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Phylogenetic analysis of the major capsid protein gene of iridovirus isolates from cultured flounders *Paralichthys olivaceus* in Korea

Jeong Wan Do[1], Seung Ju Cha[2], Jong Su Kim[2], Eun Jeong An[2], Nam Sil Lee[1], Hee Jung Choi[1], Chan Hwei Lee[1], Mi Seon Park[1], Jin Woo Kim[1], Yi Cheong Kim[1], Jeong Woo Park[2,*]

[1]Pathology Division, National Fisheries Research & Development Institute, Kijang, Busan 619-902, Korea
[2]Department of Biological Sciences, University of Ulsan, Ulsan 680-749, Korea

ABSTRACT: In 2003, 13 isolates of iridovirus were obtained from cultured flounders *Paralichthys olivaceus* during epizootics in Korea. The full open reading frames (ORFs) encoding the major capsid protein (MCP) (1362 bp) from the 13 flounder iridoviruses (FLIVs) were sequenced and the deduced amino acid sequences were phylogenetically analyzed. Phylogenetic analysis of the MCP revealed that all 13 FLIVs were the same species as rock bream iridovirus (RBIV), red sea bream iridovirus (RSIV), and infectious spleen and kidney necrosis virus (ISKNV), and were grouped into an unknown genus which was different from the 2 genera known to infect fish, *Ranavirus* and *Lymphocystivirus*. This is the first report on the isolation and phylogenetic analysis of the iridovirus of unknown genus from flounders during epizootics.

KEY WORDS: Iridovirus · Flounder · Major capsid protein · MCP

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INTRODUCTION

Iridoviruses are large cytoplasmic DNA viruses with an icosahedral morphology (Williams 1996); the family *Iridoviridae* consists of 4 genera including *Iridovirus*, *Chloriridovirus*, *Ranavirus*, and *Lymphocystivirus*. Iridoviruses infect a wide variety of cultured fish (Wolf 1988, Hetrick & Hedrick 1993). According to the International Committee on Taxonomy of Viruses (ICTVdB) (www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/), fish iridoviruses are members either of the genus *Lymphocystivirus* or *Ranavirus*. While iridoviruses belonging to the genus *Lymphocystivirus* cause the development of clusters of extremely hypertrophied fibroblasts called lymphocystis cells, other iridoviruses belonging to the genus *Ranavirus* cause systemic diseases in infected animals and are associated with high morbidity and mortality (Ahne et al. 1989, Eaton et al. 1991, Hedrick & McDowell 1995, Hedrick et al. 1992, Langdon et al. 1986, 1988, Moody & Owens 1994, Pozet et al. 1992). However, a taxonomic analysis of putative proteins suggests the presence of fish iridoviruses which belong neither to the genus *Ranavirus* nor to the genus *Lymphocystivirus* (He et al. 2001, Hyatt et al. 2000, Do et al. 2004). In addition, genetic and phenotypic variants within iridoviruses (Goldberg et al. 2003, Hyatt et al. 2000, Williams & Cory 1993) suggest the possibility of the presence of diverse variants within the fish iridoviruses.

Iridoviral epizootics have occurred recently among various kinds of cultured fish in Korea (Jung & Oh 2000, Kim et al. 2002, Do et al. 2004). In 2003, iridoviral epizootics occurred in flounders cultured in the southern part of the Korean peninsula and 13 iridoviruses were isolated from cultured flounders. In the present study, we compared the amino acid sequences of the major capsid protein (MCP) of 13 flounder iridoviruses (FLIVs) to those of other iridoviruses. Our data revealed that all of the 13 FLIVs were the same species as rock bream iridovirus (RBIV), red sea bream irido-

*Corresponding author. Email: jwpark@ulsan.ac.kr

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virus (RSIV), and infectious spleen and kidney necrosis virus (ISKNV) and were classified into a new genus, cell hypertrophy iridoviruses, proposed by He et al. (2001).

MATERIALS AND METHODS

Viruses. In 2003, epizootics occurred among juvenile of flounders cultured in the southern part of the Korean peninsula, and losses of up to 80% on affected farms were recorded. Thirteen iridoviruses were obtained from moribund flounders (3 to 11 g) cultured at 13 different fish farms located in 5 different regions of Korea (Fig. 1). Sources of the fish iridoviruses used in this study are listed in Table 1.

