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Characterization of an alloherpesvirus from wild lake sturgeon *Acipenser fulvescens* in Wisconsin (USA)

Logan Walker[1,2], Kuttichantran Subramaniam[2], Pedro H. O. Viadanna[2], Jordan A. Vann[2], Susan Marcquenski[3], Danielle Godard[3], Elizabeth Kieran[4], Salvatore Frasca Jr.[4,7], Vsevolod L. Popov[5], Karen Kerr[6], Andrew J. Davison[6], Thomas B. Waltzek[2,*]

[1]Fisheries and Aquatic Sciences, College of Agricultural and Life Sciences, University of Florida, Gainesville, FL 32611, USA

[2]Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611, USA

[3]Bureau of Fisheries Management, Wisconsin Department of Natural Resources, Madison, WI 53716, USA

[4]Department of Comparative, Diagnostic, and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611, USA

[5]Center for Biodefense and Emerging Infectious Diseases, Institute for Human Infections and Immunity, the University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555, USA

[6]MRC-University of Glasgow Centre for Virus Research, Glasgow G61 1QH, UK

[7]*Present address:* Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT 06269-3089, USA

ABSTRACT: In the spring of 2017, 2 adult lake sturgeon (LS) *Acipenser fulvescens* captured from the Wolf River, Wisconsin (USA), presented with multiple cutaneous plaques that, upon microscopic examination, indicated proliferative epidermitis. Ultrastructural examination of affected keratinocytes revealed particles in the nucleus having a morphology typical of herpesviruses. A degenerate PCR assay targeting the DNA polymerase catalytic subunit (*pol*) gene of large double-stranded DNA viruses generated amplicons of the anticipated size from skin samples, and sequences of amplicons confirmed the presence of a novel alloherpesvirus (lake sturgeon herpesvirus, LSHV) related to acipenserid herpesvirus 1 (AciHV1). The complete genome (202 660 bp) of this virus was sequenced using a MiSeq System, and phylogenetic analyses substantiated the close relationship to AciHV1. A PCR assay targeting the LSHV DNA packaging terminase subunit 1 (*ter1*) gene demonstrated the presence of the virus in 39/42 skin lesion samples collected from wild LS captured in 2017–2019 and 2021 in 4/4 rivers in Wisconsin. Future efforts to isolate LSHV in cell culture would facilitate challenge studies to determine the disease potential of the virus.

KEY WORDS: Acipenserid herpesvirus 1 · Lake sturgeon herpesvirus · *Alloherpesviridae* · Hyperplastic dermatitis · Lake sturgeon · Phylogenetics

1. INTRODUCTION

Sturgeon (family Acipenseridae) are an ancient assemblage of freshwater or anadromous fishes that are known for their primitive features, which are thought to have been maintained since the Lower Jurassic Period more than 200 million years ago (Birstein & Bemis 1997). The lake sturgeon (LS) *Acipenser fulvescens* is 1 of 27 extant sturgeon species and can reach weights of up to 140 kg (309 lb). LS are benthivorous and are broadly distributed across North America including the St. Lawrence, Hudson Bay, Great Lakes, and Mississippi River watersheds (https://www.fws.gov/species/lake-sturgeon-acipenser-fulvescens). Prior to detrimental anthropogenic activities in the late nineteenth and early twentieth

*Corresponding author: tomwaltzek@gmail.com

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centuries, LS were abundant throughout the Great Lakes system. By 1900, populations within the USA had dwindled as a result of overharvesting for caviar and isinglass. Recent studies suggest that environmental alteration and degradation, including pollution, dredging, channelization, and construction of dams and hydroelectric facilities (Kerr 2010), have negatively impacted the movement and spawning of LS populations (Auer 1996). Currently, LS are listed as endangered, threatened, or of special concern in 12 US states (Léonard et al. 2004, Holey & Trudeau 2005). Efforts to restore LS populations have increased in recent decades, and partnerships to support them have been forged between natural resource management agencies, commercial fishermen, recreational fishermen, and landowners throughout the Great Lakes watershed (Peterson et al. 2007).

Limited information is available on pathogens affecting the productivity and health of LS. To date, more than 20 different parasites have been documented in surveys of wild fish (Choudhury & Dick 1993, 1998, Choudhury et al. 1996), including descriptions of a new nematode species (*Spinitectus acipenseri*), a new gill monogenean species (*Diclybothrium atriatum*), and a new intestinal digenean of the family Deropristiidae (Choudhury & Dick 1992, Choudhury 2009). The cnidarian parasite *Polypodium hydriforme* develops in the eggs of sturgeon and paddlefish and has been reported in LS from the USA and Canada (Choudhury & Dick 1991, Raikova & Justine 1994). Bacterial pathogens of LS have not been reported, and only a single double-stranded (ds) DNA virus (Namao virus) has been described. This virus is an unclassified nucleocytoplasmic large DNA virus and was associated with morbidity and mortality in juvenile LS cultured in Manitoba, Canada, in 2013 (Clouthier et al. 2013, 2015). Other dsDNA viruses have been reported in cultured white sturgeon (WS) *A. transmontanus*, including an adenovirus (Hedrick et al. 1985, Doszpoly et al. 2019), an irido-like virus (Hedrick et al. 1990), and 2 alloherpesviruses (Hedrick et al. 1991, Watson et al. 1995).

In 1990, white sturgeon herpesvirus type 1 (WSHV1) was isolated from moribund juvenile WS using a WS skin cell line (WSSK-1). This virus was identified as the etiological agent responsible for significant losses in hatchery fry and fingerlings in California (Hedrick et al. 1991). WS infected with WSHV1 presented with no observable gross lesions. However, histopathological examination revealed hydropic degeneration, hypertrophy, and hyperplasia of keratinocytes (Hedrick et al. 1991), resulting in a diffuse hyperplastic dermatitis. Transmission electron microscopy (TEM) of infected WSSK-1 cells revealed naked hexagonal nucleocapsids in the nucleus. Numerous enveloped virus particles surrounded by a coarse electron-dense layer were observed within cytoplasmic vacuoles. WSHV1 was again isolated from cultured WS in California in 2003 in a newly established farm maintaining wild broodstock (Kelley et al. 2005), and also in Europe on an Italian farm culturing WS (Kurobe et al. 2008).

From 1991 to 1993, additional herpesvirus isolates were obtained from asymptomatic adult or subadult WS displaying cutaneous erosions and experiencing elevated morbidity and mortality on farms in California (Watson et al. 1995). Based on differences from WSHV1 in growth characteristics in cell culture and serological properties, these isolates were shown to be a second WS herpesvirus, white sturgeon herpesvirus type 2 (WSHV2). TEM of infected WSSK-1 cultures revealed numerous hexagonal nucleocapsids with electron-dense cores undergoing virion morphogenesis within both the nucleus and cytoplasm, as expected for herpesviruses.

