AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

STRUCTURE AND ASSEMBLY

Genome Characterization, Prevalence, and Transmission Mode of a Novel Picornavirus Associated with the Threespine Stickleback Fish (*Gasterosteus aculeatus*)

Megan A. Hahn,[a] Nolwenn M. Dheilly[a]

[a]School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, New York, USA

ABSTRACT The complete genome sequence of an RNA virus was assembled from RNA sequencing of virus particles purified from threespine stickleback intestine tissue samples. This new virus is most closely related to the Eel picornavirus and can be assigned to the genus *Potamipivirus* in the family *Picornaviridae*. Its unique genetic properties are enough to establish a new species, dubbed the Threespine Stickleback picornavirus (TSPV). Due to their broad geographic distribution throughout the Northern Hemisphere and parallel adaptation to freshwater, threespine sticklebacks have become a model in evolutionary ecology. Further analysis using diagnostic PCRs revealed that TSPV is highly prevalent in both anadromous and freshwater populations of threespine sticklebacks, infects almost all fish tissues, and is transmitted vertically to offspring obtained from *in vitro* fertilization in laboratory settings. Finally, TSPV was found in Sequence Reads Archives of transcriptome of *Gasterosteus aculeatus*, further demonstrating its wide distribution and unsought prevalence in samples. It is thus necessary to test the impact of TSPV on the biology of threespine sticklebacks, as this widespread virus could interfere with the behavioral, physiological, or immunological studies that employ this fish as a model system.

IMPORTANCE The threespine stickleback species complex is an important model system in ecological and evolutionary studies because of the large number of isolated divergent populations that are experimentally tractable. For similar reasons, its coevolution with the cestode parasite *Schistocephalus solidus*, its interaction with gut microbes, and the evolution of its immune system are of growing interest. Herein we describe the discovery of an RNA virus that infects both freshwater and anadromous populations of sticklebacks. We show that the virus is transmitted vertically in laboratory settings and found it in Sequence Reads Archives, suggesting that experiments using sticklebacks were conducted in the presence of the virus. This discovery can serve as a reminder that the presence of viruses in wild-caught animals is possible, even when animals appear healthy. Regarding threespine sticklebacks, the impact of Threespine Stickleback picornavirus (TSPV) on the fish biology should be investigated further to ensure that it does not interfere with experimental results.

KEYWORDS RNA virus, fish, genome organization, picornavirus, stickleback, vertical transmission, virus-host interactions

The family *Picornaviridae* encompasses positive-strand RNA viruses whose genomes encode a single large protein precursor (polyprotein). The typical genome organization comprises 6,700 to 10,100 nucleotides (nt) and is composed of a 5′ untranslated region (UTR) that possesses an internal ribosome entry site (IRES) recognized by host cell ribosomes and allowing cap-independent translation, a single open reading frame encoding the polyprotein, and a 3′ UTR with a polyadenylated tract of variable length.

Citation Hahn MA, Dheilly NM. 2019. Genome characterization, prevalence, and transmission mode of a novel picornavirus associated with the threespine stickleback fish (*Gasterosteus aculeatus*). J Virol 93:e02277-18. https://doi.org/10.1128/JVI.02277-18.

Editor Julie K. Pfeiffer, University of Texas Southwestern Medical Center

Address correspondence to Nolwenn M. Dheilly, nolwenn.dheilly@stonybrook.edu.

Received 19 December 2018
Accepted 5 February 2019
Accepted manuscript posted online 13 February 2019
Published 17 April 2019

The typical polyprotein is composed of facultative nonstructural leader (L), followed by a P1 region encoding three structural proteins (1AB that is cleaved into VP4 and VP3 in most picornaviruses, and 1C and 1D that encode proteins VP2 and VP1, respectively) and two nonstructural regions P2 and P3. Region P2 encodes proteins 2A and 2B that impair the transport of host proteins, and $2C^{ATPase}$, a multifunctional ATPase involved in vesicle formation. Region P3 encodes proteins 3A that mediate presentation of membrane proteins during viral replication, $3B^{VpG}$, a genome-linked viral protein that acts as a primer for RNA synthesis during RNA replication, $3C^{pro}$, a cysteine protease (picornain) responsible for maturation cleavage of the precursor polyprotein, and $3D^{pol}$, the RNA-dependent RNA polymerase. The gene regions coding for the capsid proteins and the nonstructural proteins 2B, 2C, $3C^{pro}$, and $3D^{pol}$ are well conserved in all described picornaviruses, whereas the number of 2A proteins varies, full-length 3A and $3B^{VpG}$ proteins can show low similarity across species and the nonstructural protein L is absent in some species (1).

The family *Picornaviridae* is comprised of 47 genera and contains more than 110 species (as of July 2018), but many of these viruses are currently awaiting classification (http://www.picornaviridae.com). Until recently, only four species of picornaviruses had been reported in fish; the Eel picornavirus 1 (EPV-1) in *Anguilla anguilla* (36), the Carp picornavirus 1 (CPV-1) in *Cyprinus carpio* (37), the Bluegill picornavirus (BGPV-1) in *Lepomis macrochirus* (38), and the Fathead Minnow picornavirus (FHMPV-1) in *Pimephales promelas* (39). BGPV, CPV, and FHMPV have recently been classified in the new genus *Limnipivirus*, whereas the EPV is the only member of the new genus *Potamipivirus*. There are also unassigned picornavirus-like species found in fish, including fisavirus (partial sequence) in *Cyprinus carpio* (40) and a partial sequence of the Tioga picorna-like virus in *Salvelinus fontinalis* (41). A recent investigation of vertebrate RNA viruses led to the discovery of an additional 27 unassigned picornavirus-like viral sequences from diverse fish species (2). The low similarity between the conserved precursor proteins and other picornaviruses suggest that within each fish species, a novel species of picornavirus was discovered.