Table 1. Sources of iridoviruses isolated from cultured flounders in Korea. FLIV: flounder iridovirus. Letters after geographical regions are fish farm initials

Isolate	Body weight of flounder (g)	Date (yr/mo)	Geographic region of fish farm (Korea)
FLIV-DS1	3–4	2003/11	Ulsan DS
FLIV-DS2	4–5	2003/12	Ulsan DS
FLIV-EJ	6–7	2003/10	Busan EJ
FLIV-JJ	6–7	2003/11	Jeju
FLIV-JHJ	6–7	2003/11	Jeju JHJ
FLIV-JJY	6–7	2003/11	Jeju JJY
FLIV-JSY	6–7	2003/11	Jeju JSY
FLIV-MI	3–4	2003/11	Busan MI
FLIV-PH	6–7	2003/10	Pohang
FLIV-SS	7–11	2003/11	Ulsan SS
FLIV-WD1	6–7	2003/10	Ulsan WD
FLIV-WD2	7–11	2003/11	Ulsan WD
FLIV-YG	6–7	2003/11	Yeonggwang

Histological examination. After external examination, an anatomical examination was carried out, and tissue samples from the kidney, spleen, liver, and gill were fixed with Bouin's solution. Fixed tissues were dehydrated in alcohol (70 and approx. 100%) and were embedded in paraffin. Tissue sections (4 µM thick) were stained with Harris' hematoxylin and eosin (H&E) solution and then examined with a light microscope (Carl Zeiss).

Ultra structural examination. Small pieces of spleen were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.2) at 4°C for 4 h, and post-fixed with 1% osmium tetroxide in phosphate buffer (pH 7.2) at room temperature for 2 h. After post-fixation, cells were dehydrated in ethanol and embedded in Epon 812. Thin sections were cut by an ultramicrotome (LKB), stained with uranyl acetate and lead citrate solution, and observed with a JEOL 1200 EX-2 transmission electron microscope (TEM).

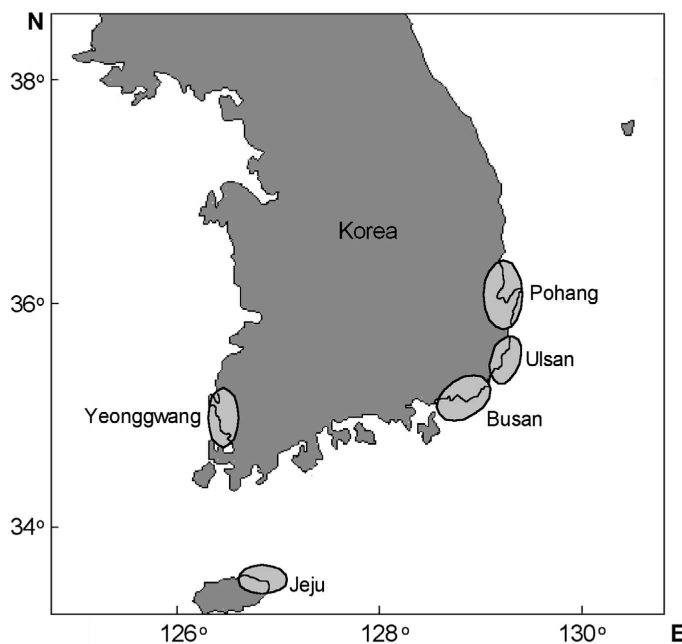

Fig. 1. Location of fish farms where epizootics occurred and iridoviruses were isolated

PCR amplification of the MCP gene. The MCP genes of iridovirus were amplified from virus-infected spleen DNA using PCR. Spleen from infected fish was homogenized in a lysis buffer (4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA, pH 7.4). After centrifugation at 1700 × g for 10 min, the supernatant was treated with proteinase K (0.2 mg ml^{-1}) and sarcosyl (1%) at 45°C for 3 h, followed by phenol/chloroform extraction and ethyl alcohol precipitation. The genomic DNA in the pellet was dissolved in TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) and used as a template for PCR. PCR primers were designed from nucleotide sequences of the MCP of rock bream iridovirus (RBIV) (AY532606, Do et al. 2004) and lymphocystis disease virus K1 (LCDV-K1) (AY303804) (Table 2). The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 35 cycles of 92°C for 30 s, 58°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. The amplified PCR products were cloned into a pGEM-T vector (Promega), and sequencing was performed at the Immunomodulation Research Center, Korea, on an automatic DNA sequencer (Applied Biosystems) according to the dye terminator procedure with forward and reverse primers and overlapping primers designed from the sequencing results.

