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Phylogenetic relationships in the family *Alloherpesviridae*

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ABSTRACT: Phylogenetic relationships among herpesviruses (HVs) of mammals, birds, and reptiles have been studied extensively, whereas those among other HVs are relatively unexplored. We have reconstructed the phylogenetic relationships among 13 fish and amphibian HVs using maximum likelihood and Bayesian analyses of amino acid sequences predicted from parts of the DNA polymerase and terminase genes. The relationships among 6 of these viruses were confirmed using the partial DNA polymerase data plus the complete sequences of the terminase, helicase, and triplex protein genes; the position of these viruses among all other sequenced HVs was also investigated using the complete terminase gene. The results established the monophyly of the fish and amphibian HVs (*Alloherpesviridae*) separate from the HVs of mammals, birds, and reptiles (*Herpesviridae*) and the single recognized HV of bivalve mollusks (*Malacoherpesviridae*) in the order *Herpesvirales*. Two major clades in the family *Alloherpesviridae* were recognized: one consisting of viruses from cyprinid and anguillid hosts and the other of viruses from ictalurid, salmonid, acipenserid, and ranid hosts. A comparison of virus and host phylogenies suggested that closely related HVs in this family may have coevolved with their hosts, whereas significant codiversification was not apparent for the more distantly related viruses.

KEY WORDS: *Alloherpesviridae* · Fish herpesviruses · Frog herpesviruses · Phylogeny · Coevolution · DNA polymerase · Terminase

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INTRODUCTION

Herpesviruses (HVs) share a characteristic virion structure, which, proceeding from the center outwards, consists of a large, linear, double-stranded DNA genome, a T = 16 (triangular number) icosahedral capsid, a proteinaceous matrix (the tegument), and a host-derived lipid envelope containing viral proteins (Davison et al. 2005a). HVs infect a wide variety of vertebrate hosts, including mammals, birds, reptiles, amphibians, and fish, and at least one invertebrate group, bivalve mollusks. Complete genome sequences are currently available for 46 distinct viruses, with 41 of these infecting mammals or birds. The remaining 5 exceptions are 2 fish viruses (ictalurid HV 1, IcHV1, Davison 1992; cyprinid HV 3, CyHV3, Aoki et al. 2007), 2 frog viruses (ranid HVs 1 and 2, RaHV1 and RaHV2, Davison et al. 2006), and a bivalve mollusk virus (ostreid HV 1, OsHV1, Davison et al. 2005b).

HV taxonomy, as determined by the International Committee on Taxonomy of Viruses (www.ictvonline.org), has recently undergone a revision in which the previous family *Herpesviridae* was raised to an order (*Herpesvirales*) and split into 3 families, one (*Her-*

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pesviridae, divided into the subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*) containing mammalian, avian, and reptilian viruses, one (*Alloherpesviridae*) containing fish and amphibian viruses, and one (*Malacoherpesviridae*) containing a single virus, OsHV1. The taxonomic structure of the family *Herpesviridae* has been well established phylogenetically, and the viruses are characterized by approximately 43 homologous genes that were presumably inherited from a common ancestor (McGeoch et al. 2006). The erection of the 2 new families was driven by the findings that these viruses are but tenuously related to the fish and amphibian HVs, which share at least 13 homologous genes with each other (Aoki et al. 2007), and that OsHV1 is related only marginally to all other HVs (Davison et al. 2005b). Indeed, the best, and perhaps only, sequence-based evidence supporting a common ancestry for all HVs resides in a single gene, that encoding the putative ATPase subunit of terminase (hereafter terminase; an enzyme complex involved in packaging DNA into capsids), in which 5 regions are conserved (Davison 1992, 2002). This protein is also conserved to a lesser extent in T4-like bacteriophages in the family *Myoviridae* (Davison 1992). Indeed, the perceived utilization of a similar enzyme complex for packaging DNA into preformed capsids by HVs and by members of the order *Caudovirales*, of which the family *Myoviridae* is a member (Maniloff & Ackermann 1998), may indicate a common evolutionary origin for these viruses of animals and bacteria.

In terms of formal taxa, the family *Alloherpesviridae* consists of one genus (*Ictalurivirus*), which contains the species *Ictalurid herpesvirus 1* (represented by IcHV1), and the species *Cyprinid herpesvirus 3* (represented by CyHV3), which has not been assigned to a genus (www.ictvonline.org). It also contains several fish and amphibian viruses that have not been assigned to any taxa: acipenserid HV 1 (AciHV1), acipenserid HV 2 (AciHV2), anguillid HV 1 (AngHV1), cyprinid HV 1 (CyHV1), and cyprinid HV 2 (CyHV2), esocid HV 1 (EsHV1), percid HV 1 (PeHV1), pleuronectid HV 1 (PlHV1), RaHV1, RaHV2, and salmonid HV 1 (SalHV1), and salmonid HV 2 (SalHV2) (Davison et al. 2005a). Thus, the classification of viruses in the family *Alloherpesviridae* is at an early stage. Nonetheless, HVs are well represented in freshwater and marine fishes (Wolf 1988, Hedrick & Sano 1989, Hetrick & Hedrick 1993), and the 2 known amphibian HVs (RaHV1 and RaHV2) were isolated from a single species, *Rana pipiens*, the leopard frog. Table 1 presents a comprehensive list of recognized fish and amphibian HVs with a summary of their features. The biology of these viruses has been examined in less detail than that of members of the family *Herpesviridae*, but the 2 groups share similar general characteristics, including narrow host specificity and establishment of long-term (perhaps latent) infections. Most fish HVs are epitheliotropic, inducing changes such as hypertrophy, hyperplasia, and necrosis of the epidermis, and, in some cases, neoplasia (Sano et al. 1985a). They can induce diseases ranging from mild subclinical infections in mature fish to severe systemic infections and mortality in young or immunologically naïve fish (Hedrick & Sano 1989).

Genetic relationships among fish and amphibian HVs have been examined in several studies. Two short sequences obtained from SalHV2 share similarities to IcHV1 genes (Bernard & Mercier 1993) and are more closely related to the corresponding genes in SalHV1 (Davison 1998). Sequence data sampled across the SalHV1 genome revealed at least 18 genes with homologs in IcHV1 (Davison 1998). Sequence comparisons of 5 genes showed that CyHV1, CyHV2, and CyHV3 are related closely to each other and more distantly to IcHV1 and RaHV1 (Waltzek et al. 2005). Comparisons of complete genome sequences demonstrated that RaHV1 and RaHV2 have 40 convincingly conserved genes, 19 of which have homologs in IcHV1 (Davison et al. 2006). IcHV1 and CyHV3 have 15 homologs (Aoki et al. 2007).

Although these studies were instrumental in the taxonomical restructuring described above, analysis of sequence data from a greater number of viruses is required to confirm the monophyly of the family *Alloherpesviridae* and to facilitate future taxonomical developments. The present study involved the generation of a molecular dataset consisting of partial sequences encoding conserved regions of DNA polymerase and terminase for 9 HVs from 5 families of fish (Acipenseridae, Cyprinidae, Anguillidae, Salmonidae, and Ictaluridae) and complete sequences encoding the terminase, helicase, and a putative intercapsomeric triplex protein (hereafter triplex protein) of CyHV2, and the terminase of CyHV1. When combined with published information, these data facilitated an evaluation of the evolutionary branching patterns of a total of 13 fish and amphibian HVs and an examination of concordance between virus and host phylogeny.

MATERIALS AND METHODS

Virus purification. Isolate and source information for the fish and amphibian HVs used in the present study are presented in Table 2. Our studies were restricted to those fish HVs where adequate infected tissue was available or propagation in fish cell lines was possible. The fish HVs marked in Table 1 were grown in the appropriate permissive cell lines until cytopathic effect

Table 1. Recognized fish and amphibian herpesviruses (HVs). Cell line abbreviations are EK: eel kidney; EP: eel epidermis; KF: koi fin; GF: goldfish fin; CCO: channel catfish ovary; WSS: white sturgeon spleen; RTG: rainbow trout gonad; CHSE: Chinook salmon embryo; ICR: leopard frog embryo; WO: walleye ovary. EM: virus observed using electron microscopy but not isolated in cell culture and thus unavailable for analysis in the present study (except SalHV3)

