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Molecular epidemiology and phylogenetic analysis of a marine fish infectious spleen and kidney necrosis virus-like (ISKNV-like) virus

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Summary

Infectious spleen and kidney necrosis virus-like (ISKNV-like) virus causes a serious systemic disease with high morbidity and mortality of freshwater and marine fishes. Based on the ISKNV putative major capsid protein (MCP), the vascular endothelial growth factor (VEGF), the mRNA capping enzyme (Capping), and the tumor necrosis factor receptor-associated protein (TNFR) genes, primers were designed and used in PCR to determine the host range of ISKNV-like viruses. From the sampling of >1600 marine fishes representing 6 orders, 25 families, and 86 species collected in the South China Sea, 13 cultured fish species (141 fish) and 39 wild fish species (102 fish) were confirmed hosts of ISKNV-like viruses. The average percentage of infection of ISKNV-like viruses was 14.6%. The results from phylogenetic analysis of these genes revealed that ISKNV-like viruses could be placed into two clusters: cluster I was more related to ISKNV; cluster II included OSGIV (orange-spotted grouper iridovirus) and RBIV (rock bream iridovirus), and was quite different from ISKNV. The results of this study can contribute to the prediction and prevention of ISKNV disease outbreaks.

Introduction

Iridoviruses are large icosahedral cytoplasmic dsDNA viruses isolated from invertebrates and vertebrates [30]. Based on the Seventh Report of the International Committee on Taxonomy of Virus (ICTV), the family *Iridoviridae* has been subdivided into four genera including *Iridovirus, Chloriridovirus, Ranavirus* and *Lymphocystivirus* [28]. Among the four genera, the viruses of the genera *Ranavirus* and *Lymphocystivirus* are recognized as causative agents of diseases in poikilothemic vertebrates including amphibia, reptiles, and fish. Additionally, another type of iridoviruses was isolated from fish, which neither belonged to lymphocystivirus nor ranavirus, but caused enlargement of cells in many tissues, especially in the spleen and kidney. These viruses were called megalocytivirus and belonged to a new genus of the family *Iridoviridae* [2]. Members in this new genus included the Red Sea bream iridovirus (RSIV) [13], the grouper sleepy disease iridovirus (GSDIV) [3], the sea bass irido-

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virus (SBIV) [19], the infectious spleen and kidney necrosis virus (ISKNV) [9], the rock bream iridovirus (RBIV) [14], the dwarf gourami iridovirus (DGIV), the African lampeye iridovirus (ALIV) [24], and the large yellow croaker iridovirus (LYCIV) [1]. They were confirmed to be pathogens associated with serious systemic diseases, and the virus infections were epidemic in cultured freshwater and marine fish and resulted in mass mortalities and huge economic losses in the South East Asia.

The infection by ISKNV of mandarin fish, *Siniperca chuatsi* (Basilewsky), is characterized by cell hypertrophy in the spleen, kidney, cranial connective tissue and endocardium. Recent research has suggested that many marine fish are infected by a virus that causes clinical signs similar to ISKNV infection [1, 29]. We have called these viruses infectious spleen and kidney necrosis virus-like (ISKNV-like) viruses in this paper. Since fish are transported worldwide for commercial, recreational, and ornamental use, it is possible that one or more fish species may be the vehicle by which the iridovirus is introduced into a new host or environment [17]. Some fish species may serve as important reservoirs or vectors of the virus for cultured marine fish. Because ISKNV and ISKNV-like viruses cause significant economic losses [6, 9, 29], the determination of host range and species susceptibility of ISKNV-like viruses can contribute to the prediction and prevention of viral disease outbreak.

In this paper, we investigated the host range of ISKNV and ISKNV-like virus in order to better understand the degree of genetic diversity and taxonomic position of the ISKNV-like virus from different hosts. We also compared the deduced amino acid sequence of the major capsid protein (MCP), the vascular endothelial growth factor (VEGF), the mRNA capping enzyme (Capping), and the tumor necrosis factor receptor-associated protein (TNFR) from these viruses to determine the phylogenetic relationships and classification of the ISKNV-like viruses.