The threespine stickleback, *Gasterosteus aculeatus* (here, "stickleback"), is a small teleost fish found widely distributed across all continents in the Northern Hemisphere (5). Sticklebacks have both anadromous and freshwater representatives, the latter of which have had recurrent adaptation in multiple different lakes (5, 6). Freshwater sticklebacks are of particular interest in ecological and evolutionary studies, because each lake comprises an independent population of fish that display population-specific traits within only a few generations (3). In particular, the region surrounding Anchorage, AK, is of great interest because of the large number of lakes and distinct lineages of sticklebacks. Indeed, over the past 20 years, this region has become a "hot spot" for evolutionary research and for studies of host-parasite coevolution (4, 7–15). However, information on stickleback viruses remains scarce. The presence and prevalence of DNA viruses have been scarcely investigated, while the presence of RNA viruses remains unknown (16–18).

Here, we investigated the presence of viruses in the guts of apparently healthy sticklebacks. Using a combination of Illumina sequencing and complementary PCR, we sequenced the full-length genome of a positive-strand RNA virus. The predicted polyprotein displays the typical organization of a picornavirus, and genome sequence composition and phylogenetic analyses suggest that it belongs to the same genus as EPV. The virus was provisionally named Threespine Stickleback picornavirus (TSPV). Additional targeted PCRs were used to test prevalence in marine and freshwater populations of sticklebacks from Alaska and to test the potential for vertical transmission of TSPV to offspring.

(This article was submitted to an online preprint archive [19].)

RESULTS AND DISCUSSION

Molecular characteristics of TSPV. The genome organization of TSPV-1 is depicted in Fig. 1 and can be described as follows: VPg plus 5′ UTR-(1AB-1C-1D-$2A1^{NPGP}$/$2A2^{NPGP}$/$2A3^{H\text{-}Box/NC}$-2B-$2C^{Hel}$/3A-$3B^{VPg}$-$3C^{pro}$-$3D^{pol}$)-3′ UTR-poly(A).

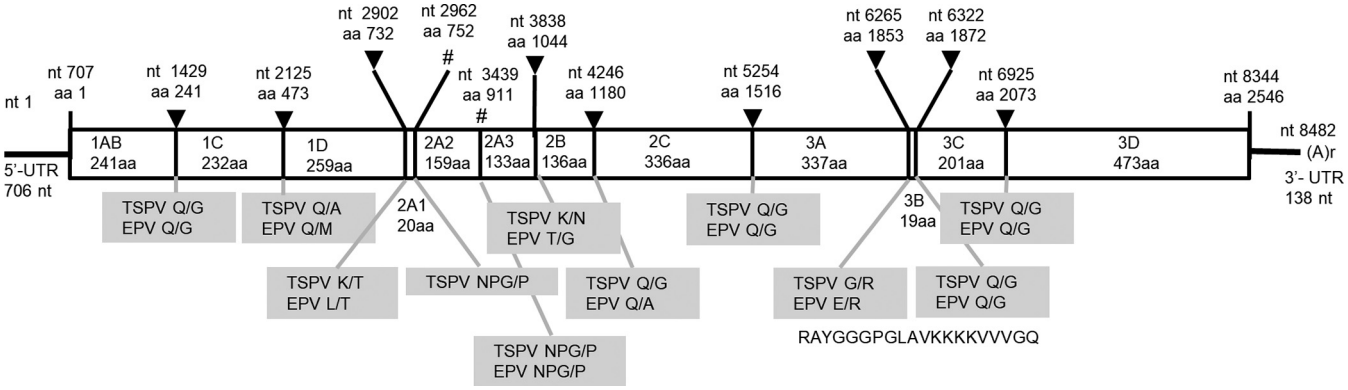

FIG 1 Schematic diagram of the predicted TSPV genome structure. Numbers on the top indicate amino acid (aa) position and nucleotide (nt) positions. The 5′ and 3′ UTRs (line) and the open reading frame (box) are displayed. Predicted protease cleavage sites are indicated with triangles () and ribosomal skip sequences are indicated with the hash (#). Gray boxes show a comparison of the cleavage sites with EPV-1.

The positive-strand RNA genome is composed of a 5′ UTR of 706 nt, an open reading frame (ORF) of 7,560 nt, a 3′ UTR of 138 nt, and a poly(A) tail of at least 50 nt and is overall more similar to EPV than to any other picornavirus (Table 1).

The deduced polyprotein precursor was 2,520 amino acids (aa) long with an overall base composition of 28.6% (A), 19.2% (C), 25.4% (G), and 26.8% (T). The genome of TSPV had no obvious leader sequence. It had three capsid proteins (1AB, 1C, and 1D). We found the conserved rhv_like hydrophobic domain of capsid (cd00205) proteins within both proteins 1AB and 1C (aa positions 103 to 228 and 294 to 465, respectively).

We identified five nonstructural proteins (2A1, 2A2, 2A3, 2B, and 2C) within the P2 region. The 2A region is complex with high similarities with EPV, CPV-1, and BGPV-1 (Fig. 2). Similarly to CPV-1 and BGPV-1, TSPV encodes two 2A proteins that end with an NPG/P ribosome skipping motif that is found in many picornaviruses and mediates in *cis* cotranslational termination-reinitiation of RNA translation. The 2A3 gene region exhibits a parechovirus-like and avihepatovirus-like structure, including the conserved H-box and NC-box. The 2B protein is also homologous to parechovirus and avihepatovirus. A conserved RNA helicase domain was found within protein 2C (aa positions 1316 to 1422, pfam00910) that contained the three domains originally identified by Koonin et al. (20).

