SCIENTIFIC REPORTS

natureresearch

Check for updates

OPEN

Mass mortality in freshwater mussels (*Actinonaias pectorosa*) in the Clinch River, USA, linked to a novel densovirus

Jordan C. Richard[1,3], Eric Leis[2], Christopher D. Dunn[3], Rose Agbalog[1], Diane Waller[4], Susan Knowles[5], Joel Putnam[4] & Tony L. Goldberg[3,6]✉

Freshwater mussels (order Unionida) are among the world's most biodiverse but imperiled taxa. Recent unionid mass mortality events around the world threaten ecosystem services such as water filtration, nutrient cycling, habitat stabilization, and food web enhancement, but causes have remained elusive. To examine potential infectious causes of these declines, we studied mussels in Clinch River, Virginia and Tennessee, USA, where the endemic and once-predominant pheasantshell (*Actinonaias pectorosa*) has suffered precipitous declines since approximately 2016. Using metagenomics, we identified 17 novel viruses in Clinch River pheasantshells. However, only one virus, a novel densovirus (*Parvoviridae*; *Densovirinae*), was epidemiologically linked to morbidity. Clinch densovirus 1 was 11.2 times more likely to be found in cases (moribund mussels) than controls (apparently healthy mussels from the same or matched sites), and cases had 2.7 (log_{10}) times higher viral loads than controls. Densoviruses cause lethal epidemic disease in invertebrates, including shrimp, cockroaches, crickets, moths, crayfish, and sea stars. Viral infection warrants consideration as a factor in unionid mass mortality events either as a direct cause, an indirect consequence of physiological compromise, or a factor interacting with other biological and ecological stressors to precipitate mortality.

Freshwater mussels (order Unionida) are important members of freshwater biomes, providing ecosystem services such as water filtration, nutrient cycling and deposition, physical habitat stabilization, and food web enhancement[1]. Mussels filter-feed on bacteria, suspended algae, detritus, phytoplankton and zooplankton[2], removing suspended particulate matter from the water column and from interstitial spaces within the substrate. During periods of low summer discharge in small rivers, mussel assemblages are capable of circulating water as it flows over them, leading to multiple cycles of filtration[3] that can strongly influence ecosystem processes, even at moderate mussel densities[4]. Unionids are also gaining attention for their ability to filter out chemical contaminants and water-borne pathogens[5–7].

Unfortunately, the order Unionida contains an exceptional number of imperiled taxa. Among North America's 298 recognized unionid species[8], >70% are considered endangered, threatened, or vulnerable[9], with 23 species having gone extinct from the Southeastern United States alone. Historically, habitat destruction (e.g., river impoundments), pollution, sedimentation, over-harvest for commercial use (most notably, pearl harvest and manufacture of shirt buttons from shells ca. 1850–1950)[10], and competition from invasive species (e.g. the Asian clam *Corbicula fluminea,* zebra mussel *Dreissena polymorpha,* and quagga mussel *D. bugensis*)[11] have greatly reduced or extirpated many native mussel fauna. These threats have been present since the early twentieth century, mirroring trends in human development and land use[12].

[1]U.S. Fish and Wildlife Service, Southwestern Virginia Field Office, 330 Cummings Street, Abingdon, VA 24210, USA. [2]La Crosse Fish Health Center, Midwest Fisheries Center, U.S. Fish and Wildlife Service, 555 Lester Ave, Onalaska, WI 54650, USA. [3]Department of Pathobiological Sciences and Freshwater and Marine Sciences Program, University of Wisconsin-Madison, 1656 Linden Drive, Madison, WI 53706, USA. [4]U.S. Geological Survey, Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Rd, La Crosse, WI 54603, USA. [5]U.S. Geological Survey, National Wildlife Health Center, 6006 Schroeder Rd, Madison, WI 53711, USA. [6]Global Health Institute, University of Wisconsin-Madison, 1300 University Avenue, Madison, WI 53706, USA. ✉email: tony.goldberg@wisc.edu

Since the late 1970s, episodic mass mortality events have been documented in unionids throughout their range, including catastrophic mortality (> 90% population declines) in some cases[12]. Unlike the aforementioned gradual declines, many mass mortality events in freshwater mussels have not been directly attributed to any specific environmental changes or events[12]. Furthermore, mass mortality events often affect only a single species of mussel within a broad ecological community. Environmental factors (e.g. chemical spills, extreme weather events) would be expected to affect many or all unionid species, in addition to other invertebrates and fishes[13]. A meta-analysis of the causes of mussel population declines found that only 48% of studies could attribute declines to any particular cause, and over 75% of studies cited multiple causes without substantial evidence of mechanisms[14].

The Clinch River watershed in southwestern Virginia and northeastern Tennessee is one of the most ecologically important and biodiverse freshwater systems in North America[15]. With 46 extant species of freshwater mussels (20 of which are federally listed as endangered) and over 100 species of fish (5 of which are federally listed as either threatened or endangered), the Clinch River supports the highest concentration of extant federally listed aquatic species in the USA[16]. Long-term quantitative monitoring has shown that mussel richness and abundance in the upper river in Virginia steadily fell from 1979 to 2014, with densities at some sites declining as much as 95%[16]. In contrast, mussel densities in the lower river in Tennessee increased from 1979 to 2014[17]. Several studies have examined Clinch River water and sediment quality and their effects on freshwater mussel assemblage in an attempt to explain this "zone of decline," but few direct links to water quality, sediment, or physical habitat quality have been identified[18].

Beginning in summer 2016, fi ld biologists began documenting mass die-offs of mussels within the "healthy" reach of the lower Clinch River[19]. Mortality episodes were characterized by large numbers of recently dead or dying mussels on the surface of the river substrate in late summer and fall. Field surveys, collection of shells from freshly dead mussels, and comparisons to known species assemblage patterns demonstrated that the pheasantshell (*Actinonaias pectorosa*) comprised a disproportionate (to their relative abundance within the community) and overwhelming majority of affected individuals[17]. These mortality events resulted in population declines of approximately 50–90% of pheasantshells at monitoring sites throughout the lower river. For example, at one monitoring site (Kyle's Ford), data from yearly quantitative surveys documented a loss of 85.4% of the pheasantshell population from 2016 to 2019, translating to a loss of approximately 80,000 individuals from this 200-m reach of the Clinch River[19]. Remarkably, similar mass mortality has not been observed in the other species of mussels inhabiting the same areas of the river. Moreover, since 2016, mass mortality of pheasantshells has occurred in upstream sites originally considered unaffected[19]. Pheasantshell are large-bodied and abundant, historically comprising over 50% of the Clinch River's mussel biomass[16]. Thus, there is great concern that this decline, if unchecked, could permanently alter the Clinch River's ecology and irreversibly affect the ecosystem services that its mussels provide.

Here, we describe a multi-year investigation into the Clinch River pheasantshell die-off focusing on infection, which has been cited as a potential—even likely—cause for unionid die-off [13,20,21] but has remained understudied[22]. Th s study is part of a broader collaborative effort to investigate potential causes for pheasantshell die-offs in the Clinch River and elsewhere[23]. We focus on viral causes because of (1) the specific ty of the die-off for pheasantshells, (2) the apparent upstream spread of pheasantshell mortality between 2016 and 2019, (3) lack of evidence for bacterial or eukaryotic etiological agents[24,25], and (4) lack of evidence of changes in physical characteristics of the environment that might explain the die-off[17]. Moreover, viruses are known to cause epidemic mortality in marine bivalves[26,27], and Lea plague virus can decimate farmed populations of Chinese triangleshell (*Hyriopsis cumingii*) freshwater mussels used for production of freshwater pearls[28,29]. We took advantage of advances in metagenomic technologies for detecting and characterizing unknown viruses and viral communities, which have proven useful for elucidating the invertebrate "virosphere"[30,31]. By applying these methodologies alongside a rigorous case–control study design in which we compared affected and unaffected animals during two consecutive years (2017 and 2018), were able to examine which constituents of the pheasantshell virome might be associated with disease.

Results

Sampling. We collected and analyzed samples from 58 pheasantshells from the Clinch River, including 26 cases (11 from 2017 and 15 from 2018) and 32 controls (8 from 2017 and 24 from 2018) at 6 sites (Fig. 1; Table S1). During sampling, we chose as cases mussels that were on the surface of the substrate, gaping, slow to respond to tactile stimuli, and able to close their valves only weakly, and we chose as controls mussels that were fi mly buried in the substrate, fast to respond to tactile stimuli, and able to close their valves strongly. In 2017, we sampled in October and November 2017 during an active mass mortality event. In 2018, we began sampling in August, before mortality was observed, and we continued sampling during September and October when mass mortality did occur. Prolonged fl od conditions immediately after the October 2018 sampling event prevented further sample collection in 2018.

Viromics and statistical analyses. Metagenomic sequencing of 58 pheasantshells from the Clinch River yielded an average of 1,921,287.6 sequence reads per hemolymph sample (standard deviation 1,127,991.5) with an average length of 118.3 nucleotides (standard deviation 10.6), after length and quality trimming. De novo assembly of these reads yielded 20,058 contiguous sequences (contigs) averaging 1,671 nucleotides in length (range 856–92,913). From these data, we identifi d 17 viruses of varied genomic compositions and taxonomic classifi ations (Table 1). Most viruses are only distantly related to known viruses phylogenetically, but many are related to viruses of freshwater and marine mollusks and other invertebrates (Fig. S1). Mussels identifi d as cases harbored an average of 4.4 (standard error = 0.66) viruses, whereas mussels identifi d as controls harbored an average of 3.2 (SE = 0.27) viruses, and this difference was statistically signifi ant (t = 1.839; df = 56; P = 0.0356).

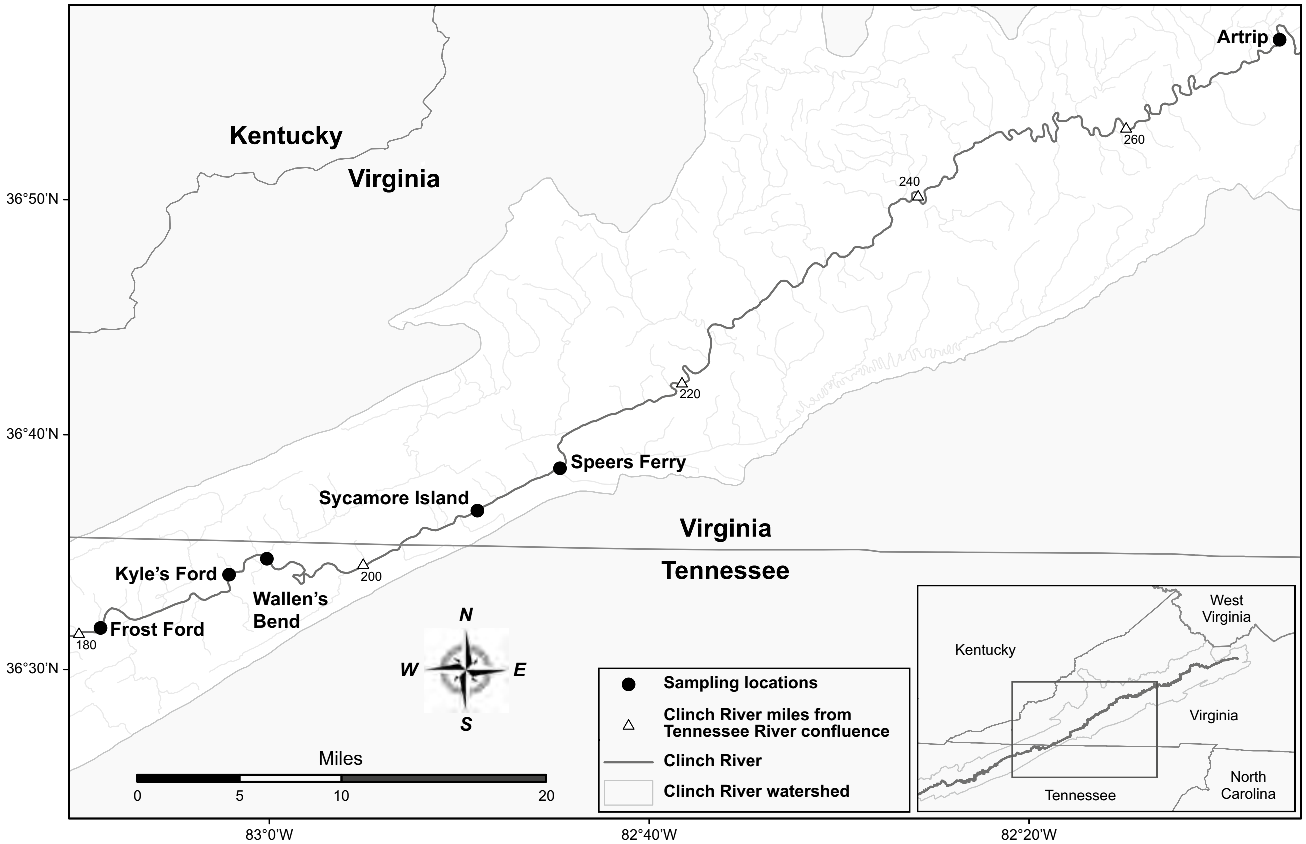


Figure 1. Map of sampling locations. The map was created using ArcMap version 10.4.1 (Esri, Redlands, California, USA; https://support.esri.com/en/products/desktop/arcgis-desktop/arcmap/10-4-1).

