Virus Research 273 (2019) 197761

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Characterization of a peribunyavirus isolated from largemouth bass (*Micropterus salmoides*)

Thomas B. Waltzek[a,*], Kuttichantran Subramaniam[a], Eric Leis[b], Ryan Katona[b], Terry Fei Fan Ng[c,d,e], Eric Delwart[d,e], Marisa Barbknecht[f], Kelly Rock[f], Michael A. Hoffman[f]

[a] University of Florida, 2187 Mowry Road, 32611 Gainesville, FL, USA
[b] La Crosse Fish Health Center - Midwest Fisheries Center, U.S. Fish and Wildlife Service, 555 Lester Ave, 54650, Onalaska, WI, USA
[c] College of Veterinary Medicine, University of Georgia, Athens, GA, USA
[d] Blood Systems Research Institute, Department of Laboratory Medicine, University of California, San Francisco, CA, USA
[e] Department of Laboratory Medicine, University of California, San Francisco, CA, USA
[f] University of Wisconsin–La Crosse, 54601, La Crosse, WI, USA

ARTICLE INFO

Keywords:
Largemouth bass
Centrachidae
Bunyavirales
Peribunyaviridae
Next-generation sequencing

ABSTRACT

We report the complete genome sequencing of the first fish peribunyavirus determined using a next-generation sequencing approach. The virus was isolated during a routine health assessment of wild largemouth bass (*Micropterus salmoides*) in Wisconsin in April of 2009. Further research is needed to determine the epidemiology and pathogenicity of the largemouth bass bunyavirus.

The order *Bunyavirales* includes 12 viral families that possess enveloped nucleocapsids with multipartite (2–8 segments), single-stranded, negative-sense or ambisense RNA genomes (Maes et al., 2018; Tatineni et al., 2014). Bunyaviruses include notable human (e.g., hantaviruses and Crimean-Congo hemorrhagic fever virus), veterinary (e.g., Rift Valley fever virus), and plant (e.g., tomato spotted wilt virus) pathogens. They may be vectored through blood-sucking (e.g., mosquitoes or ticks) or sap-sucking (e.g., thrips) arthropods (arborviruses) and rodents (rodoviruses). In 2016, the family *Peribunyaviridae* was created and now includes 4 genera: the established genus *Orthobunyavirus* with 88 species including the type species *Bunyamwera orthobunyavirus,* the genus *Herbevirus* with 3 species including the type species *Herbert herbevirus*, the genus *Pacuvirus* with 3 species including the type species *Pacui pacuvirus*, and the monotypic genus *Shangavirus* that includes the type species *Insect shangavirus* (Briese et al., 2016; Kuhn et al., 2019). Herein, we describe the ultrastructural features and complete genome of the first peribunyavirus isolated from a fish.

In April of 2009, as part of the National Wild Fish Health Survey, 30 largemouth bass were netted from Pool 10 of the upper Mississippi River near Prairie du Chien, Wisconsin (water temperature was 8 °C). Fish were processed and screened for the presence of viruses utilizing the isolation procedures outlined in the AFS-Bluebook (USFWS and AFS–FHS, 2014). Briefly, the fish were euthanized with an overdose of MS-222 and an incision was made with a sterile scalpel to allow for collection of internal organs. Each sample consisted of kidney, spleen, and swim bladder tissues pooled from five bass in Hank's Balanced Salt Solution (HBSS; USFWS and AFS-FHS, 2014). The tissues were then homogenized, centrifuged, and diluted prior to inoculation onto confluent monolayers of Chinook salmon embryo (CHSE [Lannan et al., 1984]; 15 °C), *Epithelioma papulosum cyprini* (EPC [Winton et al., 2010]; 15 °C), and Bluegill fry (BF-2 [Wolf et al., 1966]; 25 °C) cells. Cytopathic effects (CPE) were only observed in BF-2 cells (Fig. 1A). Upon infection, the BF-2 cells lost their differentiated shape as they condensed and detached from the plate, occasionally appearing refractile (Fig. 1A). Transmission electron microscopy was used to image viruses concentrated by pelleting via high-speed centrifugation. The pellet was resuspended in molecular grade water, the suspension was fixed to a Formvar copper-coated grid, and stained with sodium phosphotungstate prior to viewing on a Zeiss EM900 transmission electron microscope. Abundant spherical virus particles (≈ 85 nm in diameter), each with a prominent globular fringe indicative of an enveloped virus, were observed (Fig. 1B).

