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Rediscovering “Baculovirus-A” (Johnson, 1976): The complete genome of ‘Callinectes sapidus nudivirus’

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ABSTRACT

Callinectes sapidus, or the ‘blue crab’, supports an extensive east-coast USA fishery and was one of the first crustacean species in which viruses were observed. Pioneering research by Dr Phyllis Johnson led to these initial discoveries, one of which included the discovery of a virus termed “Baculovirus-A”. This virus was considered a potential member of the *Baculoviridae*, *Nimaviridae*, or *Nudiviridae*, in which all viral members are rod-shaped dsDNA viruses found in the nucleus of their host cell.

With the availability of genomic and bioinformatic tools, such as Illumina HiSeq and assembly programs, it is now possible to assemble the genomes of viruses and gain additional genomic insight, which can shed light on viral taxonomy. Using these tools, alongside electron micrographs and histology slides, we reveal that the hepatopancreas-infecting ‘Baculovirus-A’ from *Callinectes sapidus* is a member of the *Nudiviridae*, resembling genetic and protein similarity to other crab and lobster infecting nudiviruses from the *Gammanudivirus* genus. Histologically, the virus causes nuclear hypertrophy as observed for other *gammanuriviruses*. The genome of the virus is circular, 122,436 bp in length, and encodes a predicted 98 protein coding genes, including all of the nudivirus core genes.

The prevalence of virus from across Florida, USA, is provided alongside a genomic comparison of the new viral genome against other *Gammanudivirus* species, revealing the average prevalence to be 2.2% and that Callinectes sapidus nudivirus is distantly similar to the recently described Carcinus maenas nudivirus from Canada.

1. Introduction

Efforts to sequence the genomes of viruses from invertebrate animals has yielded millions of new isolates, expanding our view of the global virome (Shi et al. 2016; Edgar et al. 2022). In particular, our catalogue of the crustacean virosphere has also become more complete, including discoveries from across the viral realm using a range of genomic and pathological tools (Bateman and Stentiford, 2017; Bojko et al., 2019; Hooper et al. 2020; Zhao et al. 2021). Viral pathogens of crustaceans can have devastating effects on wild and cultured populations, resulting in vast economic and ecological damage. Therefore, it is of great importance to understand the emergence, impact, and treatment of crustacean diseases (Stentiford et al. 2012; Stentiford et al. 2020).

Our understanding of viral diversity has increased relatively quickly for one group of double-stranded DNA viruses, the *Nudiviridae* (Harrison et al. 2020), a family of non-occluded viruses related to the *Baculoviridae* and *Polydnaviridae*, from insect and crustacean hosts (Petersen et al. 2022). The systematics of the *Nudiviridae* family consists of five genera (*Alphanudivirus*, *Betanudivirus*, *Gammanudivirus*, *Deltanudivirus* and unofficial *Epsilonnudivirus*) from two sub-families (*Alphanudivirinae* and *Betanudivirinae*) (Bateman et al. 2021). Paleovirological studies have uncovered further diversity from the genomes of insects with endogenous nudivrius-like elements (Cheng et al. 2020; Liu et al. 2021). To date, the most diverse genus of exogenous *Nudiviridae* is the crustacean infecting *Gammanudivirus* genus, which now includes seven species with complete genomes. These species infect the penaeid shrimp, *Penaeus monodon* (Yang et al. 2014); the freshwater river prawn, *Macrobrachium rosenbergii* (Petersen et al. 2022); the European lobster, *Homarus*

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gammarus (Holt et al. 2019); the amphipod, *Dikerogammarus haemobaphes* (Allain et al. 2020); the shrimp *Crangon crangon* (Bateman et al. 2021); the European shore crab, *Carcinus maenas* (Bateman et al. 2021); and the mangrove crab, *Aratus pisonii* (Bojko et al. 2022). Others have been identified through transcriptomic mining, such as the Charybdis crab nudivirus(es) (Porter et al. 2019) and ‘Eriocheir sinensis nudivirus’ (Shen et al. 2021).

Despite a lack of genomic data, many more crustacean nudiviruses have been described using pathological tools, such as histology and electron microscopy (Bateman and Stentiford, 2017; Bojko et al. 2017). Such hosts include *Cancer pagurus* (Bateman and Stentiford, 2008); *Carcinus mediterraneus* (Tau-virus; Pappalardo et al. 1986); *Pinnotheres pisum* (Longshaw et al. 2012); *Astacus astacus* (Edgerton et al. 2004); *Cherax quadricarinatus* (Edgerton and Owens, 1997); *Pacifastacus leniusculus* (Anderson et al. 2021); *Cherax destructor* (Edgerton, 1996); *Astropotamobius pallipes* (Edgerton et al. 2002); *Farfantapenaeus* (=*Pandalus*) *montagui* (Bateman and Stentiford, 2017); *Penaeus japonicus* (Takahashi et al. 1994); *Penaeus duorarum* (Couch, 1974); *Penaeus plebejus* (Lester et al. 1987); *Paralithodes platypus* (Johnson and Lightner, 1988); *Scylla serrata* (Owens et al. 2010); multiple amphipod species (Bojko et al. 2013; Bojko and Ovcharenko, 2019; Warren et al. 2022); and finally, one of the first identified crustacean viruses, “Baculovirus-A”, from *Callinectes sapidus* (Johnson, 1976), an important and economically lucrative fishery asset from the east coast of the USA (Kennedy et al. 2007).

Callinectes sapidus hosts a range of viral pathogens across its geographic range, many associated with damage to the fishery and softshell aquaculture industry (Johnson, 1978; Bowers et al. 2010; Bateman and Stentiford, 2017; Zhao et al. 2020). Baculovirus-A in *Callinectes sapidus* was proposed as a possible nimavirus (*Nimaviridae*) (Bateman and Stentiford, 2017); however, tissue tropism specific to the hepatopancreas suggests that the virus may be a candidate for the *Nudiviridae*. In this study, we provide histopathological, ultrastructural, and genomic data for a virus infecting the hepatopancreas of *Callinectes sapidus*, which resembles Baculovirus-A, and identify it as a novel member of the *Nudiviridae* with the provisional name ‘Callinectes sapidus nudivirus’ (CsNV), pending ICTV acceptance.

