大韓獸醫學會誌(2008) 第48卷 第3號
Korean J Vet Res(2008) 48(3) : 287~293

Genogroup position of aquabirnavirus GC-1 isolated from rockfish *Sebastes schlegeli* in Korea

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(Accepted: August 7, 2008)

Abstract : The cDNA of the aquabirnavirus, GC-1 isolated from rockfish *Sebastes schlegeli* in Korea, was synthesized using the reverse transcriptase-polymerase chain reaction. The nucleotide and deduced amino acid sequences were determined from cDNA of the VP2-NS-VP3 coding region of genome segment A. The nucleotide sequences of the segment A were 3,086 base pairs (bp) in length and contained large open reading frame (ORF) and terminal sequences. The large ORF was comprised of 2,916 bp nucleotides and composed of 972 deduced amino acid sequences. Pairwise comparisons were made with other aquabirnavirus sequences published previously. The study of genetic relationships between GC-1 and aquabirnaviruses in the large ORF and VP2 coding regions demonstrated that the GC-1 has the nearest genetic relationship with the marine birnaviruses (MABV strains), and the GC-1 and MABV strains can be clustered as the same genogroup. GC-1 can be included in MABV, which is the 7th genogroup of family Aquabirnaviridae.

Keywords : marine aquatic birnavirus (MABV), phylogenetic relationship, rockfish *Sebastes schlegeli*, VP2-NS-VP3

Introduction

Aquabirnaviruses are the largest and most diverse group of viruses within the family Birnaviridae. The family comprises three main genera including the genus Aquabirnavirus, the genus Avibirnavirus and the genus Entomobirnavirus. The genus Aquabirnavirus contains the type species birnaviruses of fish and shellfish including infectious pancreatic necrosis viruses (IPNV strains) [6] and marine birnaviruses (MABV strains) [15]. All aquabirnaviruses are similar in morphological, biochemical and biophysical properties [5]. The virion consists of an unenveloped, icosahedral capsid and a bisegmented, double stranded RNA genome. The genomes consist of two segments, A and B. Segment A is a longer genome segment and consists of two open reading frames (ORFs), large and small ORFs. The large ORF encodes the major outer capsid protein (VP2), viral protease (NS) and minor inner capsid protein (VP3), and the small one encodes VP5 which function was recently identified as an anti-apoptosis protein [11]. The complete nucleotide sequences of the NH_2-VP2-NS-VP3-COOH have been reported in several IPNV and MABV strains [2, 4, 7-8, 16, 18-20].

Relationships of aquabirnaviruses have been studied at the genomic levels. The comparisons of deduced amino acid sequences have been performed for VP2/NS junction regions [9, 13], VP2 coding regions [10] and overall segment A regions containing VP2 coding regions [2, 20].

Rockfish, *Sebastes schlegeli* is one of the most important cultured fish in Korea, and the production of this species has been increased [1]. Aquabirnavirus, GC-1, isolated from rockfish *Sebastes schlegeli* was characterized physically and antigenitically [17] and investigated the genetic relationships among aquabirnaviruses on relatively short genomic fragments [13].

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In the present study, we have sequenced the full-length genome of GC-1 and investigated the genetic relationship among aquabirnaviruses based on the large ORF and VP2 coding region.

Materials and Methods

Virus and cell

The GC-1 was isolated from rockfish *Sebastes schlegeli* was grown in the Chinook Salmon Embryo-214 cell line supplemented in Earle's minimum essential medium.

RNA extraction

The virus dsRNA was extracted by the method of Heppell *et al.* [9]. Briefly, the GC-1-infected cells were being frozen and thawed three times and clarified by centrifugation. Viral dsRNA was then extracted with phenol and chloroform, followed by digestion with proteinase K.

Primers and amplification of viral RNA

Synthetic oligonucleotide primers used in this study were synthesized based on the nucleotide sequence of the YAV strain [16] (Table 1). The extracted viral RNA was denatured by the method of Charles *et al.* [3]. Extracted viral dsRNA was denatured with 100 mM methyl mercuric hydroxide (Sigma, USA). Complementary DNA was synthesized from the viral RNA with reverse-transcriptase 40 U of M-MLV (Promega, USA) in a reaction buffer containing 1 mM dNTPs, 4 mM DTT and 40 U RNase inhibitors. The DNA was amplified by polymerase chain reaction (PCR) in the GeneAmp PCR system 9600 (Perkin Elmer, USA). The resulting PCR products were confirmed by digestion of restriction enzymes and electrophoresis in a 1.5% agarose gel containing ethidium bromide.