Nucleic acid extracts from supernatant of infected BF-2 cultures tested negative against previously designed conventional PCR assays

---

[*] Corresponding author at: Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, 2187 Mowry Road, 32611, Gainesville, FL, USA.

E-mail address: tbwaltzek@ufl.edu (T.B. Waltzek).

https://doi.org/10.1016/j.virusres.2019.197761
Received 28 June 2019; Received in revised form 16 September 2019; Accepted 17 September 2019

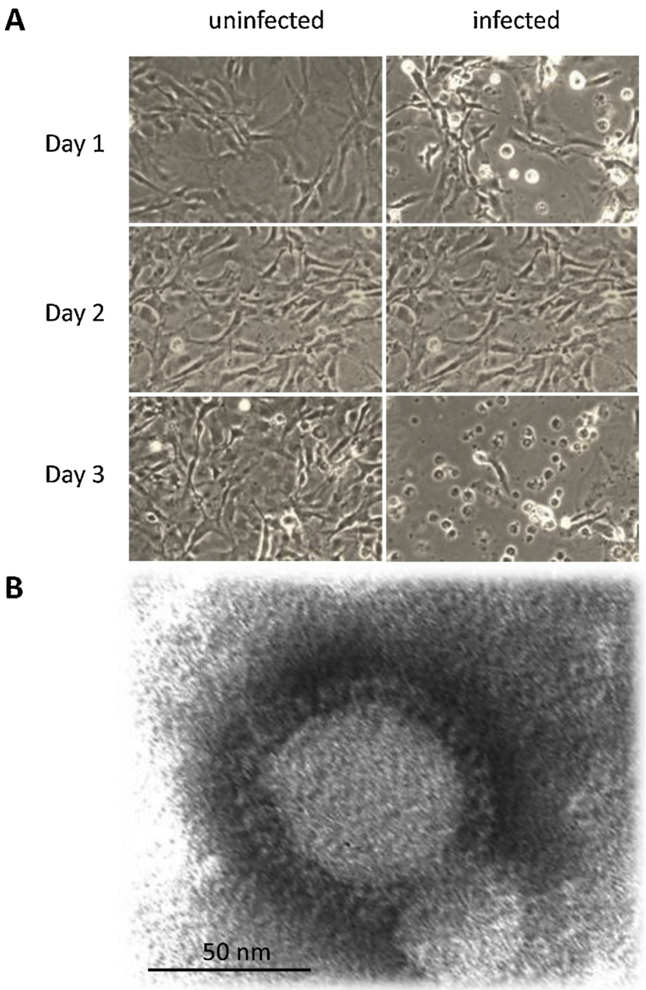


Fig. 1. *in vitro* growth characteristics and ultrastructural features of the largemouth bass bunyavirus (LBBV). (A) Cytopathic effects were not observed in uninfected control Bluegill fry (BF-2) cells. The LBBV-infected BF-2 cells occasionally appeared refractile and lost their differentiated shape as they condensed and detached from the plate at days 1, 2 and 3 post-infection. Original images taken at 40× magnification. (B) Negative stain electron photomicrograph of a circular LBBV particle displaying glycoprotein spikes.