Experimental challenge studies involving juvenile WS exposed to WSHV2 reproduced the cutaneous disease observed in farmed juvenile WS (Watson et al. 1995). Microscopic lesions of the integument included a multifocal to diffuse epithelial and mucous cell hyperplasia that progressed to erosions in severely affected regions. The nuclei of infected epithelial cells appeared lobulated and enlarged, hypochromatic, and displaying marginated chromatin. WSHV2 strains were also isolated from asymptomatic adult female WS from the Columbia River in Oregon in 1994 and the Snake River in Idaho in 1999 (Kelley et al. 2005, Doszpoly et al. 2008, Kurobe et al. 2008, Waltzek et al. 2009). Most recently, WSHV2 was isolated along with *Streptococcus iniae* from subadult WS experiencing elevated mortality and displaying cutaneous hemorrhages following transport between farms in California (Soto et al. 2017). WSHV2 strains have also been isolated from juvenile farmed shortnose sturgeon *A. brevirostrum* in Canada (Kelley et al. 2005, Kurobe et al. 2008, LaPatra et al. 2014) and farmed Siberian sturgeon *A. baeri* in Russia (Shchelkunov et al. 2009, Doszpoly & Shchelkunov 2010) that were suffering from cutaneous diseases similar to those observed in farmed WS in California.

Herpesvirus particles consist of a host-derived envelope surrounding a proteinaceous matrix (the tegument) in which is embedded an icosahedral nucleocapsid (approximately 100 nm in diameter) enclosing a large, linear, dsDNA genome. The order to which

they belong, *Herpesvirales*, includes 3 families, of which the family *Alloherpesviridae* contains frog and fish viruses. Currently, this family is composed of 4 genera and 13 species. The frog viruses are in the genus *Batrachovirus* (species *Ranid herpesvirus 1*, *Ranid herpesvirus 2*, and *Ranid herpesvirus 3*; viruses RaHV1, RaHV2, and RaHV3). The fish viruses are in the genera *Cyprinivirus* (species *Anguillid herpesvirus 1*, *Cyprinid herpesvirus 1*, *Cyprinid herpesvirus 2*, and *Cyprinid herpesvirus 3*; viruses AngHV1, CyHV1, CyHV2, and CyHV3), *Ictalurivirus* (species *Ictalurid herpesvirus 1*, *Ictalurid herpesvirus 2,* and *Acipenserid herpesvirus 2*; viruses IcHV1, IcHV2, and AciHV2), and *Salmonivirus* (species *Salmonid herpesvirus 1*, *Salmonid herpesvirus 2*, and *Salmonid herpesvirus 3*; viruses SalHV1, SalHV2, and SalHV3) (www.ictv.global/taxonomy). Among the viruses listed, IcHV1 possesses the smallest genome (134 kb, encoding 76 genes; Davison 1992), and CyHV3 has the largest (295 kb, encoding 155 genes; Aoki et al. 2007). The complete genome sequences of SalHV1, SalHV2, SalHV3, and AciHV2 have not been determined.

Alloherpesviruses share 12 core genes that exhibit convincing levels of amino acid sequence conservation. These genes are involved in DNA replication (DNA polymerase catalytic subunit [*pol*], helicase–primase helicase subunit, and helicase–primase primase subunit), DNA packaging (DNA packaging terminase subunit 1 [*ter1*]), and capsid morphogenesis (major capsid protein, capsid triplex subunit 2, and capsid maturation protease), as well as several genes encoding proteins of unknown function (Davison et al. 2013). Phylogenetic analyses based on a concatenation of the core genes have served as the basis of the current taxonomy of the family *Alloherpesviridae* (Waltzek et al. 2009, van Beurden et al. 2010, Doszpoly et al. 2011a, Davison et al. 2013). Other studies have relied on complete or partial sequences of the *pol* and *ter1* genes for determining the phylogenetic relationships of a wider array of alloherpesviruses infecting sturgeon species (Kelley et al. 2005, Kurobe et al. 2008, Waltzek et al. 2009, Doszpoly & Shchelkunov 2010, Soto et al. 2017), pilchard *Sardinops sagax neopilchardus* (Crockford et al. 2005), catfish (Doszpoly & Shchelkunov 2010, Subramaniam et al. 2019), Atlantic cod *Gadus morhua* (Marcos-Lopez et al. 2012), northern pike *Esox lucius* (Freitas et al. 2016), and European perch *Perca fluviatilis* (Garver et al. 2018).

Genetic and phylogenetic analyses based on partial *pol* and *ter1* sequences have shown that WSHV1 and WSHV2 are distantly related (Kelley et al. 2005, Doszpoly et al. 2008, Kurobe et al. 2008, Waltzek et al. 2009). WSHV2 is closely related to members of the genus *Ictalurivirus* and has been accepted as a member of this genus under the species name *Acipenserid herpesvirus 2* (AciHV2, thus becoming a synonym for WSHV2 and being used hereafter; Davison et al. 2009). Larger genome sequencing projects have further supported the placement of AciHV2 isolates from WS and Siberian sturgeon in the genus *Ictalurivirus* (Doszpoly & Shchelkunov 2010, Doszpoly et al. 2011a,b). In contrast, only the partial *pol* and *ter1* sequences have been determined for WSHV1 isolates originating from farmed or wild WS (Waltzek et al. 2009). The lack of additional sequence data has delayed the formal classification of WSHV1 (e.g. in species *Acipenserid herpesvirus 1*) as proposed previously (Kelley et al. 2005, Kurobe et al. 2008, Waltzek et al. 2009). Nonetheless, we refer hereafter to WSHV1 by its synonym AciHV1.

In this study, we report the first case of alloherpesvirus infection in LS. We present the gross, microscopic, and ultrastructural features of the observed cutaneous disease in wild adult fish captured in 2017–2019 and 2021 in 4 rivers in Wisconsin. We also describe the complete genome sequence of lake sturgeon herpesvirus (LSHV), thus facilitating genetic and phylogenetic comparisons with previously reported fish and frog herpesviruses. LSHV was found to be most closely related to AciHV1 strains isolated previously from WS from North America and Europe.