The P3 region contained four nonstructural proteins (3A, $3B^{VpG}$, $3C^{pro}$, and $3D^{pol}$). 3A showed no obvious homology and was twice as long as 3As in related picornaviruses. It showed no homology to the EPV 3A region either. The N terminus of 3B exhibits the characteristic RAY of EPV, CPV-1, BGPV-1, and parechoviruses. A conserved 3C cysteine protease was found in protein 3C (aa positions 1954 to 2056, pfam00548). The putative protease has the core domain of picornaviruses (GxCG) and the GxHxxG substrate binding pocket. A conserved RdRP domain was found in protein 3D (aa positions 2089

TABLE 1 Percent pairwise amino acid identity of TSPV proteins to other picornavirus proteins from the genera *Potamipivirus*, *Limnipivirus*, *Aquamavirus*, *Avihepatovirus*, and *Parechovirus*

TSPV-1 protein	Pairwise amino acid identity (%) to proteins from:					
	Potamipivirus (Eel picornavirus, KC843627)	Unassigned (Wuhan carp picornavirus, MG600066)	*Limnipivirus* (carp picornavirus, KF306267)	*Aquamavirus* (seal picornavirus, EU142040)	*Avihepatovirus* (duck hepatitis A virus, DQ249299)	*Parechovirus* (human parechovirus, L02971)
1AB	48.9	42.4	23.3	27.3	27	34.5
1C	44.8	40.4	19.9	27.9	30.3	29.3
1D	40.2	30.6	19.8	29.2	24.7	26.2
2B	50	48.5	18.3	14.9	18.4	22.3
2C	37.6	37.9	25.3	31.4	33.3	32.4
3C	44.9	35.2	22.8	23.6	21.4	23.4
3D	56.7	54.2	40.8	30.2	36.8	37.9

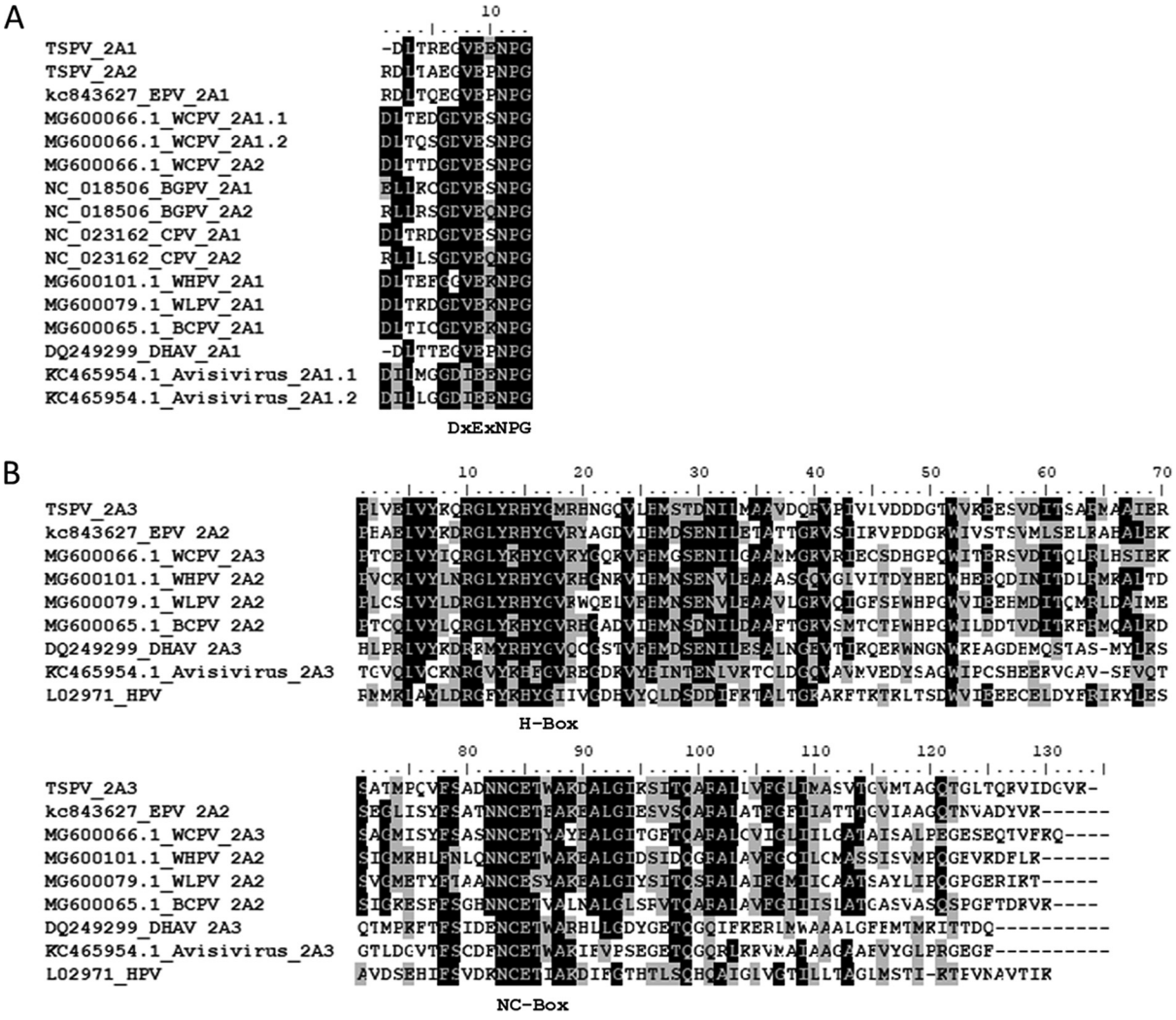

FIG 2 Sequence alignment of TSPV 2A proteins with proteins 2A from related species. (A) Alignment of the carboxy-terminal region of aphthovirus-like 2A1 and 2A2. (B) Alignment of the parechovirus-like 2A3. Characteristic functional motifs are indicated.

to 2487, cd01699, pfam00680). The RdRp had all eight motifs of the core region described by Koonin et al. (20, 21).

