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Phylogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of genome segment A cDNA

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ABSTRACT: Aquatic birnaviruses, such as infectious pancreatic necrosis virus (IPNV), cause serious diseases in a variety of fish species used worldwide in aquaculture and have also been isolated from a variety of healthy fish and shellfish species. These viruses exhibit a high degree of antigenic heterogeneity and variation in biological properties such as pathogenicity, host range, and temperature of replication. To better understand genetic and biological diversity among these viruses, the nucleotide and deduced amino acid sequences were determined from cDNA of the large open reading frame (ORF) of genome segment A of the 9 type strains of Serogroup A and 4 other representative strains of Serotype A1, the predominant serotype in the United States. In addition, nucleotide and deduced amino acid sequences were determined for the VP2 coding region of a variety of isolates representing 5 of the 9 serotypes. VP2 is the major outer capsid protein of aquatic birnaviruses. RT-PCR was used to amplify a 2904 bp cDNA fragment including all but a few bp of the large ORF of genome segment A or a 1611 bp fragment representing the entire VP2 coding region. Nucleotide and deduced amino acid sequences were determined from the PCR products. Pairwise comparisons were made among our data and 2 other aquatic birnavirus sequences previously published. Several hypervariable regions were identified within the large ORF. The most divergent pair of viruses exhibited a similarity of 80.1% in the deduced amino acid sequence encoded by the large ORF. Genomic relationships revealed in a phylogenetic tree constructed from comparison of the deduced amino acid sequences of the large ORF demonstrated that these viruses were clustered into several genogroups. Phylogenetic comparison of the deduced amino acid sequences of the VP2 coding region of 28 aquatic birnavirus isolates, including the type strains of all 9 serotypes, demonstrated 6 genogroups, some of which were comprised of several genotypes. The most divergent pair of viruses exhibited a similarity of 81.2% in the deduced amino acid sequence from the VP2 coding region. In contrast to previous studies of much shorter genomic sequences within the C-terminus-pVP2/NS junction coding region, these genogroups based on the entire large ORF or the VP2 coding region generally correlated with geographical origin and serological classification. Isolates from the major Canadian serotypes were more closely related to the European isolates than to isolates from the United States.

KEY WORDS: Aquatic birnavirus · Infectious pancreatic necrosis virus · Genogroups · Phylogenetic relationships

INTRODUCTION

The aquatic birnaviruses are the largest and most diverse group of viruses within the family Birnaviridae; they include a variety of viruses from numerous species of fish and marine invertebrates worldwide (Wolf 1988). These viruses exhibit a high degree of antigenic heterogeneity and variation in biological properties such as pathogenicity, host range, and temperature of replication. Many of these viruses have been proven or

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implicated as the etiologic agents of disease in a variety of fish species important in fish farming and aquaculture worldwide. Different strains of aquatic birnaviruses infect different species of fish and cause different diseases, such as infectious pancreatic necrosis (IPN) in salmonid species (Wolf 1988), nephroblastoma and branchionephritis in eel (Egusa 1970), and gill necrosis in clams (Lo et al. 1988).

All aquatic birnaviruses are similar in morphology and biochemical and biophysical properties (Dobos et al. 1979). The virion consists of an unenveloped, icosahedral capsid and a bisegmented, double stranded RNA genome (Cohen et al. 1973, MacDonald & Yamamoto 1977, Dobos et al. 1979, Dobos & Roberts 1983). The smaller genome segment B (2784 bp) is monocistronic and encodes an internal polypeptide VP1 (94 kDa), the putative virion-associated RNA-dependent RNA polymerase. Genome segment A (approximately 3100 bp) contains 2 partially opverlapping open reading frames (ORF) (Duncan et al. 1987, Havarstein et al. 1990). The large ORF encodes a 106 kDa polyprotein which is cleaved to produce 3 polypeptides: pVP2, the precursor of the major capsid protein VP2; VP3, a minor capsid protein; and NS, a non-structural protein. The pVP2 protein is further cleaved to VP2 during virus maturation. VP2 is the major outer capsid protein of the virus, whereas VP3 is thought to be an internal virion protein. The protease activity responsible for the cleavage has been associated with the non-structural virion protein NS (Duncan et al. 1987, Manning & Leong 1990, Manning et al. 1990, Magyar & Dobos 1994). The coding order for these polypeptides within the virion genome is NH_2-pVP2-NS-VP3-COOH. The small ORF overlaps the amino terminal end of the polyprotein ORF in a different reading frame. This small ORF encodes a 17 kDa arginine-rich minor polypeptide, designated VP5. VP5 has been detected in purified virus and infected cells (Magyar & Dobos 1994) but its function is unknown. Complete nucleotide sequences of genome segment A have been determined for only 5 strains: Jasper-Dobos (JaD) (Duncan & Dobos 1986), N1 (Havarstein et al. 1990), DRT (Chung, Lee, Lee, Ha, Lee & Kim, 1994, GenBank acession no. D265527), Sp (Mason & Leong, 1996, GenBank accession no. U48225) and West Buxton (WB) (Yao & Vakharia 1998).

The vast majority of aquatic birnaviruses, regardless of host species or geographic origin, are related antigenically and form a major serogroup (Serogroup A), (Caswell-Reno et al. 1989, Nicholson 1993, Hill & Way 1995). Relatively few antigenically unrelated aquatic birnaviruses represent a second, minor serogroup (Serogroup B). Considerable antigenic diversity exists among Serogroup A aquatic birnaviruses. Based on reciprocal neutralization tests with polyclonal antisera and enzyme immunoassays with monoclonal antibodies, Serogroup A contains 9 cross-reacting serotypes: A1 (type strain West Buxton), A2 (type strain Sp), A3 (type strain Ab), A4 (type strain He), A5 (type strain Te), A6 (type strain Canada 1), A7 (type strain Canada 2), A8 (type strain Canada 3), and A9 (type strain Jasper-ATCC VR 1325). Most aquatic birnavirus isolates from freshwater and marine fish in the United States belong to the A1 serotype. Four serotypes (A6, A7, A8, and A9) occur in Canada, and 4 serotypes (A2, A3, A4, and A5) are found in Europe. Isolates representing Serotypes A1, A2, and A3 have been found in Asia and South America.

Relationships of aquatic birnaviruses have been studied at the genomic level in only a few investigations of a limited number of virus strains and/or relatively small genomic fragments. Comparing deduced amino acid sequences of a 310 bp cDNA fragment located at the junction of the C-terminus of the pVP2 and the NS coding regions of 17 isolates, Heppell et al. (1993) suggested that only 3 major genogroups exist among the many Serogroup A viral strains. Little or no correlation was found among genogroups based on these VP2/NS cDNA sequences and the established serological groups. However, the region investigated was relatively small (310 bp) and located in the region of the junction of the VP2 and NS coding regions. More recently, Heppell et al. (1995) determined the nucleotide and deduced amino acid sequences of the VP2 coding region of 5 aquatic birnavirus strains.

In serological studies and in studies of nucleotide sequences and restriction endonuclease patterns of a 359 bp fragment of genome segment A, Berthiaume et al. (1992) indicated that Jasper strain viruses (Jasper-American Type Culture Collection ATCC VR-1325 [JaATTC] and Jasper-Dobes [JaD]) maintained in 2 different laboratories, but originally obtained from the same source, were not identical. Thus, there has been some confusion regarding the true type strain of the Jasper (A9) serotype of aquatic birnaviruses.

In order to better understand genetic relationships and diversity among these viruses, we compared the nucleotide and deduced amino acid sequences of a 2904 bp genomic fragment representing all but about 200 C-terminus nucleotides of the large ORF of genome segment A of the 9 type strains of Serogroup A and 4 other representative strains of Serotype A1, the predominant serotype in the United States. In addition, nucleotide and deduced amino acid sequences were determined for the VP2 coding region (1611 bp) of a variety of isolates (28 isolates) representing 5 of the 9 serotypes, including viruses from widely different geographical and host origins. The VP2 coding region was chosen for detailed investigation because VP2 is the major capsid protein and contains all neutralization epi-

topes (Lipipun et al. 1992). These aquatic birnaviruses were clustered into 6 genogroups, several of which were comprised of several genotypes. In contrast to previous studies of shorter genomic sequences within the pVP2/NS coding region (Heppell et al. 1993), these genogroups, based on the entire large ORF and the entire VP2 coding region, generally correlated with geographical origin and serological classification. Interestingly, isolates from the major Canadian serotypes were more closely related to the European isolates than to isolates from the United States.

