Characterization of a new picornavirus isolated from the freshwater fish *Lepomis macrochirus*

Marisa Barbknecht,1 Sol Sepsenwol,2 Eric Leis,3 Maren Tuttle-Lau,3,4 Mark Gaikowski,4 Nick J. Knowles,5 Becky Lasee3 and Michael A. Hoffman1

1Department of Microbiology, University of Wisconsin–La Crosse, 1725 State Street, La Crosse, WI, 54601, USA
2Department of Biology, University of Wisconsin–Stevens Point, 2100 Main Street, Stevens Point, WI, 54481, USA
3US Fish and Wildlife Service, La Crosse Fish Health Center, 555 Lester Avenue, Onalaska, WI, 54650, USA
4US Geological Survey–Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Road, La Crosse, WI, 54603, USA
5Pirbright Institute, Ash Road, Pirbright, Woking, Surrey, GU24 0NF, UK

The freshwater fish *Lepomis macrochirus* (bluegill) is common to North American waters, and important both ecologically and as a sport fish. In 2001 an unknown virus was isolated from bluegills following a bluegill fish kill. This virus was identified as a picornavirus [termed bluegill picornavirus (BGPV)] and a diagnostic reverse transcriptase PCR was developed. A survey of bluegills in Wisconsin waters showed the presence of BGPV in 5 of 17 waters sampled, suggesting the virus is widespread in bluegill populations. Experimental infections of bluegills confirmed that BGPV can cause morbidity and mortality in bluegills. Molecular characterization of BGPV revealed several distinct genome characteristics, the most unusual of which is the presence of a short poly(C) tract in the 3' UTR. Additionally, the genome encodes a polyprotein lacking a leader peptide and a VP0 maturation cleavage site, and is predicted to encode two distinct 2A proteins. Sequence comparison showed that the virus is most closely related to a phylogenetic cluster of picornaviruses that includes the genera *Aquamavirus*, *Avihepatovirus* and *Parechovirus*. However, it is distinct enough, for example sharing only about 38% sequence identity to the parechoviruses in the 3D region, that it may represent a new genus in the family *Picornaviridae.*

INTRODUCTION

*Lepomis macrochirus*, commonly known as the bluegill, is one of the most common freshwater fishes in North America. The native range of the bluegill includes the Great Lakes and Mississippi River basins. However, through stocking, the current range now includes most of the continental USA, southern Canada and Mexico. In many waters bluegills are the most abundant sport fish, making the species socially, ecologically and economically important (Snow et al., 1978). For these reasons healthy bluegill populations are desired and fish populations are monitored for pathogens.

Several viruses have been found to infect bluegills in the wild or in experimental settings. Bluegill hepatic necrosis reovirus and lymphocystis disease virus (an iridovirus) do cause disease in bluegills during experimental infections, but have never been isolated from wild bluegills (Meyers & Hirai, 1980; Wolf & Carlson, 1965). Conversely, largemouth bass virus (LMBV, an iridovirus) and *Spring viralemia of carp virus* (SVCV, a rhabdovirus) have been isolated from wild bluegills, but no disease has been associated with these infections (Grizzle & Brunner, 2003). Thus, none of the aforementioned viruses has been observed to cause disease outbreaks in wild bluegill populations. In contrast, *Viral hemorrhagic septicemia virus* (VHSV), an emerging rhabdovirus that infects multiple fish species, was associated with a fish kill involving bluegills (Michigan Department of Natural Resources, 2007; Goodwin & Merry, 2011). However, there is only one known instance of a fish kill of wild bluegills due to VHSV. Other bluegill fish kills have occurred, and while clear causes (environmental
and/or infectious) have occasionally been identified, at other times a cause was not confirmed, but involvement of viral agents suspected.

In 2001, the US Fish and Wildlife Service La Crosse Fish Health Center isolated an unknown virus from bluegills following a bluegill fish kill that occurred in a small lake in northern Wisconsin, USA. Here we show that the virus appears to be widespread in wild bluegill populations, and can cause morbidity and mortality in bluegills. Additionally, we report the characterization of the complete genome of the virus, which may represent a new genus in the family Picornaviridae.

RESULTS

Initial isolation and characterization

In late autumn 2001 a bluegill fish kill was reported in Montana Lake in northern Wisconsin, USA. The La Crosse Fish Health Center collected samples, made homogenates from spleen and kidney tissues, and inoculated the homogenates onto bluegill fry-2 (BF-2) cells. Unique, slow developing cytopathic effects (CPEs) appeared in the cells. The virus was passaged multiple times, but diagnostic PCRs for known viruses were negative.

Infection of BF-2 cells showed that CPE developed between 3 and 5 days post-infection, with complete detachment occurring within 12–14 days. The CPEs included cell elongation and accumulation of large cytoplasmic vesicles (Fig. 1a). Analysis of the isolate by transmission electron microscopy showed round to icosahedral virus particles averaging 30 nm in diameter, consistent with the size and shape of picornaviruses (Fig. 1b).

Initial sequence information was obtained by amplification using reverse transcriptase PCR (RT-PCR) with an oligo(dT)-containing negative-sense primer and positive-sense degenerate pan-picornavirus primers designed to bind the conserved XXYGDD motif of the 3D polymerase (Knowles, 2005). The procedure did not amplify the target region, but amplicons from the genome were cloned and sequenced. This sequence information showed that the virus was a picornavirus [termed bluegill picornavirus (BGPV)], and allowed development of a diagnostic RT-PCR. For the diagnostic RT-PCR, forward and reverse primers were designed to anneal within the 3’ UTR, producing a 180 bp amplicon. This RT-PCR was observed to be specific as reactions using nucleic acid extracts from uninfected cells and cells infected with LMBV, SVCV, infectious pancreatic necrosis virus and VHSV produced no amplicons. By performing the RT-PCR with serial dilutions of RNA from purified virions, a lower detection limit of 1.9 × 10^4 p.f.u. was determined.

