# A new species of nucleo-cytoplasmic large DNA virus (NCLDV) associated with mortalities in Manitoba lake sturgeon *Acipenser fulvescens*

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ABSTRACT: A newly discovered virus, Namao virus, associated with morbidity and mortality, was detected among juvenile lake sturgeon *Acipenser fulvescens* being propagated by a conservation stocking program for this endangered species in Manitoba, Canada. The outbreaks resulted in cumulative mortalities of 62 to 99.6 % among progeny of wild Winnipeg River or Nelson River lake sturgeon and occurred at 2 geographically separate facilities. Namao virus was detected in almost 94 % of the moribund or dead lake sturgeon according to a conventional polymerase chain reaction (cPCR) test that is based upon amplification of a 219 bp fragment of the virus major capsid protein (MCP). The virus itself was large (242 to 282 nm) and icosahedral-shaped with 2 capsids and a condensed bar-shaped core. It was found in virus factories within the host cell cytoplasm and displayed a tropism for the integument. Namao virus caused cellular changes characterized by enlarged eosinophilic epithelial cells in the gills and skin. Samples suspected of containing Namao virus did not have cytopathic effects on primary lake sturgeon or established white sturgeon cell lines. However, viral nucleic acid was detected in the former after prolonged incubation periods. Using primers designed from conserved regions of the MCP from NCLDVs, an estimated 95 to 96 % of the Namao virus MCP open reading frame was captured. Phylogenetic analysis using the MCP of Namao virus and 27 other NCLDVs suggested that Namao virus and white sturgeon iridovirus share a common evolutionary past and might be members of the family *Mimiviridae* or a new, as yet unrecognized, virus family.

KEY WORDS: Namao virus · *Mimiviridae* · Endangered species

## INTRODUCTION

Viruses classified within the families *Mimiviridae*, *Phycodnaviridae* and *Iridoviridae* as well as the Marseillevirus and megaviruses are a ubiquitous component of the aquatic environment (Chen et al. 1996, Fuhrman 1999, Monier et al. 2008, Van Etten et al. 2010). Along with members of the families *Poxviridae* and *Ascoviridae,* they form a putative monophyletic class of viruses referred to as nucleo-cytoplasmic large DNA viruses (NCLDVs; Table 1) (Iyer et al. 2001, 2006, Koonin & Yutin 2010). A common ancestral virus is suggested to have possessed a set of at least 47 conserved genes that can be mapped onto the genomes of extant NCLDVs (Koonin & Yutin 2010). As a group, they share 5 common protein-

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Table 1. Members of the nucleo-cytoplasmic large DNA virus (NCLDV) super-family

| Virus taxon | Host range | Genome size (kb) | Capsid diameter (nm) |
|---|---|---|---|
| *Iridoviridae* | Insects, cold blooded vertebrates | 100–220 | 120–350 |
| *Ascoviridae* | Insects, mainly Noctuids | 150–190 | 130 |
| *Asfarviridae* | Mammals | 170 | 170–190 |
| *Poxviridae* | Insects, reptiles, birds, mammals | 130–380 | 200 |
| *Phycodnaviridae* | Green algae; algal symbionts of paramecia & hydras | 150–400 | 130–200 |
| Marseillevirus | Amoeba | 370 | 250 |
| *Mimiviridae* | | | |
| CroV | Marine microzooplankton | 730 | 300 |
| *Mimivirus* | Amoeba | 1181 | 390–400 |
| Mamavirus | Amoeba | 1191 | Not available |
| Megaviruses | Unknown | 1259 | 440 |
| Sturgeon viruses | | | |
| Namao virus (NV) | Lake sturgeon | Unknown | 225–263 |
| White sturgeon iridovirus (WSIV) | White sturgeon | Unknown | 262 |
| Missouri River sturgeon iridovirus (MRSIV) | Pallid and shovelnose sturgeon | Unknown | 254 |
| Russian sturgeon iridovirus (RSIV) | Russian sturgeon | Unknown | 283 |

coding sequences (CDSs), including the major capsid protein (MCP), with an additional 177 shared by 2 or more families within the NCDLV super-family (Yutin et al. 2009).

NCLDVs replicate in the cytoplasm of the host cell or start their life cycle in the host nucleus and complete it in the cytoplasm (Iyer et al. 2001, 2006, Koonin & Yutin 2010). They share common biophysical characteristics such as complex, isometric virion structures (120 to 440 nm) and very large double-stranded DNA genomes (0.1 to 1.3 Mb) (Table 1). The number of CDSs of many NCLDVs exceeds that of the smallest free-living bacteria *Mycoplasma genitalium* (~470 CDS, Claverie et al. 2006). The large repertoire of genes encoding proteins involved in DNA replication, processing, maturation and packaging, transcription initiation and elongation as well as capsid proteins and chaperones for assembly provide NCLDVs with relative autonomy from the host cell. It is perhaps not surprising that they can have diverse, complex life-cycles and a broad host range that includes amoeba, algae, insects, reptiles, birds, mammals, and fish (Van Etten et al. 2010, Van Etten 2011).

Several NCLDVs of sturgeon (Acipenseridae) hitherto recognized as unclassified members of the family *Iridoviridae*, pose a potential disease risk for strategies designed to aid the recovery of threatened sturgeon populations in North America. These include the white sturgeon iridovirus (WSIV), found in wild and cultured white sturgeon *Acipenser transmontanus* (Hedrick et al. 1990, LaPatra et al. 1994, Raverty et al. 2003), as well as the Missouri River sturgeon iridovirus (MRSIV), which has been isolated from pallid sturgeon *Scaphirhynchus albus* and shovelnose sturgeon *S. platorynchus* (Kurobe et al. 2010, 2011).

Sturgeon NCLDVs can assume a complex lifecycle. In some instances they cause upwards of 95% mortality within captive populations whereas in others they are associated with a chronic debilitating wasting syndrome, resulting in severely impaired growth and reduced survival of fry and fingerlings (Hedrick et al. 1990, LaPatra et al. 1994, Kurobe et al. 2011). Outbreaks of viral disease in white sturgeon as well as pallid or shovelnose sturgeon are typically observed during the first year of growth in hatcheries (Hedrick et al. 1990, 1992, Kurobe et al. 2011). Adults can be refractory to clinical disease, but are likely subclinical carriers that may intermittently shed virus (Hedrick et al. 1990, LaPatra et al. 1994, Kurobe et al. 2011). Horizontal transmission of WSIV and MRSIV from convalescent to naïve juvenile sturgeon has been demonstrated (Hedrick et al. 1990, Drennan et al. 2006, Kurobe et al. 2011) and there are suggestions that egg-associated transmission from adult to offspring occurs during spawning (Hedrick et al. 1992, LaPatra et al. 1994, Georgiadis et al. 2001). Handling, stocking density and fluctuations in water levels, flow rates, and/or temperature are among the other risk factors known to contribute to the onset and severity of these disease outbreaks (LaPatra et al. 1994, 1996, Watson et al. 1998a, Georgiadis et al. 2000, 2001, Drennan et al. 2005, 2006).

WSIV and MRSIV have historically been diagnosed using a combination of histology and electron microscopy. Both viruses localize to the integument of the oropharynx and olfactory organs as well as the

fins and in some cases the epithelium of the gills (Hedrick et al. 1990, Watson et al. 1998b, Drennan et al. 2007, Kurobe et al. 2011). Microscopic signs of infection include hypertrophied, basophilic or amphophilic- to eosinophilic-staining cells and eccentric nuclei, as well as inclusion bodies containing isometric, doubly encapsidated virus particles displaying a condensed bar-shaped nucleoid (Hedrick et al. 1990, 1992, Kurobe et al. 2011). A number of white sturgeon cell lines have been developed that are permissive to WSIV amplification but in an isolate-specific manner and sometimes without evidence of cytopathic effect (CPE) (Hedrick et al. 1991, Watson et al. 1998b). These as well as pallid and shovelnose-derived cell lines do not support the growth of MRSIV (Kurobe et al. 2011). More recently, conven-

tional (cPCR) and/or quantitative PCR (qPCR) assays have been developed to detect WSIV or MRSIV. The current white sturgeon end-point or qPCR assays target viral DNA encoding the MCP (Kwak 2006, Kwak et al. 2006) which is one of the 5 orthologous DNA sequences common to NCLDVs (Yutin et al. 2009), while the MRSIV qPCR assay is based on amplification of DNA encoding the serpin protein (Kurobe et al. 2010). Neither of these assays is capable of detecting viral DNA from the aforementioned NCLDV found in Manitoba lake sturgeon.

In this paper we provide the first description of a new NCLDV found in populations of Manitoba (MB) lake sturgeon *Acipenser fulvescens*, a species classified as endangered by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2011). Further, we provide evidence of a new NCLDV lineage comprised of these sturgeon viruses and discuss their phylogenetic relationship to other viruses in the NCLDV clade.