MATERIAL AND METHODS

Cell cultures. The Chinook salmon embryo cell line (CHSE-214) (Lannon 1984) was propagated as monolayer cultures using Eagle's minimum essential medium (MEM, Sigma Chemical Co., St. Louis, MO) supplemented with 0.2% sodium bicarbonate (Sigma) and either 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT) for routine cell cultivation or 2% FBS for virus propagation. The cells were incubated at 20°C in a 5% CO_2 incubator as previously described (Caswell-Reno et al. 1986).

Viruses. The aquatic birnavirus isolates used for sequence comparisons in this investigation are listed in Table 1. Included were 2 viruses, each reputed to be the Jasper type strain but maintained in different laboratories. One strain studied by Duncan & Dobos (1986) and determined to belong to Serotype A1 is referred to as JaD; the other strain (ATCC, VR-1325) used in previous studies in our laboratory and determined to represent Serotype A9 is referred to as Ja-ATCC. A variety of isolates representing the Buhl Serotype of Serogroup A (91-114, 91-137, 64-93, 90-11, and CTT) were provided by Dr Paul Reno from Oregon State University. All other isolates have been maintained in our laboratory for many years.

Viral RNA extraction. The viral genomic RNA was extracted using the methods described by Blake et al. (1995). One hundred microliters of virus suspension was treated with 6 µl Proteinase K (10 mg ml^{-1}, Sigma) and lysis buffer (100 mM Tris-HCl, 1% SDS, 150 mM NaCl, 1.25 mM EDTA, pH 7.4) at 37°C for 3 h. The mixture was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) solution. The aqueous phase was then precipitated at –20°C overnight in the presence of 0.1 volume of 3 M sodium acetate and 2.5 volumes of prechilled 100% ethanol. The nucleic acid was collected by centrifugation at 14 000 × *g* for 30 min at 4°C. The pellet was washed with 70% ethanol, dried at room temperature, and resuspended in 10 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) buffer. The extracted nucleic acid was stored in –20°C.

Table 1. Aquatic birnaviruses

Virus	Geographic origin	Host of origin	Serotype
West Buxton	Maine, USA	Trout	A1
Dry Mills	Maine, USA	Trout	A1
VR299	West Virginia, USA	Trout	A1
Buhl	Idaho, USA	Trout	A1
Reno	Nevada, USA	Trout	A1
64-93	Idaho, USA	Trout	A1[a]
90-11	Idaho, USA	Trout	A1[a]
91-114	Idaho, USA	Trout	A1[a]
91-137	Idaho, USA	Trout	A1[a]
Sp	Denmark	Trout	A2
N1[b]	Norway	Atlantic salmon	A2
Fr 10	France	Trout	A2
Fr 21	France	Trout	A2
OV 2	England	Oyster	A2
DPL	Thailand	Snakehead	Not typed
Ab	Denmark	Trout	A3
EEV	Japan	Eel	A3
E1S	Taiwan	Eel	A3
PV	Taiwan	Perch	A3
CV-HB1	Taiwan	Clam	A3
He	Germany	Pike	A4
Te	England	Tellina	A5
C1	Canada	Trout	A6
ASV	Canada	Atlantic salmon	A6
C2	Canada	Trout	A7
C3	Canada	Arctic char	A8
JaD[c]	Canada	Trout	A1
JaATCC	Canada	Trout	A9

[a]Unpublished data from Paul Reno, Oregon State University
[b]Sequence from Havarstein et al. (1990)
[c]Sequence from Duncan & Dobos (1986)

Primers for PCR assays and cDNA nucleotide sequencing. Several oligonucleotide primers for PCR and cDNA sequencing were designed from the published cDNA sequence of genome segment A of the JaD (Duncan & Dobos 1986) strain of aquatic birnavirus. The primer sequences and location within genome segment A of aquatic birnaviruses are shown in Table 2. Different primer pairs were used for RT-PCR to amplify cDNA fragments that covered the large ORF of genome segment A.

Reverse transcription and polymerase chain reaction (RT-PCR). The RT-PCR procedure used in this study was a modification of the method previously described by Blake et al. (1995). Four microliters of viral RNA were mixed with 1.0 µl containing 0.5 µg of random hexamer primers and 2.2 µl of sterile distilled water. After heating at 80°C for 5 min and cooling at room temperature, the sample was mixed with 12.8 µl of RT master mix consisting of 4 µl of 5× First Strand RT buffer (Gibco BRL), 0.3 µl of 100 mM DTT, 1.0 µl (200 U) of Superscript II RNase H^- Reverse Transcrip-

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

	Primers (sequence)	Segment A position[a]	PCR product length	Coding region
Pb9	(GAGAGCTCTTACGGAGGAG)	39–547	508 bp	Noncoding–VP2
Pa8	(GACATCAAGCTGTTGTAGG)			
P8	(GGAAATACGACATCCAGAGCT)	421–1309	988 bp	pVP2
P10	(CACAGGATCATCTTGGCATAGT)			
P15	(GAACGGAGCAAGGATGAGGTG)	683–1759	1076 bp	VP2-NS
P12	(TGCACCACAGGAAAGATGACTC)			
P14	(GTATCCAACTATGAGCTGAT)	1224–2160	936 bp	pVP2-NS
P1R	(GTTCATGGGCGGCTATGGCTTT)			
P13	(GAGTCATCTTTCCTGTGGTGCA)	1738–2312	574 bp	NS
P7	(TCTCATCAGCTGGCCCAGGTAC)			
P1	(AAAGCCATAGCCGCCCATGAAC)	2139–3062	923 bp	VP3
PY	(ATCAGTCAGGAAAGAGAGTA)			
P17	(CCAGTTCATCGGAGATCTCAC)	1369–2160	791 BP	NS
P1R	(GTTCATGGGCGGCTATGGCTTT)			

[a]Map position of the primers based on the published sequence of Jasper strain (Duncan & Dobos 1986)

tase (Gibco BRL), 0.5 µl (20 U) of RNasin (Promega), and 4.0 µl of 10 mM dNTP mix (Promega). The reaction was performed at 42°C for 1 h and stopped by heating at 80°C for 5 min. The PCR assay was performed with 10 µl of cDNA, 5 µl of 10× PCR buffer (Perkin Elmer Co.), 2.5 U of *AmpliTaq* DNA polymerase (Perkin Elmer Co.), 0.1 µg of each primer (sense and antisense) and 1.0 to 2.0 mM Mg^{++}. The reaction mixture was adjusted to a final volume of 50 µl with sterile distilled water. Amplification was performed in a Programmable Thermal Controller (PCT-100, MJ Research Inc.) using the program listed in Table 3. The RT-PCR products were separated by electrophoresis through a 2% SeaPlaque agarose gel (FMC Bioproducts) containing ethidium bromide (0.5 µg ml^{-1}) in TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA). The ethidium-stained DNA bands were visualized by UV fluorescence.

Purification of RT-PCR amplification products. The amplified fragment of the expected length was cut out of the gel and transferred to a microcentrifuge tube, melted by heating at 65°C for 5 min, and treated with 5 U of Agarase (Sigma) were added to the tube. The samples were then incubated at 37°C overnight.

cDNA sequencing. The purified PCR products were cycle sequenced using *Taq* polymerase-mediated incorporation of dye-labeled dideoxy terminators. Ten microliters of PCR product (0.1 µg $µl^{-1}$) were mixed with 9.5 µl of Terminator Premix (PRISM Ready Reaction Sequencing Kit, Perkin Elmer, and 1 µl of primer (10 µM) in a 0.6 ml microcentrifuge tube. The cycling reaction was performed in a Perkin-Elmer (Model 480) thermal cycler using the following program: 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. The reaction mixture was then loaded on the top layer of a prewashed Spin-

Table 3. PCR amplification protocol for each pair of primers

Primer pair	Mg^{++} (mM)	Amplification				
		Denaturation (°C/min)	Denaturation (°C/s)	Annealing (°C/s)	Extension (°C/min)	Final (°C/min)
Pb9/Pa8	1.0					
P15/P12	1.0	93/2	92.5/45	58/45	72/1.75	72/7
P14/P1R	1.0					
P8/P10	1.5					
P17/P1R	1.5	93/2	92.5/45	59/45	72/1.75	72/7
P13/P7	1.5	93/2	92.5/45	58/45	72/1.75	72/7
P1/PY	2.0	100/5	95/45	60/45	72/1.75	72/7
		← 35 cycles →				

Column (Sepharose 100). The column was placed in a microcentrifuge tube and centrifuged at 750 × *g* for 2 min to remove unincorporated nucleotides. The sample was dried in a vacuum centrifuge and resuspended with 4 µl of loading buffer (83.3% [v/v] deionized formamide, 16.7% [v/v] 50 mM EDTA, pH 8.0). The sample was denatured at 90°C for 2 min, then immediately transferred into an ice bath and loaded onto a 7% polyacrylamide gel in an ABI Model 373A DNA sequencer at the University of Maine DNA Sequencing Facility.