Virus name (virus abbreviation)	Common name (abbreviation)	Host(s)	Cell line	Source
Anguillid HV 1 (AngHV1)[a]	HV anguillae (HVA) Eel HV in Formosa (EHVF) Gill HV of eel (GHVE) European eel HV (EEHV)	Japanese eel *Anguilla japonica* and European eel *A. anguilla* Japanese eel *A. japonica* Japanese eel *A. japonica* European eel *A. anguilla*	EK-1 EK-1 EK-1 EP-1 & EK-1	Sano et al. (1990) Ueno et al. (1992) Lee et al. (1999) Chang et al. (2002)
Cyprinid HV 1 (CyHV1) [a]	HV cyprini, carp pox HV, carp HV (CHV)	Common carp *Cyprinus carpio*	KF-1	Sano et al. (1985b)
Cyprinid HV 2 (CyHV2) [a]	Goldfish hematopoietic necrosis virus (GFHNV)	Goldfish *Carassius auratus*	GF-1	Jung & Miyazaki (1995)
Cyprinid HV 3 (CyHV3)[d]	Koi HV (KHV), carp nephritis and gill necrosis virus (CNGV)	Common carp *Cyprinus carpio*	KF-1	Hedrick et al. (2000)
Ictalurid HV 1 (IcHV1)[d]	Channel catfish virus (CCV)	Channel catfish *Ictalurus punctatus*	CCO	Fijan et al. (1970)
Ictalurid HV 2 (IcHV2) [a,c]	Ictalurus melas HV (IcmHV)	Black bullhead *Ameiurus melas* and channel catfish *Ictalurus punctatus*	CCO	Hedrick et al. (2003)
Acipenserid HV 1 (AciHV1) [a]	White sturgeon HV 1	White sturgeon *Acipenser transmontanus*	WSS-1	Hedrick et al. (1991)
Acipenserid HV 2 (AciHV2) [a]	White sturgeon HV 2	White sturgeon *Acipenser transmontanus*	WSS-1	Watson et al. (1995)
Salmonid HV 1 (SalHV1) [a]	HV salmonis (HPV) Steelhead herpesvirus (SHV)	Rainbow trout *Oncorhynchus mykiss* Rainbow trout *Oncorhynchus mykiss*	RTG-2 CHSE	Wolf & Taylor (1975) Eaton et al. (1989)
Salmonid HV 2 (SalHV2) [a]	Oncorhynchus masou virus (OMV)[a] Yamame tumor virus (YTV) [a] Oncorhynchus kisutch virus (OKV) Coho salmon tumor virus (COTV) Coho salmon HV (CSH) Rainbow trout kidney HV (RKV) Nerka Virus in Towanda Lake, Akita and Aomori Prefecture (NeVTA)[a]	Cherry salmon *Oncorhynchus masou* Cherry salmon *Oncorhynchus masou* Coho salmon *Oncorhynchus kisutch* Coho salmon *Oncorhynchus kisutch* Coho salmon *Oncorhynchus kisutch* Rainbow trout *Oncorhynchus mykiss* Sockeye salmon *Oncorhynchus nerka*	RTG RTG RTG RTG RTG CHSE RTG	Kimura et al. (1981) Sano et al. (1983) Horiuchi et al. (1989) Kimura & Yoshimizu (1991) Kumagai et al. (1994) Suzuki (1993) Sano (1976)
Salmonid HV 3 (SalHV3)[c,d]	Epizootic epitheliotropic disease virus (EEDV)	Lake trout *Salvelinus namaycush*	EM	McAllister & Herman (1989), Bradley et al. (1989)
Ranid HV 1 (RaHV1)[d]	Lucké tumor HV (LTHV)	Leopard frog *Rana pipiens*	EM	Lunger et al. (1965)
Ranid HV 2 (RaHV2)[d]	Frog virus 4 (FV-4)	Leopard frog *Rana pipiens*	ICR-2A	Rafferty (1965)
Percid HV 1 (PeHV1)[b]	HV vitreum, walleye HV	Walleye *Stizostedion vitreum*	WO	Kelly et al. (1983)
Esocid HV 1 (EsHV1)	Pike epidermal proliferative HV, pike HV	Northern pike *Esox lucius* and muskellunge *E. masquinongy*	EM	Yamamoto et al. (1983)
Pleuronectid HV 1 (PlHV1)	HV scopthalami	Turbot *Scopthalamus maximus*	EM	Buchanan & Madeley (1978)
None	Flounder HV (FHV)	Japanese flounder *Paralichthys olivaceous*	EM	Iida et al. (1989)
None	Golden ide virus	Golden ide *Leuciscus ide*	EM	McAllister et al. (1985)
None	Pacific cod HV	Pacific cod *Gadus macrocephalus*	EM	McArn et al. (1978)
None	Sheatfish HV (SHV)	Wels *Silurus glanis*	EM	Békesi et al. (1981)
None	Smelt papillomatous virus	European smelt *Osmerus eperlanus*	EM	Anders & Moller (1985)
None	None	Rainbow smelt *Osmerus mordax*	EM	Morrison et al. (1996)
None	Smooth dogfish HV	Smooth dogfish *Mustelus canis*	EM	Leibovitz & Leibovitz (1985)
None	Atlantic salmon papillomatosis virus	Atlantic salmon *Salmo salar*	EM	Schelkunov et al. (1992)
None	Angelfish HV	Angelfish *Pterophyllum altum*	EM	Mellergard & Bloch (1988)
None	Pilchard HV	Pacific sardine *Sardinops sagax*	EM	Hyatt et al. (1997)
None	None	Red striped rockfish *Sebastes proriger*	EM	Kent & Meyers (2000)

[a]Viruses isolated in cell culture, purified, and analyzed in the present study. [b]Virus has been isolated in cell culture but material was not available for analysis in the present study. [c]Tentative designation. [d]Analyzed but not isolated in the present study

Table 2. Fish and amphibian herpesvirus isolates used to construct the 4 datasets in the present study (DS15, DS6, DS4, and DS41) and their GenBank and RefSeq accession numbers. Accessions in bold italics represent new sequence data generated in the present study. NA: not applicable

Virus abbreviation (isolate)	Country of isolation	DS15	DS6	DS4	DS41	Source
AngHV1 (H. Fukuda)[a]	Japan	***EU349271, EU349278***	NA	NA	NA	Present study
AngHV1 (569116)[a]	Netherlands	***EU349272, EU349279***	NA	NA	NA	Present study
CyHV1 (H. Fukuda)[a]	Japan	AY939868, ***EU349288***	***EU349288***, AY939858, AY939860, AY939868	NA	***EU349288***	Waltzek et al. (2005), present study
CyHV2 (H. Fukuda)[a]	Japan	AY939863, ***EU349285***	***EU349285, EU349287, EU349286***, AY939863	NA	***EU349285***	Waltzek et al. (2005), present study
CyHV3 (KHV-U)	USA	NC_009127	NC_009127	NC_009127	NC_009127	Aoki et al. (2007)
IcHV1 (ATCC-VR-665)	USA	NC_001493	NC_001493	NC_001493	NC_001493	Davison (1992)
IcHV2 (761/94)[a]	Italy	***FJ641907, FJ827489***	NA	NA	NA	Present study
IcHV2 (762A)[a]	Italy	***EU349276, EU349280***	NA	NA	NA	Present study
AciHV1 (UC Davis type isolate)[a]	USA	EF685903, EF535573	NA	NA	NA	Kelley et al. (2005), Kurobe et al. (2008)
AciHV2 (UC Davis type isolate)[a]	USA	AY874416, EF535576	NA	NA	NA	Kelley et al. (2005), Kurobe et al. (2008)
SalHV1 (ATCC-VR-868)[a]	USA	***EU349273, EU349281***	NA	NA	NA	Present study
SalHV2 (OMV)[a]	Japan	***EU349275, EU349282***	NA	NA	NA	Present study
SalHV2 (YTV)[a]	Japan	***FJ641908, FJ641909***	NA	NA	NA	Present study
SalHV2 (NeVTA)[a]	Japan	***EU349274, EU349283***	NA	NA	NA	Present study
SalHV3 (Wisconsin)	USA[b]	***EU349277, EU349284***	NA	NA	NA	Present study
RaHV1 (McKinnell)	USA	NC_008211	NC_008211	NC_008211	NC_008211	Davison et al. (2006)
RaHV2 (ATCC-VR-568)	USA	NC_008210	NC_008210	NC_008210	NC_008210	Davison et al. (2006)

[a]Virus isolated in cell culture and purified in the present study. [b]Country of epidemic

was complete. All except salmonid HV 3 (SalHV3) were purified before viral genomic DNA was isolated, as described previously (Waltzek et al. 2005). For SalHV3, DNA was extracted from infected skin tissues using a DNeasy kit (QIAGEN), following the tissue extraction protocol. DNA was stored at 4°C.

Previous sequence data. The analysis focused on 4 conserved genes encoding DNA polymerase (ORF57 in IcHV1), terminase (ORF62, present as 3 coding exons), helicase (ORF25), and triplex protein (ORF27). For IcHV1, RaHV1, RaHV2, and CyHV3, the complete sequences of these, and other, genes were derived from the published genome sequences (Davison 1992, Davison et al. 2006, Aoki et al. 2007). The complete DNA polymerase, helicase, and triplex protein sequences were available for CyHV1, and partial sequences were available for CyHV2 (Waltzek et al. 2005). Sequence data for mollusk, avian, and mammalian HVs, and T4-like bacteriophages were obtained from RefSeq (www.ncbi.nlm.nih.gov/genomes/VIRUSES/35237.html); accession numbers and references are listed in Table 3.

