Systemic iridovirus from threespine stickleback *Gasterosteus aculeatus* represents a new megalcytivirus species (family *Iridoviridae*)

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ABSTRACT: Megalcytiviruses have been associated with epizootics resulting in significant economic losses in public aquaria and food-fish and ornamental fish industries, as well as threatening wild fish stocks. The present report describes characteristics of the first megalcytivirus from a wild temperate North American fish, the threespine stickleback *Gasterosteus aculeatus*. Moribund and dead fish sampled after transfer to quarantine for an aquarium exhibit had amphophilic to basophilic intracytoplasmic inclusions (histopathology) and icosahedral virions (transmission electron microscopy) consistent with an iridovirus infection. Phylogenetic analyses of the major capsid, ATPase, and DNA polymerase genes confirmed the virus as the first known member of the genus *Megalcytivirus* (family *Iridoviridae*) from a gasterosteid fish. The unique biologic and genetic properties of this virus are sufficient to establish a new *Megalcytivirus* species to be formally known as the threespine stickleback iridovirus (TSIV). The threespine stickleback is widely distributed throughout the northern hemisphere in both freshwater and estuarine environments. The presence of megalcytiviruses with broad host specificity and detrimental economic and ecological impacts among such a widely dispersed fish species indicates the need for sampling of other stickleback populations as well as other North American sympatric marine and freshwater ichthyofauna.

KEY WORDS: Iridovirus · Megalcytivirus · Threespine stickleback · Phylogeny

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INTRODUCTION

The family *Iridoviridae* is a lineage of double-stranded DNA (dsDNA) viruses that includes the genera *Cloriridovirus*, *Iridovirus*, *Lymphocystivirus*, *Megalcytivirus*, and *Ranavirus* (Chinchar et al. 2005). Members of the genera *Iridovirus* and *Chlor-*

iridovirus infect invertebrate hosts, while agents in the remaining genera infect poikilothermic vertebrates (Williams et al. 2005, Delhon et al. 2006). Although ranaviruses infect amphibians, reptiles, and fish, both lymphocystiviruses and megalcytiviruses have only been identified from fish (Chinchar et al. 2005). The ranaviruses and megalcytiviruses,

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often considered collectively as the systemic iridoviruses, are emerging aquatic animal pathogens of economic and ecological importance causing mass mortality events in endangered wild amphibian populations (ranaviruses) and losses in economically important ornamental and food-fish aquaculture industries (Chinchar 2002, Kawakami & Nakajima 2002, Bondad-Reantaso et al. 2005, Whittington & Chong 2007, Whittington et al. 2010).

Vertebrate iridoviruses generate a variety of diseases in their hosts, ranging from relatively benign localized skin diseases (e.g. lymphocystiviruses) to lethal systemic diseases (e.g. ranaviruses and megalocytiviruses). Megalocytivirus infections are characterized by a unique histopathologic change marked by the presence of large amphophilic to basophilic cytoplasmic inclusions in cytomegalic cells within multiple tissues (Gibson-Kueh et al. 2003, Lee et al. 2009). The megalocytiviruses have a broad host range, and closely related strains of the type species of the genus—infectious spleen and kidney necrosis virus (ISKNV)—have caused epizootics in wild and cultured freshwater and marine fish stocks, including numerous economically important food-fish species, in the temperate to tropical waters of Japan (Inouye et al. 1992, Matsuoka et al. 1996, Kawakami & Nakajima 2002), China (He et al. 2000, Weng et al. 2002, Chen et al. 2003, Shi et al. 2004, Lu et al. 2005, Wang et al. 2007, Dong et al. 2010), Korea (Jung & Oh 2000, YJ Kim et al. 2002, Do et al. 2005a, WS Kim et al. 2005, Jeong et al. 2006a,b), SE Asia (Miyata et al. 1997, Chou et al. 1998, Chao et al. 2002, Sudthongkong et al. 2002a, Gibson-Kueh et al. 2003, 2004, Wang et al. 2003, 2009), and Australia (Lancaster et al. 2003). Megalocytivirus epizootics have been diagnosed by histopathology, electron microscopy, and molecular techniques in ornamental fish exports from both South America (Leibovitz & Riis 1980) and Asia (Armstrong & Ferguson 1989, Anderson et al. 1993, Sudthongkong et al. 2002b, Go et al. 2006, Lyu et al. 2006, Jeong et al. 2008a, 2008b, Weber et al. 2009, Kim et al. 2010), as well as in ornamental fish breeding facilities in the USA (Fraser et al. 1993, Petty & Fraser 2005), UK (Rodger et al. 1997), and Israel (Paperna et al. 2001). Two tilapia epizootics that occurred after the importation of cultured fry from Florida into Idaho (Smith et al. 1997) and Canada (McGrogan et al. 1998) were likely due to megalocytiviruses given the observed pathology. To date, no megalocytivirus outbreaks have been reported in wild temperate North American fish species.

