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## A NOVEL PICORNA-LIKE VIRUS IN A WABASH PIGTOE (*FUSCONAIA FLAVA*) FROM THE UPPER MISSISSIPPI RIVER, USA

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### ABSTRACT

Unionid mussels are threatened by multiple environmental stressors and have experienced mass mortality events over the last several decades, but the role of infectious disease in unionid health and population declines remains poorly understood. Although several microbial agents have been found in unionids, to date only one virus has been documented—Lea plague virus (*Arenaviridae*) in propagated Triangle Shell mussels (*Hyriopsis cumingii*) in China. We used next-generation DNA sequencing to screen hemolymph of seven individuals of five unionid species from the Upper Mississippi River basin, USA for viruses. We identified the complete polyprotein gene of a novel picornalike virus in one individual of the Wabash Pigtoe (*Fusconaia flava*). The virus is a member of the Nora virus clade of picornalike viruses and is most closely related to viruses from arthropods in China. We did not detect viruses in another Wabash Pigtoe or in animals of the other four species. It is premature to make inferences about the role of this virus in the health of Wabash Pigtoes or other unionid species or the origin or transmission of this virus. Nevertheless, to our knowledge, our results represent the first report of a virus in wild North American unionids. Technologies based on next-generation DNA sequencing should prove useful for identifying new viruses and investigating their role in unionid health and disease.

**KEY WORDS:** Unionidae, *Fusconaia flava*, Wabash Pigtoe, Mississippi River, virus, next-generation DNA sequencing

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### INTRODUCTION

Freshwater mussels (order Unionida) face mounting threats from habitat loss and alteration, invasive species, poor water quality and pollutants, hydrologic changes, and other stressors (Strayer et al. 2004; Dudgeon et al. 2006; Downing et al. 2010; Haag and Williams 2014). Unexplained mortality events have been documented since at least the 1970s, but their causes remain poorly understood (Haag and Williams 2014). Unionids are susceptible to a variety of metazoan, protozoan, fungal, and viral infections (Carella et al. 2016), which may contribute to mussel mortality as primary or secondary factors. Recently, we described a coordinated effort to investigate potential pathogens associated with unionid mass mortality events (Leis et al. 2018).

Viruses are likely culprits in mass die-offs of wildlife species, accounting for a higher percentage of disease-associated events across all animal taxa than other classes of pathogens (Fey et al. 2015). Viruses are also more likely to emerge (appear in new places, new hosts, and new clinical contexts) than other classes of pathogens because of their error-prone replication and ensuing ability to mutate, evolve, and “jump” to new species (Woolhouse et al. 2005). Viruses are major causes of mortality in marine bivalves (Zannella et al. 2017). To our knowledge, the only virus described from unionids to date is Lea plague virus, an arenavirus (family *Arenaviridae*) responsible for mass mortality of Triangle Shell mussels (*Hyriopsis cumingii* Lea) in southern China (Carella et al. 2016); these mussels are cultivated at high density for freshwater pearl production. We surveyed five unionid species from the Upper Mississippi River basin, USA to investigate whether viruses may be present in North American unionids.