DNA sequence analysis. The DNA and the deduced amino acid sequences were compared with the GenBank/EMBL databases using the basic local alignment search tool (BLAST). Sequences were aligned using CLUSTAL W (Thompson et al. 1994), after which the phylogenetic tree was constructed with TreeView

Table 2. PCR primers used in gene amplification. LCDV: lymphocystis disease virus; RBIV: rock bream iridovirus; F: forward primers; R: reverse direction primers. p: primer for partial open reading frame (ORF) amplication; f: primer for full length ORF amplication

Virus	Primer	Sequence
LCDV	LCDV-pMCP-F	5′-TTGACAGCAGGCGATTTAGA-3′
	LCDV-pMCP-R	5′-GCAATTCCACCGTCAAAGAT-3′
RBIV	RBIV-pMCP-F	5′-GAGGAGGTGTCGGTGTCATT-3′
	RBIV-pMCP-R	5′-GGCATAGTCTGACCGTTGGT-3′
	RBIV-fMCP-F	5′-GAAAAACGAGGCCGATCATA-3′
	RBIV-fMCP-R	5′-TACGCTATGGCCACAATTCA-3′

(Page 1996). The phylogenetic relationships among species were determined using the neighbor-joining method (Saitou & Nei 1987) and the reliability of the NJ tree was inferred using the Felsenstein (1985) bootstrap method with 1000 replicates

Nucleotide sequence accession number. The following nucleotide sequence data reported in this paper were deposited in the GenBank (GenBank accession numbers: FLIV-DS1, AY633980; FLIV-DS2, AY633981; FLIV-MI, AY633982; FLIV-SS, AY633983; FLIV-YG, AY633984; FLIV-WD2, AY633985; FLIV-WD1, AY633986; FLIV-EJ, AY633987; FLIV-JJ, AY633988; FLIV-JSY, AY633989; FLIV-JJY, AY633990; FLIV-JHJ, AY633991; FLIV-PH, AY633992).

RESULTS AND DISCUSSION

In 2003, wide spread epizootics occurred among juvenile flounders cultured at 13 fish farms located in 5 different regions in Korea (Fig. 1, Table 1). The net losses in affected populations were up to 80%. The diseased fish were lethargic and showed severe anemia, petechiae of gills, abdominal distension, and enlargement of spleens. Histopathology revealed hypertrophied heteromorphic cells in the spleens, kidneys, and gills. Parasites and bacteria were not consistently recovered from the organs of the diseased fish. Electron microscopic observation of an ultrathin section of the spleens of the diseased fish revealed the presence of cytoplasmic inclusion body that displaced the nucleus to the periphery of the cell. The marginally located nucleus was irregular in shape. Within the inclusion body, both complete and incomplete icosahedral nucleocapsids were observed. The size of virus particles

