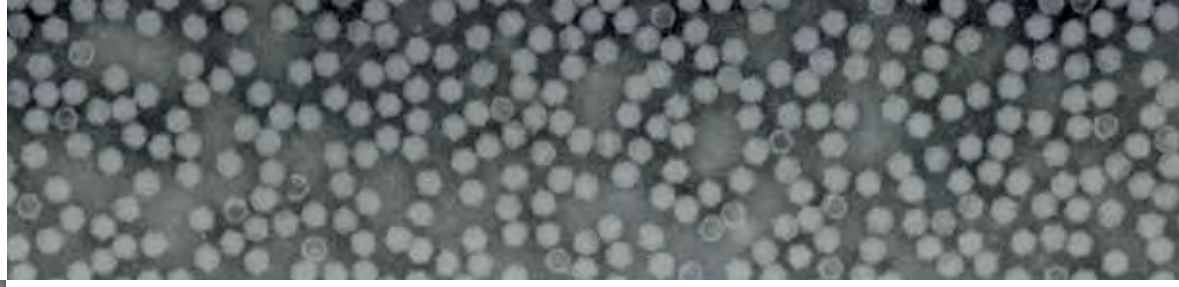

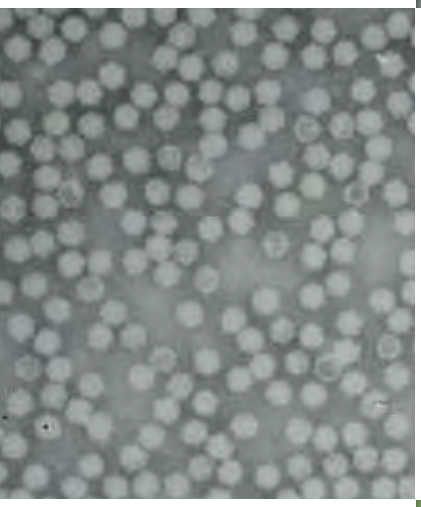

Characteristics and pathogenicity determination of insect-specific RNA and DNA viruses

CGM 2019-01

ONDERZOEKSRAPPORT

Characteristics and pathogenicity determination of insect-specific RNA and DNA viruses

COGEM Report: CGM 2019-01

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Date: March 2019

Preface

Insect-specific viruses (ISVs) replicate solely in insects or insect cell lines and are generally unable to infect vertebrate tissues or cells. Until recently, ISVs have received relatively limited attention since they have minimal or no impact on human health. In the past, research on ISVs has mainly focused on their use for crop-protection by controlling plant-feeding insects. Recently, for several reasons the interest in ISVs has increased. One reason is the growing interest in insect breeding for feed and food (insect-farming). The incursion of ISV infections in insect-farms may have devastating economic consequences. Therefore, it is imperative to avoid the introduction of ISVs. Another reason is the threat of specific ISVs to economically useful insects such as silkworms and pollinators (e.g. honey bees).

Despite their host-restriction, phylogenetic analysis of some ISVs has demonstrated that many are closely related to arboviruses of clinical and veterinary importance. This has raised important questions about their evolution and potential to adopt an arboviral lifestyle. Comparative studies on insect-specific and dual-host viruses may provide unique insights into why some viruses possess the ability to infect and cause devastating disease in animals and/or humans (arboviruses) while others do not. Given the host-range restriction of ISVs, exploitation of this intrinsic safety feature may allow e.g. for the production of safe recombinant vaccine strains to control relevant arboviral threats.

Due to the expected increase in research (including gmo research) involving ISVs, COGEM expects an increase in the number of requests for the assignment of ISVs to a specific pathogenicity-class. Until now, several ISVs have been individually assessed and assigned to pathogenicity-class 2 (PG-2). In order to examine whether, similar to plant viruses, a generic PG-2 classification can be used for ISVs, COGEM commissioned a desk study with the objective to provide a taxonomic classification of ISVs based on a number of relevant criteria. In addition, the project should provide information on the potential harmful effects of ISVs on their host(s), in particular those used for insect farming.

The author has conducted a thorough review of the scientific literature. This was not an easy task in view of the rapidly growing number of insect viruses and the many taxonomic revisions. Furthermore, for many viruses limited information is available. By using a weight-of-evidence approach the author has come to a taxonomic classification of true insect-specific viruses consisting of 11 virus families, 1 subfamily, 16 genera and two species groups, together totaling 470 viruses. Furthermore, where possible, the author compiled the available information concerning the pathogenicity of the different viruses.

The supervisory committee pays tribute to the author for this difficult task and trusts that the report constitutes a solid scientific basis for the classification of ISVs by COGEM.

Ben Peeters

Chair of the supervisory committee

Characteristics and pathogenicity determination of insect-specific RNA and DNA viruses

Executive summary

This report presents the findings of a literature review to identify virus taxa specific for insects (Order *Insecta*) to aid in the risk assessment of genetic modification ventures. For this purpose, criteria were identified and used in a weight-of-evidence approach to assess the specificity and pathogenicity of putative insect-specific viruses (ISV). Viruses that are transmitted by insect vectors to vertebrates (arboviruses) and viruses of other arthropods (ticks, mites) were also excluded from the study. Endogenous viral elements and errantiviruses were also not investigated.

The current taxonomy of viruses as determined by the International Committee on Taxonomy of Viruses (ICTV) and the available body of relevant literature up to December 2018 were used as a final point of reference for this report. The insect-specific virus taxa identified and assessed in the above study are listed at the level of the highest taxon that potentially contains ISVs.

Multiple criteria were defined and assessed using a weight-of-evidence approach. Important criteria were: ISVs (i) have a (restricted) host range in insects only, (ii) do not replicate in or have any effects on vertebrates or vertebrate cells and (iii) have an unequivocal phylogenetic position. Thirty-eight taxa (13 families, 3 subfamilies, 18 genera and 3 species groups) encompassing 518 putative ISVs in total were assessed. **Eleven virus families, 1 subfamily, 16 genera and two species groups were considered insect-specific (ISV) for a total of 470 virus species.**

Viruses of six taxa, the genera *Inshuvivirus*, *Sawastrivirus* and *Wuhivirus* (*Phasmaviridae*), *Phasivirus* and *Goukovirus* (*Phenuiviridae*) and the *dISF group* (genus *Flavivirus*) do not yet meet all of the specific criteria for ISV status due to lack of critical information. Their phylogenetic position is equivocally sandwiched between arbovirus clades. Hence, their ISV status is considered provisional (21 virus species). The members of the taxa *Carmotetraviridae*, *Betairidovirinae* and *Seadornovirus* do not meet the criteria for insect specificity, as they can replicate in vertebrates or vertebrate cells (20 virus species).

The number of mainly insect RNA viruses is rapidly increasing through next generation sequencing ventures of many new insect species. New taxa are emerging regularly, with little or no information on their biology (specificity, pathogenicity, transmission). This calls for regular review, evaluation and update of the list of ISVs and their characteristics.

Nederlandse samenvatting

Dit rapport bevat de resultaten van een literatuurstudie om virussen van insecten (Orde *Insecta*) te identificeren, als onderdeel van de risicoanalyse in geval van genetische modificatie. Voor dit doel werden criteria opgesteld en gewogen ('weight-of-evidence' benadering) om de insectspecificiteit en het ziekteverwekkend vermogen van mogelijk in aanmerking komende virussen (insectspecifieke virussen = ISV) te bepalen. Virussen die door insecten als vectoren worden overgebracht naar gewervelden (arbovirussen) en virussen van andere geleedpotigen zoals teken en mijten zijn buiten beschouwing gelaten. Hetzelfde geldt voor endogene virussen ('endogenous viral elements') en errantivirussen.

De taxonomie (ordening) van virussen, zoals vastgelegd door de International Committee on Taxonomy of Viruses (ICTV), en de beschikbare relevante literatuur ultimo 2018 vormen de basis voor het onderhavige rapport. De insect-specifieke virus taxa, die zijn geïdentificeerd en geëvalueerd, betreffen de hoogste taxons, die alleen ISV's zouden kunnen bevatten.

Meerdere criteria werden gedefinieerd en gewogen via een 'weight-of-evidence' werkwijze. Belangrijke criteria waren: ISV's (i) zijn specifiek voor insecten, (ii) vermenigvuldigen zich niet in of hebben geen effect op gewervelden of daarvan afgeleide cellen, (iii) hebben een ondubbelzinnige fylogenetische positie. Vierendertig taxa (13 families, 3 subfamilies, 18 genera en 3 virusgroepen) van in totaal 518 potentiele ISV's werden onderzocht. **Hiervan kunnen 11 virusfamilies, 1 subfamilie, 16 genera en 2 virusgroepen als insect-specifiek (ISV) worden beschouwd, voor een totaal van 470 virussoorten.**

Virussen van zes taxa, de genera *Inshuvivirus, Sawastrivirus* and *Wuhivirus* (*Phasmaviridae*), *Phasivirus* en *Goukovirus* (*Phenuiviridae*) en de dISF groep (genus *Flavivirus*) voldoen nog niet aan alle belangrijke criteria voor ISV status door gebrek aan informatie. Hun fylogenetische positie temidden van arbovirussen is nog onvoldoende duidelijk. Vandaar dat deze virussen (21) voorwaardelijk als insect-specifiek kunnen worden beschouwd. De virussen (20) van de *Carmotetraviridae, Betairidovirinae* en genus *Seadornovirus* kunnen niet als insect-specifiek worden aangemerkt omdat deze zich ook kunnen vermenigvuldigen in gewervelden.

De hoeveelheid nieuwe RNA-virussen neemt snel toe dankzij nieuwe sequentietechnieken en –analyses van bestaande en nog niet eerder onderzochte insecten. Nieuwe virustaxa worden regelmatig gerapporteerd, maar zonder informatie over de biologie (ziekteverwekkend vermogen, specificiteit, overdrachtskarakteristieken). Dit vraagt om een regelmatige herbeoordeling van nieuwe virustaxa en eventuele aanpassing van de lijst van ISV's.

Introduction

Insects are probably the largest group of terrestrial animals in the biosphere, not only by the number of species but also in absolute numbers (Stork, 2018). They form a separate class (*Insecta*) in the phylum *Arthropoda*, which also includes arachnids (mites, ticks) and crustaceans (shrimp). Insects can be infected with many different viruses, sometimes causing disease, sometimes asymptomatically. In some cases, insects can also act as vectors and transmit viruses to plants and animals (including humans). Such viruses are called arbo- (= arthropod-borne) viruses and they can cause severe diseases in humans and animals (dengue, Zika, yellow fever, chikungunya, West Nile). However, in many cases viruses circulate only within insect hosts. They are then called insect-specific viruses (ISVs), which are the subject of this report.

Viruses are found in almost every living organism on the planet (terrestrial, aquatic), including bacteria, fungi, plants and animals. About 5000 virus species are recognized by the ICTV (ICTV 2018). Most of these viruses are associated with vertebrate, plant or bacterial disease, or are of agronomic or commercial interest. This number is most likely a gross underestimation of the real number of viruses, in particular in the plant, invertebrate, bacterial and archeal world. Because insects are the largest group of terrestrial animals, a high diversity of viruses is found. Since about 80% of all insect species remain to be identified (Stork, 2018), the discovery of many new viruses is to be expected (Li *et al.*, 2015; Webster *et al.*, 2015), including ISVs.

Current climate change has a major effect on the redistribution of (sub)tropical insects into the temperate zones of the world, and hence the viruses they carry. The same holds true for the intensive trade and traveler movements, potentially introducing new or exotic insects carrying (new) viruses in new geographical areas. For example, the mosquito *Aedes albopictus*, the natural vector of chikungunya virus, recently appeared in Europe and the Netherlands (ECDC, 2018). Apart from the medical implications of viral diseases, the introduction of exotic insects poses a risk of introducing new viruses in the Dutch environment.

ISVs have been the subject of topical reviews, concentrating mainly on RNA viruses (Roundy *et al.*, 2017; Calisher and Higgs, 2018). These viruses are of interest, as they are often members of a larger family of viruses also circulating or emerging in vertebrates. The RNA ISVs may be ancestors of future arboviruses. RNA virus infections in insects often have an asymptomatic character coping with the host defense. Many insect DNA viruses are assumed to be insect-specific, primarily because they do not have counterparts in the vertebrate kingdom and have a unique 'life-cycle' involving typical invertebrate entry mechanisms *per os* (Boogaard *et al.*, 2018). However, there is as yet no comprehensive list of ISVs.

The Netherlands Commission on Genetic Modification (COGEM) is expecting an increased interest in genetic modification applications of insect viruses at large and this calls for classification of these viruses (pathogenicity classification). For microorganisms the lowest pathogenicity class is 1 (based on non-pathogenicity and/or history of safe use) and the highest 4 (based on amongst others capacity to cause serious or lethal disease and/or absence of effective prophylaxis). Host specificity, disease potential and beyond host transmission are guiding principles for this classification. Viruses in general are at least pathogenicity class 2. The latest update on animal virus pathogenicity classification for COGEM lists predominantly vertebrate viruses and a few insect viruses (CGM/170522-03). Invertebrate viruses, more specifically non-arboviruses are classified *ad hoc* and a listing of ISVs on the basis of virus

characteristics and pathogenicity information is therefore desired. The latter is the topic of this report.

There has been interest in ISVs for a long time, more specifically for the application of some of these viruses for insect pest control as alternative for chemical insecticides (Nouri *et al.*, 2018). Baculoviruses and cypoviruses are notable examples. Viruses also form a threat for insect cultures used for other purposes, such as baculovirus production (lepidopteran and hymenopteran insects), sterile insect techniques (e.g. tsetse flies) or for food and feed applications (e.g. crickets). Furthermore, a large number of RNA viruses replicate in both insects and vertebrate hosts. These arboviruses have been proposed to have evolved from ISVs. It is therefore important to understand the mechanisms of emergence and evolution of these insect arboviruses originating from insect-specific ancestors. The discovery of many new insect viruses calls for insight in the characteristics and biology of viruses of known virus taxa and for generic methodology to assess these new viruses.

Virus taxonomy

Taxonomy provides the principle framework to assign, among others, viruses (Simmonds, 2018). Classification of viruses is a key effort to organize the huge variety of viruses. The International Committee on Taxonomy of Viruses (ICTV) takes up this task. Virus experts from around the globe and with specific expertise contribute to the activities of the ICTV. The ICTV has been active since 1966 and regularly generates reports and updates on the current status of viral taxonomy and nomenclature (naming of viruses). The latest complete update is from 2011 (King *at al.*, 2011). Recent updates on individual taxa are reported via the ICTV website, when approved by the ICTV Executive Council (King *et al.*, 2018). Other taxa have not been reviewed since 2011. The generally accepted taxonomy and nomenclature as issued by the ICTV (Release December 2018) forms the basis of this report.

The ICTV has organized the viruses in so-called taxa, taxonomic ranks, at various levels, that contain related viruses with common properties. Currently, the ICTV has defined the following taxa, 1 Phylum, 2 Subphyla, 6 Classes, 14 Orders, 143 Families, 846 Genera and 4,958 Species (ICTV 2018 Release October). The NCBI database records 7512 sequenced viral genomes from all kingdoms. The species concept in virology is a continuing matter of debate (Simmonds, 2018). The current definition of a virus species by the ICTV is defined as “a monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria”. Whereas the earlier classifications were based on virus morphology, genome structure, replication and transcription strategy, and host specificity, currently comparative sequence analysis and phylogenetics of viruses and their genes are becoming leading in virus taxonomy. Gene or genome phylogenetics only reflects the genetic relatedness between viruses, not necessarily their evolutionary relatedness.

Whereas most information is available for viruses that are correlated with disease, the metagenomics era reveals a large number of potential viruses that do not have a context of disease yet or may have an asymptomatic ‘lifestyle’. These ‘metagenomic’ viruses are therefore ‘in need for a disease’ and have not been considered in this report unless they are related to ISV taxa. However, virus-like sequences from metagenomic efforts are important to make the phylogeny of viruses more robust. This report will not consider these ‘metagenomic virus’ taxa by themselves, unless they are relevant for the safety profile of invertebrate viruses. Endogenous viral elements (EVEs) are found in the genomes of many organisms

(also in insects) and are records of viral infections in the evolutionary past (Katzourakis *et al.*, 2010; Whitfield *et al.*, 2017). But whether EVEs are viruses or not is a matter of debate. They were excluded from this report. Also, errantiviruses (*Metaviridae*) (King *et al.*, 2011), retrovirus-like entities in insects, were not considered.

Insect-specific viruses (ISVs) and arboviruses

Among arthropods (animals with segmented bodies and legs) insects are the largest group. It is therefore supposed that insects harbor the largest reservoir of viruses. Two main groups of viruses can be distinguished. Viruses that replicate exclusively in insects, the so-called insect-specific viruses (ISVs), and those that also replicate in vertebrate hosts, the so-called arboviruses. This report is about defining and characterizing ISVs for the purpose of classification and risk evaluation of these viruses for genetic engineering purposes in laboratory settings.

Several recent reviews and books discuss insect viruses, however none of these cover the entire spectrum of ISVs (Bolling *et al.*, 2015; Roundy *et al.*, 2017; Calisher and Higgs, 2018; Nouri *et al.*, 2018). In most cases the focus of these reviews is different from the objective of this report. These reviews are either molecularly oriented (Maciel-Vergara and Ros, 2017; Ryabov, 2017), disease-oriented (Williams, 2018), nucleic acid oriented (Williams *et al.*, 2017a; Ryabov, 2016), species-specific (Fauver *et al.*, 2016; Halbach *et al.*, 2017; Minkeu and Vernick, 2018; McMahon *et al.*, 2018; Carrau *et al.*, 2018), with an evolutionary / ecological angle (Öhlund *et al.*, 2019) or topical (ICTV virus family descriptions) King *et al.*, 2011; ibid., 2018), but never comprehensive. Nevertheless, they are useful as a resource and input for this report.

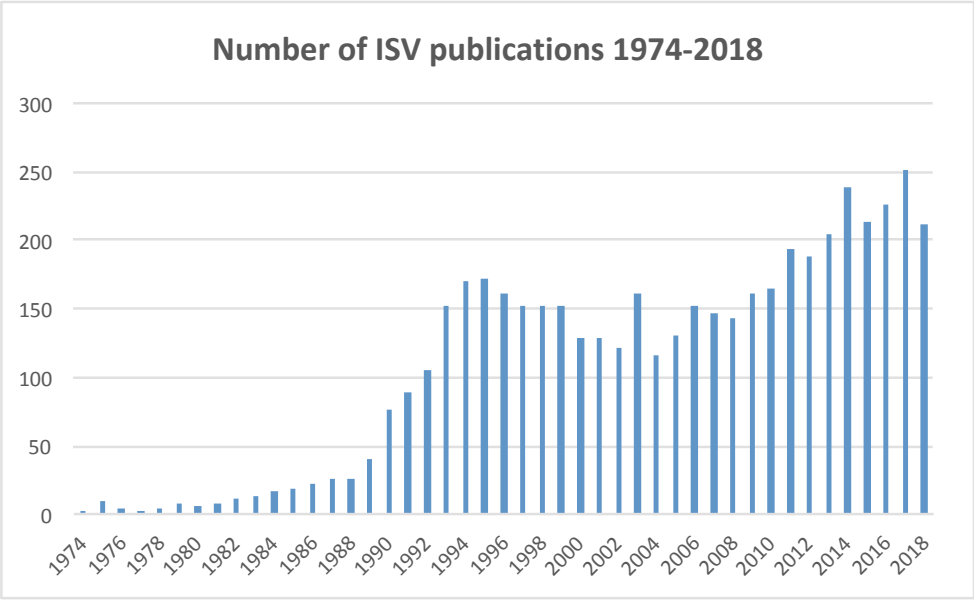

Figure 1. Time-line demonstrating the increase in ISV-related papers (PubMed December 2018). Search terms 'insect', specific' and 'virus'.

Insects for food and feed

Worldwide there is an ever-increasing interest in insects for food and feed (FAO, 2013). In Asia, Africa and Central- and South America insects are often part of the daily food package. In the developing world insects are more and more on the menu as a delicacy, but more importantly also as an alternative feed source in animal husbandry (fish culture, poultry) or as a replacement for conventional food sources (Dossey *et al.*, 2016; van Huis and Tomberlin, 2017). Insects have a high protein content, a high conversion rate of proteins and show reduced CO_2-emission relative to vertebrate-derived food sources.

Currently insects are produced at an industrial scale in the Netherlands (e.g. Koppert Biological Systems, Kreca) as well as in the European Union at large. (Ynsect, Andermatt Biocontrol). The worldwide edible insect market is likely to reach 8 billion USD in 2030 (source Meticulous Research 2019). Insects are derived from various climatic zones and often require importation from elsewhere. Major risks are (i) the introduction and emergence of microbial diseases in insect cultures, such as viruses, fungi, bacteria, rickettsia, (ii) the accumulation of heavy metals depending on the food source for insect culture, (iii) allergenicity and (iv) the accumulation of vertebrate pathogens (EFSA, 2015; Eilenberg *et al.*, 2015). In the Netherlands an initial assessment of the risk of insect consumption was made in 2014 (NVWA, 2014).

Based on this risk assessment it is assumed that insect viruses can be deleterious for the insect culture, but possibly also for the end user or consumer. Therefore, it is important to make an inventory of (possible) viruses of insects produced or to be used for food and feed, and assess to what extent they are insect-specific and safe for vertebrates. A preliminary inventory of viruses associated with insect production for food and feed has recently been made (Eilenberg *et al.*, 2015; Maciel-Vergara and Ros, 2017; Eilenberg *et al.*, 2018a, b).

Mode of operation for this report

The starting point has been the taxonomy of viruses, as published by the International Committee on Taxonomy of Viruses (https://talk.ictvonline.org/taxonomy/), more specifically the latest Virus Taxonomy release (October 2018). The use of the ICTV database has been described in detail (Lefkowitz *et al.*, 2017), and links are inserted in the text where appropriate. Background information on each taxon was derived either from the 9th Report (King *et al.*, 2011) or, when updated, from the current 10th Report (http://ictv.global/report/). The latter is an electronic report of updated and approved taxonomic proposals from Virus Study Groups (usually organized per virus family) (https://talk.ictvonline.org/ictv-reports/ictv_online_report/). An abstract of approved proposals are published in the Journal of General Virology.

For each taxon (family, subfamily, genus or species groups) a short description of virus morphology, genetics (genome structure), taxonomy including phylogeny, replication, pathobiology and ecology including specificity is given, in order to assess the generic classification of the taxon (Lemmas). All non-arbo insect viruses are considered. In some cases, multiple taxa (families, genera) are described in a single lemma, when the description is very similar (e.g. families and species within the *Bunyavirales*, tetraviruses and genera within the *Reoviridae*). Reference is made to pertinent reviews and further primary literature.

The salient observations are tabulated for each taxon and a final conclusion whether viruses are insect-specific or not is made. Endogenous viral elements (EVEs) are not considered.

Literature retrieval was carried out via (i) Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/), (ii) Google Scholar (https://scholar.google.nl/), (iii) Scopus, (iv) Web of Science / Knowledge using the principle words and terms: name taxon (family, subfamily, genus, species name) and/or one of the following: 'insect specific virus, 'specificity', 'safety/risk'. The selected sources were from national and international, peer-reviewed scientific journals, and book chapters published by well-established publishing houses for the natural sciences (e.g. Elsevier, Springer, Blackwell, Wiley, etc.)

The assessment was based on a number of criteria related to **insect specificity** and **pathogenicity** listed in Table 1). This is not an exhaustive list, but captures the main criteria related to purpose of this report. Some criteria are qualitative (e.g. isolated from insects, relation with vertebrate taxa, infection of vertebrates or vertebrate cells, hematophagous host, endemic) either from literature reporting or from experimental results. They allow a clear Yes/No answer. Other criteria (e.g. degree of pathogenicity, phylogenetic rootedness, persistence) allow a semi-quantitative or personal assessment by weighing the evidence and the relative importance (+, ++, +++). For example, when an ISV taxon has a unique genome structure and no relatives with vertebrate virus taxons (e.g. baculoviruses), this carries more weight, than an ISV taxon that has (e.g. iflaviruses). When there is conflicting evidence, this is indicated (+/-). In some cases, there is no information available (e.g. on mode of transmission) (nd). For each lemma such an assessment is carried out (assessment table), the relevant observations are listed and a final conclusion (weight-of evidence approach) as to the insect specificity of the pertinent taxon is drawn. The results of the assessment of the individual lemmas are tabulated (Table 6). For each lemma a list of proposed ISV species is produced. The final listing is an Excel file with all ISVs.

Table 1: Criteria for the asessment of insect-specific (ISV) status of virus taxa

	Yes / No	importance	specific comments
Insect-specificity		x - xxx	
only isolated from insects			
relation with vertebrate virus taxons			
evidence for infection of vertebrate animals			
replication in vertebrate cells			
monophyletic deeply-rooted clade within virus family			
insect-specific entry/release/transmission mechanism			
unique genetic traits correlating with insect-specificity			
unique genome structure			
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)			
hematophagous host(s) (Yes/No)			
within host tropism (Broad/Restricted)			
host(s) include feed/food insects (Yes/No)			
endemic in NL (Yes/No)			
persistent infections (Yes/No)			
horizontal transmission (Yes/No)			

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Observations

On the basis of their taxonomic and phylogenetic position (ICTV), alleged specificity for insects, mode of transmission and their (in)ability to infect vertebrates (or vertebrate cells) 13 virus families, as highest taxa, were investigated for the presence of potential ISVs (Table 2, yellow). Three subfamilies, 18 genera and 3 virus groups (Eilat virus group (*Togaviridae*), dISV and cISF group (*Flaviviridae*)) as highest taxa were taken into consideration (Table 2). A total of 54 genera and 518 virus species were assessed (Table 2, right 2 columns).

Table 2. Potential insect-specific virus taxa assessed

Lemma	Family	Subfamily	Genus	Species	Taxa consider			
					Family	Subfamily	Genera	Species
1	*Rhabdoviridae*		*Almendravirus*				1	5
2	*Togaviridae*		*Alphavirus*	(Eilat-group)				3
3	*[Xinmoviridae]*		*Anphevirus*				1	11
4	*Ascoviridae*				1		2	4
5	*Baculoviridae*				1		4	68
6	*Iridoviridae*	*Betairidovirinae*				1	2	16
7	*Bidnaviridae*				1		1	1
8	*Perbunyaviridae*		*Herbevirus*				1	1
8	*Perbunyaviridae*		*Shangavirus*				1	3
8	*Phasmaviridae*				1		5	15
	Phenuiviridae		*Bedeivirus*				1	1
8	*Phenuiviridae*		*Goukovirus*				1	3
8	*Phenuiviridae*		*Hudivirus*				1	1
8	*Phenuiviridae*		*Hudovirus*				1	1
8	*Phenuiviridae*		*Phasivirus*				1	3
9	*Phenuiviridae*		*Wubeivirus*				1	2
10	*Parvoviridae*	*Densovirinae*				1	4	34
11	*Dicistroviridae*				1		3	16
12	*Birnaviridae*		*Entomobirnavirus*				1	9
13	*Poxviridae*	*Entomopoxvirinae*				1	3	31
13	*Flaviridae*		*Flavivirus*	cISF group				24
14	*Flaviridae*		*Flavivirus*	dISF group				11
15	*Hytrosaviridae*				1		2	2
16	*Iflaviridae*				1		1	36
17	*Mesoniviridae*				1		1	22
18			*[Negevirus]**				1	26
19			*(Noravirus)*				1	5
20	*Nudiviridae*				1		2	7
21	*Polydnaviridae*				1		2	53
21	*Reoviridae*		*Seadornavirus*				1	3
21	*Reoviridae*		*Cypovirus*				1	74
21	*Reoviridae*		*Dinovernavirus*				1	1
21	*Reoviridae*		*Idnoreovirus*				1	5
22	*Rhabdoviridae*		*Sigmavirus*				1	7
23	*Alphatetraviridae*				1		2	11
23	*Carmotetraviridae*				1		1	1
23	*Permutotetraviridae*				1		1	2
	Taxa considered				13	3	54	518

ICTV Virus Taxonomy: 2018 Release; Ratification October 2018
https://talk.ictvonline.org/taxonomy/

( ) provisional nomenclature JMV
* Negevirus is floating taxon
[ ] not (yet) recognized by the ICTV

Nucleic acids

The genetic material of the viruses of the 23 lemmas is represented by all known types of nucleic acid (except retroviruses): ssDNA (2 lemmas), dsDNA (7 lemmas), ssRNA(-) (4 lemmas), ssRNA(+) (8 lemmas) and dsRNA(2 lemmas) (Table 3). The majority of ISV taxa (14) are RNA viruses (green), the remaining 9 taxa are DNA viruses. Viruses of 14 taxa have distant vertebrate relatives. The proposed ISV status and outcome of this assessment (from Table 7) is included for convenience (last column Table 3).