2. MATERIALS AND METHODS

2.1. Sample collection, histopathology, and TEM

In the spring of 2017, adult wild LS were caught by the Wisconsin Department of Natural Resources (WDNR; Madison, WI) during a transfer event in which LS were relocated from downstream to upstream of the Shawano dam along the Wolf River. On examination of the fish for health certificate issuance prior to transfer, focal white cutaneous plaques were noted on the ventral and dorsal skin and fins of 2 of the fish (WVL17229-01A and WVL17229-02A). Selected skin lesions were removed from both fish and fixed in 10% (v/v) neutral buffered formalin, processed routinely, and embedded in paraffin blocks. Sections of 5 µm were cut and stained with hematoxylin and eosin for light microscopic examination (Humason 1979) at the Wisconsin Veterinary Diagnostic Laboratory (Madison, WI).

For TEM, selected skin lesions were fixed at room temperature for 1 h in 15 ml modified Karnovsky's

fixative (2P+2G fixative: 2% [v/v] formaldehyde prepared from paraformaldehyde and 2% [v/v] glutaraldehyde in cacodylate buffer [0.1 M sodium cacodylate, pH 7.4]). The fixed lesions were washed in cacodylate buffer and shipped in phosphate-buffered saline overnight on ice packs to the University of Texas Medical Branch Department of Pathology Electron Microscopy Laboratory, where the samples were washed in cacodylate buffer and left overnight in 2P+2G fixative at 4°C. The next day, the samples were washed twice in cacodylate buffer, post-fixed in 1% (w/v) osmium tetroxide in cacodylate buffer, stained en bloc with 2% (w/v) aqueous uranyl acetate, dehydrated in ascending concentrations of ethanol, processed through propylene oxide, and embedded in Poly/Bed 812 epoxy plastic (Polysciences). Ultrathin sections were cut on a Leica ULTRACUT EM UC7 ultramicrotome (Leica Microsystems), stained with 0.4% (w/v) lead citrate, and examined in a JEM-1400 electron microscope (JEOL USA) at 80 kV.

2.2. DNA extraction and screening by degenerate PCR and sequencing

Skin sections from both fish were shipped overnight on dry ice from WDNR to the Wildlife and Aquatic Veterinary Disease Laboratory (WAVDL; Gainesville, FL). DNA was extracted using a DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol, and the concentrations of extractions were determined by fluorometry using a Qubit dsDNA BR assay kit. Samples were subjected to a degenerate conventional PCR assay targeting *pol* in large DNA viruses (Hanson et al. 2006; Table 1), and products were separated by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide. DNA amplicons of the anticipated size were purified using a QIAquick PCR purification kit (Qiagen), quantified fluorometrically using a Qubit 3.0 fluorometer, and sequenced in both directions on an ABI 3130 genetic analyzer (Applied Biosystems). Following removal of primer sequences, the amplicon consensus sequences were analyzed using BLASTN (https://blast.ncbi.nlm.nih.gov/)

2.3. Complete genome sequencing and annotation

The DNA extracted from the skin sample of LS WVL17229-01A was used to generate a DNA sequencing library using a Nextera XT DNA kit (Illumina) according to the manufacturer's protocol. The library was sequenced using a v3 chemistry 600-cycle kit on a MiSeq (Illumina). *De novo* assembly of the paired-end reads was performed using SPAdes v. 3.5.0 with default settings (Bankevich et al. 2012). Filling of gaps due generally to tandem reiterations of short sequences was achieved by sequencing PCR products on an ABI 3130 genetic analyzer (Applied Biosystems). The genome termini were identified from 2 sets of reads each sharing an end. The genome sequence was assessed and finalized by mapping the reads using Bowtie 2 v. 2.3.1 (Langmead & Salzberg 2012) and visually inspecting the alignment in Tablet v. 1.21.02.08 (Milne et al. 2010).

Open reading frames (ORFs) encoding functional proteins were predicted using approaches described previously (Wilkie et al. 2013, Davison et al. 2017), utilizing BLASTP (https://www.ncbi.nlm.nih.gov/) with the GenBank non-redundant protein sequence database, subsets thereof, and a custom LSHV database to detect paralogous proteins, GeneMarkS (http://exon.gatech.edu/GeneMark/), SignalP v. 5.0 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0), TMHMM v. 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0), Philius (https://www.yeastrc.org/philius/pages/philius/runPhilius.jsp), Superfamily v. 2 (https://supfam.org/), and CDD (https://www.ncbi.nlm.nih.gov/cdd/). The annotated genome sequence was deposited in GenBank (accession no. OK485036.1). BioProject, BioSample, and raw sequence data for LSHV have been deposited in the National Center for Biotechnology Information databases under accession nos. PRJNA811186, SAMN26315075, and SRR18184256, respectively.

Table 1. PCR primer pairs targeting regions of lake sturgeon herpesvirus *pol* (open reading frame [ORF]36) and *ter1* (ORF19)

Primer name	Primer sequence (5′–3′)	Target	Amplicon size (bp)	Reference
ConDNAPOLF	CGG AAT TCT AGA YCN WSN YTN TAY CC	*pol*	497	Hanson et al. (2006)
ConDNAPOLR	CCC GAA TTC AGA TCN GTR TCN CCR TA			
AciHV1ls-ter1F	TAA GAG GCT CTG CAG CAG GT	*ter1*	196	Present study
AciHV1ls-ter1R	GTC GCC GGT TAA TCA TAA CG			

2.4. Phylogenetic and genetic analyses

The sequences of the 12 core genes were retrieved for LSHV and other alloherpesviruses (Table S1 in the Supplement at www.int-res.com/articles/suppl/d149p083_supp.xlsx). The amino acid sequences were concatenated for each virus and subjected to maximum likelihood (ML) phylogenetic analysis using MEGA X 10.2.6 (Kumar et al. 2018, Stecher et al. 2020). The sequences were aligned using Muscle, and the initial tree search was generated automatically by applying the neighbor-joining and BioNJ routines to a matrix of pairwise distances estimated using the JTT model. The topology with superior log likelihood value was then subjected to nearest-neighbor-interchange ML heuristic analysis using the LG model with a discrete gamma distribution in 5 categories with invariant sites (G+I). Gaps in the alignment were treated by partial deletion (90% site coverage cutoff value), no branch swap filter was applied, and 100 bootstrap replications were carried out. The unrooted trees with the highest log likelihood values were rendered using FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

2.5. LSHV-specific PCR

Determination of the LSHV genome sequence facilitated the development of a conventional PCR assay targeting *ter1* (exon 2) (Table 1). This assay was used to detect LSHV DNA in proliferative skin lesions collected from Wisconsin LS captured in 2017–2019 and 2021 in the Wolf River, Wisconsin River, Chippewa River, and Yellow River (Table 2, Fig. 1). A standardized, non-lethal skin scrape technique using a #22 scalpel blade was employed at all sites to obtain skin cells from plaques. Samples were collected individually or pooled (up to 7 different fish sampled per pool) prior to preserving in 70–100% (v/v) ethanol. The samples were shipped overnight from WDNR to WAVDL, where DNA was extracted as described above and tested by PCR.