New sequence data. Details on the primers and the viruses from which sequences were amplified are given in Table 4. A pair of degenerate primers, HV and cons lower (Hanson et al. 2006), was used to amplify a 441 to 474 bp fragment (primer binding sites excluded) of the DNA polymerase gene, and 5 conserved primer pairs were designed on the basis of alignments to amplify regions of the terminase gene. Although amplicon sizes varied between the terminase primer sets, all amplicons contained a common 315 to 330 bp sequence located in the third region of conservation of the terminase gene (Davison 2002), and this was used for phylogenetic analyses. The PCR conditions for all primer sets were those used previously (Kelley et al. 2005). Amplicons were purified from 10 µl of the PCR products by excision from an agarose gel and cloned using a TOPA TA Cloning Kit (Invitrogen).

Table 3. RefSeq accession numbers for the sequences representing species in the family *Herpeviridae* from mammalian, avian, and oyster herpesviruses (HVs) and T4-like bacteriophages used to construct Dataset 41 (DS41)

Species name (Virus abbreviation)	RefSeq
Alcelaphine herpesvirus 1 (AlHV1)	NC_002531
Ateline herpesvirus 3 (AtHV3)	NC_001987
Bovine herpesvirus 1 (BoHV1)	NC_001847
Bovine herpesvirus 4 (BoHV4)	NC_002665
Bovine herpesvirus 5 (BoHV5)	NC_005261
Ceropithecine herpesvirus 2 (CeHV2)	NC_006560
Ceropithecine herpesvirus 9 (CeHV9)	NC_002686
Equid herpesvirus 1 (EHV1)	NC_001491
Equid herpesvirus 4 (EHV4)	NC_001844
Human herpesvirus 1 (HHV1)	NC_001806
Human herpesvirus 2 (HHV2)	NC_001798
Human herpesvirus 3 (HHV3)	NC_001348
Human herpesvirus 4 (HHV4)	NC_001345
Human herpesvirus 5 (HHV5)	NC_006273
Human herpesvirus 6 (HHV6)	NC_001664
Human herpesvirus 7 (HHV7)	NC_001716
Human herpesvirus 8 (HHV8)	NC_003409
Gallid herpesvirus 1 (GaHV1)	NC_006623
Gallid herpesvirus 2 (GaHV2)	NC_002229
Gallid herpesvirus 3 (GaHV3)	NC_002577
Macacine herpesvirus 1 (McHV1)	NC_004812
Macacine herpesvirus 4 (McHV4)	NC_006146
Macacine herpesvirus 8 (McHV8)	NC_006150
Meleagrid herpesvirus 1 (MeHV1)	NC_002641
Murid herpesvirus 2 (MuHV2)	NC_002512
Murid herpesvirus 4 (MuHV4)	NC_001826
Ovine herpesvirus 2 (OvHV2)	NC_007646
Ostreid herpesvirus 1 (OsHV1)	NC_005881
Panine herpesvirus 2 (PaHV2)	NC_003521
Psittacid herpesvirus 1 (PsHV1)	NC_005264
Saimiriine herpesvirus 2 (SaHV2)	NC_001350
Suid herpesvirus 1 (SuHV1)	NC_006151
Tupaiid herpesvirus 1 (TuHV1)	NC_002794
T4 bacteriophage (T4)	NC_000866
RB 69 bacteriophage (RB69)	NC_004928

Transformed bacterial colonies were screened using PCR, and plasmids containing relevant inserts were purified using a QIAprep Spin Miniprep Kit (QIAGEN). For each amplicon, 2 purified plasmid clones were sequenced using M13 forward and reverse primers with an ABI 377 automated sequencer (Applied Biosystems).

The complete CyHV2 helicase, triplex protein, and terminase sequences were derived from PCR, RT-PCR, and Rapid Amplification of cDNA Ends (RACE) experiments, employing primers designed from alignments of the relevant CyHV3 and CyHV1 data. Briefly, total RNA was isolated from a CyHV2-infected goldfish fin cell line (GF-1) using TRIZOL (Invitrogen), and 500 ng was transcribed into cDNA using a BD SMART RACE cDNA amplification kit (BD Biosciences). Using RNA or cDNA and primers listed in Table 4, PCR, RT-PCR, and RACE were performed with an initial denaturation step of 95°C for 5 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min, and a final extension step at 72°C for 5 min. The reactions were held at 4°C. The amplicons were cloned and sequenced as described above.

The 3′-RACE using primer CyHV2HelF and the BD SMART nested universal primer (NUP) yielded a 1996 bp amplicon. When analysed by BLASTP searches of GenBank databases, the sequence revealed the presence of both the 3′-end of the CyHV2 helicase gene and the complete triplex protein sequence, presumably as a result of their presence on the same mRNA. The 5′-RACE using primer CyHV2HelRACER1 and NUP yielded a 586 bp amplicon. The combined data from 5′- and 3′-RACE yielded a total of 2441 bp of sequence containing the complete CyHV2 helicase and triplex protein coding regions.

PCR using primers 161 and 162 yielded a 595 bp amplicon in Exon 1 of the CyHV2 terminase gene. The remainder of Exon 1, Exon 2, and the 5′-end of Exon 3 was amplified by RT-PCR using primers 155 and 118, which yielded a 1062 bp amplicon. PCR using primers 96 and 99 amplified a 721 bp sequence that extended Exon 3. Finally, 3′-RACE using primer 121 and NUP yielded an 864 bp amplicon containing the 3′-end of Exon 3. When combined, these data yielded 2628 bp of sequence containing the complete CyHV2 terminase coding region. For CyHV1, the complete terminase sequence was recovered from an incomplete shotgun sequence database (A. J. Davison & R. P. Hedrick unpubl. data).

Establishment of datasets. Table 2 summarizes the HVs, genes, and corresponding GenBank and/or RefSeq accession numbers used to generate the 4 datasets used for phenetic and phylogenetic analyses. BLASTP searches of GenBank databases using the DNA polymerase and terminase sequences were performed in order to acquire appropriate outgroups. Both partial gene sets revealed strongest similarity with other fish and amphibian HVs. Additionally, the terminase set revealed substantial but lower similarity with other HVs, and, at a lower level, with several T4-like bacteriophages. The DNA polymerase search yielded but marginal similarity with other HVs (see Table 5), thus making the choice of outgroups challenging. This is consistent with previous work indicating that the DNA polymerases of fish HVs are distinct from those of mammalian HVs (Knopf 1998). As a result of these preliminary analyses, 4 operational datasets were established.

Dataset 15 (DS15) contained 309 amino acid (AA) characters (including gaps) and consisted of the concatenated partial DNA polymerase and terminase sequences for 13 fish and amphibian HVs plus, as selected outgroups, 2 mammalian HVs, human HV 8

Table 4. Primers used for PCR and sequencing of genes from fish and amphibian herpesviruses (HVs)

Primer	Sequence (5'-3')	Target gene	Combined with	Amplicon (bp)[a]	Virus sequence amplified
HV	CGG AAT TCT AGA YTT YGC NWS NYT NTA YCC	DNA polymerase	Cons lower	460	AngHV1 (569116), AngHV1 (H. Fukuda)
				439	IcHV2 (761/94), IcHV2 (762A)
				475	AciHV1 (UC Davis type isolate)
				442	AciHV2 (UC Davis type isolate)
				451	SalHV1 (ATCC-VR-868), SalHV2 (OMV), SalHV2 (YTV), SalHV2 (NeVTA), SalHV3 (Wisconsin)
Cons lower	CCC GAA TTC AGA TCT CNG TRT CNC CRT A	DNA polymerase			
TermF-2	GCM MGR GGA CAG AWC CCM G	Terminase	TermR-2	319	IcHV2 (761/94), IcHV2 (762A)
TermR-2	SGC CWC CVG TBA RCT CSA KC	Terminase			
TermSal-R1	CGC AAA GGT GCA AAC GCC	Terminase	NeVTA-1F	506	SalHV3 (Wisconsin)
NeVTA-1F	ACT CGA GGA CAG ACG CCA G	Terminase			
TermKHV-F	GCC AGG GGA CAG AAC CCA GA	Terminase	TermSal-R1	505	SalHV1 (ATCC-VR-868)
TermAng-R2	GTG GGA TCC ATT CCG ATC ACC	Terminase	TermKHV-F	574	AngHV1 (569116)
				569	AngHV1 (H. Fukuda)
68-A	AAC TTC TGA TCT ATC CCA GC	Terminase	TermSal-R1	831	SalHV2 (OMV), SalHV2 (YTV), SalHV2 (NeVTA)
CyHV2HelF	GGA CTT GCG AAG AGT TTG ATT TCT AC	Helicase/triplex	NUP	1997	CyHV2 (H. Fukuda)
NUP	AAG CAG TGG TAT CAA CGC AGA GT	Helicase/triplex			
CyHV2HelRACER1	GCA CGT TAT TGT GAT TGA TC	Terminase	NUP	586	CyHV2 (H. Fukuda)
161	CGA GAT GAA CCT GAT GAT AC	Terminase	162	595	CyHV2 (H. Fukuda)
162	GCC GTT TTC CAA TTT CCA AGT GAG	Terminase			
155	GGT AAT ATG TGT TAC CCG GAG C	Terminase	118	1062	CyHV2 (H. Fukuda)
118	CGT GCG ATA TCC AAG AGT CG	Terminase			
96	GGA CAG AAC CCA GAT ATC TG	Terminase	99	721	CyHV2 (H. Fukuda)
99	GCR AAG GTG TTG GAC TCG AT	Terminase			
121	TGC ACG AGC TCG TGC TCA GG	Terminase	NUP	864	CyHV2 (H. Fukuda)

[a]Amplicon size excluding primer binding sites

(HHV8) from the subfamily *Gammaherpesvirinae*, and human HV 1 (HHV1) from the subfamily *Alphaherpesvirinae* in the family *Herpesviridae*. The individual genes were first analyzed separately to facilitate comparison with the concatenated analysis.