Predominantly dsDNA-containing ISV taxons are unique to insects (or invertebrates) and have no vertebrate relatives (e.g. baculoviruses, polydnaviruses, nudiviruses, negeviruses, tetraviruses, hytrosaviruses). Consequently, their genomes have unique features. When ISVs have vertebrate relatives (e.g. predominantly RNA viruses), their genome organization has distinctive features (gene organization, gene content or expression strategy) and they are phylogenetically distinct.

Table 3. Protential Insect-specific virus taxa: Type of nucleic acid

Lemma	Taxon (ICTV Release 2018)	ssDNA	dsDNA	ssRNA(-)	ssRNA(+)	dsRNA	vertebr. rel.	ISV
1	*Almendravirus*, *Rhabdoviridae*			X			N	Y
2	*Alphavirus* (1), *Togaviridae*				X		N	Y
3	*Anphevirus, Xinmoviridae*				X		Y	Y
4	*Ascoviridae*		X				Y	Y
5	*Baculoviridae*		X				Y	Y
6	*Betairidovirinae, Iridoviridae*		X				N	N
7	*Bidnaviridae*	X					Y	Y
8	*Bunyavirales* (3 families, 6 genera)			X			N	Y + P
9	*Densovirinae, Parvoviridae*	X					N	Y
10	*Dicistroviridae*				X		N	Y
11	*Entomobirnavirus, Birnaviridae*					X	Y	Y
12	*Entomopoxvirinae, Poxviridae*		X				N	Y
13	*Flavivirus* (2), *Flaviviridae*				X		N	Y + P
14	*Hytrosaviridae*		X				Y	Y
15	*Iflaviridae*				X		N	Y
16	*Mesoniviridae*				X		N	Y
17	*Negevirus, Mononegavirales*			X			Y	Y
18	*Noravirus* (3)				X		N	Y
19	*Nudiviridae*		X				N	Y
20	*Polydnaviridae*		X				N	Y
21	*Reoviridae* (4 genera)					X	Y	Y + N
22	*Sigmavirus, Rhabdoviridae*			X			Y	Y
23	*Tetraviruses* (4) (3 families)				X		N	Y + N
	Total	2	7	4	8	2		

Notes:
(1) Eilat Virus Group, three species:
(2) two subclades dISV and cDSV
(3) suggested genus author this report
(4) old name, no current taxon

vertebr. rel = vertebrate relative
Y = yes; N = No
P = provisional
ISV = Insect-Specific Virus

DNA virus
RNA virus

Host range and specificity

In most cases potential ISVs occur in a wide range of hosts from different insect families (Table 4), but the virus species themselves are often specific for a single insect species or a few related species. In some cases, ISVs have a somewhat broader host range or occurrence in a few related species with an exception for *Autographa californica nucleopolyhedrovirus*

(*Baculoviridae*, with 73+ lepidopteran species). For those insect viruses that are used for biocontrol purposes (e.g. baculoviruses, nudiviruses) host range, pathogenicity and biosafety testing has been done as a prerequisite for market registration (see refs in EFSA, 2017).

The DNA viruses are predominantly found in lepidopteran and hymenopteran insects and are mostly horizontally transmitted (*per os*). Most non-arbo invertebrate RNA viruses are found in dipterans (*Drosophila*, mosquitoes), but this may represent a sampling bias for these insect taxa. However, the picornavirus-like viruses (*Iflaviridae*, *Dicistroviridae*, '*Noravirus'*) are also found in lepidopteran, dipteran, orthopteran and coleopteran insects. Transmission of RNA viruses is not extensively studied, but it seems that most are vertically transmitted. It should be stated that the information on host range and virulence is usually scant, and not comprehensively reviewed, except for the iridoviruses (Williams, 2008).

A preliminary assessment has been made as to the virulence of viruses in the respective ISV taxa, but this turned out to be a difficult and complex task (Table 4). Viruses of some taxa (*Baculoviridae*) are highly infectious for their hosts, whereas others are only infectious when artificially inoculated. A comparative analysis or a review on this aspect was not available. Therefore, the virulence of respective virus groups is a personal assessment.

Table 4. Potential Insect-specific virus taxa: Host range and virulence (X)

Lemma	Taxon (ICTV Release 2018)	Diptera	Lepidoptera	Hymenoptera	Coleoptera	Orthoptera	ISV
1	*Almendravirus, Rhabdoviridae*	X					Y
2	*Alphavirus* (1)*, Togaviridae*	X					Y
3	*Anphevirus, Xinmoviridae*	X					Y
4	*Ascoviridae*		X	X			Y
5	*Baculoviridae*	X	XXX	XXX			Y
6	*Betairidovirinae, Iridoviridae*	X	XX				N
7	*Bidnaviridae*		X				Y
8	*Bunyavirales* (3 families, 6 genera)	X					Y + P
9	*Densovirinae, Parvoviridae*	X	X		X	X	Y
10	*Dicistroviridae*	X - XXX	X		X		Y
11	*Entomobirnavirus, Birnaviridae*	X					Y
12	*Entomopoxvirinae, Poxviridae*	X	X		X	X	Y
13	*Flavivirus* (2)*, Flaviviridae*				X		Y + P
14	*Hytrosaviridae*	X					Y
15	*Iflaviridae*	X - XXX	X-XX	X			Y
16	*Mesoniviridae*	X					Y
17	*Negevirus, Mononegavirales*	X					Y
18	*Noravirus* (3)	X	X	X			Y
19	*Nudiviridae*	X	X		X		Y
20	*Polydnaviridae*			X			Y
21	*Reoviridae* (4 genera)	X	XX	X			Y + N
22	*Sigmavirus, Rhabdoviridae*	X					Y
23	*Tetraviruses* (4)*, Alpha- and Permutotetraviridae*		XX				Y + N
	Total number	13	10	5	4	2	

Notes:
(1) Eilat Virus Group, three species:
(2) two subclades dISV and cDSV
(3) suggested genus author this report
(4) old name, no current taxon

Y = yes; N = No
P = provisional
X = virulence: X = low; XXX = high
ISV = Insect-Specific Virus

DNA virus
RNA virus

Viruses in insects for food and feed

To facilitate the evaluation of viruses from insects used for food and feed, the most frequently used insects in production systems have been listed and their putative ISVs are indicated (Table 5) (Eilenberg *et al.*, 2015; EFSA, 215). For example, in crickets, nudiviruses, densoviruses, iflaviruses, dicistroviruses and iridoviruses are found. Densoviruses are a major mortality factor in these insects. In silkworm (*Bombyx* spp, *Lepidoptera*) viruses of eight virus taxa can be present, but only baculoviruses and densoviruses are known to cause significant mortality. Infections with ifla- and dicistroviruses often go unnoticed. Of note here is that insects grown for feed (crickets) are susceptible to e.g. betairidoviruses, which may be harmful for recipient reptiles. However, having said this there are little or no studies on the viromes of these insects produced for food and feed. These may become available in the near future.

Table 5: Insect-specific virus taxa: potential agents of feed and food host

Lemma	Taxons (ICTV Release 2018)	Crickets, locusts	Flies	Caterpillars	Mealworms	ISV
	Family	*Orthoptera*	*Diptera*	*Lepidoptera*	*Coleoptera*	
	Species	*Acheta spp*	*Hermetia spp*	*Bombyx spp*	*Tenebrio spp*	
		Gryllus spp	*Musca spp*	*Galleria spp*	*Zophobas spp*	
		Locusta spp	*Chrysomya spp*	*Achroia spp*	*Albitobius spp*	
		Schistocerca spp				
1	*Almendravirus*, *Rhabdoviridae*					Y
2	*Alphavirus* (1), *Togaviridae; Eilat-group*					Y
3	*Anphevirus, Xinmoviridae*					Y
4	*Ascoviridae*			X		Y
5	*Baculoviridae*		X	XXX		Y
6	*Betairidovirinae, Iridoviridae*	X	X	X	X	N
7	*Bidnaviridae*					Y
8	*Bunyavirales* (6 genera from 3 families)				X	Y + P
9	*Densovirinae, Parvoviridae*	XXX		X		Y
10	*Dicistroviridae*	X	X	X	X	Y
11	*Entomobirnavirus, Birnaviridae*					Y
12	*Entomopoxvirinae, Poxviridae*			X		Y
13	*Flavivirus* (2), *Flaviviridae; dIFV+cIFV*					Y + P
14	*Hytrosaviridae*		X			Y
15	*Iflaviridae*	X	X	X	X	Y
16	*Mesoniviridae*					Y
17	*Negevirus, Mononegavirales*					Y
18	*Noravirus* (3)					Y
19	*Nudiviridae*	X	X			Y
20	*Polydnaviridae*		X			Y
21	*Reoviridae* (3 genera)			X	X	Y + N
22	*Sigmavirus, Rhabdoviridae*					Y
23	*Tetraviruses'* (4)			X		Y + N

Notes:
(1) Eilat Virus Group, three species:
(2) two subclades dISV and cDSV
(3) suggested genus author this report
(4) old name, no current taxon

Y = yes; N = No
P = provisional
ISV = Insect-Specific Virus
X = relative occurrence

DNA virus
RNA virus
Yes/No/provisional ISV

Weight-of-evidence assessment of ISV taxa

The various potential invertebrate virus taxa (Table 2) were assessed using a weight-of-evidence approach using the criteria outlined above. The results are tabulated for each individual taxon, including comments to support the assessment for the individual lemmas, which may contain multiple taxa (see Appendix). A comparative table is produced reflecting the individual taxon assessments (Table 6). Not all criteria could be assessed. Often no information is available ('nd' in the table). Sometimes the outcome of the assessment is ambiguous (x/- in the table). Not all criteria carry equal weight; some are qualitative (e.g. hematophagous host).

Eleven families and hence their respective genera and species are insect specific (ISV) in their entirety (Table 7). Most importantly, there is no evidence that members of these families replicate in vertebrates or vertebrate cells. In addition, these viruses have an unequivocal (deeply rooted) position in the phylogenetic trees (Table 6). Some of these 11 families are the highest taxons: *Alphatetra-* (lemma 23), *Asco-* (lemma 4), *Baculo-* (lemma 5), *Bidna-* (lemma 7), *Hytrosa-* (lemma 14), *Mesoni-* (lemma 16), *Nudi-* (lemma 19), *Permutotetra-* (lemma 23) and *Polydnaviridae* (lemma 20). Other ISV families are member of higher order taxa (orders): *Peribunyaviridae* (*Bunyavirales*) (lemma 8) and *Iflaviridae* (lemma 15) (*Picornavirales*). The latter also holds for two subfamilies and their respective genera and species, the *Denso-* (lemma 9) and *Entomopoxvirinae* (lemma 12). Some families *(Dicistroviridae*, lemma 10) or subfamilies (*Densovirinae*, lemma 9) have crustacean virus members and are invertebrate-specific.

The genera *Almendravirus* (lemma 1) *Anphevirus* (lemma 3), *Cypovirus* (lemma 21 *Dinovernavirus* (lemma 21), *Entomobirnavirus* (lemma 11), *Herbevirus* (lemma 8), *Idnoreovirus* (lemma 21), *Shangavirus* (lemma 8) and *Sigmavirus* (lemma 22) and their respective virus species are also clearly insect-specific by a combination of various criteria (Tables 6 and 7). *Negevirus* (pending ICTV approval) (lemma 17) and *Noravirus* (pending ICTV consideration) (lemma 18) have recently emerged virus taxa.

The bunyavirus families *Phenuiviridae* and *Peribunyaviridae* also contain genera with vertebrate specificity and cannot be considered insect-specific as a family (lemma 8). Of special interest are the subgroups or subclades within established vertebrate arbovirus-containing genera. Two of these subgroups (Eilat virus group, genus *Alphavirus* (lemma 2) and cISF group, genus *Flavivirus*, lemma 13) meet the criteria for insect specificity, including phylogeny (deep-rooting).

The family *Carmotetraviridae*, the subfamily *Betairidovirinae* and the genus *Seadornavirus* are not accepted as ISV, since viruses of these taxa replicate in vertebrate cells (lemmas 6, 21 and 23, Table 6). The 'dISF group' of the *Flavivirus* genus and the *Goukovirus* and *Phasivirus* genera (*Phenuiviridae*) are provisionally labeled ISV (lemmas 8 and 13b, Table 7). They are most likely ISVs, but their phylogenetic position within larger arbovirus clades is equivocal. The status of seadornaviruses is also ambiguous (lemma 21). There are mixed reports as to whether the latter replicate in vertebrate cells or not. In addition, their phylogenetic position (sandwiched between vertebrate reoviruses, in contrast to the genera *Cypovirus*, *Idnorovirus* and *Dinovernavirus*), is not in favor of insect-specificity. The latter viruses are possibly not insect-specific (Table 7).

Table 6: Weight of evidence assessment for insect-specific (ISV) status of virus taxa

Lemma	1	2	3	4	5	6	7	8	9	10	11	12	13a	13b	14	15	16	17	18	19	20	21	22	23
Insect-specificity																								
only isolated from insects	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
relation with vertebrate virus taxons	Y	Y	Y	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	N	N	N	N	Y	Y	N
evidence for infection of vertebrate animals	N	nd	nd	nd	N	Y	nd	N	N	nd	nd	nd	nd	N	N	nd	nd	N	N	N	nd	N	nd	nd
replication in vertebrate cells	N	N	N	nd	N	x/-	nd	N	N	nd	nd	N	N	N	nd	nd	N	N	nd	N	N	N	N	Y/N
monophyletic deeply-rooted clade within virus family	Y	Y	Y	Y	Y	Y	Y	Y/N	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
insect-specific entry/release/transmission mechanism	nd	nd	nd	Y	Y	N	Y	nd	Y	Y	nd	N	nd	nd	Y	nd	x/-	nd	nd	Y	Y	Y	nd	nd
unique genetic traits correlating with insect-specificity	Y	nd	N	nd	Y	N	Y	Y	nd	Y	Y	N	x/-	Y	nd	Y	nd	nd	nd	Y	Y	nd	Y	Y
unique genome structure	N	Y	N	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	N	Y	Y	N	Y	Y	Y	Y	N	N	Y
Pathogenicity																								
pathogenicity estimate (Low/Moderate/High)	L	L	L	M	H	M	M	H	H	M	L	M	L	M	L/H	L	L	L	M	M/H	L	M/H	L	L
hematophagous host(s) (Yes/No)	Y	Y	Y	N	N	Y	N	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	N	N	Y/N	N	N
within host tropism (Broad/Restricted)	nd	R	nd	R	R	B	R	nd	B	B	nd	B	B	B	R	B	nd	nd	R	R	R	R	R	R
host(s) include feed/food insects (Yes/No)	N	N	N	Y	Y	Y	Y	N	Y	Y	N	Y	N	N	N	nd	N	N	nd	N	N	Y	N	Y
endemic in NL (Yes/No)	N	N	N	nd	Y	Y	N	N	nd	Y	N	Y	N	N	x/-	Y	nd	nd	Y	N	Y	Y	N	N
persistent infections Yes/No	nd	Y	Y	N	Y	nd	nd	Y	Y	Y	Y	N	Y	Y	Y	Y	nd	Y	Y	Y	Y	Y	Y	Y
horizontal transmission (Yes/No)	Y	N	Y	Y	Y	Y	nd	Y	Y	Y	Y	Y	N	x/-	Y	Y	nd	N	Y	Y	N	Y	Y	Y
ISV status	Y	Y	Y	Y	Y	N	Y	Y/N	Y	Y	Y	Y	Y	Y/N	Y	Y	Y	Y	Y	Y	Y	Y/N	Y	Y/N

x/- = inconclusive evidence

nd = data not available

Table 7. Proposed Insect-Specific Virus Taxa

Lemma		families	families	subfamilies	subfamilies	genera	genera	groups	groups	species	species
		ISV	no / p ISV	ISV	no / p ISV	ISV	no / p ISV	ISV	no / p ISV	ISV	no / p ISV
1	*Almendravirus (rhabdo)*					1				5	
2	*Alphavirus*, Eilat group*							1		3	
3	*Anphevirus*					1				11	
4	*Ascoviridae*	1								4	
5	*Baculoviridae*	1								68	
6	*Betairidovirinae*				1		2				16
7	*Bidnaviridae*	1								1	
8	*Bunyavirales*		2 + 1p			4	4 + 6p			15	5 + 10p
9	*Densovirinae*					3	2			34	
10	*Dicistroviridae*	1								14	2
11	*Entomobirnavirus*					1				9	
12	*Entomopoxivirinae*			1						31	
13	*Flavivirus,* cISF group*							1		24	
	Flavivirus, dISF group*								1p		11p
14	*Hytrosaviridae*	1								2	
15	*Iflaviridae*	1								36	
16	*Mesoniviridae*	1								22	
17	*Negevirus**					1				26	
18	*Noravirus**					1				5	
19	*Nudiviridae*	1								7	
20	*Polydnaviridae*	1								53	
21	*Reoviridae*					3	1			80	3
22	*Sigmavirus (rhabdo)*					1				7	
23	*Tetravirales**	2	1							13	1
		11	3 + 1p	1	1	16	9 + 6p	2	1	470	27 + 21p

* not yet a taxon recognized within ICTV

p = provisional ISV

Table 7 represents the 11 families, 1 subfamily, 16 genera and 2 virus groups (and their respective genera and species), which can be assigned as ISV. This collectively encompasses 470 ISV species.

Among the investigated potential ISV taxa (Table 2) there are two special cases. The dISF and cISF flaviviruses (lemma 13) have not (yet) been considered separate flavivirus taxa, but they meet most if not all of the above criteria for being a genus. cISFs (currently 24 virus species) form a deeply rooted clade in the flavivirus phylogenetic tree and are definitely ISV. The dISF viruses (currently 11 virus species) do not (yet) meet the key criteria used for ISV (e.g. strong phylogeny). This group of viruses is in a positive 'flux' as a consequence of next-generation sequencing and biological information on these viruses is emerging. Therefore, they are proposed provisional ISV (light blue in Table 7).

Another case concerns viruses of the genera *Goukovirus* and *Phasivirus* (*Bunyavirales*, *Phenuiviridae*), which have insect specificity characteristics but are phylogenetically very close to arboviruses, thus requiring more information to meet the key criteria on insect specificity. Hence the genera *Goukovirus* and *Phasivirus* (6 viruses) and the dISF virus group (currently 11 viruses) are assigned as provisional ISV (Table 7).

Viruses of three taxa (*Carmotetraviridae*, *Betairidovirinae* and *Seadornavirus*), currently 40 species, replicate in vertebrates or vertebrate cells. Therefore, they are not considered ISV at this point (Table 7).

Conclusion

On the basis of an extensive literature review and a weight-of-evidence assessment based on predefined criteria, the following taxa (and their lower level taxa) can be considered to contain insect-specific viruses:

- Virus species of the ICTV families *Alphatetraviridae*, *Ascoviridae*, *Baculoviridae*, *Bidnaviridae*, *Dicistroviridae* (except two crustacean viruses), *Hytrosaviridae*, *Iflaviridae*, *Mesoniviridae*, *Nudiviridae*, *Permutotetraviridae* and *Polydnaviridae*.

- Virus species of the subfamily *Entomopoxvirinae*

- Virus species of the genera *Almedravirus*, *Ambidensovirus*, *Anphevirus*, *Brevidensovirus*, *Cypovirus*, *Dinovernavirus*, *Entomobirnavirus*, *Feravirus*, *Herbevirus*, *Idnoreovirus*, *Iteradensovirus*, *Jonvirus*, *Negevirus**, *Noravirus**, *Orthophasmavirus* and *Sigmavirus*

- Virus species of the *Eilat virus group (*genus *Alphavirus*, *Togaviridae*) and *cISF-group* (genus *Flavivirus*, *Flaviviridae*)

* not formally considered / approved by the ICTV

Recommendations

- To accept a list of virus species of the insect-specific taxa
- To review the literature on a regular basis because of the current and expected flux in new data
- To review the ICTV updates on a regular basis
- To review the respective lemmas when counter-indicative information becomes available
- To reappraise predefined criteria for 'IVS' specificity on a regular basis

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Appendix

23 lemmas on potential Insect-Specific Viruses (ISVs)

Appendix

Insect-Specific Viruses COGEM Report

Index

[ ] = not (yet) approved by the ICTV as taxon

1-*Almendravirus* (genus), *Rhabdoviridae* (family), *Mononegavirales* (order)

Almendraviruses belong to the *Rhabdoviridae* family. The latter is a large group of enveloped, single-stranded (ss) RNA viruses occurring in vertebrates, invertebrates and plants. Rhabdoviruses have a bullet-shaped morphology and contain a negative-sense RNA genome (Fig. 1). Among the rhabdoviruses are notable vertebrate pathogens, e.g. Lyssavirus, Vesicular stomatitis virus (VSV) and rabies virus. Many rhabdoviruses are transmitted by insects or ticks. This category of viruses is called arboviruses and will not be discussed here.

A few rhabdoviruses, more specifically members of the genus *Sigmavirus* and *Almendravirus* are only described for insects and mainly cause disease in *Drosophila* spp. and mosquito species, respectively. Almendraviruses are thought to be species-specific. The viruses were named after a city near Puerto Almendra in northern Peru, were the virus was first found (Vasilakis *et al.*, 2014). The genus *Almendravirus* was established in 2018 (ICTV 10th Report 2018) and is described here in search of ISV characteristics.

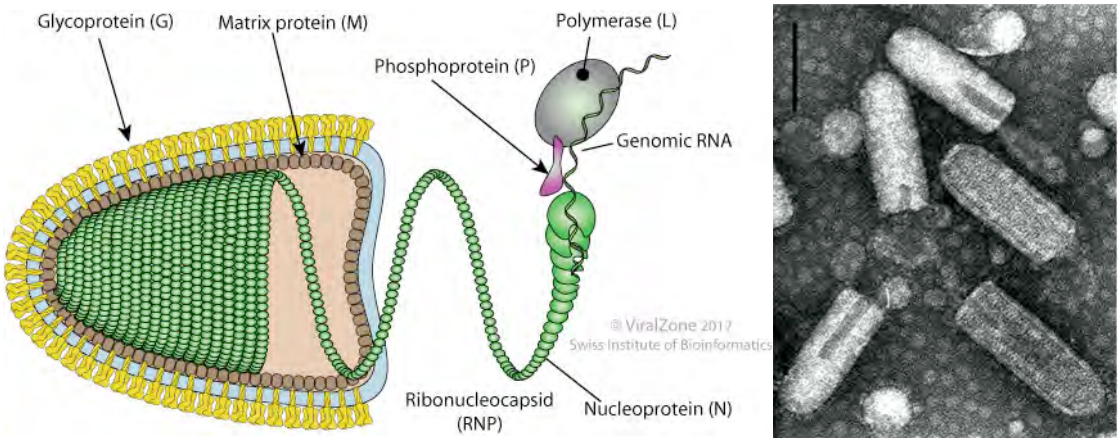

Figure 1. (Left) Diagram illustrating a rhabdovirus virion and the nucleocapsid structure (Source: ViralZone, 2018); (right) negative contrast electron micrograph of virions of an isolate of *vesicular stomatitis Indiana virus*. The bar represents 100 nm. From ICTV Taxonomy 10th Report, 2018).

Virions

Almendravirus virions are bullet-shaped, approximately a 130-300 nm long and 50 nm wide. They have an envelope with surface projections (G-protein) wrapped around a helical nucleocapsid containing a single negative-sense RNA molecule of about 11 kb in size and an RNA-dependent RNA polymerase (ICTV Taxonomy 10th Report 2018).

Genetics

The single negative-sense RNA genome is about 11.5 kb in size (Vasilakis *et al.*, 2014) with the general structure 3'-N-P-M-G-L-5 (N = nucleoprotein, P = phosphoprotein, M = matrix protein, G = glycoprotein and L = RNA polymerase). The proteins share sequence homology with cognate proteins from other rhabdoviruses. The RNA is associated with the L protein in a ribonucleoprotein complex (= RNP) in the virion in order to initiate transcription of the positive strand upon infection. A common character of almendraviruses is the presence of a small class 1a viroporin-like gene, sandwiched between the glycoprotein G and the RNA polymerase (Fig. 1, ICTV Taxonomy 10th Report, 2018).

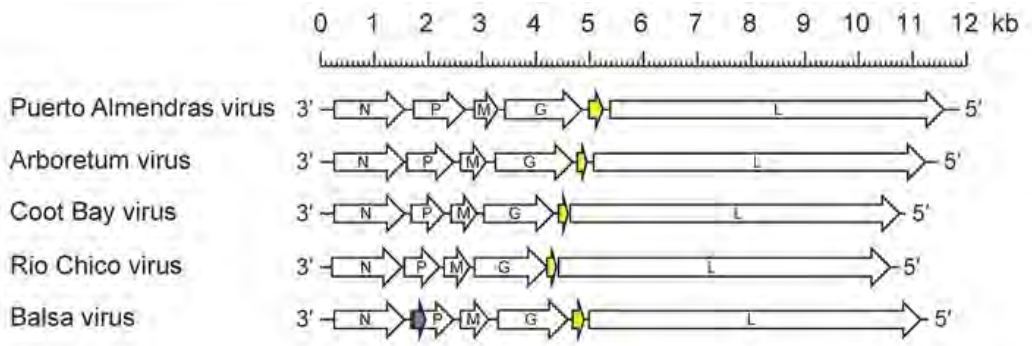

Figure 2. ***Almendravirus.*** Schematic representation of almendravirus genome organisations. N, P, M, G and L represent ORFs encoding the structural proteins. The U1 ORFs (yellow) each encode class 1a viroporin-like proteins, unique for ISV rhabdoviruses. (ICTV Taxonomy 10th Report, 2018).

Taxonomy

Rhabdoviruses are members of the *Mononegavirales* Order (Amarasinghe *et al.*, 2017; Walker *et al.*, 2018). Currently there are eighteen genera recognized within the *Rhabdoviridae* family, but members of only two genera, *Sigmavirus* and *Almendravirus*, are restricted to insects only. The almendravirus-like viruses have been described for mosquitoes in the Americas only. Five species are recognized by the ICTV (ICTV, 2018) (Table 1). *Puerto Almendras almendravirus* (PTAMV) is the type species for the genus *Almendravirus*.

Genus: *Almendravirus*
Species: *Arboretum almendravirus*
Species: *Balsa almendravirus*
Species: *Coot Bay almendravirus*
Species: *Puerto Almendras almendravirus*
Species: *Rio Chico almendravirus*

Table 1. Taxonomy of almendraviruses (ICTV Online, 2018)

Members of the genus *Almendravirus* form a monophyletic group within the *Rhabdoviridae* family (Fig. 3 in Bolling *et al.*, 2005, https://doi.org/10.3390/v7092851). However, they are well separated from arthropod (tick)-borne rhabdoviruses of vertebrates, e.g. *Bahia grande virus* and *Muir Springs virus*. An independent recent phylogenetic analysis (Fig. 5 in Contreras *et al.*, 2017, https://doi.org/10.4269/ajtmh.16-0403) confirms this observation.

Replication

Nothing is known about almendravirus replication or gene regulation, but considering their common structural genome organization with vertebrate and plant rhabdoviruses, they likely replicate and transcribe in a similar fashion. Notable is the presence of an almendravirus-specific viroporin-like ORF.

Pathobiology

Almendraviruses have been recently found in mosquitoes (Vasilakis *et al.*, 2014). Little is known about the biology of these viruses and their impact on mosquito health. Many have been isolated by their growth in mosquito C6/36 cells from mosquito extracts (Vasilakis *et al.*, 2014). Similar to most rhabdoviruses, almendraviruses replicate in the cytoplasm of infected cells and are released from the host cell by budding. It is not known whether almendraviruses are present in salivary glands of mosquitoes, potentially allowing them then to be transmitted via injection to vertebrates (hematophagy).

Ecology

Almendraviruses may be mosquito-specific. They have been found so far in the Americas and none have been reported from beyond dipterans. Nothing is known about the worldwide distribution or mode of transmission of almendraviruses, but vertical transmission is a likely due to the behavior of the mosquito.

Impact for human and animal health

Almendraviruses failed to grow in newborn mice or in a large selection of mammalian cell lines (Contreras *et al.*, 2017), but happily grow in C6/36 mosquito cells. This suggests they are insect-specific viruses.

