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Molecular characterization and genogrouping of VP1 of aquatic birnavirus GC1 isolated from rockfish *Sebastes schlegeli* in Korea

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The cDNA nucleotide sequence of genome segment B encoding the VP1 protein was determined for the aquatic birnavirus GC1 isolated from the rockfish *Sebastes schlegeli* in Korea. The VP1 protein of GC1 contains a 2,538 bp open reading frame, which encodes a protein comprising 846 amino acid residues that has a predicted MW of 94 kDa. The sequence contains 6 potential Asn-X-Ser/Thr motifs. Eight potential Ser phosphorylation sites and 1 potential Tyr phophorylation site were also identified. GC1 contains the Leu-Lys-Asn (LKN) motif instead of the typical Gly-Asp-Asp (GDD) motif found in other aquatic birnaviruses. We also identified the GLPYIGKT motif, the putative GTP-binding site at amino acid position 248. In total, the VP1 regions of 22 birnavirus strains were compared for analyzing the genetic relationship among the family *Birnaviridae*. Based on the deduced amino acid sequences, GC1 was observed to be more closely related to the infectious pancreatic necrosis virus (IPNV) from the USA, Japan, and Korea than the IPNV from Europe. Further, aquatic birnaviruses containing GC1 and IPNV have genogroups that are distinct from those in the genus *Avibirnaviruses* and *Entomo-birnaviruses*. The birnavirusstrains were clustered into 5 genogroups based on their amino acid sequences. The marine aquatic birnaviruses (MABVs) containing GC1 were included in the MABV genogroup; the IPNV strains isolated from Korea, Japan, and the USA were included in genogroup 1 and the IPNV strains isolated primarily from Europe were included in genogroup 2. Avibirnaviruses and entomobirnaviruses were included in genogroup 3 and 4, respectively.

Keywords: aquatic birnavirus, GC1, genetic characterization, rockfish *Sebastes schlegeli*, VP1

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Introduction

Members of the family *Birnaviridae* have 2-segmented genomes - A and B. This family comprises 3 main genera, including the genus *Aquabirnavirus*, *Avibirnavirus*, and *Entomobirnavirus* [4,19]. The type species of the genus *Aquabirnavirus* is the infectious pancreatic necrosis virus (IPNV); the genus comprises marine aquatic birnaviruses (MABV) of fish and shellfish [3]. Other members of the family *Birnaviridae* include infectious bursal disease virus (IBDV) belongs to the genus *Avibirnavirus*, and Drosophila X virus (DVX) that belongs to the genus *Entomobirnavirus*. Aquatic birnaviruses are the largest and most diverse group of viruses within the family *Birnaviridae*. The first reported MABV was isolated from the yellowtail *Seriola quinqueradiata* in Japan [22], other MABVs have been subsequently isolated from various marine fishes in Korea and Japan, and their characteristics have been investigated [7,8,14,18,23,24]. The genome segment B of birnaviruses encodes the VP1 protein, which is the presumptive virion-associated RNA-dependent RNA polymerase (RdRp) [13,15]. Some researchers reported the characteristics of VP1 and compared the VP1 region among birnaviruses [4, 25]. They identified several conserved domains associated with RdRps and GTP-binding proteinsin the IPNV strains; these domains were the same as those in other RNA viruses. However, they also discovered that the typical Gly-Asp-Asp (GDD) motif that is found in all RNA viruses was absent in the VP1 region of some IPNV [4] IBDV, and DXV [2] strains.

The physical, antigenic, and genetic features of the VP2/NS junction region of the aquatic birnavirus GC1 isolated from the rockfish *Sebastes (S.) schlegeli*, which is the second most important in the aquaculture industry in Korea, has been studied [8,9,20].

In the present study, we investigated the genetic characteristics of the VP1 protein and compared the genetic relationship between aquatic birnaviruses and other