Dataset 6 (DS6) contained 2356 AA characters (including gaps) and consisted of a concatenation of the complete terminase, helicase, and triplex protein sequences plus the partial DNA polymerase sequence for 6 fish and amphibian HVs. DS6, being based on longer sequences, was utilized to strengthen aspects of the DS15 analysis. As an initial step, the individual gene sequences were analyzed separately in order to facilitate comparison with the concatenated analysis.

Dataset 4 (DS4) contained 13 747 AA characters (including gaps) and consisted of a concatenation of the 13 complete genes that are convincingly conserved among the 4 sequenced fish and amphibian HVs (Aoki et al. 2007). The genes in IcHV1 (and putative characteristics or functions) are ORF25 (helicase), ORF27 (triplex protein), ORF28 (protease), ORF37 (unknown), ORF39 (major capsid protein), ORF46 (glycoprotein), ORF54 (unknown), ORF56 (unknown), ORF57 (DNA polymerase), ORF60 (unknown), ORF62 (terminase), ORF63 (primase), and ORF64 (unknown).

Dataset 41 (DS41) contained 283 AA characters (including gaps) and consisted of a concatenation of the 5 conserved regions of terminase (trimmed in the fifth region to the last conserved residue; Davison 2002) for 6 fish and amphibian HVs and 33 other HVs, plus 2 T4-like bacteriophages as outgroups. Viruses in the family *Herpesviridae* were limited to those for which complete genome sequences were available.

Phylogenetic analysis. AA sequences were aligned using MAFFT 5.8 (Katoh et al. 2005) followed by minor manual adjustments using ClustalW (Thompson et al. 1994). The E-INS-i alignment strategy was used with the following parameters: scoring matrix (BLOSUM62), gap open penalty (1.53), and offset value (0).

To assess gene concordance, likelihood (TREE-PUZZLE 5.2; Schmidt et al. 2002) and Bayesian (MrBayes version 3.1.2; Huelsenbeck & Ronquist 2001) analyses were performed independently for each gene. Preliminary analysis revealed that there was no significant incongruency among individual gene trees (defined by the presence of incompatible bipartitions that received a quartet puzzling probability >80% or a posterior probability >90%). Phylogenetic trees were constructed in TREEVIEW (Page 1996) or PAUP* (Swofford 2001). TREE-PUZZLE 5.2 was utilized to select the most appropriate model of AA sequence evolution for subsequent likelihood analysis. Statistical confidence of tree topologies was assessed using 1000 puzzling steps for quartet puzzling trees, and all other settings were default. For Bayesian analysis, a mixed prior was used on AA models and default priors for topology (uniform) and branch lengths (Exp 10). The Markov chain was run for a maximum of 10 million generations, with a stopping rule implemented so that the analysis would halt when the average deviation of the split frequencies became <0.001%. Four independent analyses were conducted, each with 1 cold and 3 heated chains with the default heating parameter (temp = 0.2). Sampling occurred every 50 generations with the first 25% of Markov chain Monte Carlo (MCMC) samples discarded as burn-in.

To test whether fish and amphibian HVs show a significant pattern of codiversification with their hosts, a host–parasite tanglegram (Page & Hafner 1996, Page & Charleston 1998) was produced using TreeMap 2.0, Hughes et al. (2007). The fish host topology (Helfman et al. 1997) was compared with the Bayesian consensus tree of the HVs. The randomization test implemented in TreeMap was used with 1000 replicates to generate the null distribution for the expected number of codiversification events.

RESULTS

The DS15 analysis demonstrated the monophyly of the 13 fish and amphibian HVs tested (Fig. 1). The DS6 and DS4 analyses (Figs. 2 & 3, respectively), which were based on longer sequences, produced a concordant phylogeny for a subset of these viruses. The more restricted DS41 analysis of this subset of viruses (Fig. 4) reiterated their monophyly, and also accorded the family *Herpesviridae* an overall taxonomic arrangement very similar to that established previously using much longer sequences (McGeoch et al. 1995, 2000, 2005, 2006). A third, monotypic group was formed by OsHV1 (*Malacoherpesviridae*).

Two major sister clades were evident in the family *Alloherpesviridae*: Clade 1, comprising HVs of cyprinid and anguillid origin, and Clade 2, comprising those from ranid, acipenserid, salmonid, and ictalurid hosts (Fig. 1). In Clade 1, the monophyly of the cyprinid HVs was supported by all analyses, with CyHV2 and CyHV3 most closely related and CyHV1 their sister group. The DS15 analysis (Fig. 1) supported AngHV1 as the sister group to the cyprinid HVs. At a more detailed level, the sequence of the DNA polymerase gene fragment of AngHV1 was identical in Dutch and Japanese isolates (Table 5), and also identical to previously reported sequences from Dutch and Japanese isolates (Rijsewijk et al. 2005). Moreover, it was 99% identical to an isolate from Japanese eels in Taiwan (Chang et al. 2002), exhibiting one synonymous and one non-synonymous difference. Two synonymous differences and 5 insertions or deletions characterized the terminase gene fragment of the Dutch and Japanese isolates. However, these isolates exhibited identical AA sequences for the smaller terminase region analyzed. Since the DNA polymerase and terminase gene fragments were identical among the Dutch and Japanese AngHV1 isolates for the regions analyzed in the present study, they are represented collectively as AngHV1 in Figs. 1, 5 & 6.

The monophyly of Clade 2, which contains HVs from ranid, acipenserid, salmonid, and ictalurid hosts, was supported by the DS15 analysis (Fig. 1). A subset of this clade consisting of IcHV1, RaHV1, and RaHV2 was also monophyletic in the DS41 analysis (Fig. 4). Within this subset, IcHV1 and IcHV2 were sister viruses and AciHV2 their closest relative (Fig. 1). At a more detailed level, the 761/94 and 762A isolates of IcHV2 exhibited identical nucleotide sequences for the regions of the DNA polymerase and terminase genes analyzed. Thus, they are represented collectively as IcHV2 in Figs. 1, 5 & 6. The monophyly of the sister group to this subset was strongly supported, with SalHV1 and SalHV2 as sister viruses and SalHV3 their nearest relative (Fig. 1). The OMV, YTV, and NeVTA isolates of SalHV2 exhibited identical nucleotide sequences for the regions of the DNA polymerase and terminase genes analyzed. Thus, they are represented collectively as SalHV2 in Figs. 1, 5 & 6. The partial DNA polymerase and terminase sequences for AciHV1 and AciHV2 (standard UC Davis strains) were identical to those deposited previously in GenBank (Kelley et al. 2005, Kurobe et al. 2008). AciHV1 was the next most closely related virus to the ictalurid and salmonid HV clades (Fig. 1). A strongly supported subset consisting of RaHV1 and RaHV2 formed the sister group to the rest of the viruses in Clade 2 (Figs. 1 to 4). The DS4 analysis supported a somewhat closer relationship between the ranid and ictalurid HVs than between viruses in either group and the cyprinid HVs (Fig. 3).