Table 2: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Genus *Almendravirus;* Family *Rhabdoviridae*, Order *Mononegavirales*

	Yes / No	importance	specific comments
Insect-specificity			
only isolated from insects	Y	xx	mosquitoes; selection on cells
relation with vertebrate virus taxons	Y	x	rhabdovirus structure
evidence for infection of vertebrate animals	N	xxx	mice
replication in vertebrate cells	N	xxx	no effect
monophyletic deeply-rooted clade within virus family	Y	xx	sandwiched between rhabdoviruses
insect-specific entry/release/transmission mechanism			nd
unique genetic traits correlating with insect-specificity	Y	xx	viroporin-like protein
unique genome structure	N	x	rhabdovirus-like
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L		no obvious pathology
hematophagous host(s) (Yes/No)	Y		mosquitoes
within host tropism (Broad/Restricted)			nd, likely salivary glands
host(s) include feed/food insects (Yes/No)	N		
endemic in NL (Yes/No)	N		
persistent infections (Yes/No)			nd
horizontal transmission (Yes/No)	Y		very likely

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Genetic engineering

Almendraviruses have not (yet) been engineered.

Conclusion

On the basis of the available literature, almendraviruses have a restricted host range (mosquitoes), form a monophyletic, deeply-rooted clade within the *Rhabdoviridae* family and have no known effects on vertebrates. The observations have been summarized in Table 2.

Proposed status *Almendravirus* (*Rhabdoviridae*) species: Insect-Specific Virus (ISV)

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Proposed genus *Almendravirus* ISV species [family *Rhabdoviridae*]

Species	Virus name	Abbreviation
• *Arboretum almendravirus*	Arboretum virus	ABTV
• *Balsa almendravirus*	Balsa virus	BALV
• *Coot Bay almendravirus*	Coot Bay virus	CBV
• *Puerto Almendras almendravirus*	Puerto Almendras virus	PTAMV
• *Rio Chico almendravirus*	Rio Chico virus	RCHV

2-*Alphavirus* (genus) (Eilat virus group), *Togaviridae* (family)

The *Togaviridae* is a family of small, enveloped viruses with a positive stranded RNA genome ranging in size from about 11 to 12 kb. Most of these viruses infect vertebrate hosts and represent very pathogenic viruses, causing diseases such as chikungunya, encephalitis, rash and arthropathy. Terrestrial vertebrate togaviruses are transmitted by insects, mainly mosquitoes, and are therefore called arthropod-borne viruses (arboviruses).

Only three togaviruses are restricted to insects, more specifically to mosquitoes (Fig. 1; Nasar *et al.*, 2012; Hermanns *et al.*, 2017). These so-called insect-specific togaviruses (so far *Eilat virus*, *Mwinilunga alphavirus*, *Tai Forest alphavirus*) are provisionally named Eilat virus group (J.M. Vlak) and the topic of this report

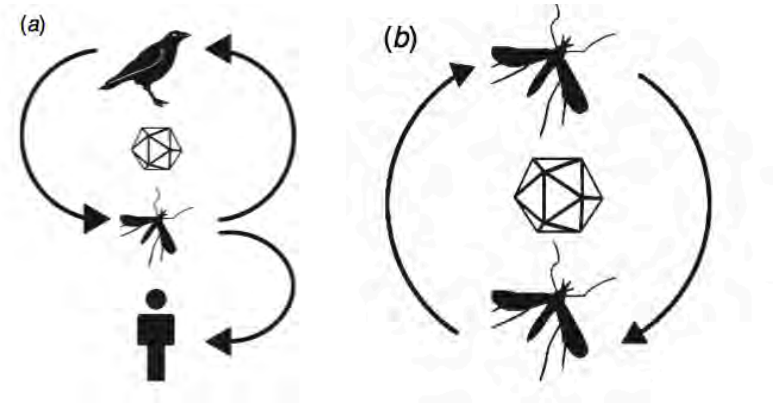

Figure 1. Mosquito-borne alphavirus transmission cycle and (b) proposed insect-specific alphavirus (ISV) transmission cycle (Source: Fig. 1a, Hall and Hobson-Peters, 2018, https://doi.org/10.1071/MA18020).

The name (*toga* = cloak) is derived from the envelope.

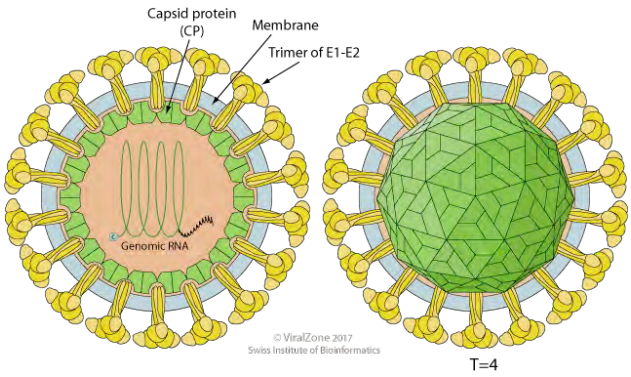

Figure 2. Togavirus virion. Source: ViralZone

The virions

Alphavirus virions are spherical in shape, about 70 nm in diameter and enveloped (Fig. 2). The core is about 40 nm in diameter. The virions consist of a lipid envelope with protein spikes, a matrix and an icosahedral nucleocapsid, the latter containing a positive, single stranded RNA molecule of about 11-12 kb in size. The virions have a one type of capsid

protein and one or more envelope proteins, which appear as spikes in a lipid bilayer (Chambers *et al.*, 1990).

Genetics

The togavirus genome encodes two large polyproteins, which are sequentially processed into monomeric proteins by host and viral-encoded proteases. The RNA molecule has a 5'-end Cap structure for translation initiation and a 3'-end polyA tail (Fig. 3). Members of the *Alphavirus* genus produce a subgenomic RNA from which structural proteins are expressed (Fig. 3; see also Jose *et al.*, 2009 and Griffin *et al.*, 2007, for review).

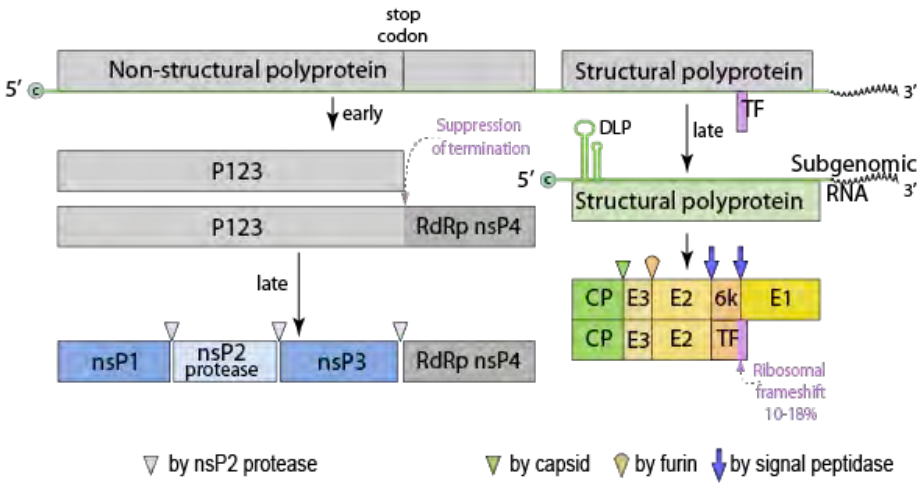

Figure 3. Genome organization and expression of members of the genus *Alphavirus*. Source: ViralZone

The insect-specific alphaviruses have low amino acid identity (ca. 30-60%) with other alphaviruses (Sindbis virus, western equine encephalitis virus) and a weak immunological cross-reaction with these exists (Nasar *et al.*, 2012). It has been suggested that the insect-specific alphaviruses lost their ability to grow / replicate in vertebrates (Nasar *et al.*, 2012).

Taxonomy

The *Togaviridae* family currently encompasses two genera: *Alphavirus* with currently 31 species (ICTV2018; Chen *et al.*, 2018) and genus *Rubivirus* (with one species: *rubellavirus*). Alphaviruses are mostly arboviruses, whereas *rubellavirus* is restricted to human hosts. Species demarcation is based on nucleotide and amino acid sequences (homology < 90%, phylogeny, Fig. 3, Hermanns *et al.*, 2917; https://doi.org/10.1099/jgv.0.000694), vector, host and disease association and ecology.

EILV and TALV are the only two ICTV-recognized alphaviruses restricted to insects, more specifically mosquitoes. Phylogenetic analyses (Fig. 4 in https://doi.org/10.1099/jgv.0.000694) place EILV and TALV in a deeply rooted monophyletic sister clade to the western equine encephalitis and *Sindbis virus* clade of mosquito-borne alphaviruses. Given the position of salmon pancreas disease virus, alphaviruses have been suggested to have a marine origin (Forester *et al.*, 2011). Recently, a third alphavirus (*Mwinilunga alphavirus* = MWAV) was identified from *Culex* mosquitoes, phylogenetically falling in the EILV group (Torii *et al.*, 2018).

The evolution of insect-specific alphaviruses (Eilat virus group) is currently unclear. This clade of viruses may have emerged from alphavirus arboviruses (Nasar *et al.*, 2012), which would fit their position in the phylogenetic tree between viruses of the Western Encephalitis Group en the Semliki Forest Virus complex. However, it cannot be excluded that insect-specific alphaviruses are basal to the more recent arboviral alphaviruses (Fig. 3 in https://doi.org/10.1099/jgv.0.000694).

Score plot analysis on the dinucleotide usage (CpG) of mosquito-borne alphaviruses with vertebrate hosts and alphaviruses with assumed insect-specific hosts (EILV, TALV, MWAV) indicate that the latter group is distinctly different from the former (Fig. 4; also Fig 3 in https://doi.org/10.1016/j.virusres.2018.04.005). Mosquito-borne alphaviruses have typical vertebrate CpG usage, whereas proposed alphavirus ISVs have a typical insect CpG usage.

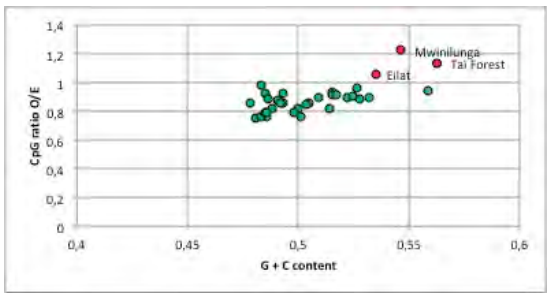

Figure 4: Score plots on the dinucleotide usage of vertebrate and mosquito-borne alphaviruses and insect-specific alphaviruses (EILV, TALV, MWAV). CpG ratio versus G+C content (courtesy Dr. J.J. Fros).

Replication

The replication of alphaviruses has been extensively studied. Much less is known about the replication of viruses of the Eilat group, but it is assumed they follow the togavirus (alphavirus) replication strategy producing polyproteins, which are subsequently processed into monomers and subgenomic RNAs encoding structural proteins. One of the proteins is a RNA-dependent RNA polymerase producing negative RNA strands, which are the templates for viral RNAs to be encapsidated into virions.

Pathobiology

EILV infects mosquitoes, primarily the mosquito posterior gut (Fig. 5; from https://en.wikipedia.org/wiki/Eilat_virus). TALV does not replicate in *Aedes* C6/36 cells nor in vertebrate VERO (monkey) cells (Hermanns *et al.*, 2017). In insect cells EILV grows to high titers (Fig. 5 in Nasar *et al.*, 2012; https://doi.org/10.1073/pnas.1204787109). No pathobiology information is available for TALV (Hermanns *et al.*, 2017).

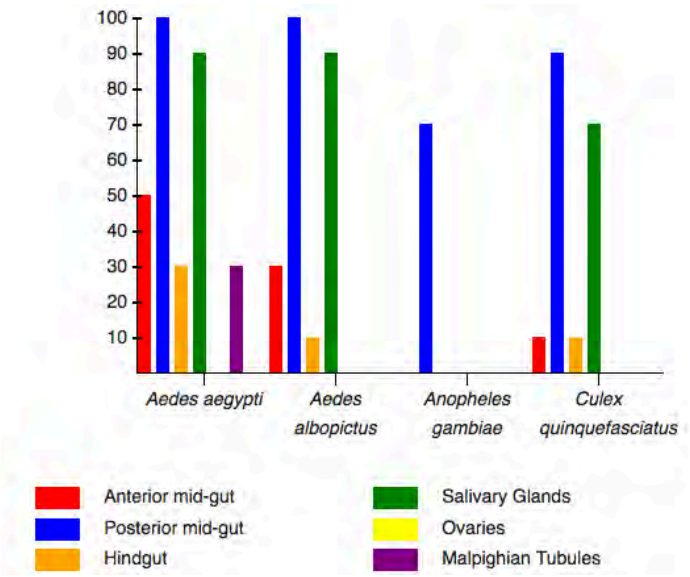

Figure 5. Tissue specificity of EILV expression. From https://en.wikipedia.org/wiki/Eilat_virus

EILV does not replicate in baby hamster and human embryonic kidney cells, duck embryo fibroblast and clawed frog cells, nor in infant mice (Fig. 5B in Nasar *et al.*, 2012, https://doi.org/10.1073/pnas.1204787109). Investigation into the nature of EILV vertebrate host range restriction demonstrated that the blocks are at the level of genomic RNA replication and at attachment and/or viral entry (Nasar *et al.*, 2015). EILV replicates in insect cells to high titers (Fig. 5A in Nasar *et al.*, 2012, https://doi.org/10.1073/pnas.1204787109), but with limited pathology. EILV has no effect on vertebrate animals (suckling mice).

Mosquitoes were susceptible for virus *per os* (EILV) at low efficiency; injections resulted in low efficiency infections (Nasar *et al.*, 2014). The viruses of the Eilat group also appear to have a narrow host range (specificity) among mosquitoes.

Ecology

Togaviruses can infect a wide range of invertebrates (vectors) and vertebrates, including fish. The terrestrial alphaviruses are insect-transmitted, mostly by mosquitoes. Transmission is mainly vertical, but at high doses also *per os*.

The insect-specific togaviruses, EILV and TALV, have been identified in Israel and Ivory Coast, respectively, and are restricted to mosquitoes (Nasar *et al.*, 2014). Recently a third virus in this group (*Mwinilunga alphavirus*) has been recently found in mosquitoes in Zambia (Tori *et al.*, 2018). Their prevalence in the field and ecological function is not known. It is likely that such mosquito-restricted viruses will be found more frequently in the (near) future, e.g. through next generation sequencing efforts or virome surveys.

Genetic engineering

Viral RNA (EILV) has been made via a cDNA clone, which was infectious for insect cells including the production of a cytopathic effect and subgenomic RNAs. Since the EILV is

mosquito restricted, it has been proposed to be used as a platform for safe human vaccines, e.g. against chikungunya (Erasmus *et al.*, 2018).

Impact for human and animal health

To date there are no indications that these insect-specific alpha viruses have any effect on humans or vertebrates as far as we know.

Relevant observations

Insect-specific Eilat virus group (Family *Togaviridae*, genus *Alphavirus*)

- encompass three species, *Eilat virus*, *Tai Forest alphavirus* and *Mwinilunga alphavirus*
- form a distinct subclade in the alphavirus phylogeny
- are species-specific (mosquitoes)
- display an insect-specific CpG profile
- have not been reported to enter / infect / replicate in vertebrate(s) / - cells specificity at various levels (entry, replication)

Conclusion

The conclusion reached is that, on the basis of available literature, the Eilat group of alphaviruses can be considered insect specific, without known effect on vertebrates (cells) and with specificity for mosquito species (restricted hosts). An insect-specific alphavirus subclade is also supported by phylogenetic analysis. Studies using chimeras between insect-specific and arboviral alphaviruses, e.g. to investigate the basis for insect specificity, may pose a higher risk. The observations have been summarized in Table 1.

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Eilat virus group, Genus *Alphavirus*, Family *Togaviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xx	mosquitoes; restricted
relation with vertebrate virus taxons	Y	x	alphaviruses, morphology, genome
evidence for infection of vertebrate animals			nd
replication in vertebrate cells	N	xxx	multiple cells, no effect
monophyletic deeply-rooted clade within virus family	Y	xxx	sandwiched between rhabdoviruses
insect-specific entry/release/transmission mechanism			nd, not investigated
unique genetic traits correlating with insect-specificity			nd
unique genome structure	Y	xxx	insect specific CpG
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L		no obvious pathology
hematophagous host(s) (Yes/No)	Y		mosquitoes
within host tropism (Broad/Restricted)	R	xx	posterior gut
host(s) include feed/food insects (Yes/No)	N		
endemic in NL (Yes/No)	N		
persistent infections (Yes/No)	Y		most likely
horizontal transmission (Yes/No)	N		very unlikely

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Proposed status 'Eilat Virus Group' species [Genus *Alphavirus*, Family *Togaviridae*]: Insect-Specific Virus (ISV)

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Proposed *Alphavirus* ISV species [family *Togaviridae*]**:**

Species	Virus name	Abbreviation
• *Eilat virus*	Eilat virus	EILV
• *Tai Forest alphavirus*	Tai Forest virus	TALV
• *Mwinilunga alphavirus*		MWAV

3-*Anphevirus* (genus), *Xinmoviridae* (family), *Mononegavirales* (order)

This group of insect-specific enveloped viruses (ISVs) represents a recently found new group of enveloped negative-strand RNA viruses in mosquitoes. They form a clear recently recognized taxon (*Anphevirus*), recognized by the ICTV within *Xinmoviridae* family, order *Mononegavirales* (Maes *et al.*, 2019). Anpheviruses are among the many new virus taxa found through next generation sequencing of invertebrates (Li *et al.*, 2015), e.g. the virome of mosquitoes (Zakrewski *et al.*, 2018).

Virions

The anpheviruses are enveloped viruses, presumably spherical and of unknown diameter. Virions have not yet been observed, so the model is still hypothetical (Fig. 1).

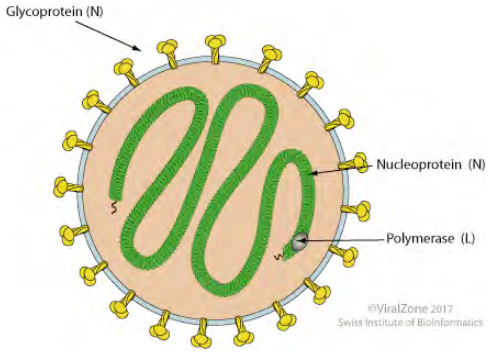

Figure 1. Hypothetical model of an anphevirus. Source: ViralZone

Genetics

The anphevirus viral RNA genome (about 12-14 kb, negative sense) contains at least three non-overlapping ORFs (ORF1-3) at the 5' end and a large RNA-dependent RNA polymerase (RdRp), called L, at the 3' end (Fig. 1a in de Giallonardo *et al.*, 2018, https://doi.org/10.1099/jgv.0.001079). ORF1 and 2 encode likely non-structural proteins, whereas ORF3, with an NXT motif, is likely an envelope protein. Three more small ORFs have been identified, but without functional significance (Fauver *et al.*, 2016). Nothing is known about the replication and expression strategy.

Anphevirus-like sequences have been identified in the *A. aegypti* genome proximal to long terminal repeat (LTR) sequences. They are currently considered endogenous virus elements (EVE's) and remnants of an earlier history of challenge with an ancestral anphevirus (Parry and Asgari, 2018).

Taxonomy

The current accepted taxonomy entails a single genus, *Anphevirus*, within the family *Xinmoviridae*, order *Mononegavirales* (ICTV 2018; Maes *et al.*, 2018). *Anphevirus* is a sigil

from Anopheles virus. So far, anpheviruses appear to be specific for mosquitoes. Seven species are recognized within the family *Xinmoviridae*, with *Xincheng anphevirus* (Li *et al.*, 2015) as type species. There are a few more tentative virus species in the *Anphevirus* genus because they are in the same phylogenetic clade as the seven recognized members of this genus (e.g. Aedes aegypti anpheviruses; (de Giallonardo *et al.*, 2018). Geographical virus lineages,exist from mosquitoes from around the world (ICTV 2017; Parry *et al.*, 2018). Viruses of the Anphevirus genus are distantly related to the recently established family *Chuviridae* (Li *et al.*, 2015; Fig. 2 in de Giallonardo *et al.*, 2018, https://doi.org/10.1099/jgv.0.001079) and distantly related (based on the RdRp) to members of the arboviral *Bornaviridae* and *Nyamiviridae* (Fig. 1C from Parry and Asgari, 2018, https://doi.org/10.1128/JVI.00224-18).

The anphevirus clade is clearly separated with bootstrap values > 85 from e.g. rhabdovirus-like viruses (Fig. 2 in de Giallonardo *et al.*, 2018, https://doi.org/10.1099/jgv.0.001079 and Fig. 1C from Parry and Asgari, 2018, https://doi.org/10.1128/JVI.00224-18. However, they are 'embedded' into a large group of yet unclassified viruses with unknown biological properties.

Pathobiology

Some mosquitoes and mosquito cell lines are persistently infected with anpheviruses. The cell-derived anphevirus is infectious for mosquitoes. Anpheviruses provoke a persistent RNAi response, which may protect mosquitoes against viral superinfections (Parry and Asgari, 2018).

Ecology

Anpheviruses are ubiquitously found in mosquitoes (*Anopheles*, *Aedes*) around the world, but also in some mosquito cell lines (Aag2 cells) (Parry and Asgari, 2018). In mosquitoes they are vertically transmitted and remain persistently present. The prevalence of anpheviruses in mosquitoes in the field is not known.

Genetic engineering

Engineering of anpheviruses via infectious RNA from cDNA has not been reported.

Impact for human and animal health

Anpheviruses (AeAV) are not infectious for vertebrate cells (Parish and Asgari, 2018). There are no reports studying effects of anpheviruses on humans or vertebrates.

Relevant observations

Anpheviruses

- encompass a monophyletic group (genus) of viruses, assigned to the *Xinmoviridae* family, *Mononegavirales* order
- form a distinct clade with distant relationship with the chuviviruses (proposed *Chuviviridae*) and rhabdovirus-like arboviruses
- persistently infect mosquitoes and mosquito cells
- are specific for *Diptera*, mosquitoes
- are unable (AeAV) to replicate in human cells
- have not (yet) been engineered

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Genus *Anphevirus*, Family *Xinmoviridae*, Order *Mononegavirales*

		importance	**specific comments**
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xx	mosquitoes; selection on cells
relation with vertebrate virus taxons	Y	x	negative strand viruses / arboviruses
evidence for infection of vertebrate animals		x	nd
replication in vertebrate cells	N	xxx	no effect
monophyletic deeply-rooted clade within virus family	Y	xx	close to arboviruses
insect-specific entry/release/transmission mechanism			nd
unique genetic traits correlating with insect-specificity	N	x	
unique genome structure	N	x	rhabdovirus-like
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L		no obvious pathology
hematophagous host(s) (Yes/No)	Y		mosquitoes
within host tropism (Broad/Restricted)			nd, likely salivary glands
host(s) include feed/food insects (Yes/No)	N		
endemic in NL (Yes/No)	N		
persistent infections (Yes/No)	Y		specific RNAi response
horizontal transmission (Yes/No)	Y		also vertical; persistent infections

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

The conclusion is that, on the basis of available literature, anpheviruses can be considered insect-specific viruses (ISVs), without any known effect on vertebrate cells and with specificity for mosquitoes (restricted hosts). The insect-specific anphevirus clade is strongly supported by phylogenetic analysis. The observations have been summarized in Table 1.

Proposed status genus *Anphevirus* (family *Xinmoviridae*) **species**: **Insect-Specific Virus**

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Proposed genus *Anphevirus* ISV species:

Species	Virus name	Abbreviation
• *Bolahun anphevirus*	Bolahun virus	BLAV
• *Dipteran anphevirus*	Hubei diptera virus	HdDV-11
• *Drosophilid anphevirus*	Drosophila unispina virus 1	DuniV-1
• *Odonate anphevirus*	Hubei rhabdo-like virus 7	HbRLV-7
• *Orthopteran anphevirus*	Hubei orthoptera virus 5	HbOV-5
• *Shuangao anphevirus*	Shuangao fly virus 2	SgFV-2-7
• *Xinchéng anphevirus**	Xinchéng mosquito virus	XcMV

Likely members of this genus (but not yet ICTV-approved)

• *Aedes aegypti anphevirus*		AeAV
• *Culex mononega-like virus 1*		CMLV1
• *Culex mononega-like virus 2*		CMLV2
•	Gambie virus	GAMV

* type species

4-*Ascoviridae* (family)

The family *Ascoviridae* accommodates a number of large, enveloped, double-stranded DNA viruses, which can infect lepidopteran larvae and their parasitic hosts. The name is derived from the virion-containing sac-like vesicles (Greek 'asco') circulating in the haemolymph upon infection. The most salient characteristic is the chronic nature of the disease, the unique vesicular pathology and the mode of transmission. A summary of the taxonomic status has been recently published by Asgari *et al.* (2017). Detailed information can be found at https://talk.ictvonline.org/ictv-reports/ictv_online_report/dsdna-viruses/w/ascoviridae.

The virions

Ascovirions are ovoidal, bacilliform or allantoid in shape and have a complex symmetry. Their size is about 200-400 nm in length and about 130 nm wide. They have a double presumably lipoprotein envelope, making these viruses sensitive to decay outside their hosts.
The virions contain a single double-stranded circular DNA molecule ranging in size from 110-200 kbp, depending on the species. In the case of the ascovirus HvAV-3i, 67 virus-encoded proteins are associated with the virions (Chen *et al.*, 2019). Ascoviruses share at least 45 open reading frames (homologous genes) with the *Betairidovirinae* (subfamily, lemma 6) and the virus family *Marseilleviridae*.

Genetics

Six ascovirus genomes have been sequenced to date (ICTV, 2018). Transcription occurs in a time-dependent manner in three temporal phases: early, late and very late (Zaghloul *et al.*, 2017). Also, multiple bi- and tricistronic mRNAs have been detected.

Replication

The ascovirus DNA replicates in the nucleus of infected cells, where the virions are assembled into minivesicles prior to nuclear rupture (Federici *et al.*, 1990). Virions are released from these vesicles into the hemolymph, where they are picked up by parasitic wasps for transmission to other larvae.

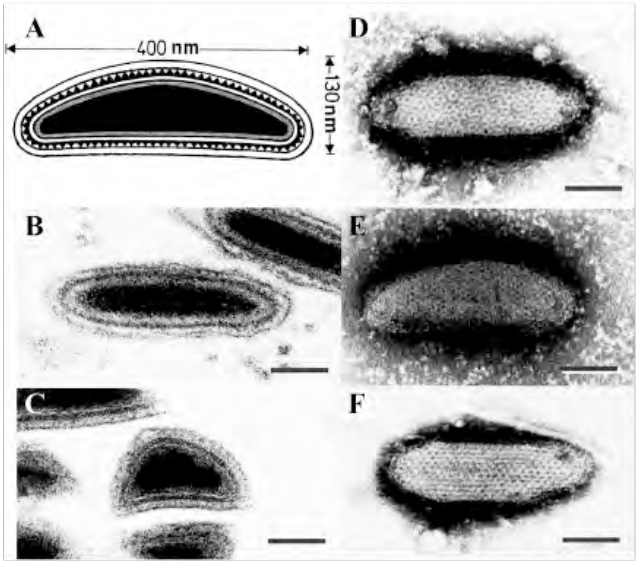

Figure 1. Ascovirus virions. Fig. 1 from *Ascoviridae* ICTV Online, 2018

Taxonomy

The taxonomy of ascoviruses is largely based on morphology and phylogenetic analysis (Asgari *et al.*, 2017). Two genera are currently recognized within the family *Ascoviridae*, *Ascovirus* and *Toursvirus*.

Family: *Ascoviridae*
Genus: *Ascovirus*
Species: *Heliothis virescens ascovirus 3a*
Species: *Spodoptera frugiperda ascovirus 1a*
Species: *Trichoplusia ni ascovirus 2a*
Genus: *Toursvirus*
Species: *Diadromus pulchellus toursvirus*

Table 1. Taxonomy of *Ascoviridae* ICTV-Online, 2018

The Genus *Ascovirus* accommodates three recognized species, *Spodoptera frugiperda ascovirus 1a* (SfAV-1a), *Trichoplusia ni ascovirus 2a* (TnAV-2a and *Heliothis virescens ascovirus 3a* (HvAV-3e). Many genetic variants of these three type members are found, e.g. HvAV-3f, HvAV-3g and HvAV3j (Arai *et al.*, 2018). The genus *Toursvirus* contains only one member so far, *Diadromus pulchellus toursvirus* (DpAV-4) (Bigot *et al.*, 1997). Whereas the members of the genus *Ascovirus* are found in (some) noctuids (*Lepidoptera*), DpAV-4, the only current member of the genus *Toursvirus*, is found in yponomeutids ('vlindermotten', 700 mostly tropical species).