2. Materials and methods

2.1. Sample collection

Wild caught blue crabs (n = 409) were collected from commercial traps in eight different locations throughout Florida. Collection habitats were diverse spanning offshore waters (Suwannee Sound), an estuarine bay (lower and upper Tampa Bay estuary), brackish river (Manatee River), salt marsh creeks (Clapboard Creek), freshwater river (Goodbys and Riverdale St. Johns River), and a freshwater lake (Lake George) (Fig. 1). Collections occurred in summer (August – September) and winter (December – February) months from August 2019 to February 2021 on contracted commercial fishing vessels. During each trip, commercial fishers hauled traps aboard where blue crabs were culled and

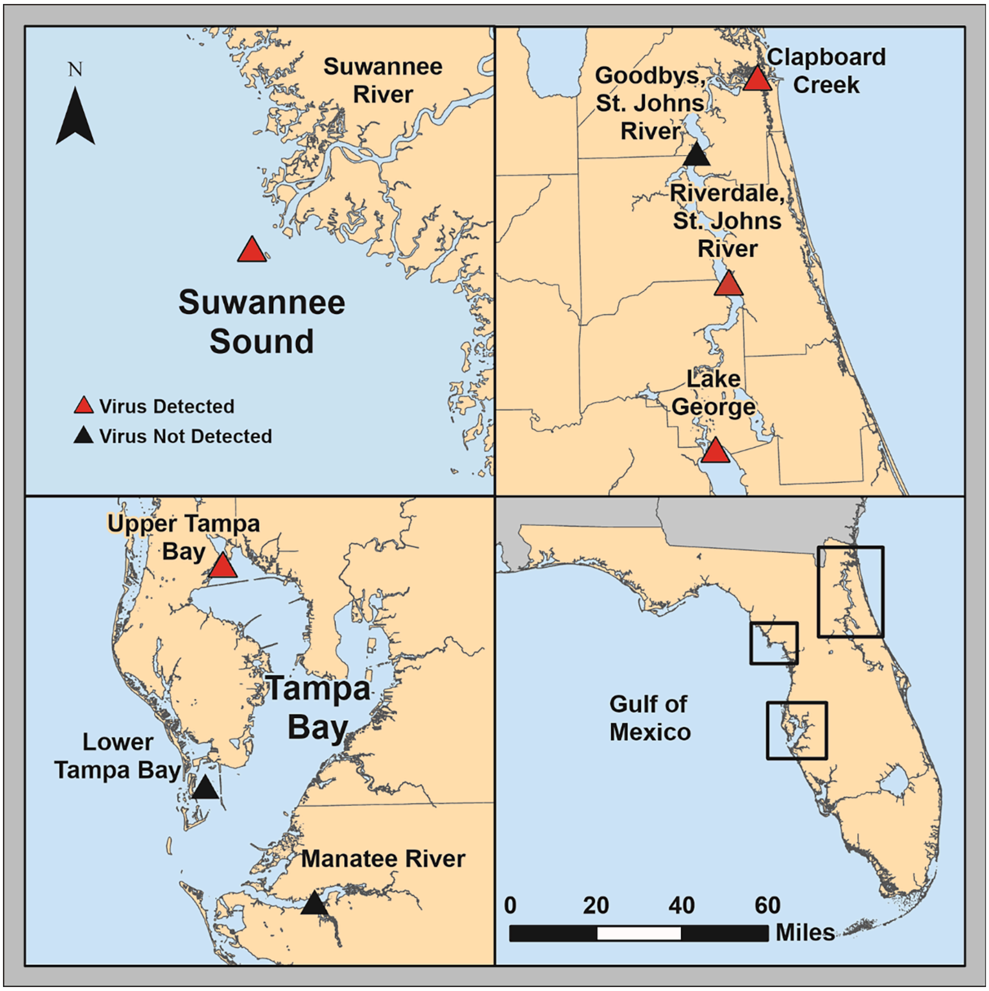

Fig. 1. Map of locations (triangles) across Florida where blue crabs were collected. Collection sites designated by red triangles indicate where Callinectes sapidus nudivirus (CsNV) positive blue crabs were found, and black triangles designate CsNV absence. The figure was developed in ESRI ArcGIS Pro mapping software.

selected for market sale. During the culling procedure on the vessel, we selected crabs at random, making sure to select legal (CW > 127 mm) and sublegal (CW > 127 mm), as well as male and female, blue crabs for histopathological analysis, and placed crabs in a wooden crate covered with wet burlap for live transport to the laboratory.

2.2. Histopathology and electron microscopy

When crabs arrived at the lab, they were placed on ice and anesthetised. Dissection occurred immediately after anesthetisation to limit tissue degradation. Hepatopancreatic tissues were collected from each crab, placed in a plastic tissue cassette and submerged in saltwater or freshwater Davidson's fixative dependent on habitat salinity crabs were pulled from, where they fixed for 48 h on a shaker table to ensure equal fixation. Tissue cassettes were then rinsed in tap water and transferred to 70 % ethanol for standard histological processing by the histology department at the Fish and Wildlife Research Institute (FWRI; Florida Fish and Wildlife Conservation Commission).

A single wax-embedded hepatopancreas sample was processed for transmission electron microscopy. The two-millimetre region of Davidson's-fixed paraffin-embedded blue crab hepatopancreas tissue was punched out of the paraffin block and deparaffinised in xylene (multiple changes). Tissues were reverse processed through 100 %, 75 %, and 50 % ethanol:water washes followed by 100 % water washes. Re-hydrated samples were then fixed in 4 % paraformaldehyde and 2.5 % glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M sodium cacodylate buffer, containing 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25 % NaCl, at a pH of 7.26. Fixed tissue was processed with the aid of a Pelco BioWave Pro laboratory microwave (Ted Pella, Redding, CA, USA) and SBT digital orbital shaker (Southwest Science, Trenton, NJ, USA). The specimen was washed in 0.1 M sodium cacodylate buffer and post-fixed with buffered 2 % osmium tetroxide, suspended in water, and dehydrated in a graded acetone series, 25 %, 50 %, 75 %, 95 %, 100 %, followed by 100 % anhydrous acetone. The dehydrated sample was infiltrated in acetone: Araldite epoxy resin with Z6040 embedding primer (Electron Microscopy Sciences, Hatfield, PA) at 30 %, 50 %, 70 %, and 100 %, and cured at 70 °C for 72 h.

Semi-thick sections (500 nm) were cut and stained with toluidine blue to define an accurate location of the source of infection. Ultrathin sections (120 nm) were collected on carbon coated Formvar 100 mesh grid and were post-stained with 2 % aqueous uranyl acetate and Reynolds lead citrate. Sections were examined under a FEI Tecnai G2 Spirit Twin TEM (FEI Corp., Hillsboro, OR) at 120 kV and digital images were acquired with a Gatan UltraScan 2 k × 2 k camera and Digital Micrograph software (Gatan Inc., Pleasanton, CA). Digital images were measured for virion size (length × width) using ImageJ software.