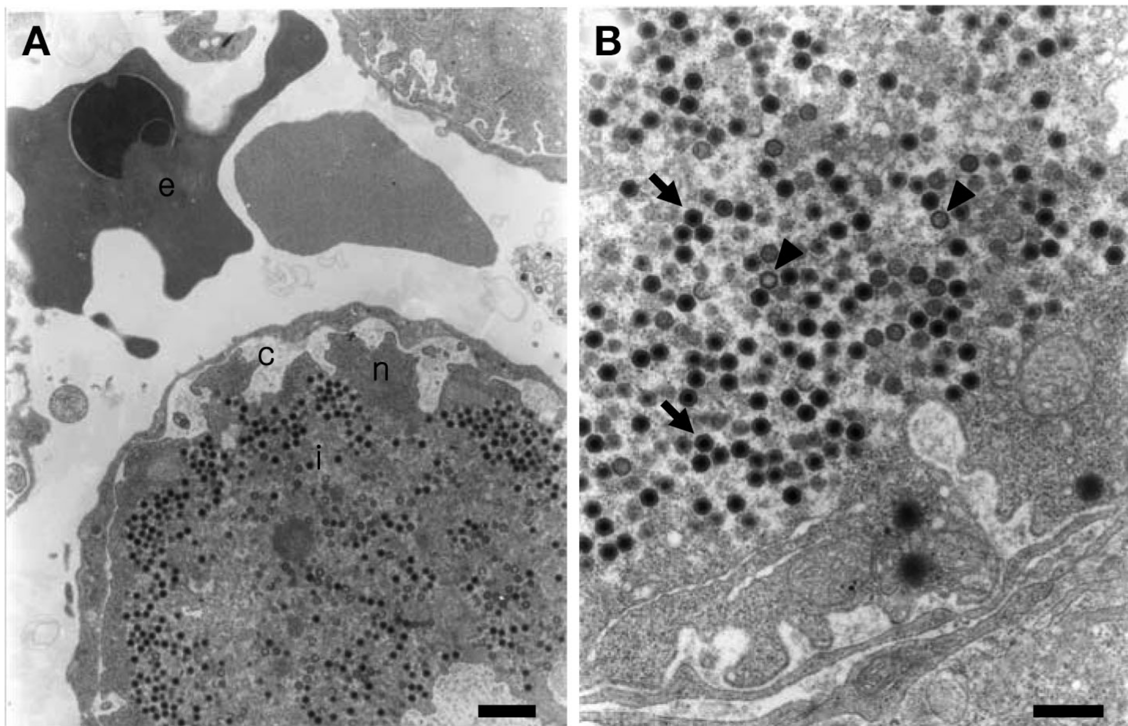

Fig. 2. *Paralichthys olivaceus.* Transmission electron micrographs of virus particles in cells of spleen from diseased flounders. (A) Virus particles in an inclusion body within the cytoplasm (bar = 1μm). (B) A high magnification of virus particles in an inclusion body (scale bar = 500 nm). Arrows and arrowheads indicate complete and incomplete nucleocapsids, respectively. c: cytoplasm of host cell; e: erythrocyte; i: inclusion body; n: nucleus of host cell

Table 3. Comparative analysis of the major capsid protein (MCP) of iridoviruses. Homology is given as percentage of amino acid sequence identity. See Table 4 for explanations of isolate abbreviations and GenBank accession numbers for protein sequences

Virus isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
1. FLIV-DS1	100																																											
2. FLIV-DS2	99	100																																										
3. FLIV-MI	99	99	100																																									
4. FLIV-SS	99	99	98	100																																								
5. FLIV-YG	99	99	98	98	100																																							
6. FLIV-WD1	98	98	97	97	97	100																																						
7. FLIV-WD2	98	98	97	97	97	98	100																																					
8. FLIV-EJ	99	99	98	98	98	98	98	100																																				
9. FLIV-JJ	99	99	99	99	99	98	98	99	100																																			
10. FLIV-JSY	99	99	98	98	98	98	97	99	99	100																																		
11. FLIV-JJY	99	99	99	99	99	98	98	99	100	99	100																																	
12. FLIV-JHJ	99	99	99	99	99	98	98	99	99	99	99	100																																
13. FLIV-PH	99	99	99	99	99	98	98	99	100	99	100	99	100																															
14. SBIV-KOR-TY	97	97	97	97	97	97	96	98	98	97	98	97	98	100																														
15. RBIV-KOR-TY1	97	97	97	96	96	96	96	98	97	97	97	97	97	99	100																													
16. RBIV-KOR-TY2	96	96	96	96	96	95	95	96	96	96	96	96	96	98	98	100																												
17. RBIV-KOR-TY3	95	95	95	94	94	94	94	96	95	95	95	95	95	97	97	96	100																											
18. RBIV-KOR-TY4	97	97	97	97	97	97	96	98	98	97	98	97	98	99	99	98	97	100																										
19. RBIV-KOR-GJ	95	95	95	95	95	95	94	96	96	95	96	95	96	97	97	96	99	97	100																									
20. RBIV-KOR-YS	95	95	95	95	95	95	94	96	96	95	96	95	96	97	97	96	99	97	100	100																								
21. RBIV-KOR-CS	99	99	98	98	98	98	98	99	99	99	99	99	99	98	97	96	95	98	96	96	100																							
22. RSIV-KOR-TY	96	96	96	96	96	96	96	97	96	96	96	96	96	98	98	97	96	98	96	96	96	100																						
23. RFIV-KOR-TY	96	96	96	96	96	96	95	97	97	96	97	96	97	98	98	97	96	98	96	96	97	97	100																					
24. RSIV	97	97	97	96	97	96	96	98	97	97	97	97	97	99	99	98	97	99	97	97	97	98	98	100																				
25. GSDIV	98	97	97	97	97	97	96	98	98	98	98	97	98	99	99	98	97	99	97	97	98	98	98	99	100																			
26. DGIV	97	97	97	97	97	97	96	98	98	97	98	97	98	98	98	96	96	98	96	96	98	97	97	98	98	100																		
27. SBIV	98	97	97	97	97	97	96	98	98	98	98	97	98	99	99	98	97	99	97	97	98	98	98	99	100	98	100																	
28. ALIV	98	97	97	97	97	97	96	98	98	98	98	97	98	98	98	97	96	98	96	96	98	97	97	98	98	99	98	100																
29. ISKNV	98	97	97	97	97	97	96	98	98	98	98	97	98	98	98	97	96	98	96	96	98	97	97	98	98	99	98	100	100															
30. TBIV	99	99	99	99	99	98	98	99	100	100	100	99	100	97	97	96	95	97	95	95	99	96	96	97	98	97	98	98	98	100														
31. LCDV-1	49	49	49	49	49	49	49	49	49	49	49	49	49	49	49	49	48	49	48	48	49	49	49	49	49	50	49	50	50	50	100													
32. LCDV-K1	48	48	48	48	48	48	48	49	49	48	49	48	49	48	48	48	47	48	47	47	49	48	49	49	49	49	49	49	49	49	87	100												
33. TFV	47	47	47	47	47	47	47	47	47	47	47	47	47	48	48	48	47	47	47	47	47	47	48	48	48	48	48	48	48	46	52	51	100											
34. ATV	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	46	47	47	47	47	47	47	47	47	47	47	48	48	46	52	51	96	100										
35. FV3	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	46	47	46	46	47	47	48	47	47	47	47	48	48	46	52	51	98	95	100									
36. EHNV	47	47	47	47	47	47	47	47	47	47	47	46	47	47	47	47	46	47	46	46	47	47	47	47	47	47	47	47	47	46	52	50	96	98	96	100								
37. BIV	47	47	47	47	47	47	47	47	47	47	47	46	47	47	47	47	46	47	46	46	47	47	47	47	47	47	47	47	47	46	51	50	98	96	98	97	100							
38. RRV	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	47	46	47	47	47	47	47	47	47	47	47	47	48	48	46	52	51	96	100	95	98	96	100						
39. GIV	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	46	46	45	52	54	72	72	72	72	73	72	100					
40. CIV	46	46	46	46	46	46	46	47	47	46	47	46	47	47	47	47	45	47	46	46	47	47	47	47	47	47	47	47	47	46	54	53	46	45	45	45	45	45	46	100				
41. CZIV	45	45	45	45	45	45	45	45	45	45	45	45	45	46	46	46	45	46	45	45	45	46	45	46	46	46	46	46	46	46	50	52	45	44	45	45	45	45	43	69	100			
42. WIV	44	44	43	43	44	43	44	44	44	44	44	43	44	44	44	44	43	44	43	43	44	44	44	44	44	44	44	44	44	45	50	51	44	43	44	43	43	43	43	66	88	100		
43. SIV	44	44	43	43	43	43	44	44	44	44	44	43	44	45	44	45	43	44	43	43	44	44	44	44	45	45	45	45	45	45	50	50	44	43	44	43	43	43	42	65	88	90	100	
44. TIV	44	44	44	44	44	44	44	44	44	44	44	44	44	45	45	45	43	45	44	44	44	45	44	45	45	45	45	45	45	45	50	51	44	43	44	44	44	43	43	67	89	92	95	100