targeting largemouth bass virus, spring viremia of carp virus (USFWS and AFS-FHS, 2014), and bluegill picornavirus (Barbknecht et al., 2014). Extracted RNA served as a template to construct a cDNA library using a NEBNext Ultra RNA Library Prep Kit (New England BioLabs® Inc.) followed by sequencing on an Illumina MiSeq sequencer using a version 3 chemistry 600 cycle Kit. *De novo* assembly of paired-end reads was performed in SPAdes 3.5.0 (Bankevich et al., 2012). In CLC Genomics Workbench V9.5, BLASTX analysis of the resulting contigs was carried out against a database created from virus protein sequences retrieved from the UniProt Knowledgebase (https://www.uniprot.org/uniprot/). Three contigs were identified with greatest BLAST scores to the large (L), medium (M), and small (S) segments of members of the genus *Orthobunyavirus* within the family *Peribunyaviridae* (Table S1). The L, M, and S segments of the largemouth bass bunyavirus (LBBV) were 6901, 3570, and 1002 bp in size with an average coverage of 1372, 2550, and 589 reads/nucleotide, respectively. The segments possess nine base pair conserved complimentary terminal sequences, with a non-canonical base pair at position eight, that enable the formation of stable panhandle structures (Fig. 2A). A single open reading frame (ORF) encoding the RNA-dependent RNA polymerase (RdRp) was identified in the L segment using CLC Genomics Workbench V9.5 (Fig. 2B and Table S1). The conserved motifs of the RdRp were predicted by comparison to the RdRps of other bunyaviruses. The LBBV RdRp contained a 124-amino acid (aa) long endonuclease in the N-terminus, six conserved motifs including premotif A, motif A, B, C, D, and E in the central region (Fig. 2B and 2C), and lacked a 500 bp insertion reported in herbeviruses (Marklewitz et al., 2013). The LBBV RdRp possessed the following conserved residues when compared to other bunyaviruses: Lysine (K), Glutamine (Q), Arginine (R), and Glutamic acid (E) in premotif A; Aspartic acid (D) in motif A; Q, Glycine (G), and Serine (S) in motif B; S and D in motif C; E in motif E (Fig. 2C). The M segment of LBBV lacks a nonstructural protein (NSm), similar to herbeviruses (Marklewitz et al., 2013), and includes a single ORF that encodes a glycoprotein precursor polyprotein that is predicted to be posttranslationally processed into glycoprotein N (Gn; aa 21–514) and glycoprotein C (Gc; aa 515–1124). A signal peptide, two zinc finger domains in Gn, and three transmembrane domains (two in Gn and one in Gc) were predicted using the Simple Modular Architecture Research Tool (http://smart.embl.de/; Fig. 2B). Seven glycosylation sites (six in Gn and one in Gc) were determined using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/; Fig. 2B). The S segment includes an ORF that encodes a nucleoprotein and lacks a nonstructural protein (NSs; Fig. 2B). The genome sequence of the LBBV has been deposited in National Center for Biotechnology Information (NCBI) GenBank database under the accession nos. MN119734-MN119736.

For the phylogenetic analyses, the aa sequences of the RdRp, glycoprotein precursor polyprotein, and nucleoprotein of 49 recognized type species in the order *Bunyavirales* were retrieved from the NCBI GenBank database and aligned along with the LBBV using the Multiple Alignment using Fast Fourier Transform (MAFFT) with the E-INS-i algorithm implemented. Poorly aligned regions were trimmed from the multiple sequence alignment using TrimAl V1.3, available in the Phylemon 2.0 webserver (http://phylemon.bioinfo.cipf.es), with the automated 1 method implemented. Three Maximum Likelihood (ML) phylogenetic analyses were performed based on the individual an alignments (i.e., RdRp, glycoprotein precursor polyprotein, and nucleoprotein) using the IQ-TREE server (http://iqtree.cibiv.univie.ac.at/) with 1000 non-parametric bootstrap replicates to determine clade support. Additionally, these alignments were concatenated in Geneious R10 and a ML phylogenetic analysis was performed using the parameters mentioned above. The LBBV was supported as the sister group to members of the family *Peribunyaviridae* (Fig. 3A and S1). A genetic analysis, based on the aa sequence alignment of the RdRp, was used to compare the LBBV to the type species of closely related genera within the *Peribunyaviridae*, *Fimoviridae*, *Cruliviridae*, and *Tospoviridae* families using the Sequence Demarcation Tool Version 1.2 (Muhire and Varsani, 2014) with the MAFFT alignment option implemented. The sequence identity of the LBBV to these bunyaviruses ranged from 23.4%–30.4% (highest identity to Herbert virus; Fig. 3B). Thus, we propose the LBBV represents the type species of a novel genus (proposed: piscibunyavirus) within the family *Peribunyaviridae*. We propose the formal species name of largemouth bass piscibunyavirus for consideration by the International Committee on Taxonomy of Viruses.