Sequence analyses. Sequences were edited using the Sequence Navigator (Applied Biosystems, Inc.) Program. Deduced amino acid sequences were derived with the DNASTAR (Lasergene, Inc.) EditSeq computer program. Multiple sequence alignments were performed using the DNASTAR (Lasergene, Inc.) MegAlign program, with suggested parameters of Unweighted Gap penalty of 10, and Gap length penalty of 10. Phylogenetic trees were constructed with the Clustal algorithm of the MegAlign program and maximum parsimony analysis using Branch and Bound algorithm of PAUP version 3.1.

Nucleotide accession numbers. The nucleotide and deduced amino acid sequence data reported in this paper have been deposited in GenBank with the following accession numbers: West Buxton, AF342727; Dry Mills, AF343571; VR299, AF343572; Buhl, AF343573; Reno, AY026345; 64-93, AY026346; 90-11, AY026347; 91-114, AY026348; 91-137, AF343570; Sp, AF342728; Fr10, AY026482; Fr21, AY026483; OV2, AY026484; DPL, AY026485; Ab, AF342729; EEV, AY026486; E1S, AY026487; PV, AY026488; CV-HB1, AY026489; He, AF342730; Te, AF342730; C1, AF342731; ASV, AY026490; C2, AF342733; C3, AF342734; Ja-ATCC, AF342735.

RESULTS

In this study, we investigated the genomic relationships of 28 viruses, including the type strains of all 9 serotypes of Serogroup A. The nucleotide and deduced amino acid sequences of cDNA of the large ORF (2904 bp) of genome segment A were determined for the type strains of the 9 serotypes. The Jasper-ATCC strain (Serotype A9) that was used in previous serological investigations in our laboratory was analyzed at the nucleic acid and deduced amino acid levels in this study. Large ORF cDNA sequences were obtained for 5 individual strains (Dry Mills [DM], VR299, Buhl, West Buxton [WB] and 91-137) of Serotype A1 (WB), the predominate serotype in the United States. In addition, nucleotide and deduced amino acid sequences were determined for the VP2 coding region (1116 bp) of a variety of isolates representing 5 of the 9 serotypes. The 28 viruses used in this investigation represented widely different geographical and host origins. The deduced amino acid sequences, numbered from the beginning of the large ORF, of the virus strains sequenced in this study are shown in Fig. 1 along with those of the JaD and N1 strain sequences reported previously by other investigators (Duncan & Dobos 1986, Havarstein et al. 1990).

With all the viruses studied, the domain corresponding to amino acid residues 243-335 (Fig. 1) was highly variable, which also was previously observed by Heppell et al. (1995). In addition, in this study, other, smaller variable regions were identified with specific variations corresponding to a specific serotype or serotypes. Moreover, many highly conserved signature amino acid residues were identified within the VP2 protein that distinguish certain serotypes (Tables 4 & 5). These highly conserved signature amino acids clearly distinguished isolates from the United States and the Jasper strains from the other 3 Canadian serotypes and the European and Asian isolates (Table 4).

Comparisons of the nucleotide and deduced amino acid sequences of the large ORF of the 9 type strains (WB, Sp, Ab, Te, C1,C2, C3, He and JaATCC) as well as 4 other isolates of Serotype A1 and the previously reported sequences of the N1 and JaD strains are shown in Tables 6 & 7. All isolates from the United States and the 2 Jasper strains exhibited 89–99.6% identity in nucleotide sequences compared to 69.0–77.4% identity to the viruses from Canada, Europe and Asia. The deduced amino acid sequences of all isolates from the United States and the 2 Jasper strains were ≥95% similar to each other but only 80.1 to 84% similar to the

Table 4. Signature amino acid residues distinguishing United States and Jasper strains from other Canadian, European and Asian strains

Amino acid position	US/Jasper strains	European, Asian and Canadian strains
12	Arg	Lys
19	Asn	Thr
72	Leu	Ala or Val
243	Thr	Asn or Ser
261	Gln	Asp or Glu
311	Ala	Ser or Thr
313	Gln	Lys
426	Ala	Val
434	Thr	Ser
448	Asp	Ile
486	Ser	Ala
543	Phe	Val
561	Val	Ala
562	Val	Ile
563	Val	Ile
537	Ala	Gly
648	Ile	Val
665	Val	Ile
688	Cys	Asn
695	Met	Glu
703	Ser	Ala
717	Gln	Lys

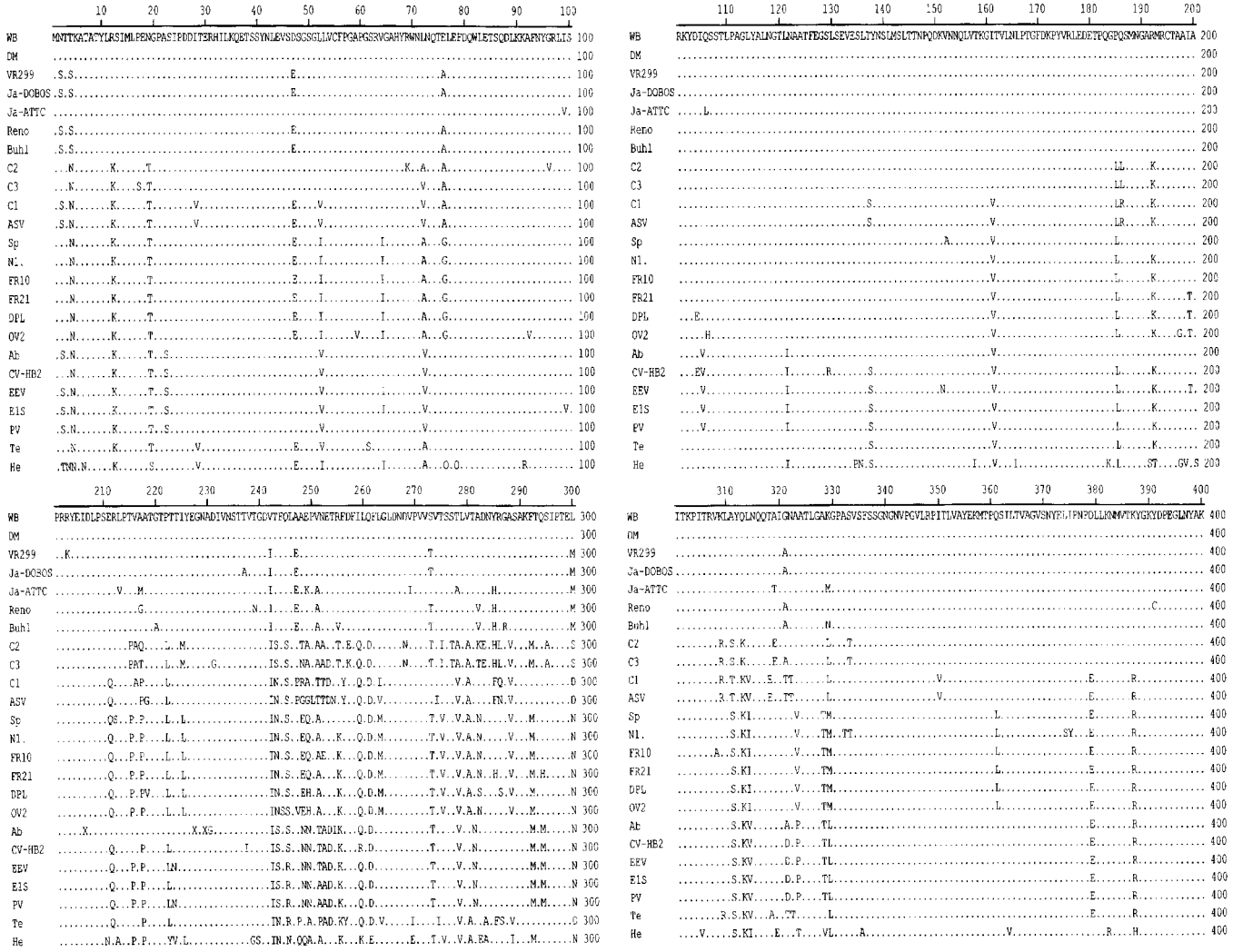

Fig. 1. (Above and facing page). Multiple alignment of deduced amino acid sequences encoded by large LORF of genome segment A of various aquatic birnaviruses. (.) indicates an amino acid residue identical to that of strain WB

viruses from Canada, Europe and Asia. The 2 most divergent pairs of viruses were Buhl and He, with a similarity in amino acid sequences of 80.1%.