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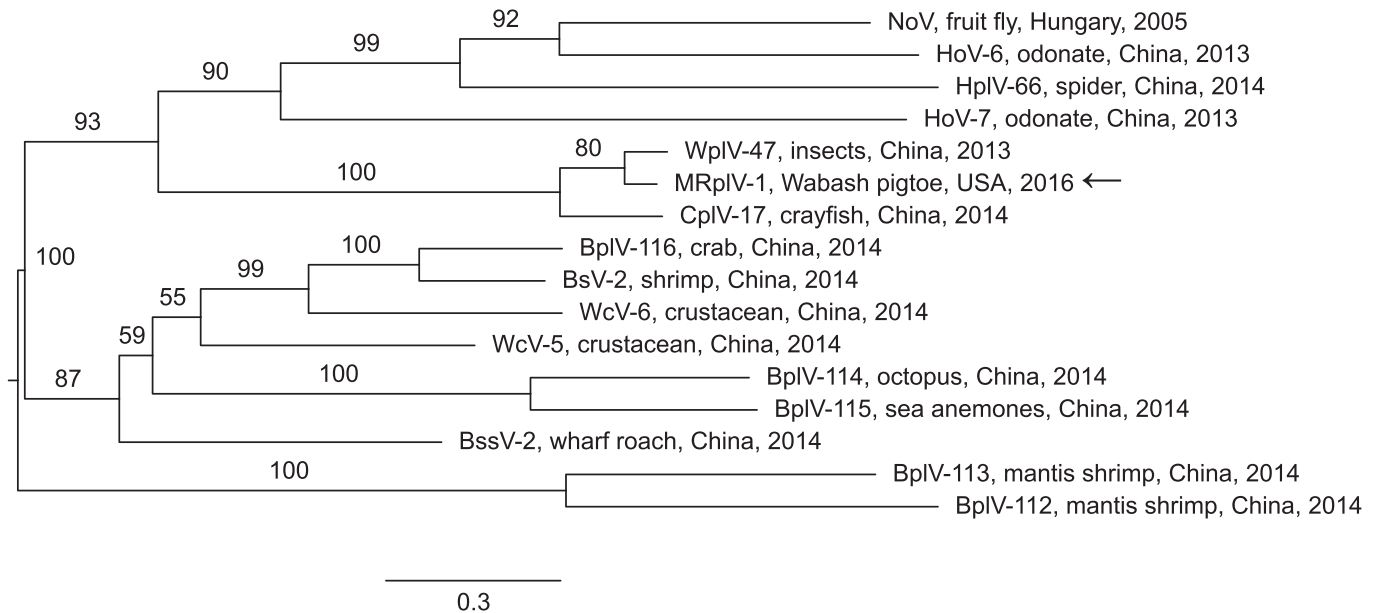


Figure 1. Phylogenetic tree of picornalike viruses. The major glycoprotein nucleic acid sequences of each virus were aligned using the codon-based Prank algorithm (Loytynoja 2014) implemented in the program TranslatorX (Abascal et al. 2010), with the Gblocks algorithm (Castresana 2000) applied to remove poorly aligned regions. The maximum-likelihood method implemented in the computer program PhyML (Guindon et al. 2010) was then applied to the resulting 1,332-position nucleic acid alignment, with the model of molecular evolution estimated from the data. Taxon names indicate abbreviated virus names (see below), host, country, and year of collection. The novel picornalike virus from the Wabash Pigtoe is indicated with an arrow. Numbers beside branches show statistical confidence of clades based on 1,000 bootstrap replicates of the data. Scale bar indicates nucleotide substitutions per site. Taxon abbreviations and GenBank accession numbers: NoV: Nora virus (NC\_007919); HoV-6: Hubei odonate virus 6 (NC\_033071); HplV-66: Hubei picornalike virus 66 (NC\_033133); HoV-7: Hubei odonate virus 7 (NC\_033232); WplV-47: Wenzhou picornalike virus 47 (NC\_033150); MRplV-1: Mississippi River picornalike virus 1 (MK301250); CplV-17: Changjiang picornalike virus 17 (KX884555); BplV-116: Beihai picornalike virus 116 (NC\_032635); BsV-2: Beihai shrimp virus 2 (NC\_032594); WcV-6: Wenling crustacean virus 6 (NC\_032810); WcV-5: Wenling crustacean virus 5 (NC\_032839); BplV-114: Beihai picornalike virus 114 (NC\_032633); BplV-115: Beihai picornalike virus 115 (NC\_032618); BssV-2: Beihai sea slater virus 2 (NC\_032622); BplV-113: Beihai picornalike virus 113 (NC\_032559); BplV-112: Beihai picornalike virus 112 (NC\_032571).

## METHODS

We sampled a total of seven individuals: one Threeridge (*Amblema plicata*) and two Wabash Pigtoes (*Fusconaia flava*), collected from the Mississippi River north of Brownsville, Minnesota (43° 43.137' N, 91° 15.373' W) on September 16, 2016, and one Threeridge, one Giant Floater (*Pyganodon grandis*), one Plain Pocketbook (*Lampsilis cardium*), and one Fatmucket (*Lampsilis siliquoidea*), collected from the La-Crosse River below Neshonoc Dam in Wisconsin (43° 54.874' N, 91° 4.586' W) on September 30, 2016. We opened the mussels slightly with reverse pliers and collected a single, approximately 1-mL hemolymph sample from each animal using a needle and syringe inserted into the anterior adductor muscle sinus, which is a nonlethal sampling method (Gustafson et al. 2005). We then transferred the hemolymph to a microcentrifuge tube, placed it on ice during transportation, and stored it at -80°C until the samples were processed for molecular analysis. This sampling was part of a pilot monitoring effort to characterize microbes in the hemolymph of mussels across the Upper Mississippi River basin.