Construction of recombinant plasmids

Each resulting products were gel-purified and then cloned into pCR2.1. TA cloning vectors (Invitrogen, USA) according to the manufacturer's instruction. All clones were amplified by transformation into competent DH5ácells. We confirmed the clones with the presence of proper insert in recombinant vector by digestion of restriction enzymes.

Nucleotide sequencing and comparison of large ORF and VP2 coding region

The nucleotide sequencing was carried out by the dideoxynucleotide chain termination method using the T7 DNA and SP6 DNA polymerase with an AB 377 (Biosystems, USA). The nucleotide and deduced amino acid sequences were analyzed by Vector NTI ver 9.0 (Hitachi, Japan) and compared with those of previously reported aquabirnaviruses. The genetic sources of reference aquatic birnaviruses used in this study were listed in Table 2.

Results

Nucleotide sequence of the segment A

Sequences from each fragment have been analyzed

Table 1. RT-PCR primer sets and amplified cDNA fragments used for sequencing

Primers (Sequence)		Position*	PCR product length	Coding region
GC1.1	AAAGAGAGTTTCAACGTTAG (ATCTCTCTGTTATGTCGTCC)	210	210 bp	Noncoding-VP2
GC1.61	CGTCGATGGCGAAAGCCCTT (AAGCTGATGTCGCCGGTCACTGTGG)	61-848	787 bp	pVP2
GC1.825	ACAGTGACCGGCGACATCAGCTTC (GGTCAGGCCTCCGATGAATTGG)	825-1562	737 bp	pVP2
GC1.1403	GATCACAGACTTCTCAAGTG (TTTGATGTCTACACCGCAGAT)	1403-2138	735 bp	pVP2-NS
GC1.1789	GGTCCCTTCTGGTAATCAT (TTTGACCATTCATA)	1789-2467	678 bp	NS-VP3
GC1.2360	GCAAAAGAGGTGAAAGACGCCGAA (GTTACACTTCTCCGTTATCTCC)	2360-3036	676 bp	VP3
GC1.2875	GACCAGATCAAGACCAGATG (CTGGGGGGCCGGGGTTGAGG)	2875-3086	211 bp	VP3

*Map position of the primers based on the published sequence of YAV strain (Cited from reference No. 16).

Table 2. Aquatic birnaviruses used and cited in this study

Virus	Geographic origin	Host of origin	Water environmetnt	Serotype	Accession number	Kind of sequence
GC1	Korea	Rockfish	Sea water	NT*	AY064396	VP2-NS-VP3
Y6	Japan	Yellowtail	Sea water	NT	AY283781	VP2-NS-VP3
YT01A	Japan	Yellowtail	Sea water	NT	AY283782	VP2-NS-VP3
H1	Japan	Flounder	Sea water	NT	AY283783	VP2-NS-VP3
NC1	Japan	Flounder	Sea water	NT	AY283784	VP2-NS-VP3
AY98	Japan	Ayu	Fresh water	NT	AY283785	VP2-NS-VP3
DRT	Korea	Rainbow trout	Fresh water	NT	D26526	VP2-NS-VP3
AM98	Japan	Amago salmon	Fresh water	NT	AY283780	VP2-NS-VP3
WB	USA	Trout	Fresh water	A1	AF342727	VP2-NS-VP3
Dry Mills	USA	Trout	Fresh water	A1	AF343571	VP2-NS-VP3
VR299	USA	Trout	Fresh water	A1	AF343572	VP2-NS-VP3
Buhl	USA	Trout	Fresh water	A1	AF343573	VP2-NS-VP3
Jasper	Canada	Trout	Fresh water	A1	M18049	VP2-NS-VP3
Sp	Denmark	Trout	Fresh water	A2	AF342728	VP2-NS-VP3
N1	Norway	Atlantic salmon	Fresh water	A2	D00701	VP2-NS-VP3
Ab	Denmark	Trout	Fresh water	A3	AF342729	VP2-NS-VP3
He	Germany	Pike	Fresh water	A4	AF342730	VP2-NS-VP3
Te	England	Tellina	Fresh water	A5	AF342731	VP2-NS-VP3
C1	Canada	Trout	Fresh water	A6	AF342732	VP2-NS-VP3
C2	Canada	Trout	Fresh water	A7	AF342733	VP2-NS-VP3
C3	Canada	Arctic char	Fresh water	A8	AF342734	VP2-NS-VP3
JaA	Canada	Trout	Fresh water	A9	AF342735	VP2-NS-VP3
1146	Spain	Trout	Fresh water	NT	AJ489222	VP2-NS-VP3
2290	Spain	Salmon	Fresh water	NT	AJ489224	VP2-NS-VP3
24R	Spain	Mussel	Fresh water	NT	AJ489227	VP2-NS-VP3
578	Spain	Turbot	Fresh water	NT	AJ489228	VP2-NS-VP3