Group 4 (isolates 1–30)

Group 3 (genus *Lymphocystivirus*) (isolates 31–32)

Group 2 (genus *Ranavirus*) (isolates 33–39)

Group 1 (genus *Iridovirus*) (isolates 40–44)

measured from vertex to vertex was about 140 nm (Fig. 2). All observations coincided with the characteristics of iridoviral diseases.

The family *Iridoviridae* consists of 4 genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus* and *Lymphocystivirus* (Willis, 1990). There have been many reports of iridoviral infection in flounders, and their causative agents have been identified as lymphocystiviruses, which are members of the genus *Lymphocystivirus* (Darai et al. 1983, Tidona & Darai 1997). To determine whether or not the causative agent of the epizootics among flounder cultured in Korea is the lymphocystivirus, PCR was performed using PCR primers specific for the MCP of LCDV designed from the nucleotide sequences in GenBank (AY303804) (Table 2). However, we could not amplify the MCP gene of LCDV from any of the spleen samples collected from the 13 fish farms (data not shown).

Wide-spread epizootics have occurred recently among various species of cultured fish, such as rock bream *Oplegnathus fasciatus*, red sea bream *Chrysophrys major*, sea bass *Lateolabrax japonicus*, and rockfish *Sebastes schlegeli* in Korea and we found that a new genus, cell hypertrophy iridoviruses, was the causative agent of the epizootics of iridoviral diseases there (Do et al. 2004). Thus, in order to amplify partial open reading frames (ORF) of MCP, PCR primers, RBIV-pMCP-F and RBIV-pMCP-R, were designed from the nucleotide sequences of the MCP of a Korean isolate, RBIV (AY532606) (Do et al. 2004) (Table 2), and PCR was performed to detect the presence of the iridovirus of the new genus. We found that the PCR amplified the MCP gene in all of the 13 spleen samples collected from the 13 fish farms (data not shown).