Average loads of all viruses were 0.135 $\log_{10}$ viral reads per million total reads per kilobase of target sequence (vRPM/kb) for cases and 0.064 $\log_{10}$ vRPM/kb for controls, and this difference was also statistically signifi ant ($t=3.706$; df = 54; $P=0.0003$). Frequency distributions of viral richness and viral load were right-skewed for cases but less so for controls, with some cases having exceptionally high viral richness and viral loads (Fig. 2).

Individual viruses varied markedly in their prevalence, load, and association with case or control status (Table 2). In univariate analyses, five viruses (Clinch densovirus 1, Clinch narna-like virus 1, Clinch noda-like virus 1, Clinch picorna-like virus 1, and Clinch CRESS virus 1) showed significantly higher prevalence and/or viral load in cases than in controls (Table 2). Two of these viruses were relatively rare: Clinch narna-like virus 1 was found in 6 cases and 1 control and Clinch noda-like virus 1 was found in 3 cases and 0 controls. Two other of these viruses (Clinch picorna-like virus 1 and Clinch CRESS virus 1) had higher viral loads in cases than in controls but showed no signifi ant differences in prevalence between cases and controls. Thus, Clinch densovirus 1 was the only virus for which both prevalence and load were signifi antly higher in cases than in controls (odds ratio (OR) = 4.30, 95% confide ce interval (CI) 1.42–13.0; $P=0.0084$, and Mann–Whitney U = 40, $P=0.0035$, respectively). The remaining 12 viruses showed no statistically signifi ant differences in prevalence or load between cases and controls overall or within years (Table 2; Fig. 3).

Based on the results described above, we conducted multivariate statistical analyses that included five viruses (Clinch densovirus 1, Clinch narna-like virus 1, Clinch noda-like virus 1, Clinch picorna-like virus 1, and Clinch CRESS virus 1) because of their significantly higher prevalence and/or load in cases than in controls. In the resulting general linear model (GLM) relating clinical status to viral infection and ecological variables, only two signifi ant factors emerged: infection with Clinch densovirus 1 [$P=0.004$, adjusted OR (95% CI) = 11.18 (2.12–58.92)] and mussel shell length [$P=0.043$, adjusted OR (95% CI) = 1.09 (1.00–1.17)]. In the GLM relating clinical status to viral load and ecological variables, the only significant factor identified was Clinch densovirus 1 load [$P=0.0287$, adjusted OR (95% CI) = 24.56 (1.39, 432.52)]; no other viruses and no ecological factors were signifi ant. The general linear model relating viral richness to ecological factors (site, sampling date, and length) had no significant terms.

Because of the strong associations of Clinch densovirus 1 prevalence and load with morbidity, we examined associations between Clinch densovirus 1 and the presence and load of other viruses using Fisher's exact tests and Student's t tests, respectively. Infection with Clinch densovirus 1 was associated with a higher frequency of infection with Clinch circular virus 1 (odds ratio = 5.9 [95% CI 1.33–37.6] Fisher's 1-tailed exact $P=0.007$) and with a higher load of Clinch CRESS virus 1 ($t=2.527$; df = 26.185; $P=0.0179$); however, no other signifi ant associations were detected.

ID[1]	Virus name	Accession	Genome	Closest relative (source, location, year, accession)[2]	Family[3]	Genus[3]	%ID (aa)[2]
A	Clinch densovirus 1	MT341473	ssDNA (linear)	Periplaneta fuliginosa densovirus (cockroach, China, 1990, AF192260)	*Parvoviridae*	*Ambidensovirus*	63.7
B	Clinch narna-like virus 1	MT341474	ssRNA(+)	Sanxia narna-like virus 2 (shrimp, China, 2014, KX883567)	Unclassifi d	Unclassifi d	45.4
C	Clinch noda-like virus 1	MT341475	ssRNA(+)	Hubei noda-like virus 2 (freshwater shellfish, China, 2014, KX883205)	Unclassifi d	Unclassifi d	51.9
D	Clinch picorna-like virus 1	MT341476	ssRNA(+)	Marine RNA virus SF-2 (wastewater, USA, 2010, NC_043518)	*Marnaviridae*	*Locarnavirus*	41.9
E	Clinch CRESS virus 1	MT341477	ssDNA (circular)	CRESS virus (minnow, USA, 2017, MH616916)	Unclassifi d	Unclassifi d	61.7
F	Clinch picorna-like virus 2	MT341478	ssRNA(+)	Hubei picorna-like virus 4 (freshwater shellfish, China, 2014, NC_033087)	Unclassifi d	Unclassifi d	65.8
G	Clinch picorna-like virus 3	MT341479	ssRNA(+)	Wenzhou picorna-like virus 7 (shrimp, China, 2013, NC_032842)	Unclassifi d	Unclassifi d	55.7
H	Clinch circular virus 1	MT341480	ssDNA (circular)	Blackfly DNA virus 6 (black flies, ew Zealand, 2015, MK433220)	Unclassifi d	Unclassifi d	70.1
I	Clinch calicivirus 1	MT341481	ssRNA(+)	Bat calicivirus (bat, USA, 2009, MH259583)	*Caliciviridae*	*Calicivirus*	80.2
J	Clinch circular virus 2	MT341482	ssDNA (circular)	Bat circovirus (bat, China, 2013, KJ641738)	*Circoviridae*	Unclassifi d	97.5
K	Clinch dicistro-like virus 1	MT341483	ssRNA(+)	Beihai picorna-like virus 105 (snails, China, 2014, NC_032604)	Unclassifi d	Unclassifi d	79.1
L	Clinch tombus-like virus 1	MT341484	ssRNA(+)	Hubei tombus-like virus 15 (centipede, China, 2013, NC_033009)	*Tombusviridae*	Unclassifi d	63.8
M	Clinch sobemo-like virus 1	MT341485	ssRNA(+)	Beihai sobemo-like virus 25 (razor shell, China, 2014, NC_032895)	*Luteoviridae*	Unclassifi d	65.6
N	Clinch dicistro-like virus 2	MT341486	ssRNA(+)	Hypsignathus monstrosus dicistrovirus (bat, Republic of the Congo, 2015, MH310078)	*Dicistroviridae*	Unclassifi d	63.0
O	Clinch picobirnavirus 1	MT341487	dsRNA (segmented)	Pink-eared duck picobirnavirus (duck, Australia, 2017, MK204418)	*Picobirnaviridae*	*Picobirnavirus*	64.1
P	Clinch picobirna-like virus 1	MT341488	ssRNA(+)	Shahe picobirna-like virus 1 (freshwater isoptera, China, 2013, KX884156)	Unclassifi d	Unclassifi d	76.5
Q	Clinch totivirus 1	MT341489	ssRNA(+)	Drosophila melanogaster totivirus (fruit fly, USA, 2009, NC_013499)	*Totiviridae*	Unclassifi d	96.0

Table 1. Viruses identifi d in Clinch River pheasantshells. [1]Letters refer to Table 2, Figs. 3, and S1. [2]Closest phylogenetic relative in the GenBank database; see Fig. S1. [3]Family, genus and percent amino acid identity to the closest phylogenetic relative in the GenBank database.

The genome of Clinch densovirus 1 (GenBank accession number MT341473) is 5,429 bases long and contains 5 open reading frames (ORFs 1–5) of lengths 735, 1,671, 1,620, 807, and 759 nucleotides in the typical arrangement of members of subfamily *Densovirinae*, encoding putative non-structural and structural proteins, which are transcribed by host cellular machinery through alternative mRNA splicing and leaky scanning[32,33]. The Clinch densovirus 1 coding genome is also flanked by inverted terminal repeats characteristic of members of this viral subfamily[32]. The amino acid sequence difference between Clinch densovirus 1 and its closest relative, periplaneta fuliginosa densovirus, a member of the genus *Ambidensivirus* (Table 1), is 63.7% within the non-structural protein NS1. Th s degree of divergence exceeds the 85% relatedness threshold accepted by the International Committee on the Taxonomy of Viruses as a species demarcation criterion within the genus *Ambidensovirus*[34].

Discussion

Clinch River pheasantshells host a diverse virome. Th ee of the 17 viruses we identifi d (Clinch picorna-like virus 1, Clinch CRESS virus 1, and Clinch circular virus 2; Table 1) are likely members of the "normal" pheasantshell virome. Such viruses would be expected to infect mussels at high prevalence (> 50% in these cases) and load, but without association with clinical disease. Th ee other viruses infected pheasantshells at moderate prevalence (between 20 and 50%) but also showed no association between case and control status (Clinch picorna-like virus 2, Clinch circular virus 1, and Clinch calicivirus 1). The other viruses we identifi d all occurred at low prevalence (sometimes in only one animal) and may be hypoendemic, sporadic, or derived from the environment. For example, the picobirnavirus detected in one case sample from 2018 is part of a group of viruses shed in the feces of mammals such as cows and marmots[35]. Although hemolymph, like mammalian blood, is not directly connected to the environment[36], filter feeding bivalves can remove viral pathogens from suspension in the water column[37,38].

Among the five viruses with prevalence or loads associated with case status by univariate analyses (Table 1), only Clinch densovirus 1 had both higher prevalence and load in cases than in controls, and these associations were the strongest observed in the study. In multivariate analyses, the other four viruses fell out as non-signifi ant with respect to both prevalence and load, as did all other factors except for mussel shell length, which was retained in the GLM examining prevalence. Clinch densovirus 1 is therefore the only of the 17 viruses identifi d that, when other variables are accounted for, is associated with disease in Clinch River pheasantshells.

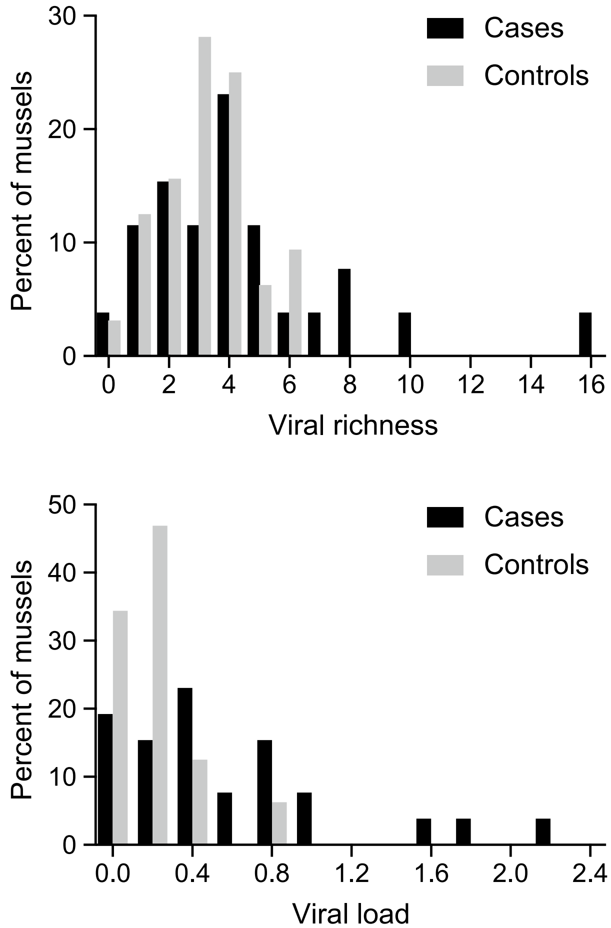


Figure 2. Frequency distribution of viral richness (number of viruses) and viral load (log_{10} viral reads per 10^6 total reads per kilobase of target sequence) in Clinch River pheasantshell cases and controls.

Densoviruses are members of the viral family *Parvoviridae*, subfamily *Densovirinae*, and can be highly host-specific and lethal[39]. Mass mortality in invertebrates is a well-characterized consequence of densovirus infection, with examples including shrimp[40], silkworms[41], cockroaches[42], mosquitos[43], crickets[44], moths[45], and crayfish[46]. In fact, so lethal are some densoviruses that they have been used commercially as powerful bioinsecticides[47]. Common signs of densovirus infection include lethargy, anorexia, development of tumors, flaccidity, and death[39]. Notably, sea star-associated densovirus, also a member of the genus *Ambidensovirus*, is the putative cause of mass mortality in another benthic invertebrate, the sunflower sea star (*Pycnopodia helianthoides*)[48]. Sea star wasting disease is characterized by loss of turgor (a "deflated" appearance), behavioral changes, and rapid degradation leading to death[48].