The 30 µl PCR reaction mixture consisted of 17.6 µl molecular grade water, 0.15 µl Platinum *Taq* DNA polymerase (Invitrogen), 3 µl 10× PCR buffer (Invitrogen), 1.2 µl 50 mM $MgCl_2$, 0.6 µl 10 mM dNTPs (Invitrogen), 1.5 µl of each 20 mM primer, and 100 ng DNA template. An initial denaturation step of 94°C for 5 min was followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min, and finally by 72°C for 10 min. Amplicons of the anticipated size were purified under ultraviolet illumination from 1% (w/v) agarose gels containing ethidium bromide by using a QIAquick PCR purification kit (Qiagen), quantified fluorometrically using a Qubit 3.0 fluorometer, and sequenced in both directions on an ABI 3130 genetic analyzer (Applied Biosystems). Only a single sample was submitted for Sanger sequencing when multiple fish from the same location and year tested positive. Following removal of primer sequences, the amplicon consensus sequences were compared by BLASTN to the LSHV genome sequence.

Table 2. PCR detection of lake sturgeon herpesvirus (LSHV) in lake sturgeon samples from Wisconsin in the spring of 2017, 2018, 2019, and 2021. BLASTN identity is based on comparison of PCR-amplified *ter1* sequences with the LSHV genome sequence. na: not applicable, as the sample was negative by PCR

Year	Location	Fish sampled (n)	Tubes (n)	Positive tubes (n)	BLASTN identity (%)	GenBank accession no.
2017	Wolf River (Highway CCC)	2	2	2	100[a]	OK485036
2018	Wolf River (Highway CCC)	5	5	4	97.4	OL440173
2018	Wolf River (Highway M)	5	5	5	97.4	OL440175
2019	Wisconsin River (Kilbourn Hydroelectric Dam)	1	1	0	na	na
2019	Wolf River (Highway CCC)	10	10	10	99.4	OL440172
2019	Chippewa River (Jim Falls)	7	1[b]	1	97.4	OL440177
2019	Wisconsin River (Nekoosa Dam)	1	1	0	na	na
2019	Yellow River (Webster)	1	1	1	98.7	OL440178
2021	Wisconsin River (Kilbourn Hydroelectric Dam)	4	3[c]	3	98.7	OL440171
2021	Wisconsin River (Prairie du Sac Hydroelectric Dam)	13	13	13	97.4	OL440170
	Total	49	42	39		

[a]Skin lesion sample used to determine the LHSV genome sequence; [b]This tube included skin lesions collected from each fish as a pool; [c]One tube included skin lesions collected from 2 fish as a pool

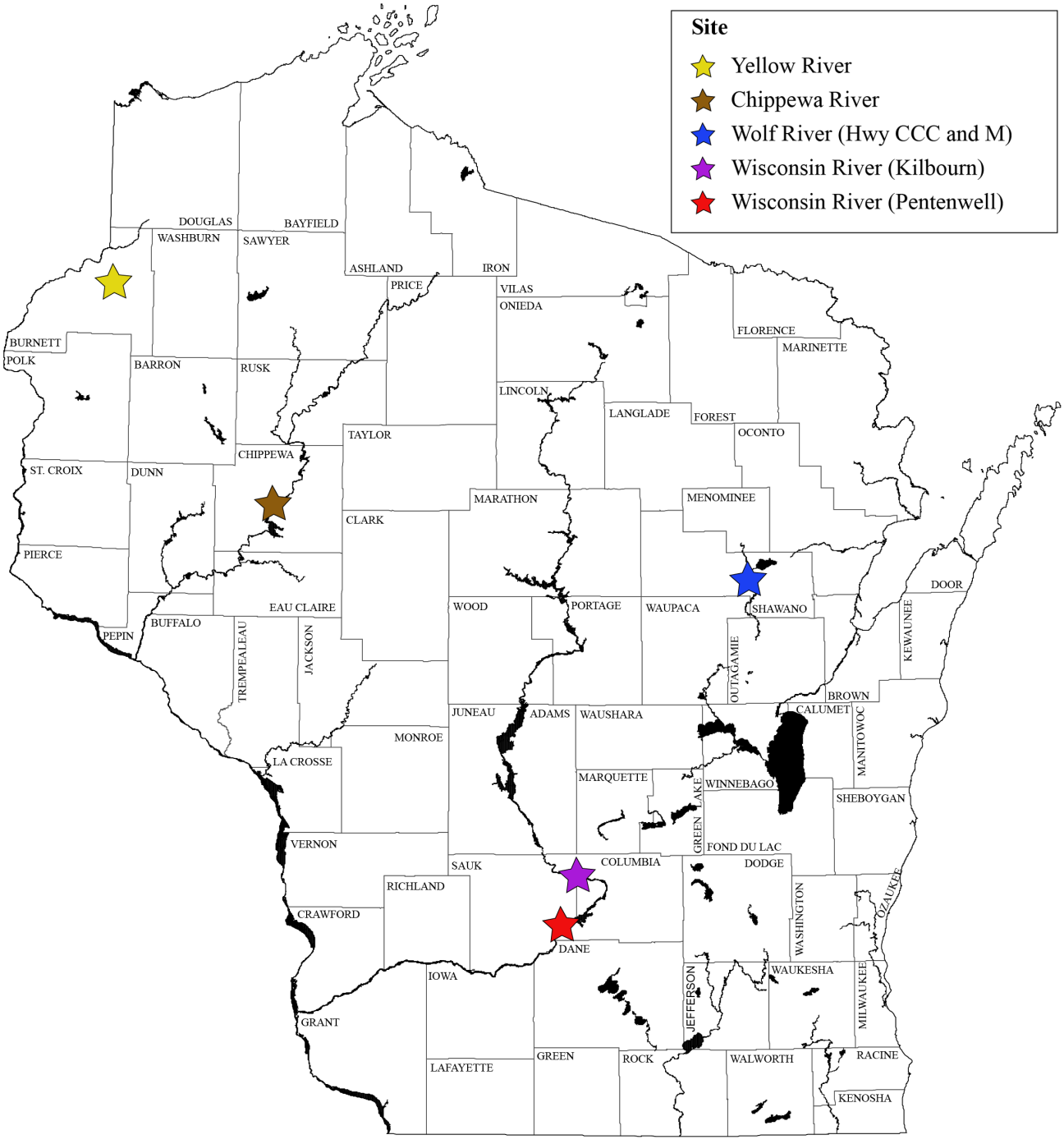

Fig. 1. Locations of positive detections of lake sturgeon herpesvirus in wild lake sturgeon from 4 Wisconsin rivers in 2017–2019 and 2021