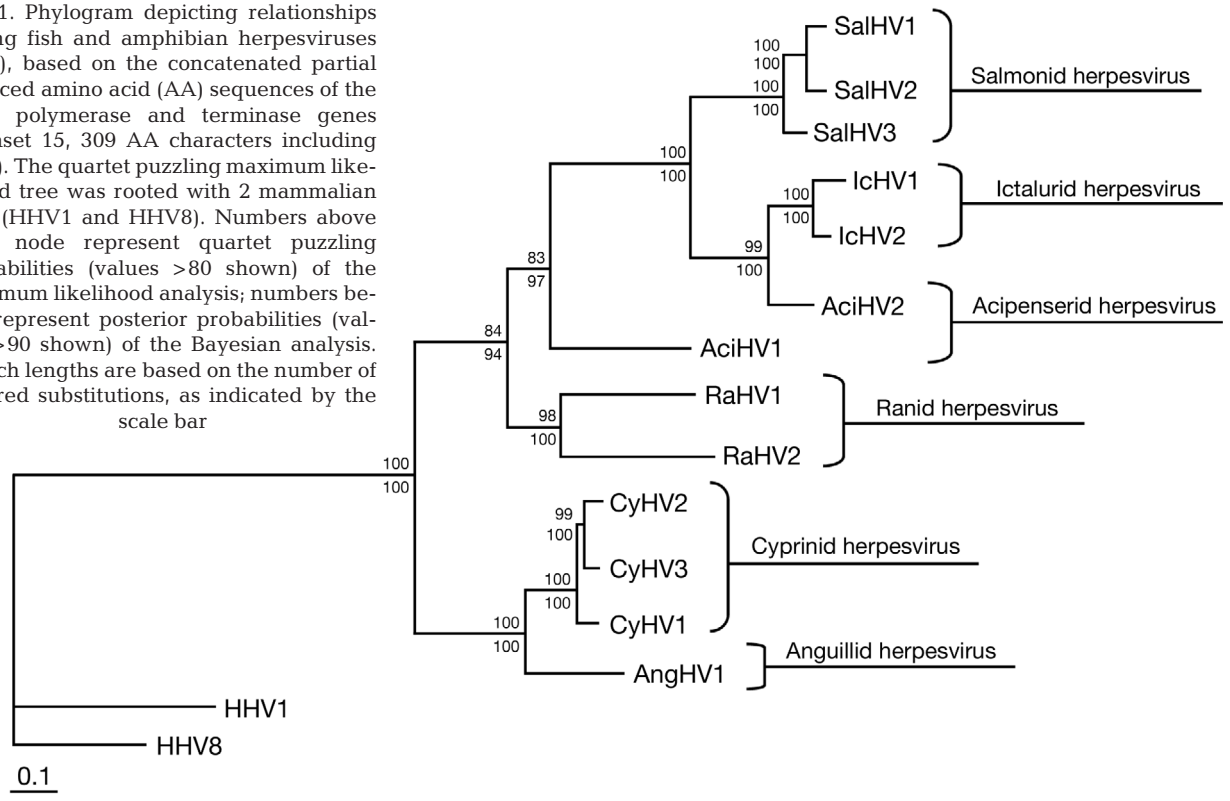

Fig. 1. Phylogram depicting relationships among fish and amphibian herpesviruses (HVs), based on the concatenated partial deduced amino acid (AA) sequences of the DNA polymerase and terminase genes (Dataset 15, 309 AA characters including gaps). The quartet puzzling maximum likelihood tree was rooted with 2 mammalian HVs (HHV1 and HHV8). Numbers above each node represent quartet puzzling probabilities (values >80 shown) of the maximum likelihood analysis; numbers below represent posterior probabilities (values >90 shown) of the Bayesian analysis. Branch lengths are based on the number of inferred substitutions, as indicated by the scale bar

The host–virus tanglegram (Fig. 5) revealed strong overall discordance between HV and host lineages, with a randomization test revealing no significant support for codiversification ($p = 0.61$).

DISCUSSION

The utility of partial sequence analyses has been established previously in resolving the evolutionary relationships among HVs of mammals (Ehlers et al. 1999, Ehlers & Lowden 2004, Wibbelt et al. 2007) and reptiles (McGeoch & Gatherer 2005), and is extended in the present study to fish and amphibian HVs. Phylogenetic analyses of partial sequences from the DNA polymerase and terminase genes, and of more extensive sequences for a subset of viruses, strongly support the monophyly of fish and amphibian HVs within a larger clade containing all HVs. In addition, analysis of the terminase, which is unique to the HVs and the

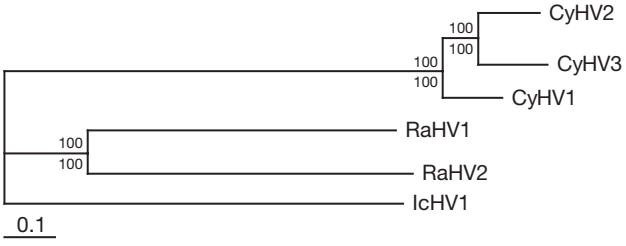

Fig. 2. Phylogram depicting relationships among fish and amphibian herpesviruses (HVs), based on the concatenated deduced amino acid (AA) sequences of the full length terminase, helicase, and triplex protein genes, plus the partial DNA polymerase gene (Dataset 6, 2356 AA characters including gaps). The tree is not rooted. Numbers above each node represent quartet puzzling probabilities (values >80 shown) of the maximum likelihood analysis; numbers below represent posterior probabilities (values >90 shown) of the Bayesian analysis. Branch lengths are based on the number of inferred substitutions, as indicated by the scale bar. See Table 1 for abbreviations

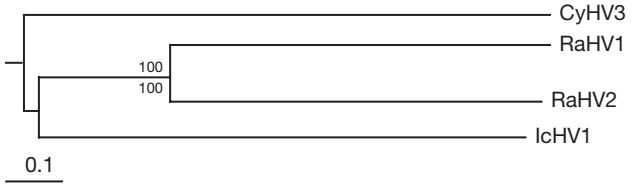

Fig. 3. Phylogram depicting relationships among fish and amphibian herpesviruses (HVs), based on the concatenated deduced amino acid (AA) sequences of the 13 full-length genes conserved among completely sequenced genomes (Dataset 4, 13 747 AA characters including gaps). The maximum likelihood tree was rooted using the midpoint rooting feature in PAUP*. Numbers above each node represent quartet puzzling probabilities (values >80 shown) of the maximum likelihood analysis; numbers below represent posterior probabilities (values >90 shown) of the Bayesian analysis. Branch lengths are based on the number of inferred substitutions, as indicated by the scale bar. See Table 1 for abbreviations