The family *Peribunyaviridae* includes the genus *Orthobunyavirus* with 88 accepted species characterized from a wide variety of mammalian hosts, including humans, as well as arthropods (Kuhn et al., 2019). The LBBV represents the first peribunyavirus characterized from an ectothermic vertebrate host. Similar to the recently sequenced fish hantaviruses and arenaviruses (Shi et al., 2018), the LBBV was found to be genetically divergent from previously characterized peribunyaviruses that infect arthropods or endothermic vertebrates. The role of fish bunyaviruses in disease has yet to be determined as previous descriptions have been based solely on metagenomic analyses (Shi et al., 2018). The isolation of the LBBV offers a unique opportunity to determine its role in disease and future challenge studies are planned. The host range of the LBBV, route(s) of transmission including potential aquatic vectors (e.g., blood-sucking crustacean parasites or leeches), and the prevalence within wild and cultured stocks of largemouth bass requires further investigation.

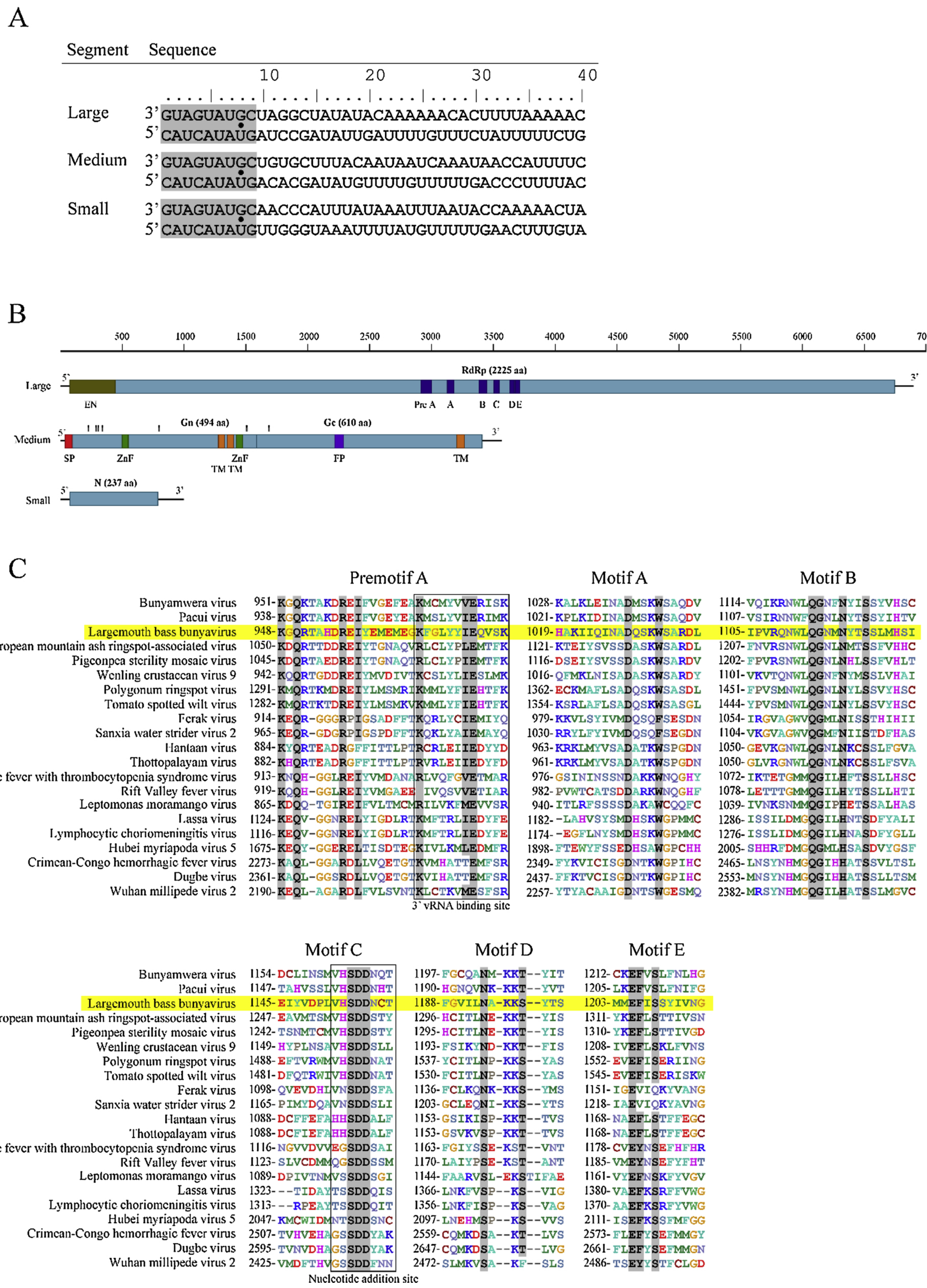
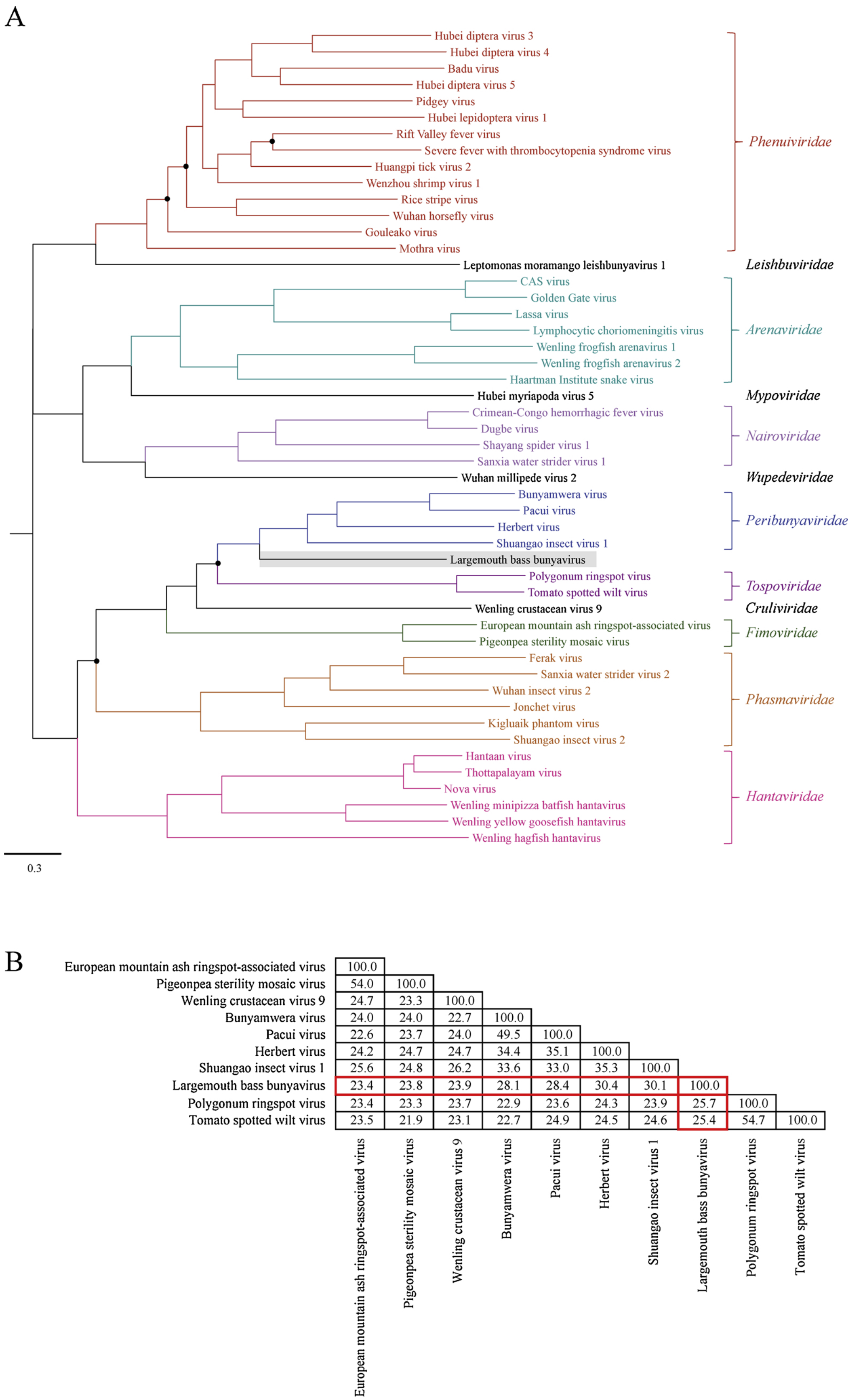