Trees were constructed using maximum parsimony analysis to describe the phylogenetic history of these viruses based on the deduced amino acid similarities of the polyprotein encoded by the large ORF. The most parsimonious tree was found using the Branch and Bound algorithm of PAUP, Version 3.1, with 1000 bootstrap replicates performed to evaluate the reliability of the reconstructions. Distance trees were also constructed using a heuristic search computed by PAUP, with 1000 bootstrap replicates, and the Clustal distance method computed by DNAStar Lasergene. The tree topologies were identical using these 3 methods. Branch lengths were drawn proportional to the number of deduced amino acid differences using the Clustal distance method and bootstrap values from the parsimony analysis were included in the branches (Fig. 2). The cladogram of these phylogenetic relationships (Fig. 2) suggests that these viruses may represent 4 or 5 distinct genomic clusters. Clearly, the isolates from the United States and the Jasper strains appear to represent one distinct group.

Comparison of the genomic relationships of the different viral isolates based on the nucleotide sequences of the VP2 coding region (Table 8) demonstrated identities of 87.9–99.7% among the isolates from the United States and the Jasper strains, compared to 76.5–80.2% with the Canadian, European and Asian strains. The deduced amino acid sequences (Table 9) of all isolates from the United States and the 2 Jasper strains were more than 94% similar to each other, but were more divergent from the Canadian, European and Asian viruses. Other pairs or groups of viruses also exhibited high similarities to each other in amino acid sequences

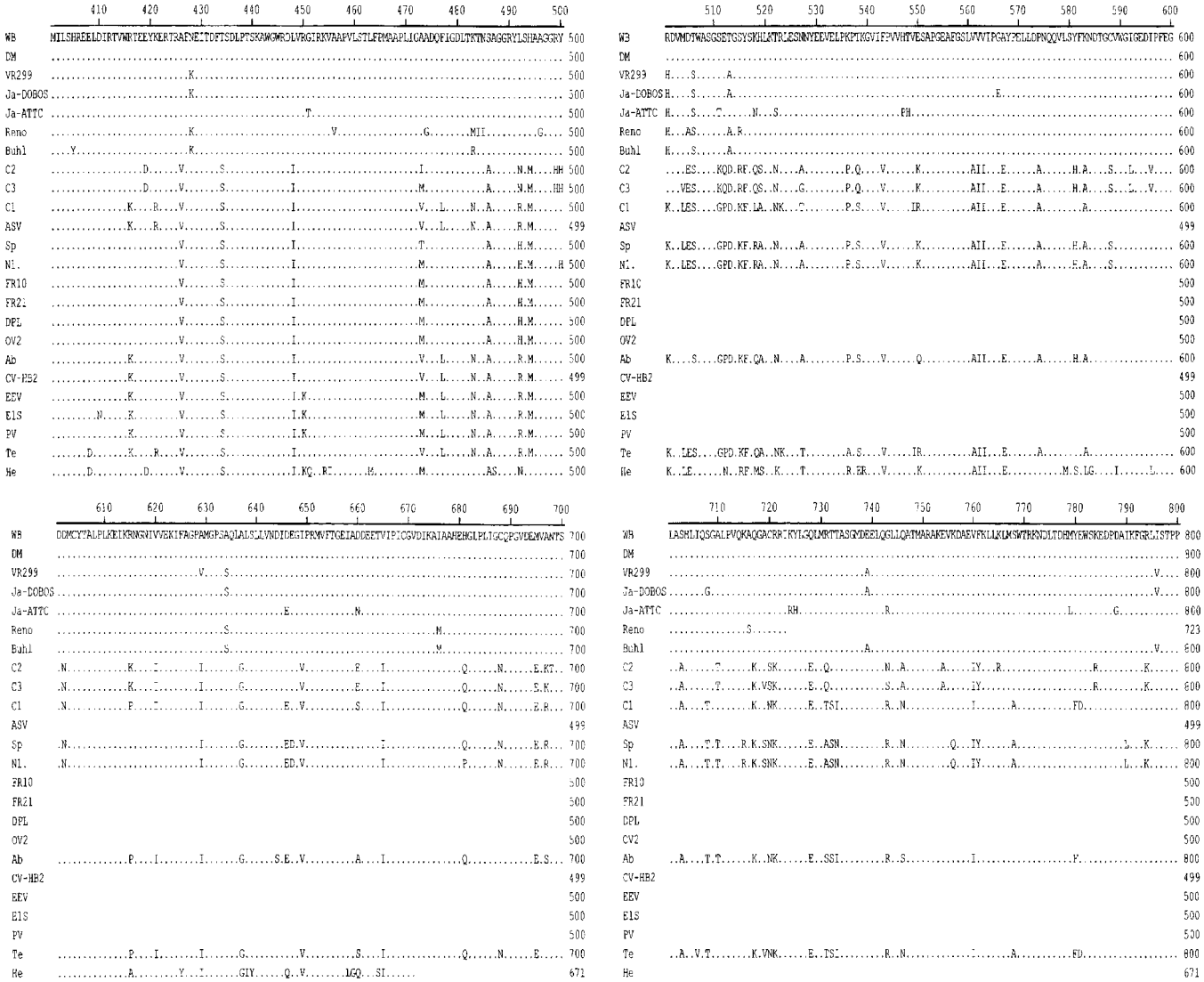
410 420 430 440 450 460 470 480 490 500
510 520 530 540 550 560 570 580 590 600
610 620 630 640 650 660 670 680 690 700
710 720 730 740 750 760 770 780 790 800
810 820 830 840 850 860 870 880 890 900
910 920 930 940 950 960 970
WB
DM
VR299
Ja-DOBOS
Ja-ATTC
Reno
Buhl
C2
C3
C1
ASV
Sp
N1.
FR10
FR21
DPL
OV2
Ab
CV-PS2
CV-HB2
EEV
E1S
PV
Te
He

Table 5. Signature amino acid residues distinguishing other groups of aquatic birnavirus isolates

Viruses	Amino acid position	Unique signature amino acid
Sp, N1, Fr10, Fr21, PDL, OV2	76	Gly
	263	Met
	314	Ile
	232	Val
	361	Leu
Ab, CV-HB1, EEV, E1S, PV	22	Ser
	105	Val
	281	Asn
	294	Met
C2, C3	225	Met
	255	Thr
	267	Asn
	275	Ile
	276	Thr
	283	Glu
	286	Leu
	295	Ala
	300	Ser
	319	Glu
C1, Te	246	Pro
	256	Tyr
	285	Phe
	288	Val
	321	Th

but were less closely related to other isolates. The highest degree of divergence (18.8%) was exhibited between the He (A1) type strain and the Reno and 90-11 strains representing the WB (A1) serotype.

A classification of these aquatic birnaviruses was determined on the basis of deduced amino acid similarities of VP2 using Megalign and PAUP as descibed previously (Fig. 3). The 28 aquatic birnavirus isolates were clustered into 6 genogroups that generally correlated with geographic origin of the virus and previous serological classifications. Genogroup 1 consisted of all isolates from the United States (Serotype A1) and the 2 Jasper strains from Canada. Genogroup 2 contained all of the isolates belonging to Serotype A3, including viruses from both Europe and Asia. Two Canadian isolates representing Serotype A6 (C1 and ASV) and the type strain (Te) of the European serotype A5 formed Genogroup 3. The type strains of the 2 Canadian Serotypes A7 (C2) and A8 (C3) comprised Genogroup 4. All members of Serotype A2, including 5 European isolates and 1 Asian isolate, formed Genogroup 5. The He strain, the only known representative of Serotype A4, represented Genogroup 6. Interestingly, viruses representing the 3 major serotypes found in Canada (A6, A7, and A8) were more closely related to the European and Asian isolates (Serotypes A2, A3, A4, and A5) than

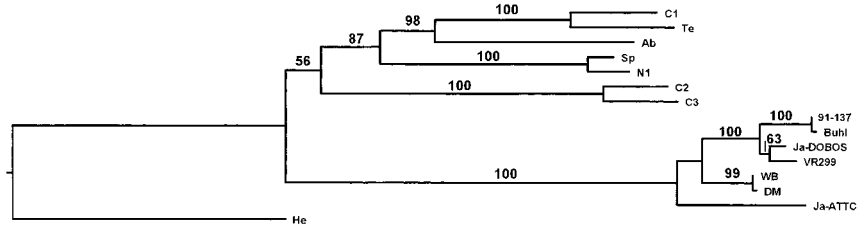

Fig. 2. Cladogram representing phylogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of VP2. The length of each pair of branches represents the distance between sequence pairs, and the numbers indicate the bootstrap values

Table 6. Pairwise distances of aquatic birnaviruses based on nucleotide sequences of large ORF of genome segment A