The complete genomic sequences of 6 megalocytiviruses have been elucidated including ISKNV, red

sea bream iridovirus (RSIV), rock bream iridovirus (RBIV), orange spotted grouper iridovirus (OSGIV), large yellow croaker iridovirus (LYCIV), and the turbot reddish body iridovirus (TRBIV) (He et al. 2001, Kurita et al. 2002, Do et al. 2004, Lu et al. 2005, Shi et al. 2010, J. Ao & X. Chen unpubl.). Genomic sequence information and numerous subsequent analyses have revealed that megalocytiviruses from a wide range of hosts share a high level of sequence homology, with 97% or greater identity at the deduced amino-acid level for the major capsid protein (Smail & Munro 2001, Chinchar et al. 2005, Wang et al. 2007). The generation of genomic sequence information for megalocytiviruses has prompted numerous phylogenetic analyses (Sudthongkong et al. 2002a, Do et al. 2005a,b, Nakajima & Kurita 2005, Go et al. 2006, Imajoh et al. 2007, Wang et al. 2007, Song et al. 2008, Shinmoto et al. 2009, Weber et al. 2009, Dong et al. 2010, Kim et al. 2010). Although the datasets, inclusion of taxa, methods, and interpretation of the phylogenetic analyses have varied considerably among these studies, a general picture of the evolutionary relationships of the megalocytiviruses has emerged. Megalocytiviruses group into 3 genotypes: Genotype 1 (RSIV group) contains isolates from marine fish in Japan, Korea, China, and SE Asia; Genotype 2 (ISKNV group) includes isolates from mandarin fish and other fish from SE Asia; and Genotype 3 (TRBIV group) is composed of isolates from Asian flatfish (Nakajima & Kurita 2005, Song et al. 2008, Kim et al. 2010).

The threespine stickleback *Gasterosteus aculeatus* is a temperate species of fish found throughout the northern hemisphere. This species can be anadromous in rivers, streams, and bays or exist solely in freshwater lakes and streams. Because threespine stickleback occupy a large geographic area and many niches, they have served as a model for evolutionary theory on reproductive isolation (McKinnon & Rundle 2002, Taylor et al. 2006). Although this species is recognized as a species of low priority on the IUCN Red List of threatened species and is of low conservation priority, there is evidence to suggest that certain genetically distinct populations of these animals, such as those in freshwater lakes in British Columbia, are at great risk for extinction (Taylor et al. 2006, Branco et al. 2008; www.iucnredlist.org).

In the present investigation we describe the lesions associated with the first megalocytivirus infection from a wild temperate North American species, the threespine stickleback. Given the importance of megalocytivirus infections in wild and cultured fish from Asia and Australia, we compare the origins,

pathogenesis, and genetic relationship of the new virus to other megalocytiviruses.

MATERIALS AND METHODS

Fish acquisition, research facilities, and husbandry

In September 2007, approximately 4000 immature adult threespine stickleback *Gasterosteus aculeatus* were captured by beach seine from the Broughton Archipelago region (Knight Inlet and Tribune Channel) of coastal British Columbia, Canada. The fish were transferred to the Pacific Biological Station of Fisheries and Oceans (DFO), Nanaimo, British Columbia. From September 2007 to March 2008, they were held in sand-filtered (to 23 μm) single-pass seawater with minor fluctuations in temperature (7.5 to 11.5°C) and salinity (28 to 30). More than 200 fish died within 24 h of arrival, and less than 10 fish died over the next few days; thereafter, mortalities were approximately 3 fish d^{-1} . Routine fish health screens were within normal limits for wild-caught fish from this area, and because these fish were no longer needed, the remaining 2808 fish were transferred to the Vancouver Aquarium (British Columbia) on 20 March 2008, where the fish were placed into a 950 l quarantine tank with a flow-through natural seawater system maintained near 10°C at a salinity of 28.5. Low-level mortality began immediately and continued for 34 d (Fig. 1). The remaining 2645 fish were humanely euthanized when increasing mortality, despite formalin bath treatments, was associated with microscopic and molecular evidence of an iridovirus infection.

Fig. 1. *Gasterosteus aculeatus*. Cumulative mortality during quarantine at Vancouver Aquarium

Histopathology, bacteriology, virology, and transmission electron microscopy

Fish health screens carried out at the DFO on 18 October 2007, 5 November 2007, 10 December 2007, and 15 January 2008 included (1) 10 fish for gill wet mounts, skin scrapings, Gram stains of kidney and gill smears, and kidney swabs plated onto trypticase soy agar (TSA) and TSA with salt; (2) 10 fish for virology: two 5-fish pools of kidney and spleen homogenates on epithelioma papillosum cyprini (EPC) and Chinook salmon embryo 214 (CHSE-214) cell lines (threespine stickleback cell lines were not available); and (3) 5 or 10 fish fixed in 10% neutral buffered formalin for diagnostic histopathology (Table A1 in Appendix 1).

At the Vancouver Aquarium, wet mounts of skin scrapes from 9 fish and fecal flotation of feces were obtained to assess external and internal pathogens. For further diagnostic analysis, clinically healthy and moribund fish were euthanized with 300 mg l^{-1} tricaine methanesulfonate for submission to the Animal Health Centre (AHC), Abbotsford, British Columbia and the Fish Health Laboratory (FHL), University of California, Davis.