Phylogenetic analysis using nine shared core genes indicates that the *Ascoviridae* form a distinct clade, well separated from closely related members of the family *Iridoviridae* and *Marseilleviridae* (Bigot *et al.*, 2009; Piegu *et al.*, 2015) (Fig. 2). Iridoviruses and ascoviruses have likely evolved from a common ancestor shared with marseilleviruses. The latter group contains (giant) viruses of amoebas, but only a single member of the latter family is found in insects (Boughalmi *et al.*, 2013).

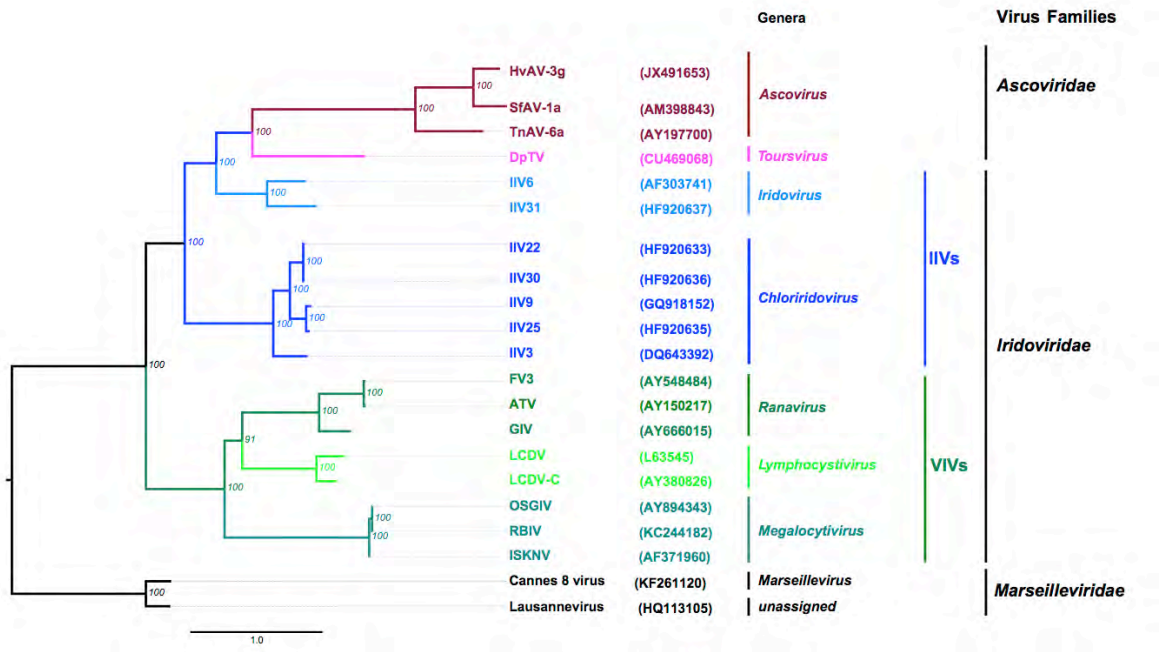

Figure 2. Phylogenetic tree (amino acids) of nine concatenated conserved genes of ascoviruses, iridoviruses and marseilleviruses. Fig. 2 from *Ascoviridae* ICTV Online, 2018

Pathobiology

Ascovirus infections in Lepidopteran larvae are usually chronic and can cause retarded larval development (stunting) and loss of appetite. Their appearance is whitish discoloured due to the accumulation of large amounts of virus-containing vesicles in the hemolymph (diagnostic tool). Therefore ascovirus-infected insects are difficult to find in nature. Ecological surveys for the presence and prevalence of ascoviruses in insects in nature have not been done so far.

A unique property of ascoviruses is the apoptotic fragmentation of infected nuclei of haemocytes into virion-containing vesicles. These vesicles circulate in the hemolymph of infected animals and give these a whitish appearance.

Ascoviruses can grow in cultured insect cells. Their effects on vertebrate cells or vertebrate animals have not been reported.

Ecology

Ascoviruses are mainly found in Lepidoptera. They are likely species-specific (Hamm *et al.*, 1986) and have only been found in members of a limited number of lepidopteran insect families: *Noctuidae* (*Helicoverpa zea, Trichoplusia ni, Spodoptera frugiperda*), *Crambidae, Plutellidae* and *Yponomeutidae* (DpAV-4) with low field prevalence The impact of ascoviruses on insect ecology has not been assessed, but is likely low.

Ascoviruses are, in contrast to other large double-stranded DNA viruses, transmitted via parasitic wasps (Tillman *et al.*, 2004, Arai *et al.*, 2018) and are hardly, if at all, transmitted *per os*. However, it is highly unlikely that they have an effect on vertebrates or lower animals other than insects. Parasitic wasps have never been associated with vertebrates or vertebrate diseases.

Genetic engineering

Engineering of ascoviruses has not been reported to date.

Impact on human and animal health

There are no specific reports on effects of ascoviruses on humans and other vertebrates or vertebrate cells *in vitro*. These viruses are only transmitted to insects by insect-specific parasitic wasps and very seldom come into contact with vertebrates (other than incidental eating). Also the entry mechanism (via injection) is highly insect-cell specific and is unlikely to occur in vertebrate cells.

Relevant observations

Ascoviruses

- occur worldwide and mainly in lepidopteran larvae.
- are rarely observed in nature (rare) and have not been reported in insect rearings as a morbidity/mortality factor.
- multiply in insects and insect cells, but their effect on vertebrate cells has not been reported.
- have been genetically characterized, but genetic engineering has not been reported.
- have only very distant vertebrate relatives (iridoviruses; gene level).
- likely have low impact on insect ecology.

Table 2: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Family *Ascoviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	lepidopteran larvae only
relation with vertebrate virus taxons	N	xxx	none, a bit to invertebr. iridoviruses
evidence for infection of vertebrate animals		x	nd, specific transmission in insects
replication in vertebrate cells		xxx	nd, unlikely; insect-dependent replication
monophyletic deeply-rooted clade within virus family	Y	xxx	family highest taxon; no vert. relative
insect-specific entry/release/transmission mechanism	Y	xx	injection; vesiculation of haemocytes
unique genetic traits correlating with insect-specificity		x	nd, replic. mechanism (large dsDNA)
unique genome structure	Y	xx	large circular dsDNA; repeat regions
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M		often chronic; slow
hematophagous host(s) (Yes/No)	N	x	only Lepidoptera
within host tropism (Broad/Restricted)	R		species-specific; haemocytes as target
host(s) include feed/food insects (Yes/No)	Y		possibly, low chance and dose
endemic in NL (Yes/No)			nd, yet possibly
persistent infections (Yes/No)	N		chronic
horizontal transmission (Yes/No)	Y	xxx	via parasitic wasps

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

The conclusion reached is that, on the basis of available literature, ascoviruses can be considered very specific viruses of insects, with no effect on organisms beyond the arthropod (insect) kingdom. The observations have been summarized in Table 2.

Proposed status *Ascoviridae* species: Insect-Specific Virus (ISV)

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Prosposed *Ascoviridae* ISV species:

Species	Virus name	Abbreviation
genus *Ascovirus*		
• *Heliothis virescens ascovirus 3a*		HvAV-3a
• *Spodoptera frugiperda ascovirus 1a*		SfAV-1a
• *Trichoplusia ni ascovirus 2a*		TnAV-2a
genus *Toursvirus*		
• *Diadromus pulchellus toursvirus*	Diadromus pulchellus virus	DpAV-4

5-*Baculoviridae* (family)

The family *Baculoviridae* accommodates large enveloped double-stranded DNA (dsDNA) viruses that infect only lepidopteran, hymenopteran and dipteran insects (Herniou *et al.*, 2011; Harrison *et al.*, 2018). The most notable feature is the occlusion body (OB), which is visible with the light microscope and contains the baculovirus virions (Fig. 1). There are about 600+ isolates described (Martignoni and Iwai, 1981), but this is likely the 'tip of the iceberg', as mostly agronomically important insect species have been investigated. Baculoviruses are successfully used as biocontrol agents of insect pests for over seven decades in agriculture, horticulture and forestry, and many are commercially available (e.g. to combat codling moth in European orchards) (Erlandson, 2008). In addition, for over three decades these viruses have been genetically modified and used as vectors for the expression of recombinant proteins e.g. for therapeutics and vaccines, for over three decades (Van Oers *et al.*, 2015). The name of this group of viruses is derived from 'baculum' reflecting the rod-shaped architecture of baculovirus virions.

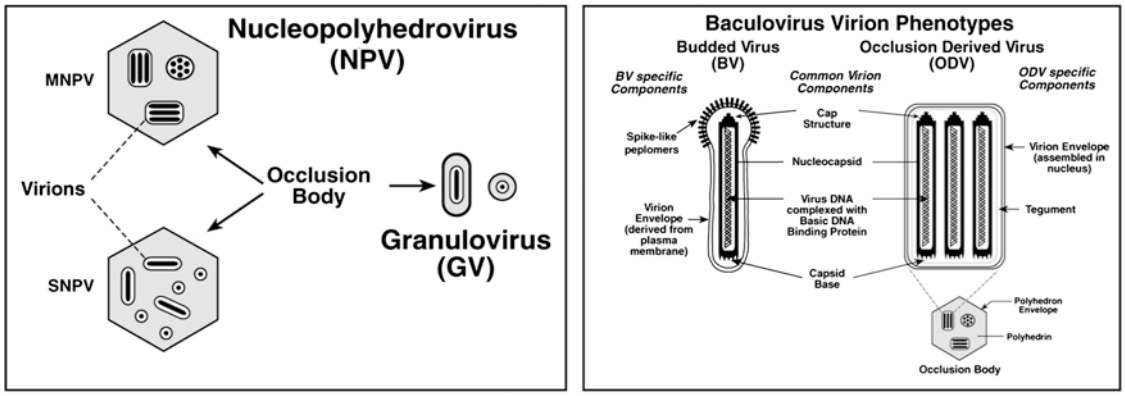

Figure 1. Baculovirus occlusion bodies, virions and nucleocapsids. (Upper left) The structures of occlusion bodies from baculoviruses in the genera *Alphabaculovirus* (nucleopolyhedrovirus, NPV) and *Betabaculovirus* (granulovirus, GV) are illustrated. Virions embedded in nucleopolyhedrovirus occlusion bodies may contain single nucleocapsids (SNPV) or multiple nucleocapsids (MNPV). (Upper right). The two baculovirus virion phenotypes are illustrated as diagrams with shared and phenotype-specific components. (Fig 1, ICTV 2011, 9th Report).

The virions

Baculovirus virions are rod-shaped particles measuring about 60 nm wide and 300 nm long. There are two types of virion phenotypes. The occlusion body-derived virions or ODVs, which are occluded in proteinaceous OBs. These OBs are responsible for the horizontal, insect-to-insect transmission. A second form is the budded virus or BV, which is produced within the infected insect and is responsible for the systemic spread of the virus within the organisms. The virions consist of a proteolipid envelope, a tegument and nucleocapsids, the latter containing a double stranded circular DNA molecule ranging in size, depending on the species, from 80-180 kbp. Genetically ODVs and BVs are identical, but their phenotype and proteomes are very different reflecting their genesis and purpose. The BV form can be used to infect insect cells. Notable is the presence of a so-called *per os* infectivity (PIFs) complex in ODV envelopes, essential for oral infection (Boogaard *et al.*, 2018), and a single type III envelope fusion protein, GP64, in the envelope of BVs to obtain entry into host cells.

Genomics

The baculoviruses genome is a large circular double-stranded DNA molecule, encodes about 70 to 160 non-overlapping open reading frames (ORFs) depending on the species, equally distributed from both strands. There are four transcriptional classes of genes: immediate early, delayed early (both before DNA replication), late and very late, finally leading to OB synthesis. Each of these transcriptional classes has a specific gene promoter, but all have polyadenylated transcripts. The baculovirus genome is further characterized by the presence of a variable number of intergenic regions, each with a variable number of homologous repeats (hrs) that are present in all hrs. They function as enhancers of transcription and origins of replication.

All currently completely sequenced baculoviruses share 38 genes that are called 'core genes' (Wennmann *et al.*, 2018). Among these are the previously mentioned PIF genes, some virion proteins and genes for DNA replication and RNA transcription. These core genes are used to infer baculovirus relatedness (Caravaglio *et al.*, 2013; Thézé *et al.*, 2018) and assist in baculovirus taxonomy. For many baculoviruses genetic variants are found both in field isolates as well as within each isolate.

Taxonomy

The taxonomy of baculoviruses is primarily based on gene content, phylogenetic relatedness and host range (Jehle *et al.*, 2006; Herniou *et al.*, 2011). Four genera are currently recognized within the family *Baculoviridae* (Table 1) Baculoviruses of the genera *Alphabaculovirus* and *Betabaculovirus* are found in lepidopteran insects and represent nucleopolyhedroviruses (multiple virions per OB, either of the single- or multiple-nucleocapsid type) and granuloviruses (a single virion per OB), respectively. Baculoviruses of the genera *Gammabaculovirus* and *Deltabaculovirus* are found in hymenopteran (sawflies) and dipteran (mosquito) insects, respectively. Sixty-eight baculovirus species are recognized in the latest (10^{th}) ICTV Report for alpha- (40), beta- (25), gamma- (2) and deltabaculoviruses (1). Recently, another 76 alphabaculoviruses and 20 betabaculoviruses are proposed as baculovirus species (Thézé *et al.*, 2018) giving a total of 165 baculovirus species to date. The species demarcation is defined by the Kimura two-parameter nucleotide distance comparison at < 0.015 (Wennmann *et al.*, 2018). Phylogenetic analysis using the 37 concatenated core genes (Caravaglia *et al.*, 2013) concatenated results in a robust baculovirus tree topology confirming the classification into four genera and providing information for the subclade structure (monophyletic groups within these genera.

The Lepidoptera-specific genera *Alphabaculovirus* and *Betabaculovirus* are sister groups and diversified after the diversification of insect orders in the mid-Mesozoic times. Gammabaculoviruses are the most ancient and are gut-restricted. Phylogenetic analysis using concatenated conserved ORFs shared among a number of large invertebrate circular double stranded DNA viruses (nudiviruses, hytrosaviruses) shows that baculoviruses form a distinct clade (Fig. 2). Fifteen of the baculovirus core genes are shared with the *Nudiviridae*, including some of the PIF genes, suggesting common ancestry. Baculoviruses are also distinct from the *Polydnaviridae*, endoparasitic dsDNA viruses.

The baculovirus *Autographa californica multiple-nucleocapsid nucleopolyhedrovirus* (AcMNPV) is the type species of the *Baculoviridae*. The latest on baculovirus taxonomy can be found at the ICTV website (2018.002D.N.v1.Baculoviridae_8sp.zip)

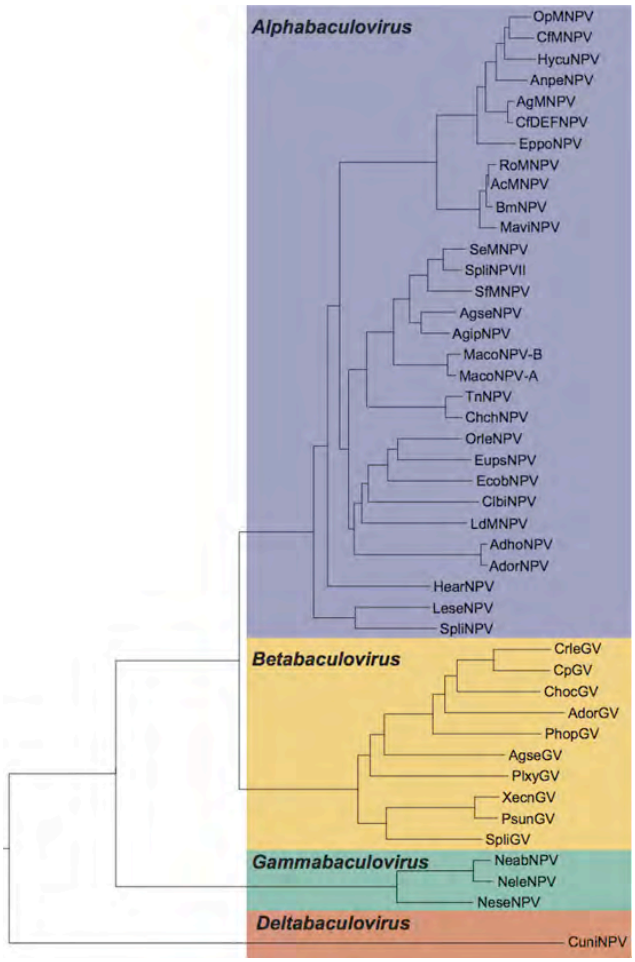

Figure 2. Phylogeny of the *Baculoviridae*. The maximum likelihood tree, based on the alignment of 30 genes, shows the relationships of the 39 species for which a completely annotated genome was available at time of analyses. (Fig. 4 in ICTV 2011, 9th Report). The most recent phylogeny (Thézé *et al.*, 2018) does not differ in main structure, but in details

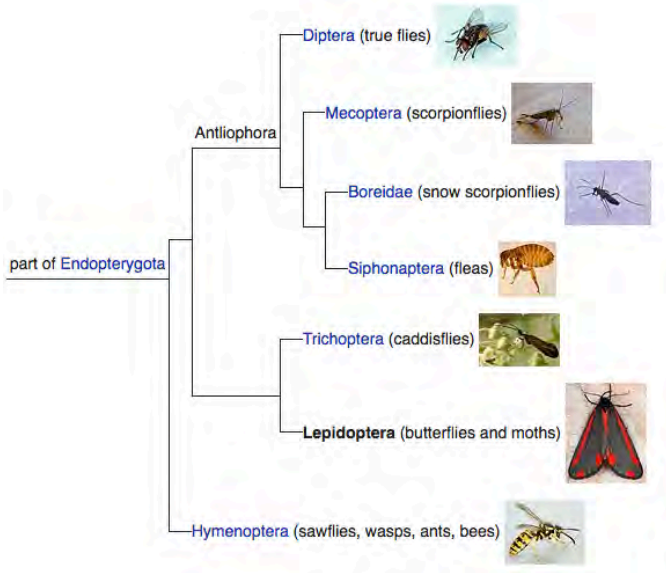

Figure 3. Cladogram of part of the *Endopterygota*, accommodating the orders *Diptera*, *Lepidoptera* and *Hymenoptera* (Yeates and Wiegman, 2016; Wikipedia, *Lepidoptera*, 2018).

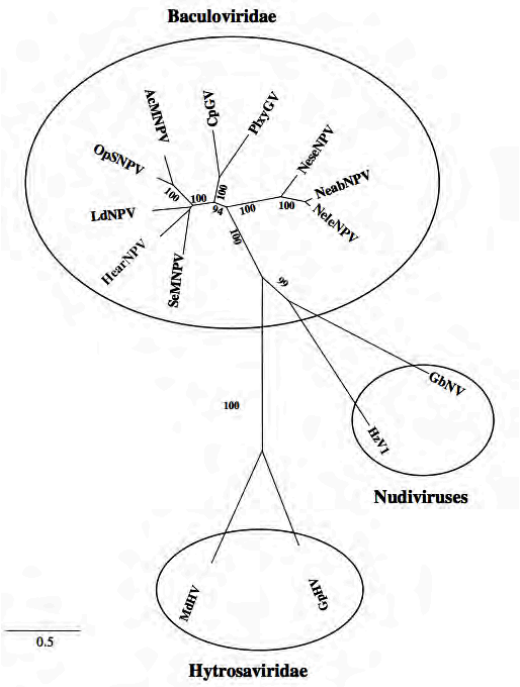

Figure 4. Neighbor-Joining (NJ) trees of concatenated predicted amino acid sequence of four *pif* genes (*p74, pif-1, pif, 2 and pif-3*) (Fig. 3 in https://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/invertebrate-official/4101).

There is no (phylo)genetic relationship with any of the vertebrate DNA viruses to date.

Biology

Baculovirus infection initiates in the midgut epithelial cells, where ODVs are released from the OBs as a consequence of the alkalinity of the midgut fluid. After entry of the ODV via the PIF complex (which is very specific for baculoviruses), the virus replicates in these midgut cells to produce BVs, which move to the interior of the insect via trachea or directly to cause a systemic infection. The major target is the fat body, where OBs are produced. At the end of the infection the larval body disintegrates and releases about 10E9 OBs per larva in the environment (4^{th}/5^{th} instar). Baculoviruses are highly infectious (for larvae only, not moths/butterflies). A few OBs are sufficient to kill 1^{st} instar larvae. This process / disease is called polyhedrosis / granulosis. These freed OBs are then able to infect other larvae to close the multiplication cycle (Blissard and Theilmann, 2018).

Within the fat body cells of the larvae (or insect cells in general) baculovirus replication occurs in two phases. In the first phase baculovirus DNA replicates in the nucleus of infected host cells, produce nucleocapsids there, which are transported to cell envelope to acquire an envelope in the exit to the exterior of the cells as BV. BVs are responsible for systemic infection of the larval body of the cell culture. In the second phase, when BV production is shut off, virions are assembled in the nucleus to be occluded into OBs. Virus-specific chitinases and proteases assist in disintegration of the cells (lysis) and release of OBs. The genesis of BVs versus ODVs is reflected in the proteome of these genetically identical phenotypes.

Ecology

Baculoviruses occur world-wide in insects and are being used as control agent of insect pests as alternative to chemical insecticides. Only larvae (caterpillars) are orally susceptible to these viruses. Baculoviruses can be transmitted horizontally (insect-to-insect) as well as vertically (to offspring) (Cory, 2015; Myers and Cory, 2015). In nature baculoviruses are usually present in insects in a latent or covert state (not yet known how), but can also 'survive' in the soil (> 20 years) (Burden *et al.*, 2003). The conditions in the field, which induce an overt infection are not well understood, but overpopulation of insects in combination of food shortage are a trigger to cause a polyhedrosis (= disease). As such baculoviruses can regulate the size of insect populations in the field. Insects can develop resistance to baculoviruses when overused, but which can easily overcome by using alternative virus strains.

Genetic engineering

Baculoviruses can in principle be easily genetically engineered in the quest for improved insecticidal activity, for gene function research and for expression of recombinant proteins (for vaccines and therapeutics). More specifically, AcMNPV, Bomyx mori NPV, Spodoptera exigua MNPV and Helicoverpa armigera MNPV have been genetically altered by virtue of their ability to be inserted into a bacterial plasmid (bacmid) for easy recombination, to be transfected into cells / larvae and their easy growth in cell culture. When used as an expression vector of foreign proteins, the baculovirus is usually 'disarmed' by removal of the polyhedrin gene, encoding the matrix of OBs (Fig. 5). Due to such deletions the baculovirus cannot produce OBs and cannot physically or ecologically survive in the environment.

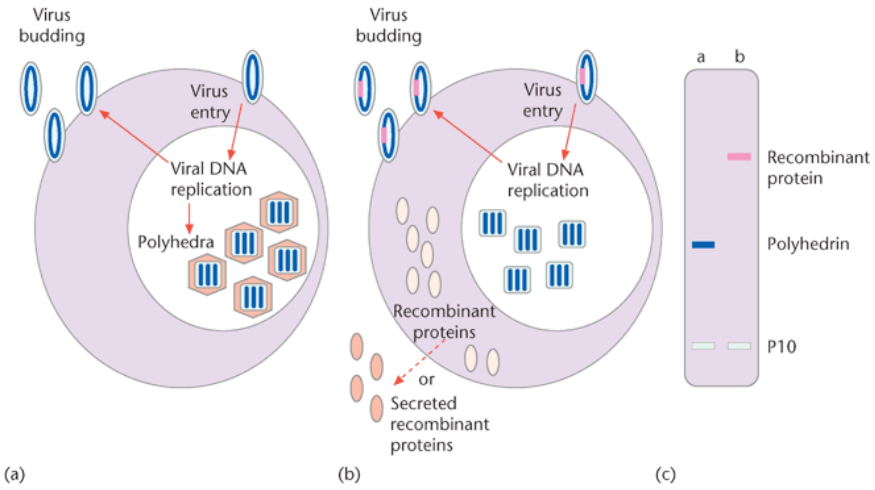

Figure 5. Baculovirus insect cell expression system. In cells infected with a wild-type virus (a) large amounts of polyhedra and *p10* are formed. In most cases in the recombinant virus (b) the polyhedrin and/or *p10* promoter is used to drive the expression of a foreign gene and the gene cassette replaces the polyhedrin gene, resulting in a recombinant with a polyhedron-negative phenotype and (c) high levels of recombinant protein are produced instead of polyhedrin (lanes 1 and 2). From: Van Oers *et al.*, 2015)

Impact for human and animal health

Baculoviruses have been extensively used for over seven decades, without any known clinical case reports in humans and other vertebrates. They have also been extensively tested for

safety for vertebrates. Baculoviruses are promoted by FAO/WHO as safe alternative to chemical insecticides and have received QPS (Qualitative Presumption of Safety) status from the European Food Safety Agency (Leuschner et al., 2010; Herman *et al.*, 2018).

Relevant information

Baculoviruses

- occur worldwide, in a restricted number of insect families (*Lepidoptera*, *Hymenoptera*, *Diptera*)
- are highly specific for (a few related) species; molecular basis specificity largely unknown
- are highly virulent and can cause large epidemics in insect species in the field
- have a unique oral entry mechanism (PIF); alkali-specific (in contrast to vertebrates),
- cannot infect / replicate in vertebrates, only entry in vertebrate cells
- have been engineered to improve efficacy, at the expense of ecological fitness
- have been used 60+ years to control target insects and have a perfect safety profile
- are widely used as vector for the expression of recombinant proteins (disarmed for oral infectivity in insects), which are considered safe for vertebrate us (vaccines)

Conclusion

The conclusion is reached that, on the basis of available literature, baculoviruses can be considered insect-specific viruses, without any known effect on vertebrates or non-target insects. The observations have been summarized in Table 1.

