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RESEARCH ARTICLE

Novel *Aquareovirus* isolated from channel catfish (*Ictalurus punctatus*) used in mussel restoration efforts in Wisconsin

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Abstract

Channel catfish (*Ictalurus punctatus*) are a food fish extensively reared in aquaculture facilities throughout the world and are also among the most abundant wild catfish species in North America, making them a popular target of anglers. Furthermore, channel catfish are important members of aquatic ecosystems; for example, they serve as a glochidial host for the endangered winged mapleleaf mussel (*Quadrula fragosa*), making them critical for conserving this species through hatchery-based restoration efforts. During a routine health inspection, a novel aquareovirus was isolated from channel catfish used in mussel propagation efforts at a fish hatchery in Wisconsin. This virus was isolated on brown bullhead cells (ATCC CCL-59) and identified through metagenomic sequencing as a novel member of the family *Spinareoviridae*, genus *Aquareovirus*. The virus genome consists of 11 segments, as is typical of the aquareoviruses, with phylogenetic relationships based on RNA-dependent RNA polymerase and major outer capsid protein amino acid sequences showing it to be most closely related to golden shiner virus (aquareovirus C) and aquareovirus C/American grass carp reovirus (aquareovirus G) respectively. The potential of the new virus, which we name genictpun virus 1 (GNIPV-1), to cause disease in channel catfish or other species remains unknown.

KEYWORDS
aquareovirus, channel catfish, *Ictalurus punctatus*, Wisconsin

1 | INTRODUCTION

Channel catfish (*Ictalurus punctatus*) are moderately sized, deep-bodied fish with a distribution ranging across eastern North America (Pfleiger, 1997). They are a prized catch for recreational anglers and, because they make excellent table fare, represent one of the most popular fish raised in commercial aquaculture operations in the United States (Pfleiger, 1997). In addition to being raised in captivity for food or angling opportunities, these fish play an important role in the propagation of at-risk mussel species.

Channel catfish serve as glochidial (larval) hosts for the winged mapleleaf (*Quadrula fragosa*), a federally endangered species that has declined to the point that only a few reproducing populations remain (Hove et al., 2012). In order to reproduce, the female mussel uses a specialized extension of its mantle that resembles a prey fish (a 'mantle lure') to entice fish to bite the mantle tissue, which triggers the release of glochidia. These parasitic larval mussels then attach to the

gills and develop over the course of months until they are ready to drop off the fish and begin filter feeding. A trial that tested 67 different fish species, as well as the common mudpuppy (*Necturus maculosus*), found that only channel catfish and blue catfish (*I. furcatus*) were suitable hosts for the winged mapleleaf (Hove et al., 2012).

Due to rapidly declining *Q. fragosa* populations, efforts are underway to augment populations through captive propagation. These efforts require that channel catfish also be brought into propagation facilities to serve as hosts. Typically, winged mapleleaf are cultured by bringing gravid females into a hatchery and manually infesting the gills of channel catfish with glochidia. The catfish are then placed into cages in areas of a river with suitable winged mapleleaf habitats (Brady et al., 2011). Healthy channel catfish populations are critical to the success of these restoration efforts.

There are several diseases associated with culturing channel catfish. Despite the routine health testing associated with holding fish in captivity, only a few viruses have been identified in this important fish species. Channel catfish virus (Ictalurid herpesvirus-1; *Alloherpesviridae*) is a herpesvirus known to result in catastrophic losses of young catfish and represents the most significant challenge to maintaining healthy aquaculture facilities (USFWS and AFS-FHS, 2020). Two reoviruses have also been identified from *I. punctatus* that caused disease outbreaks in captive populations in North America and China (Hedrick et al., 1984; Xu et al., 2013). Herein, we describe and characterize a novel aquareovirus isolated from a channel catfish population used in the propagation of freshwater mussels.

