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Journal of Invertebrate Pathology 189 (2022) 107718

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

The naked truth: An updated review on nudiviruses and their relationship to bracoviruses and baculoviruses

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ARTICLE INFO

Keywords:
Nudiviruses
Baculoviruses
Bracoviruses
DNA viruses
Classification
Phylogeny
Pathology
Insects
Crustaceans

ABSTRACT

Nudiviruses (*Nudiviridae*) are double-stranded DNA viruses with enveloped and rod-shaped virions. Several insect orders (e.g., Diptera, Lepidoptera, Coleoptera, Orthoptera) and aquatic crustaceans are susceptible to nudivirus infections, which can result in varied degrees of disease in all developmental host stages. Their pathogenicity endangers insect rearing and crustacean aquacultures, but has also proven effective in biocontrol against *Oryctes rhinoceros* infestations.

This literature review aims to present all known nudivirus species and provide a comprehensive *Nudiviridae* phylogeny by including recently described nudiviral isolates, and discuss this phylogeny in comparison to current opinions and taxonomical propositions. Moreover, we aim to clarify biological, pathological and genomic differences or similarities between nudiviruses and related entomopathogenic viruses, including baculoviruses (*Baculoviridae*) and bracoviruses (*Polydnaviridae*).

A phylogenetic analysis using 17 concatenated nudivirus core genes resulted in the expected structure with the genera *Alphanudivirus* and *Betanudivirus*, as well as the most recently recognized genera *Gammanudivirus* and *Deltanudivirus*. The hymenopteran Osmia cornuta nudivirus (OcNV) groups closest with the hymenopteran Fopius arisanus endogenous nudivirus (FaENV) and does not share a most common ancestor with the hymenopteran bracoviruses. Except for one node, all clades are highly supported.

The proposition of a recent study to assign subgroups to the alphanudiviruses might be legitimate, but more hymenopteran and orthopteran nudiviruses, especially in bees and cricket, need to be identified to resolve this proposal. In addition, freshwater and marine nudiviruses might form taxonomic subgroups among gammanudiviruses as well, but more aquatic nudiviruses need to be identified and sequenced for better resolution. Furthermore, the search for nudiviruses in insects with (semi)aquatic life stages may aid in finding the missing link that led to the manifestation of aquatic nudiviruses.

1. Nudiviruses and their societal and scientific relevance

Members of the virus family *Nudiviridae* can infect hosts from various insect orders (e.g., Diptera, Lepidoptera, Coleoptera, Orthoptera) and aquatic arthropods (e.g., lobster, shrimp, crabs). The virulence of nudiviruses poses a potential threat to a range of arthropod farms and aquacultures. For instance, PmNV causes significant mortalities in tiger shrimp (*Penaeus monodon*), endangering shrimp aquaculture (Yang et al., 2014). GbNV causes high mortalities in four field cricket species of the genera *Gryllus* and *Teleogryllus*, and poses a threat to cricket rearing (Eilenberg et al., 2015; Huger, 1985).

On the other hand, the coleopteran nudivirus, OrNV, has been successfully used for biocontrol in pest-infested agricultural regions. In Samoa and other southwest Pacific islands, OrNV was introduced to overcome the devastation produced by the coconut palm beetle (*Oryctes rhinoceros*), a key pest of young palms and coconuts (Bedford, 2014). The use of OrNV regulated and lowered the population of adult *O. rhinoceros* and their larvae (Huger, 1966; Zelazny, 1972). However, as a variety of beetle species finds use in traditional Asian medicine (Deyrup et al., 2021), the adverse effect of OrNV on this branch has to be considered when releasing this virus as a biocontrol agent. Japanese rhinoceros beetles (*Allomyrina dichotoma*) that are commercially bred in the Republic of Korea, have been shown to suffer from OrNV infection as well as from a distinct new nudivirus species, namely AdNV. Despite their use for biocontrol, these viruses pose a threat to Korean beetle industry (Lee

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https://doi.org/10.1016/j.jip.2022.107718
Received 22 October 2021; Received in revised form 14 January 2022; Accepted 17 January 2022
Available online 22 January 2022

Nomenclature

AaBV	Astacus astacus bacilliform virus
AcMNPV	Autographa californica multiple nucleopolyhedrovirus
AdNV	Allomyrina dichotoma nudivirus
AgENV	Aphis glycines endogenous nudivirus
ApBV	Austropotomobius pallipes bacilliform virus
BMN	Baculoviral mid-gut gland necrosis
CcBV	Cotesia congregata bracovirus
CcNV	Crangon crangon nudivirus
CdBV	Cherax destructor bacilliform virus
CiBV	Chelonus inanitus bracovirus
CmNV	Carcinus maenas bacilliform nudivirus
CpBV	Cancer pagurus bacilliform virus
CqBV	Cherax quadricarinatus bacilliform virus
DhNV	Dikerogammarus haemobaphes nudivirus
DiNV	Drosophila innubila nudivirus
DuhNV	Diabrotica undecimpunctata howardi nudivirus
DvvNV	Diabrotica virgifera virgifera nudivirus
EbrENV	Eurytoma brunniventris endogenous nudivirus
ENV	Esparto virus
FaENV	Fopius arisanus endogenous nudivirus
GbNV	Gryllus bimaculatus nudivirus
GpSGHV	Glossina pallidipes salivary gland hypertrophy virus
GrBV	Gammarus roeselii bacilliform virus
HgNV	Homarus gammarus nudivirus
HzNV-1	Heliothis zea nudivirus-1
HzNV-2	Helicoverpa zea nudivirus 2
KV	Kallithea virus
MdBV	Microplitis demolitor bracovirus
MdSGHV	Musca domestica salivary gland hypertrophy virus
MNV	Mauternbach virus
MrNV	Macrobrachium rosenbergii nudivirus
MsENV	Melanaphis sacchari endogenous nudivirus
NlENV	Nilparvata lugens endogenous nudivirus
OcNV	Osmia cornuta nudivirus
OrNV	Oryctes rhinoceros nudivirus
PlBV	Pacifastacus leniusculus bacilliform virus
PmBV	Pandalus montagui bacilliform virus
PmNV	Penaeus monodon nudivirus
SsBV	Scylla serrata bacilliform virus
TNV	Tomelloso virus
TnBV	Toxoneuron nigriceps bracovirus
ToNV	Tipula oleracea nudivirus
TpNV	Tipula paludosa nudivirus
VcENV	Venturia canescens endogenous nudivirus

et al., 2015; Kwak et al., 2019).

The abovementioned examples serve to highlight the societal relevance of nudiviruses and emphasise the scientific importance of gaining better insight into the biology and pathogenicity of this diverse virus family. Research on nudiviruses will help to assess their threat for arthropod production systems and to evaluate their potential as novel viral biopesticides to reduce the exposure of harmful chemicals and benefit the environment and agriculture. Additional data will also shed light on their mutual relationships and their evolutionary position in relation to other virus families.

2. The family *Nudiviridae*: General description, genomic structure and taxonomy

Before their official recognition as an own virus family (*Nudiviridae*), nudiviruses were classified as a subgroup within the family *Baculoviridae*. Baculoviruses are rod-shaped double-stranded DNA (dsDNA) viruses that infect lepidopteran, dipteran and hymenopteran species. Cells that are destined to baculovirus-induced cell lysis accumulate occlusion bodies (OBs) in their host's nuclei. OBs occlude the viral progeny responsible for primary infection, namely occlusion-derived virions (ODVs), into protective protein structures made from polyhedrin or granulin. The systemic infection of baculoviruses is coordinated by a different type of virions, called budded virus (BV) (van Oers, 2020; Ros, 2020). Contrary to the original assumption that nudiviruses are non-occluded baculoviruses (hence their designation from Latin "*nudus*" = naked, uncovered), occluded nudiviruses do exist, but were not classified as nudiviruses (OrNV, formerly Oryctes baculovirus) at the time (Huger and Krieg, 1991) or only discovered and classified later on (e.g., PmNV and ToNV) (Bézier et al., 2015; Yang et al., 2014). Today, members of the family *Nudiviridae* are assigned to the class *Naldaviricetes* together with three other families of nuclear arthropod large DNA viruses: *Baculoviridae*, *Hytrosaviridae* and *Nimaviridae*. However, nudiviruses share the same order, *Lefavirales*, only with baculoviruses and hytrosaviruses (Harrison et al., 2021a).