Table 1. Descriptions of VP1 sequences of aquabirnaviruses cited in this study

Name of virus	Genus of virus	Geographic origin	Host of origin	Accession number
GC1	Aquabirna virus	Korea	Rockfish	–
578	Aquabirna virus	Spain	Turbot	AJ489244
1146	Aquabirna virus	Spain	Trout	AJ489238
2290	Aquabirna virus	Spain	Salmon	AJ489240
AM98	Aquabirna virus	Japan	Amago salmon	AY129664
AY98	Aquabirna virus	Japan	Ayu	AY123970
DRT	Aquabirna virus	Korea	Rainbow trout	D26527
Jasper	Aquabirna virus	Canada	Turbot	M58756
NC1	Aquabirna virus	Korea	Flounder	AY129666
Sp	Aquabirna virus	Denmark	Turbot	M58757
WB	Aquabirna virus	USA	Turbot	AF078669
Y6	Aquabirna virus	Japan	Yellowtail	AY129662
YT01A	Aquabirna virus	Japan	Yellowtail	AY129663
CLV	Aquabirna virus	Viet Nam	Boltched snakehead fish	AJ459383
88R	Aquabirna virus	Spain	Oyster	AJ489245
24R	Aquabirna virus	Spain	Mussel	AJ489243
20Gld	Aquabirna virus	Spain	Deepwater redfish	AY780931
19F3b	Aquabirna virus	Spain	Greenland halibut	AY780928
6B1a	Aquabirna virus	Spain	Atlantic cod	AY780926
H1	Aquabirna virus	Japan	Flounder	AY129665
UPM97/61	Avivirna virus	Malaysia	Birds	AF527040
DXV	Entomobirna virus	Canada	Drosophila	AF196645

genuses within family *Birnaviridae*.

Materials and Methods

Virus and cell

GC1 was isolated from the rockfish *S. schlegeli*, and it was grown in the Chinook Salmon Embryo-214 cell line supplemented with Eagle's minimum essential medium. The sources of VP1 sequence cited in this study are listed in Table 1.

Viral RNA extraction and primers

The viral genomic RNA was extracted using the methods described by Joh *et al*. [8]. Briefly, GC1-infected cells were frozen and thawed 3 times and clarified by centrifugation. Viral dsRNA was then extracted with phenol and chloroform, followed by digestion with proteinase K. Seven primer pairs were used for reverse transcription- polymerase chain reaction (RT-PCR). The oligonucleotide sequences were deduced according to the published dsRNA sequences of the Western Buxton strains (AF078669) (Table 2).

cDNA synthesis by RT-PCR

The RT-PCR procedure used in this study was a modification of the method previously described by Joh *et al*. [10]. The RT-PCR solution was heated to 95°C for 3 min and passed through 35 cycles under the following conditions: 1 min at 95°C for denaturation; 1 min at 54°C to −58°C (depending on the primer) to allow annealing; 1 min at 72°C for extension and final amplification at 72°C for 3 min. The ethidium bromide-stained PCR products were electrophoresed on a 1.5% agarose gel and were visualized by UV fluorescence.

Construction of recombinant plasmids

Each resulting product was gel purified and then cloned into pCR2.1 TA cloning vectors (Invitrogen, USA) according to the manufacturer's instructions. All the clones were amplified by transformation into competent DH5 α cells. Clones with correct inserts were confirmed by restriction enzyme digestion of the recombinant vectors.

Nucleotide sequencing and analysis of the VP1

Nucleotide sequencing was carried out on an ABI 377 sequencer (Applied Biosystems, USA) by the dideoxy-nucleotide chain termination method by using T7 DNA and SP6 DNA polymerase. The nucleotide and deduced amino acid sequences were analyzedby Vector NTI ver 10.0 (Hitachi, Japan) and were compared with the corresponding sequences of previously reported cite accession numbers of aquabirnaviruses in Table 1.

Table 2. RT-PCR primer sets and amplified cDNA fragments used for sequencing

	Primers (Sequence)	Position*	PCR product length
GVP1.1F	GGAAACAGTGGGTCAACGTT	1-483	483 bp
GVP1.1R	AGAAGTGTGATGTCCGGAGC		
GVP1.2F	CCATTCCACAAGCCAGACCA	422-908	486 bp
GVP1.2R	AGGAGTCAGCCAGTACGAGC		
GVP1.3F	TCCTCAGCCGGCCTACCATA	833-1299	466 bp
GVP1.3R	GAGTACCATGTGTTGTCCTG		
GVP1.4F	AAGAGACAGCCTGGACAATG	1216-1701	485 bp
GVP1.4R	GTCTCGACGGCCTCAACGAT		
GVP1.5F	AAGATAGAGCGCGAGCTGAA	1646-2106	460 bp
GVP1.5R	ATTCCTTCTAGGTCTCCTCC		
GVP1.6F	CAAGAGGAAGAGACTGGAAG	2011-2400	389 bp
GVP1.6R	TGTTGTGCCAGTTCCTCAGT		
GVP1.7F	TACGAGATCAAGCACTAGCG	2319-2780	461 bp
GVP1.7R	TCCCTGGCGGAACCGGATGT		

*Map position of the primers based on the published sequence of Western buxton (AF078669).