2 | METHODS

2.1 | Tissue cell culture

Sampling of channel catfish for the presence of viruses followed the methods outlined in USFWS and AFS-FHS (2020). The fish, apparently healthy 11-month-old *I. punctatus* originally sourced from the upper Mississippi River drainage, were sampled as part of a routine inspection at the Genoa National Fish Hatchery (Vernon County, WI). Briefly, kidney and spleen tissue samples were collected, placed in HBSS, homogenized, diluted (1:1) and inoculated onto Chinook salmon embryo cells (CHSE-214; Lannan et al., 1984), *epithelioma papulosum cyprinid* cells (EPC; ATCC CRL-2872; Winton et al., 2010) and brown bullhead cells (BB; ATCC CCL-59). The samples inoculated onto CHSE and EPC cells were incubated at 15°C while those on BB cells were held at 25°C. The sample plates were monitored for the development of cytopathic effect (CPE) 3 days per week for 21 days. On day 14, blind passages were performed and monitored for CPE three times a week for an additional 14 days.

2.2 | PCR

The kidney and spleen samples collected from the channel catfish were screened for the presence of CCV using the qPCR assay described in Woo and Cipriano (2017). Tissues were extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Germany) and ran with IDT PrimeTime Mastermix (IDT, Iowa, USA) with primers IcHV1 forward and IcHV1 reverse, as well as two probes (IcHV1a and IcHV1b), which are able to detect CCV genotypes a and b, respectively (Woo & Cipriano, 2017). Cell culture wells displaying CPE were also tested for the presence of CCV using the same methods and qPCR assay mentioned above.

2.3 | Metagenomics and phylogenetics

Because we could not identify the putative virus using PCR, we conducted metagenomic sequencing and subsequent bioinformatic analyses as previously described for an unknown isolate from largemouth bass (*Micropterus salmoides*) (Sibley et al., 2016). Briefly, we extracted total nucleic acids from 200 µL supernatant from BB cells showing CPE using the QIAamp MinElute Virus Kit (Qiagen, Hilden, Germany), omitting carrier RNA. We then reverse-transcribed RNA to cDNA using the Superscript IV system (Thermo Fisher, Waltham, MA, USA) with random hexamers and prepared DNA-sequencing libraries using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA). We sequenced the resulting libraries on an Illumina MiSeq instrument using the 600-cycle MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA), with sequencing adapters trimmed using on-board Illumina software. We then removed sequences of known laboratory reagents using *CLC Genomics Workbench v23* (Qiagen, Hilden, Germany) and conducted de novo assembly of the remaining sequences using *SPAdes-meta v3.15.5* (Bankevich et al., 2012). We compared resulting contiguous sequences (contigs) at the translated protein level to GenBank databases of known viruses using the blastx algorithm (Altschul et al., 1997).

To place the virus phylogenetically, we collected amino acid sequences from member species (defined by the International Committee on the Taxonomy of Viruses; ICTV, https://ictv.global/taxonomy/) of the genera *Aquareovirus* and *Orthoreovirus* and used them for phylogenetic analysis targeting the RNA-dependent RNA polymerase and major outer capsid genes, which form the basis for species demarcation within the genus. We aligned sequences using *MUSCLE v.3.8* (Edgar, 2004) and trimmed the alignments using *trimAL v.1.4.1* (gap threshold = 0.8; Capella-Gutiérrez et al., 2009) with minor manual adjustments made as needed. We then inferred maximum-likelihood phylogenetic trees using *PhyML v.3.0* with Smart Model Selection (SMS) and 1000 bootstrap replicates implemented in *NGPhylogeny* (Lefort et al., 2017; Lemoine et al., 2019). Mycovirus 1 was used as an outgroup, and resulting phylogenetic trees were visualized using *FigTree v.1.4.4*.