3. RESULTS

3.1. Gross, microscopic, and ultrastructural analysis

LS sampled from various sites in Wisconsin during 2017–2019 and 2021 displayed similar skin and fin lesions, which were characterized as multifocal to coalescing, bilateral, flat, circular to irregular, smooth, well-demarcated, translucent grey to white, mucoid plaques with varying degrees of centralized circular focal grey-white opacities (pinpoint to entire; Fig. 2A). Microscopic examination of biopsied skin lesions from 2 Wolf River LS adults in 2017 revealed a prolif-

erative epidermitis resulting from hyperplasia of keratinocytes accompanied by infiltration of the epidermis by inflammatory cells and hydropic degeneration of keratinocytes (Fig. 2B,C). TEM revealed virus particles in 2 different developmental stages (Fig. 3) within the nucleus, including maturing forms (B-capsids) and more mature forms (C-capsids) displaying an electron-dense nucleoid. The mean diameter of the nucleocapsid was 85 nm (n = 20, SD = 9 nm) from opposite faces and 86 nm (n = 20, SD = 10 nm) from opposite vertices.

3.2. Degenerate PCR analysis

The degenerate PCR assay targeting the DNA polymerase catalytic subunit (*pol*) in large DNA viruses produced a 497 bp amplicon from both skin DNA samples tested. The amplicon sequences with primers trimmed were most closely related (82.1%; 389/474 nt) to an AciHV1 sequence (GenBank accession no. EF685905).

3.3. Genome sequence analysis

The LSHV genome sequence (202 660 bp; 46% G+C) was recovered from a dataset of 6 477 748 paired-end reads, of which 2 315 047 (36%) mapped to the genome with an average coverage depth of 3224 reads nt^{-1}. The genome consists of a long unique region (U_L; 198 615 bp) that is flanked by an inverted repeat (TR_L/IR_L; 241 bp), linked to a short unique region (U_S; 102 bp) that is also flanked by an inverted repeat (TR_S/IR_S; 1610 bp), with a further copy of TR_L at the right genome terminus (Fig. 4). The size of U_L, and therefore of the genome, is approximate because of the presence of 2 reiterated sequences of unresolved, and perhaps heterogeneous, sequence: one was set at minimum size and the other at size of the PCR product. The genome thus has the overall structure TR_L–U_L–IR_L–IR_S–U_S–TR_S–TR_L and is terminally redundant. This structure is reminiscent of the genome structures of some members of the family *Herpesviridae*, particularly those belonging to the

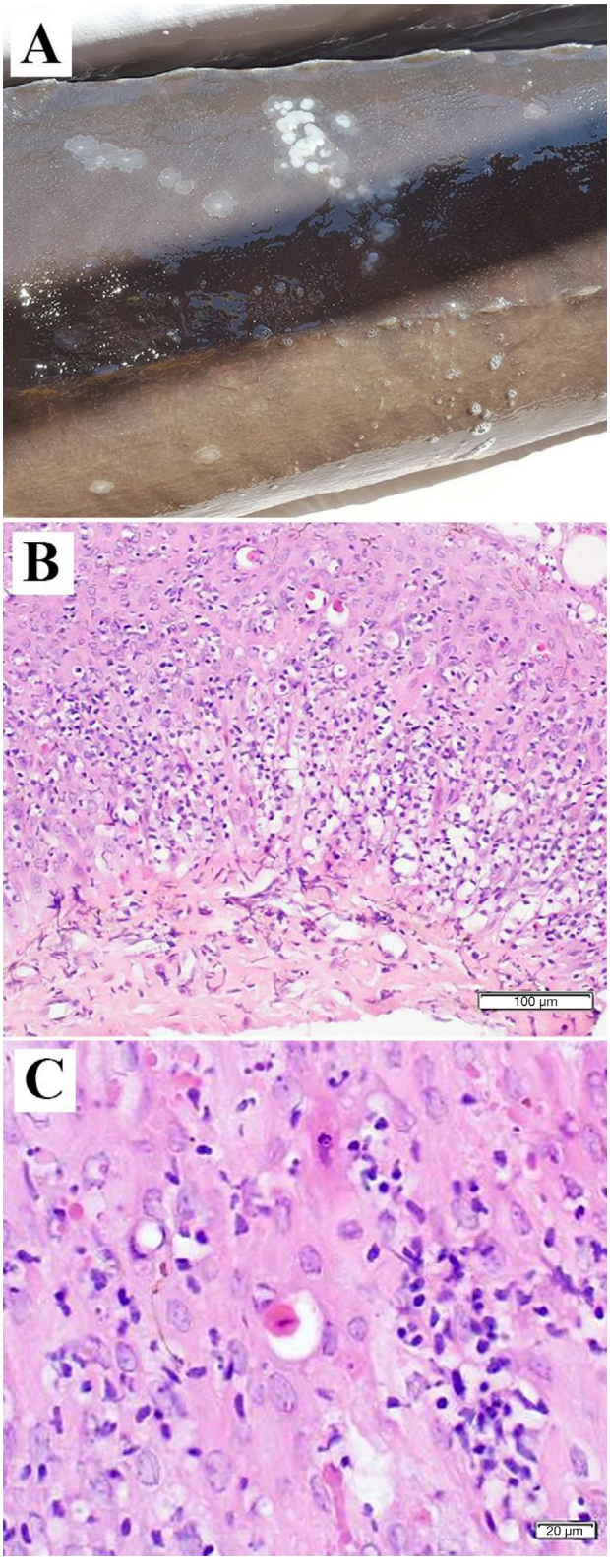

Fig. 2. Pathologic features of a skin lesion from lake sturgeon. (A) Gross appearance of a wild lake sturgeon displaying multiple cutaneous plaques. Photo credit: Ryan Koenigs. (B) Photomicrograph of affected skin illustrating proliferative epidermitis with marked hyperplasia of keratinocytes. Hematoxylin and eosin stain. Scale bar = 100 µm. (C) Photomicrograph at higher magnification illustrating inflammatory cell infiltrate of hyperplastic epidermis. Hematoxylin and eosin stain. Scale bar = 20 µm