2.3. Metagenomics, genome assembly and annotation

DNA extraction on the hepatopancreatic tissue was performed using the Wizard Genomic DNA purification kit (Promega) at the National Horizons Centre (Teesside University, Darlington). The DNA extract was frozen (-80 °C) and transported to Novogene for metagenomic sequencing. One microgram of DNA was used to prepare a NEBNext® Ultra™ DNA Library (Illumina) following manufacturer's instructions. The library was loaded onto an Illumina NovaSeq 6000 coupled with the 150 bp NovaSeq 6000 SP reagent kit (300 cycles) for paired-end sequencing. The sequencing run produced 3,075,147 forward and 2,998,046 reverse raw reads, which were trimmed using Trimmomatic v0.39 (parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36) (Bolger et al. 2014). Assembly of the trimmed paired and unpaired reads was conducted using SPAdes v3.15.3 (Bankevich et al. 2012) (N50: 1,664; N75: 1,081; L50: 85,963; L75: 172,015) (Gurevich et al. 2013). The assembly resulted in 319,689 contiguous nucleotide sequences above 500 bp, which were screened for similarity to the *Nudiviridae* using blastx against all available nudivirus protein sequences on the NCBI database. Seven contiguous sequences (all above 1000X coverage) were identified as partial nudivirus-like contigs based on the blastx analysis, which were mapped and assembled into a single contiguous sequence (122,436 bp; 1065X coverage) with the help of CLC genomics workbench v.12. Further mapping was then conducted in Bowtie2 v.2.4.5 (Langmead and Salzberg, 2012), confirming coverage of the entire circular genome. The genome was annotated using GeneMarkS (Besemer et al. 2001) and manually searched for smaller coding regions, including the p6.9 gene (via ExPASy translate; Gasteiger et al. 2003). The annotated genome was represented as a circular plot using Circa (omgenomics.com/circa/) and stored on NCBI (accession: ON638996).

2.4. Phylogenetics and genome comparisons

Nudivirus species with complete genomes (n = 12) were mined for 17 core protein sequences (*pif-1, pif-2, pif-3, pif-4, pif-5, pif-6, 38 k, ac81, DNApol, helicase, lef-4, lef-5, lef-8, lef-9, p74, vp39* and *vp91*) as well as the new isolate from *C. sapidus*, and a baculovirus (Lonomia obliqua multiple nucleopolyhedrovirus) (outgroup). Each protein from each genome was aligned with the same protein from other species using MAFFT XSEDE v7.402 (CIPRES science gateway; Miller et al. 2012). Individual protein alignments were then concatenated manually into a single concatenated file and then analysed using IQ-Tree v.2.1.3 (Nguyen et al. 2015). IQ-Tree produced a maximum-likelihood (ML) phylogenetic tree based on 14,569 columns, 10,463 distinct patterns (7272 parsimony-informative), 2831 singleton sites, and 4466 constant sites. The best-fitting evolutionary model was Q.pfam + F + I + G4 according to Bayesian Information Criterion (BIC). The final consensus tree had a log-likelihood of −260059.396 and was visualised and annotated in Figtree v.1.4.4. In addition, the concatenated MAFFT protein alignment file was used with the Sequence Demarcation Tool (SDT v1.2) to produce a similarity plot for all the Gammanudivirus and Epsilonnudivirus isolates used in the phylogenetic analysis. A nucleotide comparison was also conducted for each genome to explore gene arrangement between different viruses using MAUVE (progressive MAUVE alignment; match seed weight: 15; determine LCBs: on; scoring matrix: HOXD) (Darling et al. 2004).

3. Results

3.1. Histopathology and ultrastructure of virally infected cells

Histopathological analysis of wax-infiltrated blue crab tissues determined an overall prevalence of 2.2 % (9/409) of CsNV in crabs from five locations in Florida, including: Clapboard Creek (n = 4); Riverdale St. Johns River (n = 1); Lake George (n = 1); Suwannee Sound (n = 1); and upper Tampa Bay (n = 2) (Fig. 2). Infections were found in both summer (n = 2) and winter (n = 7) season collections and in a wide range of habitat salinities (0.5 ‰ – 29.1 ‰). Nudivirus infections in histological section were found in the hepatopancreatic epithelia and discernible via enlarged nuclei, with basophilic inclusions and marginated chromatin (Fig. 2A). A single hepatopancreas sample processed for transmission electron microscopy revealed that these enlarged nuclei were housing rod-shaped virions (Fig. 2B-C). Due to wax-processing and subsequent re-processing for TEM, the putative viral envelope of the nudivirus was not discernible; however, observable virions (nucleocapsids) were measured. The average virion length measured 0.262 μm ± 0.022 μm (standard deviation) and the width measured 0.036 μm ± 0.003 μm (Fig. 2B-C). Virion measurements were comparable to Baculovirus-A descriptions in Johnson and Lightner (1988), where virions measured 0.26 μm to 0.3 μm in length and 0.06 μm in width; and nucleocapsids: 0.043 μm in diameter and 0.24 μm to 0.254 μm in width.