2.4 | Transmission electron microscopy

After CPE was observed on BB cells, 1.5 mL of the cell culture isolate was clarified by centrifugation at 1200 rcf for 10 min, and free viral particles from 450 µL of the supernatant were pelleted by air-driven ultra-centrifugation at 30 psi for 30 min (equivalence to 95,000 rpm) in an Airfuge (Beckman Coulter, Brea, CA). After removal of the

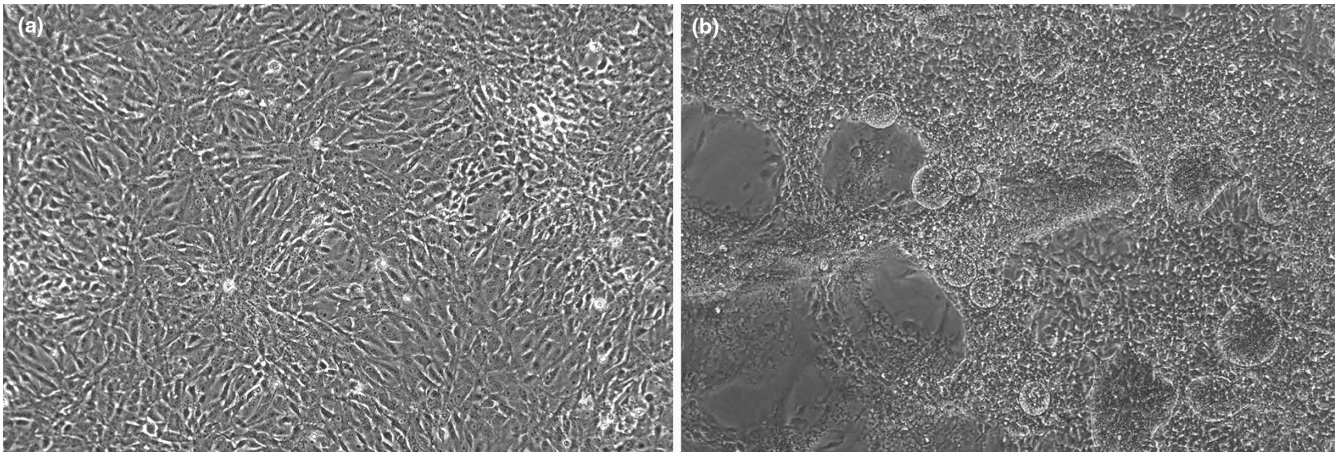


FIGURE 1 (a) Uninoculated brown bullhead cells; (b) brown bullhead cells 2 days after being inoculated with channel catfish reovirus.

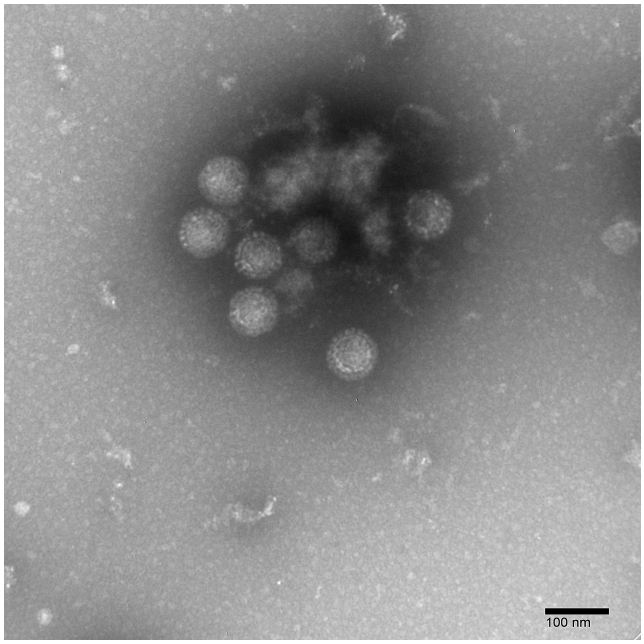


FIGURE 2 Transmission electron microscopy photos of virus particles isolated from channel catfish (*Ictalurus punctatus*) at Genoa National Fish Hatchery in Wisconsin.

supernatant, pelleted sample was resuspended in 10ul of distilled water and applied to a 300-square copper mesh grid and negatively stained using a 2% phosphotungstic acid solution. The grids were viewed and photographed on a Hitachi H76000 TEM at 50,000–60,000 times magnification.