Henley et al.[24] conducted a histological study of moribund Clinch River pheasantshells collected during the beginning of the die-off in 2016 from the Kyle's Ford sampling site. Th s study documented internal organ damage, including pervasive necrosis, but was unable to link any measured factor (including parasitic trematode infestation and bacterial infection), to mortality. Certain of the histologic lesions documented, however, would be consistent with densovirus infection, as described in other invertebrates (see above). Ultimately, experimental infection and studies of pathogenesis will be necessary to resolve any causal relationship between phesantshell mussel mortality and infection with Clinch densovirus 1, as has been attempted in the case of sea star wasting disease[48] and cherax quadricarinatus densovirus[46].

In this light, we caution that our results, while suggestive, do not demonstrate direction of causality. For example, a preceding diseased state may render mussels more susceptible to infection with Clinch densovirus 1. We also note that we characterized viruses from hemolymph, because it is useful for bivalve health assessment and can be obtained non-lethally[36,49], but other tissues may host different viruses. Our focus on hemolymph may also account for our fi ding of only relatively small viruses (similar to vertebrate blood). Other (and perhaps larger) viruses may have tropisms for different tissues (e.g. mantle, gill, gonads), and these tissues also warrant investigation. Quantitative polymerase chain reaction assays could be developed to measure the tissue-specific loads of viruses determined by epidemiology and metagenomics to be linked to disease states, including Clinch densovirus 1 but not dismissing other viruses (discovered and as-yet undiscovered). Such assays could also be applied to environmental samples (e.g. water or sediment) to investigate viral transmission and persistence.

Should infection with Clinch densovirus 1 or other pathogens ultimately be a cause of pheasantshell mass mortality, this result would not exclude the possibility of "upstream drivers." Infectious diseases are often

ID[1]	Virus name	Individuals infected	Prevalence (%)[2]				Viral load (Log_{10}vRPM/kb)[3]			
			Cases	Controls	OR (95% CI)	*P*	Cases	Controls	U	*P*
A	Clinch densovirus 1	29	69.2	34.4	4.30 (1.42, 13)	**0.0084**	1.057	0.396	40	**0.0035**
B	Clinch narna-like virus 1	7	23.1	3.1	9.30 (1.041, 83.12)	**0.0267**	0.601	0.074	n/a	n/a
C	Clinch noda-like virus 1	3	11.5	0.0	9.68 (0.4771, 196.4)	**0.0360**	0.512	0.000	n/a	n/a
D	Clinch picorna-like virus 1	36	61.5	62.5	0.96 (0.3306, 2.788)	0.9999	0.992	0.225	78	**0.00415**
E	Clinch CRESS virus 1	32	57.7	53.1	1.20 (0.4241, 3.413)	0.4676	0.911	0.544	80	**0.03785**
F	Clinch picorna-like virus 2	18	34.6	28.1	1.35 (0.443, 4.132)	0.4017	0.628	0.289	21	0.0939
G	Clinch picorna-like virus 3	3	11.5	0.0	9.68 (0.4771, 196.4)	0.0843	0.657	0.000	n/a	n/a
H	Clinch circular virus 1	15	34.6	18.8	2.29 (0.6908, 7.619)	0.1423	0.737	0.592	17	0.13605
I	Clinch calicivirus 1	14	23.1	25.0	0.90 (0.2675, 3.028)	0.9999	0.630	0.254	6	0.1725
J	Clinch circular virus 2	42	69.2	75.0	0.75 (0.2363, 2.38)	0.8435	0.930	0.946	207	0.8308
K	Clinch dicistro-like virus 1	2	7.7	0.0	6.63 (0.3045, 144.5)	0.1966	0.638	0.000	n/a	n/a
L	Clinch tombus-like virus 1	4	11.5	3.1	4.04 (0.3948, 41.41)	0.2314	0.567	0.471	n/a	n/a
M	Clinch sobemo-like virus 1	4	3.8	9.4	0.39 (0.03779, 3.956)	0.7774	0.895	0.092	n/a	n/a
N	Clinch dicistro-like virus 2	3	7.7	3.1	2.58 (0.221, 30.2)	0.4213	0.859	0.101	n/a	n/a
O	Clinch picobirnavirus 1	1	3.8	0.0	3.82 (0.1494, 97.84)	0.4483	1.048	0.000	n/a	n/a
P	Clinch picobirna-like virus 1	1	3.8	0.0	3.82 (0.1494, 97.84)	0.4483	1.473	0.000	n/a	n/a
Q	Clinch totivirus 1	1	3.8	0.0	3.82 (0.1494, 97.84)	0.4483	1.727	0.000	n/a	n/a

Table 2. Univariate statistical associations between clinical classifi ation (case or control) and prevalence and loads of viruses in Clinch River pheasantshells. [1]Letters refer to Table 1, Figs. 3, and S1. [2]Percentage of mussels within each group (case or control) with reads mapping to each virus, plus odds ratios and 95% confide ce intervals. *P* values (statistically signifi ant values in bold) were calculated using Fisher's exact tests. [3]Log_{10} reads mapping to each virus per million total reads per kilobase of target sequence, Mann–Whitney U statistics, and associated *P* values (infected mussels only).

proximate causes of mortality while also being caused by other factors themselves. For example, introductions of exotic species and their pathogens, climate change, and ecologically induced physiological stressors have all been implicated as predisposing factors for infectious disease in wildlife[50]. Determining proximate causes is nevertheless important for management and conservation. For example, vaccines, probiotics, or controlled exposure to pathogens to induce resistance might be effective in conditioning mussels in captive rearing facilities, where many species are bred for restoration efforts[51].

Overall, our results show that, while diverse, the virome of Clinch River pheasantshells contains only one virus, Clinch densovirus 1, showing a strong and consistent association with disease. Mass mortality events in freshwater mussels are unfortunately accelerating worldwide[12]. Studying other species of mussels in other geographic locations using both epidemiology and metagenomics could help reveal whether infection with viruses or other agents is a generalized characteristic of unionid mass mortality events. The resulting information would be important for conserving and managing this remarkable complex of imperiled species.

Methods

Field sampling. We sampled pheasantshells in 2017 and 2018. We collected moribund mussels (cases) and apparently healthy mussels (controls) during mortality events using swim-through searches of shoals. We focused on the months of September, October and November of each year because these were the months in which mass mortality was observed, although we added a sampling event in August 2018 in anticipation of a mortality event. At four sites along the river (Frost Ford, Kyle's Ford, Wallen's Bend, and Sycamore Island; Fig. 1), we fi st located moribund individuals (lying on the surface with shells gaping and minimally responsive to tactile stimuli). We then located apparently healthy individuals (buried in the substrate, siphoning normally, with tightly closed shells and strongly resistant to being opened) at the same sites and from two additional upstream sites (Speers Ferry and Artrip) where no mortality had been observed.

We sampled hemolymph because it is useful for health and disease assessment in freshwater bivalves and can be collected non-lethally[36,49] and because (similar to vertebrate blood) it is not directly exposed to the physical environment, unlike other accessible organs (e.g. foot, mantle, gill). We fi st gently opened the valves of each animal with a sterile pediatric nasal speculum. We then disinfected the outer surface of the anterior adductor muscle with 70% isopropyl alcohol and extracted up to 1.0 ml hemolymph (depending on the size of the mussel) from the anterior adductor muscle sinus using a 1 ml tuberculin syringe. We placed hemolymph immediately in sterile tubes on dry ice in the field then stored samples at − 80 °C until further analysis. For each individual, we noted its general appearance, recorded the strength and speed of its response to tactile stimuli (opening the valves and application of isopropanol), and measured the length of its shell using digital calipers. We marked animals with FPN glue-on shellfish tags (Hallprint, Hindmarsh Valley, Australia) to prevent re-sampling during successive sampling events and then returned animals to the shoals from which they were collected.

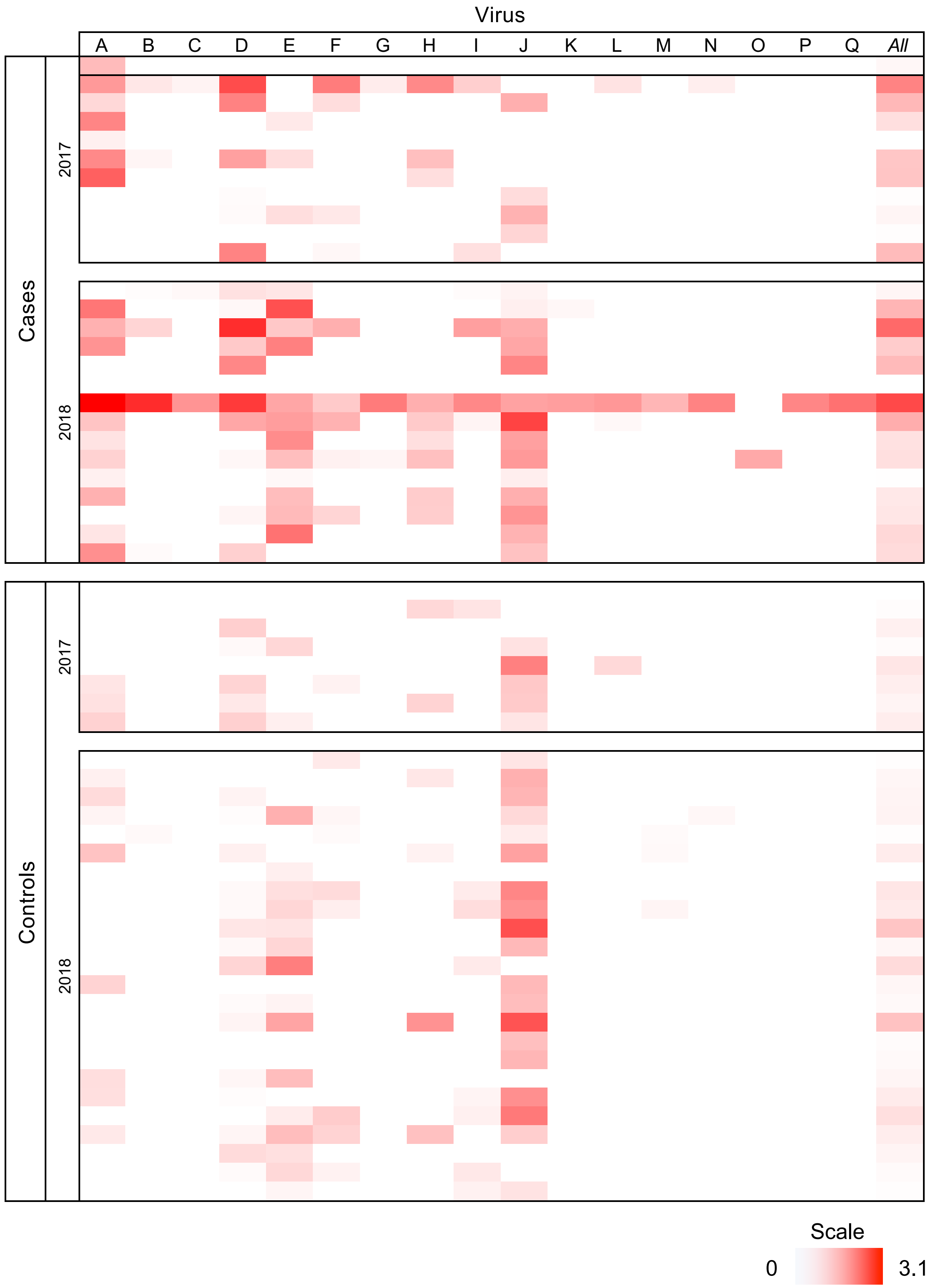


Figure 3. Heatmap of viral loads in Clinch River pheasantshells. Data are $\log_{10}$ viral reads per 10^6 total reads per kilobase of target sequence for each virus separately (viruses A–Q) and for all viruses combined (*All*). Data are presented separately for cases and controls in 2017 and 2018. Raw data on viral loads are presented in Table S2.

Metagenomic sequencing and bioinformatics. We processed hemolymph for metagenomic sequencing for virus discovery as described previously[52]. Briefly, we clarifi d hemolymph by centrifugation at 10,000×*g* for 10 min and used the QIAamp MinElute virus kit (Qiagen, Hilden, Germany) to extract total nucleic acids from the supernatant. We then converted RNA to double-stranded cDNA using random hexamers and prepared libraries using the Nextera XT DNA sample preparation kit (Illumina, San Diego, California, USA), after which we sequenced the libraries on an Illumina MiSeq instrument (V3 chemistry, 600 cycle kit; Illumina, San Diego, California, USA). Using CLC Genomics Workbench version 11.0 (CLC bio, Aarhus, Denmark), we fi st trimmed low-quality bases (phred quality score < 30) and discarded short reads (< 75 bp). We then conducted de novo assembly using the native CLC assembler with a word size of 50 and a bubble size of 5,000 and analyzed both contigs and unassembled reads for nucleotide-level (blastn) and protein-level (blastx) similarity to viruses in the GenBank database. For each mussel, we measured its infection status (positive or negative) for each virus and, for infected mussels, vRPM/kb, which is a metagenomic measure of viral load that adjusts for sequencing depth and target sequence length and is correlated with quantitative real-time PCR[52].