Material and methods

Fish

Fish (N = 1629) were collected from Daya Bay in the South China Sea and from the coastal area of Sanya (37 fish), Hainan Province, from November 2001, May–July 2002, September 2002, and January 2003 (Fig. 1). The samples (N = 1666) comprised fishes from 6 orders, 25 families, and 86 species, of which 13 were cultured fish and 73 were wild fish (Table 1). Spleen and kidney of these fish were dissected for the detection of ISKNV-like virus by a 1-step PCR and a 2-step PCR (nested-PCR).

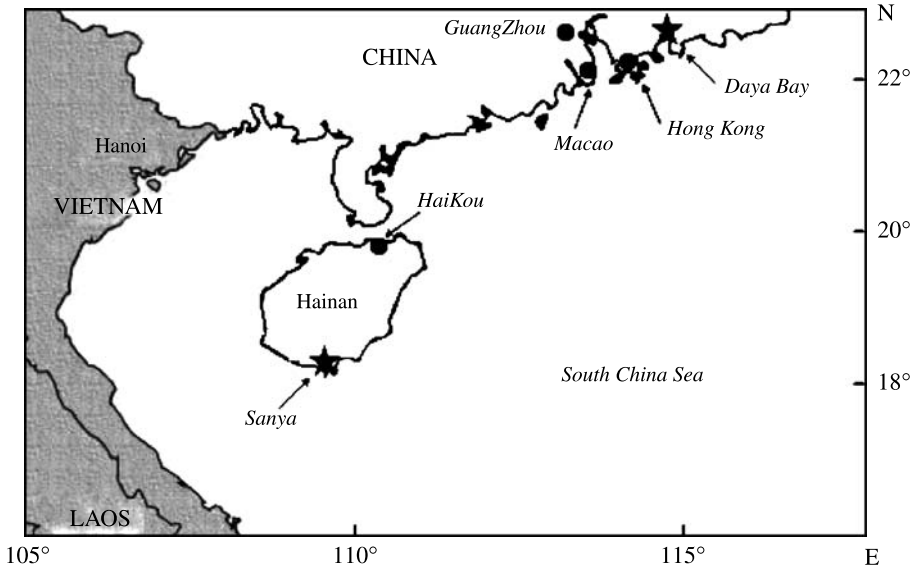

Fig. 1. Map showing the major geographic zones and the sites where the fish were collected

Table 1. The host range of the ISKNV-like viruses

Order	Family	Species	Infected fish/Total fish					Host
			Win	Sum	Aut	Spr	Total	
Perciformes	Sciaenidae	*Sciaenops ocellatus**	7/30	9/28	11/19	2/10	29/87	+
		Umbrina russelli	\	\	3/10	1/10	4/20	+
		Argyrosomus argentatus	\	\	\	2/10	2/10	+
		Argyrosomus macrocephalus	\	\	0/9	5/10	5/19	+
		Otolithes argenteus	0/6	0/10	\	1/10	1/26	+
		Miichthys miiuy	0/4	0/4	\	\	0/4	−
		Pseudosciaena crocea	\	\	3/3	\	3/3	+
	Serranidae	*Epinephelus coioides**	10/27	7/21	16/26	0/10	33/84	+
		*Epimephelus awoara**	6/16	2/8	2/3	5/11	15/38	+
		*Epinephelus alabaricus**	\	0/10	6/14	3/19	9/43	+
		*Epinephelus bleekeri**	\	5/12	1/16	2/16	8/44	+
		*Epinephelus akaara**	\	2/18	\	\	2/18	+
		Epinephelus fario	\	\	0/2	\	0/2	−
		Epinephelus fasciatomaculatus	\	\	1/1	\	1/1	+
		Epinephelus tauvina	\	5/10	1/3	3/12	9/25	+
		Epimephelus areolatus	\	0/1	\	\	0/1	−
		Epinephelus brunneus	0/1	\	\	0/1	0/2	−
		Epinephelus chlorostigma	\	\	\	1/1	1/1	+
		Epinephelus megachir	\	\	0/1	\	0/1	−
		Epinephelus epistictue	\	\	0/1	0/2	0/3	−
		Epinephelus merra	\	\	0/1	1/1	0/2	+
		Cephalopholis pachycentron	0/12	\	0/11	5/12	5/35	+
		Lateolabrax japonicus	\	\	0/1	\	0/1	−
	Lactariidae	*Lactarius lactarius*	\	\	0/10	\	0/10	−
Perciformes	Sparidae	*Acanthopagrus latus**	3/8	6/16	2/13	1/10	12/47	+
		*Pagrus major**	1/20	1/1	2/10	\	1/31	+
		*Sparus macrocephalus**	0/1	7/8	3/17	0/3	10/29	+
Perciformes	Sparidae	*Rhabdosarfus sarba**	2/11	1/20	1/10	2/11	6/52	+
		Parargyrops edita	0/8	0/2	\	\	0/10	−
	Cybiidae	*Scomberomorus commersoni*	\	2/9	\	\	2/9	+
	Rachycentridae	*Rachycentron canadum*	2/7	\	\	\	2/7	+
	Carangidae	*Caranx djeddaba*	\	\	0/10	0/10	0/20	−
		Caranx kalla	0/11	6/15	2/10	\	8/36	+
		Decapterus maruadsi	\	\	1/10	\	1/10	+
		Selaroides leptolepis	0/11	\	2/10	0/12	2/33	+
		Chorinemus hainanensis	1/19	\	\	\	1/19	+