3 | RESULTS

3.1 | Cell culture and PCR

Monolayers of BB cells did not show any signs of change after the initial 21 days of sample incubation. CPE (Figure 1) was observed during the blind passage on day 8 and the samples were reset onto a fresh monolayer which was identified as positive for a virus 3 days later. CPE was not observed on the EPC and CHSE cells at 15°C. Molecular analysis via qPCR did not identify CCV in the tissue samples nor cell culture isolates indicating the presence of a potential novel virus. The samples were sent to the University of Wisconsin-Madison for metagenomic analysis. Isolation of this virus resulted in removal of this group of hatchery fish out of an abundance of caution.

3.2 | Transmission electron microscopy

Clusters of spherical viral particles of 70–80 nm in diameter were observed under electron microscopy. The 60–70 nm inner core, or capsid, is visible within the virus structure. This dense capsid is surrounded by a thin outer shell which has pronounced 10–20 nm spiked proteins or turrets attached (Figure 2). The viral structure and size of the observed virions are consistent with the reoviruses (King et al., 2012).

3.3 | Metagenomics and phylogenetics

Metagenomic analyses identified 11 segments of a novel aquareovirus (Table 1) ranging in length from 801 to 3936 nucleotides and representing a nearly coding-complete genome (with the exception of segment 8). Depending on the segment analysed, the new virus is most closely related to either golden shiner virus (aquareovirus C) or American grass carp reovirus (aquareovirus G) (Table 1). The new virus has only 26.98% amino acid identity to its closest relative (aquareovirus C) at the VP7 major outer capsid protein and only 71.93% amino acid identity to its closest relative (aquareovirus G) at the VP2 RNA-dependent RNA polymerase, meeting the species demarcation criteria for aquareoviruses specified by ICTV. We have named the new virus genictpun virus 1 (GNIPV-1) to reflect its origin from Genoa National Fish Hatchery in *I. punctatus*. Phylogenetic analysis confirms that GNIPV-1 is most closely related to either aquareovirus C based on RNA-dependent RNA polymerase amino acid sequences or to both aquareovirus C and G based

TABLE 1 Features of 11 segments of genictpun virus 1 (GNIPV-1) from channel catfish.

Segment	GenBank accession	Segment length (nt)	Coverage	ORF length (aa)	Protein ID	Putative protein	% id to AqRV-C (aa)	% id to AqRV-G (aa)
1	PP035844	3936	105.3451	1302	VP1	Guanylyl transferase/methyl transferase	66.13	60.48
2	PP035845	3855	91.24421	1274	VP2	RNA-dependent RNA polymerase	79.83	71.93
3	PP035846	3692	127.4467	1216	VP3	NTPase/helicase	81.65	77.02
4	PP035847	1989	43.85522	648	Mu-1/Mu-1C	Major virion structural protein	70.06	71.36
5	PP035848	2212	23.44367	725	VP5	Core protein NTPase	62.73	57.56
6	PP035849	1982	86.74831	600	NS1	Non-structural protein 1	51.16	45.51
7	PP035850	1431	35.49709	315	NS4 and NS5	Non-structural proteins 4 and 5	41.54	43.36
8	PP035851	1248	15.20802	413	VP6	Core protein	67.51	58.12
9	PP035852	1071	35.24933	352	NS2	Non-structural protein 2	61.93	53.41
10	PP035853	801	18.90239	260	VP7	Major outer capsid protein	26.98	26.00
11	PP035854	810	26.44992	258	NS3	Non-structural protein 3	46.03	40.52

Note: %id is to the closest GenBank match for each segment. Segment 8 is missing the 3′ end of the VP6 gene.

on major outer capsid protein amino acid sequences (Figure 3). For both genes, the phylogenetic distance between GNIPV-1 and its closest relatives exceeds that between other pairs of recognized *Aquareovirus* species.