We inferred phylogenetic relationships among newly identified virus sequences and published sequences of the most closely related viruses in the GenBank database using viral replicase (polymerase) genes when available. We first aligned sequences using a codon-based version of the Prank algorithm[53] and applied the Gblocks algorithm[54] to remove regions with poor alignment, as implemented in the computer program TranslatorX[55]. We then inferred maximum likelihood phylogenetic trees from the resulting alignments using PhyML 3.0[56], with 1,000 bootstrap replicates to assess statistical confidence in clades. We used FigTree v1.4.4 to display fi al trees.

Statistical analyses. We used a multi-tiered statistical approach to examine associations between viral infection, load, and richness (total number of viruses infecting a mussel) and clinical status (cases versus controls). First, we used Fisher's exact tests and Mann–Whitney U tests to assess univariate statistical differences between cases and controls with respect to these measures. Based on the results of these univariate analyses (Table 2), we constructed general linear models to investigate the combined influence of viruses and other predictor variables (shell length, sampling location, and date of sampling) on clinical status (case or control). We conducted all statistical analyses using R software[57].

Ethics statement. We obtained biological samples in accordance with all federal, state, and local laws and policies.

Data availability

All data generated during the current study are available in GenBank (accession numbers MT341473–MT341489) or are included in this published article and its Supplementary Information files.

Received: 24 April 2020; Accepted: 10 August 2020
Published online: 02 September 2020

References

1. Vaughn, C. C. Ecosystem services provided by freshwater mussels. *Hydrobiologia* **810**, 15–27. https://doi.org/10.1007/s10750-017-3139-x (2018).
2. Christian, A. D., Smith, B. N., Berg, D. J., Smoot, J. C. & Findlay, R. H. Trophic position and potential food sources of 2 species of unionid bivalves (Mollusca:Unionidae) in 2 small Ohio streams. *Freshw. Sci.* **23**, 101–113 (2004).
3. Vaughn, C. C. Biodiversity losses and ecosystem function in freshwaters: emerging conclusions and research directions. *Bioscience* **60**, 25–35. https://doi.org/10.1525/bio.2010.60.1.7 (2010).
4. Howard, J. K. & Cuffey, K. M. The functional role of native freshwater mussels in the fluvial benthic environment. *Freshw. Biol.* **51**, 460–474. https://doi.org/10.1111/j.1365-2427.2005.01507.x (2006).
5. Izumi, T., Yagita, K., Izumiyama, S., Endo, T. & Itoh, Y. Depletion of *Cryptosporidium parvum* oocysts from contaminated sewage by using freshwater benthic pearl clams (*Hyriopsis schlegeli*). *Appl. Environ. Microbiol.* **78**, 7420–7428. https://doi.org/10.1128/AEM.01502-12 (2012).
6. Ismail, N. S., Müller, C. E., Morgan, R. R. & Luthy, R. G. Uptake of contaminants of emerging concern by the bivalves *Anodonta californiensis* and *Corbicula flu inea*. *Environ. Sci. Technol.* **48**, 9211–9219. https://doi.org/10.1021/es5011576 (2014).
7. Ismail, N. S. *et al.* Improvement of urban lake water quality by removal of *Escherichia coli* through the action of the bivalve *Anodonta californiensis*. *Environ. Sci. Technol.* **49**, 1664–1672. https://doi.org/10.1021/es5033212 (2015).
8. Williams, J. D. *et al.* A revised list of the freshwater mussels (Mollusca: Bivalvia: Unionida) of the United States and Canada. *Freshw. Mollusk Biol. Conserv.* **20**, 33. https://doi.org/10.31931/fmbc.v20i2.2017.33-58 (2017).
9. Lydeard, C. *et al.* The global decline of nonmarine mollusks. *Bioscience* **54**, 321. https://doi.org/10.1641/0006-3568(2004)054[0321:TGDONM]2.0.CO;2 (2004).
10. Haag, W. R. *North American Freshwater Mussels: Natural History, Ecology, and Conservation* (Cambridge University Press, Cambridge, 2012).
11. Strayer, D. L. Effects of alien species on freshwater mollusks in North America. *J. N. Am. Benthol. Soc.* **18**, 74–98. https://doi.org/10.2307/1468010 (1999).
12. Haag, W. R. Reassessing enigmatic mussel declines in the United States. *Freshw. Mollusk Biol. Conserv.* **22**, 43–60 (2019).
13. Goldberg, T. L., Dunn, C. D., Leis, E. & Waller, D. L. A novel picornalike virus in a Wabash Pigtoe (*Fusconaia fl va*) from the Upper Mississippi River, USA. *Freshw. Mollusk Biol. Conserv.* **22**, 81–84 (2019).
14. Downing, J. A., Van Meter, P. & Woolnough, D. A. Suspects and evidence: a review of the causes of extirpation and decline in freshwater mussels. *Anim. Biodivers. Conserv.* **33**, 151–185 (2010).
15. Zipper, C. E. *et al.* Freshwater mussel population status and habitat quality in the Clinch Rver, Virginia and Tennessee, USA: a featured collection. *J. Am. Water Resour. Assoc.* **50**, 807–819 (2014).
16. Jones, J. *et al.* Clinch River freshwater mussels upstream of Norris Reservoir, Tennessee and Virginia: a quantitative assessment from 2004 to 2009. *J. Am. Water Resour. Assoc.* **50**, 820–836. https://doi.org/10.1111/jawr.12222 (2014).
17. Jones, J. W. *et al.* Collapse of the Pendleton Island mussel fauna in the Clinch River, Virginia: setting baseline conditions to guide recovery and restoration. *Freshw. Mollusk Biol. Conserv.* **21**, 36–56 (2018).
18. Cope, W. G. & Jones, J. W. Recent precipitous declines of endangered freshwater mussels in the Clinch River: an *in situ* assessment of water quality stressors related to energy development and other land-use. 244 (U.S. Fish and Wildlife Service, Southwestern Virginia Field Office, 2016).
19. Richard, J. C. Clinch River mussel die-off. *Ellipsaria* **20**, 1–3 (2018).

20. Neves, R. J. Proceedings of the Workshop on Die-offs of Freshwater Mussels in the United States (U.S. Fish and Wildlife Service, Upper Mississippi River Conservation Committee, 1986).
21. Starliper, C. E., Powell, J., Garner, J. T. & Schill, W. B. Predominant bacteria isolated from moribund *Fusconaia ebena* ebonyshells experiencing die-offs in Pickwick Reservoir, Tennessee River, Alabama. *J. Shellfish Res.* **30**, 359–366. https://doi.org/10.2983/035.030.0223 (2011).
22. Grizzle, J. M. & Brunner, C. J. Infectious diseases of freshwater mussels and other freshwater bivalve mollusks. *Rev. Fish. Sci.* **17**, 425–467 (2009).
23. Leis, E. *et al.* Building a response network to investigate potential pathogens associated with unionid mortality events. *Ellipsaria* **20**, 44–45 (2018).
24. Henley, W. F., Beaty, B. B. & Jones, J. W. Evaluations of organ tissues from *Actinonaias pectorosa* collected during a mussel die-off in 2016 at Kyles Ford, Clinch River, Tennessee. *J. Shellfish Res.* **38**, 681. https://doi.org/10.2983/035.038.0320 (2019).
25. Leis, E., Erickson, S., Waller, D., Richard, J. & Goldberg, T. A comparison of bacteria cultured from unionid mussel hemolymph between stable populations in the Upper Mississippi River basin and populations affected by a mortality event in the Clinch River. *Freshw. Mollusk Biol. Conserv.* **22**, 70–80 (2019).
26. Garcia, C. *et al.* Ostreid herpesvirus 1 detection and relationship with *Crassostrea gigas* spat mortality in France between 1998 and 2006. *Vet. Res.* **42**, 73. https://doi.org/10.1186/1297-9716-42-73 (2011).
27. Arzul, I., Corbeil, S., Morga, B. & Renault, T. Viruses infecting marine molluscs. *J. Invertebr. Pathol.* **147**, 118–135. https://doi.org/10.1016/j.jip.2017.01.009 (2017).
28. Zhang, Z., Sufang, D., Yimin, X. & Jie, W. Studies on the mussel *Hyriopsis cumingii plague*. I. a new viral infectious disease. *Acta Hydrobiol. Sin.* **26**, 308–312 (1986).
29. Zhong, L., Xiao, T.-Y., Huang, J., Dai, L.-Y. & Liu, X.-Y. Histopathological examination of bivalve mussel *Hyriopsis cumingii* lea artificially infected by virus. *Acta Hydrobiol. Sin.* **35**, 666–671 (2011).
30. Shi, M. *et al.* Redefining the invertebrate RNA virosphere. *Nature* **540**, 539–543. https://doi.org/10.1038/nature20167 (2016).
31. Zhang, Y. Z., Shi, M. & Holmes, E. C. Using metagenomics to characterize an expanding vrosphere. *Cell* **172**, 1168–1172. https://doi.org/10.1016/j.cell.2018.02.043 (2018).
32. Bergoin, M. & Tijssen, P. Molecular biology of Densovirinae. *Contrib. Microbiol.* **4**, 12–32. https://doi.org/10.1159/000060329 (2000).
33. Mietzsch, M., Penzes, J. J. & Agbandje-McKenna, M. Twenty-five years of structural parvovirology. *Viruses* https://doi.org/10.3390/v11040362 (2019).
34. Cotmore, S. F. *et al.* ICTV virus taxonomy profile: *Parvoviridae*. *J. Gen. Virol.* **100**, 367–368. https://doi.org/10.1099/jgv.0.001212 (2019).
35. Ganesh, B., Masachessi, G. & Mladenova, Z. Animal picobirnavirus. *Virus Dis.* **25**, 223–238. https://doi.org/10.1007/s13337-014-0207-y (2014).
36. Gustafson, L. L. *et al.* Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a freshwater bivalve (Mollusca: Unionidae). *Dis. Aquat. Organ.* **65**, 159–165. https://doi.org/10.3354/dao065159 (2005).
37. Lees, D. Viruses and bivalve shellfish. *Int. J. Food Microbiol.* **59**, 81–116. https://doi.org/10.1016/S0168-1605(00)00248-8 (2000).
38. Faust, C., Stallknecht, D., Swayne, D. & Brown, J. Filter-feeding bivalves can remove avian influenza viruses from water and reduce infectivity. *Proc. R. Soc. B* **276**, 3727–3735. https://doi.org/10.1098/rspb.2009.0572 (2009).
39. Fédière, G. in *Parvoviruses. From Molecular Biology to Pathology and Therapeutic Uses. Contributions to Microbiology.* Vol. 4 (eds S. Faisst & J. Rommelaere) 1–11 (Karger, 2000).
40. Kalagayan, H. *et al.* IHHN virus as an etiological factor in runt-deformity syndrome (RDS) of juvenile *Penaeus vannamei* cultured in Hawaii. *J. World Aquacult. Soc.* **22**, 235–243. https://doi.org/10.1111/j.1749-7345.1991.tb00740.x (1991).
41. Ito, K., Kidokoro, K., Shimura, S., Katsuma, S. & Kadono-Okuda, K. Detailed investigation of the sequential pathological changes in silkworm larvae infected with *Bombyx densovirus* type 1. *J. Invertebr. Pathol.* **112**, 213–218. https://doi.org/10.1016/j.jip.2012.12.005 (2013).
42. Jiang, H. *et al.* Genetic engineering of *Periplaneta fuliginosa* densovirus as an improved biopesticide. *Arch. Virol.* **152**, 383–394. https://doi.org/10.1007/s00705-006-0844-6 (2007).
43. Ledermann, J. P., Suchman, E. L., Black, W. C. & Carlson, J. O. Infection and pathogenicity of the mosquito densoviruses AeDNV, HeDNV, and APeDNV in *Aedes aegypti* mosquitoes (Diptera: Culicidae). *J. Econ. Entomol.* **97**, 1828–1835. https://doi.org/10.1093/jee/97.6.1828 (2004).
44. Szelei, J. *et al.* Susceptibility of North-American and European crickets to Acheta domesticus densovirus (AdDNV) and associated epizootics. *J. Invertebr. Pathol.* **106**, 394–399. https://doi.org/10.1016/j.jip.2010.12.009 (2011).
45. Kouassi, N. *et al.* Pathogenicity of diatraea saccharalis densovirus to host insets and characterization of its viral genome. *Virol. Sin.* **22**, 53–60. https://doi.org/10.1007/s12250-007-0062-8 (2007).
46. Bowater, R. *et al.* A parvo-like virus in cultured redclaw crayfish *Cherax quadricarinatus* from Queensland, Australia. *Dis. Aquat. Organ.* **50**, 79–86. https://doi.org/10.3354/dao050079 (2002).
47. Johnson, R. M. & Rasgon, J. L. Densonucleosis viruses ('densoviruses') for mosquito and pathogen control. *Curr. Opin. Insect. Sci.* **28**, 90–97. https://doi.org/10.1016/j.cois.2018.05.009 (2018).
48. Hewson, I. *et al.* Densovirus associated with sea-star wasting disease and mass mortality. *Proc. Natl. Acad. Sci. USA* **111**, 17278–17283. https://doi.org/10.1073/pnas.1416625111 (2014).
49. Fritts, A. K., Peterson, J. T., Hazelton, P. D. & Bringolf, R. B. Evaluation of methods for assessing physiological biomarkers of stress in freshwater mussels. *Can. J. Fish. Aquat. Sci.* **72**, 1450–1459. https://doi.org/10.1139/cjfas-2014-0564 (2015).
50. Cunningham, A. A., Daszak, P. & Wood, J. L. N. One health, emerging infectious diseases and wildlife: two decades of progress?. *Philos. Trans. R. Soc. Lond. B* https://doi.org/10.1098/rstb.2016.0167 (2017).
51. Patterson, M. A. *et al. Freshwater Mussel Propagation for Restoration* (Cambridge University Press, Cambridge, 2018).
52. Toohey-Kurth, K., Sibley, S. D. & Goldberg, T. L. Metagenomic assessment of adventitious viruses in commercial bovine sera. *Biologicals* **47**, 64–68. https://doi.org/10.1016/j.biologicals.2016.10.009 (2017).
53. Löytynoja, A. Phylogeny-aware alignment with PRANK. *Methods Mol. Biol.* **1079**, 155–170. https://doi.org/10.1007/978-1-62703-646-7_10 (2014).
54. Talavera, G. & Castresana, J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst. Biol.* **56**, 564–577. https://doi.org/10.1080/10635150701472164 (2007).
55. Abascal, F., Zardoya, R. & Telford, M. TranslatorX: multiple alignment of nucleotide sequences guided by amino acid translations. *Nucleic Acids Res.* **38**, W7-13. https://doi.org/10.1093/nar/gkq291 (2010).
56. Guindon, S. *et al.* New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **59**, 307–321. https://doi.org/10.1093/sysbio/syq010 (2010).
57. R Core Team. *R: A language and environment for statistical computing*, version 3.6.3. https://www.R-project.org (R Foundation for Statistical Computing, Vienna, 2019).