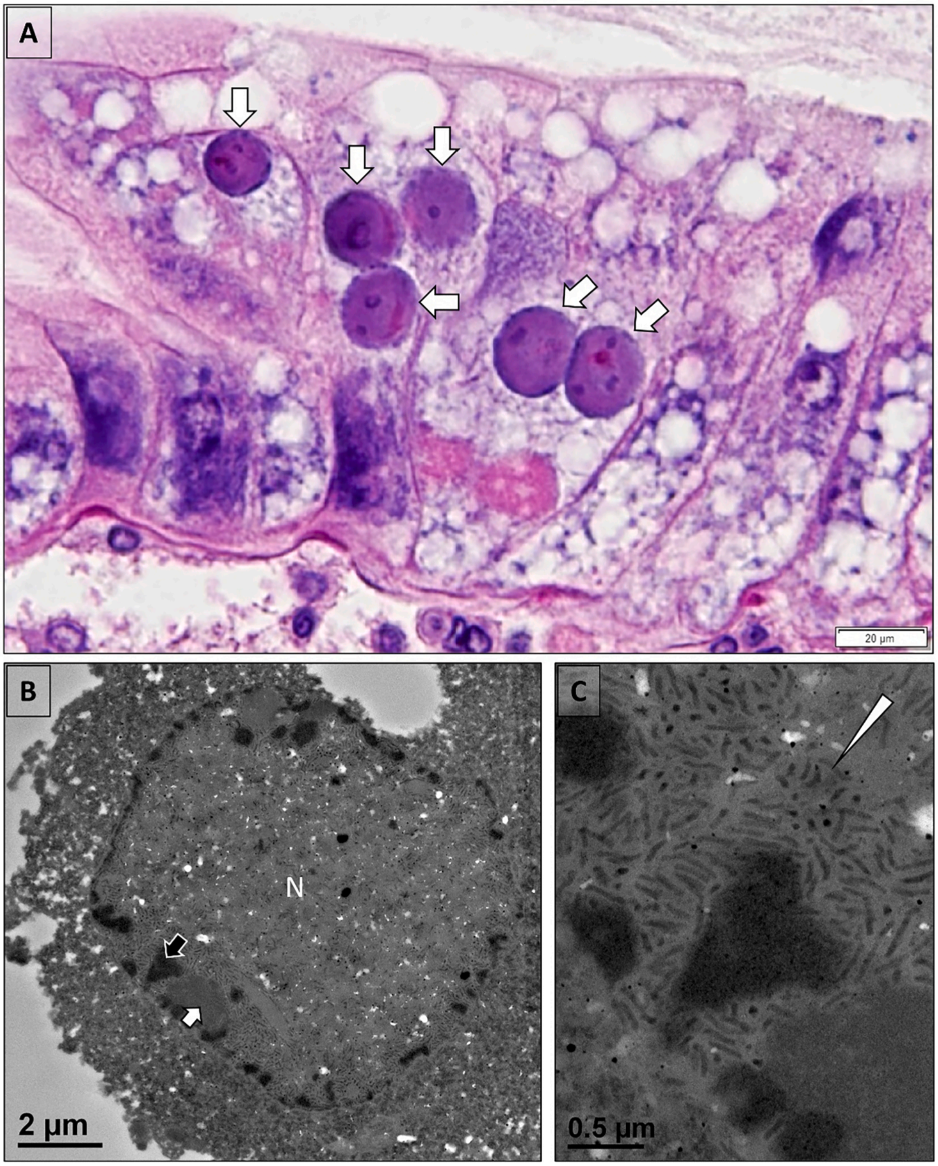

Fig. 2. Histopathology and ultrastructure of Callinectes sapidus nudivirus. A) Hypertrophic nuclei (arrow) of hepatopancreatic epithelial cells display marginated chromatin and a granular, basophilic, inclusion, typical of nudivirus infection. B-C) Transmission electron micrographs developed from the same wax-embedded tissue was used to visualise the putative virus. An infected nucleus (N) harbours a growing viroplasm with supposed viral factories (white arrow), in close association with host chromatin (black arrow). Rod-shaped, slightly curved, virions are visible (white triangle).

3.2. Viral genomics and phylogenetic relationships

The CsNV genome is a single, circular, molecule of 122,436 bp and in our assembly the genome had over 1000X coverage (Fig. 3). The genome encodes 98 protein coding genes, including all genes considered to be conserved for the *Nudiviridae* [*pif-1, pif-2, pif-3, pif-4* (*odv-e28*)*, pif-5* (*odv-e56*)*, pif-6, 38 k, ac81, ac92* (*p33*)*, DNApol, helicase, lef-4, lef-5, lef-8, lef-9, p74* (*pif-0*)*, vp39* (*31 k*)*, vp91* (*pif-8*)*, p6.9*, two copies of *helicase 2*, and two copies of *vlf-1*]. The genome also encodes multiple genes common among the *Gammanudivirus* members, such as *tk1* (CsNV_58)*, tk2* (CsNV_33)*, tk3* (CsNV_40)*, fen-1* (CsNV_12), Serine-Threonine protein kinase (CsNV_28), methyltransferase (CsNV_02) and several apoptosis inhibitor proteins (e.g. CsNV_93, CsNV_95) (Table 1). Nineteen predicted genes of the 98 annotated onto the CsNV genome, and their hypothetical protein products, had no discernible similarity to other known sequences.

Some genetic conservation is observable between the nucleotide sequence of each known nudivrius genome, with most of the decapod infecting nudiviruses having 19 broad clusters of genomic similarity to the new CsNV genome (Fig. 4). The most similar genome to that of CsNV, sequenced to date, is the genome of Carcinus maenas nudivirus (CmNV), which shared 15 broad clusters of genome similarity and synteny (Fig. 4) as well as 66 proteins with greatest similarity to those encoded by CsNV (Table 1). Other crab infecting nudiviruses lack some larger areas of nucleotide similarity, such as the lack of a large cluster shared between CsNV (~106 kb – ~122 kb region) and CmNV (~95 kb - ~115 kb region). In addition, some smaller inversions and rearrangements that are not observable in the Aratus pisonii nudivirus (ApNV) genome (Fig. 4), are also noticeably lacking in the Homarus gammarus nudivirus (HgNV) genome, relative to CsNV (Fig. 4).

Using 17 conserved nudivirus genes, a concatenated ML phylogenetic tree was developed to determine similarity and evolutionary relationships among the viruses sequenced to date (Fig. 5). The two viruses Dikerogammarus haemobaphes nudivirus (DhNV) and Crangon crangon nudivirus (CcNV), both branched together on the tree forming the putative *Epsilonnudivirus* genus, which represents nudiviruses of crustacean hosts lacking the conserved *p6.9* gene with its Serine-Argenine (‘SRSRSR’) repeat motif. The remaining crustacean infecting nudiviruses branched in the ICTV accepted *Gammanudivirus* genus (Fig. 5). No particular clustering among these viruses was observed, other than CsNV branching alongside CmNV (bootstrap: 100). Bootstrap support for Macrobrachium rosenbergii nudivirus (MrNV) is relatively low (58);