Results

Nucleotide and amino acid sequences of the VP1 protein

The nucleotide sequence of GC1 was found to be 2,776 bp long. The VP1 open reading frame (ORF) gene starts at nucleotide 101 and ends with a single TAA termination codon at nucleotide 2,638. The predicted molecular weight of this virus is 94,263 Daltons, and it contains a single large ORF encoding the 846-amino acid VP1 protein. The VP1 sequence starts with the nucleotide sequence 'GGAAA' and contains the inverted terminal repeats 'GGGTCAA-GTTGGTGG' and 'GTGCCACCAAC-TGACCC' near the 5' and 3' terminal sequences, respectively.

Characterization of the VP1 protein

The amino acid composition of VP1 was determined. The VP1 amino acid sequence was scanned for several functional motifs, and the results are summarized in Table 3. We observed that the VP1 sequence contained 6 potential Asn-X-Ser/Thr motifs. These motifs were presumed to contain an *N*-linked glycosylation site. There were 8 potential Ser phosphorylation sitesand 1 Tyr phophorylation site. The amino acid sequence of VP1 did not contain the GDD motif, which exists commonly in the RdRps of RNA viruses; however, we could identifythe Leu-Lys-Asn (LKN) motif at position 521 (Table 3). Further, we confirmed the 'GLPYIGKT'motif at amino acid position 248; this motif is the putative GTP-binding site that is commonly found in other aquatic birnaviruses.

Table 3. Kinds of potential motif exist in VP1 of GC1

Kinds of motif	NO. of sites	Position of sites in amino acid sequences
N-linked glycosylation site	6	184, 226, 409, 437, 658, 677
Serine phosphorylation site	8	13, 21, 236, 245, 375, 635, 701, 802
Thyrosine phosphorylation site	1	399
GDD motif	1	521
GTP-binding site (GLPYIGKT)	1	248

Comparative studies of nucleotide and amino acid sequences of the VP1 protein

On comparing the nucleotide sequences of VP1 in 22 birnavirus strains, it was found that GC1 shares 97-98% homology with MABVs; 86% homology with the IPNV strains of aquabirnaviruses isolated mainly from the USA, Japan, and Korea; 80-82% homology with the IPNV strains of aquabirnaviruses from Spain; 54-56% homology with the avibirnaviruses; and 46% homology with entomobirnaviruses (Table 4). On comparing the amino acid sequence of VP1, it was found that GC1 shares 97-98% homology with MABVs; 94% homology with the IPNV strains of aquabirnaviruses found mainly in the USA, Japan, and Korea; 87-89% homology with the IPNV strains of aquabirnaviruses from Spain; 46-47% homology with the avibirnaviruses; and 29% homology with the entomobirnaviruses (Table 5).

Table 4. Pairwise similarity and distances among the VP1 nucleotide sequences of 22 birnavirus strains

Percent identity nucleotide sequence of VP1

Percent identity nucleotide sequence of VP1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
1		98	98	89	88	88	88	88	55	56	46	80	81	82	82	81	80	80	80	80	80	80	1	1146
2	2		98	89	88	88	88	88	54	56	46	80	80	82	81	81	80	80	80	80	80	80	2	88R
3	2	2		89	87	87	87	88	54	55	46	80	80	81	81	80	80	80	80	80	80	80	3	Sp
4	11	11	11		99	99	98	99	54	55	45	81	81	81	81	81	81	82	82	82	82	82	4	20Gl
5	12	12	13	1		100	100	100	54	56	45	81	81	81	81	81	81	82	82	82	82	82	5	2290
6	12	12	13	1	0		100	100	54	56	45	81	81	81	81	81	81	82	82	82	82	82	6	24R
7	12	12	13	1	0	0		99	54	56	45	81	81	81	81	81	81	82	82	82	82	82	7	578
8	12	12	13	1	0	0	0		53	55	44	80	80	81	81	81	81	82	82	82	82	82	8	6Bla
9	45	45	46	46	45	45	45	46		58	44	54	54	55	55	54	54	55	55	55	54	55	9	UPM976
10	44	44	45	45	44	44	44	45	42		44	56	56	56	56	56	56	56	56	56	56	57	10	CLV
11	54	54	54	55	54	54	54	56	56	56		46	46	47	46	46	46	46	46	46	46	46	11	DVX1
12	19	20	20	19	19	19	19	19	46	44	54		100	91	91	90	86	85	85	86	86	86	12	19F3b
13	19	20	20	19	19	19	19	19	46	44	54	0		91	91	90	86	85	85	86	86	86	13	WB
14	18	18	19	18	19	19	19	19	45	44	53	9	9		98	89	86	86	86	86	86	86	14	AM98
15	18	18	19	18	19	19	19	19	45	44	53	9	9	2		90	86	86	86	86	86	86	15	DRT
16	19	19	19	18	18	18	18	18	46	44	54	10	10	10	10		86	85	85	85	86	86	16	Jasper
17	20	20	20	18	19	19	19	19	46	44	54	14	14	14	14	13		98	97	98	97	98	17	GCl
18	19	20	19	18	18	18	18	18	45	44	54	14	14	14	14	15	2		98	99	99	99	18	YT01A
19	20	20	20	18	18	18	18	18	45	43	54	14	14	14	14	15	3	2		99	99	99	19	NC1
20	20	20	20	18	18	18	18	18	45	43	54	14	14	14	14	15	2	1	1		99	100	20	H1
21	20	20	20	18	18	18	18	18	45	44	54	14	14	14	14	15	2	1	1	0		100	21	AY98
22	20	20	20	18	18	18	18	18	45	43	54	14	14	14	14	15	2	1	1	0	0		22	Y6
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		