		Percent identity													
		WB	DM	JaD	VR299	91137	Buhl	JaA	Sp	N1	Ab	C1	C2	C3	Te	He
Percent divergence	WB		99.6	91.1	91.0	90.7	89.0	90.6	77.1	77.0	77.4	76.8	76.1	75.7	75.8	70.1
	DM	0.4		91.1	91.0	90.8	88.9	90.6	77.0	76.8	77.2	76.8	76.1	75.8	75.7	70.3
	JaD	8.5	8.6		99.6	98.1	96.4	91.4	76.9	76.7	77.2	76.2	76.2	75.8	75.8	70.8
	VR299	8.6	8.6	0.4		98.0	96.2	91.2	76.9	76.7	77.1	76.3	76.2	75.7	75.8	70.7
	91137	8.9	8.9	1.9	2.0		97.5	90.7	77.0	77.0	77.0	76.3	76.4	76.0	76.0	71.0
	Buhl	9.2	9.4	2.2	2.3	1.1		89.1	75.3	75.3	75.4	74.6	74.7	74.3	74.3	69.0
	JaATCC	8.8	8.8	8.4	8.5	8.9	9.1		76.8	76.7	76.7	76.4	76.4	76.3	75.7	70.5
	Sp	20.8	20.7	20.8	20.8	20.7	21.0	20.8		98.2	85.9	85.9	83.4	83.1	86.3	75.1
	N1	20.9	20.9	21.0	21.0	20.7	21.0	20.9	1.7		85.8	86.2	83.6	83.5	86.8	75.1
	Ab	19.9	20.0	20.3	20.4	20.4	20.6	20.5	13.1	13.2		87.0	81.5	81.7	86.3	73.2
	C1	21.0	21.0	21.2	21.1	21.1	21.5	21.2	13.3	13.0	12.3		82.4	83.0	91.0	73.0
	C2	21.7	21.7	21.7	21.7	21.6	21.8	21.3	15.2	15.1	17.0	16.4		97.0	81.4	73.3
	C3	22.0	21.8	22.0	22.0	21.8	22.1	21.4	15.4	15.0	16.9	15.9	3.0		81.6	72.9
	Te	21.5	21.5	21.4	21.4	21.4	21.7	21.5	12.8	12.5	12.8	8.7	17.1	16.8		73.8
	He	25.9	25.7	25.5	25.5	25.3	25.8	25.6	22.6	22.5	24.1	24.2	23.9	24.4	23.6	

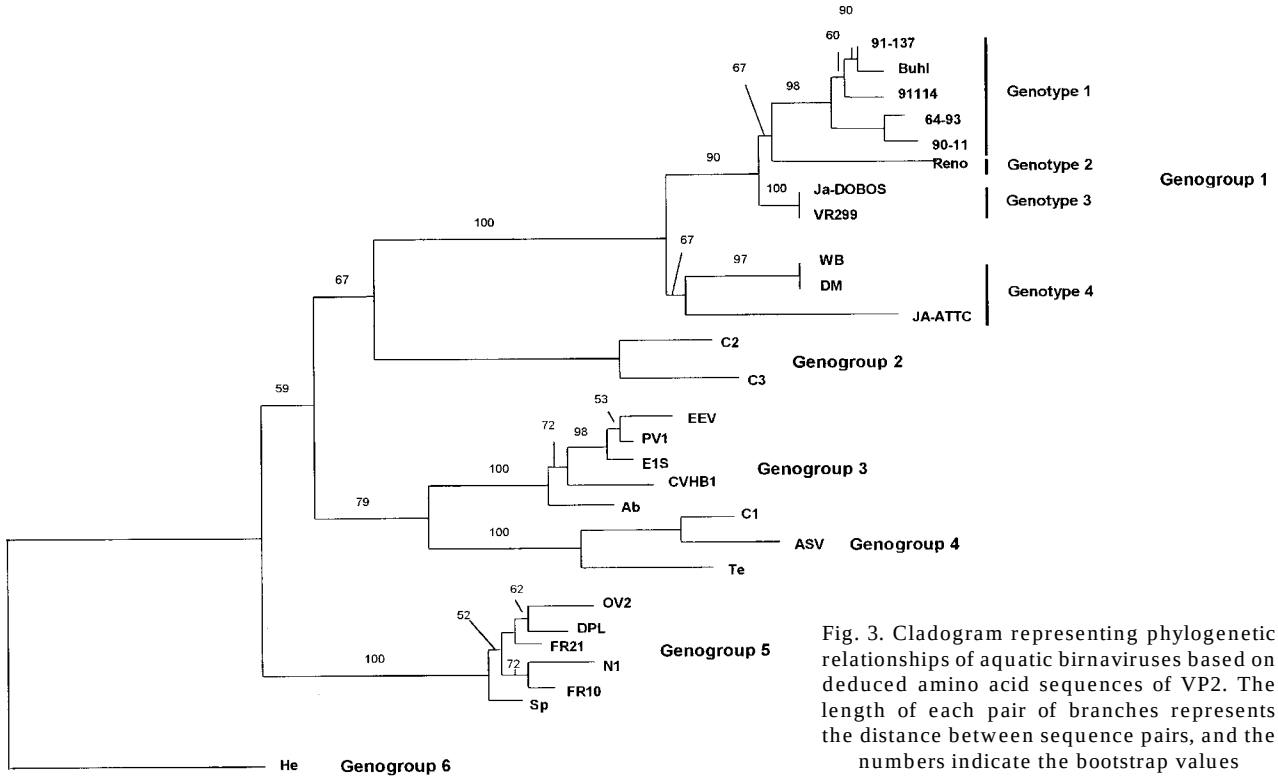

Fig. 3. Cladogram representing phylogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of VP2. The length of each pair of branches represents the distance between sequence pairs, and the numbers indicate the bootstrap values

to isolates representing the prevalent serotype (A1) in the United States.

Some genogroups comprised of a number of viruses appeared to contain 2 or more genotypes. This is particularly evident in Genogroup 1, in which 4 genotypes can be identified that corresponded to previously identified specific serological strains. Previous investigations using monoclonal antibodies have shown that at least 4 distinct strains of aquatic birnaviruses can be identified within Serotype A1: WB strain, VR299 strain, Buhl strain, and Reno strain (Caswell-Reno et al. 1986, 1989, P. Reno, Oregon State University, pers. comm.). Genotype 1 contained 5 viruses (Buhl, 91-114, 91-137, 64-93, and 90-11) that were previously shown using monoclonal antibodies to belong to a single serological strain (Buhl strain) within the A1 serotype. Three, or possibly 4, other genotypes were also identified within Genogroup 1. Genotype 2 included strain VR299 and the

Table 7. Pairwise distances of aquatic birnaviruses based on deduced amino acid sequences of large ORF of genome segment A

		Percent similarity													
		WB	DM	JaD	VR299	91137	Buhl	JaA	Sp	N1	Ab	C1	C2	C3	Te	He
Percent divergence	WB		99.9	97.5	97.3	96.9	96.8	96.3	85.3	84.9	84.9	84.2	84.3	83.8	84.2	81.1
	DM	0.1		97.4	97.2	96.9	96.8	96.2	85.2	84.8	84.8	84.1	84.2	83.7	84.1	81.0
	JaD	2.5	2.6		99.2	98.6	98.5	95.5	84.5	84.1	84.5	84.2	83.7	83.4	83.6	80.6
	VR299	2.7	2.8	0.8		98.3	98.2	95.2	84.4	84.0	84.4	84.1	83.7	83.4	83.5	80.4
	91137	3.1	3.1	1.4	1.7		99.9	95.1	84.0	83.7	84.0	83.7	83.6	83.3	83.2	80.2
	Buhl	3.2	3.2	1.5	1.8	0.1		95.0	83.9	83.6	83.9	83.6	83.5	83.2	83.1	80.1
	JaATCC	3.7	3.8	4.5	4.8	4.9	5.0		84.2	83.8	83.7	83.4	83.7	83.2	83.4	80.2
	Sp	14.7	14.8	15.5	15.6	16.0	16.1	15.8		98.8	90.5	91.1	88.5	88.2	90.3	84.6
	N1	15.1	15.2	15.9	16.0	16.3	16.4	16.2	1.2		90.1	90.8	88.7	88.5	90.2	84.5
	Ab	14.8	14.9	15.2	15.3	15.8	15.9	16.1	9.2	9.6		92.0	86.5	87.0	91.5	82.1
	C1	15.8	15.9	15.8	15.9	16.3	16.4	16.6	8.9	9.2	7.7		87.0	87.3	96.5	82.3
	C2	15.7	15.8	16.3	16.3	16.4	16.5	16.3	11.5	11.3	13.3	13.0		97.4	86.5	82.6
	C3	16.2	16.3	16.6	16.6	16.7	16.8	16.8	11.8	11.5	12.7	12.7	2.6		86.8	82.3
	Te	15.8	15.9	16.4	16.5	16.8	16.9	16.6	9.7	9.8	8.2	3.5	13.5	13.2		82.9
	He	18.8	18.9	19.3	19.5	19.8	19.9	19.8	15.3	15.4	17.5	17.6	17.3	17.6	17.1	