## MATERIALS AND METHODS

### Case history

Unusual mortalities occurred in groups of juvenile lake sturgeon reared at the Grand Rapids Hatchery (GRH) (Grand Rapids, MB) and the University of Manitoba Aquatic Animal Holding Facility (UMAAHF) (Winnipeg, MB) (Fig. 1) in 2009 and 2010.

The lake sturgeon reared at UMAAHF were derived from multiple crosses of eggs and sperm collected from sexually mature, wild Winnipeg River (MB) lake sturgeon (16 males, 8 females) captured at Slave Falls in May 2008. The fertilized eggs were hatched at the temporary Pinawa Hatchery (Pinawa, MB) and the fry (15 to 20 mm in length) were subsequently transported on June 12, 2008 to UMAAHF. Tissue samples were collected for diagnostic evaluation on December 7, 9, 11 and 17, 2008 from 7 apparently healthy lake sturgeon housed in 6 different tanks. On December 22, 2008, after a series of transfers to successively larger tanks, approximately 1758 lake sturgeon (mean body weight 5.7 g, length not recorded) were placed in four
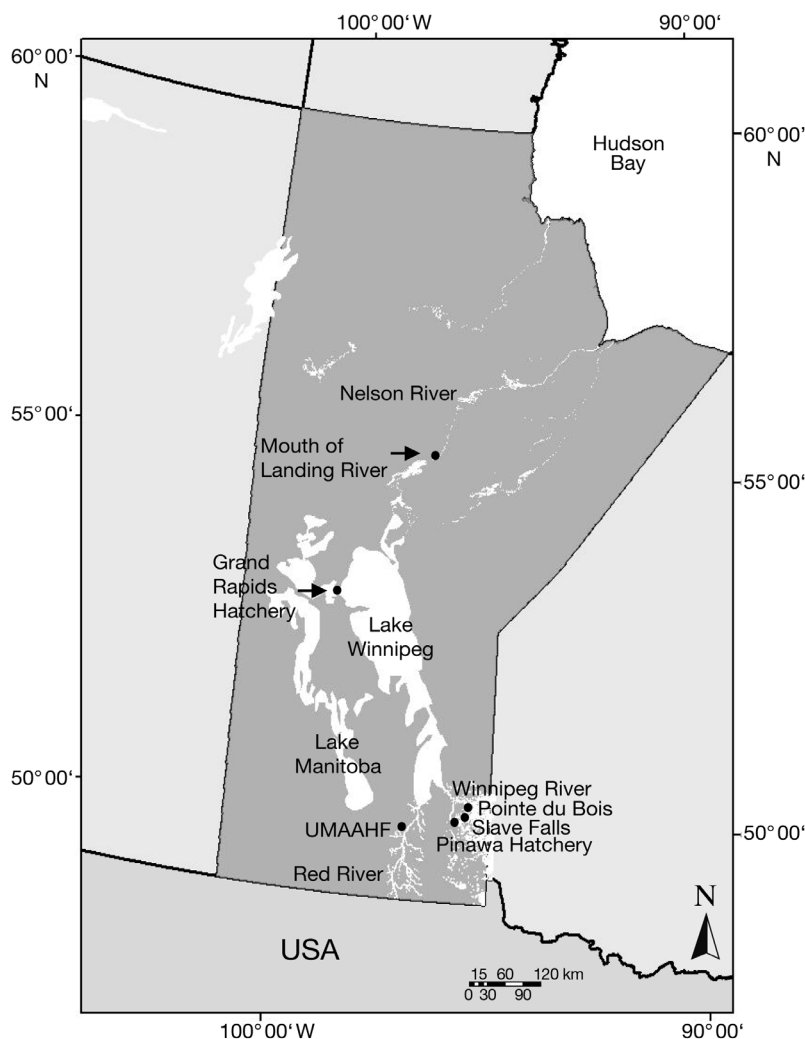


Fig. 1. Location of rivers in Manitoba (dark grey shading), Canada where wild lake sturgeon *Acipenser fulvescens* broodstock were collected and hatcheries where progeny were held. UMAAHF: University of Manitoba Aquatic Animal Holding Facility

600 gallon tanks receiving de-chlorinated 16°C municipal water at 1 to 2 l min$^{-1}$. In February 2009, mortalities occurred amongst 606 lake sturgeon (mean body weight 3.0 g, cumulative percent mortality [CPM] 64%) held in Tank 600-1 (Fig. 2A). Then in May 2009, mortalities were observed in Tanks 600-3, 600-4 and 600-5 stocked with 569, 195 and 388 lake sturgeon (mean body weight 19.3 g, mean fork length 174 mm), respectively. CPM in these 3 tanks ranged from 62 to 99%. Tissue samples from dead or moribund fish were submitted for diagnostic evaluation.

The GRH received fertilized eggs derived from wild Nelson River (MB) lake sturgeon (6 males, 4 females) on June 15, 2009 and fry from wild Winnipeg River (Pointe du Bois) lake sturgeon (17 males, 5 fe-
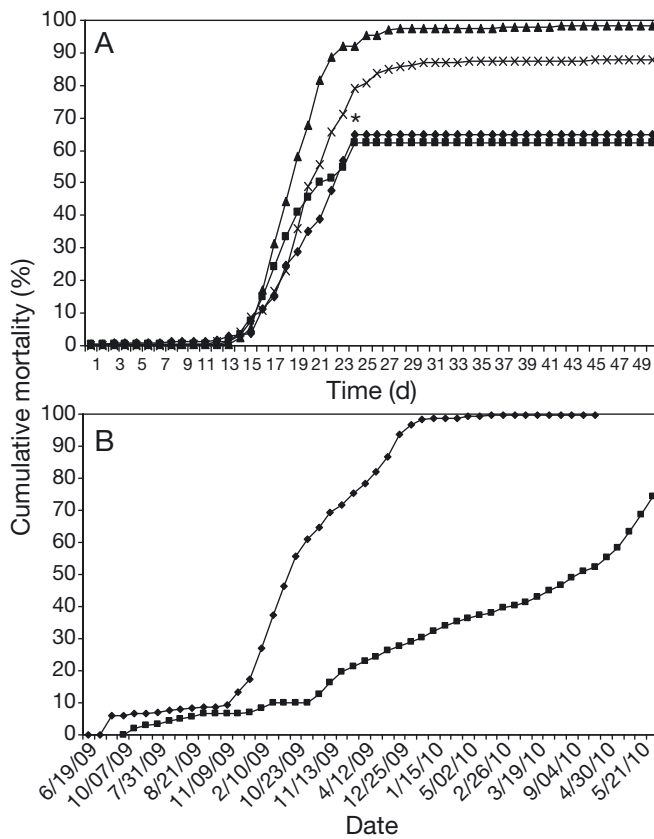


Fig. 2. *Acipenser fulvescens*. Cumulative mortality among juvenile lake sturgeon. (A) Sturgeon held at University of Manitoba Aquatic Animal Holding Facility: progeny (2008 year class) from wild Winnipeg River broodstock collected at Slave Falls. Time zero was normalized to 16 d prior to onset of significant mortality within each of 4 tanks: (♦) Tank 600-1, Day 16 = Feb 11, 2009; (■) Tank 600-3, Day 16 = May 9, 2009; (▲) Tank 600-4, Day 16 = May 27, 2009; (×) Tank 600-5, Day 16 = May 12, 2009. (⋆) Date when fish remaining in tanks 600-1 and 600-3 were removed. (B) Sturgeon held at Grand Rapids Hatchery: progeny (2009 year class) from (♦) feral Nelson River broodstock collected near Landing River and (■) Winnipeg River broodstock collected at Pointe du Bois

males) on July 9, 2009. The 2 populations of fish were held separately in long, shallow troughs (L × W × D ≈ 3.7 × 5.2 × 3.1 m) receiving untreated, ambient temperature surface water from the Saskatchewan River (Cross Bay, Cedar Lake) (average temperature 10.1°C June through September, 4.9°C October through May). Mortalities began to increase among approximately 1974 Nelson River lake sturgeon (Nov 4, 2009: mean body weight 17.3 g, mean fork length 15.5 cm) in late September 2009 and reached CPM of 99.6% by February 19, 2010 (Fig. 2B). Similarly, mortalities began to increase among approximately 14 608 Winnipeg River lake sturgeon (mean body weight 11 g, mean fork length 13.9 cm) in early November 2009 and reached a CPM of 74% on May 28, 2010 at which time the remaining fish were euthanized. Tissue samples were submitted from each group of lake sturgeon for diagnostic evaluation.

The UMAAHF also received fry derived from the wild Winnipeg River (Pointe du Bois) lake sturgeon broodstock described in the previous paragraph. The fish were received on October 8, 2009 and held in 600 gallon tanks receiving dechlorinated 16°C municipal water at 1 to 2 l min$^{-1}$. Sporadic mortality occurred in these tanks throughout 2010 (body weight range 11 to 32 g, lengths not recorded) and tissue samples from dead or moribund fish were submitted for diagnostic evaluation.