In order to determine the genetic characteristics of the 13 FLIVs, a full-length ORF of the MCP of the 13 FLIVs was amplified by PCR. PCR primers, RBIV-fMCP-F and RBIV-fMCP-R, were designed from the nucleotide sequences in the GenBank nucleotide sequence database of RBIV (AY532606) (Table 2). Here we used the PCR to directly amplify the MCP DNA sequences from uncultured (frozen) spleen obtained from moribund fish in order to avoid any random genetic changes accumulated during viral passage in cell cultures. The PCR products were cloned into a pGEM-T vector and the nucleotide sequence of the cloned DNA fragments was determined by using M13 forward and M13 reverse sequencing primers in the vector and internal sequencing primers. Every nucleotide position in the MCP gene was determined at least twice from each DNA strand. The MCP ORF was 1362 bp in length and codes for a protein of 453 amino acids.

To determine the relationship between the 13 FLIVs and previously reported iridoviruses, we compared the full amino acid sequences of the MCP of 13 FLIVs to those of 31 other iridoviruses available in GenBank including *Chilo* iridescent virus (CIV, type-species of the genus *Iridovirus*), FV3 (type-species of genus *Ranavirus*), and LCDV-1 (type-species of genus *Lymphocystivirus*). As shown in Table 3 (see Table 4 also), the 13 FLIVs showed 97 to 100%

Table 4. Viral isolates and GenBank accession numbers for protein sequences in Table 3 and Fig. 3

Virus isolate	GenBank accession no.
ALIV, African lampeye iridovirus	AAP37442
ATV, *Ambystoma tigrinum* virus	AAP33191
BIV, Bohle iridovirus	AAO32316
CIV, *Chilo* iridescent virus	NP_149737
CZIV, *Costelytra zealandica* iridescent virus	O39164
DGIV, Dwarf gourami iridovirus	AAP37441
EHNV, Epizootic haematopoietic necrosis virus	AAO32315
FLIV-DS1, Flounder iridovirus	AY633980
FLIV-DS2	AY633981
FLIV-EJ	AY633987
FLIV-JJ	AY633988
FLIV-JHJ	AY633991
FLIV-JJY	AY633990
FLIV-JSY	AY633989
FLIV-MI	AY633982
FLIV-PH	AY633992
FLIV-SS	AY633983
FLIV-WD1	AY633986
FLIV-WD2	AY633985
FLIV-YG	AY633984
FV3, frog virus 3	Q67473
GIV, grouper iridovirus	AAM00286
GSDIV, grouper sleepy disease iridovirus	AAP37443
ISKNV, infectious spleen and kidney necrosis virus	NP_612228
LCDV-1, lymphocystis disease virus 1	NP_044812
RBIV-KOR-CS, rock bream iridovirus	AY532611
RBIV-KOR-GJ	AY532609
RBIV-KOR-TY1	AY532606
RBIV-KOR-TY2	AY533035
RBIV-KOR-TY3	AY532067
RBIV-KOR-TY4	AY532608
RBIV-KOR-YS	AY532610
RFIV-KOR-TY, rockfish iridovirus	AY532614
RRV, Regina ranavirus	YP_003785
RSIV, red sea bream iridovirus	BAC66968
RSIV-KOR-TY	AY532612
SBIV, sea bass iridovirus	BAC77297
SBIV-KOR-TY	AY532613
SIV, Simulium iridescent virus	P22166
TBIV, turbot iridovirus	BAD12494
TFV, Tiger frog virus	NP_572010
TIV, Tipula iridescent virus	P18162
WIV, Wiseana iridescent virus	O39163