Eleven dead threespine sticklebacks were received by the AHC for diagnostic evaluation: 2 fish (natural deaths) on 9 April 2008 and 5 fish (3 natural deaths and 2 euthanized) on 17 April 2008 had already been preserved in 10% neutral buffered formalin for histopathology; 4 other fish were submitted dead on 17 April 2008 on ice (these fish varied from 6.2 cm and 1.6 g to 7.7 cm and 3.6 g). Prior to shipping the 17 April samples, sterile swabs of the coelomic cavities from 2 fish were performed at the aquarium for plating on blood and TSA agars at the AHC. For virology, the head kidney and spleen were removed from each of these 4 fish, pooled, processed by standard methods (USFWS & AFS-FHS 2007), and inoculated onto CHSE-214 and EPC cell lines (threespine stickleback cell lines were not available). The remainders of these fish were preserved in 10% neutral buffered formalin for histopathology. The 2 preserved fish received on 9 April were decalcified overnight in an 8% formic acid solution (Protocol 1, Fisher Scientific) and an additional 2 h in a 10% HCl solution (Protocol B, Fisher Scientific). The 9 fish received on 17 April were decalcified in a 10% EDTA solution for 6 d. For all fish following decalcification, the trimming protocol was adjusted based on whether the tissues would fit in a standard histocassette and microscope slide. The smaller fish were transected mid-sagittally and processed routinely

into a single slide, 1 fish slide⁻¹. Larger fish were first cut transversely just behind the head, and then the resultant 2 pieces were transected mid-sagittally and processed onto 2 to 4 slides each. Paraffin sections were cut at 3 µm and stained routinely with hematoxylin and eosin (H&E). The slides from the 40 fish sampled during the health screens at DFO were processed similarly.

Based on histopathology findings consistent with an iridovirus, a GenBank search of various fish iridovirus major capsid protein (MCP) sequences facilitated the design of primers for a nested PCR assay using external primers Irid-SB-1 (5'-GGC GAC AAT GCC GTG AC-3') and Irid-SB-4 (5'-CGC ATG CCA ATC ATC TTG-3'), and internal primers Irid-SB-2 (5'-ATG TGT GGC TGC GTG TTA AG-3') and Irid-SB-3 (5'-AAT GAC ACC GAC ACC TCC TC-3'). Viral DNA was extracted from tissue culture fluid from EPC cells inoculated with tissue extracts using a DNeasy Blood and Tissue Kit (QIAGEN) following the protocol for viruses in suspension and stored at 4°C until used for PCR. The DNA sequence of the amplified product was compared with other known iridovirus sequences. After the general fish iridovirus PCR identified a unique iridovirus, 6 fish euthanized and frozen at -80°C on 22 April 2008 were shipped from the aquarium to the FHL for further molecular diagnostic testing.

A single H&E-stained slide with characteristic megalocytivirus pathology and the source paraffin block were sent from the AHC to the Electron Microscopy Laboratory (EML), Department of Medical Pathology and Laboratory Medicine, School of Medicine, University of California at Davis for transmission electron microscopy (TEM). An area of interest in the spleen was removed from the paraffin block to 100% xylene and after clearing overnight, rehydrated and processed using a standard protocol (Johannessen 1977). Briefly, osmium was followed sequentially by a 0.1% tannic acid solution for 30 mins and then a 2% uranyl acetate solution prior to embedding in an Epon substitute-Araldite (Hayat 1989). Ultrathin sections were cut and stained with uranyl acetate and lead citrate before viewing.

DNA extraction, PCR amplification, cloning, and sequencing

At the FHL, DNA was extracted from the spleens from 2 of 6 frozen fish samples using a DNeasy Blood and Tissue Kit following the protocol for animal tissues. The extracted DNA was subjected to a previ-

ously described degenerate PCR protocol (Hanson et al. 2006) that targets a portion of the viral DNA-dependent DNA polymerase (DPOL). These 2 samples were then used in attempts to amplify the full-length MCP and ATPase genes. Amplification of the full-length MCP gene amplification was attempted using primers MCPF1 (5'-CTG TTG GTC TTG CTG AGT GC-3') and ISKNVMCPR (5'-TTA CAG GAT AGG GAA GCC TGC-3') and for the ATPase amplification using primers ATPFIXEDF1 (5'-CAA ACC ACA GCG CGG CAA GT-3') and ATPASER2 (5'-GCT GGG CGA CCC CAT TCG-3'). Each 50 µl PCR reaction contained 1× PCR buffer (Invitrogen), 1.5 mM MgCl₂ (Invitrogen), 400 µM deoxynucleoside triphosphate, 0.5 units *Taq* polymerase (Invitrogen), 40 pmol of each primer, and 100 ng of DNA. The mixture was subjected to a precycle at 95°C for 3 min, then 40 cycles of amplification (94°C for 1 min, 50°C for 1 min, 72°C for 2 min), followed by a postcycle extension at 72°C for 10 min.

Amplicons were purified from 10 µl of the PCR product by excision of bands from agarose gels, and then cloned using a TOPO TA cloning kit and plasmid pCR2.1-TOPO (Invitrogen). Transformed bacterial colonies were screened by PCR with M13 primers, and plasmids containing relevant inserts were purified using a QIAprep Spin Miniprep Kit (QIAGEN), and sequenced by the dideoxynucleotide chain termination method using M13 forward and reverse primers and an automated sequencer (Sanger et al. 1977).

BLASTP, molecular dataset, sequence alignment, and phylogenetic analysis

Following sequence assembly and removal of primer sequences, general BLASTP searches (www.ncbi.nlm.nih.gov/blast/Blast.cgi) using the deduced amino-acid sequences for the threespine stickleback iridovirus (TSIV) DPOL, MCP, and ATPase genes were performed (Altschul et al. 1997). The results were used to find appropriate iridoviruses for the phylogenetic analyses. Sequence alignments were performed using Mafft 5.8 (Katoh et al. 2005) followed by minor manual adjustments in ClustalW (Thompson et al. 1994). The E-INS-I alignment strategy was used with the following parameters: scoring matrix (BLOSUM62), gap open penalty (1.53), and offset value (0). For each gene alignment, the sequence was trimmed to the first conserved amino acid at the 5' and 3' ends prior to analyses.