Nudiviral virions consist of single nucleocapsids surrounded by an envelope. They have an ellipsoidal or rod-shaped form of variable length and width (Supplementary file 3, Tables S1 & S2). The variety in length of rod-shaped virions correlates with a general mechanism that adapts the packaging and particle formation to match diverse viral genome sizes (Velamoor et al., 2020). The dsDNA genome of nudiviruses is a single, covalently closed circular molecule of about 96 to 232 kilobase pairs (kbp) encoding between 87 and 154 proteins depending on the virus. Open reading frame (ORF) content and order can vary greatly among different nudivirus species (Harrison et al., 2020).

Genome sequencing and phylogenetic analyses strengthened the revised view that nudiviruses are closely related to baculoviruses, but form a distinct clade of viruses (Harrison et al., 2020). The phylogenetic analyses were based on a set of genes that are present in all nudiviral genomes, named core genes. The nudiviral core genes are partly conserved in members of *Baculoviridae, Hytrosaviridae* and bracoviruses (*Polydnaviridae*). A total of 32 core genes are currently assigned to all sequenced nudivirus genomes (Harrison et al., 2020) and their partial conservation in baculoviruses (Table 1) and bracoviruses (Table 2) will be described in the course of this review. Phylogenies inferred from core genes of nudivirus species helped to assign clades within the family *Nudiviridae*. The first officially recognized *Nudiviridae* clades were the genera *Alphanudivirus* and *Betanudivirus* (Adams et al., 2014), and most recently the International Committee on Taxonomy of Viruses (ICTV) ratified the two additional genera: *Gammanudivirus* and *Deltanudivirus* (Harrison et al., 2021a). Members of *Nudiviridae* are now officially categorized into the four genera *Alphanudivirus*, *Betanudivirus*, *Gammanudivirus* and *Deltanudivirus* (Fig. 2). An additional genus *Epsilonnudivirus* was recently proposed based on the description of the demon shrimp infecting *Dikerogammarus haemobaphes* nudivirus (DhNV). It was shown that DhNV is most closely related to the gammanudiviruses with a low level of protein similarity at most loci (<50%) (Allain et al., 2020).

For the purpose of this review, a phylogenetic tree was inferred using the concatenated amino acid sequences of up to 17 aligned core gene products from 20 nudivirus species (Fig. 2). Nudiviruses with too few available gene sequences (e.g., AdNV, MrNV and Charybdis crab nudivirus) were excluded from the analyses. Although the four nudiviruses ENV, KV, MNV and TNV all share the same host (*D. melanogaster*), they are classified as individual species. These viruses have been named after their different places of origin (ENV = USA: Esparto, CA; KV = Ukraine: Kharkiv; MNV = Austria: Mauternbach, TNV = Spain: Tomelloso).

As an aid for experts, a collection of well-studied nudivirus species with extra information (classification, former names, host, virion

Table 1
The 32 core genes currently assigned to all nudivirus species distributed over functional groups. The collection of the core genes is based on coinciding results of different publications (Burke, 2019; Cheng et al., 2020; Zhang et al., 2020). The genes *tk1 - 3* and *GbNV gp58-like* are absent in CcNV (Bateman et al., 2021) and written in brackets to indicate their putative removal from the list of nudiviral core genes.*

Transcription	Infectivity	Packaging, assembly, morphogenesis	DNA replication, repair, recombination	Nucleotide metabolism	Unknown function
lef-4	***pif-0/ p74***	***vp91/ pif-8***	***dnapol***	(*tk1*)	*GbNV gp19-like*
lef-5	***pif-1***	***38K***	***helicase***	(*tk2*)	*GbNV gp51-like*
lef-8	***pif-2***	***p33/ ac92***	*helicase-2*	(*tk3*)	(*GbNV gp58-like*)
lef-9	***pif-3***	***p6.9***	*integrase*		*GbNV gp67-like*
p47	***pif-4/ 19kDa***	***vlf-1***	*fen-1*		*11K-like*
	pif-5/ odv-e56	***vp39***			
	pif-6/ac68	***ac81***			

* Abbreviations: *lef*, late expression factor; *pif, per os* infectivity factor; *vlf,* very late expression factor; *dnapol*, DNA polymerase; *fen*, FLAP endonuclease; *tk*, thymidine kinase. Genes in bold indicate core genes shared with the *Baculoviridae*. Slashes signify alternative gene names.

morphology, ORFs, genome size) is presented in Supplementary Table S1. Putative nudiviral agents can be found in the Supplementary Table S2.

3. Nudiviral biology and infection cycle compared to closely related virus clades

Nudiviruses and baculoviruses share two-thirds of their core genes (Table 1), but their biology and infection cycle differ in many aspects (Wang and Jehle, 2009; Zhao et al., 2019). While baculoviruses are able to efficiently infect all tissues of their host, nudiviruses are usually associated with cell type-specific pathogenesis (Fig. 3). The localized pathology of nudiviruses is likely related to their inability to form budded virions specialized for carrying out secondary infection, as is known from baculoviruses (Burand, 1998).

Upon budding out of the cell, BVs lose the envelope derived from the nuclear membrane to gain a new envelope with the incorporated GP64 or F-protein. Those fusion proteins facilitate the cell entry of BVs via endocytosis during systemic infection (Long et al., 2006). In contrast to BVs, nudiviral virions miss homologs of the envelope fusion proteins GP64 or F-protein (Wang et al., 2007). The virion structure of nudiviruses (Fig. 1C) possibly resembles those of baculovirus ODVs with a single nucleocapsid and infections spread either by living cell egress or after cell lysis (Burand, 1998).

ODV midgut epithelial cell infection or permeation of the peritrophic membrane, or both, are facilitated by a complex of *per os* infectivity (PIF) proteins and other envelope proteins (Wang et al., 2019; Boogaard et al., 2018), many of which are conserved between baculoviruses and nudiviruses (Table 1). Whether nudivirus virions enter host cells via receptor-mediated fusion, as it has been described for baculoviral ODVs (Horton and Burand, 1993), is yet unclear. Interestingly, an early study by Crawford and Sheehan (1985) showed that single OrNV virions enter the cells via pinocytosis. Pinocytosis is an endocytosis-related cell entry mechanism (Holter, 1959). Endocytosis-related cell entry is also known from baculoviral BVs during systemic infection, but instead of pinocytosis BVs enter the host cell via clathrin-mediated endocytosis (Long et al., 2006).

Cell egression mechanisms can differ among nudivirus species. For instance, single OrNV virions can egress from an infected cell, but also single or multiple OrNV virions encapsulated in one or multiple membrane vesicles were observed (Velamoor et al., 2020). High quantities of virion-filled vesicles have also been observed in the “waxy plug” of *Helicoverpa zea* during HzNV-2 infection, which is the main substance to spread HzNV-2 infections from one individual to another during mating (Hamm et al., 1996).

A general infection or life cycle model for all nudiviruses has so far not been exemplified, but Velamoor et al. (2020) used an electron microscopy approach to describe a unique mechanism of virion assembly and egress during OrNV infection. Although the extrapolation of this model for other members of the *Nudiviridae* should be interpreted with care, these findings highlight valuable directions for future research on other nudivirus species. The addition of genomic and proteomic data is required to extend our knowledge of the exact mechanisms of viral entry, replication, assembly and egress used by nudiviruses.

