



Short communication

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

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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 (arboviruses) 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

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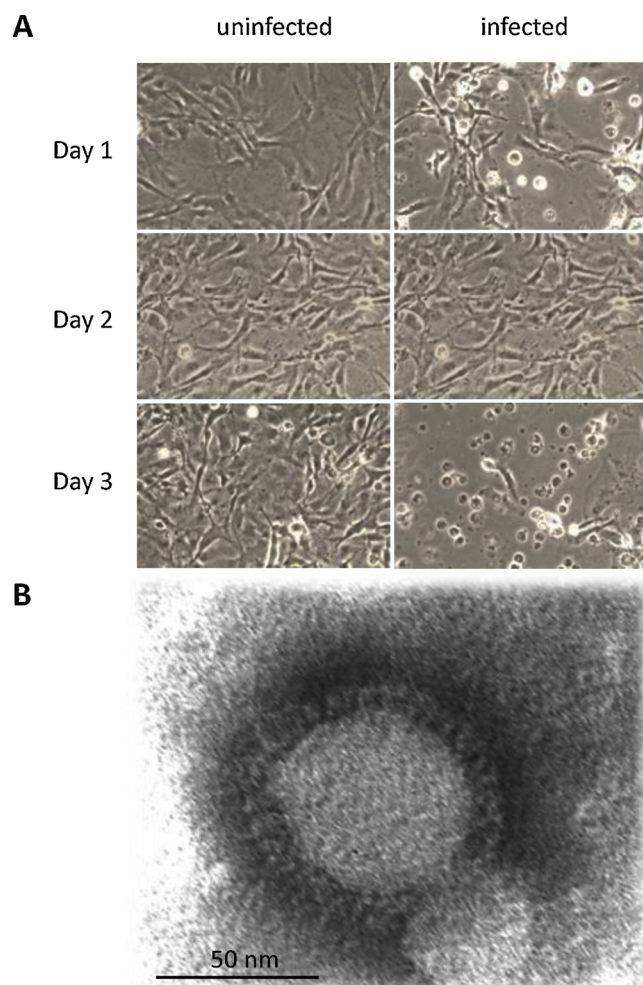


Fig. 1. *in vitro* growth characteristics and ultrastructural features of the largemouth bass bunyavirus (LBBV). (A) Cytopathic effects were not observed in uninfected control Bluegill fry (BF-2) cells. The LBBV-infected BF-2 cells occasionally appeared refractile and lost their differentiated shape as they condensed and detached from the plate at days 1, 2 and 3 post-infection. Original images taken at 40 \times 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 complementary 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.

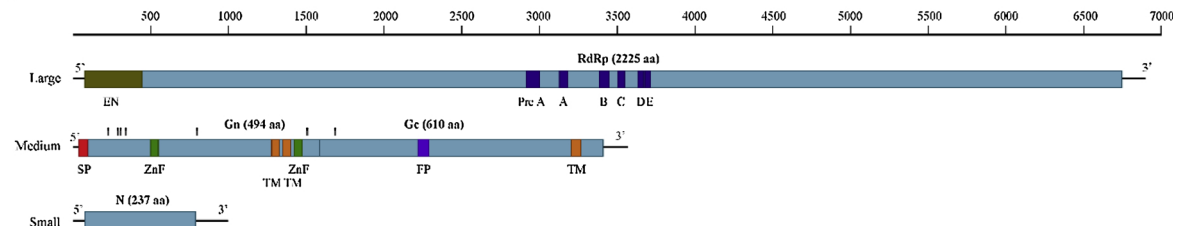
For the phylogenetic analyses, the aa sequences of the RdRp, glycoprotein precursor polyprotein, and nucleoprotein of 49 recognized type species in the order *Bunyavirales* were retrieved from the NCBI GenBank database and aligned along with the LBBV using the Multiple Alignment using Fast Fourier Transform (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 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.

A

Segment	Sequence
	10 20 30 40
Large	3' GUAGUAUGCUAGGCUAUUAACAAAAACACUUUAAAAAC 5' CAUCAUAUGAUCGGAUAUUGAUUUUGUUUCUAUUUUUCUG
Medium	3' GUAGUAUGCUGUGCUUUACAUAUACAAUAUACCAUUUUC 5' CAUCAUAUGACACGAUAUUGUUUUUGUACCCUUUAC
Small	3' GUAGUAUGCAACCCAUUUUAAAAUUAAUACAAAAACUA 5' CAUCAUAUGUUGGGUAAUUUUUUGUUUUUGAACUUUGUA