*Not typed.

and have been deposited as accession numbers AY064396 for large ORF in a genome database (i.e., GenBank) of the National Center for Biotechnology Information, National Institutes of Health, USA. The nucleotide sequence of the full-length genome of segment A of GC-1 was 3,086 bp long including a large ORF, a small ORF and non-coding regions at both ends.

Characterization of the large ORF

The sequence revealed a large ORF of 2,916 bp long extending from the start codon (ATG) at position 117 and a stop codon (TAA) at position 3033. This ORF encodes a precusor polyprotein of 972 amino acids. The non-coding region at 5'- and 3'-ends of the segment A consisted of 109 bp and 51 bp in length, respectively. The postulated cleavage sites were located at aa 486-503 between VP2 and NS and at aa 721-729 between NS and VP3, and consisted of residues AAGGRYPTHAAGGRYTDV and RRIKYLGELMRTTASG, respectively.

Among amino acids consisted of large ORF, fifty one percent of amino acids were hydrophobic in the VP2 region and forty nine percent of amino acids were hydrophilic in the VP3 region. Especially, the basic amino acids such as arginine and lysine were appeared highly in the VP3 region.

Eight glycosylation sites were found in predicted amino acids encoded by the large ORF, of which 6 were in the VP2 and 2 were in the NS region.

Comparison of nucleotide and amino acids sequences in large ORF and VP2

In the comparison of nucleotide sequences in large ORF, the GC-1 has 98-99% homology with MABV strains. MABV cluster including GC-1 has a homology of 83-84% with Genogroup 1 (IPNV strains) representing serotype A1 and A9. Homology with other Genogroups

Percent identity of nucleotide sequences of large ORF coding region

Percent identity of amino acid sequences of large ORF coding region

	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		
1		98	98	87	87	87	86	86	87	84	84	77	79	79	79	79	78	78	78	79	80	80	80	80	80	79	1	1146
2	98		98	87	87	87	87	87	87	85	85	77	79	79	79	78	79	79	79	78	79	79	79	79	79	79	2	Sp
3	98	98		88	88	88	86	86	87	84	84	77	79	79	79	79	78	78	78	79	79	80	80	80	80	79	3	N1
4	90	90	90		100	100	97	88	87	83	83	75	78	78	79	79	78	78	78	78	79	79	79	79	79	79	4	2290
5	90	90	90	100		100	97	88	87	83	83	75	78	78	79	79	78	78	78	78	79	79	79	79	79	79	5	24R
6	90	90	90	100	100		97	88	87	83	83	75	78	78	79	79	78	78	78	78	79	79	79	79	79	79	6	578
7	90	90	90	98	98	98		88	87	83	83	76	79	80	79	79	79	79	80	79	79	79	79	79	79	79	7	Ab
8	90	91	90	92	92	92	92		91	84	84	76	78	79	78	78	79	79	79	78	78	78	79	79	79	78	8	CA1
9	90	90	90	92	92	92	92	96		83	83	76	78	79	78	78	78	78	78	78	79	79	79	79	79	79	9	Te
10	88	88	88	86	86	86	86	87	86		97	76	78	78	78	78	78	78	78	78	79	79	79	79	79	79	10	CA2
11	88	88	88	87	87	87	87	87	87	97		76	78	78	78	78	78	78	78	78	78	78	78	78	78	78	11	CA3
12	84	85	84	82	82	82	82	82	83	83	82		74	74	74	74	74	74	74	74	74	74	74	74	74	74	12	He
13	83	84	83	83	83	83	84	83	82	83	83	80		97	98	98	97	90	90	91	84	84	84	84	84	84	13	AM98
14	83	84	83	83	83	83	84	83	83	84	83	80	98		98	98	98	91	91	90	84	84	84	84	84	83	14	Buhl
15	84	84	84	83	84	84	84	84	83	84	83	80	98	98		99	98	91	91	91	84	84	84	84	84	84	15	DRT
16	84	84	84	84	84	84	84	84	83	83	83	80	98	98	99		99	91	91	91	84	84	84	84	84	84	16	Jasper
17	84	84	84	83	83	84	84	84	84	84	83	80	97	98	98	99		91	91	91	84	84	84	84	84	83	17	VR299
18	83	84	83	83	83	83	84	83	83	84	83	80	94	95	95	95	95		91	90	83	83	84	84	84	83	18	JaA
19	84	85	84	84	84	84	85	83	84	84	84	81	96	97	97	97	97	96		99	84	84	84	84	84	84	19	DM
20	84	85	85	84	84	84	84	84	84	84	83	80	96	96	97	97	97	99	99		84	84	84	84	84	84	20	WB
21	85	85	85	84	85	85	84	84	84	84	83	80	89	88	89	89	89	89	90	90		99	98	99	99	98	21	GC1
22	85	85	85	85	85	85	85	84	85	83	83	81	89	89	90	90	89	89	90	90	99		98	99	99	98	22	YT01A
23	85	85	85	84	85	85	84	84	84	84	83	81	88	88	89	89	89	88	90	90	98	99		99	99	98	23	NC1
24	85	85	85	85	85	85	85	84	85	84	83	81	89	88	90	89	89	89	90	90	99	99	99		99	99	24	AY98
25	85	85	85	85	85	85	85	84	85	84	83	81	89	88	89	89	89	89	90	90	99	99	99	99		99	25	H1
26	84	84	84	84	84	84	84	83	84	83	83	80	88	88	88	88	88	88	89	89	98	98	98	98	99		26	Y6
	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		