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Family *Baculoviridae*

		importance	**specific comments**
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	lepidop-, dip-, hymenopterans only
relation with vertebrate virus taxons	N	xxx	none
evidence for infection of vertebrate animals	N	xxx	none, OBs acid-insensitive
replication in vertebrate cells	N	xx	only erratic transcription; no integrat
monophyletic deeply-rooted clade within virus family	Y	xxx	family highest taxon; no vert. relative
insect-specific entry/release/transmission mechanism	Y	xxx	specific entry (PIF); alk.sens OBs
unique genetic traits correlating with insect-specificity	Y	xxx	replic. mechanism (large dsDNA)
unique genome structure	Y	xxx	large circular dsDNA; repeat regions
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	H		for target insects; age-depend larvae
hematophagous host(s) (Yes/No)	N		
within host tropism (Broad/Restricted)	R	xx	species-specific; fatbody as target
host(s) include feed/food insects (Yes/No)	Y		contaminated cultures
endemic in NL (Yes/No)	Y		some baculovirus host species
persistent infections (Yes/No)	Y		mechanism unknown
horizontal transmission (Yes/No)	Y		very common; basis for biocontrol

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Proposed status *Baculoviridae* species: Insect-Specific Virus (ISV)

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Proposed *Baculoviridae* ISV species:

Species	Abbreviation
Genus *Alphabaculovirus*	
• *Adoxophyes honmai nucleopolyhedrovirus*	AdhoNPV
• *Agrotis ipsilon multiple nucleopolyhedrovirus*	AgipNPV
• *Agrotis segetum nucleopolyhedrovirus A*	AgseNPV-A
• *Agrotis segetum nucleopolyhedrovirus B*	AgseNPV-B
• *Antherea pernyi nucleopolyhedrovirus*	AnpeNPV
• *Anticarsia gemmatalis multiple nucleopolyhedovirus*	AgMNPV
• *Autographa californica multiple nucleopolyhedrovirus**	AcMNPV
• *Galleria mellonella multiple nucleopolyhedrovirus*	GmMNPV
• *Plutella xylostella nucleopolyhedrovirus*	PlxyNPV
• *Rachiplusia ou multiple nucleopolyhedrovirus*	RoMNPV
• *Spodoptera exempta nucleopolyhedrovirus*	SpexNPV
• *Trichoplusia ni multiple nucleopolyhedrovirus*	TnMNPV
• *Bombyx mori nucleopolyhedrovirus*	BmNPV
• *Buzura suppressaria nucleopolyhedrovirus*	BuzuNPV
• *Catopsilia pomona nucleopolyhedrovirus*	CapoNPV
• *Choristoneura fumiferana DEF multiple nucleopolyhedrovirus*	CfDefNPV
• *Choristoneura fumiferana multiple nucleopolyhedrovirus*	CfMNPV
• *Choristoneura murinana nucleopolyhedrovirus*	ChmuNPV
• *Choristoneura rosaceana nucleopolyhedrovirus*	ChroNPV
• *Chrysodeixis chalcites nucleopolyhedrovirus*	ChchNPV

- *Chrysodeixis includens nucleopolyhedrovirus* ChinNPV
- *Clanis bilineata nucleopolyhedrovirus* ClbiNPV
- *Ectropis obliqua nucleopolyhedrovirus* EcobNPV
- *Epiphyas postvittana nucleopolyhedrovirus* EppoNPV
- *Euproctis pseudoconspersa nucleopolyhedrovirus* EupsNPV
- *Helicoverpa armigera nucleopolyhedrovirus* HearNPV
    - *Helicoverpa zea multiple nucelopolyhedrovirus* HzNPV
- *Hyphantria cunea nucleopolyhedrovirus* HycuNPV
- *Lambdina fiscellaria nucleopolyhedrovirus* LafiNPV
- *Leucania separata nucleopolyhedrovirus* LeseNPV
- *Lymantria dispar multiple nucleopolyhedrovirus* LdMNPV
- *Lymantria xylina nucleopolyhedrovirus* LyxyNPV
- *Mamestra brassicae multiple nucleopolyhedrovirus* MbMNPV
    - *Pannolis flammea nucleopolyhedrovirus* PaflNPV
- *Mamestra configurata multiple nucleopolyhedrovirus A* MacoNPV-A
- *Mamestra configurata multiple nucleopolyhedrovirus B* MacoNPV-B
- *Maruca virtrata nucleopolyhedrovirus* MaviNPV
- *Mythimna unipuncta nucleopolyhedrovirus* MyunNPV
- *Orgyia leucostigma nucleopolyhedrovirus* OrleNPV
- *Orgya pseudotsugata multiple nucleopolyhedrovirus* OpMNPV
- *Orgya pseudotsugata single nucleopolyhedrovirus* OpSNPV
- *Spodoptera exigua multiple nucleopolyhedrovirus* SeMNPV
- *Spodoptera frugiperda multiple nucleopolyhedrovirus* SfMNPV
- *Spodopteras littoralis nucleopolyhedrovirus* SpliNPV
- *Spodoptera litura nucleopolyhedrovirus* SpltNPV
- *Sucra jujuba nucleopolyhedrovirus* SujuNPV
- *Thysanoplusia orichalcea nucleopolyhedrovirus* ThorNPV
- *Trichplusia ni single nucleopolyhedrovirus* TnSNPV
- *Wiseana signata nucleopolyhedrovirus* WisiNPV

Genus *Betabaculovirus*

- *Adoxophyes orana granulovirus* AdorGV
- *Agrotis segetum granulovirus* AgseGV
- *Artogeia rapae granulovirus* ArGV
- *Choristoneura fumiferana granulovirus* ChfuGV
- *Choristoneura occidentalis granulovirus* ChocGV
- *Clostera anachoreta granulovirus* ClanGV
- *Clostera anastomosis granulovirus A* ClanGV-A
- *Clostera anastomosa granulovirus B* ClanGV-B
- *Cnaphalocrocis medinalis granulovirus* CnmeGV
- *Cryptophlebia leucotreta granulovirus* CrleGV
- *Cydia pomonella granulovirus** CpGV
- *Diatrea saccharalis granulovirus* DisaGV
- *Epinotia aporema granulovirus* EpapGV

- *Erinnyis ello granulovirus* ErelGV
- *Harrisina brillians granulovirus* HabrGV
- *Helicoverpa armigera granulovirus* HearGV
- *Lacanobia oleracea granulovirus* LaolGV
- *Mythimna unipuncta granulovirus A* MyunGV-A
- *Mythimna unipuncta granulovirus B* MyunGV-B
- *Pieris rapae granulovirus* PiraGV
- *Phtorimaea operculella granulovirus* PhopGV
- *Plodia interpunctella granulovirus* PiGV
- *Plutella xylostella granulovirus* PlxyGV
- *Pseudoletia unipuncta granulovirus* PsunGV
- *Spodoptera frugiperda granulovirus* SpfrGV
- *Spodoptera litura granulovirus* SpliGV
- *Trichoplusia ni granulovirus* TnGV
- *Xesta c-nigrum granulovirus* XcGV

Genus *Gammabaculovirus*

- *Neodiprion abietis nucleopolyhderovirus* NeabNPV
- *Neodiprion lecontei nucleopolyhedrovirus** NeleNPV
- *Neodiprion sertifer nucleopolyhedrovirus* NeseNPV

Genus *Deltabaculovirus*

- *Culex nigripalpus nucleopolyhedrovirus** CuniNPV

Member viruses are regularly added to the list. The NCBI lists 80 species (as of January 1 2019).
Some baculoviruses have a different name (historically), but appear to be genotypic variants of other baculoviruses (e.g. AcMNPV and GmNPV) > indicated indented (not exhaustive).

6-*Betairidovirinae* (subfamily), *Iridoviridae* (family)

Members of the family *Iridoviridae* cause chronic to acute disease both in a wide range of vertebrates (fish, reptiles) and invertebrates (insects, crustaceans). Iridescence is a hallmark phenotype of this group of viruses (Williams and Smith, 1957). The family accommodates large, linear, enveloped, double-stranded DNA (dsDNA) viruses (see also Ince *et al.*, 2018; Williams *et al.*, 2018, for review). Infection can be chronic or acute. There are about 100+ iridoviruses known from insects and crustaceans, but few have been investigated in detail. Iridoviruses are of ecological importance for fish and amphibians.

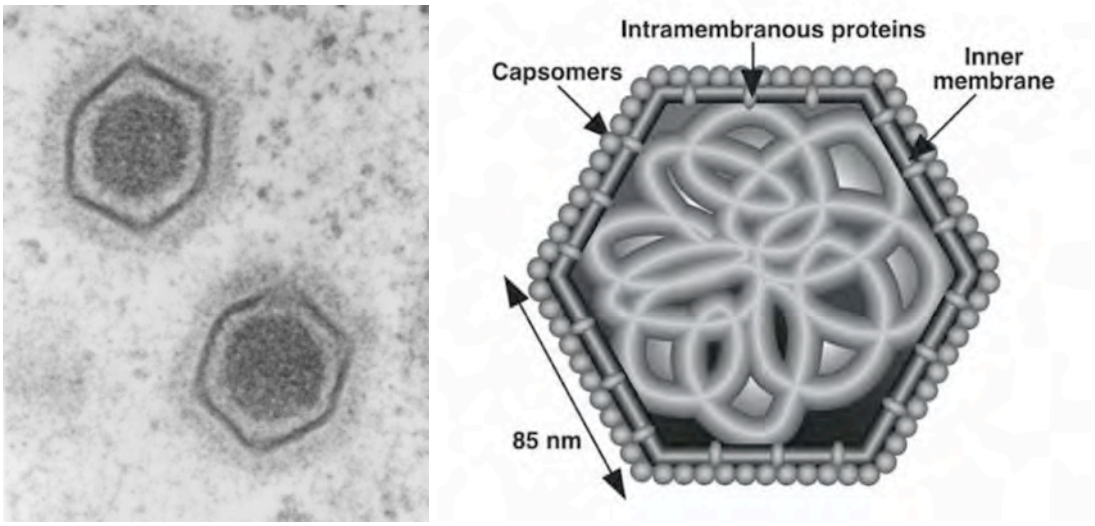

Figure 1. Iridovirus virions. EM-picture: Source: UBWeb; Right: schematic representation (Fig 1 in ICTV, *Iridoviridae*, 2018 release)

The virions

Iridovirus virions are about 185 nm in diameter with an icosahedral capsid surrounded by an inner lipid membrane and an outer envelope (Fig. 1). The iridovirus virions contain about 50 proteins (Ince *et al.*, 2010) including the major capsid protein (MCP) and a linear double-stranded DNA molecule.

Genetics

The iridovirus genome has a size of 103-220 kbp, depending on the species, encoding 92-211 putative non-overlapping open reading frames (ORFs). The ORFs are about equally distributed over both strands of the DNA molecule. Iridovirus DNA is circularly permuted, with terminally redundant sequences at the very ends (Murti *et al.*, 1985). Transcription of iridovirus is temporally regulated, as is usual for large double-stranded DNA viruses of invertebrates.

There is no evidence for genomic integration of iridovirus sequences into the genome of their hosts.

Taxonomy

The taxonomy of *Iridoviridae* is primarily based on gene content, phylogenetic relatedness and monophylogeny versus other large invertebrate dsDNA viruses, e.g. baculoviruses, hytrosaviruses, and host range (Chinchar *et al.*, 2018; Ince *et al.*, 2018). The *Iridoviridae* family now consists of two subfamilies, *Alphairidovirinae* and *Betairidovirinae*, representing iridoviruses from vertebrates and invertebrates (insects), respectively. For this report only the betairidoviruses are considered. Type species for the family is the vertebrate *Frog Virus 3* (*Ranavirus*) (ICTV, 2012). Viruses of the *Iridoviridae* family sharing 95% or greater amino acid sequence identity are tentatively considered as belonging to the same species.

Two genera are currently recognized within this subfamily, *Iridovirus* and *Chloriridovirus*. Seven *Betairidovirinae* species are accepted by the ICTV (2012; ibid 2018). The genus *Chloriridovirus* contains five species, with *Invertebrate iridescent virus 3* (IIV-3) as type species. The genus *Iridovirus* contains two species, *Invertebrate iridescent virus 6* (IIV-6) as type species. A few more are proposed for each genus in the *Betairidovirinae* subfamily (Fig. 2) (ICTV, 2018). In some cases the betairidovirus is known under more than one name (e.g. IIV6 and CIV)

There is evidence to suggest that members of the genus *Ranavirus* (*Alphairidovirinae*) (fish hosts) shifted hosts recently (Jancovich *et al.*, 2010), explaining the recent iridovirus epidemics among reptiles. Although this host switch initially occurred for viruses within the *Ranavirus* genus, it possible that a switch across invertebrate iridovirus genera might occur in the future.

Figure 2. Phylogenetic analysis of family *Iridoviridae*, subfamily *Betairidovirinae.* Accession numbers are shown for the viruses. Exemplars of accepted species are shown in boldface. The tree was constructed using maximum likelihood analysis in IQTREE and the concatenated amino acid (aa) sequences of 26 core genes (19,773 aa characters including gaps) from 45 completely sequenced genomes of members of the family. The tree was midpoint rooted and branch lengths are based on the number of inferred substitutions, as indicated by the scale bar. All branch points (i.e., nodes) separating genera are supported by bootstrap values greater than 99%. For other branch points all bootstrap values are >70%. (Fig. 6 in ICTV *Iridoviridae,* 2018b Release); Figure provided by K. Subramaniam and Waltzek, 2018).

Iridoviruses and ascoviruses are thought to share a common ancestor with marseilleviruses (Bigot *et al.*, 2015). However, the betairidoviruses are deeply rooted in the phylogenetic tree (Fig. 3 in Boyer *et al.*, 2009, https://doi.org/10.1073/pnas.0911354106).

Pathobiology

Replication of betairidoviruses is temperature-dependent and occurs in poikilothermic invertebrates (insects, crustaceans). These viruses infect both larvae and adults *per os* and target the general body, but predominantly epidermis, muscle and adipose tissue (fat body) (Federici, 1980). Viral entry is via cell receptors and is not mediated by peroral infectivity factor proteins or PIF complex, as is the case for baculovirus, hytrosavirus, nudivirus and polydnavirus. Betairidoviruses replicate in the nucleus of infected cells and virions are assembled in the cytoplasm and released by cell lysis (Fig. 3). Iridovirus virions can form crystalline arrays, which are responsible for the iridescence upon radiation by visible light (Fig. 4). Iridoviruses can replicate in various cultured insect cells allowing detailed studies on gene regulation and expression (Ince *et al.*, 2018).

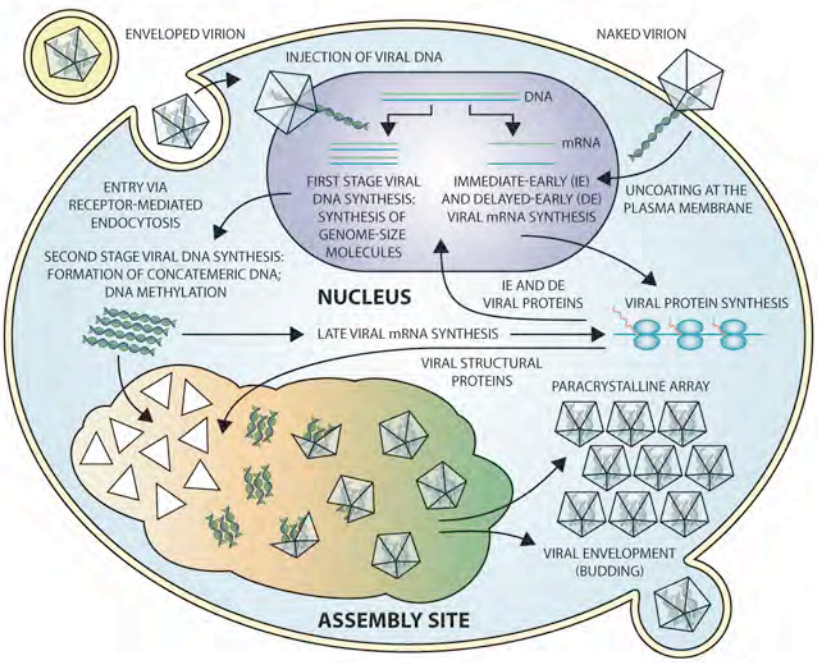

Figure 3. Schematic and generic representation of iridovirus replication (Fig. 4 in ICTV *Iridoviridae,* 2018b Release; Figure provided by Chinchar *et al.*, 2017)

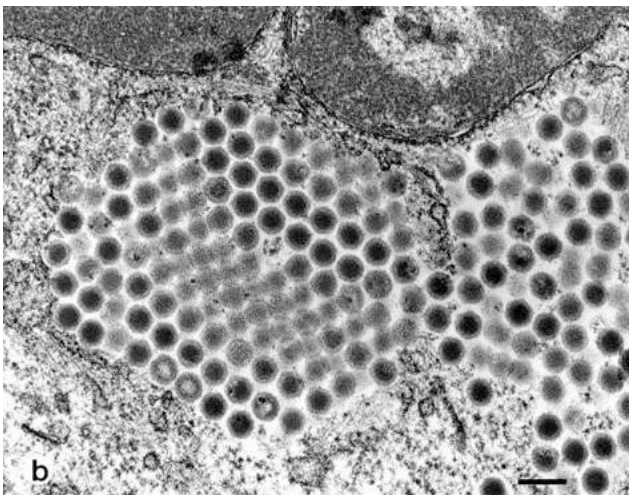

Figure 4. Paracrystalline array of iridoviruses in infected insect tissue (fat body)

Ecology

Over a 100 iridoviruses have been isolated worldwide from terrestrial and aquatic invertebrates (Williams, 2018). About 60 are insect-specific, mainly from dipterans (44 species), coleopterans (9 species), lepidopterans (9 species) and orthopterans (4 species). Insect iridoviruses are relatively species-specific *per os*, but less so per injection. Transmission is usually by predation or via faeces, but also vertical transmission has been recorded. Infection is relatively slow, almost chronic, but at some fitness costs (lower fecundity). Insect iridoviruses do not persist long in the environment (Williams, 2018). Due to their slow activity, insect iridoviruses have never considered a biocontrol agent of insect pests.

Genetic engineering

Betairidoviruses (IIV-6) have been engineered (Nalcacioglu *et al.*, 2014) via classical recombination in cell culture.

Impact for human and animal health

Insect iridoviruses can infect poikilothermic reptiles and amphibians (Weinmann *et al.*, 2007; Marschang, 2011; Stöhr *et al.*, 2016), but also mice (when injected) and can induce mortality. There are also several reports indicating that *Chilo iridescent virus* replicates in a poikilotherm vertebrate cell lines (McIntosh and Kimura, 1974; Ohba *et al.*, 1981; ibid., 1982). However, the yields are about 2 logs lower than in insect cells. Furthermore, *Invertebrate Iridescent virus 6* (IIV-6) can activate a canonical RNA sensing pathway (IFN-beta) in the innate immune response of mouse embryonic fibroblasts or human A549 lung epithelial cells (Ahlers *et al.*, 2016). However, in the latter case no virus replication was reported. This all suggests that the so-called invertebrate iridoviruses may also infect lower vertebrates.

Relevant information

Betairidovirinae

- occur world-wide, in members of various insect families (as well as crustaceans)
- are likely specific for insects
- are not very virulent and are not known to cause major epidemics in insects
- can infect poikilothermic vertebrates
- are reported to be able to enter / infect vertebrates cells and produce immune responses
- have been engineered for pathogenesis studies (IIV-6)
- have not been used as biocontrol agent for insect pests in the field

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Subfamily *Betairidovirinae*, Family *Iridoviridae*

		importance	**specific comments**
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	wide insect host range; low specificty
relation with vertebrate virus taxons	Y	xxx	vertebrate iridoviruses (fish)
evidence for infection of vertebrate animals	Y	xxx	poikilothermic vertebrates
replication in vertebrate cells	x/-	xx	specific vertebrate host responses
monophyletic deeply-rooted clade within virus family	Y	xx	robust
insect-specific entry/release/transmission mechanism	N	xxx	no, infect vertebrate cells
unique genetic traits correlating with insect-specificity	N	xx	
unique genome structure	Y	xxx	large linear dsDNA; terminal repeats
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M		often chronic
hematophagous host(s) (Yes/No)	Y		quite a few viruses
within host tropism (Broad/Restricted)	B		fat body, muscle, epithelia
host(s) include feed/food insects (Yes/No)	Y		crickets as pet food
endemic in NL (Yes/No)	Y		some iridovirus host species
persistent infections (Yes/No)			nd, mechanism unknown
horizontal transmission (Yes/No)	Y		very common

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

The conclusion is that, on the basis of available literature, members of the subfamily *Betairidovirinae* cannot be considered strictly insect-specific viruses, as they have profound effect(s) on vertebrates or vertebrate cells. Since molecular phylogeny is leading in *Betairidovirinae* (and *Iridoviridae*) taxonomy, classification of novel iridoviruses from invertebrates should be closely watched for possible switches to vertebrates. The observations have been summarized in Table 1.

Proposed status subfamily *Betairidovirinae* species: not insect-specific (no ISV)

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Betairidovirinae species: not proposed insect specific (ISV)

Species	Virus name	Abbreviation
Genus *Chloriridovirus*		
• *Invertebrate iridescent virus 3*		IIV3 (MIV)
• *Invertebrate iridescent virus 9*		IIV9
• *Invertebrate iridescent virus 22*		IIV9
• *Invertebrate iridescent virus 22A*		IIV22A
• *Invertebrate iridescent virus 25*		IIV25
• *Invertebrate iridescent virus 30*		IIV30
• *Anopheles minimus iridovirus*		AMIV
Genus *Iridovirus*		
• *Invertebrate iridescent virus 1*		IIV1
• *Tipula iridescent virus*		TIV
• *Invertebrate iridescent virus 2*		IIV1
• *Invertebrate iridescent virus 6*		IIV6 (CIV)
• *Chilo iridescent virus*		CIV
• *Invertebrate iridescent virus 16*		IIV16
• *Invertebrate iridescent virus 23*		IIV23
• *Invertebrate iridescent virus 24*		IIV24
• *Invertebrate iridescent virus 29*		IIV29
• *Invertebrate iridescent virus 31*		IIV31
• *Gryllus bimaculatus iridovirus*		GbIV

7-*Bidnaviridae* (family)

The *Bidnaviridae* is a family of small, unenveloped isometric viruses containing two single-stranded DNAs (Bando *et al.*, 1992), causing fatal disease in silkworm cultures. They are look-alikes of *Densovirinae* in size and microscopy, but have a distinct genome architecture and coding strategy. The name (Bidna-) is derived from the property that the genetic information of bidnaviruses is divided over two single-stranded ambisense DNA molecules (Hu *et al.*, 2013). Bidnaviruses infect (so far) only silkworm (*Lepidoptera*).

Virions

Bidnavirus virions are icosahedral in shape, 20-25 nm in diameter. The virions contain two virion proteins (see Fig. 2 in Hu *et al.*, 2013, https://link.springer.com/article/10.1007/s11434-013-5876-1. Bidnavirus populations consist of four types, depending on the single-stranded DNA inserted. The DNAs are of ambisense polarity.

Genetics

The genetic information of bidensoviruses is distributed over two non-homologous DNA segments, VD1 and VD2, each about 6 kb in size with terminal inverted repeats (Wang et al., 2010; Hu *et al.*, 2013; Krupovic and Koonin, 2014) (see Fig. 6 in Hu *et al.*, 2013, https://link.springer.com/article/10.1007/s11434-013-5876-1). The two DNA segments are of ambisense polarity and their two complementary strands are encapsidated separately each in virions; so four types of virions exist (AB, A'B, AB', A'B').

Most interestingly, the open readings frames (ORFs) seem to be related (derived) from densoviruses (VD1 ORF1-3, polintovirus (VD1 ORF4, DNA polymerase), reovirus (VD2 ORF1, virion protein) and baculoviruses (VD2 ORF2) (Krupovic and Koonin, 2014). Thus, their genome structure and gene composition is very different from the densoviruses (lemma 9), hence the establishment of a new family *Bidnaviridae*. A salient difference with other animal single-stranded DNA viruses is the presence of a virus-encoded DNA polymerase in bidnaviruses.

Krupovic and Koonin (2014) suggest that bidnaviruses are derived from polintoviruses, by capturing various genes over time.

Taxonomy

The family *Bidnaviridae* is a recently established (ICTV, 2012), with a single genus, *Bidensovirus*, and a single (type) species *Bombyx mori bidensovirus* (BmBDV) until now. Bidnaviruses are characterized by the presence of genetic information from four other virus groups (reoviruses, baculoviruses, polintoviruses and densoviruses) in their genome. When the parvovirus ORFs are used, bidnaviruses cluster with the densoviruses (see Fig. 1 in Krupovic and Koonin, 2014, https://doi.org/10.1038/srep05347). However, when reovirus segments were taken, the bidnaviruses cluster with reoviruses of insects. So, bidnaviruses are clearly borne from elements from different viruses.

The *Bidnaviridae* family has been proposed in 2010, ratified by the ICTV in 2012 and confirmed in 2018.

Replication

Bidnaviruses do not replicate via a rolling circle and replication is not initiated by a virus-encoded endonuclease cleavage, as is the case for densoviruses. More likely, they replicate by a protein-initiated mechanism, as observed for adenoviruses (Tijssen *et al.*, 2016).

Pathobiology

Bombyx mori bidensovirus (BmBDV) is the causative agent of a sometimes chronic, sometimes fatal disease (‘flacherie’), in silkworm larvae and the virus replicates predominantly in the columnar cells of the digestive tract of silkworm larvae (Kadono-Okuda *et al.*, 2004; Hu *et al.*, 2013; Tijssen *et al.*, 2016). The symptoms further include anorexia, diarrhea and a flaccid appearance. The virus replicates in the hypertrophied nucleus of infected tissue cells (but also in BmN insect cell culture). After discharge of the infected epithelium, the virus particles are released and secreted via the bead-like feces excretions (see Fig. 7 in Zhang *et al.*, 2016, https://doi.org/10.1186/s12985-016-0576-5)

Resistant silkworm showed an altered receptor for bidensovirus, encoded by the *nsd-2* gene of the host (Qin and Yi, 1996; Ito *et al.*, 2016)).

Ecology

Bidnaviruses have been detected from silkworm in India, Japan and China. Very little is known about the ecology of bidnaviruses, even in the case of the silkworm, *Bombyx mori*. Several strains have been detected, but only in *Bombycidae*.

Genetic engineering

An infectious clone of a bidensovirus (BmBDV) has been made and a recombinant virus has been isolated and characterized in insect cells and insects (Zhang *et al.*, 2016). A recombinant BmBDV with a fluorescent marker (GFP) has been used to monitor infection and pathogenesis in silkworm cells.

Impact on human and animal health

To date, bidnaviruses have not been tested for their ability to grow in vertebrate cells or their effect on vertebrate animals. Any effect is unlikely considering the number of insect virus-related genes present in these viruses and due to the specificity for silkworm.

Relevant observations

Bidensoviruses

- occur in Asia and only in bombycid insects (silkworms)
- are an important mortality factor in silkworm rearing
- are related to insect densoviruses, but also contain elements from other insect viruses
- multiply in insects and insect cells, but effects on vertebrate animals or cells are unknown
- can be genetically engineered
- have unknown impact on insect (*Bombyx*) ecology.

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Family *Bidnaviridae*

		importance	**specific comments**
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	only *Bombyx mori* as known host
relation with vertebrate virus taxons	Y	xx	parvoviruses, but not virus-dependent
evidence for infection of vertebrate animals			nd
replication in vertebrate cells			nd
monophyletic deeply-rooted clade within virus family	Y	xxx	related to insect *Densovirinae*
insect-specific entry/release/transmission mechanism	Y	xx	nuclear replication
unique genetic traits correlating with insect-specificity	Y		independ. replicat.; protein-initiated
unique genome structure	Y	xxx	small multiple ssDNA molecules
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M		chronic to fatal
hematophagous host(s) (Yes/No)	N		
within host tropism (Broad/Restricted)	R		digestive tract
host(s) include feed/food insects (Yes/No)	Y		silkworm as food
endemic in NL (Yes/No)	N		
persistent infections (Yes/No)			nd
horizontal transmission (Yes/No)			nd, likely oral since gut is target

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

The conclusion reached is that, on the basis of available literature, bidnaviruses can be considered genuine insect-specific viruses, with unlikely effects on organisms beyond the insect order. The observations have been summarized in Table 1.

Proposed status *Bidnaviridae* species: Insect-Specific Virus (ISV)

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Bidensovirus Species

Species	Abbreviation
• *Bombyx mori bidensovirus*	BmBDV

8-*Bunyavirales* (order), families *Peribunyaviridae*, *Phasmaviridae* and *Phenuiviridae* (specific genera)

The order *Bunyavirales* (previously family *Bunyaviridae*) is a large order of small, enveloped viruses with a single-stranded negative-sense RNA genome divided over 2-6 RNA segments, three segments, L, M and S in most cases (Elliott and Schmaljohn, 2013; Adams *et al.*, 2017; ICTV, 2017). Most known bunyaviruses infect mammals or rodents (vertebrates) and represent very pathogenic viruses, causing lethal diseases in humans such as Rift Valley fever, Crimean Congo haemorrhagic fever and Sin Nombre, but also in vertebrate animals. Members of one bunyavirales family (Family *Tospoviridae*) infect > 1000 plant species world-wide. Many bunyaviruses are transmitted by mosquitoes, midges, sandflies or ticks, and are therefore called arthropod-borne viruses or arboviruses.

An increasing number of bunyaviruses has been reported, that has an insect-restricted host range. The number has increased fairly recently, among others through next generation sequencing efforts (Li *et al.*, 2015; Shi *et al.*, 2016; ICTV 2017). These insect-specific bunyaviruses (feraviruses, jonviruses, phasiviruses, herbeviruses, goukoviruses and phasiviruses) are the topic of this report as potential insect-specific viruses (ISV).

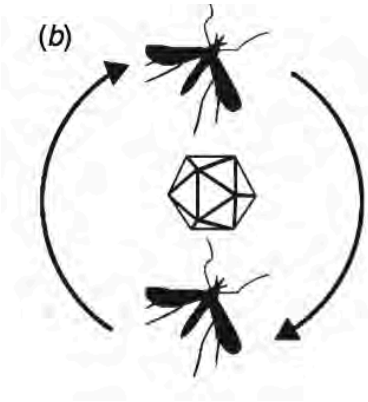

Figure 1. (a) proposed insect-specific *Bunyavirales* transmission cycle (From Hall and Hobson-Peters, 2018).

The name (‘Bunya-‘) is derived from Bunyamwera, an area within the Semliki Forest of Uganda). The host range of bunyaviruses is restricted.