Acknowledgements

We thank the U.S. Fish and Wildlife Service (USFWS) Virginia Field Offic for logistic support during fi ld sampling, and the USFWS La Crosse Fish Health Center, USFWS Midwest Fisheries Center, and U.S. Geological Survey (USGS) National Wildlife Health Center for assistance with sample processing and analyses, and especially Sara Erickson for invaluable assistance with sample collection and associated logistics. Th s work was supported by USFWS (Award Number G18AC00334), USGS (Award Number 140F0518P0079), and by the University of Wisconsin-Madison through the John D. MacArthur Professorship Chair. The findings and conclusions in this article are those of the author(s) and do not necessarily represent the views of the U.S. Fish and Wildlife Service. The use of trade, fi m, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Author contributions

J.C.R., E.L., D.W., S.K., J.P., and T.L.G. designed the study; J.C.R., R.A., and T.L.G. performed field work. C.D.D and T.L.G. performed laboratory experiments; J.C.R., C.D.D. and T.L.G. analyzed the data; J.C.R. and T.L.G. wrote the manuscript. All authors reviewed and improved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-71459-z.

Correspondence and requests for materials should be addressed to T.L.G.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

SUPPLEMENTARY INFORMATION[1]

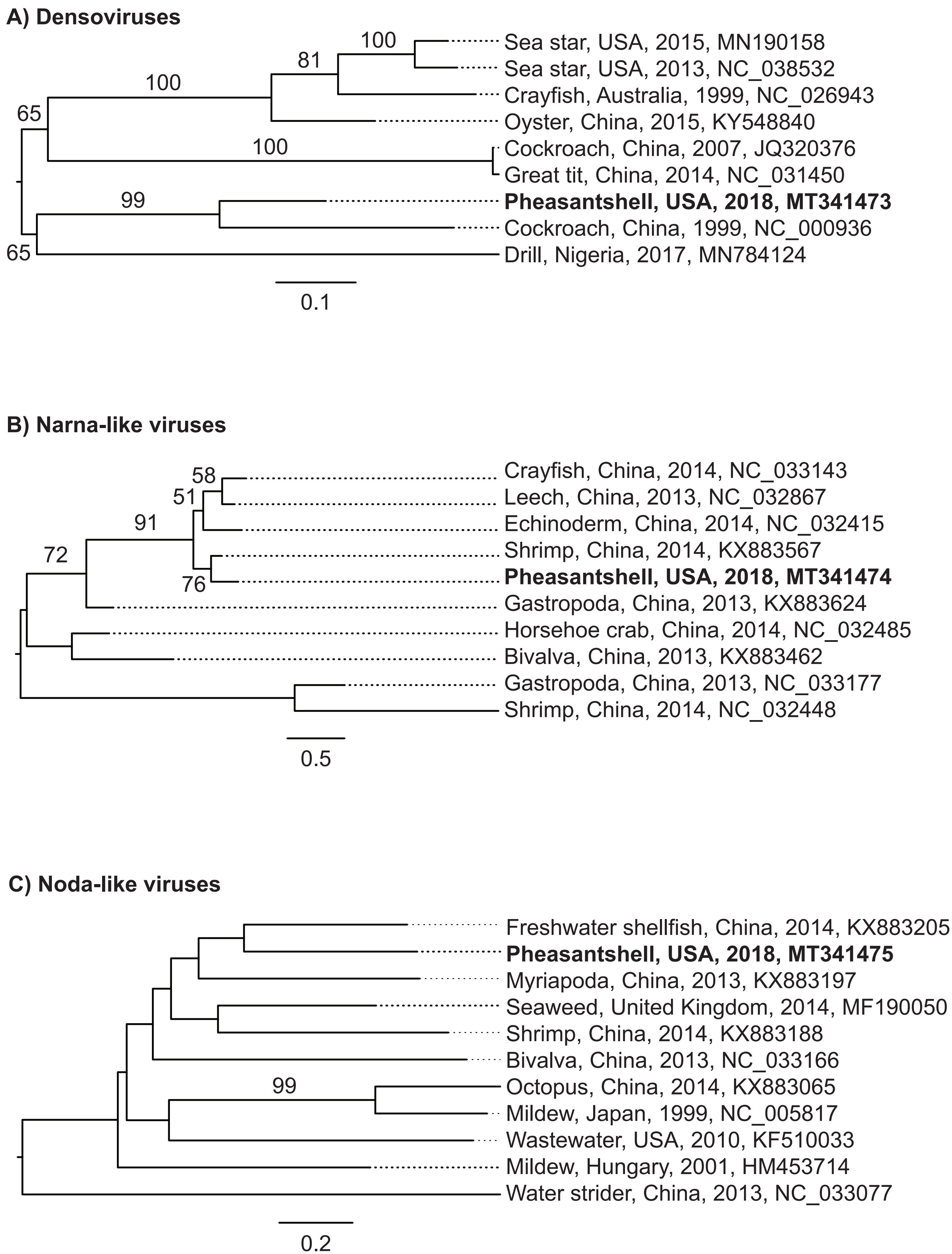
Mass mortality in freshwater mussels (*Actinonaias pectorosa*) in the Clinch River, USA, linked to a novel densovirus

Jordan Richard[1,3], Eric Leis[2], Christopher D. Dunn[3], Rose Agbalog[1], Diane Waller[4], Susan Knowles[5], Joel Putnam[4], and Tony L. Goldberg[3,6]*

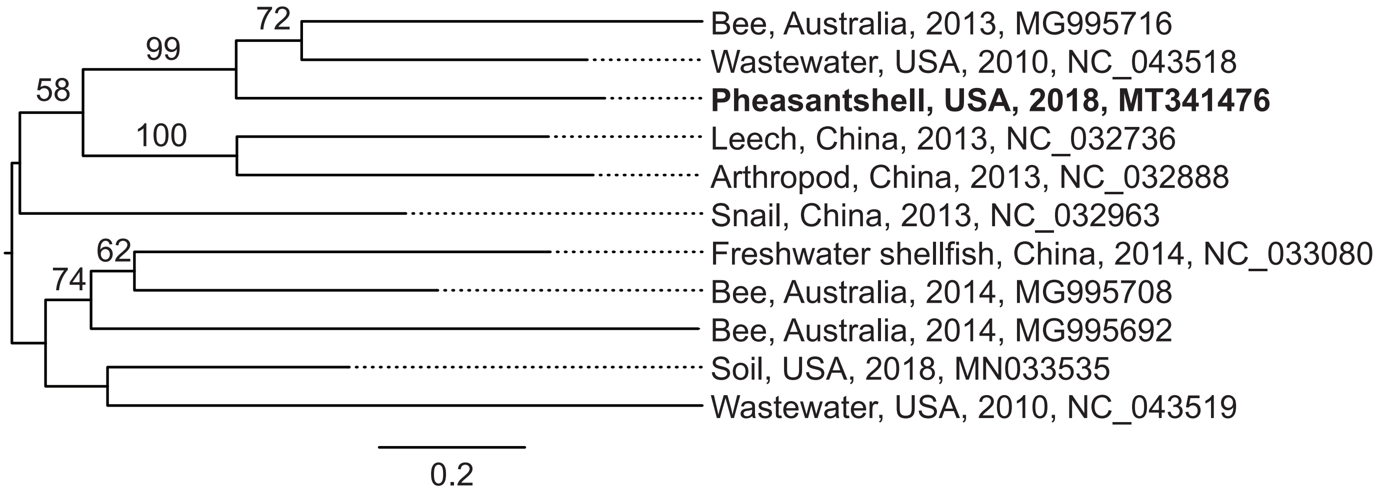
[1]U.S. Fish and Wildlife Service, Southwestern Virginia Field Office, 330 Cummings Street, Abingdon, VA 24210. [2]La Crosse Fish Health Center, Midwest Fisheries Center, U.S. Fish and Wildlife Service, 555 Lester Ave, Onalaska, WI, 54650. [3]Department of Pathobiological Sciences, University of Wisconsin-Madison, 1656 Linden Drive, Madison WI 53706. [4]U.S. Geological Survey, Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Rd, La Crosse WI, 54603. [5]US Geological Survey, National Wildlife Health Center, 6006 Schroeder Rd, Madison, WI 53711. [6]Global Health Institute, University of Wisconsin-Madison, 1300 University Avenue, Madison WI 53706

*Correspondence and requests for materials should be addressed to Tony L. Goldberg, Department of Pathobiological Sciences, University of Wisconsin-Madison, 1656 Linden Drive, Madison, Wisconsin 53706, USA. Email: tony.goldberg@wisc.edu; Phone: +1-608-890-2618; Fax: +1-608-262-7420.

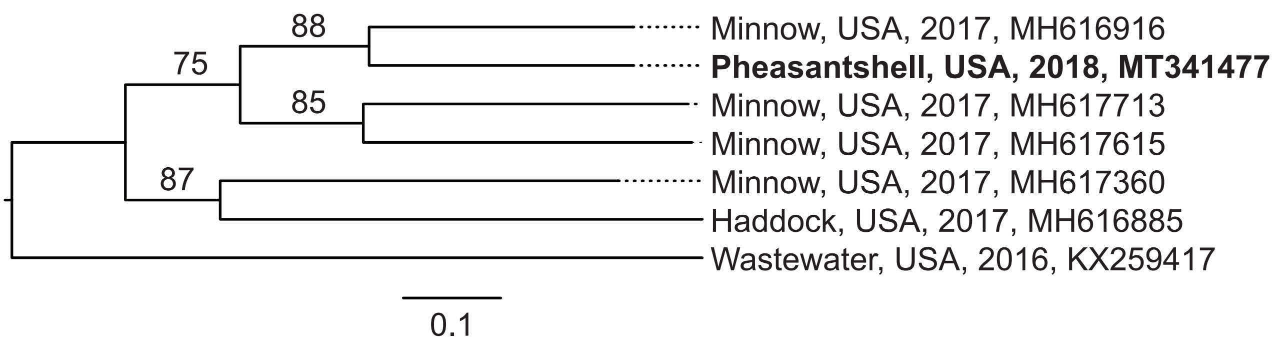
[1]Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.