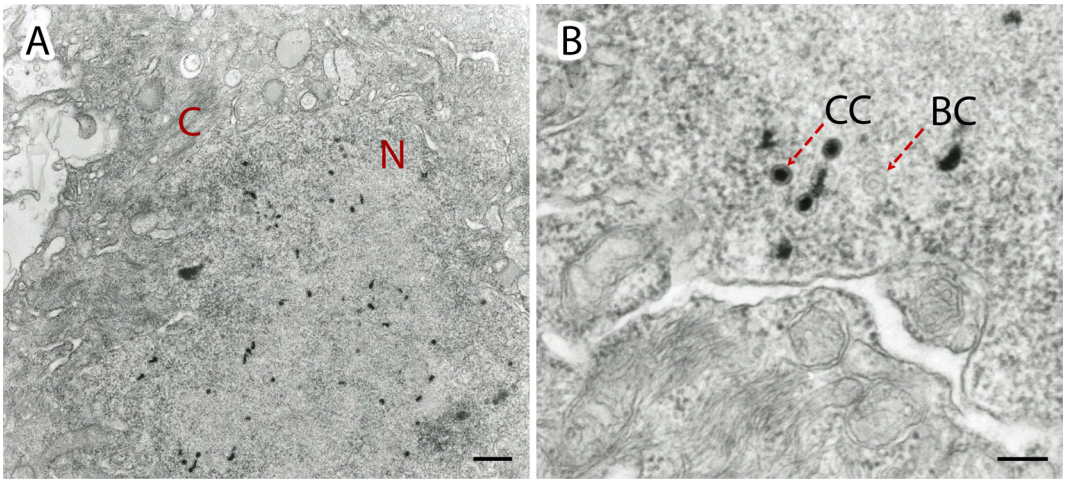

Fig. 3. Ultrastructural features of a skin lesion from lake sturgeon. (A) Photomicrograph of an infected cell in the epidermis illustrating the boundary between the nucleus (N) and cytoplasm (C). Scale bar = 500 nm. (B) Higher magnification photomicrograph showing B-capsids (BC) and C-capsids (CC) within the nucleus. Scale bar = 200 nm

genera *Simplexvirus* and *Cytomegalovirus* (Gatherer et al. 2021) and was presumably arrived at independently. Analysis of the sequence dataset produced no evidence for the presence of tandem copies of TR_L/IR_L at the ends of the genome or internally, or for the presence of the inverted form of U_L. It was not possible to determine whether the inverted form of U_S is present.

A total of 133 genes encoding functional proteins were predicted (Fig. 4; GenBank accession no. OK485036.1). These include the 12 core genes and 32 genes grouped into 6 families, each of which encodes a set of related proteins. The families are the protein kinase (PK; 6 members), ORF37 (3 members), immunoglobulin domain (IgD; 7 members), ORF77 (2 members bearing similarities to the spike glycoprotein of toroviruses), ORF81 (2 members), and ORF100 (12 members) families. Members of gene families, particularly the PK and IgD families, are not necessarily paralogous and may have been captured independently from the host. Several LSHV genes encode potential immune evasion proteins, including proteins with similarities to an inhibitor of apoptosis (ORF3), a tumor necrosis factor receptor (TNFR; ORF87), a TNFR-associated factor (ORF86), a US22 protein (ORF125), and the members of the IgD family.

3.4. Phylogenetic and genetic analyses

Three phylogenetic analyses of amino acid sequences were conducted using the same set of parameters (Fig. 5). The first (Fig. 5A) was based on a concatenation of the 12 core genes of 15 alloherpesviruses for which complete sequences were available, and the second (Fig. 5B) included 2 additional viruses (AciHV2 and ranid herpesvirus 4 [RaHV4]) for which complete sequences were lacking for only 1 gene (*ter1* and *pol*, respectively). These analyses produced well-resolved and well-supported phylogenies, with LSHV being most closely related to AciHV1. The closest relative to these 2 viruses is present in another sturgeon species, the sterlet *Acipenser ruthenus*. The clade of sturgeon alloherpesviruses is next most closely related in these analyses to the catfish viruses (genus *Ictalurivirus*). The third analysis was based on a concatenation of partial *pol* and *ter1* sequences for 23 alloherpesviruses, and therefore depended on far fewer aligned positions. This analysis placed the sturgeon viruses closer to the frog viruses (genus *Batrachovirus*) than to the catfish viruses (Fig. 5C), thus indicating their deep branching history.

3.5. LSHV-specific PCR assay

The PCR assay for part of exon 2 in the LSHV *ter1* gene (ORF19) produced the expected 196 bp amplicon for 39/42 skin lesion samples collected from wild LS captured in 4/4 rivers in Wisconsin during 2017–2019 and 2021 (Table 2). All but 1 LS sampled from the Wolf River locations tested positive. Fish collected from the Chippewa and Yellow Rivers tested positive in 2019. Two LS captured from dif-

ferent localities along the Wisconsin River tested negative in 2019, but all fish from the same river tested positive in 2021. The amplicon sequences with primers removed (156 bp) were 97.4–99.4% identical to the corresponding region of the LSHV genome.