(*continued*)

Table 1 (*continued*)

Order	Family	Species	Infected fish/Total fish					Host
			Win	Sum	Aut	Spr	Total	
		Chorinemus moadetta	\	\	1/12	\	1/12	+
		Caranx sexfasciatus	\	\	\	1/3	1/3	+
		Alectis indica	\	\	\	0/10	0/10	−
	Stromateidae	*Pampus argenteus*	5/7	\	\	\	5/7	+
	Leiognathidae	*Leiognathus insidiator*	0/13	0/12	1/10	0/13	1/48	+
		Leiognathus berbis	0/7	0/8	0/6	0/3	0/24	−
		Leiognathus bindus	\	\	1/12	\	1/12	+
		Leiognathus dussumieri	0/10	\	\	0/10	0/20	−
		Leiognathus rivulatus	0/10	\	\	0/10	0/20	−
		Leiognathus brevirostris	\	0/12	\	3/10	3/22	+
	Theraponidae	*Terapon jarbua*	\	\	2/11	1/11	3/22	+
		Pelates quadrilineatus	\	\	1/10	2/9	3/19	+
	Lutjanidae	*Lutjanus sanguineus**	3/20	\	1/10	2/10	6/40	+
		*Lutjanus argentimaculatus**	4/5	\	\	\	4/5	+
		Lutjanus russelli	\	\	1/11	2/18	3/29	+
		Lutjanus johnii	\	2/5	\	1/6	3/11	+
		Lutjanus fulviflamma	0/10	0/10	0/10	0/10	0/40	−
		Lutjanus rivulatus	\	0/4	\	\	0/4	−
	Siganidae	*Siganus oramin*	3/11	0/13	0/10	0/10	0/44	+
	Pomadasyidae	*Plectorhynchus pictus*	0/16	0/5	1/5	1/4	2/30	+
	Priacanthidae	*Priacanthus hamrur*	0/7	0/12	0/10	0/11	0/40	−
	Apogonidae	*Apogon kinesis*	\	0/2	\	\	0/2	−
Pleuronectiformes	Cynoglossidae	*Cynoglossus sinicue*	0/10	2/10	1/13	1/10	4/43	+
		Cynoglossus puncticeps	0/10	0/10	0/13	0/10	0/43	−
	Soleidae	*Brachirus orientalis*	\	0/2	0/6	0/3	0/11	−
	Paralichthyidae	*Tarphops oligolepis*	\	\	\	0/10	0/10	−
		Pseudorhombus cinnamomeus	\	\	\	0/8	0/8	−
		Pseudorhombus levisquamis	\	\	\	0/8	0/8	−
		Tephrinectes sinensis	\	\	1/7	0/10	0/17	+
		Pseudorhombus arsius	\	\	0/1	\	0/1	−
Myctophiformes	Synodontidae	*Saurida elongate*	\	3/11	0/10	\	3/21	+
Clupeiformes	Engraulidae	*Thrissa vitirostris*	\	0/10	0/10	\	0/20	−
Clupeiformes	Engraulidae	*Thrissa mystax*	\	2/12	\	\	2/12	+
	Clupeidae	*Clupanodon punctatus*	0/9	1/15	2/12	0/9	3/45	+
		Mugil cephalus	0/11	\	\	0/11	0/22	−
		Osteomugil ophuyseni	\	3/10	0/10	\	3/20	+
		Clupea harengus pallasi	\	0/13	0/10	\	0/23	−