A) Densoviruses
100
81
100
65
100
99
65
Sea star, USA, 2015, MN190158
Sea star, USA, 2013, NC_038532
Crayfish, Australia, 1999, NC_026943
Oyster, China, 2015, KY548840
Cockroach, China, 2007, JQ320376
Great tit, China, 2014, NC_031450
Pheasantshell, USA, 2018, MT341473
Cockroach, China, 1999, NC_000936
Drill, Nigeria, 2017, MN784124
0.1
B) Narna-like viruses
58
51
91
72
76
Crayfish, China, 2014, NC_033143
Leech, China, 2013, NC_032867
Echinoderm, China, 2014, NC_032415
Shrimp, China, 2014, KX883567
Pheasantshell, USA, 2018, MT341474
Gastropoda, China, 2013, KX883624
Horsehoe crab, China, 2014, NC_032485
Bivalva, China, 2013, KX883462
Gastropoda, China, 2013, NC_033177
Shrimp, China, 2014, NC_032448
0.5
C) Noda-like viruses
Freshwater shellfish, China, 2014, KX883205
Pheasantshell, USA, 2018, MT341475
Myriapoda, China, 2013, KX883197
Seaweed, United Kingdom, 2014, MF190050
Shrimp, China, 2014, KX883188
Bivalva, China, 2013, NC_033166
99
Octopus, China, 2014, KX883065
Mildew, Japan, 1999, NC_005817
Wastewater, USA, 2010, KF510033
Mildew, Hungary, 2001, HM453714
Water strider, China, 2013, NC_033077
0.2

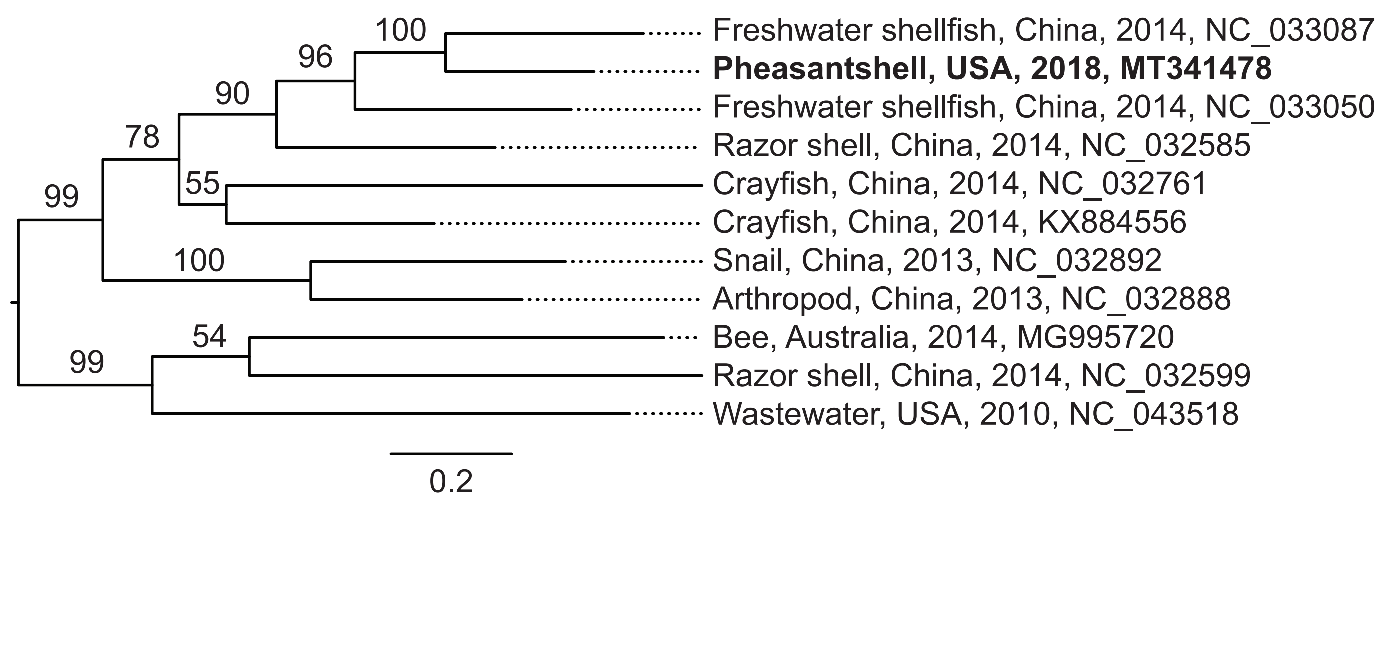
D) Picorna-like viruses (1)



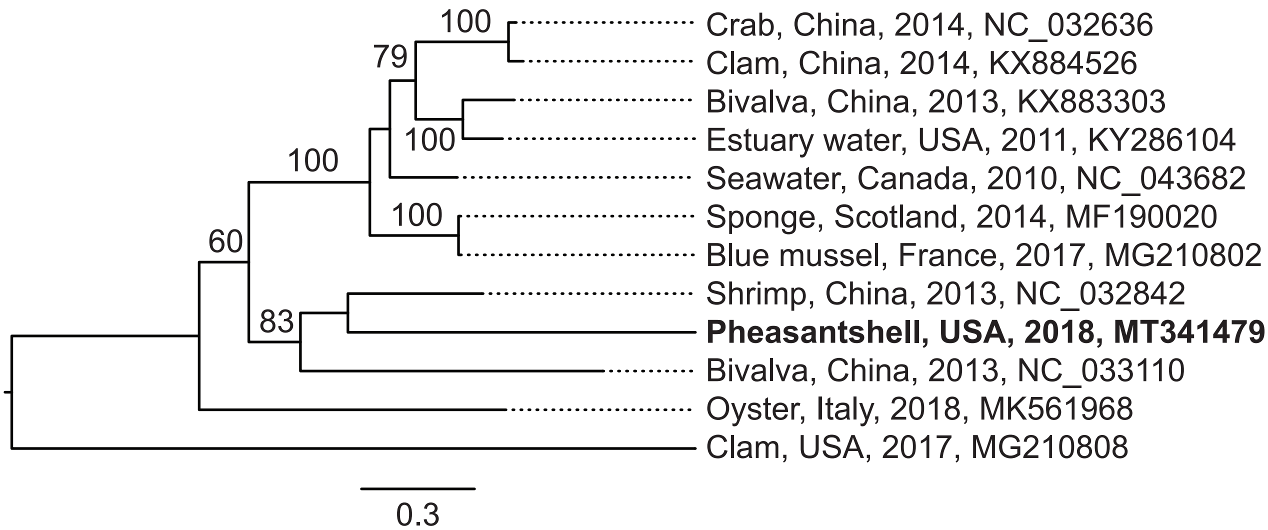
E) CRESS viruses



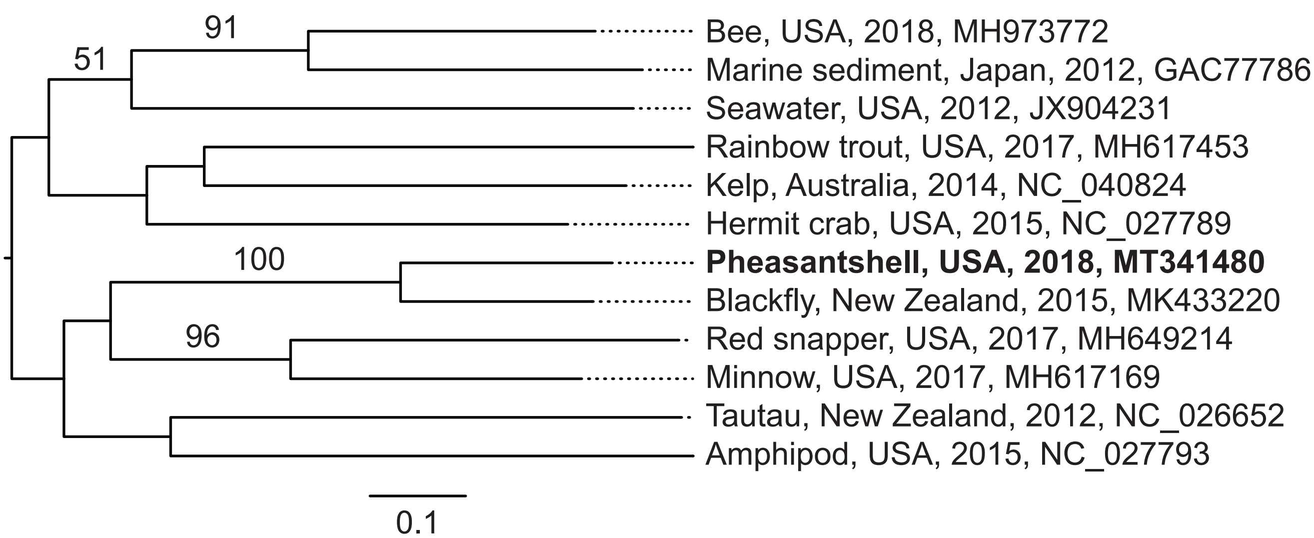
F) Picorna-like viruses (2)



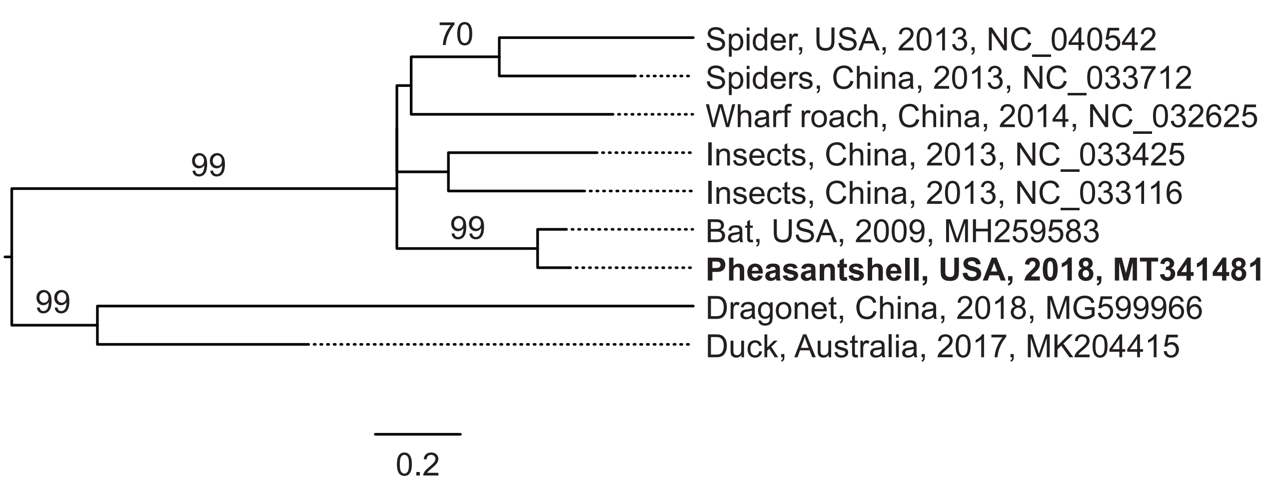
G) Picorna-like viruses (3)



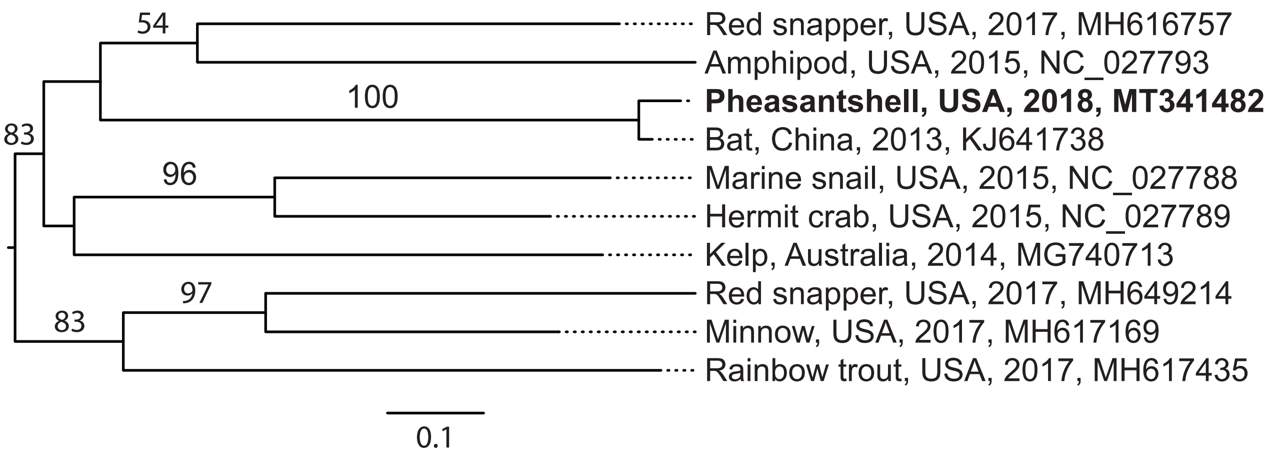
H) Circular viruses (1)