Distribution of BGPV

In the years after 2001, presumptive isolations, based on observation of characteristic tissue culture CPEs, were made from 13 additional water bodies in Wisconsin, indicating that the virus may be fairly widespread. To better understand how common the virus is within Wisconsin bluegill populations, we surveyed 17 bodies of water throughout the state, including 4 sites of previous presumptive isolations (Fig. 2). Thirty bluegills were harvested from each site for detection of the virus by culture. If CPEs were observed in the original culture and after passage, the presence of BGPV in culture was confirmed by PCR. The virus was isolated from 5 locations in total: from 2 of the 4 presumptive isolation sites and 3 of 13 additional sites. No fish exhibited clinical signs of infection, and no other viruses were isolated.

Fig. 1. Microscopic analysis of BGPV. (a) CPEs associated with BGPV infection of BF-2 cells. For phase-contrast microscopy at ×10 magnification cells were imaged at 10 days post-infection. For the images with phase-contrast microscopy at ×60 magnification, and differential interference contrast (DIC) microscopy, cells were fixed (3 % paraformaldehyde and 0.1 % glutaraldehyde in PBS) and examined at 14 days post-infection. Arrows indicate large vesicles in infected cells. Bars, 30 mm. (b) Transmission electron micrograph of negatively stained, concentrated extracellular BGPV particles. Particles were stained with 1 % aqueous uranyl acetate and examined using a Hitachi H-600 transmission electron microscope, operating at 75 kV. Bar, 100 nm.
Experimental infection

As noted, while the original isolate came from diseased fish, later presumptive isolations were made from apparently healthy fish. To determine the ability of BGPV to cause disease, groups of 20 juvenile bluegills were injected intraperitoneally with PBS (mock), or $1.5 \times 10^4$ p.f.u. (low-dose) or $1.5 \times 10^7$ p.f.u. (high-dose) BGPV (Table 1). To monitor the progression of disease and determine whether fish could clear the infection, five fish from each group were sampled weekly to examine for symptoms and to isolate virus by culture from kidney, spleen and liver tissues. If CPEs were observed in culture from a weekly sampling, medium was collected from one positive tissue culture well for diagnostic RT-PCR.

The frequency of disease was significantly higher in the infected fish compared with the mock-infected fish. Only 1 mock-infected fish exhibited minor haemorrhaging around its vent, while 5 of 20 low-dose fish showed signs of disease (including one mortality) and 9 of 20 high-dose fish showed signs of disease (including two mortalities). The percentage of infected fish showing signs of disease was most likely underestimated because fish harvested in early weeks may have gone on to develop symptoms. No clear pattern of disease emerged as fish collected each week included asymptomatic fish and those showing a variety of symptoms. In the fish showing signs of disease ($n=14$), the most common symptom was inflammation and redness at the base of fins ($n=8$). Other observed symptoms included haemorrhaging at additional external locations, exophthalmia, abdomen distension, internal haemorrhaging and ascites. Regardless of whether the fish showed signs of disease, virus was recovered in culture as determined by observation of CPEs from 39 of 40 infected fish. Diagnostic RT-PCR with eight CPE-positive cultures confirmed the presence of BGPV.

Molecular characteristics of BGPV

To complete the sequencing of the BGPV genome, gaps between previously obtained sequences were bridged by RT-PCR, while 5′- and 3′-terminal sequences were obtained using RACE procedures. The complete BGPV genome is 8050 nt, excluding the poly(A) tail, with a 5′ UTR of 712 nt, a coding region of 7008 nt (2336 aa) and a 3′ UTR of 330 nt (GenBank accession no. JX134222; Table 2, Fig. 3). The 3′ UTR is one of the longest of the picornaviruses. This length is in part due to the presence of a poly(C) tract of at least 21 nt. To confirm the presence of the poly(C) tract, additional PCR amplicons encompassing the poly(C) region were cloned and sequenced. All ten amplicons sequenced had poly(C) tracts that ranged from 12 to 21 Cs.

5′ UTR

The 5′ UTRs of picornaviruses contain RNA elements with roles in replication, pathogenicity and translation. The dominant RNA element within the 5′ UTR of picornaviruses is the internal ribosomal entry site (IRES), which is critical for translation initiation and comprises a series of RNA secondary structures and sequence elements. In picornavirus type I and II IRES elements a Yn-Xm-AUG sequence motif is conserved at the 3′ side of the IRES. In this motif, Yn represents a polypyrimidine tract of 7–12 bases positioned 15–25 nt (Xm) upstream of an AUG (Palmenberg et al., 2010). For BGPV, a Yn-Xm-AUG motif is present. An AUG at nucleotide 713 is predicted to be the start codon for the polyprotein based on its being 25 nt downstream of an 11 nt polypyrimidine tract (677UUUUCCUACU687).
To further analyse the 5′ UTR of BGPV, secondary structure analysis of the BGPV 5′ UTR was done using MFOLD (Zuker, 2003). Because no closely related viruses have been sequenced, it was impossible to compare structures between viruses (looking for compensatory mutations that restore Watson–Crick base pairings) as a way to validate potential BGPV stem–loop structures. Therefore, most of the structures depicted in Fig. 4 are those with high pairing fidelity (low P-num). Clearly present in BGPV is a 5′-terminal stem–loop consisting of 56 nt. Such an unbranched terminal stem–loop is common to most picornaviruses and is believed to function in genome replication (Palmenberg et al., 2010).