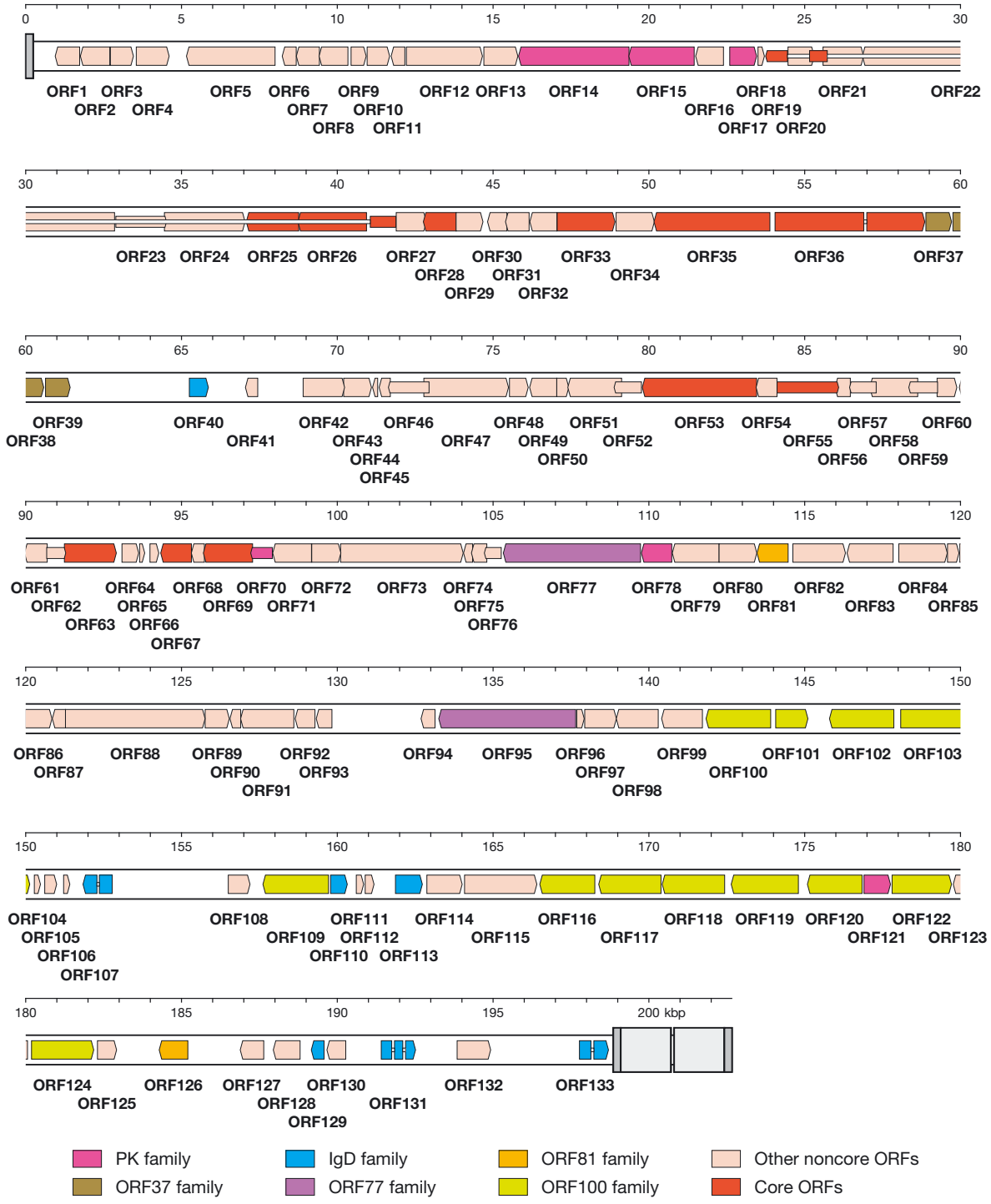

Fig. 4. Lake sturgeon herpesvirus (LHSV) genome map. The inverted repeats (TR_L/IR_L and IR_S/TR_S) are shown in a thicker format than the unique regions (U_L and U_S), with U_L and U_S shaded white, TR_L/IR_L shaded dark gray, and IR_S/TR_S shaded light gray. Predicted functional open reading frames (ORFs) are indicated by open arrows colored according to the key, with names indicated below. Some ORFs are shown by narrow arrows to make their locations clearer. Introns connecting ORFs are shown as narrow white bars

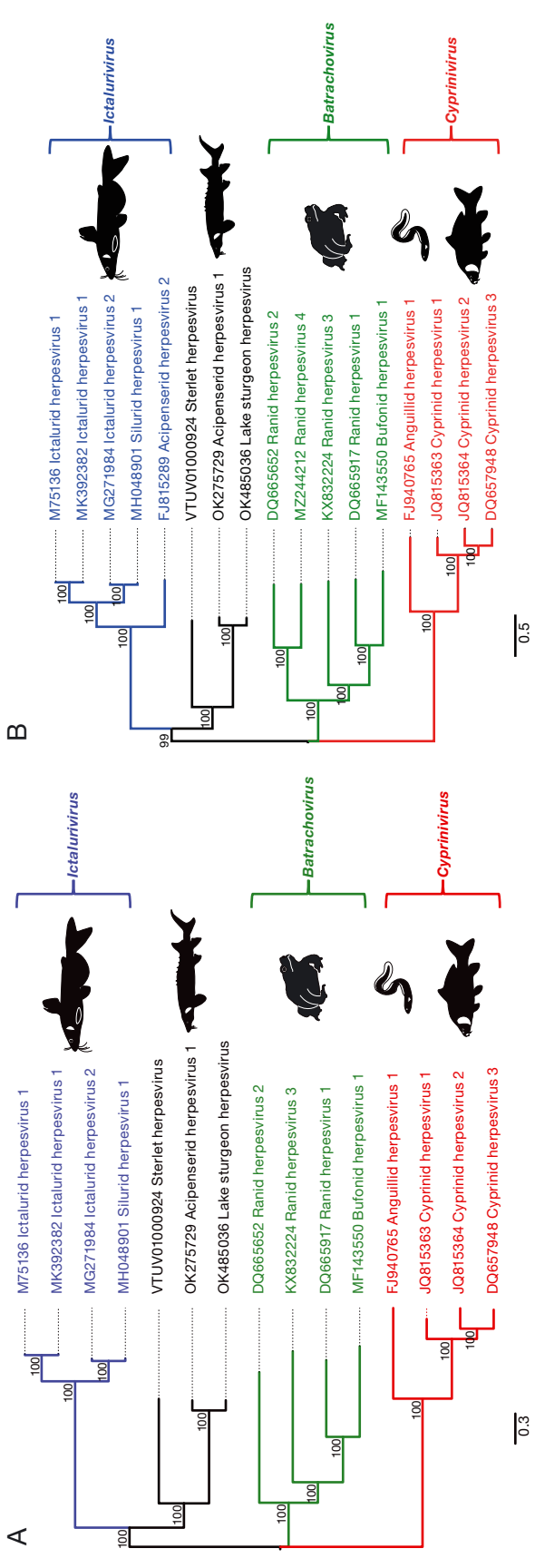

Fig. 5. Alloherpesvirus phylogeny. Maximum likelihood trees of the amino acid sequences listed in Table S1 are shown for (A) 15 viruses for which the complete sequences of the 12 core genes were available, (B) these 15 viruses plus 2 others for which the sequence of 1 gene in each was incomplete, and (C) 23 viruses for which partial sequences of the *pol* and *ter1* genes were available. The viruses are indicated by GenBank accession no. and name, and the genera to which they belong or potentially belong are indicated on the right. It is not known if the sterlet herpesvirus and ranid herpesvirus 4 sequences represent replicating viruses or integrated virus genomes. Bootstrap values are shown at the nodes as a fraction of 1. Scale bar = substitutions per site. The statistics were (A) log likelihood value = −199 189.69, G = 1.8933, I = 0.0363, and positions analyzed in the alignment = 7598; (B) log likelihood value = −226 080.98, G = 1.7967, I = 0.0354, and positions analyzed = 7546; and (C) log likelihood value = −7702.64, G = 0.7547, I = 0.0063, and positions analyzed in the alignment = 282