Table 5. Pairwise similarity and distances among the VP1 amino acid sequences of 22 birnavirus strains

Percent identity amino acid sequence of VP1

Percent identity amino acid sequence of VP1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
1		100	98	95	95	96	96	96	47	47	28	90	90	90	90	90	89	90	90	90	90	90	1	1146
2	0		98	95	96	96	96	46	46	28	90	90	90	90	88	90	88	90	90	90	90	90	2	88R
3	2	2		94	95	95	95	46	46	28	89	89	89	89	89	87	89	89	89	89	89	89	3	Sp
4	5	5	6		99	98	98	98	47	46	27	89	89	89	89	90	88	90	90	90	90	90	4	20Gl
5	5	5	6	1		99	99	99	47	46	27	89	89	89	89	90	88	90	89	90	90	90	5	6Bla
6	4	4	5	2	1		100	100	48	47	28	90	90	90	90	91	89	91	90	91	91	91	6	2290
7	4	4	5	2	1	0		100	48	47	28	90	90	90	90	91	89	91	90	91	91	91	7	24R
8	4	4	5	2	1	0	0		48	47	28	90	90	90	90	91	89	91	90	91	91	91	8	578
9	53	53	54	53	53	52	52	52		50	28	47	47	47	47	47	47	47	47	47	47	47	9	CLV
10	53	53	54	54	53	53	53	53	50		27	46	46	46	47	46	46	46	46	46	46	46	10	UPM976
11	72	72	72	72	73	72	72	72	72	73		29	29	29	29	29	29	29	29	29	29	29	11	DVX1
12	10	10	11	11	11	10	10	10	53	53	71		100	98	98	97	94	95	95	96	96	96	12	19F3b
13	10	10	11	11	11	10	10	10	53	53	71	0		98	98	97	94	96	95	96	96	96	13	WB
14	10	10	11	10	11	10	10	10	53	53	71	2	2		99	98	94	96	96	96	96	96	14	AM98
15	10	10	11	11	11	10	10	10	53	54	71	2	2	1		98	94	96	96	96	96	96	15	DRT
16	10	10	11	10	10	9	9	9	53	53	71	3	3	2	2		94	95	95	96	96	96	16	Jasper
17	11	11	12	12	12	11	11	11	53	54	71	6	6	6	6	6		97	97	97	97	98	17	GCl
18	9	10	11	10	10	9	9	9	53	53	71	5	4	4	4	5	3		98	99	99	99	18	NC1
19	10	10	11	10	10	9	9	9	53	54	71	5	4	4	4	5	3	1		100	99	99	19	AY98
20	9	10	11	10	10	9	9	9	53	54	71	4	4	4	4	4	3	1	0		100	100	20	Y6
21	9	10	11	10	10	9	9	9	52	53	71	4	4	4	4	4	3	1	1	0		100	21	H1
22	9	10	11	10	10	9	9	9	52	54	71	4	4	4	4	4	2	1	1	0	0		22	YT01A
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		

Phylogenetic relationships

In the phylogenetic cladograms that were based on both nucleotide and amino acid sequences, the genetic relationships among the 22 birnaviruses were established and the viruses, including GC1, were clustered into 5 genogroups that generally correlated with the geographic origin of the viruses and the water environment of the host. The MABV genogroup consisted of strains such as GC1 and NC1 from Korea and YT01A, H1, AY98, and Y6 from Japan. Genogroup 1 mostly consisted of strains from the Pacific coastal nations; DRT is from Korea, WB from the USA, Jasper from Canada, and AM98 from Japan. The isolates of 1146, 88R, 20G1, 2290, and 6B1A from Spain and Sp from Denmark comprised genogroup 2. The 2 avibirnaviruses UPM976/61 from Malaysia and CLV from Vietnamformed genogroup 3, and 1 entomobirnavirus, DVX, formed genogroup 4 (Fig. 1).