A series of stem–loop structures are present in what would be the IRES region of the 5′ UTR, from nt 301 to 666 (Fig. 4). The nt 301–322 stem–loop did not have high pairing fidelity, but was included in the predicted structure because the loop sequence of ACAA fits a consensus RNRA loop (Jang, 2006). The RNRA tetraloop (often recognized as GNRA) is critical in the structure and function of type I and II IRES elements, and is typically found at the 5′ side of the IRES (Fernández-Miragall et al., 2006; Kaminski et al., 1994; López de Quinto & Martinez-Salas, 1997; Robertson et al., 1999). No other potential RNRA tetraloop sequences were apparent. Comparison of the BGPV stem–loop structures with established type I and II IRES structures shows that the BGPV structures contain sequences that are conserved in other picornaviruses, but also include unique elements that may contribute to the specificity of the BGPV IRES.

### Table 1. Experimental intraperitoneal infection of juvenile bluegills with BGPV

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mortality</th>
<th>Morbidity</th>
<th>Tissue culture CPE</th>
<th>Positive PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>Week 1</td>
<td>0</td>
<td>1/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>0</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Week 3</td>
<td>0</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>0</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0/20</td>
<td>1/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Low dose (1.5×10⁴ p.f.u. per fish)</td>
<td>Week 1</td>
<td>1</td>
<td>1/4</td>
<td>4/5</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>0</td>
<td>0/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Week 3</td>
<td>0</td>
<td>0/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>0</td>
<td>3/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1/20</td>
<td>4/19</td>
<td>19/20</td>
</tr>
<tr>
<td>High dose (1.5×10⁷ p.f.u. per fish)</td>
<td>Week 1</td>
<td>1</td>
<td>1/4</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>0</td>
<td>3/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Week 3</td>
<td>1</td>
<td>2/4</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>0</td>
<td>1/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>2/20</td>
<td>7/18</td>
<td>20/20</td>
</tr>
</tbody>
</table>

### Table 2. Genome organization of BGPV

<table>
<thead>
<tr>
<th>Region</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>Downstream cleavage*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Begin</td>
<td>End</td>
<td>Total</td>
</tr>
<tr>
<td>5′ UTR</td>
<td>1</td>
<td>712</td>
<td>712</td>
</tr>
<tr>
<td>1AB</td>
<td>713</td>
<td>1426</td>
<td>714</td>
</tr>
<tr>
<td>1C</td>
<td>1427</td>
<td>2113</td>
<td>687</td>
</tr>
<tr>
<td>1D</td>
<td>2114</td>
<td>2998</td>
<td>885</td>
</tr>
<tr>
<td>2A1</td>
<td>2999</td>
<td>3049</td>
<td>51</td>
</tr>
<tr>
<td>2A2</td>
<td>3050</td>
<td>3457</td>
<td>408</td>
</tr>
<tr>
<td>2B</td>
<td>3458</td>
<td>4237</td>
<td>780</td>
</tr>
<tr>
<td>2C</td>
<td>4238</td>
<td>5263</td>
<td>1026</td>
</tr>
<tr>
<td>3A</td>
<td>5264</td>
<td>5575</td>
<td>312</td>
</tr>
<tr>
<td>3B</td>
<td>5576</td>
<td>5656</td>
<td>81</td>
</tr>
<tr>
<td>3C</td>
<td>5657</td>
<td>6250</td>
<td>594</td>
</tr>
<tr>
<td>3D</td>
<td>6251</td>
<td>7720</td>
<td>1470</td>
</tr>
<tr>
<td>3′ UTR</td>
<td>7721</td>
<td>8050</td>
<td>330</td>
</tr>
</tbody>
</table>

*The first two cleavage sites were determined experimentally, remaining cleavage sites are predicted.
II IRES structures did not reveal any noticeable structural similarities.

**Polyprotein**

A combination of sequence analysis and N-terminal sequencing was used to predict/determine the amino and carboxy termini of processed proteins. To determine cleavage sites in structural proteins, proteins from purified virus particles were gel purified and analysed by N-terminal sequencing. In the P1 region there is no apparent leader protein, but 1AB (VP0), 1C (VP3) and 1D (VP1) polypeptides are encoded. The VP0/VP3 junction was determined to be at a VLE↓G site while the VP3/VP1 junction was at a KFQ↓A site. Because there are no viruses closely related to BGPV for which cleavage locations have been assigned experimentally, additional predicted cleavage sites were determined by similarity to established NPG↓P ribosome skipping or 3Cpro cleavage sites (Donnelly et al., 2001; Ryan et al., 1999). These predictions are therefore tentative.

The P2 region is similar to seal aquamavirus 1 (SeAV-1; genus *Aquamavirus*), *Duck hepatitis A virus* (DHAV; genus **Fig. 3.** Genome organization of BGPV (GenBank accession no. JX134222) showing the location of the poly(C) tract.

**Fig. 4.** Putative RNA secondary structures formed in the 5′ UTR of BGPV. RNA secondary structure elements were predicted using the MFOLD program. The RNRA tetraloop, 310ACAAG313, is denoted by a bold curved line, and the Yn (nt 669–679) and AUG sequences of the Yn-Xm-AUG motif are underlined.
Avihepatovirus) and Ljungan virus (LV; genus Parechovirus) in encoding multiple 2A proteins (Johansson et al., 2002; Kapoor et al., 2008; Tseng et al., 2007). Unusually, BGPV encodes two 2A proteins that end in an NPG ↓P ribosome skipping motif. In BGPV, this motif, D(V/I)ExNPGP, is represented by DVESNPQP and DVEQNPQP. Since ribosome skipping at NPG ↓P sites only occurs in cis during translation this implies that BGPV contains an extra primary processing site not present in other known picornaviruses. The first 2A protein is predicted to be 17 aa and analogous to the foot-and-mouth disease virus (FMDV) 2A, while the second 2A is predicted to contain 136 aa. However, it is possible that the first 2A motif is actually part of VP1 and not trimmed off by 3Cpro. Carp picornavirus is also predicted to possess two 2A polypeptides both with NPG ↓P motifs, the first an FMDV-like short 2A and the second 133 aa in length (Knowles et al., 2012; Lange et al., 2014).