B



C

	Premotif A	Motif A	Motif B
Bunyamwera virus	951-KGQKTAKDREIFVGEFEAKMCMVVERISK	1028-KALKLEINADMSKWSAQDV	1114-VQIKRNWLGQGNFYISVYVHSC
Pacui virus	938-KGQKTAKDREIFVGEFEAKMCMVVERISK	1021-KPLKIDINADMSKWSAQDV	1107-VSIRNNWFGQNLNTSSYIHTV
Largemouth bass bunyavirus	948-KQRTAHDRREIYEMEMEQKFLGYIEQVSK	1019-HKKIQTINADQSKWSARDL	1105-TPVRQNWLGQGNFYISVYVHSC
European mountain ash ringspot-associated virus	1050-KDQRTDDREIYTGNAQVRLCLYPLEMTFFK	1121-KTEIYVSSSDASKWSARDL	1207-FNVRSNWLGQGNFYISVYVHSC
Pigeonpea sterility mosaic virus	1045-KDQRTAHDRREIYTGNAQVRLCLYPLEMTFFK	1116-DEIYVSSSDASKWSARDV	1202-FPVRSNWLGQGNFYISVYVHSC
Wenling crustacean virus 9	942-KQRTGDDREIYMDIVTKCSLYLIESIMK	1016-QFMKINISADASKWSARDY	1101-VKVTQNWLGQGNFYISVYVHSC
Polygonum ringspot virus	1291-KMQRTKMDREIYLMEMKIMMLYFIEHFFK	1362-ECKMAFLSADQSKWSASDL	1451-FPVRSNWLGQGNFYISVYVHSC
Tomato spotted wilt virus	1282-KMQRTKMDREIYLMEMKIMMLYFIEHFFK	1354-KSRLAFLSADQSKWSASGL	1444-FPVRSNWLGQGNFYISVYVHSC
Ferak virus	914-KEQR-GGGRPIGSADFFTKQRLYCIEMTIQ	979-KKVLSTYVMDQSQFSESDN	1054-IRGVAGWVGQGNFYISVYVHSC
Sanxia water strider virus 2	965-KEQR-GGGRPIGSADFFTKQRLYCIEMTIQ	1030-RRLYFYVMDQSQFSESDN	1104-VKGVAGWVGQGNFYISVYVHSC
Hantaan virus	884-KYQRTEDRGGFFITLPTRCLEIIEIDYFD	963-KRKLMYVSADATKWSFGDN	1050-GLVGRNWLGQGNFYISVYVHSC
Thottopalayam virus	882-KYQRTEDRGGFFITLPTRCLEIIEIDYFD	961-KRKLMYVSADATKWSFGDN	1050-GLVGRNWLGQGNFYISVYVHSC
Severe fever with thrombocytopenia syndrome virus	913-KNQH-GGLREIYVMDANARLVQFVETMAR	976-GSININSSNDAKKNQGHY	1072-IKTETGMQGLIFHTSSLLHSC
Rift Valley fever virus	919-KQKH-GGLREIYVMDAEE IVQSVVETIAR	982-PVWTCATSDDAKKNQGHY	1078-LETTTGMQGLIFHTSSLLHSC
Leptomonas moramango virus	865-KDQK-TGIREIFVLMCMRILVKMEVVSAR	940-ITLRFSSSSDAKWCQPMK	1039-IVNKNMCGQILHATSSLLHSC
Lassa virus	1124-KEQV-GGNRELYIGDLRTKMFTRLIEDYFE	1182-LAHVSYSDMSKWSGPMK	1286-ISSILDMGQGLIFHTSSLLHSC
Lymphocytic choriomeningitis virus	1116-KEQV-GGNRELYIGDLRTKMFTRLIEDYFE	1174-EGFLNYSMDMSKWSGPMK	1276-ISSILDMGQGLIFHTSSLLHSC
Hubei myriapoda virus 5	1675-KEQY-GGERELTISDTCKIVLKLMDMFR	1898-FTEWYFSSSEDSAWGPMK	2005-SHHRFDMGQGLIFHTSSLLHSC
Crimoean-Congo hemorrhagic fever virus	2273-KAQL-GGARDLLVQETGKVMHATTEMFSR	2349-FYKVICISGDNKKGPIHC	2465-LNSYNHMGQGLIFHTSSLLHSC
Dugbe virus	2361-KAQL-GGARDLLVQETGKVIHATTEMFSR	2437-FYKVICISGDNKKGPIHC	2553-MNSYNHMGQGLIFHTSSLLHSC
Wuhan millipede virus 2	2190-KEQL-AGARDLLVSVNKLCTKVMESFSR	2257-YTYCAAIGDNTSGWESMQ	2382-MRSYNHMGQGLIFHTSSLLHSC
	3' vRNA binding site		
	Motif C	Motif D	Motif E
Bunyamwera virus	1154-DCLINSMVHSDDNQT	1197-FGCQANM-KKT--YIT	1212-CKEFVSLFNLHG
Pacui virus	1147-TAHVSSIVHSDDNQT	1190-HGNQVNM-KKT--YVT	1205-LKEFVSLFNIFG
Largemouth bass bunyavirus	1145-EYVDPILVHSDDNQT	1188-FGVILNA-KKS--YTS	1203-MMEFISYIVNG
European mountain ash ringspot-associated virus	1247-EAVMTSMVHSDDSTY	1296-HCITLNE-KKT--YIS	1311-YKEFLSTTIVSN
Pigeonpea sterility mosaic virus	1242-TSNMTSMVHSDDSTY	1295-HCITLNE-KKT--YIS	1310-YKEFLSTTIVGD
Wenling crustacean virus 9	1149-HYPLNSVHSDDSL	1193-FSIKIND-KKT--FIS	1208-IVEFLSKLFVNS
Polygonum ringspot virus	1488-EFTVRMVHSDDNAT	1537-YCITLNP-KKS--YAS	1552-EVEFISERILNG
Tomato spotted wilt virus	1481-DQTRWIVHSDDNAT	1530-FCITLNP-KKS--YAS	1545-EVEFISERISKW
Ferak virus	1098-QVEVDHIVHSDDSFA	1136-FCLKQNK-KKS--YMS	1151-IGEVICQKYVANG
Sanxia water strider virus 2	1165-PIMVDQVHSDDSLI	1203-GCLEQNI-KKS--YTS	1218-IAEVIQKYAVNG
Hantaan virus	1088-DCFEFAHHSDDALF	1153-GSIKISP-KKT--TVS	1168-NAEFLSTFFEGC
Thottopalayam virus	1088-DCFEFAHHSDDALF	1153-GSVKISP-KKT--TVS	1168-NAEFLSTFFEGC
Severe fever with thrombocytopenia syndrome virus	1116-NGVVDVHSDDSAI	1163-FGIYSSE-KST--VNT	1178-CVEYNSEFFHFR
Rift Valley fever virus	1123-SLVCDMMQSDSSMI	1170-LAIYPSE-KST--ANT	1183-VMEYNSEFFHFT
Leptomonas moramango virus	1089-DPIVTNMVHSDDSGI	1144-FAARVSL-EKSTIFAE	1161-VYEENSKFYVVG
Lassa virus	1323--TIDATSSDDQIS	1366-LNKFVSP--KS--VIG	1380-VAEFKSRFFVVG
Lymphocytic choriomeningitis virus	1313--RPEAYTSSDDQIT	1356-LNKFISP--KS--VAG	1370-AAEFKSRFFVVG
Hubei myriapoda virus 5	2047-KMCWIDMNTSDDSNK	2097-LNEHMSK--KS--VVS	2111-ISEFKSFFMFGG
Crimoean-Congo hemorrhagic fever virus	2507-TVHVEHAGSSDDYAK	2559-CQMKDSA--KT--LVS	2573-FLEFYSEFMFGY
Dugbe virus	2595-TVHVEHAGSSDDYAK	2647-CQMKDSA--KT--LVS	2661-FLEFYSEFMFGN
Wuhan millipede virus 2	2425-VMDTFHVSDDENN	2472-SLMKUSA--KF--SLS	2486-TSEYYSFCLGD
	Nucleotide addition site		