Phylogenetic analyses. To investigate the relationship of TSPV with EPV and other picornaviruses, protein sequences from the P1 and 3CD regions were aligned with representatives from each described genus and additional unassigned candidate picornavirus species from fish (Fig. 3). Maximum likelihood phylogenetic trees confirmed that TSPV was most closely related to EPV and belongs to the genus *Potamipivirus*. It is the first known RNA virus of threespine sticklebacks.

Distribution and transmission. Diagnostic PCR on RNA extracted from intestines of sticklebacks from anadromous and freshwater populations in Alaska revealed high prevalence of TSPV: 100% prevalence was observed in anadromous fish collected from Rabbit Slough (20 individuals) and in freshwater fish from Cheney Lake (32 individuals) and Loberg Lake (15 individuals) and 88% in freshwater fish from Wolf Lake (17 individuals). The amplicons were sequenced using Sanger sequencing and showed no variation in sequence of this fragment of the genome regardless of the origin of the fish. However, given that stickleback populations are geographically isolated in lakes in America and in Europe, it is likely that different strains of TSPV infect fish from different populations and/or that other virus species infect threespine sticklebacks from these populations. Further studies would be necessary to compare full-length virus genome sequences, and the virome, of sticklebacks across its geographic range.

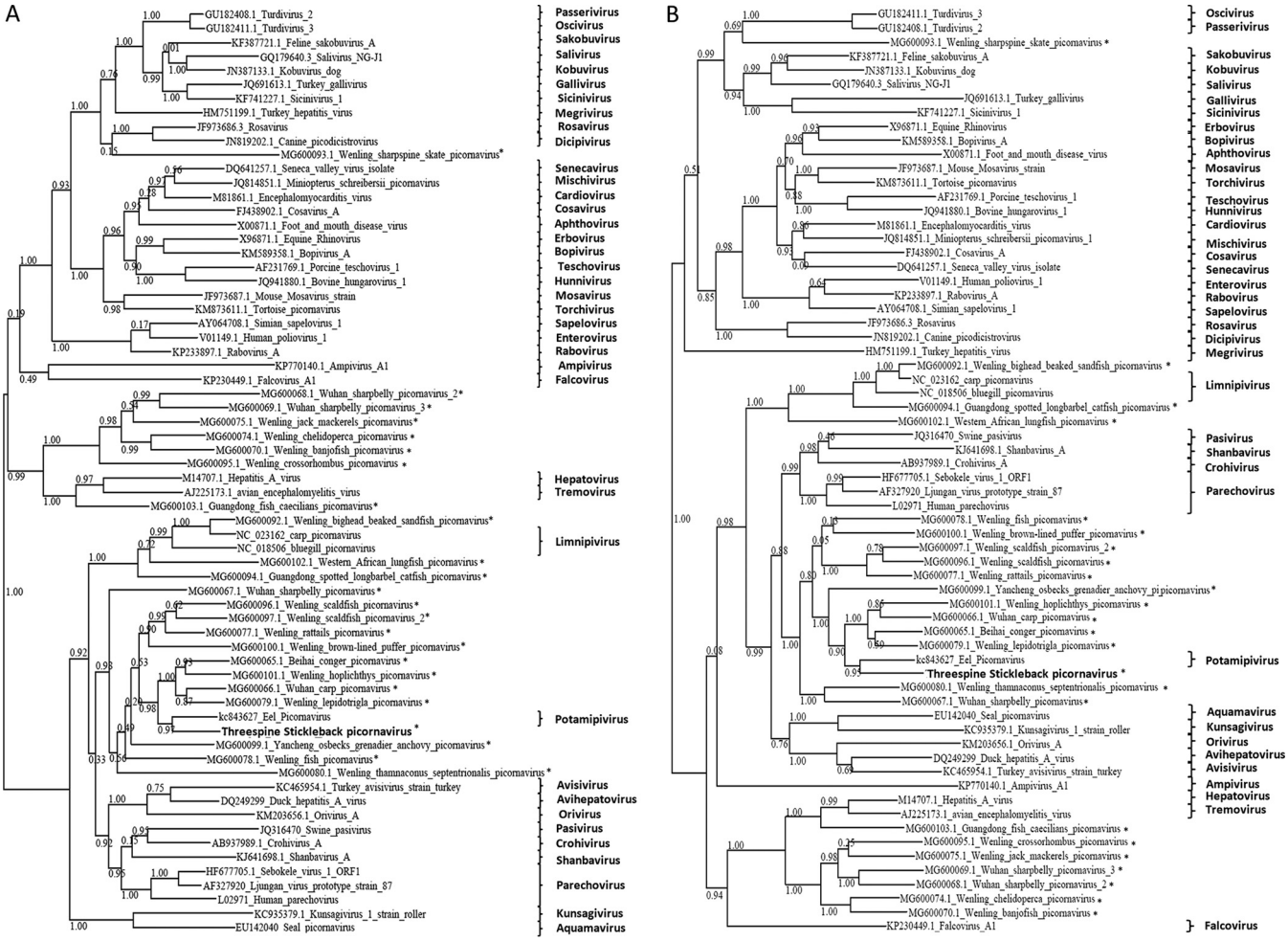

FIG 3 Phylogenetic analysis of picornavirus 3CD (A) and P1 (B) gene regions. One representative sequence of each of the 40 described genera and an additional 22 sequences of fish-associated viruses obtained from GenBank and the TSPV were included. The trees were inferred with PhyML using the LG substitution model. Values next to the branch indicate the result of a Shimodaira-Hasegawa branch test.