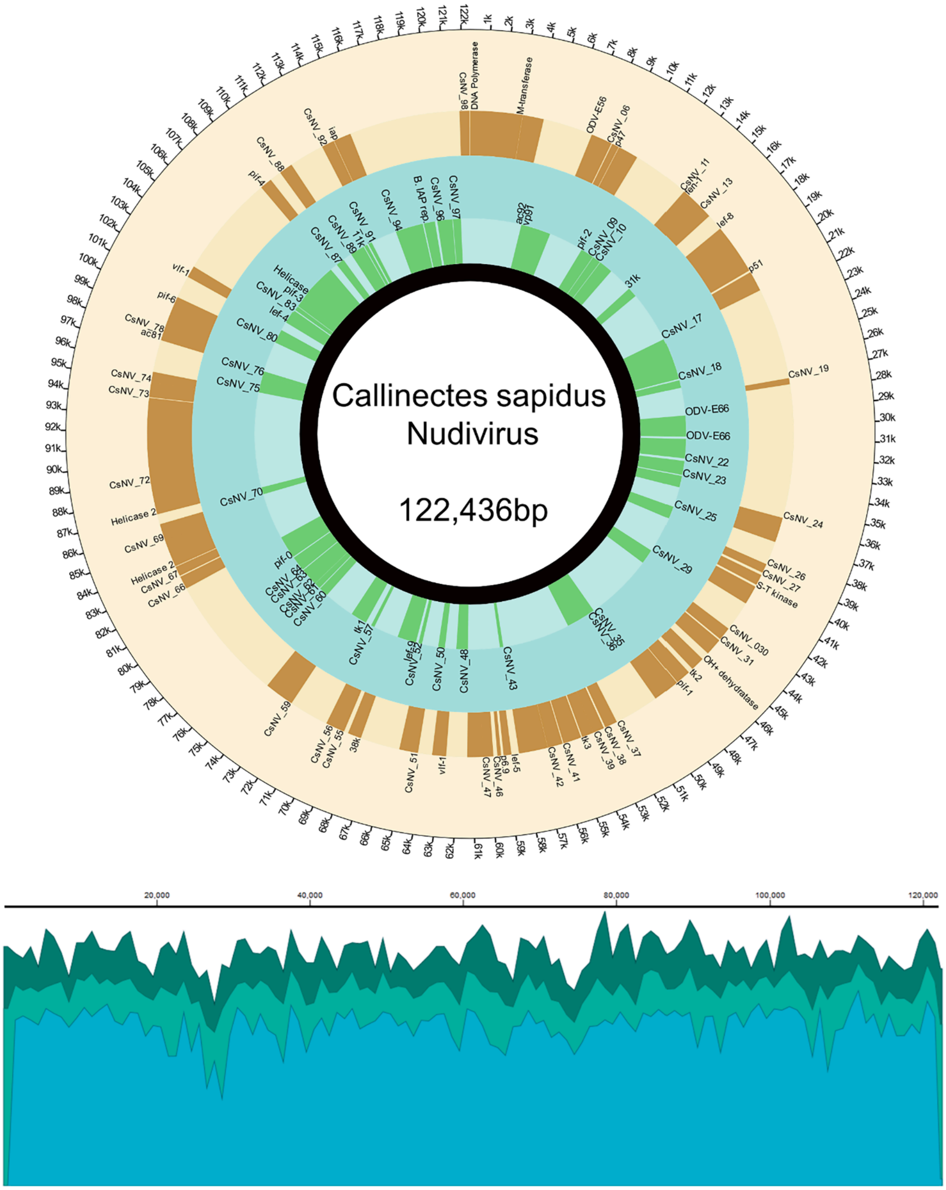

Fig. 3. Architecture of the Callinectes sapidus nudivirus genome, including protein coding genes on the positive strand (brown) and negative strand (green) of the dsDNA circular genome. The name of each gene name is listed at the start codon of the gene. Below the circa plot is a coverage plot highlighting the continuous mapping of trimmed read data across the genome. Ticks are provided every 1000 nt for scale.

however, in alternative trees this isolate can branch with PmNV; the remaining nodes are well supported (>92). Despite their grouping and clustering, the protein similarity across the crustacean-infecting nudiviruses is relatively low, with the greatest similarity between CmNV and CsNV, at an average of ~ 60 % based on the proteins used in the concatenated ML tree. The least similar crustacean infecting nudivirus to those now sequenced is CcNV, which is most similar to the amphipod infecting, DhNV.

4. Discussion

In 1976, Dr Phyllis T. Johnson, a researcher at the National Marine Fisheries Laboratory in Oxford, Maryland (USA), presented her research on the viruses of *Callinectes sapidus*, some of the first viruses identified from a marine invertebrate (Johnson, 1976; Stentiford, 2016). ‘Callinectes sapidus nudivirus’ (CsNV) (previously, ‘Baculovirus-A’) was one of the first viruses discovered by Dr Johnson. Here, we present the complete genome of this iconic finding as part of the ongoing advancement of crustacean virology. To date, this virus has the second largest genome of a crustacean-infecting nudivirus from the *Gammanudivirus*. The genome of CsNV consists of a circular dsDNA molecule, which is 122,436 bp in size and encodes a predicted 98 proteins. We discuss the importance of this discovery for viral taxonomy as well as the putative role of this virus within the blue crab fishery across the eastern USA.

4.1. *‘Callinectes sapidus nudivirus’ within the Nudiviridae and blue crab fishery*

Callinectes sapidus is the third crab species to have its nudivirus sequenced, which possesses a relatively large genome compared to other *Gammanudivirus* species (Bateman et al. 2021; Bojko et al. 2022). CsNV incites a similar pathology in this crustacean host as compared to other nudiviruses, causing nuclear hypertrophy of host hepatopancreatocytes with no sight of an occlusion body. The observed rod-shaped, slightly curved virions are coherent with the virion morphology of other

Table 1
Protein-level similarity table for the 98 predicted protein coding genes of the Callinectes sapidus nudivirus genome.