## Bacteriology, virology, histology and electron microscopy

Gill, spleen and kidney tissues from a small number of dead or moribund juvenile lake sturgeon collected during the mortality events at UMAAHF (n = 10, Winnipeg River stock) or GRH (n = 15; 10 Winnipeg River stock, 5 Nelson River stock) were tested for the presence of bacteria by inoculation of tryptic soy agar (TSA) supplemented with 5% sheep blood and MacConkey agar followed by incubation at 22°C and 35°C under aerobic as well as anaerobic conditions. Gill tissue was also used to inoculate chocolate agar as well as TSA containing 5% sheep blood followed by incubation in a $CO_2$ candle jar at 22°C and 35°C. Further, skin, gill and intestinal wet mounts as well as Gram stains of kidney and gill smears were performed on samples from 8 fish from UMAAHF (Winnipeg River stock) and 15 fish from GRH (10 Winnipeg River stock, 5 Nelson River stock).

Barbells, gill arches, operculum, head skin, anal and/or pectoral fins as well as kidney, liver, spleen and/or pyloric caeca tissues were aseptically collected

from 33 Winnipeg River and 15 Nelson River lake sturgeon and held at 4°C prior to analyses by virology on epithelioma papulosum cyprini (EPC), Chinook salmon embryo 214 (CHSE-214), white sturgeon skin (WSSK-1), white sturgeon spleen (WSS-2), lake sturgeon gill (LSGI) and/or lake sturgeon gonad (LSGO) cell lines (Fijan et al. 1983, Lannan et al. 1984, Hedrick et al. 1991). Some of these samples were also submitted to the British Columbia Freshwater Fisheries Society (BCFFS) (Duncan, British Columbia). Each cell line was propagated at 16°C in minimum essential medium with Earle's salts (MEM-E) or Hanks salts (MEM-H) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Life Technologies). Replicate wells of a 24-well tissue culture plate containing cell monolayers were inoculated with 0.1 ml of a 1:50 dilution of clarified homogenate generated from pools of tissue from a single fish. After an absorption period of 1 h, 1 ml of MEM-E or MEM-H supplemented with 2% FBS (MEM-E2, MEM-H2), 2 mM L-glutamine and 1× antibiotic/antimycotic (Life Technologies) was added to each well of the plate, the plate was incubated at 16°C and the monolayers were observed daily for evidence of CPE. At the time of harvest, each inoculated monolayer was scraped from the bottom of the well into the culture fluid and the cell suspension was transferred to a storage vial and placed at −80°C.

In preparation for histological examination, an incision was made in the abdomen of 9 moribund lake sturgeon from the Winnipeg River stock (5 from GRH, 4 from UMAAHF) and 5 from the Nelson River stock and whole fish were placed in 10% buffered formalin. After 24 to 48 h, portions of the fixed tissues were processed for light microscopy into paraffin embedded blocks, sectioned at 5 µm, deparaffinized and then stained with hematoxylin and eosin (H&E).

For transmission electron microscopy (TEM), formalin-fixed gill tissue from a single juvenile Winnipeg River lake sturgeon (MAAHF) was rinsed in buffer and post-fixed in 1% aqueous osmium tetroxide, dehydrated through a graded ethanol series, infiltrated and then embedded in Araldite 502/Embed 812 resin. Thin sections (about 100 nm) were stained with uranyl acetate and lead citrate prior to being examined using a Philips CM 120 transmission electron microscope at 80 kV. Digital imaging was performed with an AMT XR-611 camera system.

## Establishment of lake sturgeon cell lines

Gill and gonad tissues were aseptically removed from 2 juvenile lake sturgeon (52.5 and 62.7 g),

washed 5 times with sterile phosphate buffered saline (PBS), minced into smaller pieces and then resuspended in 0.25% porcine trypsin/EDTA (HyClone). Cells were dispersed from the tissue fragments by mechanical agitation for 20 min at 22°C. The resulting cell suspension was diluted 1:1 (v/v) with Dulbecco's Modified Eagles Medium (DMEM)/Ham's F-12 supplemented with 10% FBS, 200 IU penicillin and 200 µg streptomycin ml$^{-1}$ (Life Technologies), centrifuged at 1228 × $g$ for 15 min at 4°C and then resuspended in fresh media prior to being transferred to 25 cm$^2$ flasks. The lake sturgeon cell monolayers were propagated at 16°C in MEM-H supplemented with 10% FBS, 2 mM L-glutamine and 2× antibiotic/antimycotic (Life Technologies).

## DNA synthesis and plasmid purification

DNA representing the gene encoding the WSIV MCP (Genbank accession number DQ897645) was synthesized and ligated into the cloning vector pJ204 (DNA2.0). The construct was then transformed into *Escherichia coli* DH5α competent cells and plasmid DNA was purified with a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

## DNA extraction

Tissue samples including ventral head skin, abdominal skin, gill arches, barbells, operculum, kidney, liver as well as pectoral and anal fins were collected from dead, moribund or frozen lake sturgeon from mortality events occurring at GRH and UMAAHF. Homogenates of 50 mg portions of these tissues were generated in lysis buffer (Qiagen buffer ATL) using a 5 mm stainless steel bead (Qiagen) and TissueLyser (Qiagen) operated twice for 2 min at 30 Hz. DNA was extracted from the tissue homogenates using the DNeasy Blood and Tissue Kit (Qiagen) as outlined by the manufacturer and then quantified using the Nanodrop 8000.

## PCR amplification, cloning and DNA sequence analysis

DNA samples were tested for the presence of WSIV and MRSIV using qPCR protocols described by Kwak (2006) and Kurobe et al. (2010), respectively. Samples were also submitted to the Animal Health Cen-

ter (AHC) in Abbotsford, BC for additional molecular testing.

Primers targeting the Namao virus MCP were designed based on conserved regions identified from sequence alignments of the MCP from representative members of the NCLDV family of viruses (Table 2). Portions of the Namao virus MCP gene were amplified by cPCR using pairwise combinations of the primers listed in Table 3 and DNA was extracted from tissue samples collected from populations of lake sturgeon experiencing mortality at either GRH or UMAAHF. The origin of the amplicon was tested using DNA from Namao virus-infected

Table 3. Oligonucleotides used in the present study for amplification of DNA encoding lake sturgeon Namao virus major capsid protein (partial). Nucleotide positions are shown relative to white sturgeon iridovirus major capsid protein coding region (GenBank accession no. DQ897645)

| Primer | Sequence (5′ to 3′) | Nucleotide position (bp) |
|---|---|---|
| speF | CCA GAA ATG ACT TAC TTC AAG | 64 |
| ginF | GGT ATC AAC GTA TAT TCG TTT GC | 1369 |
| ginR | GCA AAC GAA TAT ACG TTG ATA CC | 1391 |
| pekR | CCA GAA GGT TGG TGC TTT TCA GG | 1421 |
| glaR | GTA TGC CAG ACC CGC TAG ACC | 1587 |

Table 2. Major capsid protein of representative members of the nucleo-cytoplasmic large DNA virus (NCLDV) superfamily. Capsid NCLDV domains were identified by the Conserved Domain Database (see 'Materials and methods')