4. Nudiviral host genome integration

Nudiviruses have the ability to durably integrate into their host’s genome. For instance, genome integration was observed for HzNV-1 under laboratory conditions as part of latent infection (Lin et al., 1999) and a variety of endogenous nudiviral sequences (NlENV, VcENV, AgENV, MsENV, FaENV, EbrENV) are permanently integrated into insect genomes. Two of those (VcENV and FaENV) can produce virus-like particles (VLPs), while the others (NlENV, AgENV, MsENV and EbrENV) seem to be non-functional or at least unable to produce particles (Burke et al., 2018; Cheng et al., 2020; Cheng et al., 2014; Leobold et al., 2018; Liu et al., 2020; Zhang et al., 2020). Notwithstanding the distinction between functional and non-functional endogenous nudiviral agents, it suggests that some nudiviruses may have integration mechanisms or life cycle-related features that facilitated those endogenization events. In addition, it was shown that an ancient nudiviral genome integration in the genetic material of parasitoid wasps gave rise to the endogenous virus clade of bracoviruses of the family *Polydnaviridae*. This family consists of the two genera, *Bracovirus* and *Ichnovirus*. In both genera, exogenous viruses were domesticated by the parasitoid wasps for the production of viral particles. Those are injected into parasitized caterpillars along with wasp eggs and are necessary for successful host parasitism. However, the two genera originated from independent viral integration events that underwent convergent evolution after endogenization (Pichon et al., 2015; Drezen et al., 2017). It is still unknown what ancient virus laid the foundation for ichnoviruses in ichneumonid wasps (Volkoff and Cusson, 2020) since conserved ichnovirus genes involved in particle production do not share similarities with currently known virus genes. On the other hand, it was shown that the domestication of an ancestral nudivirus with close relation to the currently known betanudiviruses led to the clade of bracoviruses in braconid wasps (Bézier et al., 2009a; Bézier et al., 2013; Burke et al., 2013). The endogenization of alphanudiviruses in the genomes of braconid (Burke et al., 2018) and ichneumonid wasps (Pichon et al., 2015) resulted in the manifestation of other hymenopteran endogenous nudiviral agents (FaENV and VcENV). In contrast to exogenous nudiviruses, genes required for DNA replication or particle production are not packaged into bracovirus particles (Dupuy et al., 2012) and therefore the production of virions in the parasitized larvae is not provided (Belle et al., 2002; Bézier et al., 2009a; Bézier et al., 2009b). Thus, members of *Polydnaviridae* have been associated with the term “viriforms” to

Table 2

Distribution of nudivirus core genes in bracovirus species, hymenopteran endogenous nudiviral agents and EbrENV. Genes present in the virus genome are indicated with filled circles "●". The presence of pseudogenes is indicated with circles in a square "◙". Open circles "○" indicate no detection of the core genes. No data on the presence or absence of genes, or pseudogenes, are indicated with empty areas. Core gene functions are labelled on the left and slashes signify alternative gene names. The genes *tk1 - 3* are written in brackets to indicate their putative removal from the nudiviral core genes. The table is based on the findings from (Zhang et al., 2020) and was complemented with studies from (Gauthier et al., 2021; Burke et al., 2018; Pichon et al., 2015; Leobold et al., 2018).

	Gene	Bracoviruses			Endogenous nudiviruses		EbrENV
		CcBV	CiBV	MdBV	VcENV	FaENV	EbrENV-β
Transcription	*lef-4*	●	●	●	●	●	●
	lef-5	●		●	●	●	
	lef-8	●	●	●	●	●	
	lef-9	●		●	●	●	●
	p47	●		●	●	●	●
Infectivity	*pif-0/p74*	●	●	●	●	●	
	pif-1	●	●	●	●	●	◙
	pif-2	●	●	●	●	●	●
	pif-3	●		●	●	●	◙
	pif-4/19kDa	●	●	●	●	●	◙
	pif-5/odv-e56	●	●	●	●	○	◙
	pif-6/ac68	●		●	●	●	
Packaging, assembly, morphogenesis	*vp91/pif-8*	●	●	●	●	●	◙
	38K	●	●	●	◙	●	●
	p33/ac92	●		●	●	●	
	p6.9	●			◙		
	vlf-1	●	●	●	◙	○	
	vp39	●	●	●	◙	●	
	ac81	○		○	●	●	●
DNA replication, repair, recombination	*dnapol*	○		○	◙		
	fen-1	●		●	◙		●
	helicase	●		●	●	●	●
	helicase-2	○		○	○		●
	integrase	●	●	●	◙		
Nucleotide metabolism	(*tk1*)						
	(*tk2*)						
	(*tk3*)						
Unknown function	*GbNV gp19-like*	●		●	●	●	
	GbNV gp51-like	○		○	●	●	
	(*GbNV gp58-like*)	○		○	●	●	
	GbNV gp67-like	●			●	●	
	11K-like	●	●	●	●	○	

distinguish them from true viruses that have replication competent virions (Kuhn et al., 2020). However, viruses are specified by the ICTV as a group of mobile genetic elements (MGEs) that packages their nucleic acid core in virions assembled from at least one virus-encoded component, or MGEs that clearly descend from a virus ancestor (Kuhn et al., 2020). At least for bracoviruses that originate from nudiviruses, the second definition applies. Recent literature suggests to classify braco- and ichnoviruses as Domesticated Endogenous Viruses (DEVs) (Drezen et al., 2021) to emphasise that they retained many features of their virus ancestors.

In hymenopteran ovary cells where bracovirus DNA is amplified, a viral factory is produced to assemble particles in a manner resembling nudivirus replication. Late genes coding for particle components are under the transcriptional control of the nudiviral RNA polymerase (Burke et al., 2013) as during a baculovirus infection. Once injected into the host, bracovirus particles enter its cells via PIF-mediated entry and