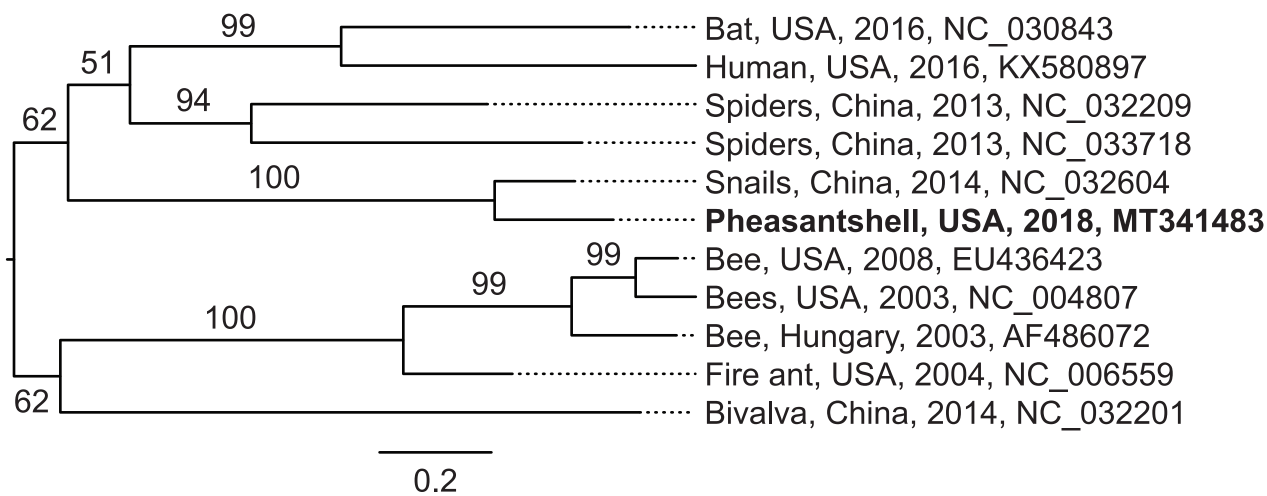
I) Caliciviruses



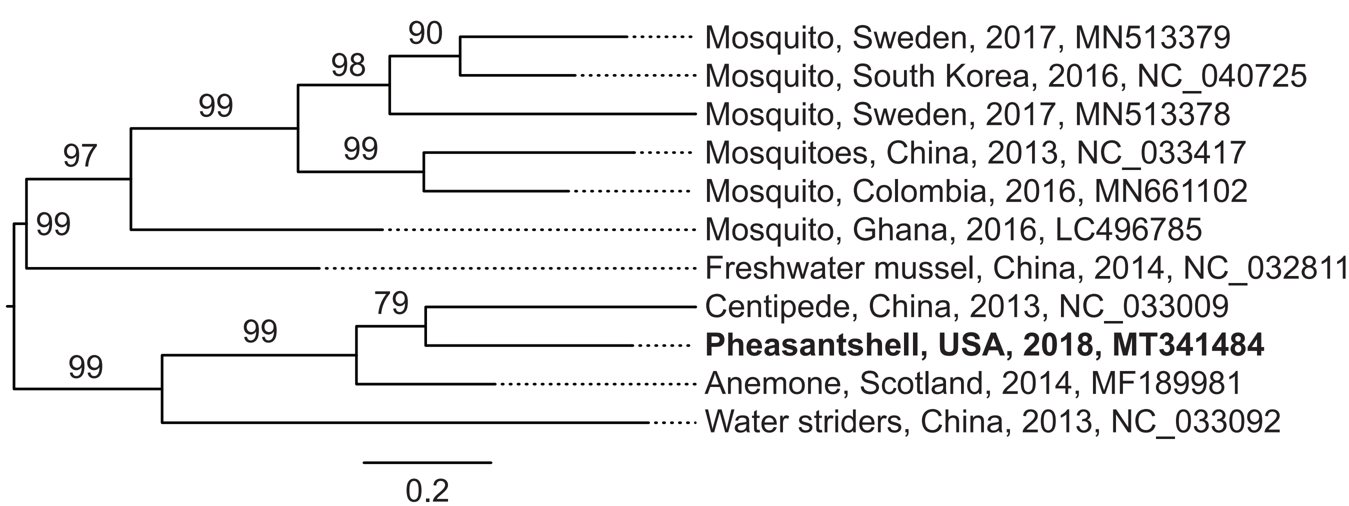
J) Circular viruses (2)

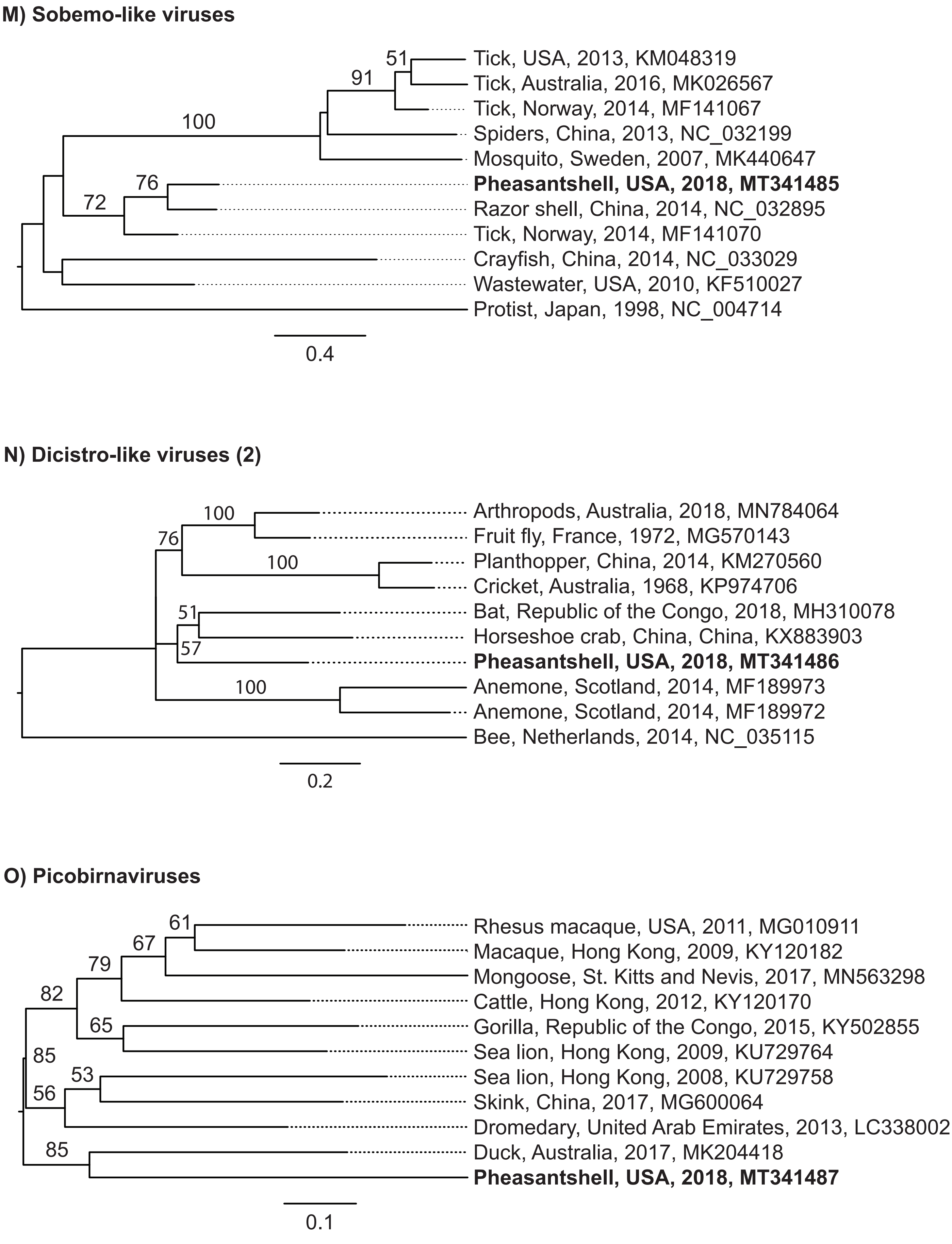


K) Dicistro-like viruses (1)

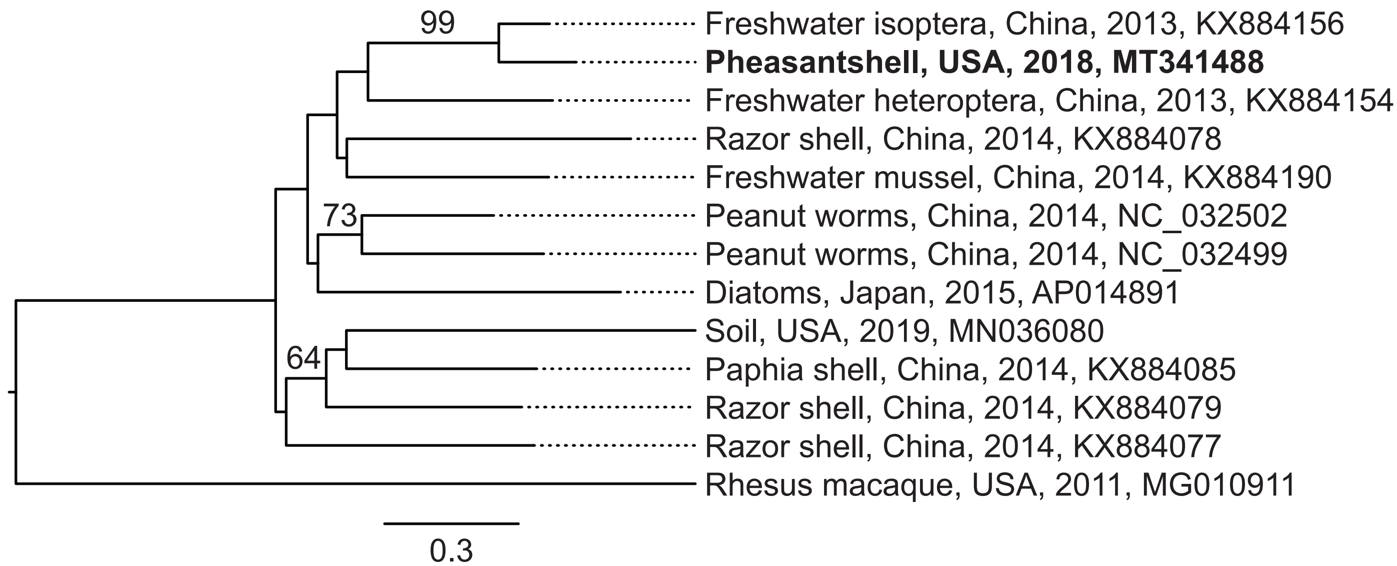


L) Tombus-like viruses




M) Sobemo-like viruses
51
91
100
76
72
Tick, USA, 2013, KM048319
Tick, Australia, 2016, MK026567
Tick, Norway, 2014, MF141067
Spiders, China, 2013, NC_032199
Mosquito, Sweden, 2007, MK440647
Pheasantshell, USA, 2018, MT341485
Razor shell, China, 2014, NC_032895
Tick, Norway, 2014, MF141070
Crayfish, China, 2014, NC_033029
Wastewater, USA, 2010, KF510027
Protist, Japan, 1998, NC_004714
0.4
N) Dicistro-like viruses (2)
100
76
100
51
57
100
Arthropods, Australia, 2018, MN784064
Fruit fly, France, 1972, MG570143
Planthopper, China, 2014, KM270560
Cricket, Australia, 1968, KP974706
Bat, Republic of the Congo, 2018, MH310078
Horseshoe crab, China, China, KX883903
Pheasantshell, USA, 2018, MT341486
Anemone, Scotland, 2014, MF189973
Anemone, Scotland, 2014, MF189972
Bee, Netherlands, 2014, NC_035115
0.2
O) Picobirnaviruses
61
67
79
82
65
85
53
56
85
Rhesus macaque, USA, 2011, MG010911
Macaque, Hong Kong, 2009, KY120182
Mongoose, St. Kitts and Nevis, 2017, MN563298
Cattle, Hong Kong, 2012, KY120170
Gorilla, Republic of the Congo, 2015, KY502855
Sea lion, Hong Kong, 2009, KU729764
Sea lion, Hong Kong, 2008, KU729758
Skink, China, 2017, MG600064
Dromedary, United Arab Emirates, 2013, LC338002
Duck, Australia, 2017, MK204418
Pheasantshell, USA, 2018, MT341487
0.1