Fig. 2. Genomic characterization of the largemouth bass bunyavirus (LBBV). (A) The LBBV genome terminal sequences displaying conserved complementary nine bases are highlighted in grey, including a non-canonical base pairing at position eight (indicated by •). (B) Schematic representation of the LBBV genome. Open reading frames (ORFs) are shown as light blue boxes, the endonuclease domain in the RdRp is indicated by an olive green box and the conserved motifs are indicated by dark blue boxes. The glycosylation sites in the glycoprotein are marked by grey thick lines above the ORF, the signal peptide is indicated by a red box, the zinc finger motifs in the glycoprotein N are indicated by green boxes, the fusion peptide in glycoprotein C is indicated by a purple box, and the transmembrane domains are indicated by orange boxes. Abbreviations: Gn, glycoprotein N; Gc, glycoprotein C; EN, endonuclease; Pre A, premotif A; A, motif A; B, motif B; C, motif C; D, motif D; E, motif E; SP, signal peptide; ZnF, Zinc finger; TM, transmembrane domain; FP, fusion peptide. (C) Amino acid sequence alignment displaying conserved domains of the LBBV RdRp (highlighted in yellow) when compared to other bunyaviruses. Amino acid positions with a threshold of conservation > 85% are highlighted in grey.



	European mountain ash ringspot-associated virus	Pigeonpea sterility mosaic virus	Wenling crustacean virus 9	Bunyamwera virus	Pacui virus	Herbert virus	Shuangao insect virus 1	Largemouth bass bunyavirus	Polygonum ringspot virus	Tomato spotted wilt virus
European mountain ash ringspot-associated virus	100.0									
Pigeonpea sterility mosaic virus	54.0	100.0								
Wenling crustacean virus 9	24.7	23.3	100.0							
Bunyamwera virus	24.0	24.0	22.7	100.0						
Pacui virus	22.6	23.7	24.0	49.5	100.0					
Herbert virus	24.2	24.7	24.7	34.4	35.1	100.0				
Shuangao insect virus 1	25.6	24.8	26.2	33.6	33.0	35.3	100.0			
Largemouth bass bunyavirus	23.4	23.8	23.9	28.1	28.4	30.4	30.1	100.0		
Polygonum ringspot virus	23.4	23.3	23.7	22.9	23.6	24.3	23.9	25.7	100.0	
Tomato spotted wilt virus	23.5	21.9	23.1	22.7	24.9	24.5	24.6	25.4	54.7	100.0