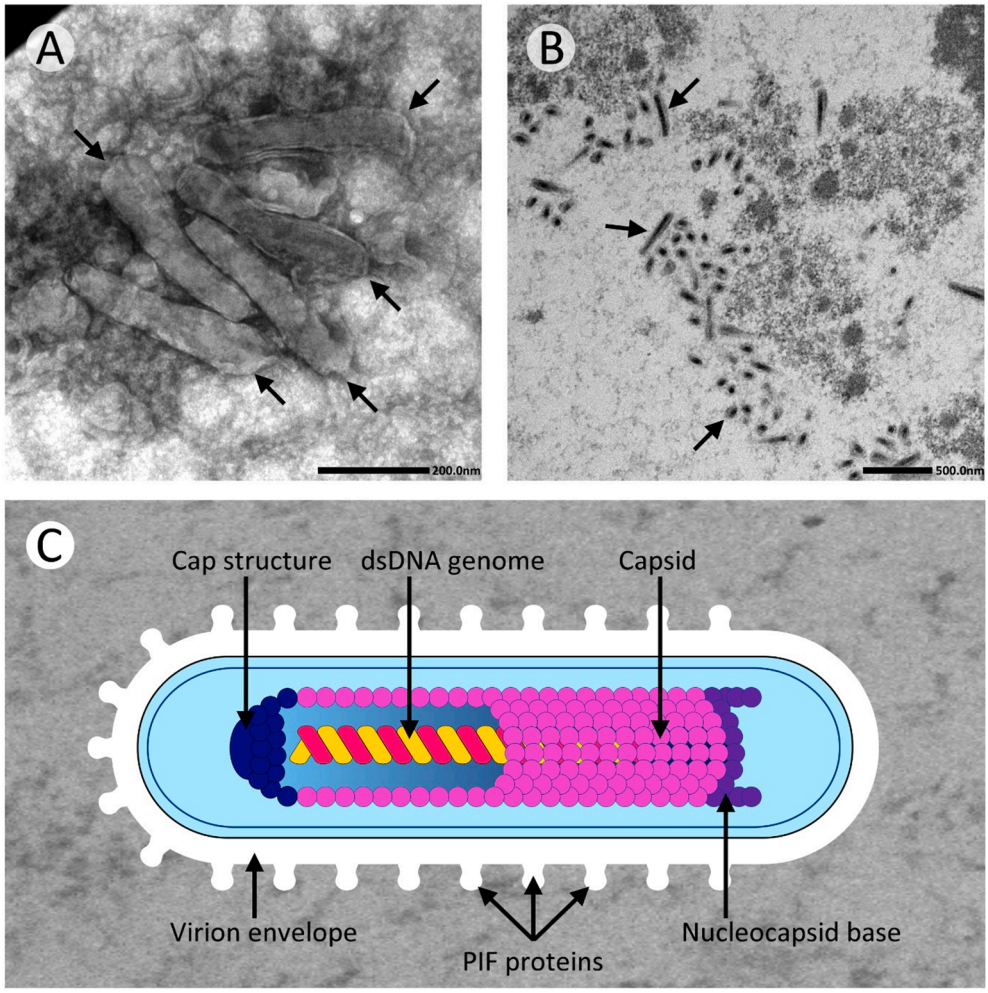

Fig. 1. Virion structures of the betanudivirus HzNV-1. (A) Electron microscopy (EM) image of rod-shaped HzNV-1 virions negatively stained with uranyl acetate from supernatant of infected HzAM1 cells (72 h post infection, hpi). Bar, 200 nm, **(B)** EM image of an ultrathin section made from HzNV-1 infected HzAM1 cells (60 hpi) using standard methods. Bar, 500 nm. **(C)** Exemplary illustration of nudiviral virion structure. EM image (B) courtesy Jan van Lent, Wageningen Electron Microscopy Centre (WEMC).

express virulence factors to facilitate the parasitism in the lepidopteran host and the development of the wasp eggs. Thus, most of the virus cycle is conserved, but distributed over two hosts allowing the wasp species to reproduce, and the viral entity to be maintained both functionally (i.e. producing infectious particles) and as integrated sequences in the wasp chromosomes (Gauthier et al., 2021). Although at first glance the biology of bracoviruses seems to resemble those of the endogenous nudiviral agents VcENV and FaENV, the latter are unable to package DNA into their particles. Instead, both hymenopteran ENVs wrap virulence proteins made by the wasp into viral envelopes containing PIF proteins (VLPs), similarly produced in a nudivirus-like viral factory in the nucleus (Fig. 3).

5. Genetic relationship between *Nudiviridae*, *Baculoviridae* and *Hytrosaviridae*

Currently, there are 32 core genes shared between all sequenced nudivirus species (Harrison et al., 2020), 21 are homologs to baculovirus (Table 1) and 16 to hytrosavirus genes. Due to the ongoing discovery and full sequencing of new nudivirus genomes, the number of nudivirus core genes might need adaptation in the future. For example, the recently sequenced CcNV lacks the three thymidine kinase (*tk*) genes, which would bring the total number of core genes back to 29 (Bateman et al., 2021).

In addition to the 32 core genes, accessory genes, such as DNA *ligase*, the ribonucleotide reductases *rr1* and *rr2*, *GbNV gp74, odv-e66* and inhibitor of apoptosis protein (*iap-3*), are commonly found in nudiviruses or only present in some species (Wang and Jehle, 2009; Bézier et al., 2015; Yang et al., 2014). The entirety of the nudiviral core genes and accessory genes make up the nudiviral pangenome.

Partial conservation of genes between nudiviruses, baculoviruses and hytrosaviruses indicates similarities in their biology. For example, all five of the genes in the transcriptional group (*lef-4, lef-5, lef-8, lef-9, p47*) are conserved between the nudiviruses (including the domesticated VcENV and FaENV), baculoviruses and bracoviruses, which suggests that these viruses share a similar mode of late gene transcription (Yang et al., 2014; Pichon et al., 2015; Wang et al., 2011; Burke, 2019). The same applies to the eight conserved PIF factors (*pif-0/p74*, *pif-1*, *pif-2*, *pif-3*, *pif-4/19kDa*, *pif-5/odv-e56*, *pif-6/ac68, pif-8/vp91*) in nudiviruses, baculoviruses, bracoviruses, VcENV, but not FaENV, which lacks the *pif-5* homolog. PIF proteins are all essential for the infectivity of baculoviral ODVs and believed to play a similar role for nudivirus and bracovirus virions as well as VcENV particles (Burke et al., 2013). Neither PIF-4 nor PIF-6 have been identified as components of FaENV particles yet (Burke et al., 2018). In baculoviral ODVs they facilitate specific binding or fusion to the midgut cells (P74, PIF-1 and PIF-2, PIF-3) (Haas-Stapleton et al., 2004; Ohkawa et al., 2005; Boogaard et al., 2020; Burke, 2019), stabilize the ODV-entry core-complex to resist proteolytic activity (PIF-4 and PIF-6) (Boogaard et al., 2017) or contribute to oral infection by a mechanism that has not been determined yet (PIF-5 and PIF-8) (Jiantao et al., 2016). Despite the absence of OBs in bracoviruses and most nudiviruses, the presence of conserved *per os* infectivity factors implies a similar mechanism of cell entry. Four homologous genes with relatively high identity and similarity (*pif-0/p74*, *pif-1*, *pif-2*, *pif-3 and pif-5/odv-e56*) are also present in members of the family *Hytrosaviridae,* infecting Dipteran species (Abd-Alla et al., 2008). The conservation of those