Several conserved motifs present in picornavirus genomes are apparent in BGPV, while others are absent. The 2A2 protein does not have the H-box/NC motif that is present in the Parechovirus, Kobuvirus, Tremovirus and Avihepatovirus genera and the proposed ‘Avisivirus’ genus (Boros et al., 2013a; Hughes & Stanway, 2000; Ng et al., 2013). This motif is also lacking in SeAV-A1, carp picornavirus, swine pasivirus 1 (SPA-1) and greplavirus A1 (Boros et al., 2013b; Kapoor et al., 2008; Lange et al., 2014; Sauvage et al., 2012). The 2C protein contains an NTP binding motif consisting of three conserved sequences, termed A, B and C motifs (Gorbunova et al., 1990; Kadare & Haenni, 1997). In BGPV the A (GXXGXGKS), B (hyhhyDD) and C (hhyhhyhN) motifs are \(^{138}{\text{Gepgq}}{\text{qkt}}^{145}, \quad ^{182}{\text{Hyide}}^{186}\) and \(^{236}\text{Vititsn}^{232}\), respectively.

The P3 region encodes the expected 3A, 3B, 3C and 3D polypeptides. Only a single 3B (VPg) is predicted as is present in most members of the group, except for SeAV-1, which is predicted to possess two in tandem copies of 3B (Palmenberg et al., 2010). The 3Cpro protein contains a motif \(^{157}\text{gcdg}^{160}\) that is part of the proteolytic active site, while 3Dpro contains multiple conserved motifs associated with RNA polymerase activity. The conserved motifs and respective BGPV sequences are: KDE[LI][JR] \(^{160}\text{kdelr}^{173}\), GLMMPSG \(^{299}\text{gmpsg}^{303}\); YGDD \(^{348}\text{ygdd}^{349}\) and FLKR \(^{394}\text{flk}^{389}\).

Phylogenetic analysis of BGPV

The family Picornaviridae currently comprises 37 species grouped into 17 genera, viz. Aphthovirus, Aquamavirus, Avihepatovirus, Cardiovirus, Cosavirus, Dicippivirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Megrivirus, Parechovirus, Salivirus, Sapelovirus, Senecavirus, Teschovirus and Tremovirus (Adams et al., 2013; Knowles et al., 2012). Additionally, approximately 30 candidate picornavirus species have been suggested to belong to as many as 20 more genera (see www.picornaviridae.com). To investigate the relationship of BGPV with other picornaviruses, BGPV protein sequences from the P1, P2, P3 and 3D regions were aligned with representatives from each genus of the family. Maximum-likelihood phylogenetic trees were constructed using the rREV + G + I + F model which gave the lowest Bayesian information criterion values (Dimmic et al., 2002). In all analyses BGPV was most closely related to members of the genera Aquamavirus, Avihepatovirus and Parechovirus, as well as to the newly described Rhinolophus affinis picornavirus 1 (partial P1 sequence only), SPaV-1, turkey avisivirus 1, greplavirus A1, and eel and carp picornaviruses (Boros et al., 2013a, b; Fichtner et al., 2013; Ng et al., 2013; Sauvage et al., 2012; Wu et al., 2012) (Fig. 5).

Alignment of the P1, 2C and 3C polypeptide sequences showed that BGPV was an outlier of the other members of the group. In P1, BGPV shared 22.8 % identity with human parechovirus (HPeV)-1, 21.8 % with SeAV-1, 21.0 % with LV-1, 20.1–20.4 % with SPaV and 20.0 % with DHAV-1, while other picornaviruses shared between 14.6 and 19.2 % amino acid identity (Fig. 5a, Table 3). In 2C, BGPV shared 29.0 % amino acid identity with LV-1, 28.4 % with SPaV, 27.3 % with SeAV-1, 27.0 % with DHAV-1 and 26.3 % with HPeV-1, and between 18.6 and 24.5 % amino acid identity with other picornaviruses (Fig. 5b, Table 3). In 3C, BGPV shared 19.3–20.4 % amino acid identity with SPaV, 18.5 % with HPeV-1, 18.1 % with SeAV-1, 18.0 % with LV-1 and 16.7 % with DHAV-1. However, a number of other picornaviruses were more closely related to BGPV, e.g. encephalomyocarditis virus (EMCV)-1 (21.1 %) and cosavirus-A1 (20.1 %) (Fig. 5c, Table 3). The 3D protein of BGPV shared 37.8 % amino acid identity with LV-1, 37.7 % with HPeV-1, 36.9 % with DHAV-1, 29.0–29.9 % with the two SPaVs and 27.4 % identity with SeAV-A1. Thus, phylogenetic analysis with the 3D alignment shows BGPV being most closely related to the genus Parechovirus (Fig. 5d, Table 3). These relationships suggest that a progenitor of BGPV may have been a recombinant virus, with the 3D region being donated by a parecho-like virus.