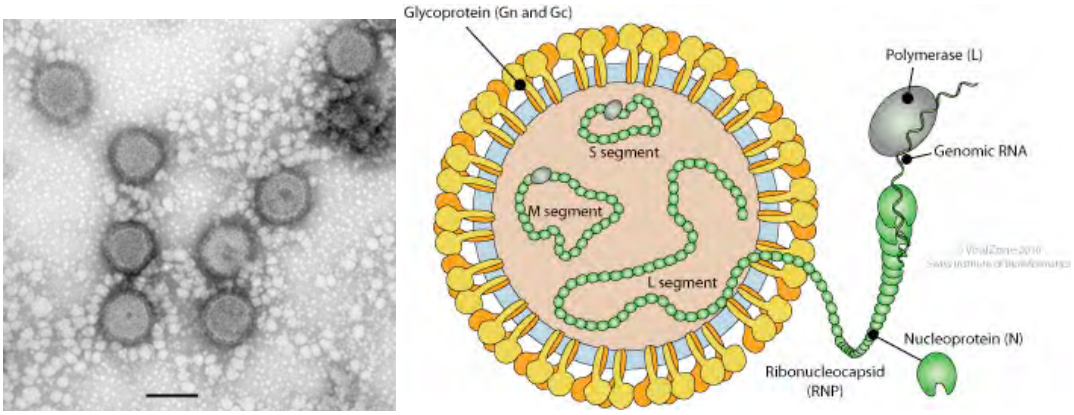

Figure 2. Left: TEM of Rift Valley fever virus. Source: Robert Koch Institute, Germany; Right: Schematic representation of a bunyavirus. Source: ViralZone

The virions

Bunyavirus virions are, in general, spherical in shape, 80-120 nm in diameter and enveloped (Fig. 2). The virions has a lipid envelope containing protein spikes. Two to six RNA molecules are embedded into nucleocapsids inside the virions. The RNAs are single-stranded and of negative polarity. Sometimes (tospoviruses, tenuiviruses) the RNA can be of ambi-sense polarity. The virions have a single capsid protein and two envelope proteins, but no matrix protein (Elliott and Schmaljohn, 2013).

Genetics

The bunyavirus genome is divided over three segments, **L**, encoding an RNA-dependent RNA polymerase to make positive-sense RNA, **M**, encoding two envelope proteins, Gc and Gn, and a non-structural NSm protein, and **S**, encoding a nucleoprotein (N) wrapped around the RNA and a non-structural protein called NSs (in frame-shift). The UTRs of the RNA segments are highly conserved and can form hairpins to initiate transcription and replication. The RNA-dependent RNA polymerase is enclosed in the mature virions to initiate positive-sense RNA synthesis.

The positive-sense RNA molecules have a 5'-end cap structure for translation initiation, which is 'snatched' from host messenger RNAs, and a strong hairpin structure at the 3'-end (no polyA) (Fig. 3).

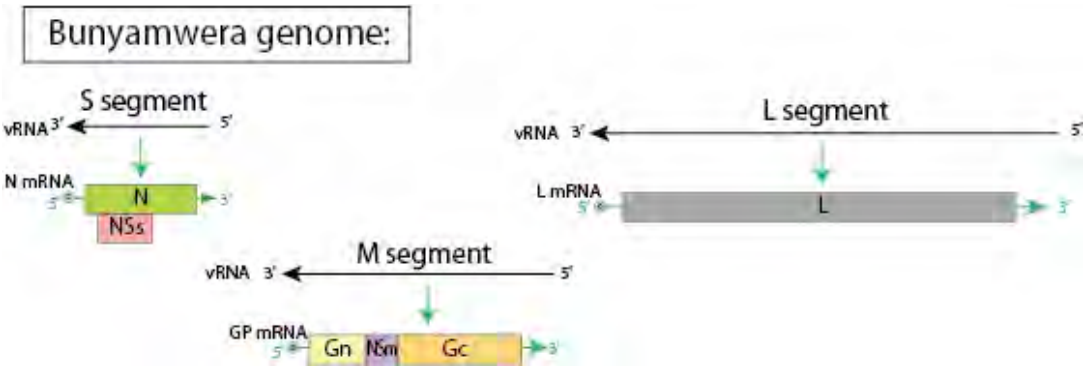

Figure 3. Genome organization and expression of members of the order *Bunyavirales*. (Source: ViralZone)

Taxonomy

At least 550 bunyaviruses are described, but not all of these have been classified (yet) by the ICTV (2018). The classification is evolving (= changing) rapidly upon the influx of next generation sequences of organismal viromes. The *Bunyavirales* Order (new since 2017) currently encompasses twelve families (ICTV 2018; Maes *et al.*, 2018), among these many arboviruses (e.g. *Hantaviridae*). For this report, only families or genera likely to contain insect-specific viruses are considered: *Peribunyaviridae* (two genera), *Phasmaviridae* (six genera) and *Phenuiviridae* (5 genera). Phylogenetic associations with confirmed cases form the basis for this recent classification (Table 1; ICTV 2017, 2018)). A bunyavirus species is based on complete genome composition and on nucleotide and amino acid homology (phylogeny), host and vector associations and geographical distribution (ICTV 2017). Due to the increased number of bunyavirus species the resolution of the phylogeny increased significantly (ICTV 2018).

The bunyavirus phylogeny is based on full genome sequences. The most comprehensive

phylogeny was published in the 2018 ICTV update. The names and taxonomy of the various virus families and genera have changed over time (ICTV 2018), but the general architecture of the trees is still valid. The bunyavirus taxonomy continues to evolve (Guterres *et al.*, 2017) and the 2019 taxonomy may contain even more insect-specific virus families, genera and species (Kuhn *et al.*, 2019).

− **Order:** *Bunyavirales* Member of *Ellioviricetes*	12 families, 1 genus
+ **Family:** *Arenaviridae* Member of *Bunyavirales*	4 genera
+ **Family:** *Cruliviridae* Member of *Bunyavirales*	1 genus
+ **Family:** *Fimoviridae* Member of *Bunyavirales*	1 genus
+ **Family:** *Hantaviridae* Member of *Bunyavirales*	4 subfamilies
+ **Family:** *Leishbuviridae* Member of *Bunyavirales*	1 genus
+ **Family:** *Mypoviridae* Member of *Bunyavirales*	1 genus
+ **Family:** *Nairoviridae* Member of *Bunyavirales*	3 genera
− **Family:** *Peribunyaviridae* Member of *Bunyavirales*	4 genera
− **Genus:** *Herbevirus* Member of *Peribunyaviridae*	3 species
★ **Species:** *Herbert herbevirus* Member of *Herbevirus*	
Species: *Kibale herbevirus* Member of *Herbevirus*	
Species: *Tai herbevirus* Member of *Herbevirus*	
+ **Genus:** *Orthobunyavirus* Member of *Peribunyaviridae*	88 species
+ **Genus:** *Pacuvirus* Member of *Peribunyaviridae*	3 species
− **Genus:** *Shangavirus* Member of *Peribunyaviridae*	1 species
★ **Species:** *Insect shangavirus* Member of *Shangavirus*	
− **Family:** *Phasmaviridae* Member of *Bunyavirales*	6 genera
− **Genus:** *Feravirus* Member of *Phasmaviridae*	1 species
★ **Species:** *Ferak feravirus* Member of *Feravirus*	
− **Genus:** *Inshuvirus* Member of *Phasmaviridae*	1 species
★ **Species:** *Insect inshuvirus* Member of *Inshuvirus*	
− **Genus:** *Jonvirus* Member of *Phasmaviridae*	1 species
★ **Species:** *Jonchet jonvirus* Member of *Jonvirus*	
− **Genus:** *Orthophasmavirus* Member of *Phasmaviridae*	10 species
Species: *Culex orthophasmavirus* Member of *Orthophasmavirus*	
Species: *Ganda orthophasmavirus* Member of *Orthophasmavirus*	
★ **Species:** *Kigluaik phantom orthophasmavirus* Member of *Orthophasmavirus*	
Species: *Nome phantom orthophasmavirus* Member of *Orthophasmavirus*	
Species: *Odonate orthophasmavirus* Member of *Orthophasmavirus*	
Species: *Qingling orthophasmavirus* Member of *Orthophasmavirus*	
Species: *Seattle orthophasmavirus* Member of *Orthophasmavirus*	
Species: *Wuchang cockroach orthophasmavirus 1* Member of *Orthophasmavirus*	
Species: *Wuhan mosquito orthophasmavirus 1* Member of *Orthophasmavirus*	
Species: *Wuhan mosquito orthophasmavirus 2* Member of *Orthophasmavirus*	
− **Genus:** *Sawastrivirus* Member of *Phasmaviridae*	1 species
★ **Species:** *Sanxia sawastrivirus* Member of *Sawastrivirus*	
− **Genus:** *Wuhivirus* Member of *Phasmaviridae*	1 species
★ **Species:** *Insect wuhivirus* Member of *Wuhivirus*	

Table 1. Current taxonomy of the order *Bunyavirales* (ICTV 2018; Maes *et al.*, 2018).

The genus *Herbevirus* (***Peribunyaviridae***) has three mosquito-specific virus species *Herbert-*, *Kibale-* and *Tai herbevirus*. This genus forms a clade with the genus *Orthobunyavirus*, which accommodates a.o. *Bunyamwera virus* and *La Crosse virus*, both mosquito arboviruses of humans (Fig. 5). This likewise holds for the one and type species of the genus *Shangavirus* (*Insect shangavirus*) also within the *Peribunyaviridae* family. The herbe- and shangaviruses are positioned close to arbovirus clades, but are separated by sufficiently deep rooting. They also seem to branch at the base of the peribunyavirus clade (Fig. 6). The herbe- and shangaviruses may have a yet unknown vertebrate host or never have acquired replicative

ability in vertebrates since they are at the base of the tree (Fig. 6) (Marklewitz *et al.*, 2015).

−	**Family:** *Phenuiviridae* Member of *Bunyavirales*	15 genera
+	**Genus:** *Banyangvirus* Member of *Phenuiviridae*	3 species
−	**Genus:** *Beidivirus* Member of *Phenuiviridae*	1 species
★	**Species:** *Dipteran beidivirus* Member of *Beidivirus*	
−	**Genus:** *Goukovirus* Member of *Phenuiviridae*	3 species
	Species: *Cumuto goukovirus* Member of *Goukovirus*	
★	**Species:** *Gouleako goukovirus* Member of *Goukovirus*	
	Species: *Yichang insect goukovirus* Member of *Goukovirus*	
+	**Genus:** *Horwuvirus* Member of *Phenuiviridae*	1 species
−	**Genus:** *Hudivirus* Member of *Phenuiviridae*	1 species
★	**Species:** *Dipteran hudivirus* Member of *Hudivirus*	
−	**Genus:** *Hudovirus* Member of *Phenuiviridae*	1 species
★	**Species:** *Lepidopteran hudovirus* Member of *Hudovirus*	
+	**Genus:** *Kabutovirus* Member of *Phenuiviridae*	2 species
+	**Genus:** *Laulavirus* Member of *Phenuiviridae*	1 species
+	**Genus:** *Mobuvirus* Member of *Phenuiviridae*	1 species
−	**Genus:** *Phasivirus* Member of *Phenuiviridae*	3 species
★	**Species:** *Badu phasivirus* Member of *Phasivirus*	
	Species: *Phasi Charoen-like phasivirus* Member of *Phasivirus*	
	Species: *Wutai mosquito phasivirus* Member of *Phasivirus*	
+	**Genus:** *Phlebovirus* Member of *Phenuiviridae*	10 species
+	**Genus:** *Pidchovirus* Member of *Phenuiviridae*	1 species
+	**Genus:** *Tenuivirus* Member of *Phenuiviridae*	7 species
+	**Genus:** *Wenrivirus* Member of *Phenuiviridae*	1 species
−	**Genus:** *Wubeivirus* Member of *Phenuiviridae*	2 species
★	**Species:** *Dipteran wubeivirus* Member of *Wubeivirus*	
	Species: *Fly wubeivirus* Member of *Wubeivirus*	

Table 1. Current taxonomy of the order *Bunyavirales* (ICTV 2018; Maes *et al.*, 2018), continued.

The family ***Phasmaviridae*** contains six genera (*Feravirus*, *Inshuvivirus*, *Jonvirus*, *Orthophasmavirus*, *Sawastrivirus* and *Wuhivirus*) (Table 1) and their members all seem to be associated with insects (invertebrates). *Feravirus*, *Inshuvivirus*, *Jonvirus*, *Sawastrivirus* and *Wuhivirus* each contain one species, whereas *Orthophasmavirus* has ten associated species. These orthophasmaviruses cluster with species of the insect-specific *Fera-*, *Jon-*, *Wuhi- and Inshuviviruses*, but not closely to arthropod-borne bunyaviruses (Fig. 5, e.g. *Hantavirus*). The *Phasmaviridae* are a phylogenetically distinct clade within the *Bunyavirales* order.

The family ***Phenuiviridae*** accommodates fifteen genera, six of which seem (*Beidivirus* (1 species), *Goukovirus* (3 species), *Phasivirus* (3 species)) or may be (*Hudivirus* (1 species), *Hudovirus* (1 species) and *Wubeivirus* (2 species) insect-specific. This is a complex family of viruses, as some members are transmitted by insect vectors to humans (Rift Valley fever virus, *Phlebovirus*), or to plants (tenuivirus). The clade structure of the *Phenuiviridae* is equivocal e.g. with goukoviruses close to phleboviruses (arbovirus). However, viruses of these genera are monophyletic, but not deeply rooted in the tree (ICTV2018; Fig. 5, 6).

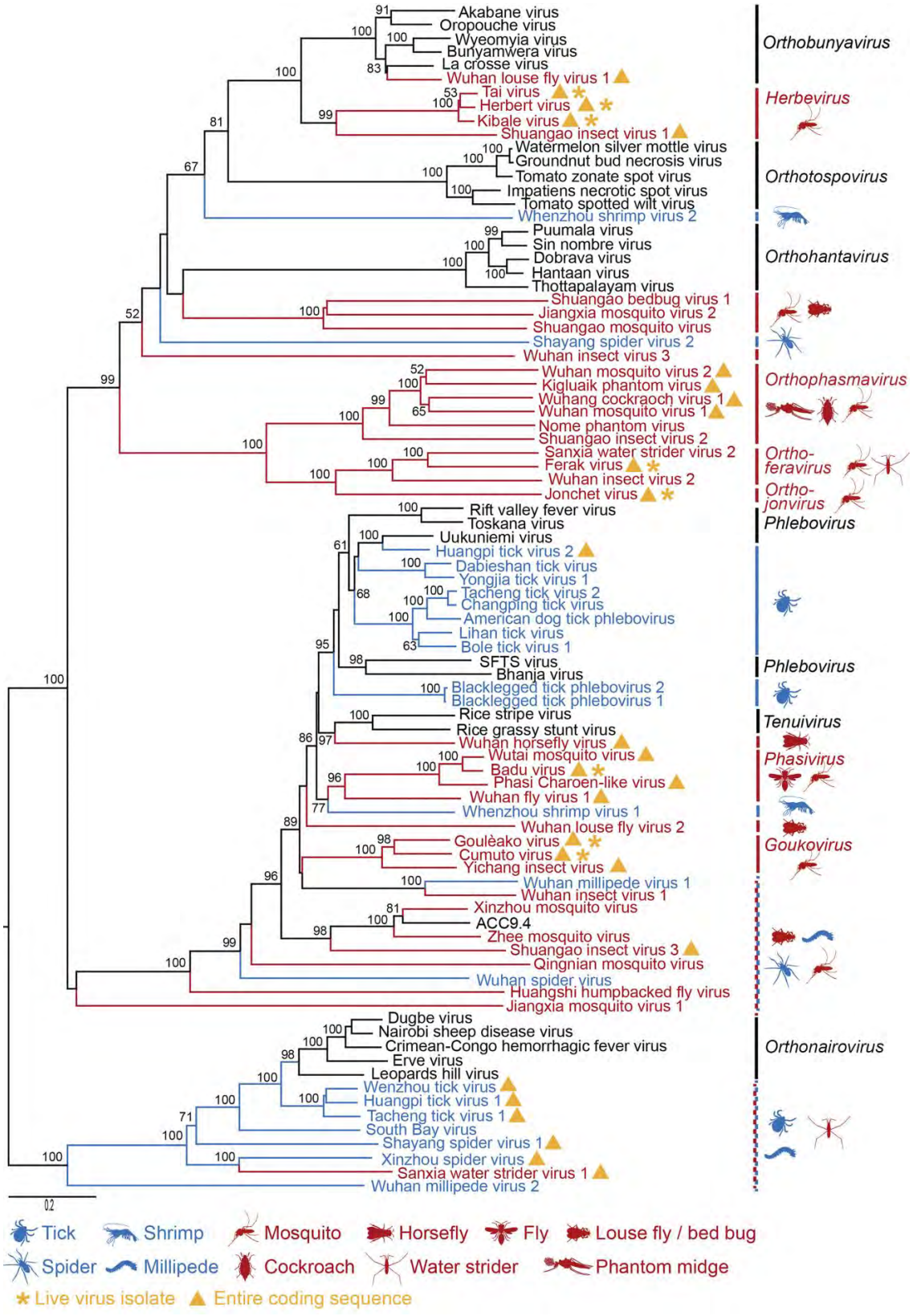

Figure 4: Phylogenetic relationship of bunyaviruses. Phylogenetic analyses were based on RdRp proteins. Complete RdRp proteins were aligned using MAFFT (E-INS-I algorithm). Alignment columns were stripped to 10% gaps in Geneious. Maximum likelihood (ML) analyses were performed on a 508 amino acid alignment guided by the Blosum62 amino acid substitution matrix with 4 gamma categories and a gamma shape parameter of 1. Confidence testing was performed by 1000 bootstrap replicates. Only bootstrap values over 50 are shown. (Fig. 1 in Virus Taxonomy Release 2017, 2016.021a-dM.A.v2.Orthoferavirus.pdf).

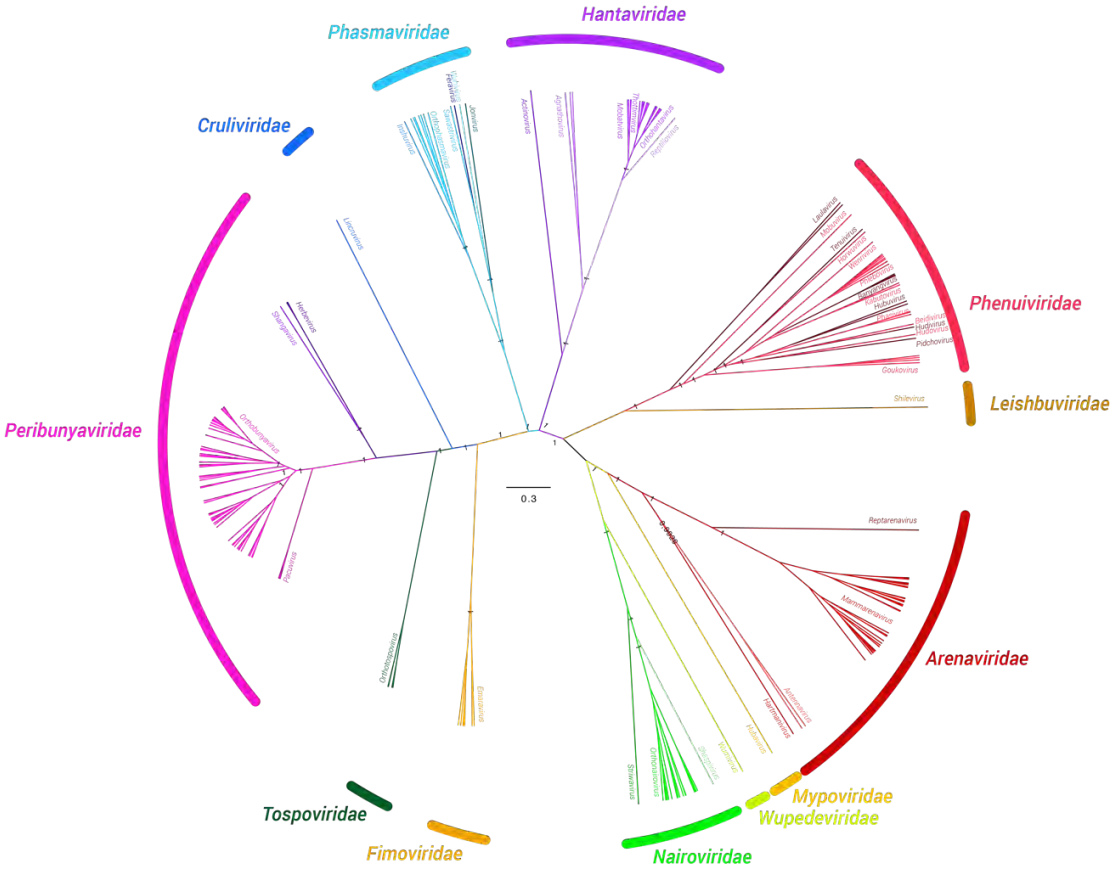

Figure 6. Bayesian tree of *Bunyavirales* families and genera (ICTV, 2018.017M.A.v1.bunyavirales_2fam5gen). Note the equivocal position of insect-specific genera with in the *Phenuiviridae* family and the unequivocal position of herbe-/shangavirus.

The genus *Goukovirus* (***Phenuiviridae***) accommodates three species: *Gouleako goukovirus* (GOLV), *Cumuto goukovirus* (CUMV) and *Yichang insect goukovirus* (YcIV) (ICTV, 2017, 2018). The goukoviruses form a monophyletic clade, but cluster with lower bootstrap values with the phasiviruses (Fig. 5). The genus *Phasivirus* contains three species: *Badu phasivirus* (BADUV), *Phasi-Charoen-like phasivirus* and *Wutai mosquito phasivirus*, and this clade is sandwiched between tenui- and goukoviruses, and well away from the phleboviruses (Fig. 5) (ICTV 2017). However, the taxonomic position of *Badu phasivirus* is a mater of debate, as Hobson-Peters *et al.* (2016) places this virus as belonging to the *Goukovirus* genus. In addition a few more possibly insect-specific genera (*Hudovirus*, *Hudivirus*, *Wubeivirus*) within the *Phenuiviridae* family have been identified (ICTV 2018; Kuhn *et al.*, 2019) (Fig. 6). The current phylogeny within the *Phenuidae* virus family is equivocal at best and needs further substantiation (Fig. 5 and 6). There is biological information on gouko- and phasiviruses, but none for the other possibly insect-specific genera within the *Phenuiviridae*.

It is of interest to note that the proposed insect-specific bunyaviruses are thought to be ancestors of the different lineages of bunyaviruses (Marklewitz *et al.*, 2015; Junglen, 2016).

Replication

Bunyaviruses enter cells via attachment of the viral surface proteins Gn/Gc (encoded by the M segment) and a host cell receptor and replicate in the cytoplasm of infected cells. The

virions carry a RNA dependent RNA polymerase (L protein), which transcribes the negative strands into mRNA, which in turn 'snatches' the CAPs of host cell mRNAs. Progeny viral RNA is encapsidated via the N- proteins into virions. Viral progeny is assembled in the Golgi apparatus of the cell, where the surface glycoproteins are also glycosylated. Progeny virions are released via exocytosis.

Pathobiology

The insect-specific viruses have no clear pathology in their host. The tissue location of these viruses is also not clear. Jonviruses, feraviruses, herbeviruses, phasiviruses and goukoviruses were unable to replicate in insect cells at temperatures above 30 ^{o}C, which is a strong indication that these are indeed insect-specific viruses. Indeed, they were unable to replicate in a range of vertebrate cells at 37 ^{o}C (Marklewitz *et al.*, 2015).

For the genus *Goukovirus*, the four species listed above have been shown to replicate in mosquito cells (C6/36), but not in vertebrate cells (ref in Hobson-Peters *et al.*, 2016). Phasiviruses have a similar response to insect versus vertebrate cells, i.e. insect specificity (Marklewitz *et al.*, 2015).

The molecular mechanism of insect-specificity and which virus functions are involved is not known, although NSs and NSm paralogs may be involved (Marklewitz *et al.*, 2013).

Ecology

Arthropod bunyaviruses are of significant health importance in vertebrates. They are often transmitted by mosquitoes (*Aedes*, *Culex*, *Anopheles*) and sandflies, and can become epidemic. Much less is known about the ecology of presumed insect-specific bunyaviruses.

Members of the genus *Goukovirus* show vertical transmission, but only the females appear to carry the virus (Hobson-Peters *et al.*, 2016). For other insect-specific viruses the mode of transmission has not (yet) been studied.

Genetic engineering

Bunyaviruses have been genetically engineered, Bunyamwera virus being the first (Bridgen and Elliot, 1996). This technology is based on transfection of vertebrate cells with appropriate bunyavirus plasmids driven by T7 or PolI polymerases, and rescue of viruses, often with a fluorescent marker. This technology is now widely used, for example to engineer replication-defective or attenuated (non-pathogenic) bunyaviruses for vaccine purposes (Bouly and Flick, 2009; Wichgers Schreur *et al.*, 2017). To date, the insect-specific virus members of the six bunyavirus families have not (yet) been engineered.

Impact for human and animal health

Jonviruses, feraviruses, herbe-, phasi- and goukoviruses are unable to replicate in insect cells at temperatures above 30 ^{o}C. They are unable to replicate in a range of vertebrate cells (Marklewitz *et al.*, 2015; Hobson-Peters *et al.*, 2016) at vertebrate temperatures. Orthophasma- and inshuviviruses have not been tested yet in insect cells, but they are highly monophyletic with the tested fera- and jonviruses (Fig. 5). As far as we know, no experiments

have been performed in vertebrates (e.g. evaluation of the presence of virus-specific antibodies).

Relevant observations

Insect-specific bunyaviruses

- occur world-wide, potentially derived from 3 families and 13 genera of *Bunyavirales*
- seem specific for insects (some genera), but the molecular basis of specificity is unknown
- are phylogenetically distinct (except phenuiviruses)
- are not pathogenic for insects
- are not known to cause epidemics in insects
- have not been reported to replicate in vertebrate(s) / - cells at any temperature
- have not (yet) been engineered

Conclusion

The conclusion is that, on the basis of available information (specificity, biology, taxonomy), members of some virus taxa of the *Peribunyaviridae* family (genera *Herbevirus* and very likely *Shangavirus*) and *Phasmaviridae* family (genera *Orthophasmavirus*, *Jonvirus*, *Feravirus* and very likely (= provisional) *Inshuvirus*, *Sawastrivirus and Wuhivirus*) can be considered insect-specific (ISV) (Table 2a). Although members of some phenuivirus genera (*Goukovirus*, *Phasivirus*) show insect specificity, their taxonomic position is inconclusive (equivocal taxonomy) and not strong enough to warrant full ISV status at this point (Table 2b; provisional ISV). No biological information is available on the phenuivirus genera *Bedivirus*, *Hudivirus*, *Hudovirus* and *Wubeivirus*), hence they cannot yet be considered ISV (Table 2c; no ISV).