Fig. 3. (A) Phylogram depicting the relationship of the largemouth bass bunyavirus (LBBV; highlighted in grey) to 49 recognized type species in the order *Bunyavirales*. The Maximum Likelihood tree was generated using the concatenated amino acid (aa) sequence alignments of the RNA-dependent RNA polymerase (RdRp), glycoprotein precursor polyprotein, and nucleoprotein. Branch lengths are based on the number of inferred substitutions, as indicated by the scale. The nodes supported by bootstrap values < 75% are indicated by black circles. (B) Sequence identity matrix based on the RdRp aa alignment of the LBBV (red box) compared to the type species within genera of the families *Peribunyaviridae*, *Fimoviridae*, *Cruliviridae*, and *Tospoviridae*.

Declaration of Competing Interest

None.

Acknowledgements

The authors would like to thank the Wisconsin Department of Natural Resources for collecting the largemouth bass sampled in this study as well as Jessica Mollison and Sarah Leis (La Crosse Fish Health Center) for their assistance in the virology laboratory.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197761.

References

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., et al., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455–477.

Barbknecht, M., Sepsenwol, S., Leis, E., Tuttle-Lau, M., Gaikowski, M., Knowles, N.J., et al., 2014. Characterization of a new picornavirus isolated from the freshwater fish Lepomis macrochirus. J. Gen. Virol. 95, 601–613.

Briese, T., Alkhovskiy, S., Beer, M., Calisher, C.H., Charrel, R., Ebihara, H., et al., 2016. Create a New Order, *Bunyavirales*, to Accommodate Nine Families (Eight New, One Renamed) Comprising Thirteen Genera. Accessed 19 Apr 2019. https://talk.ictvonline.org/ictv/proposals/2016.030a-vM.A.v6.Bunyavirales.pdf.

Lannan, C.N., Winton, J.R., Fryer, J.L., 1984. Fish cell lines: establishment and characterization of nine cell lines form salmonids. In Vitro 20, 671–676.

Maes, P., Alkhovsky, S.V., Bào, Y., Beer, M., Birkhead, M., Briese, T., et al., 2018. Taxonomy of the family *Arenaviridae* and the order *Bunyavirales*. update 2018. Arch. Virol. (163), 2295–2310.

Marklewitz, M., Zirkel, F., Rwego, I.B., Heidemann, H., Trippner, P., Kurth, A., Kallies, R., Briese, T., Lipkin, W.I., Drosten, C., Gillespie, T.R., Junglen, S., 2013. Discovery of a unique novel clade of mosquito-associated bunyaviruses. J. Virol. 87, 12850–12865.

Muhire, B.M., Varsani, A., 2014. Martin DP. SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. PLoS One 9, e108277.

Shi, M., Lin, X.D., Chen, X., Tian, J.H., Chen, L.J., Li, K., et al., 2018. The evolutionary history of vertebrate RNA viruses. Nature 556, 197–202.

Tatineni, S., McMechan, A.J., Wosula, E.N., Wegulo, S.N., Graybosch, R.A., French, R., et al., 2014. An eriophyid mite-transmitted plant virus contains eight genomic RNA segments with unusual heterogeneity in the nucleocapsid protein. J. Virol. 88, 11834–11845.

USFWS and AFS-FHS (U.S. Fish and wildlife service and American fisheries society-fish health section), 2014. Standard procedures for aquatic animal health inspections. AFS-FHS Blue Book: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens, 2016 ed. .

Winton, J., Batts, W., deKinkelin, P., LeBerre, M., Bremont, M., Fijan, N., 2010. Current lineages of the *epithelioma papulosum cyprini* (EPC) cell line are contaminated with fathead minnow, *Pimephales promelas*, cells. J. Fish Dis. 33, 701–704.

Wolf, K., Gravell, M., Malsberger, R.G., 1966. Lymphocystis virus: isolation and propagation in centrarchid fish cell lines. Science 151, 1004–1005.