ClustalW was used to generate individual gene sequence identity matrices for analyses. To assess gene concordance, Bayesian analyses were performed independently for each gene. Phylogenetic trees were constructed using MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001). A mixed prior was used on amino-acid models and default priors for topology (uniform) and branch lengths (Exp and 10). The Markov chain was run for a maximum of 10 million generations, with a stopping rule implemented so that the analysis would halt when the average deviation of the split frequencies was <0.001%. Four independent analyses were conducted, each with 1 cold and 3 heated chains with the default heating parameter (temperature = 0.2). Every 50 generations were sampled and the first 25% of MCMC samples discarded as burn-in. Bayes factors were calculated in Tracer 1.4 (<http://tree.bio.ed.ac.uk/software/tracer/>) to determine if partitioning the data by gene (2 partitions) or concatenating the amino-acid matrix (1 partition) produced a substantial improvement in model fit.

RESULTS

Disease signs, histopathology, bacteriology, virology, and TEM

Histopathology, wet mounts, and stained smears from the DFO fish health screens revealed that fish consistently had low numbers of endemic infectious agents (*Bomolochus* sp., *Ergasilus* sp., *Myxobilatus* sp., *Sphaerospora* sp., and chlamydia-like bacteria that cause epitheliocystis), and 1 fish had an unidentified protozoan in the kidney (Table A1 in Appendix 1). Although evidence of a viral infection was not noted before transfer of fish to the Vancouver Aquarium, reexamination of the archived DFO histopathology slides after fish were transferred to the aquarium revealed that in 3 out of the 4 fish health screens, at least 1 fish had few-to-abundant iridovirus inclusions in the spleen, kidney, liver, gill, or heart (Table A1 in Appendix 1). Fish sampled for bacteriology and virology at the DFO were negative.

At the aquarium, moribund fish exhibited exophthalmos, dermal hyperemia, and skin lesions ranging from erosions to perforating ulcers that exposed coelomic contents. Several fish were missing part of their caudal fin. Whole mounts of skin scrapes revealed a mixed population of bacteria, including motile rods and filamentous bacteria. Fecal flotation was negative for parasites.

At the AHC, no cytopathic effect (CPE) occurred on either CHSE-214 or EPC cells after 21 d of observation. A blind passage again revealed no CPE for either cell line after 21 d. The original infected tissues and tissue culture fluids from both cell lines (first and second passage) were positive by PCR using the consensus iridovirus nested PCR assay. We do not know whether unobserved low-level replication occurred in the cells or whether we were simply detecting virus originally inoculated from the fish. An extract from the original tissues remained positive through 10^{-5} dilution (10^{-6} was negative).

Of the 11 fish sent from the aquarium to the AHC, 9 contained large, amphophilic or pale-basophilic, intracytoplasmic inclusions in multiple organs characteristic of infection with an iridovirus (Figs. 2 to 5). Organs with viral inclusions included the head kidney, trunk kidney including glomeruli, spleen, liver, heart, ovary, testis, gastrointestinal tract and surrounding mesenteries, skeletal muscle, skin (dermis), gas gland and rete mirabile, choroid plexus, cerebral and spinal meninges, and loose connective tissues of the head, including gill arches and filaments. The morphology and location of cells with inclusions is most consistent with leukocytes or endothelial cells as the affected cell type. In some fish, viral inclusions comprised nearly 40% of the volume of the head kidney (Fig. 2A).

Iridoviral infected fish also had other lesions. Four fish had focal or multifocal coagulative hepatocellular necrosis (e.g. Fig. 3E,F). Pansporoblasts and pre-spore stages of myxosporeans were common in the kidney (Fig. 2B–D), and anisakid parasites were common in the coelomic cavity (not shown). One fish had copepod parasites and large numbers of filamentous bacteria in the branchial cavity (Fig. 5A–C). One fish had several small multicellular parasites, possibly a myxosporean, on the surface of the intestinal epithelium (Fig. 5G). One fish had sporulated and unsporulated oocysts of a coccidian in the liver (not shown).

Two of the 11 fish had no evidence of iridovirus infection; instead, these fish had other significant microscopic lesions. One fish had disseminated granulomatous inflammation with intralesional resting spores characteristic of *Ichthyophonus hoferi*. The other fish (one that died of natural causes) had multiple intravascular colonies of Gram-positive bacterial cocci arranged in long chains; colonies were most abundant in the choroid plexus and spleen, ranging up to 700 μm in diameter. The Gram-positive bacterial colonies also occurred in branchial vessels, loose connective tissue around the brain, cardinal veins,

Fig. 2. *Gasterosteus aculeatus*. Sections of kidney and ovary with abundant amphophilic intracytoplasmic iridovirus inclusions (*) and myxosporean pansporoblasts (arrows); H&E stain. (A) Iridovirus inclusions comprise about 40% of the head kidney volume. (B) Ovary and trunk kidney. (C) Trunk kidney; black rectangle outlines area shown in (D). (D) Iridovirus inclusions in interstitium and glomeruli