Table 8. Pairwise distances of aquatic birnaviruses based on nucleotide sequences of VP2 coding region of genome segment A

Percent divergence	Percent identity	WB	DM	Buhl	91114	6493	91137	9011	Reno	VR299	JaA	JaD	C1	ASV	C2	C3	Ab	EEV	E1S	CVHB	PV1	Sp	N1	DPL	Fr10	Fr21	OV2	He	Te
WB			99.7	88.3	88.7	87.9	88.8	87.9	88.5	89.3	89.9	89.4	78.0	76.7	77.5	77.5	80.2	76.7	77.1	76.9	77.3	78.4	78.0	78.3	78.7	78.5	77.7	69.4	76.2
DM	0.3			88.6	88.9	88.2	89.0	88.2	88.6	89.5	89.9	89.6	78.1	76.9	77.5	77.5	80.1	76.7	77.1	76.9	77.3	78.5	78.0	78.3	78.7	78.5	77.7	69.6	76.4
Buhl	11.1	10.9			99.2	98.1	99.1	98.2	97.9	97.6	89.9	97.6	77.2	76.4	76.7	76.8	78.5	77.0	77.4	77.0	77.4	78.2	77.9	78.0	77.9	78.5	77.6	69.8	76.5
91114	10.8	10.6	0.8			98.5	99.6	98.4	97.5	97.8	90.2	97.8	77.7	76.9	77.2	77.2	78.8	77.4	77.7	77.4	77.8	78.5	78.3	78.3	78.2	78.8	77.9	70.3	76.9
6493	11.2	11.0	1.8	1.5			98.2	99.4	97.0	97.3	90.0	97.3	77.2	76.4	76.5	76.5	78.3	76.8	77.2	76.8	77.3	78.0	77.8	77.7	77.5	78.2	77.4	70.9	76.0
91137	10.6	10.5	0.9	0.4	1.7			98.4	97.4	97.8	90.2	97.8	77.7	77.0	77.2	77.4	78.9	77.4	77.8	77.4	77.8	78.5	78.4	78.4	78.3	78.8	78.1	70.4	76.9
9011	11.3	11.1	1.8	1.6	0.5	1.6			97.1	97.4	90.0	97.3	77.2	76.5	76.5	76.5	78.4	76.9	77.3	76.9	77.4	78.0	77.8	77.8	77.6	78.2	77.4	70.7	76.2
Reno	10.9	10.8	2.0	2.4	2.8	2.5	2.8			98.2	90.3	98.1	76.7	76.2	76.3	76.2	78.3	76.5	76.9	76.5	77.0	78.0	77.5	77.5	77.6	78.0	77.2	69.5	75.8
VR299	10.3	10.1	2.3	2.2	2.5	2.2	2.5	1.7			90.7	99.8	77.4	76.8	76.8	76.6	78.7	76.9	77.3	76.9	77.4	78.1	77.7	77.7	77.8	78.0	77.4	70.2	76.2
JaATCC	9.6	9.6	9.7	9.5	9.6	9.5	9.7	9.5	9.2			90.8	77.5	76.9	77.0	76.7	78.4	76.6	77.3	76.9	77.3	77.9	77.5	77.6	77.8	77.9	77.2	69.8	76.1
JaD	10.2	10.0	2.4	2.2	2.6	2.2	2.6	1.7	0.2	9.1			77.3	76.8	76.8	76.7	78.7	76.9	77.4	77.0	77.4	78.2	77.7	77.8	77.9	78.1	77.5	70.3	76.3
C1	20.3	20.2	20.6	20.2	20.4	20.2	20.5	20.5	20.9	20.4	20.3	20.5		95.0	82.2	82.9	86.1	86.4	86.6	87.5	86.9	85.1	85.3	85.1	85.3	85.2	84.5	71.5	90.7
ASV	21.2	21.0	21.2	20.9	21.2	20.8	21.2	21.2	21.4	20.9	20.6	20.8	4.7		81.9	82.5	85.5	86.0	86.1	86.8	86.4	84.6	84.7	85.0	85.0	84.8	84.7	71.4	90.7
C2	20.7	20.7	21.3	21.1	21.6	21.1	21.5	21.7	21.6	21.4	20.9	21.4	16.8	17.0		96.6	80.9	81.8	82.0	82.6	82.2	82.5	82.9	82.8	83.1	83.1	82.4	72.5	81.3
C3	20.9	20.9	21.5	21.2	21.7	21.2	21.7	21.7	22.0	21.7	21.1	21.6	15.9	16.1	3.4		81.2	82.4	82.5	83.0	82.7	82.4	83.2	82.8	82.7	83.0	82.2	72.0	81.6
Ab	17.6	17.7	19.2	19.0	19.4	19.0	19.4	19.4	19.5	19.1	18.7	19.0	13.1	13.4	17.5	17.2		94.4	94.6	94.4	94.5	85.4	85.2	85.7	85.8	85.8	85.2	70.9	84.7
EEV	21.0	20.9	20.7	20.6	20.9	20.5	20.9	21.2	20.8	20.7	20.7	20.7	12.8	12.9	16.7	16.2	5.1		99.3	97.3	99.2	87.3	87.3	87.7	87.3	87.6	87.1	72.3	86.0
E1S	20.7	20.6	20.4	20.3	20.6	20.2	20.6	20.6	20.9	20.5	20.3	20.4	12.7	12.8	16.6	16.1	5.1	0.6		97.5	99.3	87.7	87.7	88.0	87.7	87.9	87.4	72.5	86.3
CVHB	20.8	20.7	20.7	20.6	20.9	20.4	20.7	20.9	21.2	20.7	20.4	20.7	12.0	12.5	16.0	15.7	5.1	2.5	2.5		97.5	87.1	87.3	87.3	87.1	87.2	86.9	71.7	86.5
PV	20.6	20.6	20.4	20.3	20.6	20.2	20.6	20.6	20.9	20.5	20.3	20.4	12.5	12.6	16.6	16.0	5.1	0.7	0.6	2.5		87.6	87.6	87.9	87.6	87.7	87.3	72.6	86.7
SP	19.9	19.8	19.8	19.7	20.0	19.6	20.0	20.0	20.0	19.8	20.0	19.7	14.0	14.1	15.7	15.6	13.2	11.7	11.4	12.0	11.5		97.7	98.1	98.1	98.7	98.1	74.5	85.5
N1	20.3	20.2	19.9	19.8	20.1	19.7	19.7	20.1	20.4	20.2	20.4	20.1	13.8	14.2	15.7	15.2	13.3	11.6	11.3	11.7	11.4	2.2		97.7	97.7	98.1	97.6	74.6	86.0
DPL	19.9	19.9	19.8	19.8	20.1	19.7	20.1	20.1	20.3	20.1	20.3	20.0	14.0	14.0	15.6	15.4	13.0	11.3	11.1	11.7	11.2	1.9	2.2		98.6	98.8	98.4	74.1	86.1
Fr10	19.8	19.6	19.9	19.8	20.3	19.8	20.2	20.2	20.0	20.0	20.1	19.9	13.9	13.9	15.3	15.4	12.7	11.5	11.2	11.7	11.3	1.8	2.2	1.3		98.6	98.2	74.4	86.3
Fr21	19.9	19.8	19.5	19.4	19.8	19.4	19.8	19.8	20.0	19.9	20.1	19.8	14.0	14.1	15.4	15.3	12.9	11.4	11.2	11.8	11.3	1.3	1.8	1.2	1.2		98.9	74.3	86.0
OV2	20.4	20.3	20.1	20.0	20.3	19.8	20.4	20.4	20.4	20.2	20.6	20.1	14.5	14.3	15.8	15.7	13.3	11.7	11.5	12.0	11.6	1.9	2.4	1.6	1.7	1.1		74.4	85.4
He	26.4	26.3	26.1	25.9	25.4	25.8	25.6	25.6	26.6	26.1	26.2	26.0	25.8	25.7	24.4	25.3	25.8	24.8	24.8	25.5	24.7	23.2	23.0	23.4	23.3	23.4	23.3		72.2
Te	21.4	21.2	21.1	20.9	21.5	20.9	21.5	21.5	21.7	21.2	21.3	21.2	9.0	9.0	17.1	16.7	13.9	13.1	12.9	12.7	12.6	13.4	13.1	13.0	12.8	13.2	13.6	25.2	

JaD strain. The Reno strain constituted Genotype 3. Genotype 4 was formed by WB and DM, both of which represent the same strain identified by monoclonal antibody reaction patterns. The JaATCC strain, which currently constitutes a separate serotype may or may not represent a fifth genotype within Genogroup 1.