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 the 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.

Phylogenetic tree showing the relationships between 32 insect-associated bunyaviruses, color-coded by family. The tree is rooted on the left and branches to the right. A scale bar of 0.3 is located at the bottom left.

Phenuiviridae (Red):

- Hubei diptera virus 3
- Hubei diptera virus 4
- Badu virus
- Hubei diptera virus 5
- Pidgey virus
- Hubei lepidoptera virus 1
- Rift Valley fever virus
- Severe fever with thrombocytopenia syndrome virus
- Huangpi tick virus 2
- Wenzhou shrimp virus 1
- Rice stripe virus
- Wuhan horsefly virus
- Gouleako virus
- Mothra virus

Leishbuviridae (Black):

- Leptomonas moramango leishbunyavirus 1

Arenaviridae (Teal):

- CAS virus
- Golden Gate virus
- Lassa virus
- Lymphocytic choriomeningitis virus
- Wenling frogfish arenavirus 1
- Wenling frogfish arenavirus 2
- Haartman Institute snake virus

Mypoviridae (Black):

- Hubei myriapoda virus 5

Nairoviridae (Purple):

- Crimean-Congo hemorrhagic fever virus
- Dugbe virus
- Shayang spider virus 1
- Sanxia water spider virus 1

Wupedeviridae (Black):

- Wuhan millipede virus 2

Peribunyaviridae (Blue):

- Bunyanwera virus
- Pacui virus
- Herbert virus
- Shuangao insect virus 1
- Largemouth bass bunyavirus

Tospoviridae (Purple):

- Polygonum ringspot virus
- Tomato spotted wilt virus

Cruliviridae (Green):

- Wenling crustacean virus 9

Fimoviridae (Green):

- European mountain ash ringspot-associated virus
- Pigeonpea sterility mosaic virus

Phasmaviridae (Brown):

- Ferak virus
- Sanxia water strider virus 2
- Wuhan insect virus 2
- Jonchet virus
- Kigluaik phantom virus
- Shuangao insect virus 2

Hantaviridae (Pink):

- Hantaan virus
- Thottapalayam virus
- Nova virus
- Wenling minipizza batfish hantavirus
- Wenling yellow goosefish hantavirus
- Wenling hagfish hantavirus

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

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.

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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>.

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