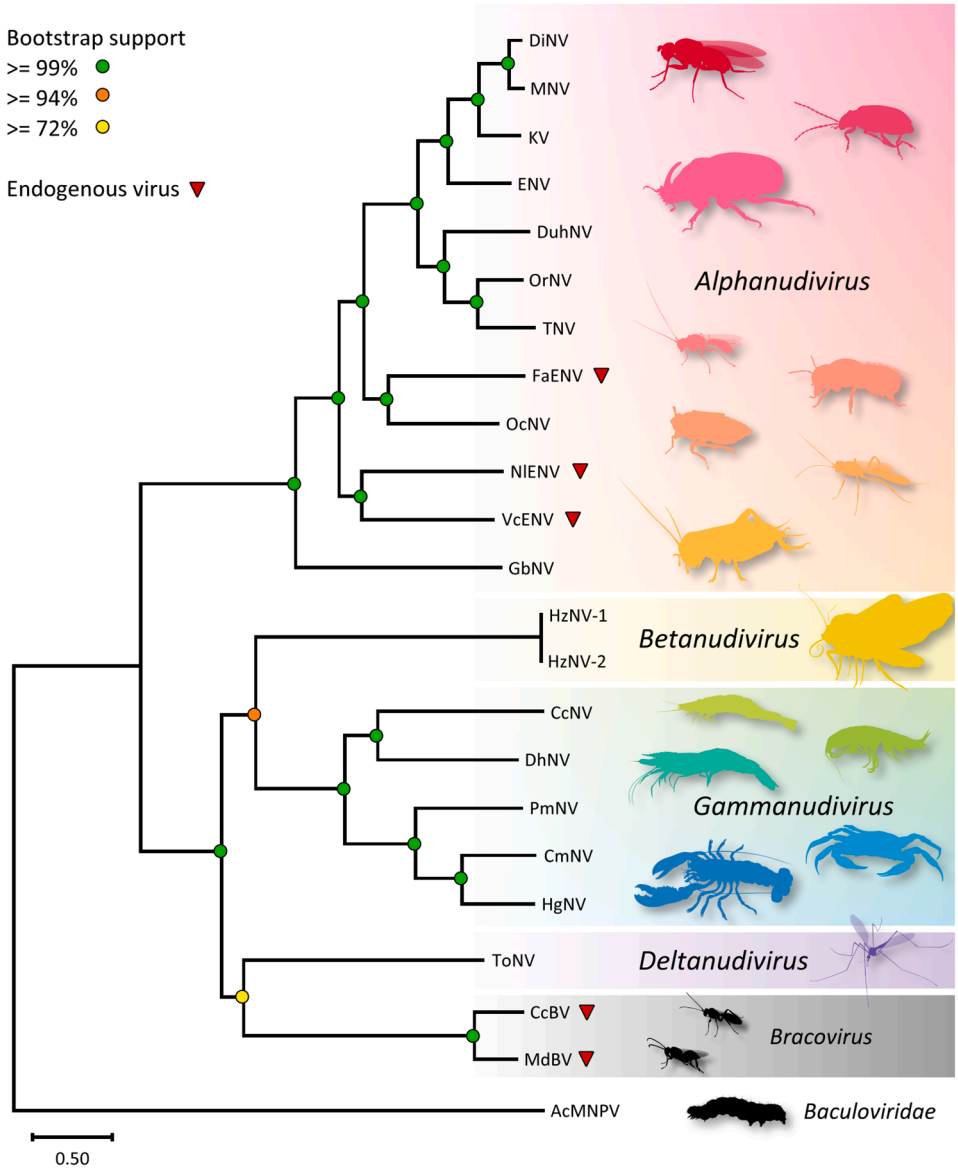

Fig. 2. Phylogenetic tree of 17 concatenated nudivirus core gene products from 17 exogenous nudiviruses, three endogenous nudiviruses, two bracoviruses and the baculovirus Autographa californica nucleopolyhedrovirus (AcMNPV) as outgroup. The tree was inferred in MEGAX using maximum likelihood with the WAG + G + F + I model from an alignment of 17 concatenated amino acid sequences. The final dataset had a total of 8545 positions. Except for one node, all clades have bootstrap values of at least 94% after 1000 replicates. Percentage values of bootstrap supports are indicated as colored circles. The evolutionary time (i.e., 0.50 substitutions *per* sequence site) between two nodes is represented by the branch length. The nudivirus species are grouped into the four officially recognized genera of *Nudiviridae*: *Alphanudivirus*, *Betanudivirus*, *Gammanudivirus* and *Deltanudivirus*. The two bracoviruses represent the *Bracovirus* genus of *Polydnaviridae*. The original phylogenetic tree and genes used from each virus can be found in Supplementary file 1 (Fig. S1) and Supplementary file 2.

proteins between baculoviruses, nudiviruses, and hytrosaviruses supports these virus families share a similar virus infectivity model (Yang et al., 2014).

The core genes *p33*, *vp91*, *vlf-1*, *38K, p6.9, vp39* and *ac81* are involved in viral particle formation. VP39 (formerly annotated as 31k-like protein in PmNV) (Holt et al., 2019) and 38K are major viral capsid proteins in baculoviruses, nudiviruses and in the bracovirus CiBV (Wang et al., 2007; Wetterwald et al., 2010; Wu et al., 2006). P33 is described as an essential component of BVs and ODVs of baculoviruses (Wu and Passarelli, 2010). VP91/PIF-8 (also) associates with the nucleocapsid and envelope, but is majorly involved in baculovirus primary infection (Jiantao et al., 2016). The condensation of the viral genome into the nucleocapsid and formation of infectious virions is facilitated by the *P*6.9 protein (Wang et al., 2010), while the processing of the viral DNA to nucleocapsid length is coordinated by the lambda family integrase VLF-1 (Vanarsdall et al., 2006).

The protein sequences of DNA polymerase and helicase are more diverse among the nudivirus species than other core genes, which is consistent with previous findings that described *helicase* as one of the most rapidly evolving genes across baculoviruses and nudiviruses (Hill and Unckless, 2017). In previous studies, genetic mapping demonstrated that the sequence variation in the helicase sequence supports variations in host range and host swapping in baculoviruses (Argaud et al., 1998; Croizier et al., 1994). Phylogenetic analyses have shown greater evolutionary distances for both *dnapol* and *helicase* among the nudiviruses than among baculovirus species (Yang et al., 2014). The *helicase-2* gene is scarcely present in baculovirus genomes (Rohrmann, 2019a), but is a core gene in nudiviruses and was found in both sequenced hytrosaviruses (Jehle et al., 2013).

Besides the genes that closely interact with the viral DNA, there is another particularity when it comes to viral non-coding DNA sequences involved in DNA replication. Homologous regions (*hrs*) in baculoviruses have a palindromic structure and function as origins of viral DNA replication (Pearson and Rohrmann, 1995) and support viral transcription (Guarino and Summers, 1986). Despite the absence of *hrs* in nudivirus genomes, most nudiviruses contain tandem direct repeat sequences (*drs*), which are proposed of being associated with viral replication (Holt et al., 2019). The presence of *drs* is a common feature that members of *Nudiviridae, Baculoviridae, Hytrosaviridae* and bracoviruses share (Garcia-Maruniak et al., 2008; Abd-Alla et al., 2008).

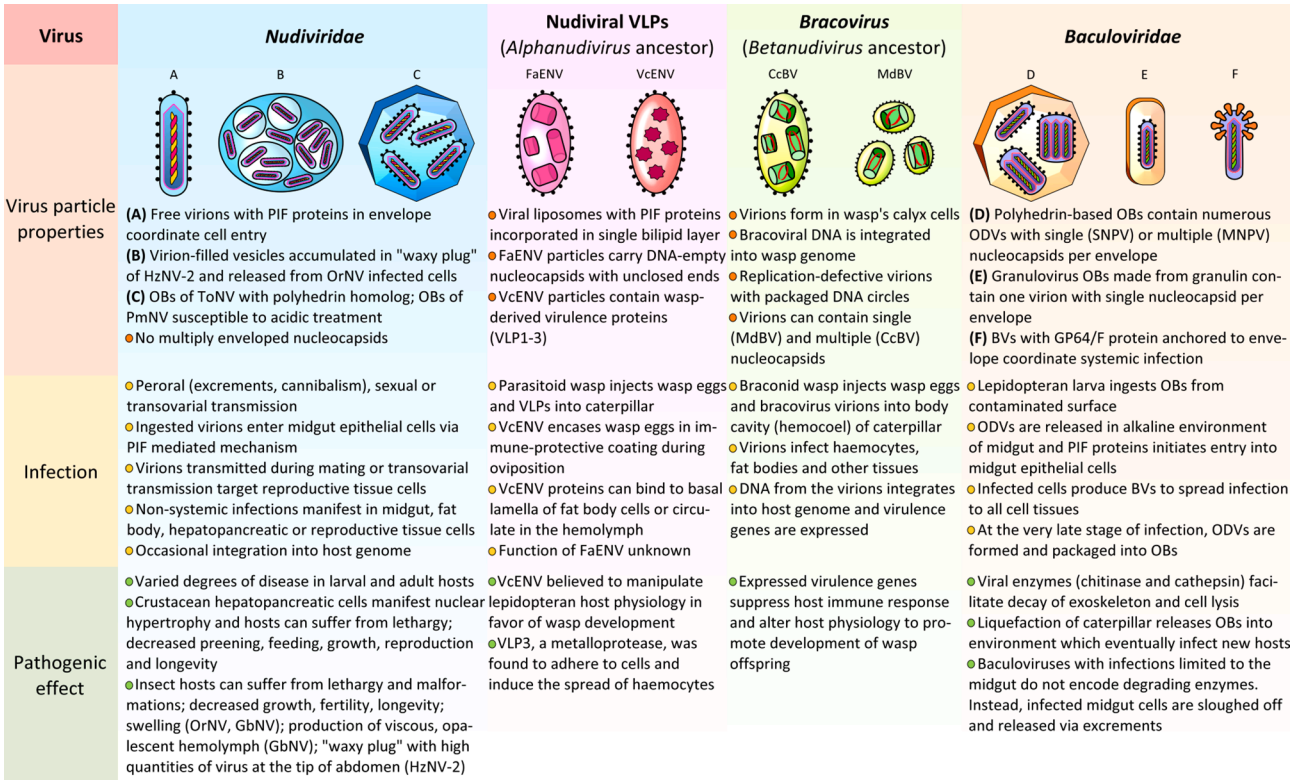

Fig. 3. Comparative overview of virus characteristics in the family *Nudiviridae*, nudiviral VLPs, bracoviruses and viruses in the family *Baculoviridae*. Abbreviations: SNPV, single nucleopolyhedrosis virus; MNPV, multiple nucleopolyhedrosis virus. References can be found in Supplementary file 1.