**DISCUSSION**

**BGPV distribution**

BGPV is probably widespread in bluegill populations in Wisconsin and perhaps across the entire range of bluegills. We detected BGPV in 29 % (5/17) of waters sampled, but because we only sampled 30 fish per water body, it is possible that we missed positive fish in waters where the prevalence of the virus was low. Our inability to detect the virus in two of four waters that were sites of previous presumptive isolations would seem to indicate that the prevalence is relatively low and a 30-fish sampling may not be enough to reliably detect the virus in waters where it is present. If true for the average lake, this would indicate that well over 50 % of the waters sampled may harbour the virus. Sampling greater numbers of bluegill from waters previously testing positive, and from additional waters, will help to better understand the prevalence and distribution of BGPV.
Since the development of the diagnostic PCR and the survey of Wisconsin waters, the La Crosse Fish Health Center has begun screening for BGPV during routine pathogen testing of fish. In addition to Wisconsin, bluegills from waters in Illinois and Ohio have tested positive for BGPV. Additionally, BGPV has been isolated from pumpkinseed (Lepomis gibbosus), green sunfish (Lepomis cyanellus), black crappie (Pomoxis nigromaculatus) and largemouth bass (Micropterus salmoides). Since all these species are from the fish family Centrarchidae, to which the bluegill also belongs, it appears that BGPV is capable of infecting many members of this family. Of these isolations, one, from black crappie, was associated with a fish kill. In this case successive fish kills involving thousands of bluegills and black crappie occurred in winter and spring of 2009 in Lake Monona in Madison, WI, USA. Diseased fish of both species tested positive for BGPV (B. Lasee, unpublished).

The La Crosse Fish Health Center also isolated BGPV from common carp (Cyprinus carpio) and channel catfish (Ictalurus punctatus). No disease was associated with these single isolations. As these are commercially important species, further experimental infections could be helpful in assessing the potential risk from BGPV infections in these species.

Pathogenicity of BGPV

Because BGPV was isolated from bluegills during fish kills, but more often from apparently healthy fish during routine pathogen surveys of fish, it was unclear as to whether BGPV could cause disease. It was possible that, during fish kills in which BGPV was found, environmental factors or other pathogens could have been the primary cause of disease. Thus, we experimentally infected juvenile bluegills with BGPV, and observed morbidity and mortality in the infected fish but not in the mock-infected fish, indicating that BGPV can cause disease in bluegills independently of other factors. Significantly, the pathology observed, in particular the most common symptom, inflammation and redness at the base of fins, mirrored previous observations made during fish kills.

We were surprised that there was not a greater increase in the incidence of disease between infections with low (1.5 $\times$ 10^4 p.f.u.) and high (1.5 $\times$ 10^7 p.f.u.) doses of BGPV. It is possible that the pathology of BGPV is highly dependent on the immune status of the fish. Stressors such as other infections, rapid temperature change, low dissolved O2, crowding and spawning have been associated with bluegill fish kills in the past, and may be important factors in making fish susceptible to infection and disease (Rottmann et al., 1992). Additional experimental infections with BGPV can help to better understand the influence of stressors on the susceptibility of fish to disease, the pathology of the disease, and possible routes of infection. Of note, we did try to infect fish by immersion in water containing 1.5 $\times$ 10^5 p.f.u. BGPV ml^-1 for 1 h, but no fish became infected (data not shown).

BGPV genome

The most unusual feature of the BGPV genome is the presence of a poly(C) tract in the 3’ UTR. The longest poly(C) tract we recovered was 21 nt in length. However, because of polymerase slippage during DNA synthesis from homopolymeric templates, and difficulty cloning such sequences, it is possible that the true poly(C) length in BGPV is greater (Duke & Palmenberg, 1989). FMDV (genus Aphthovirus) and EMCV (genus Cardiovirus) have large poly(C) tracts (50–450 nt) in their 5’ UTRs, between the 5’-terminal stem-loop and the IRES, while other picornaviruses have shorter (10–12 nt) C-rich regions in the same location (Palmenberg et al., 2010). The poly(C) tracts appear to play a role in the pathogenesis of some picornviruses as truncation or deletion of these tracts can reduce virus virulence (Duke et al., 1990; Toyoda et al., 2007). Poly(C) tracts may mediate this effect via sequestration of innate immune response factors. Should the poly(C) tract of BGPV function similarly a possible approach for the production of a BGPV vaccine, if needed, would be the recombinant generation of a BGPV strain with the poly(C) tract deleted.

Viruses related to BGPV have type II (Parechovirus) or type IV IRES elements (SeAV-A1 and DHAV) (Ding & Zhang, 2007; Ghazi et al., 1998; Kapoor et al., 2008). BGPV does appear to have a Yn-Xm-AUG motif similar to those found in type II IRES elements since the Xn spacer of the motif is relatively short (25 nt). However, comparison of the predicted BGPV IRES structure with those of previously characterized related viruses did not reveal any obvious structural similarities. Thus, definitive classification of the BGPV IRES awaits sequencing of more closely related viruses and more refined secondary structure analysis.