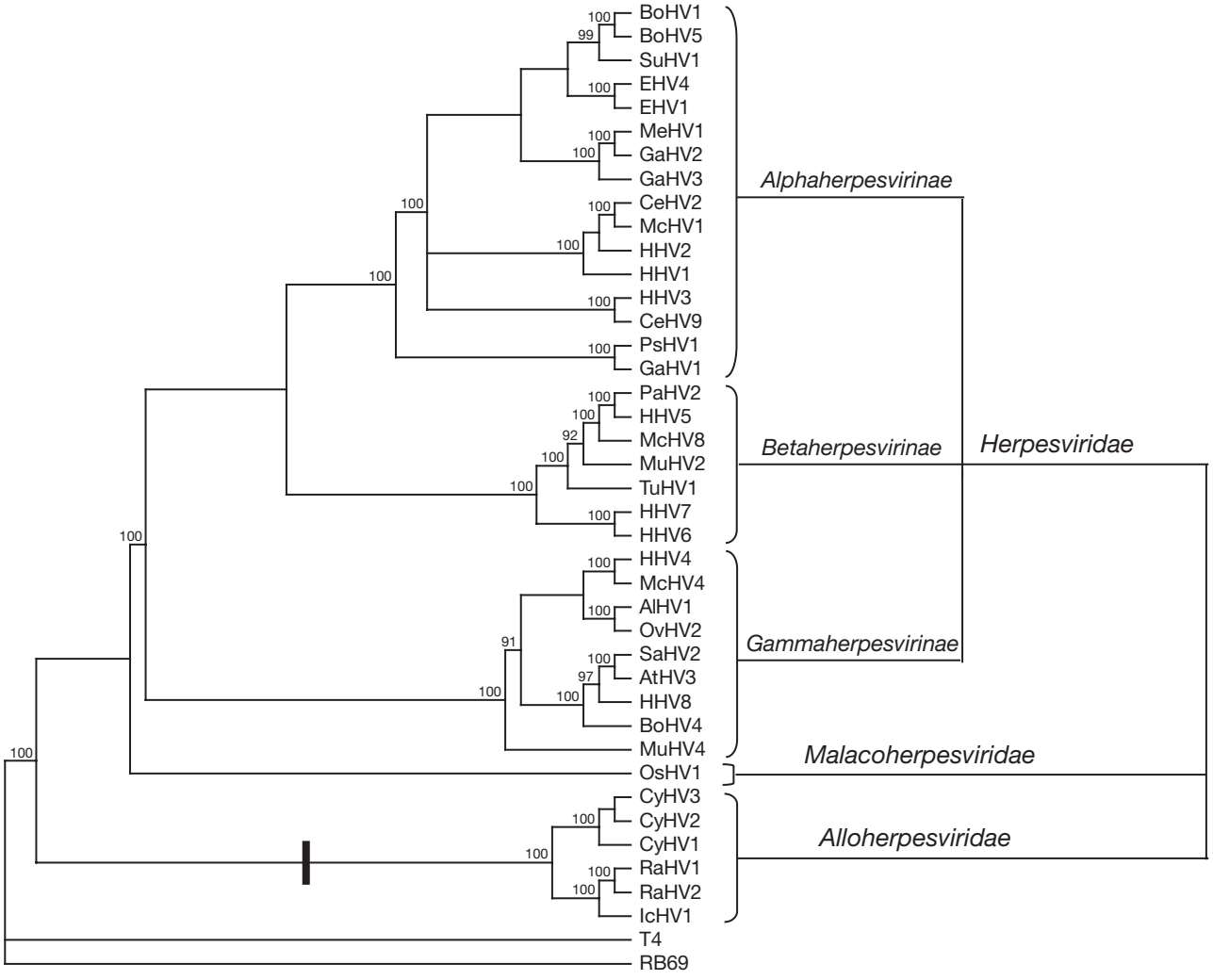

Fig. 4. Cladogram depicting relationships among viruses in the order *Herpesvirales*, based on the deduced amino acid (AA) sequences of the concatenated 5 conserved regions of the terminase gene (Dataset 41, 283 AA characters including gaps). The Bayesian maximum likelihood tree was rooted using bacteriophages T4 and RB69. Numbers at each node represent the posterior probabilities (values >90 shown) of the Bayesian analysis. The vertical bar indicates that the cohesion of the family *Alloherpesviridae* is also supported by the detectable conservation of at least 13 genes (Aoki et al. 2007). See Tables 1 & 3 for abbreviations

T4-like bacteriophages, provides the first formally assessed insights into the relationships among all HVs. The findings are in full accord with the recent reclassification of HVs into 3 families. The general lack of detectable sequence relationships among the 3 families implies that future progress in understanding the broad evolutionary relationships among HVs will have to rely on other lines of evidence.

The cladistic (Figs. 1 to 4) and phenetic (Fig. 6, Table 5) analyses facilitated subdivision of the family *Alloherpesviridae* into 2 sister clades. Clade 1 includes HVs from anguillid and cyprinid hosts, which possess the largest genomes found among HVs, ranging from about 245 kbp in the Dutch and Japanese isolates of AngHV1 (Rijsewijk et al. 2005) to 295 kbp in CyHV3 (Aoki et al. 2007). Our analyses of this clade significantly extend previous preliminary work based upon smaller datasets and incomplete statistical methodologies, and support cohesion of the anguillid and cyprinid HVs (Rijsewijk et al. 2005, Doszpoly et al. 2008). In addition, we note that CyHV3 ORF45 may represent a clade-specific gene, in that a 1500 bp region of a Taiwanese isolate of AngHV1 (Shih et al. 2003; sequence not deposited in a public database) has convincing AA similarity to CyHV3 ORF45 and its counterpart in CyHV1 but not to genes in IcHV1, RaHV1, or RaHV2. Further sequence data are likely to define additional Clade 1-specific genes. Clade 2 contains the remaining fish HVs and the frog HVs, which appear to possess smaller genomes: IcHV1 has the

Table 5. Phenetic analysis of the Dataset 15 alignment, which was composed of the deduced amino acid (AA) sequences for a portion of the DNA polymerase and terminase genes from representative fish, amphibian, and mammalian herpesviruses (HVs). Comparisons of DNA polymerase (193 AA characters including gaps) are above the diagonal and those of terminase (116 AA characters including gaps) are below. Values are expressed as a percentage of identity. See Table 1 for abbreviation

	Virus abbreviation (isolate)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	AngHV1 (H. Fukuda)		100.0	62.3	63.6	63.6	32.2	31.6	31.6	33.1	34.1	33.5	32.9	32.9	32.9	31.0	35.0	33.9	15.2	12.2
2	AngHV1 (569116)	100.0		62.3	63.6	63.6	32.2	31.6	31.6	33.1	34.1	33.5	32.9	32.9	32.9	31.0	35.0	33.9	15.2	12.2
3	CyHV1 (H. Fukuda)	69.0	69.0		86.3	87.6	27.7	28.3	28.3	34.7	32.7	29.6	29.0	29.0	29.0	30.3	34.1	37.6	15.7	14.3
4	CyHV2 (H. Fukuda)	70.0	70.0	95.2		90.2	29.0	28.3	28.3	32.9	32.7	30.3	29.6	29.6	29.6	28.4	32.9	35.7	15.7	13.2
5	CyHV3 (KHV-U)	69.0	69.0	95.2	98.1		28.3	27.1	27.1	33.5	32.0	30.3	29.6	29.6	29.6	29.6	34.8	36.3	15.2	13.8
6	IcHV1 (ATCC-VR-665)	36.3	36.3	36.7	38.6	38.6		84.9	84.9	37.5	70.0	57.3	56.6	56.6	56.6	56.6	33.1	29.9	15.0	11.3
7	IcHV2 (761/94)	38.1	38.1	36.7	38.6	38.6	97.1		100.0	38.1	72.7	56.0	56.0	56.0	56.0	58.0	32.4	31.2	12.7	11.9
8	IcHV2 (762A)	38.1	38.1	36.7	38.6	38.6	97.1	100.0		38.1	72.7	56.0	56.0	56.0	56.0	58.0	32.4	31.2	12.7	11.9
9	AciHV1 (UC Davis type)	45.4	45.4	45.2	46.2	45.2	50.0	50.9	50.9		41.2	39.2	41.1	41.1	41.1	37.4	35.4	33.1	15.7	17.1
10	AciHV2 (UC Davis type)	40.0	40.0	37.7	38.6	38.6	86.6	87.6	87.6	46.2		56.6	58.6	58.6	58.6	60.6	34.4	31.2	15.5	15.3
11	SalHV1 (ATCC-VR-868)	35.1	35.1	38.3	39.2	39.2	54.7	52.8	52.8	44.8	53.7		90.6	90.6	90.6	82.0	34.0	34.0	12.5	10.0
12	SalHV2 (OMV)	36.0	36.0	39.2	40.1	40.1	56.6	54.7	54.7	46.7	55.6	97.1		100.0	100.0	82.6	33.3	34.6	12.5	10.0
13	SalHV2 (YTV)	36.0	36.0	39.2	40.1	40.1	56.6	54.7	54.7	46.7	55.6	97.1	100.0		100.0	82.6	33.3	34.6	12.5	10.0
14	SalHV2 (NeVTA)	36.0	36.0	39.2	40.1	40.1	56.6	54.7	54.7	46.7	55.6	97.1	100.0	100.0		82.6	33.3	34.6	12.5	10.0
15	SalHV3 (Wisconsin)	36.9	36.9	41.1	42.0	42.0	57.5	55.6	55.6	47.6	56.6	95.2	96.1	96.1	96.1		35.3	33.3	12.5	10.6
16	RaHV1 (McKinnell)	36.0	36.0	41.1	41.1	40.1	38.3	35.5	35.5	54.2	36.4	40.1	41.1	41.1	41.1	42.0		47.4	15.6	13.6
17	RaHV2 (ATCC-VR-568)	40.5	40.5	42.9	43.9	42.9	40.1	39.2	39.2	55.1	40.1	40.1	41.1	41.1	41.1	42.0	58.8		13.3	15.4
18	HHV1	21.7	21.7	21.6	19.8	18.9	19.8	18.9	18.9	22.5	20.7	17.8	18.7	18.7	18.7	18.7	23.2	22.3		44.5
19	HHV8	26.9	26.9	32.4	30.6	29.7	23.4	23.4	23.4	25.2	25.2	20.5	21.4	21.4	21.4	20.5	31.2	28.5	46.2	

smallest genome at 134 kbp, and RaHV2 the largest at 232 kbp (Davison 1992, Davison et al. 2006). Of the 19 genes that are convincingly conserved between IcHV1, RaHV1, and RaHV2, 5 appear not to be conserved in CyHV3 (IcHV1 ORF34, ORF43, ORF44, ORF70, and ORF78) and 1 (ORF53) has a marginal counterpart. Clades 1 and 2 might eventually deserve recognition in the taxonomy of the family *Alloherpesviridae*, perhaps at the level of subfamily. However, it would be wise to gather additional data before taking this step.

HVs typically exhibit a pattern of cospeciation with their hosts, but exceptions have been noted among the mammalian (McGeoch et al. 2006) and fish HVs (Kelley et al. 2005). Comparison of the phylogeny of fish and amphibian HVs with that of their hosts does not support coevolution between host and virus at deeper nodes of the tree (Fig. 5). For example, the cyprinid and ictalurid fishes are members of the superorder Ostariophysi, and yet the analysis did not reveal a sister species relationship between their respective HVs. The family Acipenseridae (sturgeons) is an ancient fish lineage, and yet the sturgeon HVs (AciHV1 and AciHV2) are not sister taxa, with AciHV2 being the sister group of the ictalurid HVs. The eel HV, AngHV1, also did not show evidence of host cospeciation, as it grouped tightly with the cyprinid HVs. Finally, frogs are the sister group to all of the fish, but frog HVs are sister to only one of the fish HV clades.