Next, we tested the presence of TSPV in various organs of one field-sampled stickleback from Cheney Lake, and in two lab-reared sticklebacks from Loberg Lake and Rabbit Slough. For the three tested individuals, we found the TSPV in all tested fish tissues (muscle, liver, head kidney, intestine, stomach, gonad) except the eye. This result indicates a multiorgan tropism of TSPV. Because we found the TSPV in lab-reared sticklebacks obtained from *in vitro* fertilization, we suspected that it might be vertically transmitted, meaning that transmission occurs from parents to offspring, in contrast to horizontal transmission that is nonparental. The multiorgan tropism and absence of obvious detrimental effect of TSPV infection supports the hypothesis that TSPV is vertically transmitted.

We used *in vitro* fertilization to cross male and female fish from Rabbit Slough, Cheney Lake, and Loberg Lake. We then tested TSPV presence in newly hatched unfed larvae from six families for each population and found TSPV in all samples. This result indicates that the virus could be vertically transmitted from one or both of the parents to the offspring. In invertebrates, picorna-like viruses can be vertically transmitted (22–25), but the vertical transmission of picornaviruses of vertebrates has never been observed. Further studies are necessary to determine the physical route of transmission from parents to offspring, as it remains possible that transmission occurs via the mucus or when testes are chopped during the *in vitro* fertilization process. Indeed, picornavirus-like particles were previously observed in the ovarian fluids of cutthroat,

brown, and brook trout (26). However, it should be noted that exclusive vertical transmission is rare, and most microorganisms exhibit a mixed mode of transmission (27). Microbes with strict vertical transmission must be beneficial to their host, on which they totally depend and tend to reach a 100% prevalence in population (28). The prevalence of TSPV in tested populations is high but does not reach 100%, which suggests a mixed-mode transmission of TSPV. In order to test for horizontal transmission, it will be necessary to obtain virus-free stickleback families in laboratory settings. Currently, none of the families of sticklebacks produced in the Dheilly lab were free of the virus and could be used for this purpose. Also, virus-free families could be used to conduct experimental infections with culture of purified viruses to assess TSPV effect on fish development and overall health.

Viruses in fish have most commonly been investigated when mortality is occurring in aquaculture stocks or fisheries, but it is also necessary to investigate the presence of viruses in healthy fish to better reconstruct virus phylogenies and understand processes of pathogenicity (29). Threespine sticklebacks are extensively used in evolutionary ecology; in particular, populations from Alaska have been used to study the transition from marine to freshwater and resulting adaptations (3, 13, 14, 30). In addition, lab-reared sticklebacks are used in laboratory conditions to study fish development, the genetic basis of their evolution of phenotypes suited to freshwater, and coevolution with parasites (3, 15, 31). Given the high prevalence of TSPV in our field-caught samples and demonstration of transmission to the fish offspring in laboratory crosses, it is very likely that field-caught and lab-raised individuals from these populations commonly carry the virus. In other locations, populations may be infected with TSPV or carry different virus species that could influence their ecology and evolution. We investigated this question by using Blastn to search for reads that map against the TSPV genome in Sequence Read Archives. TSPV was absent in genome sequences but was found in transcriptomic data from various tissues associated with several Bioprojects (PRJDB7242, RNA-seq of hybrid testis; PRJDB2384, Japanese sympatric sticklebacks brain miRNA; PRJEB8677, habitat-specific gene expression in stickleback immune tissues from four parametric lake-river population pairs; and PRJNA252659, transcriptome analysis of threespine sticklebacks acclimated in different salinities). It is therefore crucial to monitor virus presence and investigate the impact of TSPV and other highly prevalent viruses on threespine stickleback behavior, physiology, and immunity.

MATERIALS AND METHODS

Virus isolation. Viruses were initially purified from the intestine tissue of five apparently healthy *G. aculeatus* collected in Cheney Lake, AK (61°12′17′′N, 149°45′33′′), in June 2016. Fish were starved for 48 h prior to intestine collection to reduce the chance of isolating viruses associated with fish food. The full intestine, including content, was dissected out of the fish and immediately transferred to phosphate-buffered saline (PBS) for virus purification. Viruses were purified using a filter-chloroform nuclease virus purification protocol. Briefly, tissue samples were homogenized in sterile PBS by bead beating with 3-mm glass beads. The homogenates were centrifuged for 1 min at 6,000 rpm. The pellets were discarded, and the solutions were further diluted with 500 μl of PBS and centrifuged at 6,000 rpm for 6 min to remove remaining cell debris. The supernatants were then filtered successively through a 0.4-μm filter and 0.22-μm filter and incubated for 10 min in 0.2 volumes of chloroform. The viruses were then separated from the chloroform by centrifugation for 20 s at 20,000 rpm. A second chloroform treatment was applied to ensure removal of bacterial contaminants. The purified viruses were finally treated with 2.5 U of DNase I and 0.25 U of RNase A at 37°C for 3 h to eliminate nonviral DNA and RNA. EDTA was added at a final concentration of 20 mM.