The four species Aquamavirus A (genus Aquamavirus), Duck hepatitis A virus (genus Avihepatovirus), HPeV and LV (both genus Parechovirus), as well as the unclassified Rhinolophus affinis picornavirus [found in bats (Wu et al., 2012)], carp picornavirus (Lange et al., 2014), eel picornavirus (Fichtner et al., 2013), SPaV-1 (Sauvage et al., 2012; Yu et al., 2013), turkey avisivirus (Boros et al., 2013a; Ng et al., 2013) and the European roller grepavivirus A1 (Boros et al., 2013b), form a phylogenetically related cluster of viruses within the family Picornaviridae. They share a number of genome features: (i) the apparent lack of a 1AB cleavage resulting in only three mature capsid polypeptides; (ii) the lack of a canonical myristoylation motif at the 1AB N terminus (i.e. GxxxS/T), although it has been shown that the 3A polypeptide of some picornviruses can be myristoylated despite lacking this motif (Greninger et al., 2012); (iii) absence of a leader polypeptide; (iv) possession of a 2A-like ribosome skipping motif [D(V/I)ExNPGP] following the capsid (except for human parechoviruses); (v) possession of either a type II (HPeV, LV and possibly BGPV) or type IV (DHAV and SeAV-A1) IRES, though the IRES type of SPaV has not yet been established. Additionally, most known picornviruses having an aquatic association belong to this genetic cluster.
Fig. 5. Phylogenetic analysis of BGPV with representative species from the 17 picornavirus genera and five unassigned viruses using the P1 (a), 2C (b), 3C (c) and 3D (d) protein sequences. Sequences were aligned and used to test maximum likelihood fits of 48 different amino acid substitution models. The model with the lowest Bayesian information criterion was used in the construction of maximum likelihood phylogenetic trees using MEGA 5.2. Branching confidence was inferred by bootstrap resampling (1000 pseudo-replicates). Bars, number of amino acid substitutions per site.
BGPV is clearly most closely related to the Aquamavirus/Avihepatovirus/Parechovirus grouping of picornaviruses and shares the aforementioned genome features. The sequence alignments and phylogenetic analysis support this, as does the lack of a VP2/VP4 cleavage and the presence of two 2A proteins. However, there are some significant differences between BGPV and these related viruses. These include the presence of a poly(C) tract in the 3′ UTR, and the absence of an H-box/NC motif in the 2A2 protein of BGPV. Additionally, there is significant sequence divergence between BGPV and the related viruses, with the most highly conserved protein, 3D, sharing approximately 38–39% amino acid identity with the parechoviruses. Generally, in the Picornaviridae, genus members share
Table 3. Pairwise percentage identities of BGPV proteins and corresponding proteins of other picornaviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>1AB</th>
<th>1C</th>
<th>1D</th>
<th>2A</th>
<th>2B</th>
<th>2C</th>
<th>3A</th>
<th>3B</th>
<th>3C</th>
<th>3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carp picornavirus</td>
<td>62.2</td>
<td>67.2</td>
<td>43.8</td>
<td>21.1</td>
<td>13.6</td>
<td>44.8</td>
<td>20.2</td>
<td>3.7</td>
<td>30.5</td>
<td>49.7</td>
</tr>
<tr>
<td>Sebokele virus HJ677705</td>
<td>21.8</td>
<td>24.8</td>
<td>13.5</td>
<td>–</td>
<td>15.4</td>
<td>27.4</td>
<td>12.6</td>
<td>40.7</td>
<td>18.9</td>
<td>40.0</td>
</tr>
<tr>
<td>Human parechovirus L02971</td>
<td>23.4</td>
<td>26.8</td>
<td>16.9</td>
<td>–</td>
<td>14.8</td>
<td>25.5</td>
<td>16.5</td>
<td>29.2</td>
<td>17.9</td>
<td>39.1</td>
</tr>
<tr>
<td>Eel picornavirus KC843627</td>
<td>18.3</td>
<td>23.0</td>
<td>15.5</td>
<td>–</td>
<td>13.2</td>
<td>27.1</td>
<td>10.8</td>
<td>27.8</td>
<td>16.8</td>
<td>38.4</td>
</tr>
<tr>
<td>Ljungan virus AF327290</td>
<td>21.8</td>
<td>25.2</td>
<td>14.9</td>
<td>–</td>
<td>11.0</td>
<td>28.0</td>
<td>11.8</td>
<td>32.0</td>
<td>18.6</td>
<td>38.3</td>
</tr>
<tr>
<td>Duck hepatitis A virus DQ226541</td>
<td>21.5</td>
<td>18.1</td>
<td>13.9</td>
<td>–</td>
<td>18.5</td>
<td>24.2</td>
<td>15.7</td>
<td>22.2</td>
<td>16.1</td>
<td>35.6</td>
</tr>
<tr>
<td>Avisivirus A KC614703</td>
<td>17.8</td>
<td>18.8</td>
<td>15.4</td>
<td>–</td>
<td>13.2</td>
<td>24.0</td>
<td>11.4</td>
<td>12.0</td>
<td>14.9</td>
<td>32.0</td>
</tr>
<tr>
<td>Avisivirus A KC645954</td>
<td>19.1</td>
<td>19.4</td>
<td>15.3</td>
<td>–</td>
<td>13.2</td>
<td>24.3</td>
<td>11.4</td>
<td>12.0</td>
<td>14.3</td>
<td>31.1</td>
</tr>
<tr>
<td>Pasivirus A JX491648</td>
<td>20.8</td>
<td>24.9</td>
<td>11.5</td>
<td>–</td>
<td>9.6</td>
<td>26.6</td>
<td>6.7</td>
<td>29.2</td>
<td>20.0</td>
<td>28.8</td>
</tr>
<tr>
<td>Pasivirus A JQ316470</td>
<td>22.1</td>
<td>23.6</td>
<td>11.6</td>
<td>–</td>
<td>9.6</td>
<td>26.6</td>
<td>6.7</td>
<td>29.2</td>
<td>20.5</td>
<td>28.4</td>
</tr>
<tr>
<td>Rhinolophus affinis picornavirus JQ814853</td>
<td>19.4</td>
<td>24.8</td>
<td>11.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Grepleavirus A KC935379</td>
<td>13.6</td>
<td>20.5</td>
<td>11.7</td>
<td>–</td>
<td>9.0</td>
<td>22.7</td>
<td>9.6</td>
<td>31.8</td>
<td>17.3</td>
<td>28.0</td>
</tr>
<tr>
<td>Aquamavirus A EU142040</td>
<td>19.9</td>
<td>19.7</td>
<td>18.7</td>
<td>–</td>
<td>16.4</td>
<td>24.3</td>
<td>6.9</td>
<td>19.0/26.1*</td>
<td>18.7</td>
<td>26.4</td>
</tr>
<tr>
<td>Enterovirus C J02281</td>
<td>14.8</td>
<td>17.6</td>
<td>6.2</td>
<td>–</td>
<td>14.4</td>
<td>19.8</td>
<td>7.2</td>
<td>9.5</td>
<td>12.8</td>
<td>22.1</td>
</tr>
</tbody>
</table>