| Virus | Abbre-viation | Gene (bp) | Protein (aa) | NCLDV domains | Accession number DNA | Protein | Reference |
|---|---|---|---|---|---|---|---|
| **Unclassified mimivirus-like** | | | | | | | |
| Namao virus | NV | 1524 partial | 508 partial | 2 | JX155659 | | Present study |
| White sturgeon iridovirus | WSIV | 1596 | 531 | 2 | DQ897645 | ABK34555.1 | Direct submission |
| **Mimiviridae** | | | | | | | |
| Acanthamoeba polyphaga mimivirus (Bradford isolate) | APMV-1 | 1782 | 593 | 2 | NC 014649.1 | YP003986929 | Legendre et al. (2011) |
| Acanthamoeba polyphaga mimivirus (M4 isolate) | APMV-2 | 1776 | 591 | 2 | JN036606.1 | AEJ34665.1 | Boyer et al. (2011) |
| *Cafeteria roenbergensis* virus | CroV | 1521 | 506 | 2 | NC 014637.1 | YP003969975.1 | Fischer et al. (2010) |
| Megavirus chiliensis | MVChile | 1770 | 589 | 2 | NC 016072.1 | YP004894515.1 | Arsian et al. (2011) |
| Megavirus courdo | MVCour | 1698 | 565 | 2 | JN885991.1 | AEX61606 | Direct submission |
| *Acanthamoeba castellanii* mamavirus | ACMV | 1776 | 591 | 1 | JF801956.1 | AEQ60611.1 | Colson et al. (2011) |
| **Phycodnaviridae** | | | | | | | |
| Organic lake phycodnavirus 2 | OLPV-2 | 1611 | 536 | 1 | HQ704803.1 | ADX06358 | Yau et al. (2011) |
| *Phaeocystis pouchetii* virus | PPV | 1563 | 520 | 1 | EU006631.1 | ABU23715.1 | Larsen et al. (2008) |
| *Chrysochromulina ercina* virus | CEV | 1755 | 584 | 1 | EU006628.1 | ABU23712.1 | Larsen et al. (2008) |
| *Pyramimonas orientalis* virus | POV | 1191 | 396 | 1 | EU006630.1 | ABU23714 | Larsen et al. (2008) |
| *Heterosigma akashiwo* virus | HAV | 1323 | 440 | 1 | AB198422.1 | BAE06835 | Nagasaki et al. (2005) |
| *Acanthocystis turfacea* chlorella virus | ATCV | 1317 | 438 | 1 | NC 008724.1 | YP001426761.1 | Direct submission |
| *Paramecium bursaria* chlorella virus | PBCV | 1314 | 437 | 1 | NC 009898.1 | YP001497813.1 | Fitzgerald et al. (2007) |
| *Micromonas pusilla* virus | MPV | 1257 | 418 | 1 | HQ633072.1 | AET43572 | Direct submission |
| *Ostreococcus tauri* virus | OTV | 1269 | 422 | 1 | NC013288.1 | YP003495004.1 | Weynberg et al. (2009) |
| *Feldmannia* spp. virus | FSV | 1308 | 435 | 1 | NC011183.1 | YP002154681.1 | Schroeder et al. (2009) |
| *Ectocarpus siliculosus* virus | ESV | 1428 | 475 | 1 | FN648730.1 | CBN80416.1 | Cock et al. (2010) |
| **Iridoviridae** | | | | | | | |
| Chilo iridescent virus | CIV | 1404 | 467 | 1 | NC003038.1 | NP149737 | Jakob et al. (2001) |
| Invertebrate iridescent virus 3 | IIV3 | 1401 | 466 | 1 | NC008187.1 | YP654586 | Delhon et al. (2006) |
| Frog virus 3 | FV3 | 1392 | 463 | 1 | FJ459783.1 | ACP19256.1 | Holopainen et al. (2009) |
| Infectious spleen and kidney virus | ISKNV | 1362 | 453 | 1 | AF371960.1 | AAL98730.1 | He et al. (2001) |
| Lymphocystis disease virus | LDV | 1380 | 459 | 1 | EF103188.1 | ABL07488.1 | Direct submission |
| **Unclassified** | | | | | | | |
| Marseillevirus | MV | 1434 | 477 | 1 | NC013756.1 | YP003407071.1 | Boyer et al. (2009) |
| **Ascoviridae** | | | | | | | |
| *Diadromus pulchellus* ascovirus 4a | DPAV | 1305 | 434 | 1 | AJ312705.1 | CAC84483.1 | Stasiak et al. (2003) |
| **Asfarviridae** | | | | | | | |
| African swine fever virus | ASFV | 1941 | 646 | 1 | XM369245.2 | XP369245.2 | Direct submission |

lake sturgeon tissue, naïve lake sturgeon tissue as well as white and lake sturgeon cell lines. Each 25 µl reaction contained 1× PCR buffer (Applied Biosystems), 1.5 mM MgCl$_2$, 200 µM deoxynucleoside triphosphates, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.8 µM of each primer and between 100 to 500 ng of DNA. Thermocycling parameters were as follows: 95°C for 5 min, 40 cycles of (95°C for 30 s, 52 or 53°C for 30 s, 72°C for 1 or 1.5 min) followed by a post-cycle extension at 72°C for 10 min.

Amplicons generated from 25 to 50 µl of the cPCR product were analyzed for purity and size by electrophoresis in 1% agarose gels, purified with the QIAquick Gel Extraction Kit (Qiagen) as outlined by the manufacturer and then TA cloned into vector pGEM-T Easy (Promega). Plasmid DNA was isolated from cultures of transformed *E. coli* as outlined above and screened for inserts by cPCR using the relevant primer pairs. Both strands of DNA from at least 3 positive clones per amplicon were sequenced by the dideoxynucleotide chain termination method using an automated sequencer (Sanger et al. 1977). Analyses of the DNA sequences were performed using BioEdit v7.0.9.0 software (Hall 1999).

## BLASTP, sequence alignment and phylogenetic analysis

Sequences displaying similarity to the deduced amino acid sequence of the partial Namao virus MCP were identified from protein databases searched using the BLASTP program (Altschul et al. 1990, 1997). Protein sequence alignments were performed using T-Coffee (Notredame et al. 2000, Di Tommaso et al. 2011) with sequences that were trimmed to the first amino acid aligning with the N terminal end of the Namao virus MCP fragment and the last amino acid at the C terminal end. A percent identity and similarity matrix for the trimmed MCP sequences was created using the Matrix Global Alignment Tool (MatGAT 2.02) software (Campanella et al. 2003). Conserved domains in the truncated protein sequences were annotated through the Conserved Domain Database using the National Center for Biotechnology Information's (NCBI) Conserved Domain Search service (Marchler-Bauer et al. 2011).

Phylogenetic tree reconstructions were estimated by 2 methods: Bayesian inference analysis as implemented by MRBAYES v3.2.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) and maximum-likelihood as implemented by PHYML 3.69 (Guindon et al. 2010). The Bayesian trees were estimated using multiple independent runs of aamodelpr = mixed and default settings for 1 500 000 generations when the average deviation of the split frequencies was < 0.002. For maximum-likelihood trees, clade confidences were estimated from 1000 bootstrap replicates. The output tree produced from each method was visualized using FigTree v1.3.1 software (Rambaut 2008).

## RESULTS

### Disease signs and bacteriology

Lake sturgeon mortality events at GRH or UMAAHF during 2009 to 2010 (Fig. 2) were characterized by fish inappetence, anorexia and erratic behavior such as swimming with the tail up and head down in the water column, unstable equilibrium and gasping near the surface of the water. Petechial haemorrhaging was present at the base of the pectoral and anal fins as well as around the mouth area in some of the fish. Dark red gills with pinpoint foci, accumulations of gill mucus and exaggerated gill movement were also noted. However, bacterial growth was not observed on media inoculated with samples derived from moribund fish.

### Virology and PCR

Namao virus was detected by cPCR amplification of a 219 bp MCP DNA fragment in almost 94% (59 of 63) of those samples in this study derived from dead or moribund lake sturgeon originating from the Nelson or Winnipeg Rivers (Table 4). The ginF and

Table 4. Diagnostic test results (no. positive/no. tested) from samples taken during disease outbreaks in the 2008 and 2009 year classes of juvenile lake sturgeon, sourced from the Winnipeg (WR) and Nelson River (NR), and raised at Grand Rapids Hatchery (GRH) and the University of Manitoba Aquatic Animal Holding Facility (UMAAHF). cPCR: conventional polymerase chain reaction; qPCR, quantitative PCR. See Tables 1 & 2 for virus abbreviations

| Molecular test method | 2008 UMAAHF WR | 2009 GRH NR | 2009 GRH WR | 2009 UMAAHF WR |
|---|---|---|---|---|
| NV cPCR | 7/8 | 3/3 | 35/37 | 14/15 |
| WSIV qPCR | 0/8 | 0/3 | 0/35 | 0/12 |
| MRSIV qPCR | 0/8 | 0/3 | 0/35 | 0/12 |

glaR primer pair yielded a cPCR product of both the expected size (data not shown) and sequence (described below). Neither the WSIV MCP-based nor
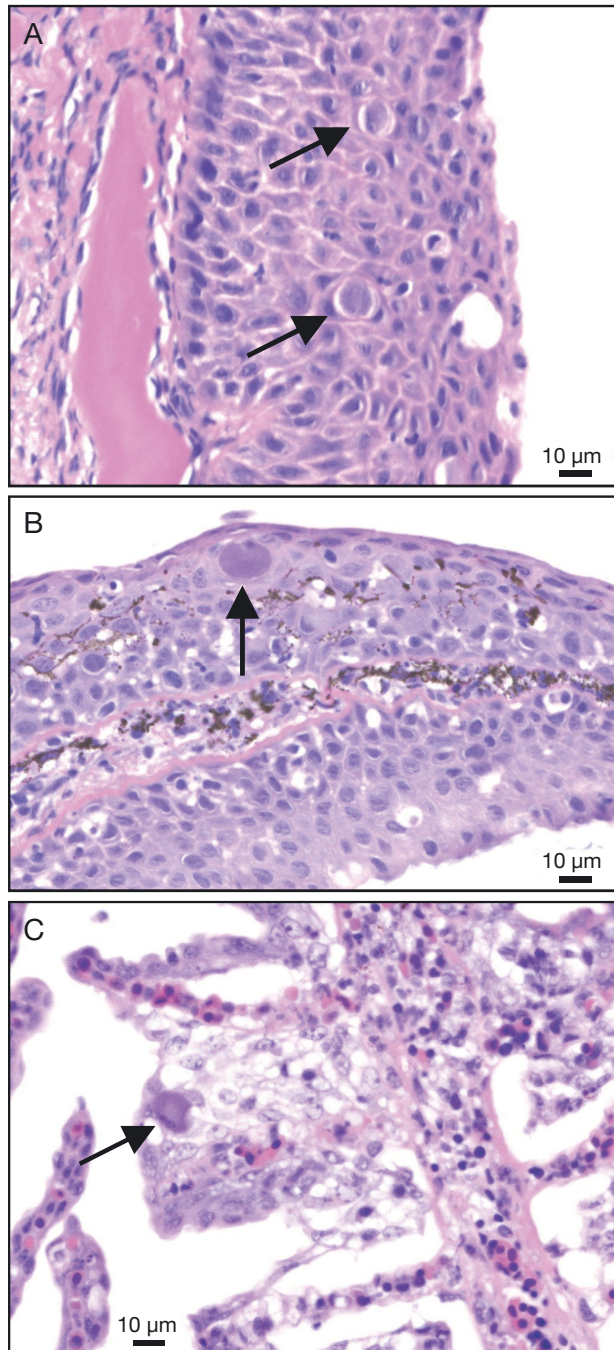