amino acid sequence identity to each other and 94 to 100% identity to 17 iridoviruses such as SBIV-KOR-TY (sea bass iridovirus), RBIV-KOR-TY1, RBIV-KOR-TY2, RBIV-KOR-TY3, RBIV-KOR-TY4, RBIV-KOR-GJ, RBIV-KOR-YS, RBIV-KOR- CS, RSIV-KOR-TY, RFIV-KOR-TY (rockfish iridovirus), RSIV, GSDIV (grouper sleepy disease iridovirus), DGIV (dwarf gourami iridovirus), SBIV, ALIV (African lampeye iridovirus), ISKNV, and TBIV (turbot iridovirus). The high degree of sequence identity suggests that the 13 FLIVs are the same species as the other 17 iridoviruses. However, the 13 FLIVs showed 46 to 49% amino acid sequence identity to 3 type-species: 46–47% to CIV, 47% to FV3, and 49% to LCDV-1 (see Table 4 for viral isolate abbreviation explanations and GenBank accession numbers). In the MCP tree, the iridoviruses used in the multiple alignments were subdivided into 4 groups: group 1, iridoviruses including CIV; group 2, ranaviruses including FV3; group 3, lymphocystiviruses including LCDV-1; group 4, unassigned viruses which did not include any of the 3 type species (Fig. 3A). Hyatt et al. (2000) have suggested the presence of 2 groups of iridoviruses, erythrocytic iridoviruses and cell hypertrophy iridoviruses, which are not closely related to any of the known genera. Our MCP tree result supports the presence of a new genus in the family *Iridoviridae*, and the members of group 4 may belong to a new genus, tentatively referred to as the cell hypertrophy iridoviruses (He et al. 2001). All the 13 FLIVs clustered within group 4 (Fig. 3A). These findings indicate that all the flounder isolates were members of the new genus, cell hypertrophy iridoviruses, and that this new genus may be responsible for the epizootics of iridoviral diseases in flounders in Korea.

In 2000 and 2002 we isolated 10 iridoviruses from rock bream, red sea bream, rockfish, and sea bass. Even though all the iridoviruses isolated from cultured fish in Korea were grouped into a new genus, cell hypertrophy iridoviruses, multiple strain variants existed among the iridoviruses, based on amino acid sequence variation (data not shown). Thus, we reexamined the phylogenetic positions of the 13 iridoviruses from flounders in Korea by comparing the full-length 453 amino acid sequences of the MCP of the 13 isolates with those of the previously isolated 10 Korean isolates and other iridoviruses within