Table 2a: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Families *Phasmaviridae (*Genera *Jon-, Fera-, Inshuvi-, Orthophasmavirus) + Peribunyaviridae*
(Genera *Herbe-*, *Shangavirus*)

		importance	**specific comments**
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	mainly mosquitoes and flies
relation with vertebrate virus taxons	Y	xx	arbobunyaviruses; similar repl. strategy
evidence for infection of vertebrate animals	N	xxx	
replication in vertebrate cells	N	xxx	even at 30 oC
monophyletic deeply-rooted clade within virus family	Y	xxx	close to arboviruses
insect-specific entry/release/transmission mechanism			nd
unique genetic traits correlating with insect-specificity	Y	xx	lack of replication > 30 oC
unique genome structure	N	x	bunyavirus-like
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M		no obvious pathology
hematophagous host(s) (Yes/No)	Y		mosquitoes
within host tropism (Broad/Restricted)			nd
host(s) include feed/food insects (Yes/No)	N		
endemic in NL (Yes/No)	N		
persistent infections (Yes/No)	Y		persistent infections likely
horizontal transmission (Yes/No)	Y		probably also vertical

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Table 2b: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Family *Phenuiviridae (*Genera *Goukovirus* and *Phasivirus),* Order *Bunyavirales*

		importance	**specific comments**
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	mainly mosquitoes and flies
relation with vertebrate virus taxons	Y	xxx	arbobunyaviruses; similar repl. strategy
evidence for infection of vertebrate animals		xxx	
replication in vertebrate cells		xxx	even at 30 oC
monophyletic deeply-rooted clade within virus family		xxx	equivocal within family (arbovirus)
insect-specific entry/release/transmission mechanism			nd
unique genetic traits correlating with insect-specificity	Y	xx	lack of replication > 30 oC
unique genome structure		x	bunyavirus-like
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M		no obvious pathology
hematophagous host(s) (Yes/No)	Y		mosquitoes
within host tropism (Broad/Restricted)			nd
host(s) include feed/food insects (Yes/No)			
endemic in NL (Yes/No)			
persistent infections (Yes/No)	Y		persistent infections likely
horizontal transmission (Yes/No)	Y		probably also vertical

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Table 2c: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Families *Phasmaviridae (*Genus *Sawastrivirus*) and *Phenuiviridae* (Genera *Bedivirus, Hudivirus, Hudovirus* and *Wubeivirus*)

		importance	**specific comments**
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	mainly mosquitoes and flies
relation with vertebrate virus taxons	Y	xxx	arbobunyaviruses; similar repl. strategy
evidence for infection of vertebrate animals			nd
replication in vertebrate cells			nd
monophyletic deeply-rooted clade within virus family		xxx	equivocal within family (arbovirus)
insect-specific entry/release/transmission mechanism			nd
unique genetic traits correlating with insect-specificity	Y		nd
unique genome structure		x	bunyavirus-like
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)			nd
hematophagous host(s) (Yes/No)	Y		mosquitoes
within host tropism (Broad/Restricted)			nd
host(s) include feed/food insects (Yes/No)			
endemic in NL (Yes/No)			
persistent infections (Yes/No)			nd
horizontal transmission (Yes/No)			nd

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

- **Proposed status *Peribunyaviridae* (genus *Herbevirus)* and *Phasmaviridae* (genera *Orthophasmavirus, Feravirus, Jonvirus)* species: Insect-Specific Viruses (ISV)**

- **Proposed status *Peribunyaviridae* (genus *Shangavirus), Phasmaviridae* (genera *Inshuvivirus, Sawastrivirus, Wuhivirus*) and *Phenuiviridae* (genera *Goukovirus* and *Phasivirus,* species: provisionally Insect-Specific Viruses (ISV)**

- **Proposed status *Bedeivirus, Hudivirus, Hudovirus* and *Wubeivirus*) species: cannot (yet) be assigned as Insect-Specific Virus (ISV)**

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Proposed Insect-Specific *Bunyavirales* species:

Species		Abbreviation
Family *Phasmaviridae*		
Genus *Jonvirus*		
• *Jonchet jonvirus**	Jonchet virus	JONV
Genus *Feravirus*		
• *Ferak feravirus**	Ferak virus	FRKV
Genus *Orthophasmavirus*		
• *Kigluaik phantom orthophasmavirus**		KIGV
• *Culex orthophasmavirus*		CPLV
• *Ganda orthophasmavirus*		GBEEV
• *Nome phantom orthophasmavirus*		NOMV
• *Odonate orthophasmavirus*		HbOV-8
• *Qingling orthophasmavirus*		HbOV-9
• *Seattle orthophasmavirus*		SEPV
• *Wuchang cockroach virus orthophasmavirus 1*		WcCV-1
• *Wuhan mosquito orthophasmavirus 1*		WhMV-1
• *Wuhan mosquito orthophasmavirus 2*		WhMV-2

Family *Peribunyaviridae*

Genus *Herbevirus*

• *Herbert herbevirus**	Herbert virus	HEBV
• *Kibale herbevirus*	Kibale virus	KIBV
• *Taï herbevirus*	Taï virus	TAIV

Provisional Insect-Specific *Bunyavirales* species:

Family *Peribunyaviridae*

Genus *Shangavirus*

• *Insect shangavirus**	Shuāngào insect virus 1	SglV-2

Family *Phasmaviridae*

Genus *Inshuvirus*

• *Insect inshuvirus**	Shuāngào insect virus 2	SglV-2

Genus *Sawastrivirus*

• *Sanxia sawastrivirus**	Sānxiá water strider virus 2	SxWSV-2

Genus *Wuhivirus*

• *Insect wuhivirus**	Wǔhàn insect virus 2	WhIV-2

Family *Phenuiviridae*

Genus *Goukovirus*

• *Gouleako goukovirus**	Gouléako virus	GOLV
• *Cumuto goukovirus*	Cumoto virus	CUMV
• *Yichang insect goukovirus*	Yíchāng insect virus	YcIV

Genus *Phasivirus*

• *Badu phasivirus**	Badu virus	BADUV
• *Phasi Charoun-like phasivirus*	Phasi Charoun-like virus	PCLV
• *Wutai mosquito phasivirus*	Wutai mosquito virus	WtMV

Possible Insect-Specific *Bunyavirales* species (no ISV yet):

Family *Phenuiviridae*

Genus *Bedeivirus*

• *Dipteran bedeivirus**	Húběi diptera virus 3	HbDV-3

Genus *Hudivirus*

• *Dipteran hudivirus**	Húběi diptera virus 4	HbDV-4

Genus *Hudovirus*

• *Lepidopteran hudovirus**	Húběi lepidoptera virus 1	HbLV-1

Genus Wubeivirus

• *Dipteran wubeivirus**	Húběi diptera virus 5	HbDV-5
• *Fly wubeivirus*	Wǔhàn fly virus 1	WhFV-1

Listing based on the latest ICTV report (December 2018, https://talk.ictvonline.org/taxonomy/bunyavirales) + the updated list of Kuhn *et al.* (2019)

9-*Densovirinae* (genera *Ambidensovirus, Brevidensovirus, Iteradensovirus*), *Parvoviridae* (family)

The *Densovirinae* (parvovirus subfamily) occur in arthropods (insects, crustaceans) accommodates small non-enveloped isometric viruses with a single-stranded DNA genome, which are highly virulent for their insect hosts (Fédière *et al.*, 2000). The name (denso-) is derived from the dense inclusion in the nuclei of infected cells.

The *Parvovirinae* subfamily encompasses viruses causing major diseases in humans (such as the 'fifth child disease' caused by parvovirus B19) and other vertebrates (dogs). The major difference between these two subfamilies is that densoviruses can grow and replicate independently, whereas this is not always the case for parvoviruses of vertebrates (e.g. adeno associated virus or AAV) (ICTV 2018).

Virions

Densovirus virions are about 24 nm in diameter in size and are icosahedral in shape with a relatively 'smooth' surface. The structure has been resolved at the Angstrom level. They contain 2-5 proteins depending on the species, ranging in size from 45 to 95 kDa. Densovirus populations have both linear positive- and negative-sense DNA molecules encapsidated, with a size of about 4-6 kb.

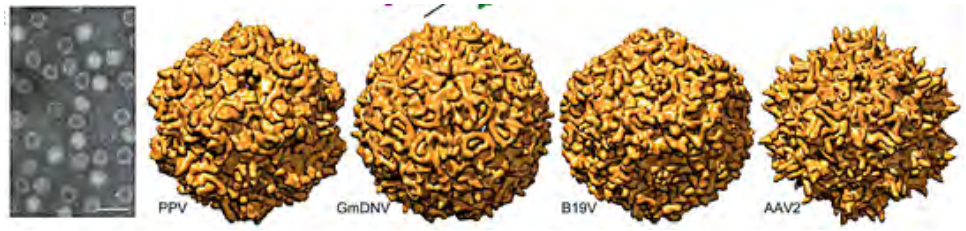

Figure 1. Densovirus virions (GmDNV). Source: ICTV, 2011, 2018.

Taxonomy

The subfamily *Densovirinae* is one of two subfamilies accommodated in the Family *Parvoviridae* (ICTV 2011, 2018; Cotmore *et al.*, 2014, ibid. 2019). This family accommodates small icosahedral viruses occurring in vertebrates (*Parvovirinae*) and invertebrates (*Densovirinae*). The subfamily *Densovirinae* currently contains five genera: *Ambidensovirus* (11 species) *Iteradensovirus* (5 species), *Hepandensovirus* (1 species), *Brevidensovirus* (2 species) and *Penstyldensovirus* (1 species) and one floating species (*Orthopteran densovirus 1*) (Table 1). The genera *Hepandensovirus* and *Penstyldensovirus* exclusively contain densoviruses from decapods. The genera *Ambidensovirus, Brevidensovirus* and *Iteradensovirus* accommodate densoviruses from a wide range of insects (dipterans, lepidopterans, hemipterans, orthopterans, hymenopterans). The current classification is supported by the host specificity of the respective viruses and the phylogeny of all known densoviruses (Table 1) (Fig. 2). The *Densovirinae* have been ratified as a subfamily by the ICTV in 2018.

Densoviruses have a complex genome arrangement and show bidirectional transcription, a property not found in the parvoviruses from vertebrates (*Parvovirinae*).

−	**Family:** *Parvoviridae*	2 subfamilies
−	**Subfamily:** *Densovirinae* Member of *Parvoviridae*	5 genera, 1 species
−	**Genus:** *Ambidensovirus* Member of *Densovirinae*	11 species
	Species: *Asteroid ambidensovirus 1* Member of *Ambidensovirus*	
	Species: *Blattodean ambidensovirus 1* Member of *Ambidensovirus*	
	Species: *Blattodean ambidensovirus 2* Member of *Ambidensovirus*	
	Species: *Decapod ambidensovirus 1* Member of *Ambidensovirus*	
	Species: *Dipteran ambidensovirus 1* Member of *Ambidensovirus*	
	Species: *Hemipteran ambidensovirus 1* Member of *Ambidensovirus*	
	Species: *Hemipteran ambidensovirus 2* Member of *Ambidensovirus*	
	Species: *Hemipteran ambidensovirus 3* Member of *Ambidensovirus*	
	Species: *Hymenopteran ambidensovirus 1* Member of *Ambidensovirus*	
★	**Species:** *Lepidopteran ambidensovirus 1* Member of *Ambidensovirus*	
	Species: *Orthopteran ambidensovirus 1* Member of *Ambidensovirus*	
−	**Genus:** *Brevidensovirus* Member of *Densovirinae*	2 species
★	**Species:** *Dipteran brevidensovirus 1* Member of *Brevidensovirus*	
	Species: *Dipteran brevidensovirus 2* Member of *Brevidensovirus*	
−	**Genus:** *Hepandensovirus* Member of *Densovirinae*	1 species
★	**Species:** *Decapod hepandensovirus 1* Member of *Hepandensovirus*	
−	**Genus:** *Iteradensovirus* Member of *Densovirinae*	5 species
★	**Species:** *Lepidopteran iteradensovirus 1* Member of *Iteradensovirus*	
	Species: *Lepidopteran iteradensovirus 2* Member of *Iteradensovirus*	
	Species: *Lepidopteran iteradensovirus 3* Member of *Iteradensovirus*	
	Species: *Lepidopteran iteradensovirus 4* Member of *Iteradensovirus*	
	Species: *Lepidopteran iteradensovirus 5* Member of *Iteradensovirus*	
−	**Genus:** *Penstyldensovirus* Member of *Densovirinae*	1 species
★	**Species:** *Decapod penstyldensovirus 1* Member of *Penstyldensovirus*	
	Species: *Orthopteran densovirus 1* Member of *Densovirinae*	
+	**Subfamily:** *Parvovirinae* Member of *Parvoviridae*	8 genera

Table 1. Taxonomy of *Densovirinae* (ICTV Online 2018).

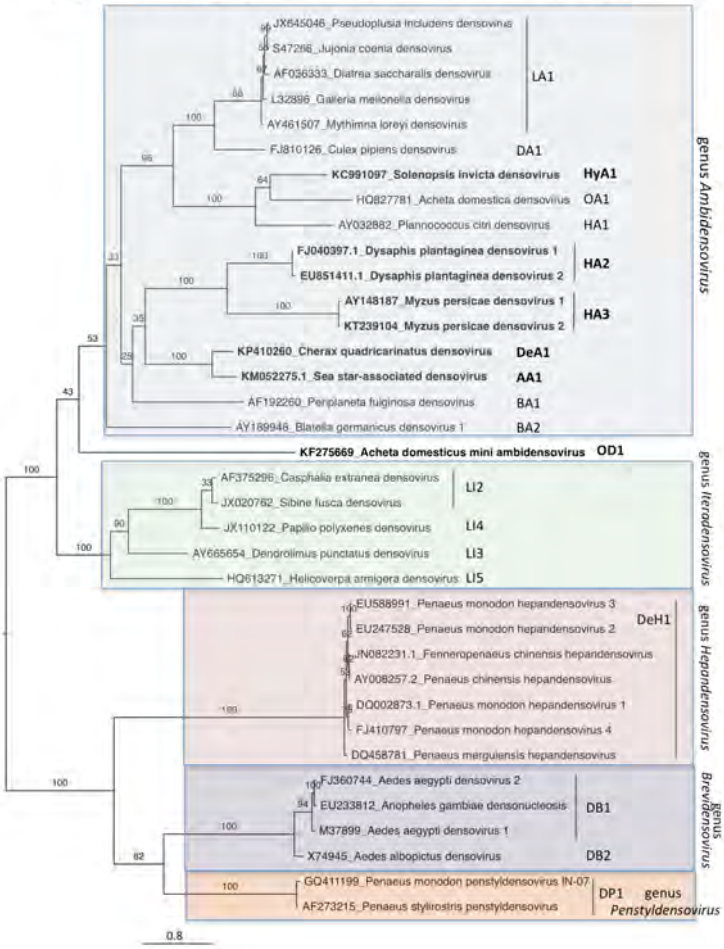

Figure 2. Phylogenetic tree (amino acids; NS1) showing the five genera within the subfamily *Densovirinae* (Fig ure in ICTV, 2016, 2016.003a,bD.A.v1.Densovirinae_6sp

Genetics

The single-stranded viral DNA of 4-6 kb has cohesive terminal ends, able to form hairpins, important for rolling circle replication. The genome of some densoviruses are mono-sense (iteraviruses and brevidensoviruses), others have an ambisense genome (densoviruses). Densoviruses encode for a replication protein and 2-5 virion proteins.

Densovirus proteins are derived from alternatively spliced mRNAs. Densoviruses do not encode for a DNA polymerase, but use a non-structural protein (NS1 or rep) for 'rolling circle' replication of the viral DNA (Fig. 3). In this aspect they differ from the *Bidnaviridae* (Lemma 7; also small single-stranded DNA viruses of invertebrates), which encode a classical DNA polymerase.

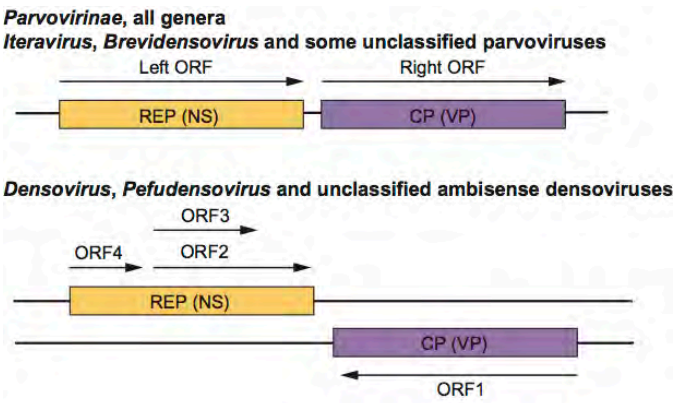

Figure 3. Genome structure of *Parvovirus* (upper) and *Densovirus* (lower) (Source: ICTV 2011)

A particular feature is that densovirus sequences have been found in the invertebrate chromosome as a result of fossilization millions of years ago (Liu *et al.*, 2011). These insertions are called 'endogenous viral elements' or EVEs.

Replication

Densovirus DNA is replicated in the nucleus of infected cells via a 'rolling circle' mode of replication, that is initiated by a virus-encoded endonuclease (Ilyina and Koonin, 1992). They replicate in the nucleus, showing hypertrophy when infected.

Pathobiology

In insect colonies densoviruses can cause feeding arrest, growth retardation, malformations and mortality. This is particularly relevant for cricket rearing as food source (e.g. *Acheta domestica densovirus* = AdNV) (Eilenberg *et al.*, 2015; Maciel-Vergara and Ros, 2017). Densoviruses are highly virulent (Tijssen and Bergoin, 1995). Some densoviruses are restricted to the gut; others also infect other tissues, such as fat body.
Persistent infection of insect cells (C6/36) with densoviruses has been reported (Jousset *et al.*, 1993).

Ecology

Densoviruses occur at least in five insect orders and in crustaceans. Some densoviruses have a restricted host range in insects (e.g. *Galleria mellonella densovirus* = GmDV), others (e.g. *Mithimna leoreyi densovirus* = MlDV) have a wider host range among the insects. The hairpins in the region encoding virion protein 1 (VP1) seem to be host determinants. Densoviruses are successfully used to control certain insect pests (Belloncik, 1990; Erlandson, 2008; Johnson and Rasgon, 2018).

Transmission of densoviruses is *per os* as well as vertical. Densovirus prevalence in the field (*Aedes aegypti* and *A. albopictus*) can reach values of 50% (Kittayapong *et al.*, 1999).

Genetic engineering

A plethora of recombinant densoviruses has been made which are infectious for insects and insect larvae and used to study densovirus pathology and insect host responses. Furthermore they have been used as expression vectors of proteins, as paratransgenesis vectors to transfer DNA into the germ line, and as improved biocontrol agents (Afanasiev *et al.*, 1994; Jiang *et al.*, 2007; Gu *et al.*, 2011; Johnson and Rasgon, 2018, for review). The limitation for this approach is the limited capacity of the densovirus genome.

Densoviruses are potential candidates for paratransgenesis of mosquitoes (Ren *et al.*, 2008; Barik *et al.*, 2016).

Impact on human and animal health

There are old reports (Kurstak *et al.*, 1969) indicating effect of densoviruses on vertebrate cells. More recently, densoviruses (*Mythimna lorreye densovirus* = MlDV) have been tested in detail and with modern techniques on their ability to enter, replicate and produce virus in vertebrate cells, but were found to only cause an abortive infection (El-Far *et al.*, 2004). The viral DNA was able to integrate into the genome of L cells, but only after transfection.

Relevant observations

Densoviruses

- occur worldwide in arthropods including insects
- are not often observed in nature, but can be a major mortality factor in insect rearings
- multiply in insects and insect cells, but are unable to grow in vertebrate cells
- are used as biocontrol agent of insect pests
- have been genetically characterized and engineered
- have low impact on insect ecology

Conclusion

The conclusion is reached that, on the basis of available literature, insect densoviruses (*Densovirinae*) can be considered true insect viruses, with no effect on organisms beyond the arthropod (insect) kingdom. The observations have been summarized in Table 2.

Table 2: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Genera *Ambidensovirus*, *Brevidensovirus, Iteradensovirus*, Subfamily *Densovirinae*, Family *Parvoviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	wide range of hosts; specific
relation with vertebrate virus taxons	Y	xx	parvoviruses, but not virus-dependent
evidence for infection of vertebrate animals	N	x	not found or experimentally tested
replication in vertebrate cells	N	xxx	only entry; abortive
monophyletic deeply-rooted clade within virus family	Y	xxx	related to parvoviruses
insect-specific entry/release/transmission mechanism	Y	x	nuclear replication
unique genetic traits correlating with insect-specificity			nd
unique genome structure	Y	xxx	small ssDNA molecule
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	H		lethal
hematophagous host(s) (Yes/No)	Y		not exclusively
within host tropism (Broad/Restricted)	B		gut, fat body, sperm
host(s) include feed/food insects (Yes/No)	Y		
endemic in NL (Yes/No)			nd, but probably yes
persistent infections (Yes/No)	Y		in cells
horizontal transmission (Yes/No)	Y		

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Proposed status genus *Ambidensovirus, Brevidensovirus* and *Iteradensovirus,* (*Densovirinae, Parvoviridae*) species: Insect-Specific Virus (ISV)

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Proposed *Densovirinae* (genera *Ambidensovirus, Brevidensovirus* and *Iteradensovirus*) ISV species

Virus species	Virus name	Abbrev.
Orthopteran ambidensovirus 1	*Acheta domestica densovirus*	AdDV
Orthopteran densovirus 1	*Acheta domestica mini ambidensovirus*	AdMADV
	Aedes aegypti densovirus 1	AeDNV-1
	Aedes aegypti densovirus 2	AeDNV
Dipteran brevidensovirus 2	*Aedes albopictus densovirus 2*	AalDV
	Agraulis vanilla densovirus	AvDNV
Dipteran brevidensovirus 1	*Anopheles gambiae densovirus*	AgDV
Blattodean ambidensovirus 2	*Blattella germanica densovirus 1*	BgDV
Lepidopteran iteradensovirus 1	*Bombyx mori densovirus 1*	BmDV
Lepidopteran iteradensovirus 2	*Casphalia extranea densovirus*	CeDV
Dipteran ambidensovirus 1	*Culex pipiens densovirus*	CpDV
	Culex pipiens pallens densovirus	CppDNV
Lepidopteran iteradensovirus 3	*Dendrolimus punctatus densovirus*	DpDV
	Diatraea saccharalis densovirus	DsDNV
Hemipteran ambidensovirus 2	*Dysaphis plantaginea densovirus 1*	DpDV
	Euxoa auxilliaris densovirus	EaDNV
Lepidopteran ambidensovirus 1	*Galleria mellonella densovirus*	GmDV
	Haemogogus equinus densovirus	HeDNV
Lepidopteran iteradensovirus 5	*Helicoverpa armigera densovirus*	HaDV
	Junonia coenia densovirus	JcDNV
	Leucorrhinia dubia densovirus	LduDNV
	Lymantria dispar densovirus	LdiDNV
	Mythimna loreyi densovirus	MlDNV
Hemipteran ambidensovirus 3	*Myzus persicae densovirus 1*	MpDV
Lepidopteran iteradensovirus 4	*Papilio polyxenes densovirus*	PpDV
Blattodean ambidensovirus 2	*Periplaneta fuliginosa densovirus*	PfDV
	Pieris rapae densovirus	PrDNV
Hemipteran ambidensovirus 1	*Planococcus citri densovirus*	PcDNV
	Pseudaletia includens densovirus	PiDNV
	Sibine fusca densovirus	SfDNV
	Simulium vittatum densovirus	SvDNV
Hymenopteran ambidensovirus 1	*Solenopsis invicta densovirus*	SiDV
	Toxorhynchites amboinensis densovirus	TaDNV
	Toxorhynchites splendens densovirus	TsDNV

Ad 1 DV = current taxonomy (2018); DNV = former taxonomy (2011)

Ad 2 New densoviruses within these genera appear continuously and have been included up to January 2019.

10-*Dicistroviridae* (family)

The *Dicistroviridae* is a family of small, isometric, positive-sense RNA viruses of invertebrates (mosquitoes, flies, bees, ants, aphids, leafhoppers, moths and shrimp) (Plus *et al.*, 1975; Christian and Scotti, 2009; Bonning and Miller, 2010). Some of these viruses cause major diseases of notable economic interest, such as acute bee paralysis in bees (Blanchard *et al.*, 2008) and the association of the virus with colony collapse disorder, paralysis in crickets (cricket paralysis virus) that are used for food and feed, and disease in crustaceans, such as Taura syndrome virus in shrimp (Valles *et al.*, 2017; ICTV 2011). The family name is derived from the presence of two cistrons on the RNA genome.

Virions

Dicistrovirus virions are small, non-enveloped icosahedral particles of about 30 nm in diameter, containing four capsid proteins and a single positive-stranded RNA molecule (Fig. 1). By electron microscopy, dicistrovirus virions cannot be discriminated from iflavirus (Lemma 15), noravirus (Lemma 18) and vertebrate picornavirus virions. The virions contain a single copy of single-stranded, positive-sense RNA molecule of about 10kb in size, which also contains a covalently linked VPg at the 5'-end of the RNA molecule.

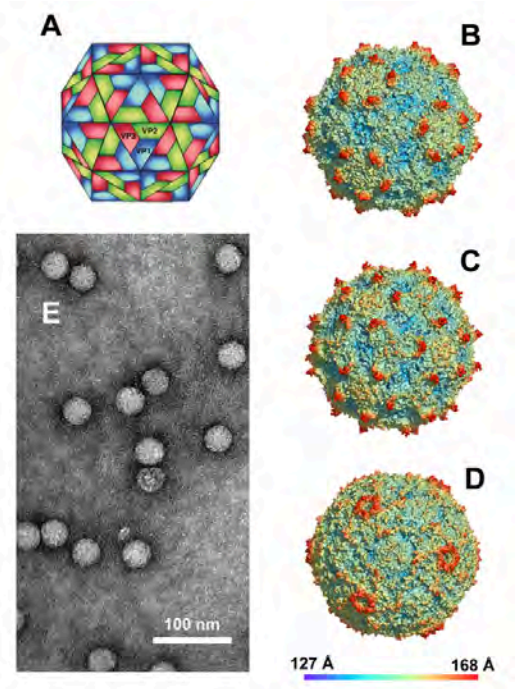

Figure 1. ICTV Online Report (2017); Fig. 1 in Valles *et al.*, 2017

Genetics

A dicistrovirus viral RNA is about 9-10 kb in size and is of positive-sense with two cistrons (Fig. 2). The RNA-dependent RNA polymerase (RdRp) along with a helicase and protease in one cistron is located at the 5'-end (ORF1). The capsid proteins (VP1-4) are located at the 3'end in a non-overlapping second cistron (ORF2). The 5' non-translated region and the region in between the two cistrons contain a highly structured sequence serving as two internal ribosomal entry sites (IRES) to allow cap-independent translation into proteins (Carter *et al.*, 2008). The coding regions are flanked by a non-translated region at the 3'-end.

The presence of an IRES and a 3'-poly A tail is a characteristic shared with ifla- (Lemma 15) and noraviruses (Lemma 18).

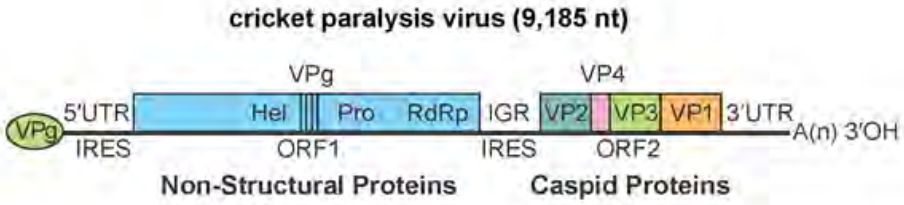

Figure 2. Genome structure of a dicistrovirus (cricket paralysis virus). ICTV Online report (2017); Fig. 2 in Valles *et al.*, 2017.

Taxonomy

Originally, these viruses were named picorna-like viruses, due to their similarity in virion shape, RNA structure, replication strategy and phylogenetic relatedness with vertebrate picornaviruses (pico = small), such as poliovirus, foot-and-mouth disease, etc.). Currently the picorna-like viruses are accommodated within the Order *Picornavirales* containing six virus families of vertebrate, algal or plant origin (Le Gall *et al.*, 2008) two of which occur in insects (*Dicistroviridae* and *Iflaviridae*).

The family *Dicistroviridae* encompasses three genera, *Aparavirus*, *Cripavirus* and *Triatovirus*, classified on the basis of phylogenetic divergence and structure of the internal ribosome entry site (Valles *et al.*, 2017). The genus *Aparavirus* has six recognized species, among others *Acute bee paralysis virus*. *Taura syndrome virus* (TSV) and Mud Crab Virus (MCV) in this genus are not from insects, but from crustaceans. The genus *Cripavirus* currently has four recognized virus species. The genus *Triatoma* accommodates virus five virus species (ICTV, 2011; Chen *et al.*, 2011).

The genome structure of dicistroviruses (Fig. 2) is a common characteristic within the family, and distinct from the other families. Also the phylogeny of members of the *Picornavirales* is very distinctive with deep rooting (Fig. 3).

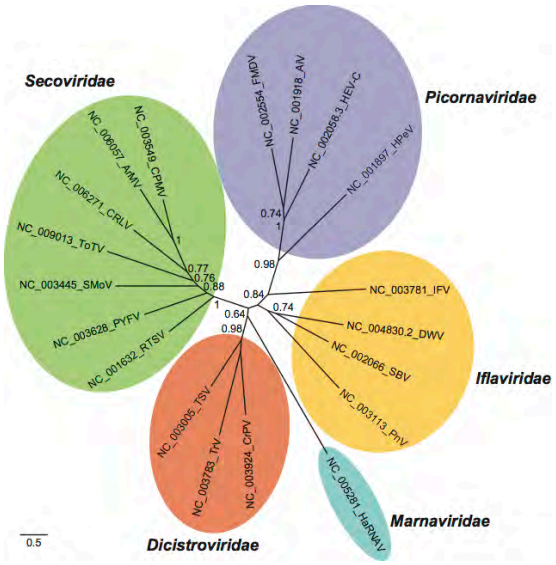

Figure 3. Phylogeny of the order *Picornavirales* using RdRP sequences (Source ICTV 10th report, 2011; https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/227/picornavirales). The noraviruses are not in here (see Lemma 18).