6. Genomic similarities between nudiviruses and bracoviruses

The bracoviral *drs*, called direct repeat junctions (DRJs) or wasp integration motifs (WIMs), are localized at the ends of the proviral segments in the wasp genome. Proviral segments make up the part of the bracoviral genome that is packaged into bracovirus virions. The proviral segments may be amplified separately or together (when contiguous in the wasp genome) within replication units (RUs terminally flanked by replication unit motifs (RUMs)) (Louis et al., 2013). The proviral segments are composed of sequences homologous to wasp genes (Gundersen-Rindal et al., 2013) as well as transposable elements and genes of unknown origin (Drezen et al., 2006; Gauthier et al., 2021). In addition to the proviral segments, the bracovirus genome consists of a second major region that presumably reflects the genome of the ancestral nudivirus that integrated into the genome of an ancestor wasp, the nudiviral cluster (Bézier et al., 2009a). The genes of the nudiviral cluster encode several of the structural proteins required for virion production, whereas the others are dispersed in the wasp genome. In contrast to the proviral segments, no RUMs were found in the nudiviral cluster, indicating a different mechanism for the amplification of genes in this genomic region (Gauthier et al., 2021; Burke et al., 2015). A study on Microplitis demolitor bracovirus (MdBV) revealed that recombination between the two flanking WIMs involves two nudiviral integrases (Burke et al., 2013) and leads to the circularization of the DNA segments packaged into bracovirus nucleocapsids (Dupuy et al., 2012). Genes of the *integrase/recombinase* superfamily that are conserved between all known bracoviruses and nudiviruses include *vlf-1* and *integrase*, whereas *HzNVorf140-like* is present in bracoviruses, but not in all nudiviruses (Bézier et al., 2009b). Even though the nudiviral cluster is amplified, it is not encapsidated into the nucleocapsids like the proviral segments. Sequence analyses of Cotesia congregata bracovirus (CcBV) revealed that all RUs of the proviral segments and nudiviral cluster comprise a conserved TA-rich sequence motif. The conserved motif was shown to have variable length (up to 100 bp) and has hairpin-forming attributes. Hairpins are secondary-structures that are associated to genome replication and can function as origins of replication (Louis et al., 2013). Further studies on CcBV associated the hairpin-forming regions with the generation of concatemeric intermediates. They revealed unexpectedly that a CcBV locus forms head-to-head/tail-to-tail concatemers (Louis et al., 2013) as predicted by a model of linear replication as described in *Poxviridae*. A more extensive study showed that both the latter and head-to-tail concatemers characteristic of rolling circle replication as described in *Baculoviridae* are formed depending on the locus in MdBV (Burke et al., 2015). The similarity between sequence motifs flanking RU junction sites whatever the replication intermediates, suggests a conservation of the mechanism involved to produce two concatemer types (Burke et al., 2015; Gauthier et al., 2021). How exactly those concatemers are produced has yet to be fully understood.

The wasp machinery is thought to amplify bracovirus DNA sequences, while the nudiviral machinery appears to be mostly associated with DNA processing and virion production (Bézier et al., 2009a; Bézier et al., 2009b; Burke and Strand, 2012). This division of tasks is coherent with the absence of the nudiviral DNA polymerase in all bracovirus species (Table 2). This table indicates the nudiviral core genes present in bracovirus species (CcBV, MdBV and CiBV) (Gauthier et al., 2021; Zhang et al., 2020) and endogenous nudiviral agents from hymenopteran hosts (VcENV and FaENV) (Burke et al., 2018; Pichon et al., 2015). The 14 nudiviral core genes (or pseudogenes) present in a presumably inactive nudivirus in the genome of the chalcid wasp *Eurytoma brunniventris* (EbrENV-β) (Zhang et al., 2020) were also included in the comparison. Phylogenetic analysis indicated that EbrENV-β was derived from a betanudivirus and is the closest known relative to bracoviruses (Zhang et al., 2020).

The presence of the above listed genes in endogenized nudiviruses does not necessarily represent their functionality or activity, as some of these genes may have obtained alternative, yet undescribed functions, or lost their functionality totally after endogenization (Francino, 2005). However, *Venturia canescens* genome analysis showed that nudiviral

genes without beneficial function for the parasitism tend to become ultimately pseudogenized, such as the nudiviral DNA polymerase and genes coding for nucleocapsid components (Leobold et al., 2018).

7. Discussion

7.1. Phylogeny and taxonomic classification

The family *Nudiviridae* comprises a rich diversity of species and their taxonomic classification is an ongoing process. Deep sequencing approaches have gradually revealed new nudiviral agents in arthropods and the full sequencing of their genomes has allowed more profound phylogenetic analyses. There are currently four genera among the family *Nudiviridae* that the ICTV officially recognized: *Alphanudivirus*, *Betanudivirus*, *Gammanudivirus*, and *Deltanudivirus* (Harrison et al., 2021b). Recently discovered nudiviral agents (AgENV, MsENV, DuhNV) demonstrate a high diversity of viruses in terrestrial hosts in the genus *Alphanudivirus*. In addition, the ongoing exploration of nudiviruses in aquatic arthropods is gradually providing higher resolution of intrafamilial relationships within the *Nudiviridae*. The official recognition of two new genera, *Gammanudivirus* and *Deltanudivirus*, corresponds to the phylogenetic analyses of 15 terrestrial and 5 aquatic nudiviruses (Fig. 2) and emphasises their evolutionary distinction. Previous phylogenetic analyses first grouped PmNV and ToNV together with the genus *Betanudivirus* (Bézier et al., 2015), then later assigned ToNV and CcNV to the genus *Deltanudivirus* (van Eynde et al., 2020), but the inclusion of more sequences and aquatic nudivirus species in the analysis invalidated these propositions. Instead, HzNV-1 and −2 now represent the only *Betanudivirus* members and ToNV makes up the genus *Deltanudivirus*, while PmNV and CcNV group with the other aquatic nudiviruses (DhNV, CmNV, HgNV) in the genus *Gammanudivirus*.

Due to the growing diversity of alphanudiviruses, Liu et al. (2021) proposed to upgrade this genus to the subfamily *Alphanudivirinae*, with three genera (*Grynudivirus*, *Orynudivirus* and *Endonudivirus*) due to the diverged lineages. *Grynudivirus* would currently only consist of GbNV that infects the field cricket *G. bimaculatus*, while *Orynudivirus* features nudiviruses that infect flies (*D. melanogaster*, *D. inubila*) and beetles (*O. rhinoceros*, *D. u. howardi*). The genus *Endonudivirus* would include the endogenous nudiviruses from aphids (AgENV and MsENV). The distinct lineage of cricket-infecting nudiviruses likely reflects the diversification of orthopteran species at the end of the Devonian period (~370 million years ago), while the common ancestor of dipteran, lepidopteran and coleopteran species manifested later between the Carboniferous and Permian period (~300 million years ago) (Thézé et al., 2011) and with this ancestral host the corresponding nudiviruses of the proposed *Orynudivirus* genus. Therefore, the placement of cricket-infecting nudiviruses in the proposed genus *Grynudivirus*, next to the flies- and beetle-infecting nudiviruses in the genus *Orynudivirus*, appears reasonable. Nevertheless, the discovery and inclusion of more genomic data from orthopteran nudiviruses, with special regard to cricket-infecting nudiviruses, is needed to carefully evaluate this proposition.