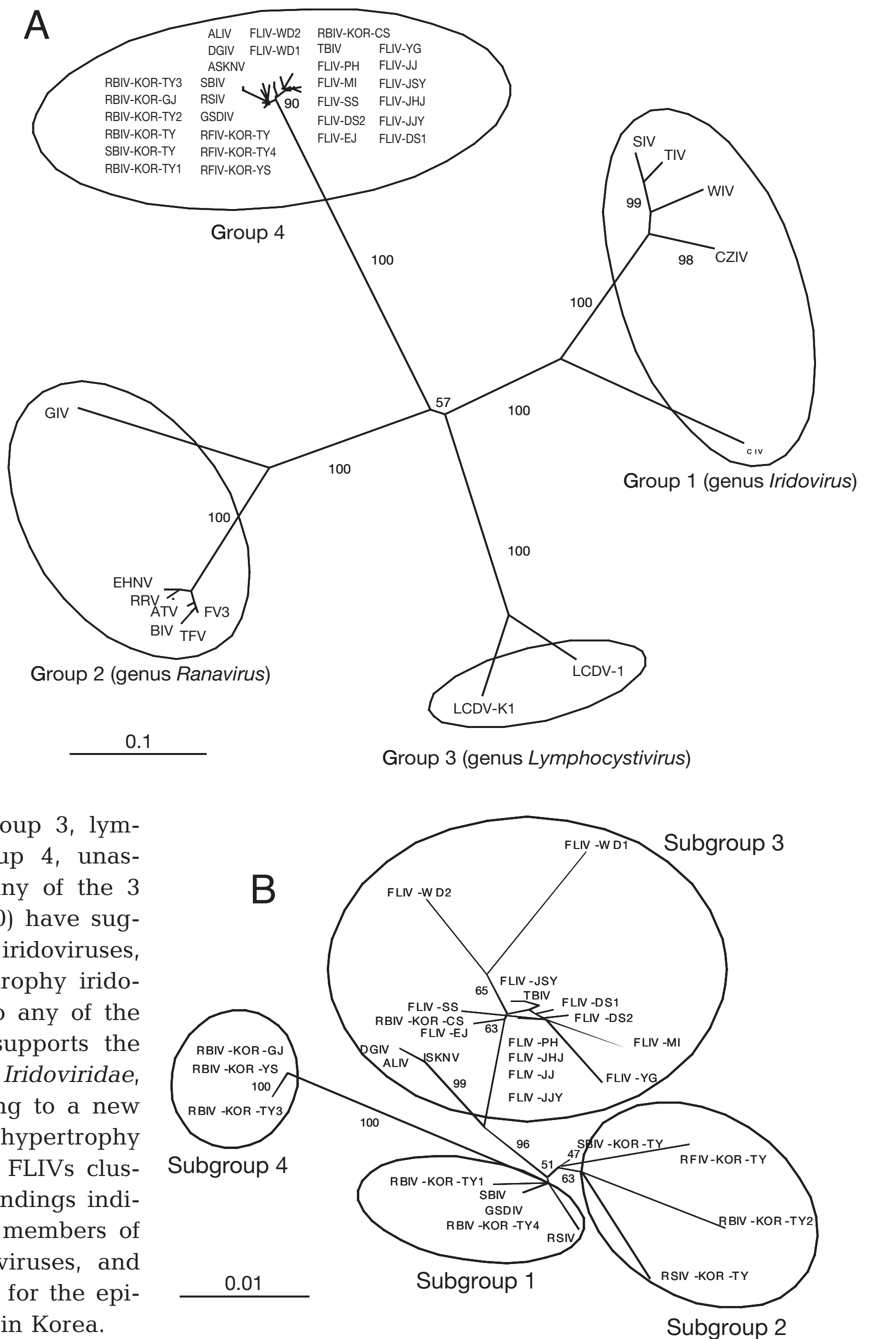

Fig. 3. Phylogenetic analysis of iridovirus major capsid protein (MCP). (A) Amino acid sequences of the MCP of 13 flounder isolates of iridovirus were compared with those from 31 iridoviruses. See Table 4 for GenBank accession numbers for the protein sequences and isolate abbreviations shown. (B) Phylogenetic position of iridovirus isolates of Group 4 from (A). The tree was derived by comparing the amino acid sequences of full-length 453 amino acids of MCP. GenBank accession numbers for protein sequences are the same as in (A). The numbers indicate the percentage bootstrap support for each node from 1000 replicates. The distances are proportional to the relative sequence deviations between individual amino acid sequences. The phylogenetic analyses were carried out using the ClustalW program

group 4. Iridoviruses within group 4 could be further divided into 4 distinct subgroups (Fig 3B). While the 10 iridovirus strains isolated in 2000 and 2002 from rock bream, red sea bream, rockfish, and sea bass were segregated into 4 different subgroups, all of the 13 iridovirus strains isolated from flounder in 2003 were segregated into one subgroup, subgroup 3 (Fig. 3B).

The 13 fish farms were geographically distant from each other and included Jeju, an island located in the southernmost region of the Korean peninsula (Fig. 1). However, the epizootics occurred simultaneously both on the Korean mainland and on Jeju island; what is more, the 13 FLIVs showed 97 to 100% amino acid sequence identity to each other (Table 3). This suggests that all of the 13 isolates of iridovirus have a single origin. In Korea, there are several flounder hatcheries and eggs and juveniles from these hatcheries are frequently transferred to other fish farms over a wide area. Thus, it is highly possible that a strain of iridovirus had been introduced originally into one or a few flounder hatcheries and that the iridovirus may then have spread rapidly through a transfer of fish from one fish farm to another. At present, the origin of the iridovirus strains infecting flounders is uncertain. However, based on the MCP tree, we can speculate about the possible source of the 13 flounder isolates. Besides the 13 flounder isolates, there were 2 more iridovirus strains isolated in Korea within subgroup 3; RBIV-KOR-CS from rock bream (GenBank accession number, AY532611) and turbot iridovirus (TBIV) (GenBank accession number, BAD12494). In addition, in subgroup 3, there were 3 foreign iridovirus strains; ISKNV from mandarin fish *Synchiropus chuatsi*, China (He et al. 2001), DGIV from dwarf gourami *Colisa lalia*, Malaysia, and ALIV from African lampeye *Aplocheilichthys normani*, Sumatra Island (Sudthongkong et al. 2002). In Korea, various species of fish are imported from foreign countries, especially from China and Japan, and more than 2 species of fish are commonly cultured in the same geographic locations; even in the same fish farm. It is likely, therefore, that an iridovirus strain was introduced into Korea via imported rock bream and/or turbot which then spread to flounders cultured in hatcheries by cross-species transmission.

In this study, we isolated 13 FLIVs which are the same species as RBIVs, RSIV, and ISKNV from diseased flounders during wide-spread epizootics in Korea. A phylogenetic analysis of the 13 FLIVs, based on the amino acid sequences of the MCP, revealed that all of the 13 FLIVs clustered within one group, a new genus, tentatively referred to as the cell hypertrophy iridoviruses. Recently, in Korea, we have observed a rapid spread of the iridovirus of this new genus among various species of cultured fish including rock bream, red sea bream, rockfish, sea bass as well as flounders. Thus, it seems urgent that a further spread of this iridovirus species to other fish species and other geographic regions be prevented and that reagents be developed to control iridoviral diseases in cultured fish.

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