(*continued*)

Table 1 (*continued*)

Order	Family	Species	Infected fish/Total fish					Host
			Win	Sum	Aut	Spr	Total	
Mugiliformes	Polynemidae	*Eleutheronema tetradactylus*	0/10	\	\	1/10	1/20	+
		Sphyraena forsteri	\	2/10	\	\	2/10	+
Tetraodontiformes	Tetraodontidae	*Takifugu xanthopterus*	\	1/8	\	\	1/8	+
		Gastrophysus spadiceus	1/8	\	0/10	5/12	5/22	+
		Fugu alboplumbeus	\	0/2	\	3/10	3/10	+
		Gastrophysus alboplumbeus	\	0/10	\	\	0/10	−
	Aluteridae	*Monacanthus chinensis*	0/10	\	\	0/10	0/20	−
		Stephanolepis japonicus	0/5	3/14	0/10	\	2/29	+
Tetraodontiformes	Aluteridae	*Monacanthus chinensis*	\	0/1	\	0/9	0/10	−
		Stephanolepis cirrhifer	\	\	\	0/1	0/1	−
		Navodon tessellates	\	\	\	0/10	0/10	−
Total infected samples/total samples			45/391	72/429	70/442	56/404	243/1666	
(%)			(11.5)	(16.78)	(15.83)	(13.86)	(14.6)	
The infected cultured fish/total culture fish			36/163	40/144	48/141	17/100	141/548	
(%)			(22.1)	(27.8)	(34.0)	(17.0)	(25.7)	
The infected wild fish/total wild fish			9/228	32/285	22/301	39/304	102/1118	
(%)			(4.0)	(11.2)	(7.3)	(12.8)	(9.1)	

Note. *, Cultured fish.
Win Winter; *Sum* summer; *Aut* autumn; *Spr* spring.
Total Total samples.
\, No sample was collected.
+, The fish was PCR-positive for an ISKNV-like virus.
−, The fish was PCR-negative for an ISKNV-like virus.

DNA isolation

Spleen and kidney samples were triturated in a homogenizer containing 10 × volumes of PBS (pH = 7.4). The resulting homogenate was centrifuged at 3000 rpm for 10 min, and the supernatant was then incubated at 55 °C for 3 h in 0.5% SDS and 0.5 mg/ml proteinase K (Merck). After three extractions with phenol/chloroform, the DNA was precipitated by adding sodium acetate to 0.3 M and 2.5 × volume of ethanol. The dried precipate was redissolved in 50 µl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.3), and stored at −80 °C until use.

Primers for PCR

Primers for the four putative genes were designed from the nucleotide sequence of ISKNV (Accession No. AF371960) [10]. Primers mF1 and mR1 were derived from the DNA sequence of MCP (ORF 6L) and were used for the first-step PCR, and the size of the expected product was about 1080 bp. Another set of primers (mF2 and mR2) derived from the 1080-bp fragment of MCP was used for the second-step PCR amplification. The primers for the VEGF (ORF 48R), Capping (ORF 64L), and TNFR (ORF 111L) were designed similarly. The primer sequences are shown in Table 2.

PCR amplification

PCR amplification was carried out in a 50-µl reaction mix containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5% Tween-20, 0.2 mM each dNTP, 10 pmol of each primer, 1 unit of Taq DNA polymerase, and 100 ng of the extracted genomic DNA. The mixture was incubated in a PTC-200 DNA thermal cycler (MJ Research Inc., USA) at 95 °C for 3 min, and then for 30 cycles (94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min) plus a final 8-min extension at 72 °C. The PCR products were analyzed in 1% agarose gels containing ethidium bromide at a final concentration of 0.5 µg/ml and visualized under a UV transilluminator.

The 1-step PCR amplifications were carried out with the primer sets mF1/mR1, vF1/vR1, cF1/cR1, and tF1/tR1, and the 2-step PCR amplifications were performed with the other primer sets mF2/mR2, vF2/vR2, cF2/cR2 and tF2/tR2, respectively. The products of the 1-step PCR amplifications were diluted 1/1000 and used as DNA template for 2-step PCR amplifications. The reaction conditions of the 2-step PCR were similar to those of the 1-step PCR described above.