Fig. 3. *Acipenser fulvescens*. Light photomicrographs of H&E stained epithelial tissue sections from moribund juvenile lake sturgeon. (A) Skin tissue from Winnipeg River lake sturgeon (2008 year class) housed at the University of Manitoba Aquatic Animal Holding Facility. (B) Skin and (C) gill tissue from Winnipeg River lake sturgeon (2009 year class) housed at Grand Rapids Hatchery. Large amphophilic intracytoplasmic inclusions are evident (arrows)

the MRSIV serpin-based qPCR tests were able to detect Namao virus in the same samples. Our Namao virus MCP-based cPCR test, which also detects WSIV (including artificially synthesized DNA encoding the WSIV MCP), MRSIV and a recently diagnosed virus from white sturgeon cultured in British Columbia, will be described in detail elsewhere. The 7 samples collected in December 2008 prior to the 2009 outbreak at UMAAHF tested negative for the presence of Namao virus nucleic acid using the ginF/glaR cPCR assay.

Inoculation of primary lake sturgeon cell lines (LSGI or LSGO) with tissue homogenates shown to be positive for Namao virus by cPCR did not result in noticeable CPE after 21 to 60 d of observation or blind passage on the same cell lines. However, some of the culture fluid containing suspended lake sturgeon cells from CPE-negative monolayers tested positive for Namao virus after the first passage (the second passage was not tested by cPCR). The cells from white sturgeon (WSSK-1, WSS-2), Chinook salmon *Oncorhynchus tshawytscha* (CHSE-214) and common carp *Cyprinus carpio* (EPC) did not display CPE within 21 d of inoculation with tissue samples derived from the same populations of lake sturgeon. The WSS-2 cells, inoculated with 4 samples, 2 of which tested positive for Namao virus by cPCR, tested negative by the same assay for the presence of Namao virus after a single passage. The cPCR did not amplify the 219 bp DNA product from any of the naïve cell lines used in this study.

### Histology and TEM

Low numbers of hypertrophic cells were found in H&E stained histological samples of gill and skin epithelia tissues from moribund lake sturgeon (Fig. 3). The cytomegalic cells were scattered throughout the epithelial layer of the gill lamellae and appeared to contain granular, cytoplasmic inclusion bodies often associated with iridovirus-like infections of white-, pallid- and shovelnose sturgeon. Histological sections from the same fish did not show evidence of significant changes in kidney, heart, brain lower intestine, pancreas or nervous tissues. Enumeration of inclusion bodies in longitudinal sections of 14 juvenile lake sturgeon collected from GRH in May 2009 indicated a tropism of the virus for epithelial cells of the integument (Table 5).

Examination of cytomegalic cells within lake sturgeon gill lamellae epithelium by TEM revealed the presence of icosahedral-shaped virus particles in the

Table 5. *Acipenser fulvescens*. Enumeration of inclusion bodies (average no.) in longitudinal sections of moribund or mildly infected juvenile lake sturgeon from the Winnipeg River (WR) and Nelson River (NR). ND: not done

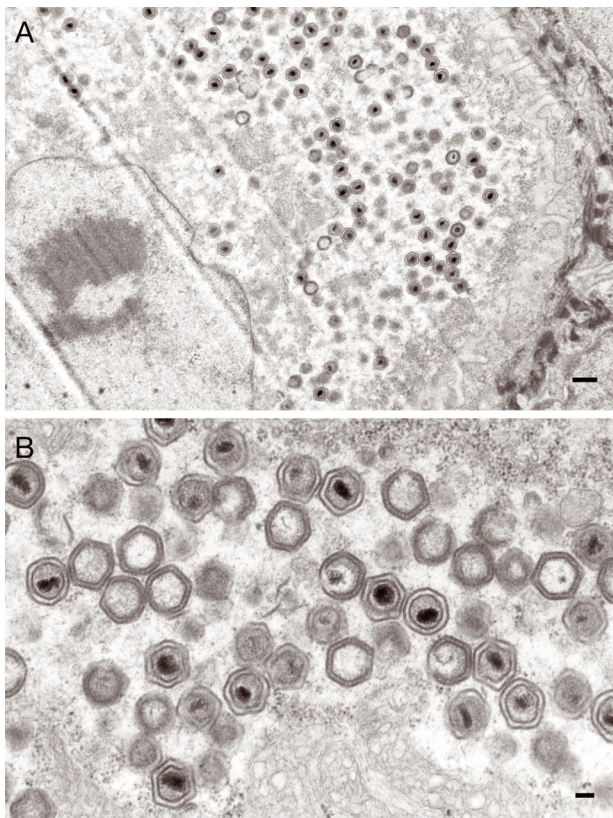| Site | Average number of inclusion bodies | | |
|------|------|------|------|
| | Moribund sturgeon | | Juvenile sturgeon |
| | WR | NR | WR |
| | (n = 5) | (n = 4) | (n = 5) |
| Nasal pits | 8.5 | 4 | 1 |
| Snout | 3.5 | 2.25 | 0 |
| Mouth | 4.3 | 1.75 | 0 |
| Gill | 3.6 | 3.5 | 0.4 |
| **Epidermis** | | | |
| Cranial | 14.1 | 8.45 | 2.0 |
| Abdominal - mid | 9.4 | 3.5 | 0.8 |
| Abdominal - distal | 4.5 | ND | 0.5 |
| **Fins** | | | |
| Pectoral | 12.3 | 2.75 | 0 |
| Dorsal | 17 | ND | ND |
| Pelvic | 4.5 | 3.5 | 0 |



Fig. 4. *Acipenser fulvescens*. Transmission electron micrographs of cytoplasmic hexagonal virus particles in epithelial cells of gill lamellae from Winnipeg River, juvenile lake sturgeon (2008 year class) held at the University of Manitoba Aquatic Animal Holding Facility. Intact virions were 225 to 263 nm (n = 18, mean ± SD = 242 ± 11 nm) in diameter, as measured from side to side, and 263 to 300 nm from vertex to vertex (n = 18, mean ± SD = 282 ± 13 nm). Scale bars: (A) 500 nm; (B) 100 nm

cell cytoplasm (Fig. 4). Some of the virions contained a condensed bar-shaped core. Amorphous virus-like particles as well as empty or incomplete capsids were also evident. Intact virions had 2 capsids and were approximately 242 nm in diameter as measured from side to side and 282 nm vertex to vertex (Fig. 4, Table 1).

## MCP alignment and analysis

We captured an estimated 95 to 96% of the Namao virus MCP open reading frame or its inferred amino acids based upon comparison with the WSIV MCP sequences (Fig. 5, Table 2; GenBank accession number JX155659). The identity of the amino acids shared between Namao virus and WSIV was 83.5% (Table 6). Namao virus and WSIV MCP sequences shared the highest E-value (7e-99) with viruses grouped in the *Mimiviridae* family and to a lesser extent *Phycodnaviridae* (3E-46) and *Iridoviridae* (1e-15) families, according to BLASTP analysis (Altschul et al. 1990, 1997). Conserved domains identified within the Namao virus MCP are presented in Fig. 5.

## Phylogenetic analyses

Namao virus and WSIV grouped more closely to each other than to any of the other viruses based upon phylogenetic analysis using the MCP amino acid sequence (Fig. 6). This was the case for tree reconstructions employing Bayesian inference (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) or maximum-likelihood methods (Guindon et al. 2010). The sturgeon viruses appeared in the *Mimiviridae* and *Phycodnaviridae* lineage but partitioned into the *Mimiviridae* clade. Both sturgeon viruses were clearly separate from and were not members of the Irido-Asco-MV clade.

Within the *Mimiviridae* family, the 2 megaviruses (proposed genera) and mimiviruses clustered together and shared a common ancestor with the mamavirus, CroV and sturgeon viruses. While this relationship was similar using either method of tree reconstruction, the relative branching topography for the latter 3 viruses was different. In the maximum-likelihood tree, CroV and mamavirus formed a lineage together that split from the sturgeon viruses after the node representing the ancestral *Mimiviridae* progenitor. However, the bootstrap values were too low (221/1000) to resolve the phylogenetic position of the sturgeon viruses relative to CroV and mamavirus.