P) Picobirna-like viruses



Q) Totiviruses

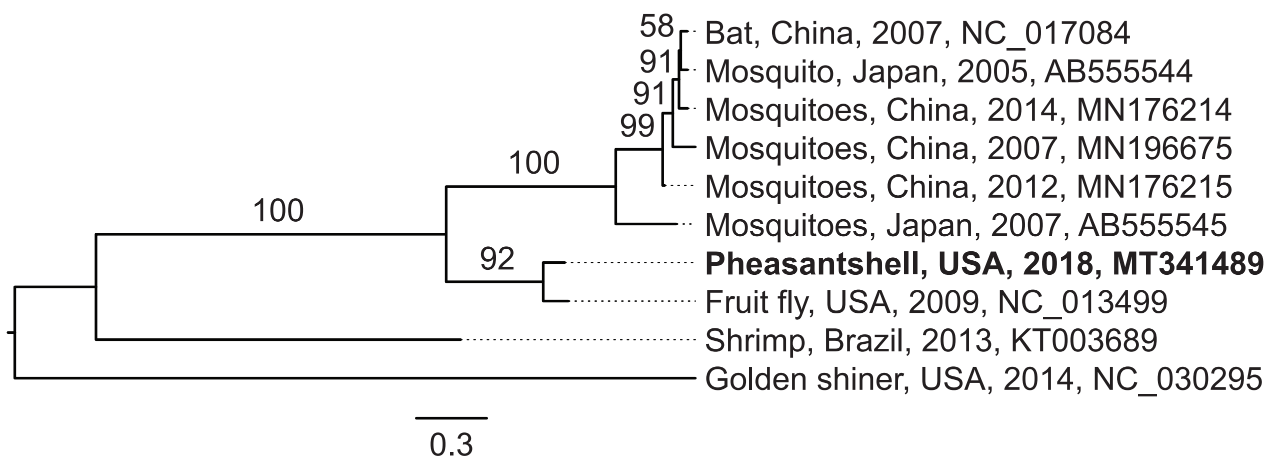


Table S1. Details of Clinch River pheasantshells (*Actinonaias pectorosa*) used in the analyses

Accession	Short ID	Status	Date	Location	Condition[1]	Length (mm)	Gravid[2]
AP102017-037	Y37	Case	10/20/17	Frost Ford		96.0	
AP102017-043	Y43	Case	10/20/17	Kyle's Ford		97.0	
AP102017-065	Y65	Case	10/20/17	Kyle's Ford		90.0	
AP102017-066	Y66	Case	10/20/17	Kyle's Ford		92.5	
AP110317-001	Z01	Case	11/3/17	Kyle's Ford		71.0	
AP110317-002	Z02	Case	11/3/17	Kyle's Ford		72.0	
AP110317-005	Z05	Case	11/3/17	Kyle's Ford		75.0	
AP110317-006	Z06	Case	11/3/17	Kyle's Ford		90.0	
AP110317-008	Z08	Case	11/3/17	Kyle's Ford		74.0	
AP110317-016	Z16	Case	11/3/17	Wallen's Bend		80.0	
AP110317-003	Z03	Case	11/3/17	Kyle's Ford		72.0	
20180925SIAPE-0007	B32	Case	9/25/18	Sycamore Island	4	95.0	Yes
20180925SIAPE-0011	B36	Case	9/25/18	Sycamore Island	5	69.0	
20180925SIAPE-0012	B37	Case	9/25/18	Sycamore Island	5	91.0	No
20180925SIAPE-0014	B39	Case	9/25/18	Sycamore Island	4	96.0	
20180925SIAPE-0015	B40	Case	9/25/18	Sycamore Island	5	101.0	Yes
20181024SFAPE-0001	C16	Case	10/24/18	Speers Ferry	4	103.0	No
20181025SIAPE-0012	C47	Case	10/25/18	Sycamore Island	4	78.0	No
20181025SIAPE-0013	C48	Case	10/25/18	Sycamore Island	4	86.0	Yes
20181025SIAPE-0021	C59	Case	10/25/18	Sycamore Island	4	97.0	No
20181025KFAPE-0007	C69	Case	10/25/18	Kyle's Ford	4	73.0	No
20181025KFAPE-0011	C73	Case	10/25/18	Kyle's Ford	4	70.0	No
20181025KFAPE-0012	C74	Case	10/25/18	Kyle's Ford	3	83.0	No
20181025KFAPE-0014	C76	Case	10/25/18	Kyle's Ford	3	88.0	Yes
20181025KFAPE-0015	C77	Case	10/25/18	Kyle's Ford	2	74.5	No
20181025KFAPE-0016	C78	Case	10/25/18	Kyle's Ford	3	94.0	Yes
AP102017-005	Y05	Control	10/20/17	Sycamore Island		104.0	
AP102017-015	Y15	Control	10/20/17	Wallen's Bend		84.0	
AP102017-027	Y27	Control	10/20/17	Frost Ford		89.0	
AP102017-044	Y44	Control	10/20/17	Kyle's Ford		85.5	
AP110317-009	Z09	Control	11/3/17	Kyle's Ford		94.0	
AP110317-010	Z10	Control	11/3/17	Kyle's Ford		61.0	
AP110317-013	Z13	Control	11/3/17	Wallen's Bend		61.0	
AP110317-015	Z15	Control	11/3/17	Wallen's Bend		84.0	
20180816SIAPE-0002	A10	Control	8/16/18	Sycamore Island	4	96.0	Yes
20180816SIAPE-0008	A16	Control	8/16/18	Sycamore Island	5	95.7	No
20180816WBAPE-0007	A23	Control	8/16/18	Wallen's Bend	4	91.6	No
20180816KFAPE-0002	A34	Control	8/16/18	Kyle's Ford		99.4	No
20180816KFAPE-0006	A38	Control	8/16/18	Kyle's Ford	5	83.8	No
20180925ARAPE-0004	B04	Control	9/25/18	Artrip	4	95.0	Yes
20180925SFAPE-0004	B17	Control	9/25/18	Speers Ferry	4	85.0	Yes
20180925SIAPE-0002	B26	Control	9/25/18	Sycamore Island	4	92.0	Yes
20180925SIAPE-0003	B27	Control	9/25/18	Sycamore Island	4	97.0	Yes
20180925SIAPE-0004	B28	Control	9/25/18	Sycamore Island	5	91.0	Yes
20180925SIAPE-0006	B31	Control	9/25/18	Sycamore Island	5	104.0	No
20180925WBAPE-0002	B44	Control	9/25/18	Wallen's Bend	4	84.4	No
20181025SIAPE-0002	C36	Control	9/25/18	Sycamore Island	5	93.0	No
20181025KFAPE-0001	C63	Control	9/25/18	Kyle's Ford	4	70.0	No
20181025KFAPE-0002	C64	Control	9/25/18	Kyle's Ford	3	88.0	Yes
20180925KFAPE-0020	B71	Control	9/26/18	Kyle's Ford	4	62.0	No
20180925KFAPE-0022	B73	Control	9/26/18	Kyle's Ford	3	78.0	No
20180925KFAPE-0024	B75	Control	9/26/18	Kyle's Ford	5	60.0	No
20181024ARAPE-0004	C04	Control	10/24/18	Artrip	4	106.0	Yes
20181024SFAPE-0013	C33	Control	10/24/18	Speers Ferry	5	82.0	No
20181025KFAPE-0004	C66	Control	10/25/18	Kyle's Ford	4	76.0	No
20181025KFAPE-0008	C70	Control	10/25/18	Kyle's Ford	3	58.0	No
20181025KFAPE-0009	C71	Control	10/25/18	Kyle's Ford	3	50.0	No
20181025KFAPE-0013	C75	Control	10/25/18	Kyle's Ford	3	88.0	Yes

[1]Body condition score was ranked on an ordinal scale of 1 (poor) to 5 (excellent). Blank cells represent missing data.

[2]Reproductive status (gravid or not gravid) was assessed during physical examintion. Bland cells represent missing data.

Table S2. Loads of 17 viruses in Clinch River pheasantshells (*Actinonaias pectorosa*)

		Virus[1]																
Short ID[2]	Status	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
Y37	Case	0.83	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Y43	Case	1.24	0.30	0.15	2.17	0.00	1.60	0.24	1.43	0.59	0.00	0.00	0.34	0.00	0.21	0.00	0.00	0.00
Y65	Case	0.48	0.00	0.00	1.52	0.00	0.42	0.00	0.00	0.00	0.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Y66	Case	1.48	0.00	0.00	0.00	0.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z01	Case	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z02	Case	1.44	0.12	0.00	1.16	0.42	0.00	0.00	0.78	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z05	Case	1.93	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z06	Case	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.43	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z08	Case	0.00	0.00	0.00	0.06	0.41	0.28	0.00	0.00	0.00	0.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z16	Case	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z03	Case	0.00	0.00	0.00	1.51	0.00	0.11	0.00	0.00	0.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B32	Case	0.00	0.04	0.09	0.37	0.31	0.00	0.00	0.00	0.05	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B36	Case	1.67	0.00	0.00	0.11	2.13	0.00	0.00	0.00	0.00	0.20	0.10	0.00	0.00	0.00	0.00	0.00	0.00
B37	Case	0.95	0.51	0.00	2.54	0.67	0.98	0.00	0.00	1.17	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B39	Case	1.32	0.00	0.00	0.66	1.54	0.00	0.00	0.00	0.00	1.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B40	Case	0.00	0.00	0.00	1.46	0.00	0.00	0.00	0.00	0.00	1.48	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C16	Case	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C47	Case	3.09	2.56	1.30	2.39	1.08	0.64	1.60	0.97	1.45	1.13	1.17	1.26	0.89	1.51	0.00	1.47	1.73
C48	Case	0.72	0.00	0.00	1.07	1.20	0.94	0.00	0.65	0.14	2.29	0.00	0.09	0.00	0.00	0.00	0.00	0.00
C59	Case	0.35	0.00	0.00	0.00	1.40	0.00	0.00	0.39	0.00	1.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C69	Case	0.55	0.00	0.00	0.11	0.79	0.18	0.13	0.77	0.00	1.24	0.00	0.00	0.00	0.00	1.05	0.00	0.00
C73	Case	0.19	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C74	Case	0.95	0.00	0.00	0.00	0.81	0.00	0.00	0.62	0.00	0.97	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C76	Case	0.00	0.00	0.00	0.13	0.84	0.52	0.00	0.62	0.00	1.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C77	Case	0.32	0.00	0.00	0.00	1.71	0.00	0.00	0.00	0.00	0.92	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C78	Case	1.36	0.07	0.00	0.57	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Y05	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Y15	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.47	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Y27	Control	0.00	0.00	0.00	0.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Y44	Control	0.00	0.00	0.00	0.08	0.49	0.00	0.00	0.00	0.00	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z09	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.54	0.00	0.47	0.00	0.00	0.00	0.00	0.00
Z10	Control	0.32	0.00	0.00	0.53	0.00	0.16	0.00	0.00	0.00	0.68	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z13	Control	0.37	0.00	0.00	0.28	0.00	0.00	0.00	0.53	0.00	0.64	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z15	Control	0.56	0.00	0.00	0.58	0.20	0.00	0.00	0.00	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A10	Control	0.00	0.00	0.00	0.00	0.00	0.27	0.00	0.00	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A16	Control	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.00	0.96	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A23	Control	0.44	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A34	Control	0.14	0.00	0.00	0.04	0.97	0.12	0.00	0.00	0.00	0.47	0.00	0.00	0.00	0.10	0.00	0.00	0.00
A38	Control	0.00	0.07	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.24	0.00	0.00	0.07	0.00	0.00	0.00	0.00
B04	Control	0.73	0.00	0.00	0.19	0.00	0.00	0.00	0.17	0.00	1.15	0.00	0.00	0.08	0.00	0.00	0.00	0.00
B17	Control	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B26	Control	0.00	0.00	0.00	0.08	0.40	0.44	0.00	0.00	0.24	1.47	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B27	Control	0.00	0.00	0.00	0.08	0.50	0.21	0.00	0.00	0.42	1.33	0.00	0.00	0.12	0.00	0.00	0.00	0.00
B28	Control	0.00	0.00	0.00	0.30	0.33	0.00	0.00	0.00	0.00	2.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B31	Control	0.00	0.00	0.00	0.09	0.51	0.00	0.00	0.00	0.00	0.85	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B44	Control	0.00	0.00	0.00	0.51	1.57	0.00	0.00	0.00	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C36	Control	0.54	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.86	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C63	Control	0.00	0.00	0.00	0.06	0.15	0.00	0.00	0.00	0.00	0.80	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C64	Control	0.00	0.00	0.00	0.14	1.10	0.00	0.00	1.33	0.00	2.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B71	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.78	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B73	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.89	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B75	Control	0.41	0.00	0.00	0.12	0.80	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C04	Control	0.39	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.14	1.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C33	Control	0.00	0.00	0.00	0.00	0.24	0.62	0.00	0.00	0.19	1.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C66	Control	0.27	0.00	0.00	0.13	0.81	0.54	0.00	0.76	0.00	0.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C70	Control	0.00	0.00	0.00	0.44	0.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C71	Control	0.00	0.00	0.00	0.06	0.48	0.17	0.00	0.00	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C75	Control	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.17	0.34	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[1]For details of viruses, see Table 1. Viral loads are presented as $\log_{10}$ viral reads per million total reads per kilobase of target sequence.

[2]See Table S1 for full details of Clinch River pheasantshells used in the analyses.