If Fig. 5 is an accurate representation of the relationships involved, the lack of observed cospeciation at deeper nodes might reflect existence within an aqueous medium of both the viruses (in relation to ease of interspecies transmission) and those of the hosts (in relation to factors such as extensive migration or international trade, intensive polyculture, and hybridization). Although the analysis revealed overall discordance between HV and host lineages, a degree of coevolution was discernable at the tips of the phylogenetic tree, with the salmonid, ictalurid, ranid, and cyprinid HVs segregating with their respective hosts. AngHV1 groups with the cyprinid HVs in Clade 1, confirming the distinctiveness of these viruses (Sano et al. 1990, Rijsewijk et al. 2005, Waltzek et al. 2005). Moreover, the sequence data confirm serological and molecular evidence that isolates of a HV causing serious disease in eel populations in Europe and Asia represent isolates of AngHV1 (Sano et al. 1990, Chang et al. 2002, Rijsewijk et al. 2005). The cyprinid HVs form a tight clade, CyHV1 and CyHV3 infecting common carp varieties (*Cyprinus carpio*) and CyHV2 infecting only goldfish *Carassius auratus* (Hedrick et al. 2004). On the basis of previous findings (Waltzek et al. 2005) and the present study, a strong taxonomic case may be made for the creation of a genus (proposed *Cypri-*

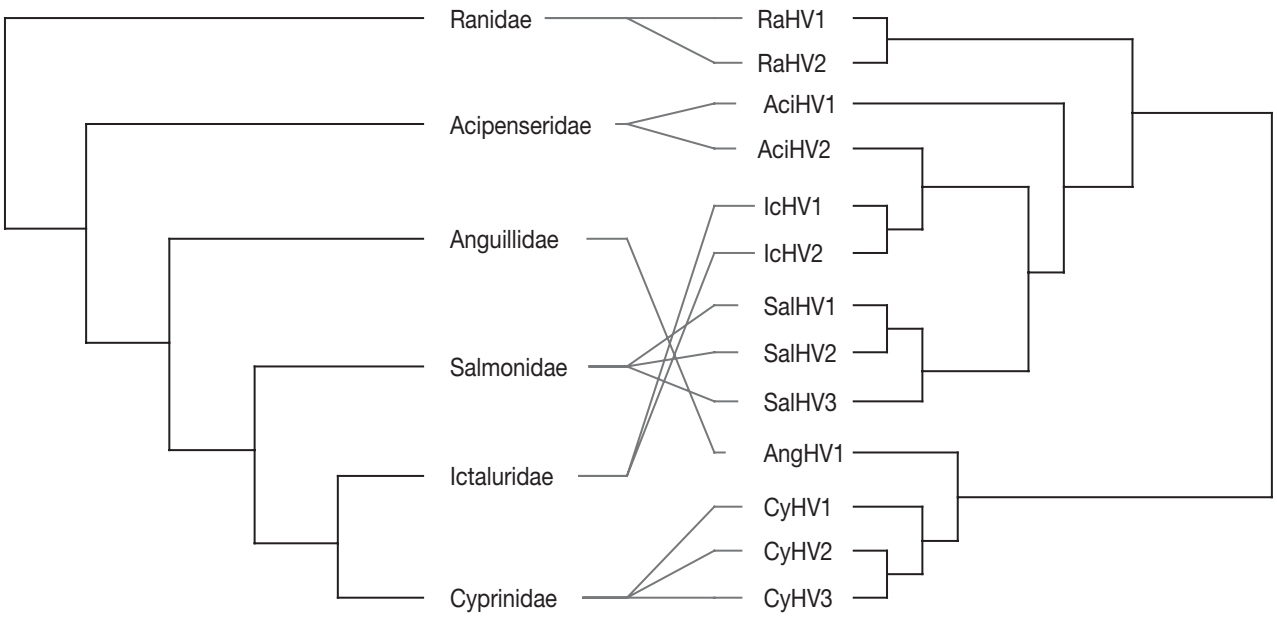

Fig. 5. Tanglegram testing correspondence between the phylogeny of fish and amphibian herpesviruses (HVs) and that of their hosts. See Table 1 for abbreviations

nivirus) to contain these viral species (*Cyprinid herpesvirus 1*, *2* and *3*).

In regard to Clade 2, the distinctiveness of the 2 ictalurid (Hedrick et al. 2003), 3 salmonid (Hedrick et al. 1987, Hayashi et al. 1989, McAllister 1993, Yoshimizu et al. 1995, Sung et al. 1996), 2 ranid (Tweedell 1989, Davison et al. 2006), and 2 acipenserid (Hedrick et al. 1991, Watson et al. 1995) HVs has been established previously. The present study supports sister status within these groupings, except for the acipenserid HVs. Both IcHV1 and IcHV2 have been associated with economically important epidemics in their respective ictalurid catfish hosts channel catfish *Ictalurus punctatus* and black bullhead *Ameiurus melas*, and the partial sequences of DNA polymerase and terminase analysed in the present study, as well as a similar analysis based solely on the partial DNA polymerase sequence (Doszpoly et al. 2008), demonstrate their affinity. Further evidence for a close relationship between these viruses is suggested by the discovery of a gene encoding a tegument-associated protein in IcHV2 that has a counterpart in IcHV1 (ORF72) but not other HVs (Doszpoly et al. 2008, T. B. Waltzek & R. P. Hedrick, GenBank accession no. 1162045). Lastly, naturally occurring and experimental infections of channel catfish with IcHV2 result in a disease and mortality similar to that induced by IcHV1 (Hedrick et al. 2003). These findings indicate that IcHV2 should be added to the genus *Ictalurivirus*. Indeed, a proposal to accomplish this by creating the new species *Ameiurine herpesvirus 1* in the genus *Ictalurivirus* was made recently (Doszpoly et al. 2008). However, we recommend the species name *Ictalurid herpesvirus 2*, which is consistent with the formal convention for naming herpesvirus species after the host family (Roizman et al. 1992, Davison et al. 2009). We also support the proposal to add AciHV2 to the genus *Ictalurivirus* (Doszpoly et al. 2008), and recommend the species name *Acipenserid herpesvirus 2*. Both SalHV1 (Wolf & Taylor 1975) and epizootic epitheliotropic disease virus (EEDV) (Bradley et al. 1989, McAllister & Herman 1989) have been associated with serious epidemics in the USA. In contrast, SalHV2 infects several salmonid species in Japan and has been associated with mortality in young fish and the induction of neoplasia among surviving fish. Our data support the proposal that EEDV represents a distinct salmonid HV, SalHV3 (McAllister 1993). This would prompt the erection of a genus (proposed *Salmonivirus*) containing these 3 viral species (*Salmonid aherpesvirus 1*, *2*, and *3*). The convincing conservation of 40 genes in the 2 frog HVs (Davison et al. 2006) and the results of the present study prompt the establishment of a genus (proposed *Batrachovirus*) for these viral species (*Ranid herpesvirus 1* and *2*). Their branching from fish HVs evidently lies deep in the clade, the phylogeny according with the somewhat greater number of genes detectably conserved between the frog HVs and IcHV1 (19 genes) as compared with the number conserved between IcHV1 and CyHV3 (15 genes). The grouping of AciHV2 isolates from white and shortnose sturgeon with IcHV1, and its more distant relationship

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AngHV1          ICVVDEAAFVSPGMLVSVLPLMTVKGTKQIHISSPGAPNSWISQVGDVKR-EDGSTLAQVIDCKYRCRAH-----AQIGDKSQAFSCVCNDIFRPDHIKIETGVESLMNLVSEGSF

CyHV1           ICVVDEAAFVGANLLVSVLPLMTVKGTKQIHISSPGSADSWISHVGDVRQ-ENGETLAQIVDYKYKCKAH-----AE----EDGISCVCNDIYKPDHIEIEAGVKELMNLVCEGAF
CyHV2           ICVVDEAAFVGANLLVSVLPLMTVKGTKQIHISSPGSADSWISHVGDVKQ-ENGETLAQIIDYKYKCKAH-----AE----EEGISCVCNDIYKPDHIEIEEGVKELMNLVCDGAF
CyHV3           ICVVDEAAFVGANLLVSVLPLMTVKGTKQIHISSPGSADSWISHVGDVKQ-ENGETLAQIIDYKYKCKAH-----AE----EDGISCVCNDIYKPDHIEIEEGVKELMNMVCDGAF
   Cyp.         ICVVDEAAFVGANLLVSVLPLMTVKGTKQIHISSPGSADSWISHVGDV.Q-ENGETLAQI.DYKYKCKAH-----AE----E.GISCVCNDIYKPDHIEIE.GVKELMN.VC.GAF