OcNV, the only described bee (*O. cornuta;* the European orchard bee, Megachilidae)-infecting nudivirus was not included in the analysis by Liu et al. (2020). Our phylogenetic analysis (Fig. 2) indicates that OcNV is an alphanudivirus that shares a most common ancestor with the endogenous nudivirus found in the hymenopteran host *Fopius arisanus*. On the other hand, OcNV is not closely related to the endogenous bracoviruses even though they both have hymenopteran hosts. Conclusively, the bee-infecting nudivirus appears to originate from an ancestral alphanudivirus, while bracoviruses share a common ancestor with the dipteran ToNV in the deltanudivirus branch, although with a rather low node support (72%) when compared to the other nodes.

ToNV, HzNV, crustacean nudiviruses and bracoviruses all seem to have diverged from a common ancestor and form a cluster distinct from the members in the proposed subfamily *Alphanudivirinae*. Therefore, Liu et al. (2021) proposed to group the betanudiviruses in the parallel subfamily *Betanudivirinae*. This proposition is further supported by the low number of homologous genes with high similarity shared between alphanudivirins and betanudivirins (Liu et al., 2021). An evolutionary connection between HzNV and bracoviruses is plausible, as their hosts live in a close biological relationship. A possible scenario could involve a lepidopteran host that was infected with an ancestral betanudivirus and was subsequently parasitized by an ancestor wasp. The hatched larvae of this wasp could have fed on the nudivirus-infected caterpillar and consequently ingested the betanudivirus. This event could have led to the endogenization of the nudivirus in the genome of the wasp and ultimately in the domestication and further evolution into bracoviruses. Alternatively, an ancestral parasitoid-wasp-infecting nudivirus with tissue-specific expression in the ovaries could have been transmitted to a lepidopteran host and then manifested itself in its reproductive tissues (compare HzNV-2). As indicated above, the only other hymenopteran-infecting nudivirus (OcNV) known to date shows a great evolutionary distance to the betanudivirus and bracovirus branch, making the latter hypothesis less likely. However, the fact that nudiviruses of distinct genera manifested in different hymenopteran hosts, indicates that alpha- and presumably beta- and deltanudiviruses have qualities that allow infection/adaptation to hymenopteran hosts. This, in turn, leads to the question whether nudiviruses diversified along with their hosts like it was shown for baculoviruses (Ikeda et al., 2015), or whether an innate ability to adapt to more distantly related hosts led to the broad host range of the *Nudiviridae* as a family.

In relation to the proposed genus *Endonudivirus* (Liu et al., 2021), it is debatable whether all endogenous nudiviruses should be grouped into one clade. Previous phylogenetic analyses showed that endogenous nudiviral agents in hemipteran species (e.g., MsENV and AgENV) are evolutionary distinct from the ones found in hymenopteran species (FaENV and VcENV) (Cheng et al., 2020; Liu et al., 2020). Endogenous nudiviruses from distantly related hosts are probably phylogenetically dispersed and possibly group close with their free-living relatives. This is supported by a phylogenetic tree of Cheng et al., (2020) inferred from the P74 protein sequences of several nudiviruses and endogenous nudiviral elements found in arthropod genomes. While seven nudiviral elements of hemipteran origin share a distinct phylogenetic group, the endogenized nudivirus of the lepidopteran species *Danaus plexippus* groups with the lepidopteran virus, HzNV-1.

Among the aquatic nudiviruses, CcNV, PmNV, CmNV and PmNV infect marine crustaceans, while DhNV infects a freshwater host. It can be assumed that the nudiviruses in marine hosts are distinct to the ones in freshwater hosts and underwent separate evolutionary events at different time periods. The low average protein similarity and disparate gene synteny of DhNV compared to the described marine nudiviruses has been cited to support classification of freshwater nudiviruses as a distinct genus, proposed as *Epsilonnudivirus* (Allain et al., 2020). The freshwater nudivirus MrNV (NCBI accession: PRJNA359633) purified from diseased giant freshwater prawn (*Macrobrachium rosenbergii*) larvae was shown to group between DhNV and PmNV when using its *iap* and *pif-2* genes for phylogenetic analysis (Allain et al., 2020). For a more accurate phylogeny of freshwater nudiviruses, the sequences of more MrNV genes are required, as well as genomic data of other putative freshwater nudiviruses, such as AaBV, ApBV, CdBV, CqBV, GrBV and PlBV. Also, the full sequences of putative marine nudivirus genomes, including BMN, Baculo-PP, CpBV, PmBV and SsBV, will add higher resolution to the phylogeny of aquatic nudiviruses. Next to marine and freshwater habitats, it may be worthwhile to look for potential nudiviruses in brackish crustaceans as well.

Particularly interesting in this regard is the yet unknown origin of aquatic nudiviruses. It is likely that close ecological interactions between (semi)aquatic insects and crustaceans may have allowed the switch of an ancestral nudivirus to either of those host groups. A number of Dipteran families including among others, Chironomidae, Culicidae, Simuliidae and Tipulidae, are terrestrial as adults, but their larvae and pupae are aquatic (Adler and Courtney, 2019). Crane fly larvae

(Tipulidae) have been shown to inhabit both land and aquatic areas (Evenhuis, 1989), such as marine, brackish and non-saline waters (De Jong et al., 2007). Some crane fly larvae are predatory and feed on other aquatic insects and invertebrates (Pritchard, 1983), while bigger aquatic invertebrates may naturally consume crane fly or dipteran larvae as well. Other aquatic insect larvae exist in the family of Dytiscidae, a taxonomic clade of water beetles. Members of this family are referred to as predaceous diving beetles and can be found worldwide in freshwater (Foster and Bilton, 2014), but some species also live in brackish waters (Yee, 2014). Predaceous diving beetles might form a possible bridge between insects and brackish or freshwater nudiviruses in Crustaceans, whereas crane flies could have been a driving force that led to the manifestation of nudiviruses in aquatic hosts as such. When one applies the alternative theory of nudiviral host-driven diversification, it is likely that an ancestor of all nudiviruses may have its roots in the water. The last common ancestor of hexapods and crustaceans belonged assumably to the class of Branchiopoda and lived in freshwater around 420 million years ago in the Late Silurian (Glenner et al., 2006). Therefore, it is likely that the colonization of aquatic hexapods to terrestrial habitats might have pressured the nudiviral ancestor to adapt accordingly and laid the foundation that established the entomopathogenic group of nudiviruses that we know today. However, it remains to discover and characterize more nudiviruses in aquatic or semiaquatic invertebrates (and beyond) to refine and unravel the evolutionary history of *Nudiviridae*.

7.2. Morphological and genomic properties

The discovery and identification of nudiviruses that form OBs (ToNV, PmNV and OrNV under facultative conditions) refutes the original observation that led to the naming of this “naked” virus family. Therefore, the absence of OBs should no longer be the main criterion to discriminate between nudiviruses and baculoviruses. Instead, the localized infection and wider host range of nudiviruses as a family and the specific collection of core genes appears to be a more discriminating feature that distinguishes them from baculoviruses. The number of nudiviral core genes will most likely require revision in the future, as more nudivirus species are being discovered.