Region markers: I (B1, C1, D1)

```
NV       1  PEMTYFKIGYKRHSLFAMTPVVQS-LGPLDFGKEVNFQIDMTGDVIYKMYIQLNISGTDTGS-------------------------------------------  61
WSIV     1  PEMTYFKIGYKRHSLFAMTPVVQS-LGPLDEGKEVNFQIDVTGDMIHRMYLQLNITGADTGS-------------------------------------------  61
CroV     1  PEVTFFKNIYRRHTNFSVESIEQTPNGTCDFGKTVTCEINRNGDLVTKMHLFLHLPAVEAVETGD----------------------------------------  65
MVChile  1  PQITFFKVYRRHTNFAVESIEQFNGNVNFGRKSTAEISRNGDLITQAFLKVVLPEVRFTGAFDK-----------------------------------------  66
APMV-1   1  PMITFFKVVYRRHTNFAVESIEQFFGGNLGFGKKSSAEINRSGDLIITQVFLKVTLPEVRYCGDFTN--------------------------------------  66
MVCour   1  ---MWTNRVVYRRHTNFAVESIEQFNGNVNFGRKSTAEISRNGDLITQAFLKVVLPEVRFTGAFDK---------------------------------------  63
APMV-2   1  RGNRVFKVVYRRHTNFAVESIQFFGGNLGFGKKSAEINRSGDLITQVFLKVTLPEVRYCGDFTN-----------------------------------------  66
ACMV     1  PQITFFKTIYRRHTNFAVESIVQHFIGDINEFGQEIVVEKMGDIMSRVYLEIDIPKINLVKNSSNWIKDKSTAKNDYDKISNFYGLVYNYISVNTDLAR       100
```

```
NV      61  -----------------------------------------------------------------------------------------------------------  61
WSIV    61  -----------------------------------------------------------------------------------------------------------  61
CroV    65  -----------------------------------------------------------------------------------------------------------  65
MVChile 66  -----------------------------------------------------------------------------------------------------------  66
APMV-1  66  -----------------------------------------------------------------------------------------------------------  66
MVCour  63  -----------------------------------------------------------------------------------------------------------  63
APMV-2  66  -----------------------------------------------------------------------------------------------------------  66
ACMV   101  KIKFLLSNNNISLNDISLIVLHNQFNSELDKAYTDLYKYINDTNSTDGFIQSQIPRTFNIINIFKSVYEKNLYIETNNPDEIGSIKKAELLKIINTRLYK       200
```

Region markers: II (E1, F1)

```
NV      61  --------------------------------------------------NDKKWGWVDNLGHAIIDCVDIKPGNTSIDKHYGDWLQVWNSLTSNKGHEDGYNRLIGNTSLM 123
WSIV    61  --------------------------------------------------VNKKWGWVDSLGHAIIECIDIKPGNTSIDKHYGDWLHVWNSLSSNKGQENGYDRLIGNTSPM 123
CroV    65  ----------------------------------------------EWGYARRLGHAVIDEVKIEIGGSEIDEHYGDWLNIWYELARRNSHDSGYDQMIGNIPEL        124
MVChile 66  -----------------------------------------FGHVEFAWVRHVGHAIVDETELEIGGSSIDKQYGDWLHIWKNVSQSKDHDHGMAKMLGDVPEL       129
APMV-1  66  -----------------------------------------FGHVEFAWVRNIGHAIVEETELEIGGSPIDKHYGDWLQIWQDVSSSKDHEKGLAKMLGDVPEL       129
MVCour  63  -----------------------------------------FGHVEFAWVRHVGHAIVDETELEIGGSSIDKQYGDWLHIWKNVSQSKDHDHGMAKMLGDVPEL       126
APMV-2  66  -----------------------------------------FGHVEFAWVRNIGHAIVEETELEIGGSPIDKHYGDWLQIWQDVSSSKDHEKGLAKMLGDVPEL       129
ACMV   201  QMYDYYIDIYRQYLSKKNIFESFENGTYEENYKFAWVEELGHAIIDYVDIKIGNQVIDKHTGDWFIIFNKLFTKIYQIENYNKMIGNVKELTIFD-----        295
```

Region markers: III (G1, H1), IV (I1)

```
NV     124  TKLV---------R-SRGGSTIYIPLQFFFNRNVGLSLPIVGMYGISFFMNIRLRPLMQCINRTANVS-------NTDIKLSIESANLIYDAIFLDSSER   206
WSIV   124  TKLV---------R-NHSGCTIYIPFQFFFNRSVGLSLPVVGMYGISFFMNIRLRALAQCINRTANVS-------VSDLRLSVESANLIYDAIFLDMSER   206
CroV   125  TNVSK--------NAKPEHKLYIPLQFYQRNNGLALPLIALQYHDVTKMILKLRPLKDVTNKKS---G------AQVPNVNVEDSFLIVDYIFLDAQER   206
MVChile130  TSISTLSWDVHDNTLLKPSYTLYIPLQFYQCRNNGLALPLIALQYHVRIYVKFRPAEQCYIASDAF-K-----SGAENFELDDVSVYVNYVVLDTEER   222
APMV-1 130  TSISTLSWDVPDNTVLKPSYTLYVPLQFYFNRNNGLALPLIALQYHQVRIYVKFRQADQCYIASDAF-K-----SGCGNLQLDDVSLYVNYVFLDTEER  222
MVCour 127  TSISTLSWDVHDNTLLKPSYTLYIPLQFYFQCRNNGLALPLIALQYHVRIYVKFRPAEQCYIASDAF-K-----SGAENFELDDVSVYVNYVVLDTEER  219
APMV-2 130  TSISTLSWDVPDNTVLKPSYTLYVPLQFYFNRNNGLALPLIALQYHQVRIYVKFRQADQCYIASDAF-K-----SGCGNLQLDDVSLYVNYVFLDTEER  222
ACMV   295  -------------DKIKKSYKLIIPFQFWFCRNTGLALPLIALRYHEVMFTIKLKELSKVCYVEDNDDIVSMENLQSLYNINLIDAKLYIDYIFLDTIER 382
```

Region markers: IV (B2), V (C2, D2)

```
NV     207  KTFTSYKQEYLVELLQCSANNPVTS-VATKLAPNFSHPVKFMVWHLRMGKYNTGHYLAYD-SSDWEEMIKKATIRFCLGMAKTINREGTLMLDIP----   300
WSIV   207  KTFTSYKQEYLVELLQCSANNPVTS-VATKLTPNFSHPVKFMIWHIKMGKYNTGKKFLGYD-SNNWEDMIKKATARFCSLAKTINREGVLMLDLP----  300
CroV   207  KKFAQATHEYLIEQLQFTGAESFKNS-NESYRLNFNHPSKAIVWAPQLAKYTTGATFVDWAWDGDWSAAKDRVAKRVWLAK-NLTLANSL-SEDDT-TEG 302
MVChile223  RRFAQVSHEYLIEQLQFTGEESIGTSNSAKYKLSYNHPVKGLYWVTKMGNYQGGK-FMVYD-HEDWERARENAAKLIILAQ-YDLDEFGY-FNDVA-SEC 317
APMV-1 223  RRFAQVSHEYLIEQLQFTGEESAGSSNSAKYKLFNFNHPVKAIIYVVTKLGNYQGGK-FMTYD-PVCWENARENAAKLLLLAQ-YDLDDWGY-EQEPGGYEC 318
MVCour 220  RRFAQVSHEYLIEQLQFTGEESIGTSNSAKYKLSYNHPVKGLYWVTKMGNYQGGK-FMVYD-HEDWERARENAAKLIILAQ-YDLDEFGY-FNDVA-SEC 314
APMV-2 223  RRFAQVSHEYLIEQLQFTGEESAGSSNSAKYKLFNFNHPVKAIIYVVTKLGNYQGGK-FMTYD-PVCWENARENAAKLLLLAQ-YDLDDWGY-EQEPGGYEC 318
ACMV   383  KRFAQSAHEYLIEVVQYQTFDNIDH-SKYNSHLNFSHPTKFIVWFVQPSHYRENPT----------------------------------------------- 437
```

```
NV     300  ------ASN-----GYIQID--------------------------PKFVTSRRTDLIDLFNAAR-PFAINTEIDIDSV------QIMGNYLTIE       351
WSIV   300  ------TNS--GFIQID--------------------------EAIVASRRADLVELFNLAH-PYAINTELDVDNV-----QIMGSYLTLD            351
CroV   303  E----AVTYNGETL-----VDA------QFVEHNDADA----------------------------------I-VLLTNNVSST               341
MVChile318  D---SNSYIGDCGVQYIGDVDPASPAEEPTYTFNDSLTADAFDGSVLIGKLAPCVPLLRKNKDVDLRDKVEGIIRIVTDFDNDKLKYPEVEKITKNDLTIT 414
APMV-1 319  EGNDGRSYVGDCGVQYTAVDPSNPSEEPSYIFNDTTTAFDGSLLIGKLAPCVPLLRKNKDVDLKDKVEGIIRIHTDFENDRMKYPEVEKITRNDLTLH  418
MVCour 315  D---SNSYIGDCGVQYIGDVDPASPAEEPTYTFNDSLTADAFDGSVLIGKLAPCVPLLRKNKDVDLRDKVEGIIRIVTDFDNDKLKYPEVEKITKNDLTIT 411
APMV-2 319  EGNDGRSYVGDCGVQYTAVDPSNPSEEPSYIFNDTTTAFDGSLLIGKLAPCVPLLRKNKDVDLKDKVEGIIRIHTDFENDRMKYPEVEKITRNDLTLH  418
ACMV   437  ----------------------------------------------------------------------------------------------------- 437
```