DISCUSSION

Complete sequences of genome segment A, which encodes the virion structural proteins and the protease responsible for cleavage of the primary polyprotein gene product, are available for only 4 aquatic birnavirus strains: Sp, N1 (Haverstein et al. 1990), JaD (Duncan & Dobos 1986), and DRT (Chung, Lee, Lee, Ha, Lee & Kim, 1994, GenBank accession no. D26527). Previous investigations of relationships of aquatic birnaviruses based on genomic nucleotide and deduced amino acid sequences focused on relatively short genomic fragments and/or a limited number of viral isolates (Heppell et al. 1993, 1995). In this study, we investigated the genomic relationships of 28 aquatic birnaviruses that represent widely different geographical and host origins, including the type strains of all 9 serotypes of Serogroup A.

Previously, Heppell et al. (1995) reported a central variable domain encompassing 2 hypervariable segments within the VP2 coding region based on a study of 7 virus strains. This variable domain corresponding to amino acid residues 243–335 was confirmed in this study of 28 viruses (Fig. 1).

High levels of identity (89 to 99.6%) were found among all isolates from the United States and the 2 Jasper strains but these viruses showed much lower identity levels (73.3 to 77.4%) when compared to viruses from Canada, Europe and Asia (Tables 6 & 7). In terms of deduced amino acid sequences, all isolates from the United States and

Table 9. Pairwise distances of aquatic birnaviruses based on deduced amino acid sequences of VP2

Percent divergence	Percent similarity	WB	DM	Buhl	91114	6493	91137	9011	Reno	VR299	JaA	JaD	C1	ASV	C2	C3	Ab	EEV	E1S	CVHB	PV1	Sp	N1	DPL	Fr10	Fr21	OV2	He	Te
WB			100.0	95.9	95.7	95.3	96.1	95.3	95.0	97.4	96.3	97.4	86.2	85.7	87.3	86.8	88.3	87.0	87.2	87.5	87.5	88.1	87.2	87.3	87.7	87.5	86.8	86.2	82.5
DM	0.0			95.9	95.7	95.3	96.1	95.3	95.0	97.4	96.3	97.4	86.2	85.7	87.3	86.8	88.3	87.0	87.2	87.5	87.5	88.1	87.2	87.3	87.7	87.5	86.8	86.2	82.5
Buhl	4.1	4.1			99.4	98.7	99.8	98.7	97.0	98.1	95.0	98.1	85.8	85.3	86.6	86.4	87.5	86.0	86.4	86.2	86.8	87.2	86.4	86.6	87.0	87.2	86.2	85.3	81.6
91114	4.3	4.3	0.6			98.9	99.6	98.5	97.0	98.0	95.0	98.0	86.0	85.5	86.6	86.4	87.3	86.0	86.4	86.2	86.8	87.2	86.4	86.6	87.0	87.2	86.2	85.5	81.6
6493	4.5	4.5	1.1	0.9			98.9	98.9	96.3	97.6	94.4	97.6	85.5	84.9	86.2	86.0	86.8	85.3	85.7	85.5	86.0	86.6	85.8	86.2	86.4	86.6	85.8	84.9	81.6
91137	3.9	3.9	0.2	0.4	0.9			98.9	97.2	98.3	95.2	98.3	86.0	85.5	86.8	86.6	87.7	86.2	86.6	86.4	87.0	87.3	86.6	86.8	87.2	87.3	86.4	85.5	81.8
9011	4.7	4.7	1.3	1.5	0.9	1.1			96.3	97.6	94.6	97.6	85.3	84.7	86.4	86.2	86.8	85.3	85.7	85.5	86.0	86.6	85.8	86.2	86.4	86.6	85.7	84.9	81.2
Reno	5.0	5.0	3.0	3.0	3.5	2.8	3.7			97.2	94.0	97.2	85.3	84.7	86.0	85.8	86.4	85.1	85.5	85.3	85.8	86.6	85.7	85.8	86.2	86.4	85.5	84.5	81.2
JaATCC	2.6	2.6	1.9	2.0	2.2	1.7	2.4	2.8			4.5	99.6	86.6	86.0	86.8	86.6	88.3	86.8	87.0	87.0	87.3	87.9	87.0	87.2	87.5	87.3	86.8	85.8	82.1
VR299	3.7	3.7	5.0	5.0	5.4	4.8	5.4	6.0	95.5			95.5	85.5	84.9	87.0	86.4	87.0	85.8	86.6	86.4	86.6	87.3	86.4	86.6	87.0	87.2	86.4	85.8	81.8
JaD	2.6	2.6	1.9	2.0	2.2	1.7	2.4	2.8	0.4	4.5			86.6	86.0	86.8	86.6	88.3	86.8	87.0	87.2	87.3	87.9	87.0	87.2	87.5	87.3	86.8	85.8	82.1
C1	13.8	13.8	14.2	14.0	14.4	14.0	14.7	14.7	14.7	13.4	14.5	13.4		98.3	88.1	88.5	90.7	91.2	91.2	91.6	91.6	90.9	90.3	90.7	91.1	90.9	90.1	95.9	82.5
ASV	14.3	14.3	14.7	14.5	14.9	14.5	15.3	15.3	15.3	14.0	15.1	14.0	1.7		87.0	87.3	90.3	90.7	90.7	91.1	91.1	90.3	89.8	90.3	90.5	90.3	89.6	95.7	81.8
C2	12.7	12.7	13.4	13.4	13.6	13.2	13.6	14.0	13.2	13.0	13.6	13.2	11.9	13.0		97.4	87.7	87.9	88.3	88.5	88.6	89.9	90.3	89.9	90.3	90.3	89.2	87.7	83.6
C3	13.2	13.2	13.6	13.6	13.8	13.4	13.8	14.2	14.0	13.4	13.6	13.4	11.5	12.7	2.6		88.1	88.3	88.6	88.6	89.0	89.2	89.8	89.4	89.8	89.8	88.6	87.7	83.2
Ab	11.2	11.2	12.0	12.2	12.6	11.8	12.7	13.1	11.2	12.5	13.6	11.2	8.8	9.2	11.8	11.4		96.3	96.3	96.6	96.6	90.9	90.3	90.5	91.1	90.9	89.9	90.3	82.3
EEV	13.0	13.0	14.0	14.0	14.6	13.8	14.7	14.9	13.2	14.2	14.2	13.2	8.8	9.3	12.1	11.7	3.2		98.9	97.4	99.3	91.6	91.6	92.2	92.4	92.6	91.6	91.2	84.0
E1S	12.8	12.8	13.6	13.6	14.2	13.4	13.2	14.5	13.0	13.4	13.4	13.0	8.8	9.3	11.7	11.4	3.2	1.1		97.4	99.3	92.0	92.0	92.2	92.7	92.6	91.6	91.4	84.2
CVHB	12.5	12.5	13.8	13.8	14.4	13.6	13.4	14.7	14.0	13.4	13.6	12.8	8.4	8.9	11.5	11.4	2.8	2.6	2.6		97.8	91.4	91.6	91.8	92.0	91.8	90.9	91.6	82.9
PV1	12.5	12.5	13.2	13.2	13.8	13.0	14.0	14.2	14.2	12.7	13.4	12.7	8.4	8.9	11.4	11.0	2.8	0.7	0.7	2.2		92.0	92.0	92.2	92.7	92.6	91.6	91.8	84.4
Sp	11.9	11.9	12.8	12.8	13.2	12.7	13.4	14.2	13.4	12.1	12.7	12.1	9.1	9.7	10.1	10.8	8.6	8.4	8.0	8.6	8.0		98.1	98.0	98.7	98.5	97.6	90.5	86.6
N1	12.8	12.8	13.6	13.6	14.0	13.4	13.2	14.3	13.0	13.6	13.4	13.0	9.7	10.2	9.7	10.2	9.2	8.4	8.0	8.4	8.0	1.9		98.0	98.7	98.5	97.6	90.3	86.2
DPL	12.7	12.7	13.4	13.4	13.6	13.2	13.8	14.2	14.2	12.8	13.4	12.8	9.3	9.7	10.1	10.6	9.0	7.8	7.8	8.2	7.8	2.0	2.0		98.5	98.7	98.1	90.9	86.4
Fr10	12.3	12.3	13.0	13.0	13.4	12.8	13.6	13.8	12.5	13.0	12.5	12.5	8.9	9.5	9.7	10.2	8.4	7.6	7.3	8.0	7.3	1.3	1.3	1.5		99.1	98.1	91.1	86.8
Fr21	12.5	12.5	12.8	12.8	13.2	12.7	13.4	13.6	12.7	12.8	12.7	12.7	9.1	9.7	9.7	10.2	8.6	7.4	7.4	8.2	7.4	1.5	1.5	1.3	0.9		98.3	90.9	86.6
OV2	13.2	13.2	13.8	13.8	14.0	13.6	14.3	14.5	13.2	13.6	14.2	14.2	9.9	10.4	10.8	11.4	9.6	8.4	8.4	9.1	8.4	2.4	2.4	1.9	1.9	1.7		90.1	86.4
Te	13.8	13.8	14.7	14.5	14.9	14.5	15.1	15.5	14.2	14.2	14.2	14.2	4.1	4.3	12.3	12.3	9.2	8.8	8.6	8.4	8.2	9.5	9.7	9.1	8.9	9.1	9.9		83.6
He	17.5	17.5	18.4	18.4	18.3	18.2	18.8	18.8	17.9	18.2	17.9	17.9	17.5	18.2	16.4	16.8	17.2	16.0	15.8	17.1	15.6	13.4	13.8	13.6	13.2	13.4	13.6	16.4	