Fig. 3. *Gasterosteus aculeatus*. Sections of mouth, thyroid gland, and liver with abundant amphophilic intracytoplasmic iridovirus inclusions (*); H&E stain. (A) Mouth; black rectangle outlines area shown in panel (B). (B) Iridovirus inclusions between and around maxillary bone and tooth (t). (C) Thyroid gland with follicles (f); black rectangle outlines area shown in panel (D). (D) Iridovirus inclusions around thyroid follicle (f) and skeletal muscle (s). (E) Liver with an irregular focus of coagulative necrosis (arrowheads); black rectangle outlines area shown in panel (F). (F) Necrotic hepatocytes (arrowheads) have hyper-eosinophilic cytoplasm, pyknosis, and sometimes karyorrhexis

Fig. 4. *Gasterosteus aculeatus*. Section of heart; H&E stain. (A) Overview of heart; black rectangle outlines area shown in panel (B). (B) Detailed view of atrium and ventricle with abundant amphiphilic intracytoplasmic iridovirus inclusions (*)

heart, liver, kidney, intestine, stomach, and adjacent mesenteries. Among the organs included in the sections, the only organs with no bacterial colonies were the swimbladder, gas gland, and testis. DNA sequencing of amplified DNA (using universal bacterial primers) confirmed the bacteria was a *Streptococcus* species, but the organism could not be further identified (data not shown). The colonies were associated with very little inflammation. Two fish with iridovirus inclusions also had a few small streptococcal colonies.

TEM at the EML revealed cytomegalic cells with markedly expanded cytoplasm containing abundant intracytoplasmic hexagonal virus particles consistent with an icosahedral virion (Fig. 6). Complete virions within splenic tissues were 134 nm ($n = 25$, $SD = 2$ nm) in diameter as measured from side to side and 158 nm vertex to vertex ($n = 25$, $SD = 3$ nm).

PCR amplification, BLASTP, molecular dataset, and phylogenetic analysis

The degenerate PCR designed to target a portion of the viral DPOL gene generated a 629 bp amplicon. The MCP primers designed to amplify the full-length MCP gene sequence generated a 1426 bp PCR amplicon with a sequence identical to the nested PCR products (external 371 bp and internal 112 bp). The ATPase primers yielded a 756 bp PCR product that contained the majority of the TSIV ATPase gene sequence.

The BLASTP searches for the TSIV DPOL, MCP, and ATPase gene sequences had highest identity

with iridoviruses belonging to the genus *Megalocytivirus*, lower identity to viruses belonging to the genera *Lymphocystivirus* and *Ranavirus*, and even lower identity to members of the genera *Chloriridovirus* and *Iridovirus* (data not shown). BLASTP searches revealed 24 additional iridoviruses for which the homologous MCP and ATPase sequences could be obtained for the phylogenetic analysis.

Preliminary phylogenetic analysis revealed that there was no significant incongruence among individual gene trees (defined by the presence of incompatible bi-partitions that received a posterior probability of >90%, respectively). For the final analysis, we concatenated the sequence for the 2 genes into 1 matrix. This dataset contained 657 amino-acid characters (including gaps) for 25 viral taxa with at least 1 representative of each of the 5 genera within the family *Iridoviridae* (Table 1). Bayes factor comparisons in Tracer 1.4 revealed that partitioning the dataset by gene (2 partitions) did not produce a substantial improvement in model fit, so we used the concatenated amino-acid matrix (1 partition) for the final analysis.

The concatenated 2-gene Bayesian analysis demonstrated with a high level of confidence that the TSIV is an iridovirus belonging to the genus *Megalocytivirus* (Fig. 7). RSIV, RBIV, OSGIV, LYCIV, and sea bass iridovirus (SBIV) were monophyletic, collectively representing Genotype 1 (Fig. 7). ISKNV, Banggai cardinalfish iridovirus (BCIV), dwarf gourami iridovirus (DGIV), dwarf gourami iridovirus 2004 (DGIV2004), and African lampeye iridovirus (AFLIV) were monophyletic, representing Genotype 2. A third branch contained TRBIV and olive

Fig. 5. *Gasterosteus aculeatus*. (A) Branchial cavity contains sections of a copepod parasite (arrowheads); black rectangle outlines area shown in panel (B). (B) Gills contain foci of filamentous bacteria (arrows) that extend into the branchial cavity around eggs of parasitic copepods. (C) Higher magnification of a step section near the same cluster of eggs in panel (B); bacteria are Gram-negative (arrows). (D) Pharynx with teeth (t) and abundant iridovirus inclusions (*) in the propria-submucosa. (E) Choroid plexus and perineural connective tissue contains iridovirus inclusions (*). (F) Stomach contains iridovirus inclusions (*) in all layers. (G) Intestine contains iridovirus inclusions (*) in all layers; surface of epithelium has scattered myxosporean parasites (arrowheads); black box outlines area in inset. H&E stain for all micrographs except panel (C), which is a Twort's Gram stain

Table 1. Iridovirus samples and sequences used in the present study. DPOL: DNA polymerase, MCP: major capsid protein