4 | DISCUSSION

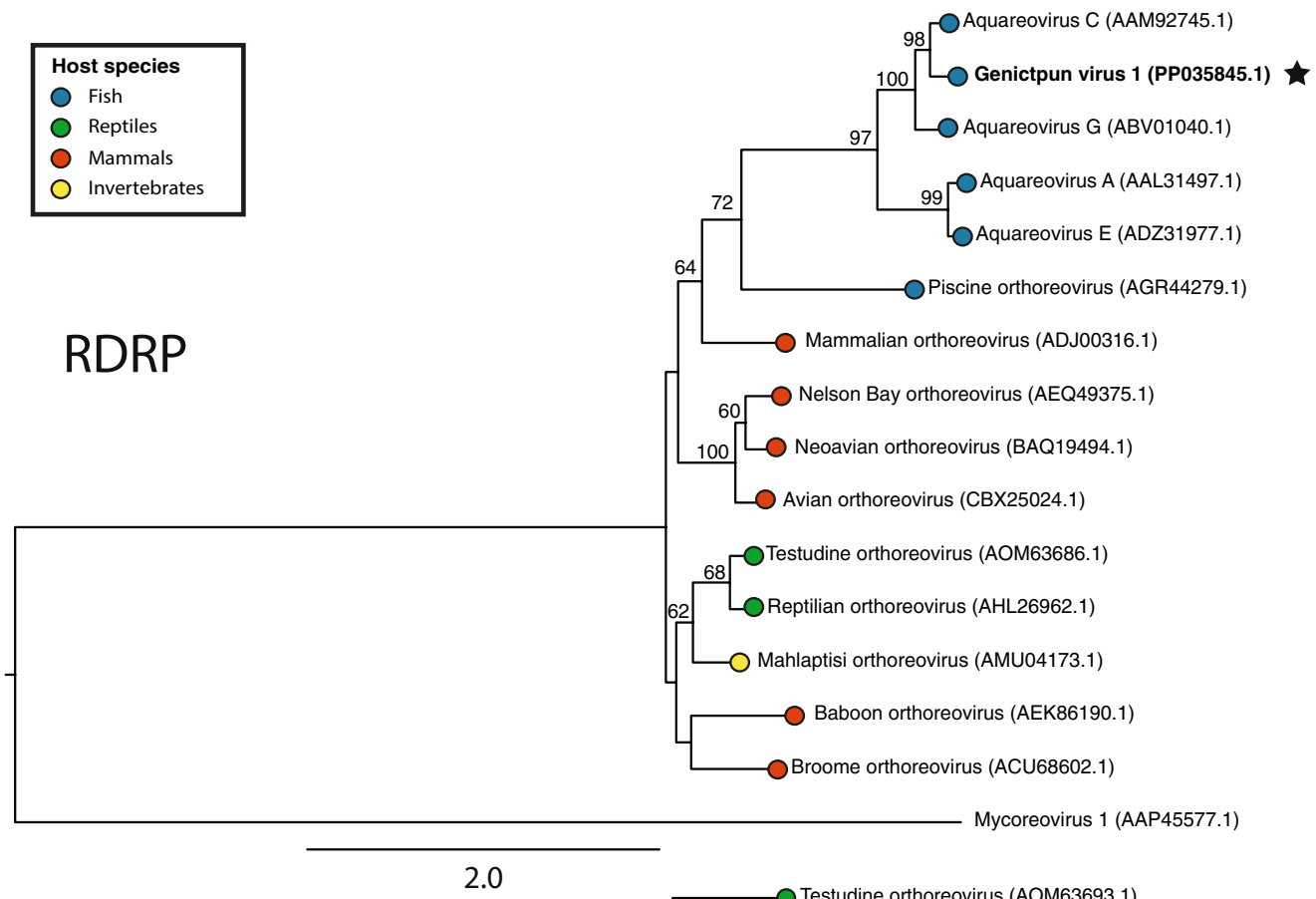
The channel catfish examined during this investigation appeared otherwise healthy and it is unknown whether GNIPV-1 is pathogenic. However, over the past several years, there have been recurrent disease outbreaks in hatchery channel catfish when these hosts were infected with glochidia. During periods of morbidity, diagnostic investigations were not able to identify any viruses. Future work, including diagnostic assays to identify this virus rapidly and cheaply, would be valuable not only to confirm its presence in samples screened using cell culture but also in surveys for this virus in captive and wild populations as well as for in vivo trials evaluating its growth kinetics and pathogenicity.