DNA sequencing

The PCR products were purified using QIAquick PCR purification kit (QIAGEN). DNA sequence determination was performed using an ABI PRISM 377 DNA sequencer with a BigDye Terminator Kit (Applied Biosystems, Inc.). The nucleotide sequences obtained from individual sequencing reactions were assembled using the Data Collection and Sequence Analysis software (Applied Biosystems, Inc.).

Phylogenetic analysis

Nucleotide and amino acid sequences were compiled and analyzed using Lasergene software (DNASTAR Inc., USA). Protein alignments were generated using Clustal-X 1.80

Table 2. Primers used for 1-step and 2-step PCR analysis

Gene product	Primers	Oligonucleotide sequence (5′ to 3′)	Size of PCR product (bp)
MCP	mF1	AGACCCACTTGTACGGCG	1080
(ORF 6L)	mR1	CCCATGTCCAACGTATAGC	
	mF2	CGTGAGACCGTGCGTAGT	562
	mR2	AGGGTGACGGTCGATATG	
VEGF	vF1	CATCAGCAGACCGAGCGTAA	596
(ORF 48R)	vR1	CCACCTGCTGTTTGCGAGT	
	vF2	GCTATCATGGCTGTCGCGT	207
	vR2	CCGTCACGTTTACCGCG	
Capping	cF1	TATGTGGCTTCATTACGC	1370
(ORF 64L)	cR1	GGATGATGCTCTCGTACAC	
	cF2	GCTGTGTGTCAAGTACAACG	504
	cR2	ACACACATATACACCGCG	
TNFR	tF1	GTGTCAAACCCCGATGCG	1016
(ORF 111L)	tR1	CAGTGGGCAGAACGTCGG	
	tF2	TTGTCGTGTCACGACGGC	318
	tR2	AAACCTGGCCGCGATTG	

[27]. The phylogenetic relationships among species were determined using the neighbor-joining (NJ) [22] and maximum parsimony methods as implemented in Mega2.1 [15].

Results

The natural host range of ISKNV and ISKNV-like viruses

Based on the optimized 1- and 2-step PCR methods, many marine fish were confirmed to carry ISKNV-like viruses, and the host species of ISKNV-like viruses were determined. ISKNV-like viruses had a wide range of natural hosts among marine fish. In a collection of >1600 marine fish, 13 cultured fish species (141 fish), and 39 wild fish species, 102 fish species were confirmed hosts of ISKNV-like viruses (Table 1). The average percentage of infection in marine fish was 14.6% (243/1666). Moreover, the percentage of infection in the 13 cultured species was much higher than that of the 39 wild species fish. In cultured fish, the average percentage of annual infection was 25.7% (141/548): 22.1% (36/163) for winter, 27.8% (40/144) for summer, 34.0% (48/141) for autumn, and 17.0% (17/100) for spring; while the average percentage of annual infection of wild fish was 9.1% (102/1118): 4.0% (9/228) for winter, 11.2% (32/285) for summer, 7.3% (22/301) for autumn, and 12.8% (39/304) for spring (Table 1). The results indicate that the percentage of infection of ISKNV-like viruses varied in different seasons.

Acute infection of ISKNV-like viruses could be found in orange-spotted grouper (*Epinephelus coioides*), red drum (*Sciaenops ocellatus*), large yellow croaker (*Pseudosciaena crocea*), banded grouper (*Epimephelus awoara*), and Malabar grouper (*Epinephelus malabaricus*). The mortality was up to 60–80%. They showed symptoms of depression, lethargy, pale body pigmentation, unresponsiveness, cessation of feeding, and gill pallor typical of that of ISKNV infection [9]. The result of 1-step PCR showed positive results for the viruses from these fish. Of the 52 species of fish (219), 47 species showed no clinical signs of disease, but ISKNV-like viruses could be detected by 2-step PCR. These fish were considered to be latently infected with the viruses.