Virus (abbreviation)	Genus	Genes (GenBank accession no.)	Reference
Invertebrate iridescent virus 3 (IIV3)	<i>Chloriridovirus</i>	Complete genome (NC_008187)	Delhon et al. (2006)
Invertebrate iridescent virus 6 (IIV6)	<i>Iridovirus</i>	Complete genome (NC_003038)	Jakob et al. (2001)
Ambystoma tigrinum virus (ATV)	<i>Ranavirus</i>	Complete genome (NC_005832)	Jancovich et al. (2003)
Frog virus 3 (FV3)	<i>Ranavirus</i>	Complete genome (NC_005946)	Tan et al. (2004)
Grouper iridovirus (GIV)	<i>Ranavirus</i>	Complete genome (AY666015)	Tsai et al. (2005)
Singapore grouper iridovirus (SGIV)	<i>Ranavirus</i>	Complete genome (NC_006549)	Song et al. (2004)
Tiger frog virus (TFV)	<i>Ranavirus</i>	Complete genome (AF389451)	He et al. (2002)
Soft-shelled turtle iridovirus (STIV)	<i>Ranavirus</i>	Complete genome (NC_012637)	Huang et al. (2009)
Epizootic hematopoietic necrosis virus (EHNIV)	<i>Ranavirus</i>	Complete genome (FJ433873)	Jancovich et al. (2010)
Lymphocystis disease virus 1 (LCDV1)	<i>Lymphocystivirus</i>	Complete genome (NC_001824)	Tidona & Darai (1997)
Lymphocystis disease virus isolate China (LCDVC)	<i>Lymphocystivirus</i>	Complete genome (NC_005902)	Zhang et al. (2004)
Infectious spleen and kidney necrosis virus (ISKNV)	<i>Megalocytivirus</i>	Complete genome (NC_003494)	He et al. (2001)
Large yellow croaker iridovirus (LYCIV)	<i>Megalocytivirus</i>	Complete genome (AY779031)	J. Ao & X. Chen (unpubl.)
Orange spotted grouper iridovirus (OSGIV)	<i>Megalocytivirus</i>	Complete genome (AY894343)	Lu et al. (2005)
Rock bream iridovirus (RBIV)	<i>Megalocytivirus</i>	Complete genome (AY532606)	Do et al. (2004)
Red sea bream iridovirus (RSIV)	<i>Megalocytivirus</i>	Complete genome (BD143114)	Nakajima & Kurita (2005)
Turbot reddish body iridovirus (TRBIV)	<i>Megalocytivirus</i>	Complete genome (GQ273492)	Shi et al. (2010)
Olive flounder iridovirus (OFLIV)	<i>Megalocytivirus</i>	ATPase (EU276418), MCP (EU276417)	J. B. Jeong et al. (unpubl.)
Dwarf gourami iridovirus (DGIV)	<i>Megalocytivirus</i>	ATPase (AY319288), MCP (AY285744)	Sudthongkong et al. (2002a,b)
African lampeye iridovirus (AFLIV)	<i>Megalocytivirus</i>	ATPase (AB043979), MCP (AY285745)	Sudthongkong et al. (2002a,b)
Sea bass iridovirus (SBIV)	<i>Megalocytivirus</i>	ATPase (AB043977), MCP (AY310917)	Sudthongkong et al. (2002a,b)
Dwarf gourami iridovirus 2004 (DGIV2004)	<i>Megalocytivirus</i>	ATPase (AY989902), MCP (AY989901)	Go et al. (2006)
Murray cod iridovirus (MCIV)	<i>Megalocytivirus</i>	ATPase (AY936204), MCP (AY936203)	Go et al. (2006)
Banggai cardinalfish iridovirus (BCIV)	<i>Megalocytivirus</i>	ATPase (EU753256), MCP (HQ857787), DPOL (EU753254)	Weber et al. (2009), T. B. Waltzek (unpubl.)
Threespine stickleback iridovirus (TSIV)	<i>Megalocytivirus</i>	ATPase (HQ857786), MCP (HQ857785), DPOL (HQ857784)	Present study

flounder iridovirus (OFLIV), representing Genotype 3. The dataset did not resolve the relationships of the aforementioned 3 clades (genotypes) but together they formed a monophyletic group to which TSIV was found to be the sister group.

The sequence identity for the Genotype 1 isolates revealed the highest percent identity with each other for all 3 genes, next highest with the Genotype 2 isolates, followed by the Genotype 3 isolates, and

substantially lower sequence identity with TSIV (Table 2). TSIV consistently had the lowest sequence identity irrespective of gene or clade compared, illustrating its obvious genetic distance from the other megalocytiviruses. The isolates from the 3 genotypes exhibited between 90.7 and 99.8% nucleotide sequence identity to each other for the 3 genes, whereas a similar comparison of these genotypes with TSIV revealed 72.6 to 84.4% identity (Table 2).

Fig. 6. *Gasterosteus aculeatus*. (A) Electron micrographs of splenic parenchyma include erythrocytes (e) and numerous virus-infected cells, including thin processes of endothelial cells (arrowheads). (B) A representative cell from the middle of panel (A) contains abundant cytoplasmic icosahedral virions that are consistent with an iridovirus

Fig. 7. Phylogram depicting the relationship of the threespine stickleback iridovirus (TSIV) to representatives from each of the 5 genera in the family *Iridoviridae*, based on the concatenated deduced amino-acid sequences of the major capsid protein and ATPase genes (657 amino-acid characters including gaps). Members of the genera *Chloriridovirus* (IIV3) and *Iridovirus* (IIV6) were included as outgroups to root the tree. Numbers above or below each node represent posterior probabilities (values >90 shown) of the Bayesian analysis. Branch lengths are based on the number of inferred substitutions, as indicated by the scale. See Table 1 for virus abbreviations

DISCUSSION

Systemic iridovirus infections were associated with morbidity and mortality among wild-caught threespine stickleback during captivity. Infections were characterized by megalocytic cells in numerous tissues and organs. TEM revealed abundant cytoplasmic icosahedral virions consistent with an iridovirus. Phylogenetic analyses indicated the TSIV to be a previously unknown iridovirus species in the genus *Megalocytivirus*. Megalocytiviruses, such as TSIV, are emerging aquatic animal pathogens of economic and ecologic importance.