Sample preparation, sequencing, and assembly. Initially, both viral DNA and viral RNA were extracted using the QIAamp Mini Elute Virus Spin kit according to manufacturer's instructions. DNA was then removed using a Turbo DNase treatment. The True Seq mRNA library preparation kit was used before single-end sequencing, for 100 cycles, on an Illumina Hi-Seq 4000 (Institute of Biotechnology at Cornell University). For each data set, adapters were removed using Trimmomatic, and PhiX174 contaminants were removed using Bowtie 2 (–very-sensitive-local). We obtained 2.38, 4.32, 6.98, 8.18, and 9.70 million high-quality reads for each respective sample. Then, for each sample, d*e novo* assembly was completed using Trinity (32). Partial sequences of a virus that showed high similarity to EPV were assembled independently from all five samples. Reads from the five samples were thus pooled to improve viral *de novo* assembly. Eight contigs of more than 500 nt showed high similarity to EPV. The two

longest contigs had 6,741 nt and 1,536 nt and were assembled in a single draft genome of 8,267 nt nucleotides, with potentially missing nucleotides at the 5′ and 3′ ends.

Fragment amplification and Sanger sequencing. First strand cDNA was synthesized by reverse transcribing 500 ng of RNA from purified viruses and mixed with 0.2 μg/μl random hexamer primer in a 20 μl reaction mixture volume containing 40 U/μl Ribolock RNase Inhibitor, 1 mM deoxynucleoside triphosphates (dNTPs), 200 U/μl RevertAid H Minus reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), and water, as per the manufacturer's recommendations. PCR was conducted using the Advantage 2 PCR system (Invitrogen) using primers targeting the conserved RNA-dependent RNA polymerase gene (forward [F], 5′-TCT CCT ACC AAA CCC GCA AC-3′; reverse [R], 5′-TTC CTC CGC CAC CAG ATA GA-3′). Amplicon presence was assayed with 1% agarose gel with SyBR Safe. To complete the 3′ ends of the genome sequence, we performed rapid amplification for cDNA ends (RACE) using Invitrogen 3′RACE systems according to the manufacturer's instructions with either primers 5′-GGC GGA CGC AAC CTT CCT CA-3′ or 5′-CCC AGT TTG GCA TGG AGG CG-3′. PCR products were sequenced using Sanger sequencing.

Phylogenetic analyses. To investigate the relationship of TSPV with other related picornaviruses, TSPV protein sequences from the P1, 2C, and 3CD regions were aligned using clustalo with representatives from each genus and with all known picornaviruses of fishes. Maximum likelihood phylogenetic trees were constructed in Seaview4 (33) using the LG substitution model (34) implemented in PhyML (35) with the nonparametric Shimodaira-Hasegawa-like procedure. Pairwise identity matrices were obtained using Clustal-Omega online (https://www.ebi.ac.uk).

Sampling, sample preparation, and diagnostic PCR. In June of 2018, we collected intestines of sticklebacks from an anadromous marine population in Rabbit Slough (61°32′08.1′′N, 149°15′10.0′′W) and from isolated freshwater populations from Cheney Lake, Wolf Lake (61°38′36′′N, 149°16′32′′W), and Loberg Lake (61°33′33.5′′N, 149°15′28.9′′W). Upon dissection, intestines were immediately transferred in RNAlater and preserved at −80°C until use. Total RNA was extracted using Trizol according to the recommended protocol.

We also collected mature male and gravid female fish from Rabbit Slough, Cheney Lake, and Loberg Lake and completed crosses *in vitro* to obtain families. These crosses were conducted under sterile lab conditions on the day the parents were captured. Eggs were expressed from the female fish into a few drops of sterile water. Male fish were euthanized with an overdose of MS222, and testes were chopped in sterile water to liberate the sperm. The testis preparation was then pipetted onto the eggs in a petri dish. After 20 min, eggs were checked to confirm that the chorion had separated from the plasma membrane. Testicular debris and ovarian mucus were then removed, and eggs were washed in methylene blue to prevent fungal growth. Eggs were maintained in individual petri dishes with daily water changes until hatching. Immediately following hatching, six larvae from each lake were collected and preserved in RNAlater. Stickleback larvae do not feed until a few days later, and no food was added to the petri dishes before collection. Total RNA was extracted using the RNeasy kit (catalog number 74104) according to the manufacturer's guidelines.

Ethics statement. Stickleback collection followed guidelines for scientific fish collection by the State of Alaska Department of Fish and Game in accordance with fish sampling permit P17-025 and P-18-008 and fish transport permits 17A-0024 provided to N. M. D. Fish were maintained at Stony Brook University under the license to collect or possess number 1949 provided by the New York State Department of Environmental Conservation to N. M. D. Fish experiments were conduct according to protocols described in Institutional Animal Care and Use Committee (IACUC) numbers 237429 and 815164 to Michael Bell and N. M. D., respectively. Fish euthanasia was conducted using MS222, and decapitation was performed before parasite and tissue sampling. All experiments were performed in accordance with relevant guidelines and regulations in the Public Health Service Policy (PHS) on Humane Care and Use of Laboratory Animals.

Data availability. The TSPV genome sequence is available in GenBank under nucleotide accession number MK189163. Sequencing data were submitted to the NCBI Sequence Read Archive and are available under BioProject accession number PRJNA501850.

ACKNOWLEDGMENTS

We acknowledge the expertise and assistance of Michael Bell in fish collection and dissection techniques.