Table 3. Host range of ISKNV-like viruses

Host	Isolates	Region	Host	Isolates	Region
A. latus	Alat[b]	Daya Bay	*L. argentimaculatus*	Larg[a]	Daya Bay
A. macrocephalus	Amac[a]	Daya Bay	*L. brevirostris*	Lbre[a]	Daya Bay
C. hainanensis	Chai[a]	Daya Bay	*L. insidiator*	Lins[a]	Daya Bay
C. kalla	Ckal[b]	Daya Bay	*L. johnii*	Ljoh[a]	Daya Bay
C. pachycentron	Cpac[a]	Daya Bay	*L. russelli*	Lrus[b]	Daya Bay
C. punctatus	Cpun[b]	Daya Bay	*L. sanguineus*	Lsan[a]	Daya Bay
C. sinicue	Csin[a]	Daya Bay	*O. argenteus*	Oarg[a]	Daya Bay
C. sexfasciatus	Csex[a]	Daya Bay	*P. crocea*	Pcro[b]	Sanya[c]
D. maruadsi	Dmar[a]	Daya Bay	*P. pictus*	Ppic[b]	Daya Bay
E. awoara	Eawo[b]	Daya Bay	*P. quadrilineatus*	Pqua[a]	Daya Bay
E. bleekeri	Eble[b]	Daya Bay	*S. commersoni*	Scom[a]	Daya Bay
E. chlorostigma	Echl[a]	Daya Bay	*S. japonicus*	Sjap[a]	Daya Bay
E. coioides	OSGIV[b]	Daya Bay	*S. leptolepis*	Slep[a]	Daya Bay
E. fasciatomaculatus	Efas[a]	Daya Bay	*S. macrocephalus*	Smac[a]	Daya Bay
E. malabaricus	Emal[b]	Daya Bay	*S. ocellatus*	Soce[b]	Daya Bay
E. merra	Emer[a]	Daya Bay	*T. mystax*	Tmys[a]	Daya Bay
E. tauvina	Etau[b]	Daya Bay	*T. xanthopterus*	Txan[a]	Daya Bay
G. spadiceus	Gspa[a]	Daya Bay	*U. russelli*	Urus[a]	Daya Bay

[a] The nucleotide sequences of these isolates were the same as that of ISKNV.
[b] The nucleotide sequences of these isolates were different from that of ISKNV.
[c] All isolates were collected from Daya Bay except for Pcro which was from Sanya.