Discussion

The viral B segment encodes VP1, which is approximately 90 kDa in weight [2,11-13]. The estimated molecular weight of VP1 ranges from 95 kDa for the Jasper isolate [4] to 89 kDa for the Sp and Ab isolates of IPNV [6]. The molecular weight of GC1 has been estimated as 94 kDa and has been shown to be similar to that of the Jasper strain.

Some researchers have reported that the sequence GXXXXGKS/T is a constant motif in GTP-binding proteins [1,16] and is observed in several viral proteins that have a tentative role in RNA replication [15]. The same motif was present in the IPNV strains [4] and in GC1 between residues 248 and 255 (GLPYIGKT). We believe that this motif represents a potential GTP-binding site in the VP1 protein, and has been conserved in GC1, including aquatic birnaviruses.

As reported previously [1,5,17], the GDD sequence is a highly conserved motif that is present in almost all putative RdRps. Researchers have found that the Asp-Asp (DD) sequence lacking Gly, is conserved in IBDV, and also that IPNV does not contain the typical GDD motif in the corresponding region of its VP1 [4,21]. Some IPNV strains containedthe Leu-Lys-Asp (LKD) or LKN motifs instead of the typical GDD motif [4]. GC1 contains the LKN motif instead of the typical GDD motif, which is present in other aquatic birnaviruses.

The study of genetic relationships using a phylogenetic cladogram revealed that GC1 is more closely related to genogroup 1 than genogroup 2. This result indicatesthat genetic relationships may be influenced by the geographical distributions of the isolates. Aquatic birnaviruses, including GC1 and IPNV, also belong to genogroups that are distinct from those of the avibirnaviruses and Entomo-birnaviruses. This result may thus indicate that the genus *Birnavirus* has evolved in different ways resulting in the formation of distinct genogroups.

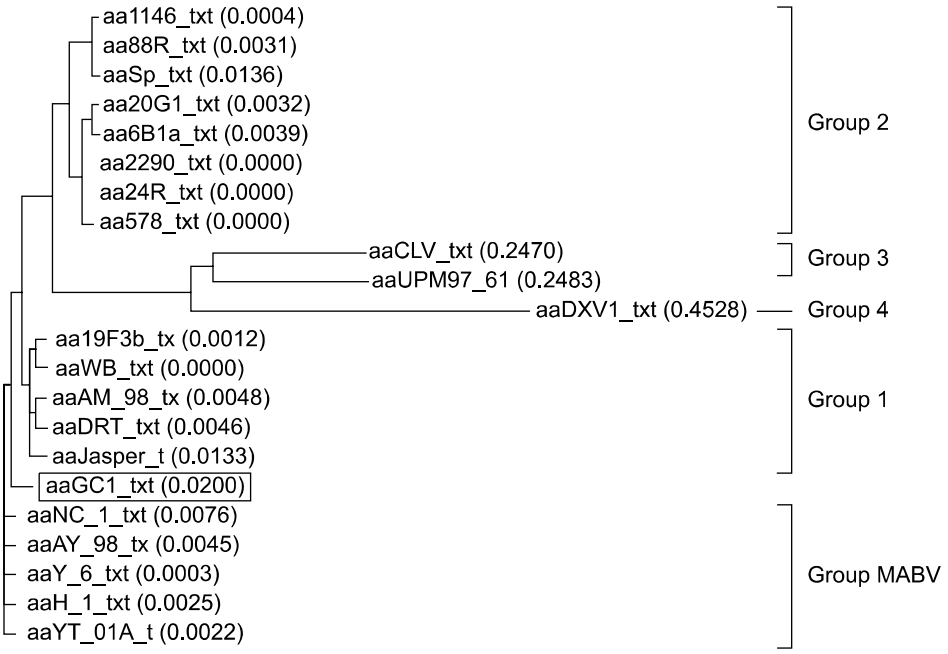

Fig. 1. Cladogram representing phylogenetic relationships between birnaviruses based on deduced amino acid sequences of VP1. The length of each pair of branches represents the distance between the sequence pairs, and the numbers in parentheses indicate the bootstrap values.

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