This project was supported by the Eppley Foundation for Research and the Laurie Landeau Foundation LLC.

REFERENCES

1. Zell R, Delwart E, Gorbalenya AE, Hovi T, King AMQ, Knowles NJ, Lindberg AM, Pallansch MA, Palmenberg AC, Reuter G, Simmonds P, Skern T, Stanway G, Yamashita T, Consortium IR. 2017. ICTV virus taxonomy profile: *Picornaviridae*. J Gen Virol 98:2421–2422. https://doi.org/10.1099/jgv.0.000911.
2. Shi M, Lin X-D, Chen X, Tian J-H, Chen L-J, Li K, Wang W, Eden J-S, Shen J-J, Liu L, Holmes EC, Zhang Y-Z. 2018. The evolutionary history of vertebrate RNA viruses. Nature 556:197–202. https://doi.org/10.1038/s41586-018-0012-7.
3. Kristjánsson BK. 2005. Rapid morphological changes in threespine stickleback, *Gasterosteus aculeatus*, in freshwater. Environ Biol Fish 74:357–363. https://doi.org/10.1007/s10641-005-1487-2.
4. Weber JN, Kalbe M, Shim KC, Erin NI, Steinel NC, Ma L, Bolnick DI. 2017. Resist globally, infect locally: a transcontinental test of adaptation by