Region markers: VI (E2, F2), VII (G2)

```
NV     352  KVSTPIDVLFAEVESFRENKGDGSPEYDIVLYQWDNYGMFLDKTKNPISHSKLFFSNGERLPEM-PGAYENLVQPYCHFSNTPPIGINVYSFAIYPEKHQ 450
WSIV   352  KVSTPVDILFADVQSVRDHKGDGSPQYDIVLYQWDNYGMFIDKSKNPISYSKIFFSNGERLPEL-PCTYYNLVQPYCHFTNTPPEGINVYSFAIYPEKHQ 450
CroV   342  HLSWRVDDWV------ANTNV-GSNLTGFVVKDYMTYSLHADGTGNPVKSALLQLNGHDRFQKR-DGTYFNYVQPYCHWANTPAEGINSYSFALNPMDHQ 433
MVChile415  DLSIPVDKFDD------EDNRVEYIKRFDITVWQHHNYGLLIDGTVNPVSTVELQLNGQSRQSKR-SCFWHDTVEPYMHHTRTPTEGLNVFSFALNPEEHQ 507
APMV-1 419  DLSVPISKYD------VDNRVDYIKKFDVTWQHNNEGLLIDGSGNPTHEAELQLNGQPRQGGK-GIWYDTVNPTVHHTKSPREGVNVFSFALNPEEHQ   511
MVCour 412  DLSIPVDKFDD------EDNRVEYIKRFDITVWQHHNYGLLIDGTVNPVSTVELQLNGQSRQSKR-SCFWHDTVEPYMHHTRTPTEGLNVFSFALNPEEHQ 504
APMV-2 419  DLSVPISKYD------VDNRVDYIKKFDVTWQHNNEGLLIDGSGNPTHEAELQLNGQPRQGGK-GIWYDTVNPTVHHTKSPREGVNVFSFALNPEEHQ   511
ACMV   437  ------------------------------GKTKCQWNNFGTNPDLTGQPLNNTHLRINSYERTDPNLGVGYNYVQPYWYFISTPTEGLYVYSFSLKPTEHQ 510
```

Region markers: VII (H2), VIII (I2)

```
NV     451  PSGSANFSRFESIRIECRLGDDDVQFKN-TYLNDDTRATVYCVGYNLRFLYTGLAGLAY  508
WSIV   451  PSGSANFSRFESIRIECRLGDDDVVNFKT-TYLDDDTRATVYCVGYNLRFLYTGLAGLAY 508
CroV   434  PSCTCNFSRIDNTTLKLELAQVQ---------NDDSRIRIYDINYNYLRVMSGMCGLAY  483
MVChile508  PSCTCNFSRIDTAQLNLWFNHFSSNNKYADIFADNDNRVMIFAINYNVLRIMSGMCGLAY 566
APMV-1 512  PSCTCNFSRIDTAQLNLWFQHFTNHKFADVFADNDNKVLIFAVNYNVLRMLSGMAGLAY  570
MVCour 505  PSCTCNFSRIDTAQLNLWFNHFSSNNKYADIFADNDNRVMIFAINYNVLRIMSGMCGLAY 563
APMV-2 512  PSCTCNFSRIDTAQLNLWFQHFTNHKFADVFADNDNKVLIFAVNYNVLRMLSGMAGLAY  570
ACMV   511  PSSTLNLSRIHDFGIYLEFSKELSKIIE-S-NCSSIFFGTYIMSYNLRFMSGMAGLGF   567
```

Fig. 5. Alignment of major capsid protein (MCP) sequences from 8 viruses found to cluster together in the phylogenetic tree presented in Fig. 6. The MCP sequences were trimmed to the first and last amino acids aligning with the N and C terminal ends, respectively, of the Namao virus (NV) MCP fragment targeted by primers from the present study. Identical amino acid residues shared by 2 or more sequences are shaded black whereas similar residues are shaded grey. The numbers preceding and following the alignments indicate the positions of the first and last residues of the aligned regions in the corresponding protein sequences. Bars above the sequence delineate the conserved nucleo-cytoplasmic large DNA virus domains identified in the NV MCP through the Conserved Domain Database and National Center for Biotechnology Information's Conserved Domain Search service. The beta strands predicted in the first and second jelly-roll motifs are designated B1 to I1 and B2 to I2, respectively. The 8 conserved capsid domains reported by Larsen et al. (2008) for viruses classified as members of family *Phycodnaviridae* are boxed and labelled I to VIII. Virus abbreviations are defined in Table 2

Table 6. Analysis of the major capsid protein from Namao virus and other nucleo-cytoplasmic large DNA viruses. Data are values or ranges of % identity/% similarity of amino acid sequences. WSIV: white sturgeon iridovirus; IAMV: Irido-asco-Marseilleviruses

| Virus taxon/clade | Sturgeon viruses | | Mimi-mama-megaviruses | Phycodnaviruses | IAMV |
| --- | --- | --- | --- | --- | --- |
| | Namao virus | WSIV | | | |
| WSIV | 83.5 | | | | |
| | 94.1 | | | | |
| Mimi-mama-megaviruses | 20.6–34 | 20.8–32 | 22.1–99.1 | | |
| | 41.6–54.7 | 40–54.1 | 43.5–99.1 | | |
| Phycodnaviruses | 20.5–29.8 | 19.1–30.2 | 20.3–42.3 | 25–77.4 | |
| | 40.2–53.2 | 38.4–51.9 | 37.4–60.0 | 39.1–86.7 | |
| IAMV | 17.8–21.6 | 17.7–20.9 | 15.4–22.2 | 16.8–26 | 27.7–60.1 |
| | 35.8–37.4 | 33.9–38.0 | 29.8–40.0 | 32.3–48.2 | 47.2–73.4 |
| *Asfarviridae* | 17.8 | 16.9 | 15.8–17.7 | 14.4–18 | 14.5–17.7 |
| | 33.9 | 32.9 | 30.7–38.3 | 25.6–34.6 | 29–33.2 |
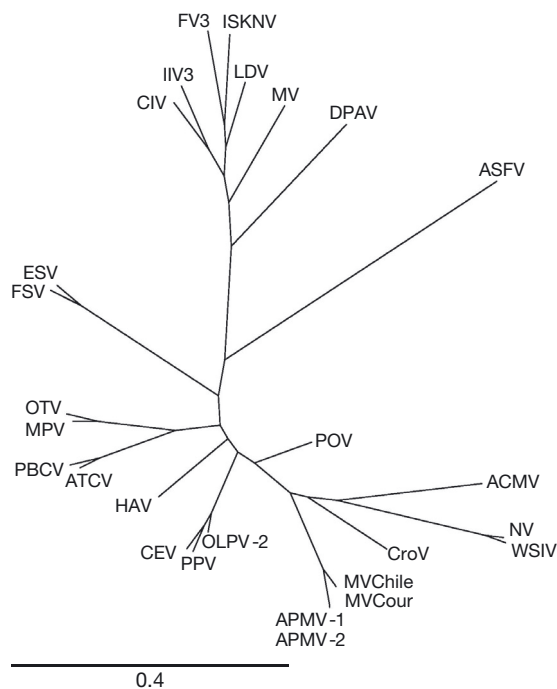


Fig. 6. Phylogenetic tree reconstruction of the major capsid protein (MCP) of 27 nucleo-cytoplasmic large DNA viruses, including Namao virus (NV) and white sturgeon iridovirus (WSIV). The tree was generated using Bayesian inference (BI) from MCP sequences with nodes labeled with BI posterior probabilities (Materials and methods). Abbreviations of other viruses and accession numbers are shown in Table 2

## DISCUSSION

We have discovered a new virus, Namao virus, associated with die-offs among juvenile lake sturgeon being reared as part of a conservation stocking program for endangered populations of sturgeon from the Nelson and Winnipeg rivers, MB. The ultrastructure of Namao virus, including its relatively large size (242 nm), was consistent with those reported for WSIV, MRSIV and a virus from Russian sturgeon *Acipenser guldenstadi* (Hedrick et al. 1990, 1992, Adkison et al. 1998, Kurobe et al. 2011). The virus also displayed a tropism for the integument of the body and gills and caused cellular abnormalities consistent with pathognomonic changes used to presumptively diagnose sturgeon NCLDVs. This information, in conjunction with the phylogenetic relationship and tree topology we observed, suggests that Namao virus and WSIV as well as other sturgeon NCLDVs (authors' unpubl. results) share a common evolutionary past and might be members of the *Mimiviridae* or a new, as yet, unrecognized virus family.