Gene	Start	Stop	Protein hit	Similar species and accession	Similarity (%)	e-value
CsNV_01	1	3195	DNA Polymerase	CmNV; UBZ25591	56.2	0.0
CsNV_02	3202	4488	Methyltransferase	CmNV: UBZ25592	41.7	8.8e-103
CsNV_03	4689	5366	Ac92-like	CmNV: UBZ25594	58.4	4.1e-94
CsNV_04	5353	7422	Vp91	CmNV: UBZ25595	50.7	0.0
CsNV_05	7532	8830	ODV-E56	HgNV; YP_010087646	61.2	8.3e-177
CsNV_06	8886	9323	CmNV_007	CmNV; UBZ25597	45.5	2.8e-39
CsNV_07	9348	10,595	P47	CmNV; UBZ25598	56.4	1.1e-174
CsNV_08	10,616	11,821	PIF-2	CmNV; UBZ25597	63.7	1.7e-179
CsNV_09	11,863	12,615	CmNV_010	CmNV; UBZ25600	41.3	5.8e-52
CsNV_10	12,659	13,924	CmNV_011	CmNV; UBZ25601	36.8	8.4e-96
CsNV_11	14,065	14,472	CmNV_012	CmNV; UBZ25602	34.2	6.8e-23
CsNV_12	14,430	15,806	FEN-1	CmNV; UBZ25603	51.8	1.8e-153
CsNV_13	15,698	16,186	KM727_gp14	HgNV; YP_010087654	38.6	6.9e-27
CsNV_14	16,183	17,070	31 K	CmNV; UBZ25605	42.2	4.0e-93
CsNV_15	17,188	20,271	LEF-8	CmNV; UBZ25606	62.3	0.0
CsNV_16	20,393	21,634	P51	CmNV; UBZ25607	49.6	8.5e-133
CsNV_17	21,745	25,563	CmNV_018	CmNV; UBZ25607	51.1	0.0
CsNV_18	25,695	26,390	–	–	–	–
CsNV_19	27,219	27,596	–	–	–	–
CsNV_20	28,929	30,842	ODV-E66	CmNV; UBZ25647	57.5	0.0
CsNV_21	31,054	32,823	ODV-E66	MrNV; UHB41759	63.3	0.0
CsNV_22	33,065	34,336	–	–	–	–
CsNV_23	34,438	35,490	–	–	–	–
CsNV_24	35,728	37,254	–	–	–	–
CsNV_25	37,324	38,451	–	–	–	–
CsNV_26	38,689	39,255	–	–	–	–
CsNV_27	39,414	40,070	–	–	–	–
CsNV_28	40,201	41,370	Serine-threonine protein kinase	CmNV; UBZ25611	32.0	2.7e-46
CsNV_29	41,743	43,089	–	–	–	–
CsNV_30	43,207	44,016	–	–	–	–
CsNV_31	44,109	45,155	–	–	–	–
CsNV_32	45,620	46,477	Dihydroxy-acid dehydratase	HgNV; YP_010087666	32.5	6.1e-15
CsNV_33	46,771	47,685	TK2	CmNV; UBZ25616	47.4	1.4e-98
CsNV_34	47,723	49,300	PIF-1	CmNV; UBZ25617	58.7	0.0
CsNV_35	49,313	49,993	CmNV_028	CmNV; UBZ25618	28.6	9.0e-11
CsNV_36	49,983	52,010	CmNV_029	CmNV; UBZ25619	51.9	0.0
CsNV_37	52,028	52,795	KM727_gp31	HgNV; YP_010087671	40.8	7.3e-61
CsNV_38	52,893	53,582	KM727_gp32	HgNV; YP_010087672	39.2	1.3e-49
CsNV_39	53,579	54,307	CmNV_032	CmNV; UBZ25622	54.1	3.1e-93
CsNV_40	54,376	55,500	TK3	CmNV; UBZ25623	46.7	2.9e-108
CsNV_41	55,553	56,473	CmNV_034	CmNV; UBZ25624	50.0	3.9e-103
CsNV_42	56,479	58,251	CmNV_035	CmNV; UBZ25625	51.8	0.0
CsNV_43	58,201	58,506	CmNV_036	CmNV; UBZ25626	41.6	1.4e-28
CsNV_44	58,711	59,187	LEF-5	CmNV; UBZ25628	48.2	6.8e-29
CsNV_45	59,339	59,564	Putative p6.9	–	–	–
CsNV_46	59,805	60,458	CmNV_039	CmNV; UBZ25630	43.7	1.7e-49
CsNV_47	60,449	61,387	CmNV_040	CmNV; UBZ25631	67.3	2.0e-156
CsNV_48	61,409	62,467	–	–	–	–
CsNV_49	62,665	63,519	VLF-1	CmNV; UBZ25632	60.5	5.0e-107
CsNV_50	63,510	64,121	–	–	–	–
CsNV_51	64,379	65,527	–	–	–	–
CsNV_52	65,611	65,943	–	–	–	–
CsNV_53	66,186	67,904	LEF-9	CmNV; UBZ25637	68.3	0.0
CsNV_54	67,942	68,769	38 K	CmNV; UBZ25638	56.0	1.5e-100
CsNV_55	69,031	69,747	CmNV_049	CmNV; UBZ25640	56.5	4.3e-94
CsNV_56	69,731	70,207	CmNV_050	CmNV; UBZ25641	49.0	4.5e-25
CsNV_57	70,204	70,521	CmNV_051	CmNV; UBZ25642	42.9	3.6e-20
CsNV_58	71,061	72,560	TK1	CmNV; UBZ25644	54.7	2.7e-157
CsNV_59	72,632	74,443	CmNV_054	CmNV; UBZ25645	41.1	8.3e-94
CsNV_60	75,383	76,234	CmNV_058	CmNV; UBZ25649	32.4	7.8e-22
CsNV_61	76,310	76,867	KM727_gp58	HgNV; YP_010087698	39.5	5.1e-35
CsNV_62	76,882	77,901	CmNV_060	CmNV; UBZ25651	54.7	4.3e-115
CsNV_63	77,883	78,482	CmNV_061	CmNV; UBZ25652	51.8	1.4e-69
CsNV_64	78,562	79,905	CmNV_062	CmNV; UBZ25653	40.6	7.2e-105
CsNV_65	80,001	82,064	PIF-0	CmNV; UBZ25654	64.6	0.0
CsNV_66	82,111	82,905	–	–	–	–
CsNV_67	82,974	83,567	CmNV_065	CmNV; UBZ25656	37.1	4.1e-25
CsNV_68	83,607	85,304	Helicase 2	CmNV; UBZ25657	59.0	0.0
CsNV_69	85,331	86,311	–	–	–	–
CsNV_70	86,301	86,939	CmNV_068	CmNV; UBZ25659	53.8	8.5e-67
CsNV_71	86,929	88,899	Helicase 2	CmNV; UBZ25660	54.2	0.0
CsNV_72	88,886	94,015	CmNV_070	CmNV; UBZ25661	35.6	5.8e-78
CsNV_73	94,055	94,975	CmNV_071	CmNV; UBZ25662	43.7	3.6e-79
CsNV_74	94,966	95,604	CmNV_072	CmNV; UBZ25663	38.2	1.1e-30

(*continued on next page*)

Table 1 (*continued*)