   Clade1       ICVVDEAAFV....LVSVLPLMTVKGTKQIHISSPG...SWISHVGDV..-E.G.TLAQ..D.KY.C.AH-----A.----....SCVCNDI..PDHI.IE.GV..LMN.V..G.F

IcHV1           FVLIDEAAFVNPASLLSLLPLLAVKNRKQIHISSHI-AKSWVTRVETIMG-SDGKRAFHVINQRFKCDGH-----AH----LPGMMCPCSAVFCPTHIDLNESIQELINNITPGGC
IcHV2           FVLIDEAAFVNPASLLSLLPLLAVKNRKQIHISSHI-PKSWVTRVETIMG-SDGKRAFHVINQRFKCEGH-----AH----LPGMMCPCSAIFCPTHIDLNESIQELINNITPGGC
   Ict.         FVLIDEAAFVNPASLLSLLPLLAVKNRKQIHISSHI-.KSWVTRVETIMG-SDGKRAFHVINQRFKC.GH-----AH----LPGMMCPCSA.FCPTHIDLNESIQELINNITPGGC

AciHV1          LVLIDEAGFINLPALLSVLPLMAINGTKQIHISSPVNHNAWISRVEDIKD-ADGQQAVHVAVHRFKCKYH-----AD----APGATCSCSDIYCPDHITVNHLLQSLLNLVQPGSF
AciHV2          FVLVDEAAFVNPASLLSLLPLLAVKNRKQIHISSHI-PKSWVNKVGNIMA-CDGKRAFHVINQRFKCDGH-----TQ----LPGMMCPCAAIFCPTHISLNNSIQELINNITPGGC

SalHV1          FILVDKAAFVNLTSITSLIPLMLVAGRKQIHISSHV-AKSWMNNVGDIIDETTGEPAFHVISQKFKCAAH-----MH----LPSLTCPCESVYCPSHIDMNPATQALLSCVAPGG-
SalHV2          FILVDEAAFVNLTSITSLIPLMLVAGRKQIHISSHV-AKSWMNNVGDIIDEATGEPAFHVISQKFKCAAH-----MH----LPSLTCPCEAVYCPSHIDMNPATQALLSCVAPGG-
SalHV3          FILVDEAAFVNLTSITSLIPLMLVAGRKQIHISSHV-AKSWINNVGDIIDETTGEPAFHVISQKFKCGAH-----MH----LPGLTCPCEAVYCPSHIDMNPATQALLSCVAPGG-
   Sal.         FILVD.AAFVNLTSITSLIPLMLVAGRKQIHISSHV-AKSW.NNVGDIIDE.TGEPAFHVISQKFKC.AH-----MH----LP.LTCPCE.VYCPSHIDMNPATQALLSCVAPGG-

RaHV1           LVIVDEAAFICMPALLSLIPLMAIDGTKQIHTTSPVDAGAWISRLSELRDVDTGEPHVHMIAQQFKCDYH-----AQ----DADITCPCMDVYRPDHMTVDVQLKDILNLISPGAF
RaHV2           LVIVDEAGFVNKGAFLSVLPLMAVKGTKQIHISSHVDKDAWLSRLGEVIDPITGKAGVHLIKSQFKCDMH-----AA----DSGVTCACNDYLCPDHMTVDDVLKQILELVVPGSF
   Bat.         LVIVDEA.F....A.LS..PLMA..GTKQIH..S.VD..AW.SRL.E..D..TG...VH.I..QFKCD.H-----A.----D...TC.C.D...PDHMTVD..LK.IL.L..PG.F

   Clade2       ....D.A.F.......S..PL......KQIH..S......W...........G....H.....FKC..H-----..----.....C.C.....P.H.............PG..

   Allo.        ....D.A.F.......S..PL......KQIH..S......W...........G...........C..H-----..----.....C.C.....P.H.............G..

HHV1            LLFVDEANFIRPDAVQTIMGFLNQANCKIIFVSSTNTGKASTSFLYNLRG--AADELLNVVT--YICDDHMPRVVTH----TNATACSCYILNKPVFITMDGAVRRTADLFLADSF
HHV8            LLYVDEANFIKKDALPAILGFMLQKDAKLIFISSVNSSDRSTSFLLNLRN--AQEKMLNVVS--YVCADHREDFHLQ----DALVSCPCYRLHIPTYITIDESIKTTTNLFMEGAF
   Herp.        LL.VDEANFI..DA...I.GF..Q...K.IF.SS.N....STSFL.NLR.--A....LNVV.--Y.C.DH.....----.....C.CY.L..P..IT.D.....T..LF....F

   Herpesv.     ....D.A.F.................K.I...S.............................C..H.............C.C.....P.....................
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Fig. 6. Partial amino acid (AA) sequence alignment of the first region of conservation in the terminases of fish and mammalian herpesviruses (HVs) (Davison 2002), corresponding to residues 296 to 396 of the IcHV1 protein (NC_001493). Consensus sequences are indented, italicized, and include: *Clade1*, composed of HVs from cyprinid and anguillid hosts; *Clade2*, composed of HVs from ictalurid, acipenserid, salmonid, and ranid hosts; *Alloherpesviridae* (*Allo.*), composed of the fish and amphibian HVs from Clades 1 and 2; *Herpesviridae* (*Herp.*), represented by 2 mammalian HVs; and *Herpesvirales* (*Herpesv.*), represented by HVs from fish, amphibian, and mammalian hosts. Additional consensus sequences were generated for the taxa comprising each of the 4 genera in the *Alloherpesviridae* proposed in the present study and at www.ictvonline.org: *Cyprinivirus* (*Cyp.*), *Ictalurivirus* (*Ict.*), *Salmonivirus* (*Sal.*), and *Batrachovirus* (*Bat.*). (–): gapping characters; (….): positions of nonconservation. See Tables 1 & 3 for abbreviations

to isolates of AciHV1 from white sturgeon, has been described previously (Kelley et al. 2005, Doszpoly et al. 2008, Kurobe et al. 2008).

In respect of biological properties, the invocation of latent infection as a hallmark of HVs (Roizman & Pellett 2001) raises the question of whether this is true of the family *Alloherpesviridae*. The best evidence comes from studies of the Lucké tumor, in which RaHV1 nucleic acid and proteins have been demonstrated in non-infectious, virus-free tumor tissue that, upon temperature manipulation, can yield infectious virus (Biggs 1972, Naegele & Granoff 1980, Tweedell 1989). Moreover, evidence for a long-term carrier state, which may or may not involve latency, has been described in several fish HVs, including IcHV1 (Wise et al. 1985, Boyle & Blackwell 1991, Baek & Boyle 1996, Gray et al. 1999, Thompson et al. 2005), SalHV2 (Gou et al. 1991, Kimura & Yoshimizu 1998), CyHV1 (Sano et al. 1993), AngHV1 (van Nieuwstadt et al. 2001, Shih 2004), and CyHV3 (Adkison et al. 2005, Bercovier et al. 2005, St-Hilaire et al. 2005). These reports, and others of an anecdotal nature, indicate the existence of long-term infections (perhaps latency) caused by a wide selection of viruses in the family *Alloherpesviridae*.

Members of the family *Alloherpesviridae* often induce proliferative epithelial lesions in their respective hosts. HV particles have been observed by electron microscopy and on occasion isolated from neoplastic or hyperplastic epithelial lesions in salmonid, cyprinid, anguillid, osmerid, esocid, silurid, percid, pleuronectid, and acipenserid fishes (Hedrick et al. 1991, Anders & Yoshimizu 1994, Watson et al. 1995). Neoplastic lesions have been associated with the following fish and amphibian HV infections: several isolates of SalHV2 (i.e. OMV, YTV, and CSTV, but not NeVTA and RKV), RaHV1, and CyHV1, as well as golden ide HV, sheatfish (wels) HV, salmon papillomatosis virus, and smelt papillomatous virus (Tweedell 1989, Anders & Yoshimizu 1994). Most of these viruses induce papillomas or papilloma-like lesions, except for RaHV1, which is associated with renal adenocarcinoma, and SalHV2, which induces cutaneous carcinomas (Yoshimizu et al. 1987, 1988). Given the phylogeny of the family *Alloherpesviridae*, and the fact that several viruses induce neoplasia (CyHV1, RaHV1, and SalHV2) or proliferative epithelial lesions (AciHV2 and SalHV3) in their respective hosts, it is likely that the evolution of these biological properties has been complex.

In conclusion, the deduced phylogenetic relationships of 13 fish and amphibian HVs strongly support the monophyly of the family *Alloherpesviridae*, with 2 major clades evident. The first contains cyprinid and anguillid taxa and the second contains ictalurid, salmonid, acipenserid, and ranid taxa. A pattern of coevolution between virus and host was not supported at deeper nodes but was supported at the tips of the tree. The findings are fully in accord with the recent taxonomic revision of the HVs and will assist in defining additional taxa in the family *Alloherpesviridae*.

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