai, Guangdong Province, China, in November 1998. The samples were examined by gross anatomy, PCR, and light and electron microscopy for confirmation of the disease as described previously (Weng *et al.,* 1998; Deng *et al.,* 2000).

Virus and viral DNA

Virus purification and DNA extraction were performed as described by Deng *et al.* (2001a). Spleen and kidney were removed from moribund mandarin fish and pulverized by a mortar and pestle in liquid nitrogen. The powdered tissue was gradually added to 10 vol of PBS (pH 7.4) and centrifuged at 5000 *g* for 20 min at 4°C. The supernatant was centrifuged at 35,000 *g* for 30 min at 4°C. The pellet was suspended in PBS and differential centrifugation was repeated. The resuspended pellet was layered on a 20–50% (w/w) sucrose gradient and further purified by centrifugation for 2 h at 90,000 *g* in a SW40 Ti rotor (Beckman). The viral band in the gradient was removed, diluted threefold with PBS buffer, and centrifuged for 30 min at 90,000 *g* at 4°C. The final pellet containing the nucleocapsid was resuspended in PBS buffer and incubated with 0.5% SDS and 0.5 mg/ml proteinase K at 55°C for 3 h. The suspension was extracted by phenol–chloroform and the DNA was precipitated by ethanol (Sambrook *et al.,* 1989).

DNA sequencing

For these studies, two genomic libraries were used. One library was generated by cloning restriction endonuclease *Bam*HI, *Hin*dIII, and *Kpn*I fragments into appropriate sites of pBluescript KS plasmids using standard methods as described by Sambrook *et al.* (1989; see also Deng *et al.,* 2001b). The other library was constructed by random cloning of sonicated viral DNA fragments into the *Sma*I site of a pUC-derived plasmid. Briefly, viral genomic DNA fragments were randomly generated by sonication at 0°C. The sheared DNA (1–2 kb) was ethanol precipitated, and blunt ends were generated using T4 and Klenow polymerase. Then the DNA fragments were blunt-end ligated into the *Sma*I site of the pUC18 vector. The ligation mix was transformed into *Escherichia coli* XL1-blue competent cells. Recombinant plasmids were screened for inserts (1–2 kb) by restriction analysis.

The cloned fragments of viral DNA were sequenced in both directions with the universal forward and reverse M13 primers using an ABI PRISM 3700 automated DNA sequencer (Applied Biosystems, Inc.). The average read-length obtained from individual sequencing reactions

was over 550 bp. The nucleotide sequences obtained from the sheared fragments of ISKNV DNA were assembled using InnerPeace software. The gaps were linked by the primer walking method.

Computer-assisted analysis

Nucleotide and amino acid sequences were compiled and analyzed using the DNASTAR and Omiga 2.0 programs. Amino acid sequences were scanned for known active site motifs and protein family signatures (PROSITE 13.0; Bairoch, 1991). The DNA and the deduced amino acid sequences were compared with the updated GenBank/EMBL/DDBJ, SWISSPROT, and PIR databases using FASTA and BLAST network service (Pearson, 1990; Altschul *et al.,* 1990). Protein alignments were generated using the CLUSTAL program (Higgins and Sharp, 1988).

ORFs were identified by the following criteria: (1) they were not less than 120 bp and (2) they were not located within larger ORFs. In addition, ISKNV ORFs that were likely to be expressed were also identified on the basis of significant identity to known protein sequences within the databases.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the GenBank/EMBL/DDBJ and NCBI nucleotide sequence databases under Accession No. AF371960.

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