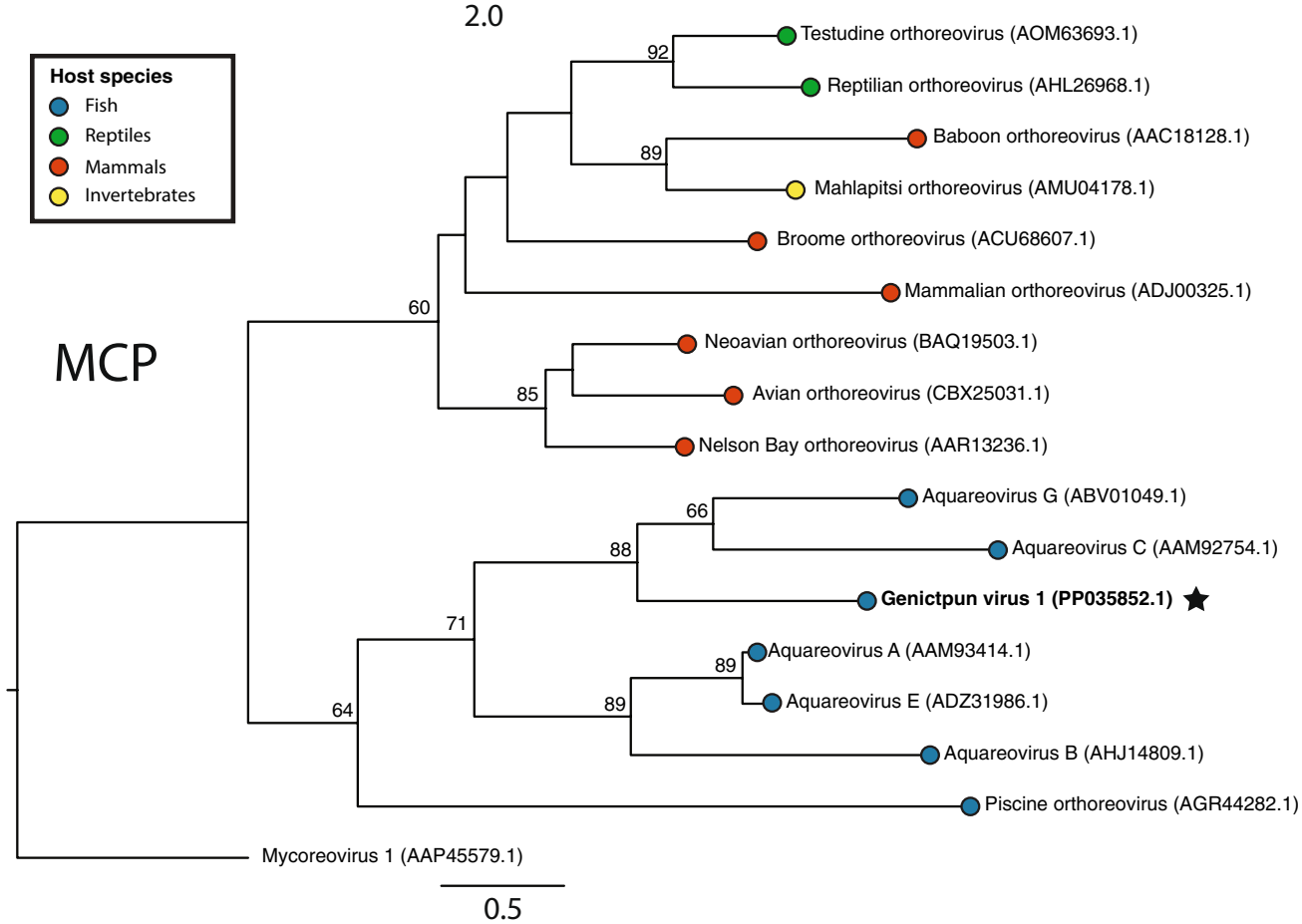
GNIPV-1 likely represents a novel species within the genus *Aquareovirus*. We note that aquareovirus D, referred to by ICTV as 'channel catfish reovirus', appears to have been identified in the same host species as GNIPV-1. Unfortunately, no reference sequences for aquareovirus D exist in GenBank or any other public database, to our knowledge. Also unfortunately, and despite extensive efforts in conjunction with members of the ICTV Reovirales subcommittee, we were unable to identify any data pertaining to the origin of aquareovirus D or its genomic sequence. It is therefore currently impossible to ascertain whether GNIPV-1 is the same virus as aquareovirus D or whether GNIPV-1 is another virus isolated from the same host species. If source and sequence data for aquareovirus D cannot be located and made public, we recommend that GNIPV-1 be designated aquareovirus D and that data pertaining to GNIPV-1 be substituted for data currently associated with aquareovirus D. This change would cause minimal disruption to virus taxonomy, since both GNIPV-1 and the currently designated aquareovirus D share the same host species.