Fig. 1. Pairwise distances of aquatic birnaviruses based on nucleotide and amino acid sequences of large open reading frame (ORF) coding region of genome segment A.

Percent identity nucleotide sequences of VP2 coding region

Percent identity amino acid sequences of VP2 coding region

	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		
1		98	97	88	88	88	86	86	87	85	86	77	81	82	81	81	81	81	81	81	84	84	84	84	84	83	1	1146
2	98		98	88	88	88	87	86	87	85	85	77	81	82	82	81	81	81	82	82	83	83	83	83	83	83	2	Sp
3	98	98		88	88	88	86	86	87	85	86	77	81	81	81	81	81	81	81	81	83	83	83	83	84	83	3	N1
4	92	92	92		100	100	96	88	87	84	84	75	80	81	81	81	80	80	80	80	82	82	82	82	82	82	4	2290
5	92	92	91	100		100	96	88	88	84	84	75	80	81	81	81	81	81	80	80	82	82	82	82	82	82	5	24R
6	92	92	92	99	100		96	88	88	84	84	75	80	81	81	81	81	81	80	80	82	82	82	82	82	82	6	578
7	90	91	90	98	98	98		86	86	83	83	74	81	82	82	82	82	82	84	83	82	82	82	82	82	82	7	Ab
8	90	91	90	92	92	92	91		91	84	85	75	81	81	81	81	81	81	81	81	82	82	82	83	83	82	8	CA1
9	90	90	90	92	92	92	90	96		84	84	76	80	80	80	80	80	80	80	80	82	82	82	83	83	82	9	Te
10	90	90	90	88	88	88	88	88	88		96	76	80	80	80	80	80	80	81	81	82	82	82	82	82	82	10	CA2
11	89	89	90	89	88	88	88	88	88	97		75	80	80	80	80	80	80	81	81	82	82	82	82	82	82	11	CA3
12	86	86	86	82	83	83	82	82	83	83	83		74	74	74	74	74	74	74	74	76	76	76	76	76	76	12	He
13	86	87	86	86	86	87	87	86	85	86	86	81		97	98	97	97	90	89	88	83	84	84	84	84	83	13	AM98
14	87	87	86	87	87	87	88	86	85	86	86	81	97		98	98	98	90	89	89	84	85	84	85	85	84	14	Buhl
15	87	88	87	87	87	88	88	86	86	87	87	82	98	99		99	99	91	89	89	84	84	84	84	84	84	15	DRT
16	87	88	87	87	87	88	88	86	86	87	86	82	98	98	99		100	91	90	89	84	84	84	85	84	84	16	Jasper
17	87	88	87	87	87	88	87	86	86	87	86	82	95	98	99	100		91	90	89	84	84	84	85	84	84	17	VR299
18	86	87	86	86	86	86	88	85	86	87	86	81	96	95	96	96	100		90	90	84	85	85	85	85	84	18	JaA
19	87	88	87	87	87	88	88	86	86	87	87	82	95	96	97	97	96	96		100	85	85	85	86	85	84	19	DM
20	87	88	87	87	87	87	88	86	86	87	86	82	95	96	97	97	97	96	100		85	85	85	85	85	84	20	WB
21	88	89	88	88	89	89	88	87	88	87	87	82	88	89	89	89	97	90	90	90		99	99	99	99	98	21	YT01A
22	88	88	88	88	88	88	88	87	88	87	87	82	88	88	89	89	89	89	90	90	98		99	99	99	98	22	GC1
23	88	89	88	88	89	89	88	87	88	87	87	82	88	89	89	89	89	90	90	90	99	99		99	99	98	23	NC1
24	88	89	88	88	89	89	88	87	88	88	87	82	88	89	89	89	89	90	90	90	99	99	99		100	99	24	AY98
25	88	89	88	88	89	89	88	87	88	87	87	82	88	89	89	89	89	90	90	90	99	99	100	99		99	25	H1
26	88	88	88	87	88	88	87	86	87	87	87	82	87	88	88	88	88	88	89	89	98	98	98	98	98		26	Y6
	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		