To identify viruses in hemolymph, we used a virus discovery method based on next-generation DNA sequencing (NGS). NGS methods are “agnostic”—they can detect not

only known viruses but also unknown viruses that are genomically similar to known viruses, without prior knowledge of which viruses may be present (Munang'andu et al. 2017). These methods have revolutionized the study of invertebrate viruses, revealing their extraordinary diversity and deep evolutionary history (Shi et al. 2016; Wolf et al. 2018).

We used published methods optimized for detecting viruses of all genomic compositions in fluids and tissues, including those of aquatic organisms (Sibley et al. 2016; Toohey-Kurth et al. 2017). Briefly, we extracted total nucleic acids from 200 µL of hemolymph using the QIAamp MinElute virus spin kit (Qiagen Inc., Valencia, CA, USA) and converted RNA to double-stranded complementary DNA (dscDNA) using the Superscript dscDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) with random hexamer priming. We then prepared dscDNA for paired-end NGS on an Illumina MiSeq instrument (MiSeq Reagent Kit v3, 2x150 cycle, Illumina, San Diego, CA, USA) using the Nextera XT DNA sample prep kit (Illumina). NGS reads were quality trimmed and analyzed for similarity to viruses in the GenBank database as described by Sibley et al. (2016) and Toohey-Kurth et al. (2017).

## RESULTS

We obtained a total of 31,907,949 sequence reads (average 4,558,278 reads per individual mussel) with an average length of 109 base pairs after quality trimming. We did not detect any viruses in the animals from the La Crosse River or in the Threeridge and one Wabash Pigtoe from the Mississippi River. Sequences from the other Wabash Pigtoe mapped to a picornalike virus with approximately 12-fold coverage, yielding a complete open reading frame of 6,990 nucleotides encoding a putative viral polyprotein gene of 2,329 amino acids (GenBank accession number MK301250). The virus is a member of the Nora virus-related clade of picornalike viruses, named for the Nora virus of *Drosophila* fruit flies (Habayeb et al. 2006), which have genomes of approximately 10,000 bases of single-stranded, positive-sense RNA and infect a diverse array of aquatic, marine, and terrestrial invertebrates (Shi et al. 2016). The virus is most closely related to the Wenzhou picornalike virus 47 strain WHCCII11151 (GenBank accession number NC\_033150) found in unspecified insects in China in 2013 (Shi et al. 2016). It is more distantly related to the Changjiang picornalike virus 17 strain CJLX30705 (GenBank accession number KX884555) found in unspecified crayfish in China in 2014 (Shi et al. 2016) (Fig. 1).

## DISCUSSION

The presence of a virus in a North American unionid is not surprising, given the ubiquity of invertebrate viruses worldwide (Shi et al. 2016; Munang'andu et al. 2017; Wolf et al. 2018). At present, no inferences should be made about the role, if any, of this virus in the health of Wabash Pigtoes or any other species it may infect. The phylogenetic similarity of the Mississippi River picornalike virus 1 to arthropod viruses from China is interesting as evidence of the global distribution of the Nora virus clade of picornalike viruses, but because current data on these viruses are geographically biased, inferences about transmission or geographic spread also are premature. However, our detection of this virus in the hemolymph of only one mussel of seven indicates that such viruses are not present in all animals, even of the same species at the same place and time. We have not previously detected a virus similar to the Mississippi River picornalike virus 1 in any other sample sequenced in our laboratory despite analyzing hundreds of samples from diverse sources, supporting the conclusion that our results do not represent contamination.

Our results suggest that NGS-based methods will be useful for identifying viruses in unionids and for investigating the role, if any, of viruses in mortality events. We are currently applying such methods to investigate unionid mass mortality events in the Clinch River, Tennessee (Leis et al. 2018). Applying these methods to carefully selected groups of mussels of different health and disease states across different geographic regions should provide useful information for understanding how viruses may contribute to unionid declines in general.

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