Further tests will be required to establish causality between infection with Namao virus and lake sturgeon morbidity and mortality. However, observations in this study point to this effect and the link is coherent with our general understanding of sturgeon

NCLDVs. Apart from the pathognomonic changes we observed, the virus was present in almost all the fish samples (93.6%) that were linked to the die-offs and in a suitable condition for analysis by cPCR. Initial attempts to isolate the virus in pure culture on white sturgeon cell lines were unsuccessful. This is similar to MRSIV (Kurobe et al. 2011) and some isolates of WSIV (Hedrick et al. 1990, Drennan et al. 2006). We were able to detect Namao virus, although weakly, by cPCR when suspect tissues were inoculated and then incubated for prolonged periods on primary lake sturgeon cell lines but without evidence of CPE. This could mean that the cells supported virus replication or that the virus present in the original inoculum was being detected. The difficulty we encountered culturing the virus has prompted our interest in ascertaining whether Namao virus and related sturgeon NCLDVs might be amenable to propagation on cultured amoebae similar to members of the family *Mimiviridae* (La Scola et al. 2003, 2008) or the unclassified Marseillevirus (Boyer et al. 2009). This follows from the proposal that amoeba function as melting pots for these and possibly other NCLDVs (Boyer et al. 2009). Successful culturing of Namao virus would open a convenient avenue for examination of the disease dynamics and enable fulfillment of the criteria required to establish causality of disease (Koch 1890, Hill 1965, Evans 1976).

WSIV and MRSIV display signs of a complex life-cycle that can manifest as an acute or chronic infection in juvenile sturgeon depending on rearing conditions during the first year of growth (Hedrick et al. 1990, 1992, LaPatra et al. 1994, 1996, Watson et al. 1998a,b, Drennan et al. 2005, 2006, Kurobe et al. 2011). Our observations suggest that Namao virus is similar in this respect. We suspect that the die-offs at UMAAHF and GRH signaled the switch between the chronic and acute phases of the virus life-cycle. In this scenario, the fish could have been infected either horizontally or vertically at the time of fertilization or subsequently by horizontal transmission prior to transport to UMAAHF. However, we cannot rule out the possibility that filtered and dechlorinated municipal water at UMAAHF was the source of infection in lake sturgeon at the facility, especially since Marseillevirus, mamavirus and mimivirus were all isolated from samples of water collected from cooling towers in Europe (La Scola et al. 2003, 2008, Boyer et al. 2009). From a parsimonious standpoint, however, it seems more likely that the fish were infected prior to transport to UMAAHF. This would point to infection occurring in the 2008 year class of Winnipeg River lake sturgeon at the broodstock collection site

(Slave Falls) or subsequently at Pinawa Hatchery (site of larval rearing) which used untreated water from the Winnipeg River. The source of the virus at GRH in the 2009 year class of Nelson River and Winnipeg River is less clear and will require completion of our ongoing epidemiological study. In addition to the sources already noted, the virus at GRH could have been introduced from the Nelson River lake sturgeon or the Saskatchewan River which supplies water to the hatchery.

The cumulative mortality in lake sturgeon populations harboring Namao virus reached a high level, approaching 100% in the 2009 Nelson River year class held at GRH. This is similar to the level of mortality caused by WSIV in white sturgeon or MRSIV in pallid or shovelnose sturgeon naturally exposed to the virus (Hedrick et al. 1990, 1992, Kurobe et al. 2011). Qualitatively, the kinetics of the die-offs were distinct when comparisons were made between UMAAHF and GRH but the outcome was similar. The die-offs at UMAAHF occurred over the course of 2 to 4 wk whereas those at GRH occurred over the course of approximately 1 mo in fish derived from the Nelson River and 6 mo in the Winnipeg River stock. Such variation in mortality kinetics is not without precedent among sturgeon NCLDV infections, and has been attributed to risk factors including handling, stocking density, and water conditions (LaPatra et al. 1994, 1996, Watson et al. 1998a, Drennan et al. 2005, 2006). Each of these factors was different between the 2 facilities as were the stocks of fish held at GRH. Specific studies designed to assess the role or influence these variables have on Namao virus disease dynamics could be used to avoid or minimize outbreaks caused by the virus in hatcheries. Further, it will be important to control viral trafficking of Namao virus in lake sturgeon through the management of factors related to virus source, prevalence and transmission much like what has already been accomplished for WSIV in white sturgeon through the Kootenai River White Sturgeon Conservation Aquaculture Program (LaPatra et al. 1999, KTOI 2007).

In the present study, 7 fish collected 2 mo prior to disease onset at UMAAHF tested negative for Namao virus by cPCR. The absence of detection does not rule out the presence of Namao virus in these fish. Especially given the limited sensitivity of the test compared to qPCR, absence of verification by a different test method such as cell culture, and the possibility that the virus might persist at extremely low levels as a localized chronic infection in asymptomatic fish. In addition, differences in the ability to

detect sturgeon NCLDVs might reflect the choice of tissues sampled or differences in disease susceptibility of the host (Kwak et al. 2006, Drennan et al. 2007, Kurobe et al. 2010). The outbreaks at UMAAHF were temporally asynchronous but acute, indicating that a large percentage of the fish were either harboring the virus at the onset of mortality or that the fish were exposed to a high dose of virus on 4 separate occasions. Again, the latter would indicate that an occasional large amount of virus was present in the treated municipal water or that a highly contaminated fomite was present and led to infection of fish in the different tanks on 4 different occasions. None of these possibilities can be ruled out from having occurred in the present study but, as we mentioned earlier, the presence of the virus in treated, municipal water seems unlikely, though not impossible. In fact, Drennan et al. (2006) reported that the accidental introduction of as little as 100 ml of Kootenai River water resulted in fish testing positive for WSIV during the course of an experiment conducted in tanks receiving municipal water.

The classification of sturgeon NCLDVs might become clearer with our phylogenetic analysis coupled with commonalities observed among their life-cycles. The general topology of the phylogenetic tree we reconstructed was strongly supported by Bayesian inference analysis and was consistent with virtually all those trees reported in the literature pertaining to NCLDVs regardless of the method used to reconstruct the tree or whether the tree was generated with the MCP or concatamers of conserved NCLDV protein sequences (Tidona & Darai 2000, Raoult et al. 2004, Iyer et al. 2006, Larsen et al. 2008, Monier et al. 2008, Boyer et al. 2009, Yutin et al. 2009, Fischer et al. 2010, Koonin & Yutin 2010, Arslan et al. 2011). It must be kept in mind that our phylogenetic tree was reconstructed using only the MCP, one of the 5 nucleo-cytoplasmic virus orthologous genes (Yutin et al. 2009). The genomes of these giant viruses possess a complex repertoire of genes from various origins including viruses, bacteria and eukaryotes, and reflect a rich evolutionary history (Filée 2009) and it is possible that the genome of Namao virus and related sturgeon NCLDVs, aside from their MCP genes, are more distantly related to the *Mimiviridae* and *Phycodnaviridae* families than indicated by our results. For instance, there is some evidence of gene exchange between the distantly related Marseillevirus and mimivirus (Boyer et al. 2009). Our ongoing metagenomic studies in conjunction with the work of others may provide further insight into the taxonomy of these viruses.

Our results indicate that Namao virus and WSIV shared a common lineage with CroV and mamavirus within the *Mimiviridae* family. The close phylogenetic relationship between the latter 2 viruses was reported previously by Fischer et al. (2010). The genome of mamavirus is approximately 1.6-fold larger than that of CroV. In this context, the sturgeon NCLDVs' capsid diameters were most similar to that of CroV in size and were approximately half the size of that for megaviruses, the largest known members of the family *Mimiviridae*. The Namao virus and WSIV MCPs both contained 2 conserved NCLDV capsid domains (Marchler-Bauer et al. 2011) separated by an insertion of approximately 135 or 91 amino acids, respectively (Fig. 5, Table 2). This insertion was similar to that described for the APMV-1 capsid protein 1 (Azza et al. 2009) and like the mimivirus, this insertion occurred in the predicted DE loop of the second jelly roll along the sturgeon virus capsid proteins. The Namao virus and WSIV MCPs also contained the 8 conserved capsid domains reported by Larsen et al. (2008) for viruses classified as members of the family *Phycodnaviridae*. The mamavirus, like most of the viruses belonging to the *Phycodnaviridae* and all of the irido-asco-MV-asfarviruses listed in Table 2, did not contain this insertion and instead had a single, fused conserved capsid domain. Further, the mamavirus MCP contained an N-terminal insertion of 162 amino acids between MCP Domains 1 and 2 of the phycodnavirus-like capsid proteins.

If Namao virus and related sturgeon viruses are indeed members of the *Mimiviridae* family, then the host range of this group of viruses would be extended from unicellular eukaryotes to lower vertebrates. Likewise, the names as well as the taxonomic grouping of suspected 'irido-like' sturgeon NCLDVs will likely require amendment based on the results of this study. We believe this might be the case regardless of the final taxonomic designation of the sturgeon NCLDVs because comparison of iridovirus MCP sequences to that of Namao virus and WSIV reveal fundamental differences in their evolutionary relationship.

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