Gene	Start	Stop	Protein hit	Similar species and accession	Similarity (%)	e-value
CsNV_75	95,616	97,175	CmNV_073	CmNV; UBZ25664	33.8	1.2e-73
CsNV_76	97,169	97,573	KM727_gp75	HgNV; YP_010087715	44.4	9.5e-36
CsNV_77	97,563	98,051	Ac81-like	CmNV; UBZ25666	75.2	2.0e-82
CsNV_78	98,038	99,954	CmNV_076	CmNV; UBZ25667	42.7	1.6e-176
CsNV_79	99,917	100,348	PIF-6	CmNV; UBZ25668	53.1	7.1e-55
CsNV_80	100,374	101,636	CmNV_078	CmNV; UBZ25669	50.0	9.0e-126
CsNV_81	101,720	102,442	VLF-1	CmNV; UBZ25670	53.9	7.2e-85
CsNV_82	102,439	103,740	LEF-4	CmNV; UBZ25671	54.0	7.2e-162
CsNV_83	103,842	104,231	CmNV_081	CmNV; UBZ25672	39.8	1.3e-21
CsNV_84	104,277	104,894	PIF-3	CmNV; UBZ25673	63.6	3.4e-91
CsNV_85	104,887	108,735	Helicase	CmNV; UBZ25674	58.5	0.0
CsNV_86	108,734	109,474	PIF-4	CmNV; UBZ25675	58.8	1.1e-93
CsNV_87	109,431	110,183	CmNV_085	CmNV; UBZ25676	42.4	5.6e-58
CsNV_88	110,182	111,018	CmNV_086	CmNV; UBZ25677	67.7	7.5e-125
CsNV_89	111,038	112,354	KM727_gp88	HgNV; YP_010087728	43.8	4.0e-51
CsNV_90	112,425	112,724	11 K	MrNV; UHB41834	71.3	1.0e-48
CsNV_91	112,847	113,182	CmNV_089	CmNV; UBZ25680	45.8	7.8e-28
CsNV_92	113,160	113,942	CmNV_090	CmNV; UBZ25681	60.1	6.5e-115
CsNV_93	113,964	115,037	Apoptosis inhibitor	CmNV; UBZ25682	29.1	3.4e-47
CsNV_94	115,579	118,104	–	–	–	–
CsNV_95	118,216	119,232	Baculoviral IAP repeat-containing protein	HgNV; YP_010087733	32.5	2.2e-31
CsNV_96	119,454	120,827	–	–	–	–
CsNV_97	120,878	121,600	CmNV_097	CmNV; UBZ25688	60.1	1.9e-104
CsNV_98	121,793	122,404	KM727_gp97	HgNV; YP_010087737	50.7	3.6e-70

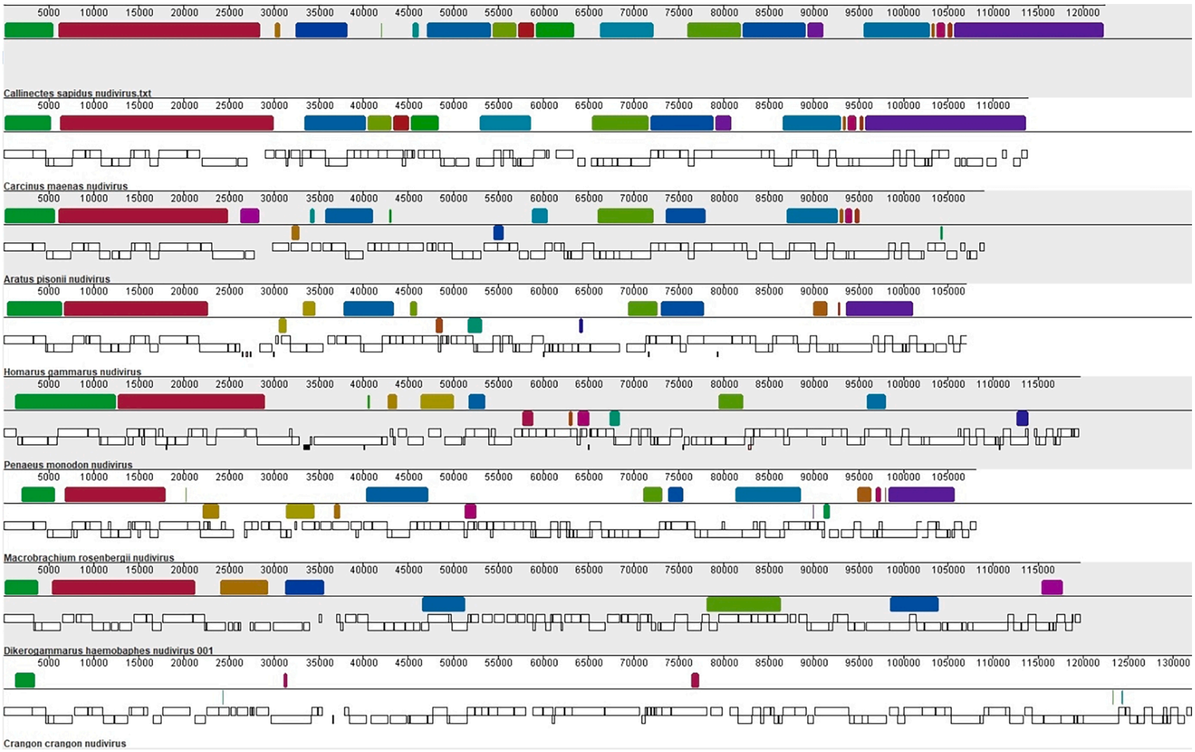

Fig. 4. The genomes of eight available crustacean-infecting nudiviruses are compared using MAUVE. The coloured blocks signify similar regions of nucleotide sequence across the genomes used in the analysis; regions in a 5′-3′ orientation are displayed above a representative line and sequences in the opposite orientation are displayed below. The open reading frames annotated onto the genomes are presented beneath the coloured blocks, where 5′-3′ coding genes are displayed above and 3′-5′ coding genes below. Ticks every 5000 nt are provided for scale.

nudiviruses (Fig. 2). Further, comparisons between the original Baculovirus-A measurements and CsNV indicate that we likely observed the same virus from Florida blue crab as was initially seen in Chesapeake blue crabs ∼ 40 years ago (Johnson and Lightner, 1988).