Fig. 2. Pairwise distances of aquatic birnaviruses based on nucleotide and amino acid sequences of VP2 coding region of genome segment A.

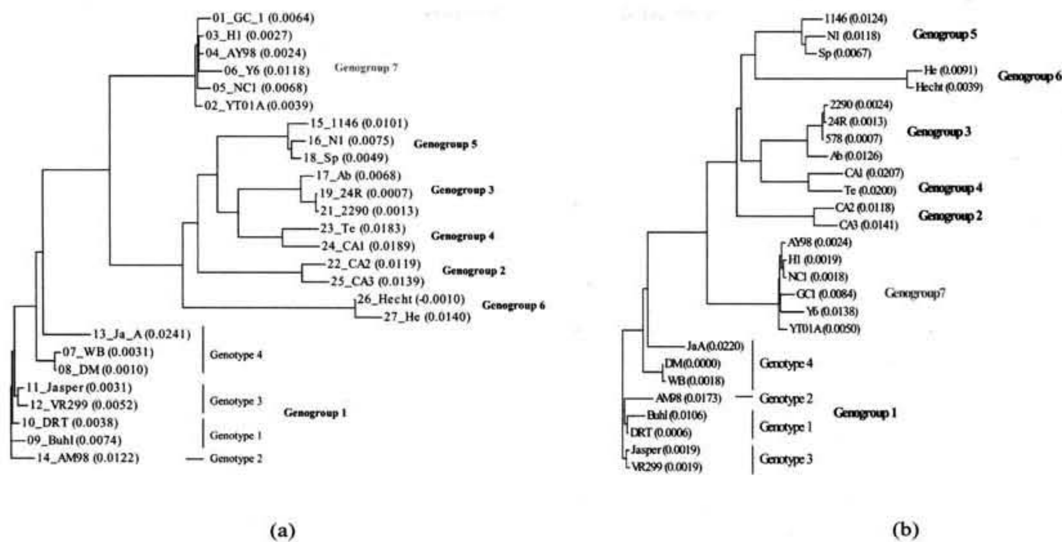

Fig. 3. Cladogram representing phylogenetic relationships of aquabirnaviruses based on deduced amino acid sequences of large open reading frame (a) and VP2 (b). The length of each pair of branches represents the distance between sequence pairs, and the numbers in parenthesis indicate the bootstrap values.

was much less (74-79%). In the comparison amino acid level, the GC-1 has a homology of 98-99% with MABV strains, 89-90% with Genogroup 1, 83-84% with Genogroup 2, 84% with Genogroup 3, 84% with Genogroup 4, 85% with Genogroup 5 and 80% with Genogroup 6, respectively (Fig. 1).