ND, Not determined.
*Seal aquamavirus has two 3B proteins.

>50 % amino acid identity in the P3 region (Knowles et al., 2010, 2012). Based on these differences, we suggest that BGPV be considered as a representative of a new species (suggested name lenavirus – from Lepomis macrochirus) within a novel genus of the family Picornaviridae.

METHODS

Cells and virus. BF-2 cells were cultured in minimal essential medium (with Hank’s salts) supplemented with 10% FBS and incubated between 15 and 25 °C. La Crosse Fish Health Center BGPV isolate 04-032 (Montana Lake, WI, USA) was used for virus characterization and experimental challenge. A concentrated, purified stock of virus was prepared. BF-2 cells in 175 cm² dishes were inoculated with 2 ml clarified medium harvested from infected BF-2 cells. When fewer than 20% of cells remained attached, medium was harvested and clarified via low-speed centrifugation (1500 g) for 10 min at 4 °C. Supernatants were centrifuged (131 000 g) through a 40% (w/w) sucrose/PBS cushion for 3 h at 4 °C and pellets resuspended in sterile PBS.

Microscopic observation of BGPV-infected cells. To monitor BGPV infection in tissue culture by phase-contrast microscopy, BF-2 cells in six-well plates (95% confluent) were inoculated with clarified medium [0.4 μl diluted in 800 μl Hank’s balanced salt solution (HBSS)] from either infected or uninfected BF-2 cells, and incubated at 25 °C. For observation at higher magnifications, BF-2 cells (30% confluent) growing on sterile, poly-L-lysine-coated coverslips were incubated between 15 and 25 °C, and examined for CPE for 21 days.

Electron microscopy. Transmission electron microscopy was performed on negative-stained virus particles from the concentrated/purified BGPV preparation. Particles were diluted 1:10 in HEPES- NaCl buffer (50 mM HEPES, 100 mM NaCl, pH 7.0), UV-treated, ultrathin Formvar–carbon grids (Pella) were placed onto 2.5 μl virus suspension for 2 min and then onto 50 μl drops of HEPES–NaCl buffer to rinse (four times), blotting between rinses. Grids were transferred to 30 μl 1% aqueous uranyl acetate, incubated for 5 min, blotted and dried. Grids were examined with a Hitachi H-600 transmission electron microscope operating at 75 kV. Images were recorded on Kodak EM film and negatives were scanned at 1200 dpi resolution on an Epson V-750 scanner.

Bluegill survey. Between August and November 2008, 30 bluegills from each of 17 sites in Wisconsin were captured by hook-and-line angling, fyke-netting or electrofishing. Captured bluegills were euthanized in a lethal concentration of tricaine methane sulfonate (MS-222) and placed on ice. Within 48 h of capture, kidney and spleen tissue (approx. 0.05 g each) was removed from each fish and combined in five-fish pools. The pools of tissue were diluted 1/10 in HBSS, homogenized in a stomacher (1 min), and clarified via low-speed centrifugation (2300 g) for 15 min at 4 °C. Clarified homogenates from each pool were diluted 1/2 in HBSS and used to infect (0.1 ml) duplicate wells of BF-2 cells in 24-well plates. Following adsorption, complete culture medium (0.5 ml) was added to each well, and cells were incubated at 25 °C and examined for CPE for 21 days.

If CPEs were observed, isolates were repassaged to confirm the presence of virus. Medium from wells exhibiting CPEs was filtered through a 0.2 μm filter, diluted 1/6 in HBSS and used to infect cells (0.1 ml, in duplicate) in 24-well plates. If no CPE was observed within 14 days of primary inoculation, culture media were pooled, filtered, diluted 1/6 and repassaged to confirm the absence of virus. Medium from cells exhibiting CPE after repassage was harvested for diagnostic RT-PCR.

Experimental challenge. Juvenile bluegills (39–54 mm) obtained from the Upper Midwest Environmental Sciences Center (La Crosse, WI, USA) were challenged with virus by intraperitoneal injection. Fish were anaesthetized in 100 mg MS-222 l¹⁻¹ before injection with 50 μl virus preparation (n=40) or sterile PBS (n=20). Of the fish receiving virus, 20 received 1.5 × 10⁴ p.f.u. and 20 received 1.5 × 10³ p.f.u. Fish from each treatment group were held in separate aerated 15 l tanks at 12–14.5 °C. For 28 days post-infection, fish were...
monitored daily for morbidity and mortality. Each week a total of five fish, including mortalities, was removed from each tank and live fish were euthanized in a lethal concentration of MS-222. Approximately 0.2 g total of kidney, spleen and liver tissue was removed from each fish, diluted in 1.5 ml HBSS and homogenized with a stomacher (2 min). Clarified homogenates were screened in BF-2 cells as described for the bluegill survey. Medium from one of every five wells exhibiting CPE was harvested for BGPV diagnostic RT-PCR.