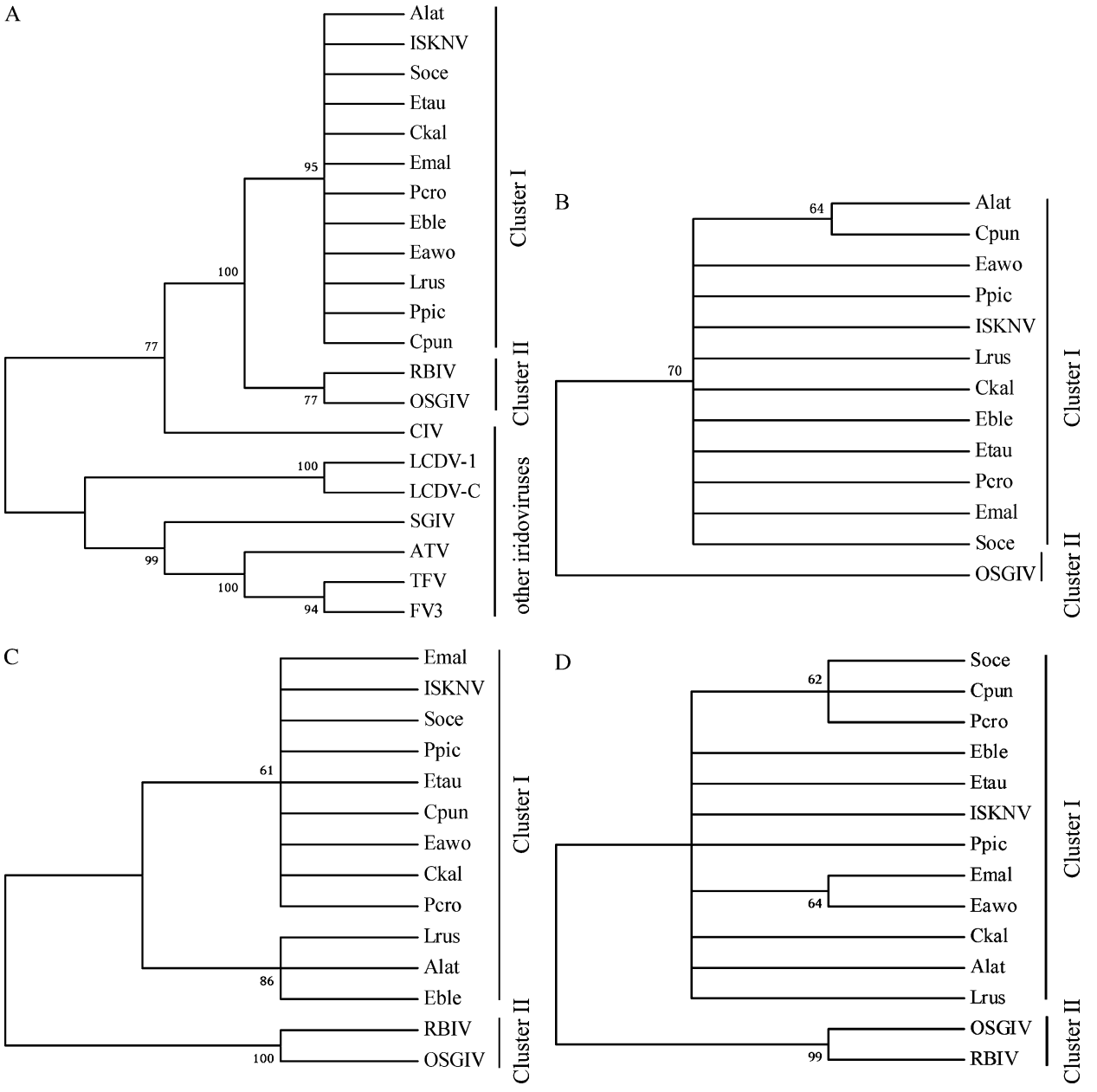

Fig. 2. Phylogenetic relationships of iridovirus obtained using 4 protein sequence alignments: **A** major capsid protein (MCP), **B** vascular endothelial growth factor (VEGF), **C** tumor necrosis factor receptor-associated protein (TNFR), **D** mRNA capping enzyme (Capping). The alignments were carried out by Clustal X 1.80 and the neighbor-joining trees obtained using MEGA2.1 were shown with the statistical support indicating the robustness of the inferred branching pattern as assessed using the bootstrap test. Accession numbers: **A** ATV, YP_003785; CIV, NP_149737; FV3, YP_031669; ISKNV, AAL98730; LCDV-1, NP_044812; LCDV-C, YP_025102; RBIV, AAS44553; SGIV, YP_164167; TFV, NP_572010; **B** ISKNV, AAL98772; **C** ISKNV, AAL98835; RBIV, AY532606; (D) ISKNV, AAL98788; RBIV, AAT71875

Sequence analysis and phylogenetic tree

To determine the taxonomic relationship of these ISKNV-like viruses, we compared the deduced amino acid sequence of the four putative genes of these viral isolates to that of ISKNV. The coding sequence of the MCP gene is 1359 bp in length, encoding 453 amino acid residues. With primers mF1 and mR1 in the 1-step PCR, the amplified product was about 1080 bp; with primers mF2 and mR2 (corresponding to two highly conserved regions with the MCP gene) in the 2-step PCR, a product of 562 bp was amplified. The results indicated that five cultured fish species were PCR positive with primers mF1 and mR1, whereas other fish species were confirmed to carry ISKNV-like viruses by 2-step PCR. Multiple sequence alignment of the deduced amino acid sequences of MCP (186 amino acid residues), VEGF (68 amino acid residues), TNFR (103 amino acid residues), and Capping (166 amino acid residues) revealed that the viruses from the detected fish were highly homologous. There was a 98% overall amino acid identity (with 7 amino acid differences) of MCP between ISKNV and the viral strain OSGIV from orange-spotted grouper. Similarly, high amino acid sequence identities of 88.6, 91.7 and 95.7% for TNFR, VEGF, and Capping, corresponding to 8, 4, and 7 amino acid differences, respectively, were observed between OSGIV and ISKNV.

In the analysis of the MCP, VEGF, Capping, and TNFR genes amplified from 40 of the fish species, they showed 100% nucleotide identity with ISKNV. So these 40 species of marine fish can be considered to be hosts of ISKNV. However, the viral gene sequences amplified from the rest (12 out of 52 species) were different from ISKNV at the nucleotide level although they had high homology with ISKNV. Moreover, the sequences of the four viral genes amplified from these 12 species were not identical, and strain variants existed (Table 3). Thus, the viral strain OSGIV might belong to a different species of ISKNV.