In the comparison of nucleotide sequence in the VP2 coding region, the GC-1 has a homology of 98-99% with MABV strains, 84-85% with Genogroup 1, 82% with Genogroup 2, 82% with Genogroup 3, 82% with Genogroup, 83% with Genogroup 5 and 76% with Genogroup 6, respectively. In the comparison amino acid sequence, the GC-1 has a homology of 98-99% with MABV strains, 88-90% with Genogroup 1, 87% with Genogroup 2, 88% with Genogroup 3, 87-88% with Genogroup 4, 88% with Genogroup 5 and 82% with Genogroup 6, respectively (Fig. 2).

Phylogenetic relationship

In the phylogenetic cladograms, the genetic relationships among the 26 aquatic birnaviruses including GC-1 were investigated on the base of the amino acids sequences of the large ORF (Fig. 3a) and VP2 (Fig. 3b) region respectively. All of them were clustered into seven genogroups. Genogroup 1 consisted of all isolates from USA (Serotype A1), two Jasper strains from Canada, DRT strain from Korea and AM98 strain from Japan. The type strains of the two Canadian Serotypes A7 (C2) and A8 (C3) comprised Genogroup 2. One Denmark isolate representing Serotype A3 (Ab) and three Spain isolates formed Genogroup 3. One type strain of the Canadian Serotype A6 (C1) and one type strain of the England Serotype A5 (Te) comprised Genogroup 4. All members of serotype A2, including European isolate formed Genogroup 5. The He strain, the only known representative of Serotype A4, represented Genogroup 6. Five isolates of Japan and one isolate of Korean (GC-1) comprised another genogroup, Genogroup 7. The Genogroup 7 was found to be more close relationship to Genogroup 1 than other genogroups of aquabirnaviruses.

Discussion

Previously, we and other researchers investigated the genetic relationships among aquabirnaviruses on relatively short genomic fragments [9, 12-14, 18]. Hosono *et al.* [12], Joh and Heo [13] and Nishizawa *et al.* [14] investigated the VP2/NS junction region of aquabirnaviruses including MABV isolated from marine fish in Japan and in Korea, respectively. They confirmed that the aquabirnaviruses were divided 4 genogroup and the MABV isolates were constructed distinct genogroup from IPNV strains. Nishizawa *et al.* [14] studied the

genogroups of the Japan isolates and suggested that the Japan isolates and Korean isolate which deposited the GC-1 nucleotide as accession number AY064396 [13] were grouped as genogroups VII. Later, some researchers studied the full genomic structure of segment A and investigated the genetic relationships of aquabirnaviruses. Blake *et al.* [2] studied the phylogenetic relationships among 28 aquabirnaviruses and suggested the aquabirnaviruses (IPNV strains) were clustered into 6 genogroups. They also suggested that the genetic relationships generally correlated with geographic origin of the virus and serological classifications. Zhang and Suzuki [20] investigated the genetic relationship of aquabirnaviruses added the MABV to the IPNV strains. They confirmed that MABV strains isolated from marine fish in Japan constructed the distinct group from IPNV strains.

In the present study, we investigated the genetic relationships on 26 aquabirnaviruses including GC-1 that represent widely different geographical and host origins including INPV and MABV strains. Our studies focused on how similar are the nucleotide and deduced amino acid sequences between GC-1 and the MABV strains and the GC-1 strain could divide the same cluster with the MABV strains. In terms of nucleotide sequence, high levels of homology in large ORF regions were found between the GC-1 and the MABV strains. This result showed that the GC-1 and MABV strains might belong to the same genogroup. Comparison with other aquabirnaviruses, the GC-1 showed 83-84% homology with IPNVs from USA, Japan and Korea, but showed much lower levels (80 to 74%) with those of from Europe and Canada. In terms of deduced amino acid sequences, GC-1 showed 89 to 90% homology with the viruses from United States and 83 to 85% from the Europe and Canada. The most divergent virus was He strain with a similarity of 80%. We also studied genomic relationship more detail in the VP2 coding region. There were 98-99% similarities between GC-1 and MABV strains. The GC-1 showed similarity levels of 84-85% with the IPNV strains from United States, Korea and Japan, compared to 82-84% with the Canadian and European strains. The lowest degree of similarity (82%) with the IPNV strain was exhibited the He strain.

The phylogenetic tree based on the ORF and VP2 coding region confirmed that GC-1 belongs to MABV strains and the GC-1 and MABV strains found to be classified in a genogroup different from the other six aquabirnaviruses [20]. We suggest the aquabirnaviruses isolated from marine aquaculture fish in Japan and Korea should be classified, as the seventh genogroup adding previously reported the six genogroups [2].

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