Our phylogenetic analysis shows that CsNV clades with other marine decapod nudiviruses, particularly those from the *Gammanudivirus* genus. The nudivirus of C. sapidus shares a well-supported most common ancestor with CmNV and the similarity of their core proteins corresponds to ∼ 60 %.“ (Fig. 5). Gammanudiviruses from diverse host groups appear to branch in different places (e.g. the two crab nudiviruses from *C. maenas* and *A. pisonii* do not branch together, but are instead separate on the tree), suggesting that the evolution of the group may have involved multiple host switching events and did not rely on the evolutionary radiation of host taxa. To date, little work has been done to determine the host range of different *Gammanudivirus* or *Epsilonnudivirus* species; however, such work could help to uncover the

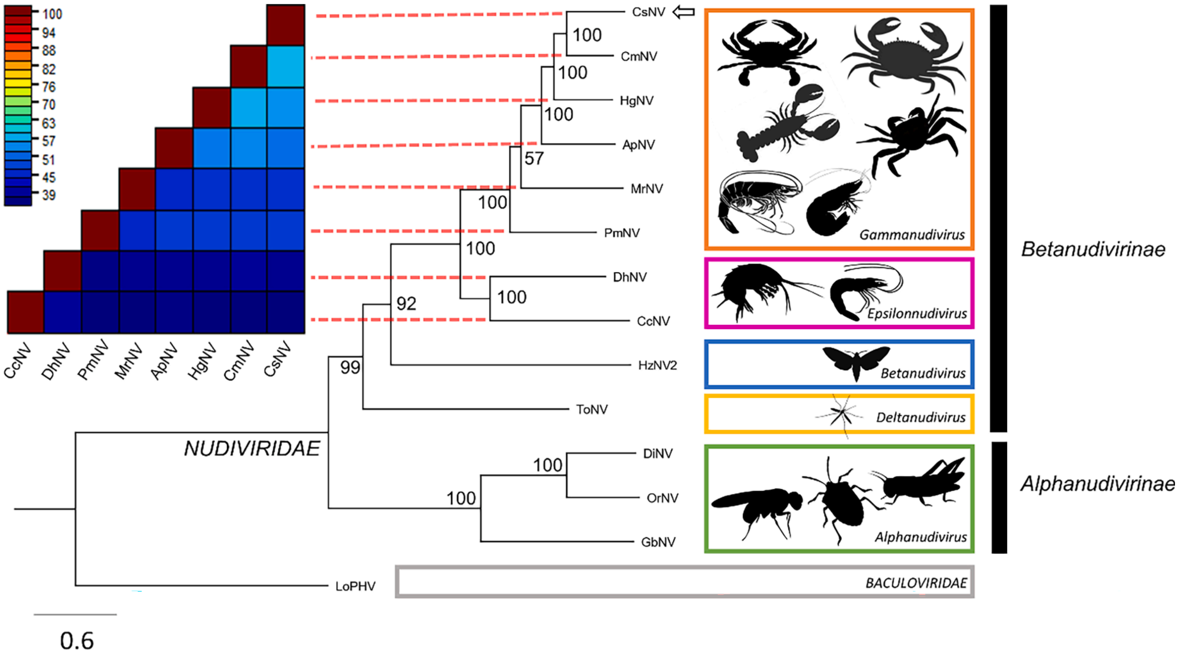

Fig. 5. A maximum-likelihood concatenated protein phylogeny (*pif-1, pif-2, pif-3, pif-4, pif-5, pif-6, 38 k, ac81, DNApol, helicase, lef-4, lef-5, lef-8, lef-9, p74, vp39* and *vp91*) accompanied by a sequence demarcation plot of the average protein sequence similarity across the same genes for the *Gammanudivirus* and *Epsilonnudivirus* members. Five genera are represented on the tree: *Alphanudivirus*, *Betanudivirus*, *Gammanudivirus*, *Deltanudivirus,* and *Epsilonnudivirus* from two sub-families (*Alphanudivirinae* and *Betanudivirinae*) within the *Nudiviridae*. A baculovirus is used as an outgroup to root the tree.

capacity for nudiviruses to infect distantly or closely related hosts and further determine levels of risk for other fished or economically important species.

The original finding of CsNV (Baculovirus-A) from the Chesapeake and Chincoteague Bays, Maryland (USA), reported a prevalence virus prevalence of 4–20 % per study site, but an overall prevalence of 6.0 % (total study population, 1,500 crabs) (Johnson and Lightner, 1988). In our study, we find this virus at 2.2 % across marine and freshwater Floridian waters from a sample size of 409 individuals. The virus was identified in hosts collected from both marine and freshwater environments across Florida (Fig. 1). The statistical prevalence of crustacean-infecting nudiviruses differs among the species: DhNV (UK, 77.7 %); CcNV (Belgium, 90.0 %); HgNV (UK, 12.7 %); ApNV (Florida, 6.5 %); CmNV (Canada, 17.4 %) (Bojko et al., 2019; Holt et al. 2019; Bateman et al. 2021; Bojko et al. 2022). Interestingly, the two putative epsilonnudiviruses appear to persist in their hosts at a higher prevalence than the gammanudiviruses, which appear to present in around 2–20 % of hosts.

To date, the *Nudiviridae* have been isolated from hosts in terrestrial, freshwater, marine, and a combination of environments. For instance, *C. maenas* and *A. pisonii* (sub-tropical) found both a niche in semi-marine and semi-terrestrial habitats (temperate), while *C. sapidus* can be found in semi-marine and semi-freshwater surroundings. Other members solely inhabit the marine or freshwater biosphere without venturing onto land (e.g. *H. gammarus*, *P. monodon*, and *M. rosenbergii*). With the growing diversity of nudiviruses, environmental and host range parameters may help to unravel the selective factors that have driven nudivirus evolution. It will be increasingly important to build aquacultural and other anthropogenic factors into studies to determine if these are now influencing the future evolution of nudiviruses.

4.2. Conclusions - crustacean pathology after 50 years

Approximately 50 years after the first discovery of a crustacean-infecting virus (Vago, 1966), we find ourselves in a new era where viral genomes can be sequenced and assembled within a few days, and researchers discover thousands of new viruses in one single study (Shi et al. 2016; Edgar et al. 2022). Baculovirus-A was first considered to be a member of the *Baculoviridae* or *Nimaviridae* (Bateman and Stentiford, 2017), we now provide strong evidence that this virus is a member of the *Nudiviridae*. The expansion of this viral family through the inclusion of crustacean-infecting nudiviruses has increased our perception of their importance and impact in wild and farmed systems, sparking a wealth of research into diagnostic development, systematics, and ecological research (Yang et al. 2014; Holt et al. 2019; Allain et al. 2020; Bateman et al. 2021; Bojko et al. 2022).

A diverse array of nudiviruses from crustacean hosts have been identified through genomic, transcriptomic, or pathological means, but we must reach a capacity where we begin to associate these viruses with their effects on farmed hosts and wild host ecology. Sequencing viruses from fishery and aquaculture species is vital to explore their capacity for virulence, and multiple fisheries species are known to harbour nudiviruses (previously bacilliform viruses), such as *C. pagurus*, that require genome sequencing. Sequencing nudivirus genomes from ecologically diverse hosts, such as the parasitic *P. pisum*, or the terrestrial crab *Aratus pisonii* (Bojko et al. 2022), may help to reveal how host life history shaped the evolution of these intriguing dsDNA viruses.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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