In addition, individuals from the same fish species could be infected by two different strains of ISKNV-like viruses, and different marine fishes could be the hosts of the same ISKNV-like virus. For example, in the cultured orange-spotted grouper, OSGIV was the causative agent of serious systemic diseases, while ISKNV could be detected in these fish without any symptoms of disease. Therefore, latent infection of ISKNV could occur in the orange-spotted grouper.

Based on the amino acid sequences of the MCP, VEGF, TNFR, and Capping genes, four phylogenetic trees were constructed (Fig. 2). The trees revealed that the ISKNV-like viruses can be grouped into two clusters: cluster I includes ISKNV and other isolates; cluster II includes OSGIV and RBIV, another megalocytivirus from Korea [7].

Discussion

In recent years, a growing number of megalocytiviruses have been isolated from different fish species. They are considered as causative agents of diseases that cause significant economic losses of the fish in aquaculture [1, 3–5, 9, 13, 14, 18–20, 23–25, 29]. In 2003, Chinchar et al. proposed a new genus in the family *Iridoviridae*, *Megalocytivirus*, recognizing ISKNV as the type species of this genus. In this proposed classification 5 distinct genera within the family *Iridoviridae* were established. These genera were *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystisvirus*, and *Megalocytivirus* [2].

The understanding of host range of a virus is important for the control of disease outbreaks. By molecular epidemiology study in 86 marine fish species, we have provided evidence that ISKNV-like virus could infect 52 natural hosts. The number of hosts was larger than that previously shown by experimental infection with ISKNV [11]. Of the experimentally infected fish, only the large-mouth bass (*Micropterus salmoides*) was highly susceptible to ISKNV infection. ISKNV did not cause mortality in grass carp (*Ctenopharyngodon idella*) infected with the virus by bathing and/or intraperitoneal infection [11]. Similarly, no deaths or clinical or histopathological signs were found in 18 other species exposed to virus by immersion or injection [11]. During our investigation, acute infection of ISKNV-like virus was observed in five cultured species (*E. coioides*, *S. ocellatus*, *P. crocea*, *E. awoara*, and *E. malabaricus*), but latent infection occurred in other species.

Water temperature was a major factor influencing the outbreaks of ISKNV [31]. Outbreaks of ISKNV-like virus usually occurred from May to November (20–32 °C). Acute infection by ISKNV-like virus in five cultured species usually occurred in summer and autumn, with 60–80% overall mortality. In spring and winter, ISKNV-like virus could be detected by 2-step PCR in fish with no external clinical signs. This suggested that fish species that are latently infected by virus might serve as carriers of ISKNV-like virus at temperatures below 20 °C.

Conserved genes, used commonly in molecular epidemiological studies to unravel the evolutionary relationships by phylogenetic analysis, were too homologous to use in the case of iridovirus [12, 16]. MCP is a suitable target for phylogenetic studies as it is highly conserved in iridoviruses [12, 27]. The MCP sequences obtained from these ISKNV-like viruses showed a high degree of identity with ISKNV and RBIV. On the other hand, we used the other three genes (VEGF, Capping and TNFR) that were as conserved as the MCP gene to classify new ISKNV isolates. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a potent mitogen in vasculogenesis and angiogenesis with a unique specificity for endothelial cells [20, 26]. The pivotal role of VEGF in vasculogenesis was exemplified in recent studies on targeted inactivation of the VEGF gene in mice that resulted in fatal deficiencies in vascularization [8]. An important insight from the results came from the gene product analysis. From the phylogenetic tree, we found that the MCP is highly conserved. The sequences were the same in 11 fish species detected with the MCP primers, but differences were identified in the fish, using primers to the VEGF, TNFR, and Capping genes.

Phylogenetic analysis also showed that two clusters were observed in the ISKNV-like viruses from the infected fish (Fig. 2). Cluster I included the ISKNV-like isolates that were more related to ISKNV, and they should be considered variants of ISKNV. Cluster II consisted of OSGIV from orange-spotted grouper and RBIV, which were different from ISKNV. ISKNV, RBIV and OSGIV were considered to belong to three different species of megalocytivirus, but more evidence is needed to confirm this.

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