the 2 Jasper strains also were very closely related (≥95% similarity) but were less closely related (83 to 84% similarity) to the viruses from Canada, Europe and Asia. The 2 most divergent pairs of viruses were Buhl and He with a similarity in amino acid sequences of 80.1%. A phylogenetic tree based on the deduced amino acid similarities of the polyprotein encoded by the large ORF (Fig. 2) suggested that these viruses may represent 4 or 5 distinct genomic clusters. Clearly, the isolates from the United States and the Jasper strains appeared to represent a distinct group.

More detailed information about the genomic relationships of these aquatic birnaviruses was obtained by comparison of the nucleotide and deduced amino acid sequences of the VP2 coding region (1611 bp) of a variety of isolates representing all 9 serotypes of Serogroup A and widely different geographical and host origins. The VP2 coding region was chosen for detailed investigation because VP2 is the major outer capsid protein and contains all neutralization epitopes (Lipipun et al. 1992) and cell attachment sites that determine host/cell range. Comparison of the nucleotide sequences of the VP2 coding region of these 28 viruses (Table 8) demonstrated identities of 87.9–99.7% among the isolates from the United States and the Jasper strains, compared to 76.5–80.2% with the Canadian, European and Asian strains. The deduced amino acid sequences (Table 9) of all isolates from the United States and the 2 Jasper strains were more than 94% similar to each other but exhibited less similarity with the Canadian, European and Asian viruses. Other pairs or groups of viruses also exhibited high similarities to each other in amino acid sequences but less similarity to other isolates. The highest degree of divergence (18.8%) was exhibited between the He (A4) type strain and the Reno and 90-11 strains representing the WB (A1) serotype.

Distinguishing the US isolates and the Jasper strains from the other Canadian, European and Asian strains were a number of highly conserved signature amino acid residues identified within the VP2 protein. A number of signature amino acids were shown to be universally conserved at specific positions in the deduced amino acid sequences of VP2 of isolates from the United States (Serotype A1) and the 2 Jasper strains (Table 4). Sequences from all other isolates contained different amino acid residues at these positions. Similarly, unique signature amino acids were identified within VP2 for other groups or pairs of isolates (Table 5).

Classification on the basis of deduced amino acid similarities of VP2 demonstrated that the 28 aquatic birnavirus isolates clustered into 6 genogroups (Fig. 3). Genogroup 1 consisted of all isolates from the United States (Serotype A1) and the 2 Jasper strains. Genogroup 2 contained all of the isolates belonging to Serotype A3, including viruses from both Europe and Asia.

Two Canadian isolates representing Serotype A6 (C1 and ASV) and the type strain (Te) of European Serotype A5 formed Genogroup 3. The type strains of the 2 Canadian Serotypes A7 (C2) and A8 (C3) comprised Genogroup 4. All members of Serotype A2, including 5 European isolates and 1 Asian isolate, formed Genogroup 5. The He strain, the only known representative of Serotype A4, represented Genogroup 6. In general, these genogroups, based on deduced amino acid sequences of cDNA of the VP2 coding region of genome segment A, corresponded with geographic origin of the virus and previous classifications based on antigenic relationships. Genogroups 3, 5, and 6 corresponded exactly to 3 (A3, A2, A4, respectively) of the 9 serotypes. The other 3 genogroups (Genogroups 1, 2, and 4) each included members of 2 aquatic birnavirus serotypes (A1 and A9, A7 and A8, A5 and A6, respectively). The phylogenetic tree also clearly shows that the viruses representing the 3 major serotypes found in Canada (A6, A7, and A8) are more closely related to the European and Asian isolates (Serotypes A2, A3, A4, and A5) than to isolates representing the prevalent serotype (A1) in the United States and the Jasper strains.

This correlation of genogroups based on the deduced amino acid sequences of the large ORF and the VP2 coding region with serological classification differs from a previous report by Heppell et al. (1993) which failed to demonstrate a clear correlation between genogroups based on genomic sequence similarities and serotypes of aquatic birnaviruses. However, their investigation focused on relatively short genomic sequences within the C-terminus-pVP2/NS junction coding region. NS protein is a non-structural protease. Our study concentrated on the entire large ORF or the entire VP2 coding region of genome segment A. Virion protein VP2 is the major outer capsid protein and the site of all neutralization epitopes. Examination in this study of the much larger genomic fragment encoding the entire VP2 protein from a relatively large number of isolates provides a more complete analysis of the genomic relationships of these viruses. Considering that VP2 is the outer capsid protein, it is not surprising that genogroups based on the deduced amino acid sequence of the entire VP2 protein generally correlates with serological classification and geographical origin. These results also are consistent with our previous study demonstrating a correlation between genogroups constructed on the basis of restriction fragment length polymorphisms (RFLP) of the VP2 coding region and serological classification based on reaction patterns with a panel of monoclonal antibodies (Lee et al. 1996).

The use of a relative large number of isolates representing a single serotype (A1) of aquatic birnaviruses in this study also indicates that classification of these viruses based on genomic sequences of the VP2 coding region is informative and correlates with serological classification of specific strains within a given serotype. Several genogroups identified in this study are comprised of 2 or more apparent genotypes. This is particularly evident in Genogroup 1, in which 4 genotypes can be identified that correspond to previously identified specific serological strains. Previous investigations using monoclonal antibodies have shown that at least 4 distinct strains of aquatic birnaviruses can be identified within Serotype A1: WB strain, VR299 strain, Buhl strain, and Reno strain (Caswell-Reno et al. 1986, 1989, P. Reno, Oregon State University, pers. comm.). In this study, 5 viruses (Buhl, 91-114, 91-137, 64-93, and 90-11) that previously were shown to belong to a single serological strain (Buhl strain) within the A1 serotype were clustered in Genotype 1. Three, or possibly 4, other genotypes also were identified within Genogroup 1. Genotype 2 included strain VR299 and the JaD strain. This grouping also agrees with previous serological studies. VR299 represents a second distinct strain within Serotype A aquatic birnaviruses based on monoclonal antibody reaction patterns. Furthermore, Berthiaume et al. (1992) showed that VR299 and the JaD strain are very similar antigenically based on ELISA with both polyclonal and monoclonal antibodies and they exhibited 100% homology in nucleotide sequences of a 359 bp fragment of genome segment A. The Reno isolate, another serologically distinct strain, constituted Genotype 3. Genotype 4 was formed by WB and DM, both of which represent the same strain identified by monoclonal antibody reaction patterns. The JaATCC strain, which currently constitutes a sep-

arate serotype, clearly was clustered in Genogroup 1 with the isolates from the United States and was more closely related to the WB strain. However, in conjunction with the previous serological data, the branching structure of the phylogenetic tree in this study suggests that the Jasper-ATCC strain may represent a fifth genotype within Genogroup 1.

In summary, the results of this investigation of the genomic relationships of a relatively large number of aquatic birnaviruses from a variety of geographical areas worldwide demonstrate that these viruses represent at least 6 major genogroups. Furthermore, in contrast to previous studies of smaller genomic fragments from relatively few virus isolates, these genogroups generally correlate with geographical origin and previous serological classifications of these viruses based on reaction with polyclonal and monoclonal antibodies. This information and the availability of this sequence database of genome segment A will be useful in future studies of aquatic birnaviruses (e.g., epidemiological investigations) as well as in fish disease diagnostic programs. For example, currently we are typing new aquatic birnavirus isolates based on automated DNA sequencing of 300 to 500 bp RT-PCR amplification products within the hypervariable region of the VP2 coding region and the comparison of these sequences with the sequence data developed in this investigation. This is relatively rapid (1 to 2 d) and cost effective compared to isolation in cell culture and the use of a panel of monoclonal antibodies for identification of serotypes.

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