Mussel restoration efforts require healthy fish hosts to either produce progeny in a hatchery setting or allow infested host fish to be placed in cages where suitable mussel habitat is present (Lima et al., 2012). Methods for in vitro cultivation of glochidia have been described where glochidia are transformed in a laboratory setting beyond the parasitic stage to a free-living juvenile stage without the use of a fish host (Lima et al., 2012). Such methods have been shown to be successful to the extent that when glochidia were reared in vitro, no differences were observed when compared to those cultured using traditional in vivo methods. Furthermore, the young raised through in vitro methods were able to successfully produce progeny capable of infecting a fish host (Douda et al., 2021). These methods have been successful for a variety of mussel species; however, others remain difficult to raise in vitro (Lima et al., 2012). Future work expanding these techniques to allow for the in vitro culture of additional species could allow for hatcheries to meet rearing goals while circumventing the risks associated with bringing fish hosts onto a hatchery.

Recently, a novel rhabdovirus was described from the western pearlshell mussel (*Margaratifera falcata*), representing the first

FIGURE 3 Maximum-likelihood phylogenetic trees of RNA-dependent RNA polymerase (RDRP; top) and major outer capsid protein (MCP; bottom) amino acid sequences for members of the genera *Aquareovirus* and *Orthoreovirus*. The virus identified in this study is indicated with a star. Virus names are followed by GenBank accession numbers in parentheses. Bootstrap values are based on 1000 replicates. Scale bar is proportional to the number of amino acid substitutions per site.





member of the subfamily *Gammarhabdovirinae* to infect a host other than a finfish (Goldberg et al., 2023). Goldberg et al. (2023) hypothesized that transfer of viruses could occur during the glochidial stage where larval mussels are actively feeding on fish tissue/blood. While we do not suspect this sort of transmission has occurred with channel catfish aquareovirus, and no aquareoviruses have been described from mussels thus far (Knowles et al., 2023), the potential for viruses to be transferred between fish and mussels is worthy of future study and consideration.

AUTHOR CONTRIBUTIONS

Eric Leis: Conceptualization; writing – original draft; writing – review and editing; methodology. **Ryan Katona:** Investigation; writing – original draft; writing – review and editing; formal analysis. **Sara Dziki:** Investigation; writing – original draft; writing – review and editing; methodology; formal analysis. **Rebekah Mccann:** Investigation; writing – original draft; writing – review and editing; formal analysis. **Isaac Standish:** Investigation; writing – review and editing; writing – original draft; formal analysis. **Eryn Opgenorth:** Visualization; writing – review and editing; writing – original draft; formal analysis. **Audrey Dikkeboom:** Visualization; writing – review and editing; writing – original draft; formal analysis. **Ailam Lim:** Writing – original draft; writing – review and editing; visualization; formal analysis. **Charlotte E. Ford:** Writing – original draft; writing – review and editing; formal analysis. **Tony L. Goldberg:** Conceptualization; writing – original draft; writing – review and editing; methodology.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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