Our report is the first description of a megalocytivirus from a wild temperate North American fish species, extending the range of these viruses into the family *Gasterosteidae*.

Mao et al. (1999) described a North American ranavirus epizootic involving sympatric populations of threespine stickleback and red-legged frog tadpoles, but this virus is clearly different from TSIV. The first North American report of a systemic iridoviral dis-

Table 2. Analysis of the major capsid protein (MCP) (1362 nucleotide/454 amino-acid characters), ATPase (501 nucleotide/167 amino-acid characters), and DNA polymerase (DPOL) (570 nucleotide/190 amino-acid characters) among megalocytiviruses. The values are expressed as a range of percent identities. Genotype 1 includes RSIV*, RBIV*, OSGIV*, LYCIV*, and SBIV; Genotype 2 includes ISKNV*, BCIV*, DGIV, DGIV2004, MCIV, and AFLIV; Genotype 3 includes TRBIV* and OFLIV (only taxa with an asterisk* could be compared for DPOL). See Table 1 for virus abbreviations

	Genotype 1	Genotype 2	Genotype 3	TSIV
MCP				
Genotype 1	97.2–99.9/98.2–100	94.0–95.2/97.1–98.6	93.6–94.5/96.6–98.0	78.7–79.2/84.7–85.4
Genotype 2		98.0/99.5–100	93.6–94.0/98.0	80.0–80.3/85.6
Genotype 3			99.1/99.5	79.9/85.2–85.6
ATPase				
Genotype 1	98.2–100/99.4–100	94.8–99.8/98.8–99.4	95.4–96.0/97.6–98.8	83.0–83.6/93.4–94.0
Genotype 2		95.2–100/98.8–100	95.8–96.8/97.6–99.4	83.4–84.4/93.4
Genotype 3			99.6/99.4	84.2/92.2–92.8
DPOL				
Genotype 1	97.7–99.8/97.8–100	94.2–94.7/95.7–96.8	91.9–92.1/95.7	72.6–73.1/76.3
Genotype 2		99.2/100	90.7/95.2	74.9–75.4/78.4
Genotype 3				74.5/76.8

ease associated with high mortality was restricted to a cichlid (ram cichlid *Mikrogeophagus ramirezi*) imported from South America (Leibovitz & Riis 1980). Other reports of systemic iridoviral infections in North America include a South American cichlid (angelfish *Pterophyllum scalare*) obtained from a Canadian ornamental fish retailer (Schuh & Shirley 1990) as well as Florida farmed-raised gourami species (Fraser et al. 1993, Petty & Fraser 2005) and cichlids (tilapia *Oreochromis niloticus*, oscar *Astronotus ocellatus*, and angelfish *Pterophyllum scalare*) (Smith et al. 1997, McGrogan et al. 1998, Petty & Fraser 2005). Finally, a systemic iridovirus closely related to ISKNV was recently characterized from several commercial Asian imports of a marine ornamental apogonid (Banggai cardinalfish *Pterapogon kauderni*) into the USA (Weber et al. 2009). Isolation of viruses from a freshwater poeciliid (guppy *Poecilia reticulata*) and a marine labrid (cleaner wrasse *Labroides dimidiatus*) as part of routine diagnostic screening of ornamental fishes originating from SE Asia at the FHL facilitated the initial characterization (Hedrick & McDowell 1995) and latter confirmation of these isolates as ranaviruses (Holopainen et al. 2009). Thus, before the present study, North American megalocytiviruses have always been associated with exotic tropical fishes and in most cases were associated with a recent history of importation from Asia. Furthermore, the unregulated international movement of live fish in the ornamental fish industry has long been recognized as a risk to the health of global aquaculture and aquatic ecosystems (Gratzek et al. 1978, Leibovitz & Riis 1980, Hedrick 1996, Whittington & Chong 2007).

In contrast to the highly virulent (i.e. mortality up to 100%) megalocytiviruses from exotic tropical and subtropical fishes, TSIV was associated with much lower mortality (Fig. 1), occurring at cooler water temperatures. Evidence suggesting that TSIV contributed to the low-level mortality at the Vancouver Aquarium includes the fact that 9 of the 11 sampled dead or moribund fish had severe viral-induced lesions and few other lesions to explain their disease or death. Further, while all groups of fish examined in the present study were infected with other organisms, most of the parasites were common among fish sampled as part of the DFO fish health screens when mortality was low. Additionally, mortality at the aquarium was not curbed following regular formalin baths nor were bacterial pathogens grown throughout the study. Interestingly, some seemingly healthy fish sampled during the DFO fish health screens had abundant viral-induced lesions. It is unknown if these seemingly healthy viral-infected fish were in fact weaker and perhaps may have comprised the majority of the low-level daily mortality observed at the DFO because daily mortalities were not sampled. Ultimately, future studies aimed at isolation of the virus would facilitate controlled experimental studies to elucidate important biologic properties including pathogenicity, host range, and permissive temperature range.