The greater evolutionary distances of the nudiviral DNA polymerase and helicase compared to their baculovirus orthologues implies that the host range within the nudivirus family is greater than the host range among baculovirus species. Indeed, this is consistent with previous findings that described the *helicase* gene as one of the most rapidly evolving genes across baculoviruses and nudiviruses (Hill and Unckless, 2017). The sequence variation of the *helicase* gene was further shown to correspond with the ability of baculoviruses to switch to new hosts (Argaud et al., 1998; Croizier et al., 1994). The corresponding broader distribution of nudiviruses over more distantly related arthropod hosts may reflect a greater evolutionary diversification that nudiviruses underwent to adjust to the manifestation of new insect orders in the course of millions of years. The nudiviral helicase is likely to facilitate a crucial function in nudivirus replication, as it has been proposed for poxviruses. It is believed that the poxviral helicase initiates DNA replication by inducing a nick in the linear dsDNA to expose a free 3′-OH group that functions as a primer for the DNA polymerase (Boyle and Traktmann, 2009). A similar role of the nudiviral helicase in DNA replication might be expected. The scarce presence of the nudiviral core gene *helicase-2* in baculoviruses might hint at a replication mechanism that further distinguishes the two virus families.

The ability of nudiviruses to maintain their replication and particle formation after host genome integration is another particularity that distinguishes them from baculoviruses. This nudivirus feature led to the formation of nudivirus-derived endogenous viral elements in genomes of arthropods and gave rise to endogenous nudiviral agents (NlENV, AgENV, MsENV, FaENV, VcENV, EbrENV) and bracoviruses. Moreover, HzNV-1 can occasionally integrate its genetic material into the host genome as part of latent infection, whereas most genome copies of HzNV-1 are suggested to persist as episomes. Latent HzNV-1 infections may be reactivated for productive and lytic infection (Lin et al., 1999), but to which extent this reactivation is bound to integrated or episomal viral DNA has not been examined yet. Two differences might hint at the possibility that nudiviruses can maintain replication activity in their host genome, while baculoviruses presumably cannot: differences in the structure of nudiviral and baculoviral origins of replication (i.e. *hrs* or *drs*), and the absence of the nudivirus core gene *integrase* in baculovirus genomes (or even the multiplicity of coding genes with integrase/recombinase functions). On the other hand, these aspects are shared between nudiviruses and bracoviruses. In bracoviruses, *drs* are loci (also called WIMs or DRJs) where recombination and excision events occur. While the nudiviral integrase probably ensures the excision of the DNA destined for circularization from previously formed concatemeric intermediates, it can be assumed that other nudiviral genes from the integrase/recombinase superfamily (*vlf-1*, *integrase and HzNVorf140-like*) are involved in the integration of the packaged DNA circles into the genome of infected lepidopteran cells (Wang et al., 2021). The formation of head-to-head/tail-to-tail concatemers during bracovirus replication leads to the question whether this is a trait that bracoviruses “inherited” from their nudiviral ancestor, or if this replication model newly evolved within the clade of bracoviruses. A unique sequence in the genome of HzNV-1, comparable to conserved direct repeat motifs at the RU boundary junction of bracoviruses, may play a similar role in HzNV-1 DNA replication. It is hypothesized that the *drs* originate from the duplication of a single nudivirus sequence that manifested as multiple loci over the wasp genome allowing the resolution of viral concatemers into individual genomes. The bracoviral VLF-1 and nudivirus-like integrase (INT-1) are both tyrosine recombinases that potentially bind and interact with the *drs* recombination sites. Possible *drs*-regulated mechanisms that might be associated with nudivirus replication and/or host genome integration have yet to be understood.

The conservation of *drs* and genes involved in DNA processing (*vlf-1*, *integrase and HzNVorf140-like*) between nudiviruses and bracoviruses hint at shared properties to maintain their replication mechanisms and to form virus particles after integration into their host’s genome. The absence of an integrase homolog and/or differences in the competence of baculoviral origins of replication may be part of the explanation why no replication competent endogenous baculovirus have been described, despite the fact that endogenized baculovirus genes were found in host genomes (Gilbert et al., 2014).

7.3. Infection mechanisms

Besides the uncertainties regarding the nudiviral pangenome and replication mechanisms, there are other gaps to be filled when it comes to the infection mechanisms of nudiviruses. The nuclear exit of nudiviruses involves passing a double membrane while retaining the viral envelope to keep PIF and other envelope proteins needed for the infection of subsequent cells (Velamoor et al., 2020). Therefore, it can be assumed that the virion-filled vesicles observed in HzNV-2 and OrNV serve as a viral-envelope-preservation mechanism to make up for the lack of GP64 or F-protein induced endocytosis. OrNV seems to circumvent this hurdle by encapsulating its virions in multiple membrane vesicles (MMVs) that egress from the outer nuclear membrane. The utilization of multiple vesicles may enable sequential cell-to-cell transmission of OrNV virions in exchange for a vesicle membrane each time it fuses with the plasma membrane of a different cell (Crawford and Sheehan, 1985). This may lead to dissemination into deeper tissue layers without the need for intermediate replication. The virion-filled vesicles of HzNV-2 may play a major role in horizontal transmission as infectious agents. In addition to OrNV virions that are encapsulated in MMVs, vesicle-free virions were also observed to egress from infected cells. The cell entry mechanisms of OrNV, and presumably other nudiviruses, might differ between vesicle-free virions and virion-filled vesicles. However, it has yet to be uncovered if these vesicle-free virions lose their

envelopes as part of a membrane fusion and only release their nucleocapsids into the extracellular space or have a special mechanism of maintaining their viral envelope.

Moreover, the exact mechanism behind nudiviral nucleus entry still needs to be unraveled. For baculoviruses, different mechanisms have been observed. Several studies describe a docking process of nucleocapsids, followed by their entry into the nucleus through the nuclear pore complex (Granados and Lawler, 1981; Ohkawa et al., 2010), while another study observed the release of the viral genome into the nucleus with empty nucleocapsids remaining on the cytoplasmic side (Au et al., 2013). Given the fact that nuclear pores can have a diameter of 38–78 nm (Alber et al., 2007) and the diameter of baculoviral nucleocapsids ranges from 30 to 60 nm (Jehle et al., 2006), it is reasonably assumed that their entry through these pores is attainable (Rohrmann, 2019b). The diameter of nudivirus nucleocapsids ranges from 30 nm (DiNV) to ~80 nm (HzNV) and it is known that the nucleocapsids of AcMNPV are able to transit nuclear pores, though via actin polymerization (Ohkawa et al., 2010). Moreover, virally-induced modification of nuclear pores can occur to allow the entry of oversized nucleocapsids into the nucleus, as it was shown for a system with AcMPNV and *Xenopus* oocytes (Au and Panté, 2012). Conclusively, most nudiviral nucleocapsids should be capable of entering the nucleus through nuclear pores as well. Whether nudiviruses are also able to reorganize the structure of nuclear pores to suit their nucleocapsids dimensions has not been described yet.

8. Conclusion

The intrafamilial phylogeny of nudiviruses is obtaining higher resolution as more nudivirus species are discovered and their genomes sequenced. In particular this includes aquatic bacilliform viruses that were originally classified as baculoviruses, but are now reclassified as nudiviruses. Additionally, the ever-growing diversity of alphanudiviruses may lead to a revision of the genus *Alphanudivirus* in the future. Upcoming studies should focus on discovering and sequencing the genomes of potential aquatic nudiviruses, also in regard to marine, brackish and non-saline habitats. Moreover, the search for more nudiviruses in orthopteran and hymenopteran hosts, including bee and cricket species, will provide more profound phylogenetic insight on the family of *Nudiviridae*. The same applies for yet undescribed endogenized forms in insect and crustacean genomes to retrace the evolutionary history of ancient viruses. The genome sequencing of new nudiviruses will further aid in understanding the nudiviral pangenome and eventually revise their set of core genes.

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.

Acknowledgements

We wish to thank the workgroup of Prof. Dr. Dirck C. de Graaf for providing the transcriptomic data of the Osmia cornuta nudivirus (OcNV). We also want to thank Dr. Ronny van Aerle for his valuable remarks and support on the phylogenetic analyses. The writing of this paper has received funding from the European Union's Horizon 2020 research and innovation programme INSECT DOCTORS under the Marie Skłodowska-Curie grant agreement No 859850.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2022.107718.

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