stickleback and their tapeworm parasite. Am Nat 189:43–57. https://doi.org/10.1086/689597.
5. Bell MA, Foster SA. 1994. The evolutionary biology of the threespine stickleback. Oxford University Press, New York, NY.
6. Wootton RJ. 1976. Biology of the sticklebacks. Academic Press, New York, NY.
7. Barber I, Scharsack J. 2010. The three-spined stickleback-*Schistocephalus solidus* system: an experimental model for investigating host-parasite interactions in fish. Parasitology 137:411–424. https://doi.org/10.1017/S0031182009991466.
8. Hahn MA, Dheilly NM. 2016. Experimental models to study the role of microbes in host-parasite interactions. Front Microbiol 7:1300. https://doi.org/10.3389/fmicb.2016.01300.
9. Erin N, Kalbe M, Reusch TB. 2017. Multiple ecological scales of host-parasite interactions using the three-spined stickleback and Schistocephalus solidus model system. Max Planck Institute for Evolutionary Biology, Department of Evolutionary Ecology, Plön, Germany.
10. Scharsack JP, Franke F, Erin NI, Kuske A, Büscher J, Stolz H, Samonte IE, Kurtz J, Kalbe M. 2016. Effects of environmental variation on host–parasite interaction in three-spined sticklebacks (*Gasterosteus aculeatus*). Zoology (Jena) 119:375–383. https://doi.org/10.1016/j.zool.2016.05.008.
11. Kurtz J, Schulenburg H, Reusch TB. 2016. Host-parasite coevolution-rapid reciprocal adaptation and its genetic basis. Zoology (Jena) 119:241–243. https://doi.org/10.1016/j.zool.2016.06.011.
12. Bell MA, Heins DC, Wund MA, von Hippel FA, Massengill R, Dunker K, Bristow GA, Aguirre WE. 2016. Reintroduction of threespine stickleback into Cheney and Scout Lakes, Alaska. Evol Ecol Res 17:157–178.
13. Bell MA, Aguirre WE. 2013. Contemporary evolution, allelic recycling, and adaptive radiation of the threespine stickleback. Evol Ecol Res 15:377–411.
14. Bell MA, Aguirre WE, Buck NJ. 2004. Twelve years of contemporary armor evolution in a threespine stickleback population. Evolution 58:814–824. https://doi.org/10.1111/j.0014-3820.2004.tb00414.x.
15. Jones FC, Grabherr MG, Chan YF, Russell P, Mauceli E, Johnson J, Swofford R, Pirun M, Zody MC, White S, Birney E, Searle S, Schmutz J, Grimwood J, Dickson MC, Myers RM, Miller CT, Summers BR, Knecht AK, Brady SD, Zhang H, Pollen AA, Howes T, Amemiya C, Baldwin J, Bloom T, Jaffe DB, Nicol R, Wilkinson J, Lander ES, Di Palma F, Lindblad-Toh K, Kingsley DM. 2012. The genomic basis of adaptive evolution in threespine sticklebacks. Nature 484:55–61. https://doi.org/10.1038/nature10944.
16. Waltzek TB, Miller DL, Gray MJ, Drecktrah B, Briggler JT, MacConnell B, Hudson C, Hopper L, Friary J, Yun SC, Malm KV, Weber ES, Hedrick RP. 2014. New disease records for hatchery-reared sturgeon. I. Expansion of frog virus 3 host range into *Scaphirhynchus albus*. Dis Aquat Organ 111:219–227. https://doi.org/10.3354/dao02761.
17. Gagné N, MacKinnon AM, Boston L, Souter B, Cook-Versloot M, Griffiths S, Olivier G. 2007. Isolation of viral haemorrhagic septicaemia virus from mummichog, stickleback, striped bass and brown trout in eastern Canada. J Fish Dis 30:213–223. https://doi.org/10.1111/j.1365-2761.2007.00802.x.
18. Waltzek TB, Marty GD, Alfaro ME, Bennett WR, Garver KA, Haulena M, Weber IIIES, Hedrick RP. 2012. Systemic iridovirus from threespine stickleback *Gasterosteus aculeatus* represents a new megalocytivirus species (family Iridoviridae). Dis Aquat Org 98:41–56. https://doi.org/10.3354/dao02415.
19. Hahn MA, Dheilly NM. 2018. Genome characterization, prevalence and transmission mode of a novel picornavirus associated with the threespine stickleback (*Gasterosteus aculeatus*). BioRxiv https://doi.org/10.1101/502195.
20. Koonin EV, Dolja VV, Morris TJ. 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit Rev Biochem Mol Biol 28:375–430. https://doi.org/10.3109/10409239309078440.
21. Koonin EV. 1991. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J Gen Virol 72:2197–2206. https://doi.org/10.1099/0022-1317-72-9-2197.
22. Chen Y, Evans J, Feldlaufer M. 2006. Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. J Invertebr Pathol 92:152–159. https://doi.org/10.1016/j.jip.2006.03.010.
23. Jousset F, Plus N. 1975. Study of the vertical transmission and horizontal transmission of "Drosophila melanogaster" and "Drosophila immigrans" picornavirus (author's transl). Ann Microbiol (Paris) 126:231–249. (In French.)
24. Shen M, Cui L, Ostiguy N, Cox-Foster D. 2005. Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite. J Gen Virol 86:2281–2289. https://doi.org/10.1099/vir.0.80824-0.
25. Habayeb MS, Cantera R, Casanova G, Ekström J-O, Albright S, Hultmark D. 2009. The Drosophila Nora virus is an enteric virus, transmitted via feces. J Invertebr Pathol 101:29–33. https://doi.org/10.1016/j.jip.2009.02.003.
26. Batts WN. 2016. Hepeviruses of fish, p 365–369. *In* Kibenge SB, Godoy MG (ed), Aquaculture virology. Academic Press, Cambridge, MA.
27. Ebert D. 2013. The epidemiology and evolution of symbionts with mixed-mode transmission. Annu Rev Ecol Evol Syst 44:623–643. https://doi.org/10.1146/annurev-ecolsys-032513-100555.
28. Fine PE. 1975. Vectors and vertical transmission: an epidemiologic perspective. Ann N Y Acad Sci 266:173–194. https://doi.org/10.1111/j.1749-6632.1975.tb35099.x.
29. Zhang Q, Gui J-F. 2015. Virus genomes and virus-host interactions in aquaculture animals. Sci China Life Sci 58:156–169. https://doi.org/10.1007/s11427-015-4802-y.
30. Barber I, Nettleship S. 2010. From "trash fish" to supermodel: the rise and rise of the three-spined stickleback in evolution and ecology. Biologist 57:15–21.
31. Kingsley D. 2003. Sequencing the genome of threespine sticklebacks (Gasterosteus aculeatus). National Human Genome Research Institute White Paper, Bethesda, MD.
32. Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, Couger MB, Eccles D, Li B, Lieber M, MacManes MD, Ott M, Orvis J, Pochet N, Strozzi F, Weeks N, Westerman R, William T, Dewey CN, Henschel R, LeDuc RD, Friedman N, Regev A. 2013. *De novo* transcript sequence reconstruction from RNA-Seq using the Trinity platform for reference generation and analysis. Nat Protoc 8:1494–1512. https://doi.org/10.1038/nprot.2013.084.
33. Gouy M, Guindon S, Gascuel O. 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27:221–224. https://doi.org/10.1093/molbev/msp259.
34. Le SQ, Gascuel O. 2008. An improved general amino acid replacement matrix. Mol Biol Evol 25:1307–1320. https://doi.org/10.1093/molbev/msn067.
35. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. System Biol 59:307–321. https://doi.org/10.1093/sysbio/syq010.
36. Fichtner D, Philipps A, Groth M, Schmidt-Posthaus H, Granzow H, Dauber M, Platzer M, Bergmann SM, Schrudde D, Sauerbrei A, Zell R. 2013. Characterization of a novel picornavirus isolate from a diseased European eel (*Anguilla anguilla*). J Virol 87:10895–10899. https://doi.org/10.1128/JVI.01094-13.
37. Lange J, Groth M, Fichtner D, Granzow H, Keller B, Walther M, Platzer M, Sauerbrei A, Zell R. 2014. Virus isolate from carp: genetic characterization reveals a novel picornavirus with two aphthovirus 2A-like sequences. J Gen Virol 95:80–90. https://doi.org/10.1099/vir.0.058172-0.
38. Barbknecht M, Sepsenwol S, Leis E, Tuttle-Lau M, Gaikowski M, Knowles NJ, Lasee B, Hoffman MA. 2014. Characterization of a new picornavirus isolated from the freshwater fish Lepomis macrochirus. J Gen Virol 95:601–613. https://doi.org/10.1099/vir.0.061960-0.
39. Phelps NB, Mor SK, Armien AG, Batts W, Goodwin AE, Hopper L, McCann R, Ng TF, Puzach C, Waltzek TB, Delwart E, Winton J, Goyal SM. 2014. Isolation and molecular characterization of a novel picornavirus from baitfish in the USA. Plos One 9:e87593. https://doi.org/10.1371/journal.pone.0087593.
40. Iwanowicz LR, Iwanowicz DD, Adams CR, Galbraith H, Aunins A, Cornman RS. 2017. Draft genome sequence of a picorna-like virus associated with gill tissue in clinically normal brook trout, *Salvelinus fontinalis*. GenomeA 9:e01022-17. https://doi.org/10.1128/genomeA.01022-17.
41. Reuter G, Pankovics P, Delwart E, Boros Á. 2015. A novel posavirus-related single-stranded RNA virus from fish (*Cyprinus carpio*). Arch Virol 160:565–568. https://doi.org/10.1007/s00705-014-2304-z.