Based on the biological properties, electron microscopy, histopathology, and phylogenetic findings, we propose that TSIV should be considered for approval by the International Committee on the Taxonomy of Viruses as a second species within the genus *Megalocytivirus*. Currently, the genus *Megalocytivirus*

contains the type species ISKNV (Chinchar et al. 2005). Given the high sequence identity for several key genes, including for MCP and ATPase, all other described megalocytiviruses are currently considered strains of ISKNV. The results of our phylogenetic analysis are in good agreement with previous phylogenetic analyses aimed at understanding the relationships of these ISKNV strains (Sudthongkong et al. 2002a, Do et al. 2005a,b, Nakajima & Kurita 2005, Go et al. 2006, Imajoh et al. 2007, Wang et al. 2007, Song et al. 2008, Shinmoto et al. 2009, Weber et al. 2009, Dong et al. 2010, Shi et al. 2010). Interestingly, TSIV has the greatest genetic distance from the rest of the ISKNV strains (Table 2). Additional evidence of the distinctness of TSIV is provided by the phylogenetic analysis that supports inclusion of this novel virus in the genus *Megalocytivirus* separated on a long branch by itself as the sister group to the rest of the ISKNV strains (Fig. 7).

ISKNV strains have primarily been observed in Asian aquaculture, Asian wild stocks, or from ornamental fish exported from Asia. It is interesting to speculate whether TSIV represents the North American equivalent, having eluded previous detection due to the inherent difficulties involved with viral discovery in wild fish and/or the presumed lower pathogenicity of this new agent. Regardless, the genetic distance separating TSIV from ISKNV implies that these viruses split early in the evolutionary history of the megalocytiviruses. If the observed low virulence of TSIV is confirmed, future comparative genomic studies including TSIV might provide insight into the genetic basis of the marked pathogenicity observed among more tropically occurring megalocytiviruses.

Systemic iridoviruses belonging to the genera *Ranavirus* and *Megalocytivirus* have resulted in significant losses in wild and cultured fish stocks (Chua et al. 1994, Matsuoka et al. 1996, Ahne et al. 1997, Chou et al. 1998, Jung & Oh 2000, Kawakami & Nakajima 2002, Goldberg et al. 2003, Lancaster et al. 2003, Shi et al. 2004), ornamental fish stocks (Leibovitz & Riis 1980, Armstrong & Ferguson 1989, Schuh & Shirley 1990, Anderson et al. 1993, Fraser et al. 1993, Rodger et al. 1997, Paperna et al. 2001, Sudthongkong et al. 2002b, Petty & Fraser 2005, Go et al. 2006, Lyu et al. 2006, Whittington & Chong 2007, Jeong et al. 2008a,b, Weber et al. 2009, Kim et al. 2010), as well as cultured and wild reptile and amphibian populations (Chen et al. 1999, Daszak et al. 1999, 2003, Chinchar 2002, Whittington et al. 2010). Given the widespread distribution of the threespine stickleback in the northern hemisphere,

its anadromous nature, and the low host specificity of other megalocytiviruses, future sampling of stickleback populations as well as other sympatric marine and freshwater ichthyofauna seems warranted.

During final acceptance of our manuscript, Marcos-Lopez et al. (2011) reported microscopic and ultrastructural features from a single threespine stickleback obtained from the same source as our fish (DFO Nanaimo); the findings were consistent with a megalocytivirus, but gene sequencing was not reported.

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Appendix 1. Fish health screens

Table A1. *Gasterosteus aculeatus*. Summary of results from diagnostic evaluations conducted during holding of threespine stickleback before transfer to Vancouver Aquarium on 20 March 2008. EPC: epithelioma papillosum cyprini cell line, CHSE-214: Chinook salmon embryo 214 cell line, TSA: trypticase soy agar, TSIV: threespine stickleback iridovirus

Method	No. of fish examined	18 Oct 2007	5 Nov 2007	10 Dec 2007	15 Jan 2008
Virology (EPC and CHSE-214)	10 (2 × 5-fish pools)	All negative	All negative	All negative	All negative
Bacteriology (kidney swab on TSA and on TSA with salt)	10	No significant growth	No significant growth	No significant growth	No significant growth
Gross exam; gill wet mounts; Gram stain of kidney and gill smears	Variable	1 of 20 with anisakid parasite in coelomic cavity	1 of 10 gills with <i>Bomolochus cuneatus</i> (copepod); 1 of 10 kidneys with <i>Myxobilatus</i> sp. (myxosporean)	1 of 10 kidneys with <i>Myxobilatus</i> sp.	2 of 10 gills with <i>Ergasilus</i> sp.; 1 of 10 kidneys with <i>Sphaerospora</i> sp. (probably <i>S. elegans</i>); 1 of 10 kidney with unidentified microparasite
Histopathology	Variable	1 of 5 with nematode in coelomic cavity; 1 of 5 livers with a coccidian (<i>Eimeria</i> sp.)	2 of 10 gills with epitheliocystis	2 of 5 gills with <i>Dermocystidium</i> sp. (?)	2 of 10 gills with epitheliocystis; 1 of 10 gills with a myxosporean; 3 of 7 kidneys with intratubular myxosporean pansporoblasts (<i>Myxobilatus gasterostei</i> ?); 2 of 9 intestines with intraluminal bacterial overgrowth; 1 of 7 intestines with digenetic trematode
TSIV inclusions (histopathology)	Variable	0 of 5	3 of 10 spleens	4 of 5 gills, livers, spleens, kidneys, hearts	2 of 3 spleens