4. DISCUSSION

The present study is the first report of an alloherpesvirus in LS. Infected wild adult LS exhibited cutaneous plaques that, upon histopathological examination, revealed hyperplasia of keratinocytes and hydropic degeneration, similar to the microscopic lesions reported previously for AciHV1 infections in farmed WS in California (Hedrick et al. 1991) (Figs. 2 & 3). Overall, AciHV1 and LSHV are similar in observed pathology and virion ultrastructure and morphogenesis. Phylogenetic analyses, facilitated by determination and annotation of the complete genome sequence of LSHV (Fig. 4), demonstrated that these viruses are also closely related genetically (Fig. 5). Although the criteria for creating genera and species in the family *Alloherpesviridae* are not well established, these analyses underscore the distinctiveness of LSHV and AciHV1 from each other and from other alloherpesviruses. The 2 most closely related viruses currently classified into different species are CyHV2 and CyHV3, and it may prove possible to make a case for the classification of AciHV1 and LSHV, which are more closely related, into 2 species within a new genus, particularly since they infect different sturgeon species.

Included in the phylogenetic analyses were alloherpesvirus sequences present in a 182 248 bp contig generated from genome data for the sterlet (Du et al. 2020), although these required annotation in the present study. However, it is not known whether these sequences represent a replicating virus or an integrated virus genome in this sturgeon species. The latter possibility may be the most likely, as the sequences were detected in 3 independent studies (International Nucleotide Sequence Database Collaboration BioProjects PRJNA561280, PRJNA491785, and PRJEB35912) and, more generally, alloherpesvirus sequences that are integrated or potentially integrated into fish genomes have been reported previously (Inoue et al. 2017). Nonetheless, these observations suggest that the association between LSHV-like viruses and their sturgeon hosts is ancient. Also included in the phylogenetic analyses were alloherpesvirus sequences present in 6 contigs generated from transcriptome data for the Chinese tiger frog *Hoplobatrachus rugulosus* (Chen et al. 2021), although these required improvement in the present study. Again, it is not clear whether these sequences represent an infectious virus or an integrated virus genome.

Herpesviruses have typically coevolved with their hosts, although exceptions have been noted among members of the families *Herpesviridae* (McGeoch et al. 2006) and *Alloherpesviridae* (Kelley et al. 2005, Waltzek et al. 2009). Thus, the phylogeny of alloherpesviruses in relation to that of their hosts does not support coevolution at deeper branches of the tree (Waltzek et al. 2009) but does support it to a degree at the tips, with the catfish, salmon, carp, and frog alloherpesviruses segregating in separate genera (*Ictalurivirus*, *Salmonivirus*, *Cyprinivirus*, and *Batrochovirus*, respectively), as confirmed in the present study (Fig. 5). However, the sturgeon alloherpesviruses are not closely related, as AciHV2 is the sister of *Ictalurivirus* and does not group with the other sturgeon alloherpesviruses. These findings are in accord with earlier studies, based on comparative analyses of shorter sequences (Doszpoly et al. 2008, 2011a, Waltzek et al. 2009) showing that the reported alloherpesviruses of catfish and sturgeon (specifically AciHV2) have a close evolutionary relationship, despite the fact that their host species are genetically very distant from each other (Doszpoly et al. 2011b). Thus, we speculate that AciHV1 and LSHV have coevolved with sturgeon over long periods of time and that AciHV2 is the result of a more recent interspecies transmission from a catfish or similar host.

In the spring of 2017–2019 and 2021, epidermal lesions on the skin and fins were observed in male and female LS adults in several Wisconsin rivers. To date, these lesions have only been observed in adult LS, anecdotally more often in females, during spawning (D. Godard pers. obs.). Similar seasonal proliferative lesions with a presumptive or proven alloherpesvirus etiology occur in other wild fish when they become immunosuppressed as a result of cooler water temperatures experienced in late fall, winter, or early spring months (Anders & Yoshimizu 1994). CyHV1, the causative agent of the proliferative skin disease known as carp pox, occurs seasonally in managed (e.g. outdoor koi) and wild common carp (*Cyprinus carpio*) populations as water temperatures decrease below 20°C (Wolf 1988, Viadanna et al. 2017). When water temperatures increase seasonally or through anthropogenic manipulation, it is thought that immune function rebounds and the proliferative skin lesions resolve (Morita & Sano 1990, Sano et al. 1993, Viadanna et al. 2017). Alloherpesviruses associated with proliferative cutaneous lesions observed during spring spawning have been reported in European smelt *Osmerus eperlanus* (Anders & Möller 1985), walleye *Sander vitreus* (Bowser et al. 1988), rainbow smelt *O. mordax* (Herman et al. 1997), northern pike (Freitas et al. 2016), and European perch (Garver et al. 2018). Among

these viruses, only the genome of CyHV1 has been completely sequenced, and it was predicted to encode numerous immune evasion proteins and a gene family specifying potential JunB oncoproteins (Davison et al. 2013). Although LSHV does not encode a predicted oncoprotein, it does encode several proteins with potential immune evasion functions. Future functional studies are needed to define how these and other proteins influence the manifestation of the proliferative skin lesions associated with LSHV. Beyond potential environmental or virus-induced immunosuppression, the physical, metabolic, and endocrinologic stressors of spawning likely influence host–virus dynamics in favor of virus replication and virus transmission during spawning congregations (Anders & Yoshimizu 1994).

AciHV1 has been isolated and shown to be the etiologic agent responsible for significant losses in hatchery-reared WS fry and fingerlings in California (Hedrick et al. 1991). In the case of LSHV, virus particles were visualized in infected epidermal cells; however, attempts were not made to isolate the virus in cell culture, and thus, its pathogenic properties remain to be demonstrated. Infection of LSHV in adult LS appeared to cause cutaneous lesions that were nonfatal; however, the effect on internal organs and the effect on other, e.g. younger, age classes is undetermined. Isolation of LSHV would facilitate the challenge studies needed to fulfil Koch's postulates, as part of the process of elucidating the disease potential of this virus in hatchery-reared fry and fingerlings and wild adult LS. The LSHV genome sequence generated in this study permitted the development of a conventional PCR assay for LSHV for use as a rapid screening tool. Future efforts are needed to optimize this and other molecular tools (e.g. quantitative PCR, *in situ* hybridization, and virus-specific antibodies) for use in determining the prevalence and pathogenicity of LSHV in wild and managed LS stocks.

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