**Genome sequencing.** To obtain initial sequence information from the virus, RT-PCR was performed using primers from a previously described pan-picornavirus RT-PCR protocol (Knowles, 2005). RNA was extracted from concentrated BGPV particles using the UltraSens virus kit (Qiagen) or Trizol (Invitrogen). Varying amounts of extract were reverse-transcribed using 200 U Moloney murine leukemia virus (M-MuLV) reverse transcriptase in standard buffer with 0.01 M DTT, 0.5 mM dNTPs and 0.4 μM reverse primer 3′ RACE A (5′-CGCTCGAGTACGGGIIGGGIGGGGIGG-3′), in a total volume of 25 μl for 45 min at 37 °C. PCR was performed with 2 μl cDNA, 2.5 U Taq polymerase in standard buffer with 0.2 mM dNTPs, 0.4 μM reverse primer and 0.4 μM of either the IAYGDD (5′-ATIGCITAYGGIGAYGA-3′) or ICYGDD (5′ATTIGTAYGGIGA-GYA-3′) forward primer. PCR was performed using the previously described thermal profile (Knowles, 2005). DNA smears ranging in size from 300 to 2000 bp were gel purified. Gel extracts and amplicons were cloned into the pCR8/GW/TOPO vector (Invitrogen), and assembled into contigs. Contigs were translated using Sixframe WorkBench (http://workbench.sdsc.edu) (Subramaniam, 1998). Contigs were bridged using additional RT-PCR.

To sequence the 3′ terminus of the genome preceding the poly(A), reverse transcription was performed on genomic RNA using the 3′ RACE A primer and the Transcriptor high fidelity cDNA synthesis kit (Roche) according to manufacturer’s specifications. PCR was performed using the 3′ RACE B reverse primer (5′-CGATCGCTCGAGAATAGCCC-3′) and the forward BGPV diagnostic PCR primer.

To sequence the 5′ terminus of the genome, a reverse transcription was performed using the virus-specific primer (5′-CGGTGTTCCACCGGTTGTGC-3′) and the Thermoscript RT-PCR kit (Invitrogen) according to manufacturer specifications, including RNase H treatment. The resulting cDNA was purified using a PCR purification kit (Qiagen) and a poly(C) tract was added to the 3′ end of denatured cDNA using dCTP and terminal deoxyribonucleotide transferase for 30 min at 37 °C. PCR was performed using the reverse transcription primer and a previously described 5′ RACE primer (5′-GGCTACCGCGGTCGACTAGTACGGGIIGGGIGGGGIGG-3′) (Kapoor et al., 2008). Samples were amplified for 35 cycles at 94 °C for 1 min, 58 °C for 30s, 72 °C for 2 min, and a final extension at 72 °C for 7 min.

**Diagnostic PCR.** For the BGPV diagnostic RT-PCR, forward and reverse primers were designed to anneal within the 3′ UTR, producing a 180 bp amplicon. For the RT-PCR, nucleic acid was extracted from 1 ml clarified culture medium using the UltraSens RNA extract, 200–300 U M-MuLV reverse transcriptase (New England Biolabs) in standard buffer plus 0.01 M DTT, 0.5–1.0 mM dNTPs and 0.4 μM reverse primer (5′-CATTGGGTTCACACTCACTAACA-3′) in a total volume of 25 μl for 1 h at 37 °C. Reactions were sometimes run in the absence of DTT with comparable results. PCRs were performed with 5 μl cDNA, 2.5 U Taq polymerase in standard buffer, 0.2 mM dNTPs, and 0.2 μM of both forward (5′-CTCAGATAATGTTACGACTCGAGACC-3′) and reverse primers in a total volume of 50 μl. PCR cycle parameters were: 94 °C for 4 min; 35 cycles of 94 °C for 20 s, 58 °C for 30s, 72 °C for 25 s; and final extension at 72 °C for 5 min. During the bluegill survey, separate routine diagnostic PCR-based assays on all extracts were performed for detection of LMBV, SVCV and VHSV (American Fisheries Society, 2009). Positive and negative control PCRs were performed alongside all reactions. Products were electrophoresed through 1.6–2% agarose gels and visualized by ethidium bromide staining.

**Sequence analysis.** Picornavirus P1-capsid, 2C, 3C and 3D amino acid sequences representative of the 37 recognized species, along with the sequences of 5 unclassified viruses determined to be phylogenetically related to BGPV (data not shown), were aligned individually using MUSCLE multiple sequence alignment software (Edgar, 2004). MEGA 5.2 (Tamura et al., 2011) was used to test maximum-likelihood fits of different amino acid substitution models for each of the sequence alignments. The model with the lowest Bayesian information criterion was used in the construction of maximum-likelihood phylogenetic trees using MEGA 5.2. Branching confidence was inferred by bootstrap resampling (1000 pseudo-replicates).

**ACKNOWLEDGEMENTS**

We thank John Whitney (La Crosse Fish Health Center) for his initial observation of CPEs from BGPV, Roland Zell (Friedrich Schiller University, Jena, Germany) for the sequences of eel and carp picornaviruses prior to publication, the Wisconsin Department of Natural Resources for providing fish samples for the BGPV survey, and Sarah Leis (La Crosse Fish Health Center) for technical support. N. J. K. is partially supported by core funding provided by the Biotechnology and Biological Sciences Research Council (BBSRC), UK. This work was also supported by a UW–La Crosse Research, Service and Educational Leadership grant to M. B.

**REFERENCES**


Michigan Department of Natural Resources (2007). Fish disease discovered in Budd Lake, Clare County. Press Release. Newberry, MI: Michigan Department of Natural Resources.