Family: *Dicistroviridae*
Genus: *Aparavirus*
Species: *Acute bee paralysis virus*
Species: *Israeli acute paralysis virus*
Species: *Kashmir bee virus*
Species: *Mud crab virus*
Species: *Solenopsis invicta virus 1*
Species: *Taura syndrome virus*
Genus: *Cripavirus*
Species: *Aphid lethal paralysis virus*
Species: *Cricket paralysis virus*
Species: *Drosophila C virus*
Species: *Rhopalosiphum padi virus*
Genus: *Triatovirus*
Species: *Black queen cell virus*
Species: *Himetobi P virus*
Species: *Homalodisca coagulata virus 1*
Species: *Plautia stali intestine virus*
Species: *Triatoma virus*

Table 1. Virus taxonomy 2018 Release

Replication

Dicistrovirus replication occurs in the cytoplasm of infected insect cells. After attachment into clathrin-coated vesicles, the viral RNA is translated into two polyproteins via the IRES, which are then further cleaved by viral and host-specific proteases into single proteins, such as RNA-dependent RNA polymerase, protease and virion proteins. The viral RNA serves as a template for complementary negative-strand synthesis, which in turn serves as a template to run off viral RNAs available for encasing into virions. Replicase protein associates with cytoplasmic vesicles and Golgi. Replicated virions can be seen as paracrystalline arrays in cells (Bonning *et al.*, 2010; Bonning and Johnson, 2010).

Pathobiology

Dicistroviruses infect a wide array of terrestrial and aquatic arthropods. In insects, these viruses have been predominantly found in dipterans and lepidopterans. Infection occurs mostly in cells of the digestive tract, but other tissues can be infected as well. Replication is cytoplasmic. Transmission is largely *per os*, but vertical transmission has been reported. Infection can range from chronic to acute. Often dicistrovirus infection reduces the lifespan of insect populations. Infections are often covert.

Cricket paralysis virus (CrPV) and Drosophila C virus (DCV) are known to replicate in insect cell culture (Christian and Scotti, 1997), other dicistroviruses are sometimes only semi-permissive for these cells.

Dicistroviruses provoke a specific innate immunity response in insects by RNA interference and a humoral response mediated by JAK-Stat. DCV and CrPV encode suppressors of the antiviral RNAi pathway of insects.

Ecology

Dicistroviruses widely occur in insects, mites, acarids and crustaceans, but most of the time unnoticed (asymptomatic) (Bonning and Johnson, 2010). For example, about 30% of the wild and laboratory populations of *Drosophila melanogaster* is infected by Drosophila C virus. Some of the dicistroviruses can cause severe disease, such as cricket paralysis virus or Israeli acute paralysis virus in bees (Blanchard *et al.*, 2008). Dicistroviruses have not been used as biocontrol agent.

Genetic engineering

An infectious clone of cricket paralysis virus was constructed producing infectious virions in Drosophila S2 cells (Kerr *et al.*, 2015), whereas infectious transcripts were obtained from Black queen cell virus (Benjiddou *et al.*, 2002). To obviate the need for dicistrovirus growth in insects, a dicistrovirus cDNA has been engineered, as a model, into a baculovirus vector and produced in insect cells (Pal *et al.*, 2007).

Impact on human and animal health

There have been no reports on the effect of dicistroviruses on vertebrates or vertebrate cells. However, the dicistrovirus IRES has been extensively studied for its ability to promote expression of genes in vertebrate cells (Gropelli *et al.*, 2007), but appeared to be inferior to picornavirus IRES motifs.

A dicistrovirus has been found in bat guano in Europe and goose feces in Canada, but this may have been a 'passenger' since bats feed on insects and is unlikely to affect bats (Reuter *et al.*, 2014; Greninger and Jerome, 2016).

Table 2: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Family *Dicistroviridae*, Order *Picornavirales*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	wide range of insects (and crustaceans)
relation with vertebrate virus taxons	Y	xx	picornaviruses; virion structure; RNA size
evidence for infection of vertebrate animals		x	nd, unlikely
replication in vertebrate cells		x	nd; only IRES tested
monophyletic deeply-rooted clade within virus family	Y	xxx	sandwiched between rhabdoviruses
insect-specific entry/release/transmission mechanism	Y	x/-	common to picornavirales (cytoplasm.repl)
unique genetic traits correlating with insect-specificity	Y	x	IRES; species specific
unique genome structure	Y	xxx	two cistrons
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M		no gross pathology; chronic to lethal
hematophagous host(s) (Yes/No)	Y		mosquitoes among many other insects
within host tropism (Broad/Restricted)	B		gut, fat body
host(s) include feed/food insects (Yes/No)	Y	x/-	nd, very likely
endemic in NL (Yes/No)	Y		probably
persistent infections (Yes/No)	Y	x/-	likely
horizontal transmission (Yes/No)	Y		per os

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Relevant observations

Dicistroviruses

- occur ubiquitously in nature and worldwide
- are a mortality factor in insect colonies, mostly dipteran and lepidopteran, and crustaceans
- do not readily replicate in insect cell culture, except for CrPV and DCV
- do not infect or affect vertebrate hosts as far as literature reports
- have been genetically well characterized and form a distinct phylogenetic clade
- has been genetically engineered via infectious cDNA clones
- can have high impact on bee ecology

Conclusion

Dicistroviruses are a distinct family of viruses within the *Picornavirales* order. Except for two species (TSV and MCV, genus *Aparavirus*) they exclusively infect insects. Members of the family are invertebrate-specific. There is no evidence to date that members of the *Dicistroviridae* family infect or has effect on vertebrate hosts, including man. The observations have been summarized in Table 2.

Proposed status species genus *Cripavirus* and *Triatovirus* (*Dicistroviridae*): Insect-Specific Virus (ISV)

Proposed status species genus *Aparavirus* (*Dicistroviridae*): Insect-Specific Virus (ISV), except crustacean viruses

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Proposed *Dicistrovirus* ISV species

Virus name	Genus	Abbreviation
Genus *Aparovirus (1)*		
• *Acute bee paralysis virus**	*Aparovirus*	ABPV
• *Israeli acute paralysis virus*	*Aparovirus*	IAPV
• *Kashmir bee virus*	*Aparovirus*	KBV
• *Solenopsis invicta virus-1*	*Aparovirus*	SINV1
Genus *Cripavirus*		
• *Anopheles C virus*	*Cripavirus*	AnCV
• *Aphid lethal paralysis virus*	*Cripavirus*	ALPV
• *Cricket paralysis virus**	*Cripavirus*	CrPV
• *Drosophila C virus*	*Cripavirus*	DCV
• *Nilaparvata lugens C virus*	*Cripavirus*	NlCV
• *Plautia stali intestine virus*	*Triatovirus*	PSIV
Genus *Triatovirus*		
• *Black queen cell virus*	*Triatovirus*	BQCV
• *Himetobi P virus*	*Triatovirus*	HiPV
• *Homalodisca coagulatavirus-l*	*Triatovirus*	HoCV
• *Triatoma virus**	*Triatovirus*	TrV

* Type species

(1) *Taura Syndrome Virus* (TSV) and *Mud Crab Virus* (MCV) are not from insects but from crustaceans.

11-*Entomobirnavirus* (genus), *Birnaviridae* (family)

Birnaviruses are pathogens of fish, poultry and insects. In fish, birnaviruses cause infectious pancreatic necrosis in salmon, a serious disease with high economic losses. In young chickens birnaviruses cause infectious bursal disease. In insects, mainly from dipterans, they have been found by coincidence from insect cell cultures and via next generation sequencing (NGS).

Virions

The virions are non-enveloped and about 65 nm in diameter, and with isosahedral symmetry. The proteinaceous capsid is single shelled (in contrast to reoviruses).

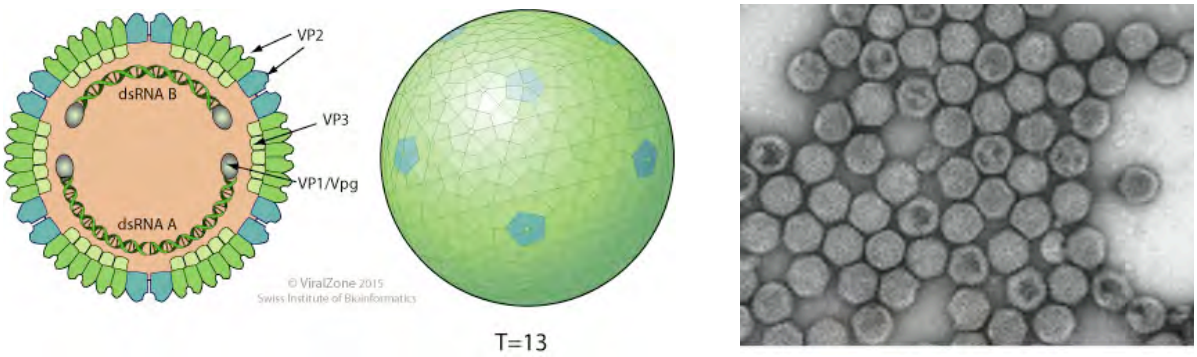

Figure 1. Left: Drosophila X virus particle consisting of two linear dsRNA molecules of about 3.0 and 3.4 kbp in size (source: ViralZone 2015), Right: Infectious bursal disease virus (EM, source: ICTV, 2011).

Genetics

The birnavirus (e.g. *Drosophila X virus*) contains two dsRNA segments, of 2.9 and 3.6 kbp in size (ICTV 2018). The larger segment encodes a polyprotein that is cleaved into virion proteins. The smaller segment encodes an RNA dependent RNA polymerase linked to the 5' end of the RNAs. Viral RNAs have no polyA at the 3' end. VP3 is a dsRNA binding protein that specifically suppresses the RNAi-based antiviral defense in insect cells (Van Cleef *et al.*, 2014).

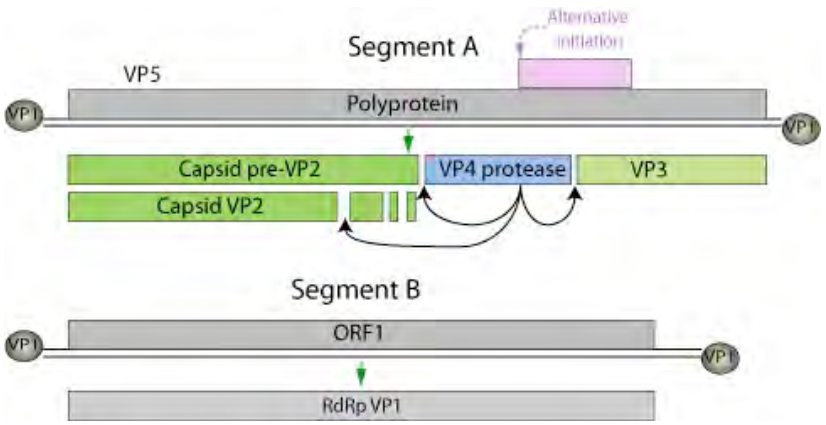

Figure 2. Segmented linear dsRNA genome: 2 segments (A,B) encode for 5 proteins. VP1 is found in a free form and covalently attached at the 5' genomic RNA end (VPg). Segments size is about 3 kbp and 3.4 kbp, respectively, in size. Genome total size is about 6.5 kb. (ViralZone, 2015)

Taxonomy

The *Birnaviridae* is a family of double stranded, segmented RNA viruses. Currently this family has four genera: *Aquabirnavirus* (3 species), *Avibirnavirus* (1 species), *Blosnavirus* (1 species) and *Entomobirnavirus* (1 species) accommodating birnaviruses from fish, birds, fish and insects, respectively (ICTV 2011; Delmas *et al.*, 2011; ibid, 2019). The only current member of the genus *Entomobirnavirus* occurs in *Drosophila* spp. (Brun *et al.*, 1980), but putative new members have been found in other dipteran species, such Culex Y virus (CYV) and a sister virus Espirito Santo Virus (ESV) from mosquitoes (Vancini *et al.*, 2012). The genus *Entomobirnavirus* is phylogenetically a deeply rooted clade among the birnaviruses (Fig. 3).

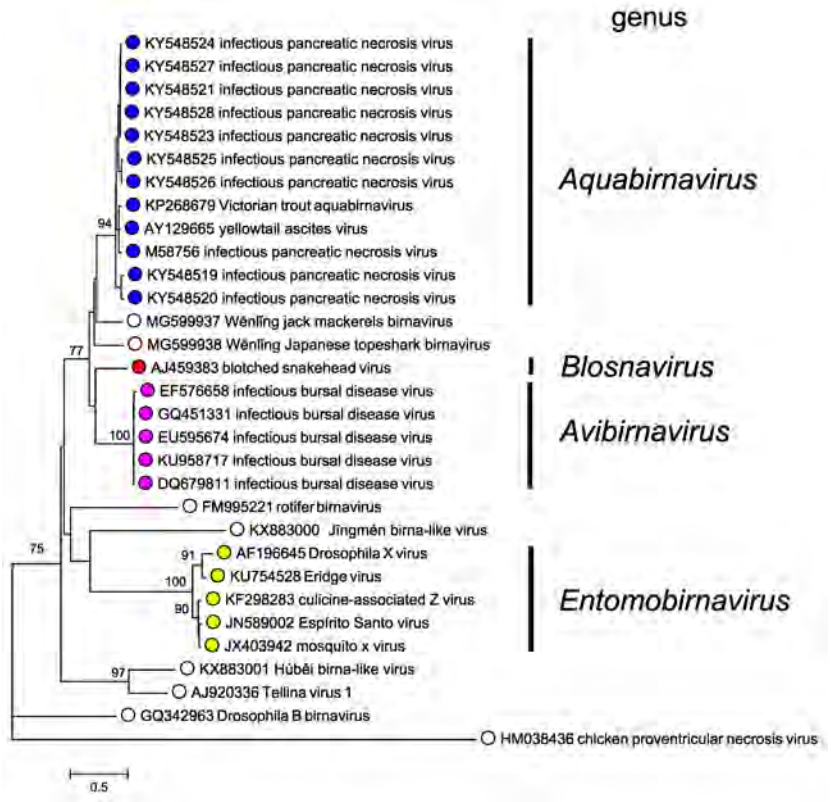

Figure 3. Phylogeny of birnaviruses based on virion protein 1 (VP1). Fig 4 in ICTV, 2011, https://talk.ictvonline.org/ictv-reports/ictv_online_report/dsrna-viruses/w/birnaviridae.

Pathobiology

Drosophila X virus was initially found in a *Drosophila* cell culture (persistent infection) as a contaminant (Teninges *et al.*, 1979; Brun *et al.*, 1980), but also in *Drosophila* flies by NGS of whole flies (Fig. 1D in Webster *et al.*, 2016, https://doi.org/10.4137/EBO.S39454). Culex Y virus was also found in natural flies (*Culex pipiens*) (Marklewitz *et al.*, 2012). Infection causes severe vacuolization and chromatin rearrangement.

The virus induces sensitivity to carbon dioxide and can cause mortality of the flies. Drosophila X virus does not replicate in vertebrate cells or mouse brain, suggesting that it is insect-specific. A sister virus, Espirito Santo virus does replicate in insect cells to high titers, but not at all in Vero cells (Vancini *et al.*, 2012).

Ecology

Nothing is known about the transmission of Drosophila X virus, neither is its role in (worldwide) ecology. Drosophila X virus is widely distributed in nature around the world Vancini *et al.*, 2012). This virus is horizontally transmitted (ICTV 2018), but the mechanisms (e.g. possible vectors?) are not known.

Genetic engineering

Vertebrate birnaviruses have been synthetically made using cDNA as template (Mundt and Vakharia, 1996), but entomobirnaviruses have not (yet) (van Cleef *et al.*, 2014). Thus, the technology is in place.

Impact on human and animal health

There is no information on the effect of entomobirnaviruses on vertebrates or vertebrate cells.

Relevant observations

Insect-specific birnaviruses (ISVs, genus *Entomobirnavirus*, family *Birnaviridae*,)

- occur world-wide, mainly in *Drosophilidae* and mosquitoes
- are species-specific, but the (molecular) basis of specificity unknown,
- are not very virulent and are not known to cause epidemics
- have not been reported to enter / infect / replicate in vertebrate(s) / - cells
- have not (yet) been engineered

Table 2: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Genus *Entomobirnavirus;* Family *Birnaviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	dipterans (mosquitoes)
relation with vertebrate virus taxons	Y	xx	part of a larger family
evidence for infection of vertebrate animals			nd, unlikely
replication in vertebrate cells			nd
monophyletic deeply-rooted clade within virus family	Y	xxx	
insect-specific entry/release/transmission mechanism			nd
unique genetic traits correlating with insect-specificity	Y	xxx	VP3 in suppressing RNAi
unique genome structure	Y	x	
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L	x/-	
hematophagous host(s) (Yes/No)	Y		dipterans
within host tropism (Broad/Restricted)			nd, gut?
host(s) include feed/food insects (Yes/No)	N	x/-	may be in Culex
endemic in NL (Yes/No)	N		may be in Culex
persistence (Yes/No)	Y		in cells
horizontal transmission (Yes/No)	Y		

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

On the basis of available literature, members of the genus *Entomobirnavirus* (Family *Birnaviridae*) can be considered classical insect-specific viruses (ISVs), without any known effect on vertebrates and with high specificity for dipteran hosts. This group of viruses is strongly supported by phylogenetic analysis of its members to be a well-defined clade within the *Birnaviridae* family. The observations have been summarized in Table 2.

Proposed status *Entomobirnavirus* (genus): Insect-Specific Virus (ISV)

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Proposed *Entomobirnavirus* ISV Species (1)

Species	Abbreviation
• *Culex Y virus*	CuYV
• *Culicine-associated Z virus*	CAZV
• *Drosophila melanogaster birnavirus*	
• *Drosophila B virus*	
• *Drosophila X virus**	DXV
• *Eridge virus (Dimm)*	EV
• *Espirito Santo virus*	ESV
• *Eurytemora affinis virus*	
• *Mosquito X virus*	MXV

* type species
(1) Although these species are not all formally listed by the ICTV, they are included here to reflect the advance in the field.

12-*Entomopoxvirinae* (subfamily), *Poxviridae* (family)

Entomopoxviruses (*Entomopoxvirinae*) are insect viruses that cause chronic or slow disease in coleopteran, dipteran, lepidopteran and orthopteran insect larvae (ICTV, 2011; Skinner *et al.*, 2012). The first entomopoxvirus was discovered in 1963 (Vago *et al.*, 1963; King *et al.*, 1998; Bergoin, 2002)). The entomopoxviruses are relatives of the vertebrate chordopoxviruses of humans, goats, sheep, birds and rabbits, swine and deer. Hence, poxviruses as such can affect a wide range of vertebrates and invertebrates and have a number of common and distinctive characteristics.

Virions

The entomopox virions are brick- or ovoid-shaped, of considerable size (70-250 x 350 nm) (Fig. 1), enveloped and containing a very large (250-380 kpb) linear, double stranded DNA molecule. Unlike all other large double stranded DNA viruses, which replicate in the nucleus, replication of entomopoxvirus occurs entirely within the cytoplasm.

Like baculoviruses and insect reoviruses, entomopoxviruses are embedded in a protein matrix or spheroid. The matrix of entomopoxviruses contains two major proteins, fusolin and spindolin. The amino acid sequence of these proteins is entirely different from the occlusion body protein of baculoviruses of insect reoviruses. The entomopoxvirus occlusion bodies have a paracrystalline structure and are sensitive to alkali, a situation in the insect digestive tract.

In addition to proteins, the virions also contain lipids and carbohydrates (ICTV 2011).

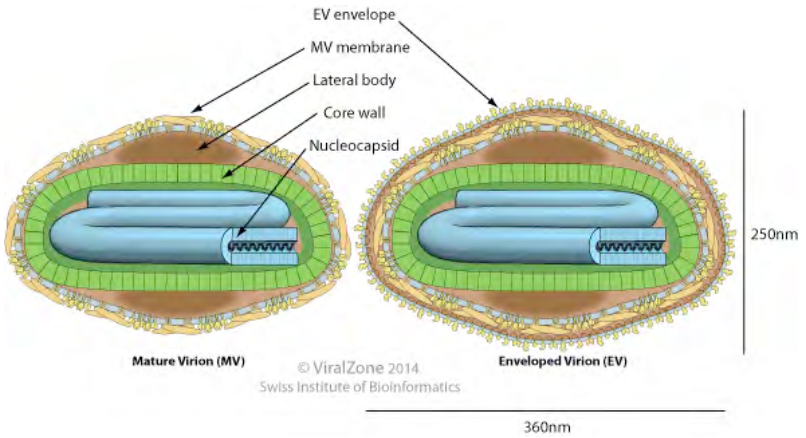

Figure 1. Entomopoxvirus virion with 'kidney-shaped nucleoid. Source: Viral Zone 2014.

Taxonomy

The large ovoid- to brick-shaped phenotype is a common characteristic of poxviruses. Also their large genome size (> 200 kbp), genome organization, gene content and homology, replication strategy and subcellular localization (cytoplasm) are typical feature of poxviruses.

The family *Poxviridae* has been subdivided into two subfamilies: *Chordopoxvirinae* to accommodate the vertebrate poxviruses from o.a. humans, sheep, birds, etc., and *Entomopoxvirinae* for viruses of insects. A putative member of the poxvirus family has been

found in molluscs. The taxonomy of *Poxviridae* has been revised in 2009 (ICTV 2011) and the most recent update is ratified October 2018 (ICTV, 2018).

The entomopoxviruses are subdivided into three genera, based on core structure, host range and phylogeny (ICTV 2011). The alpha-, beta- and gammaentomopoxviruses occur in *Coleoptera* (beetles), *Lepidoptera* (butterflies, moths) and *Orthoptera* (crickets, locusts, grasshoppers) and *Diptera* (flies), respectively. As of 2018, seven species are recognized with the genus *Alphaentomopoxvirus*, sixteen in the genus *Betaentomopoxvirus* and six in the genus *Gammaentomopoxvirus*. Two species are also recognized within this subfamily and to be classified (Afonso *et al.*, 1990).

Phylogenetically, the *Entomopoxvirinae* are well separated from the vertebrate poxviruses (*Chordopoxvirinae*) and deeply rooted. The relation among the entomopoxviruses is somewhat ambiguous (Fig. 2) due to the lack of sufficient sequence information.

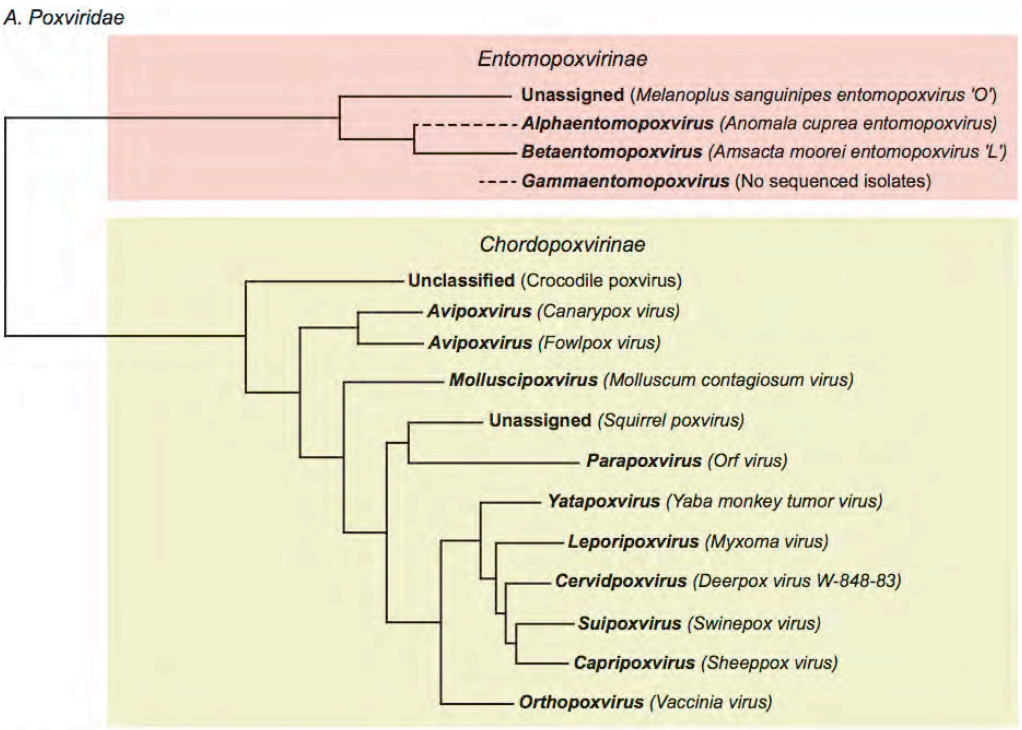

Figure 2. Phylogenetic relationship between poxviruses based on the amino acid sequences of 19 conserved poxvirus genes (ICTV, 2011).

Entomopoxviruses share quite a few genes suggesting common ancestry. The entomopoxviruses are genetically more diverse than the orthopoxviruses, suggesting they are older and had a longer evolutionary history (Barrett and McFadden, 2008).

Genetics

The genomes of entomopoxviruses range from 230 to 380 kbp, are very AT-rich and contain repeats and 200+ open reading frames (see Fig. 1 in Bawden et al., 2000, https://doi.org/10.1006/viro.2000.0449). There is considerable homology among the entomopoxvirus genes, but their order along the genome is highly scattered (Williams *et al.*, 2017). The linear DNA is circularized by cohesive hairpin ends.

Entomopoxviruses share at least 49 genes with the chordopoxviruses suggesting common ancestry. The entomopoxvirus genomes are generally larger than those from orthopoxviruses (small pox) of vertebrates, as they contain additional genes for their specific

'life cycle' in invertebrates, such as DNA repair enzymes, protective proteins against UV damage and occlusion body proteins. Only four entomopoxviruses are completely sequenced to date (GeneBank Nov 2018).

Replication

Entomopoxviruses replicate entirely in the cytoplasm of infected cells in cytoplasmic factories. The virions contain a virus-specific DNA-dependent RNA polymerase to transcribe the genome in a temporal fashion. Messenger RNA is polyadenylated at the 3' end and capped at the 5' end. The first proteins produced are required for replication of viral DNA, to carry out functions for late transcription and expression and for capturing a.o. the host protein synthesis machinery.

Pathobiology

Entomopoxviruses are infectious for larvae *per os*. They initially infect midgut columnar cells before other tissues such as fat body are infected (Pereira *et al.*, 2010, and refs therein). The virions are released from the spheroids due to the alkalinity in the gut. Entomopoxviruses encode an inhibitor of apoptosis in order for the infection to proceed. Infection is often chronic (slow) and prevents the target insects to undergo metamorphosis. Infection is ultimately lethal after weeks or months. Hence, they cause epizootics that advance slowly. Typical signs of infection are lethargy and flaccidity and whitening of the body (Williams *et al.*, 2017).

Entomopoxviruses can also infect insect cells (Langridge and Greenberg, 1981; Marlow *et al.*, 1993) and this allows detailed molecular studies of these viruses.

Ecology

Entomopoxviruses occur predominantly in *Lepidoptera*, *Diptera*, *Coleoptera* and *Orthoptera* larvae (Granados, 1981) and are a mortality factor of low significance. Their special phenotype (paracrystalline spheroidin capsule encasing the virions) protects the entomopoxviruses from rapid environmental decay and allows them to survive for some time outside their host. Their role in the ecosystem has not been studied.

Entomopoxviruses have been considered as biocontrol agents of insect pests, but their speed of action is too slow, as is the case for ascoviruses (Lemma 4).

Genetic engineering

Entomopoxviruses can be engineered by homologous recombination in insect cell culture using plasmids with marker genes (chloramphenicol acetyltransferase, green fluorescent protein) and wild type virus (Palmer *et al.*, 1995). Poxvirus DNA by itself is noninfectious in cells (Moss *et al.*, 1990). Recombinant entomopoxviruses are obtained after plaque purification (Olszewski and Dall, 2002). The promoters of the spheroidin genes have been used to drive the expression of foreign genes. GFP-containing viruses were used to study the pathology of entomopoxviruses (Pereira *et al.*, 2010).

Impact for human and animal health

Entomopoxviruses (*Amsacta morei*) can grow in insect cell culture, when applied as virions not occluded in occlusion bodies. Infection of vertebrate (murine cells) with entomopoxvirus (*Amscata morei*) did not result in increase of virus titers (Langridge, 1983), but apparently they can enter vertebrate cells and carry out early transcription, but without replication of the virus and no cytopathology (Li *et al.*, 1997).

Entomopoxviruses have not been tested in vertebrate animals for infectivity, but any effect is unlikely since these viruses have to be released first from their occlusion body, requiring highly alkaline environments (as in insects). This condition is not the case in the vertebrate digestive system (acid environment).

Relevant observations

Entomopoxviruses

- encompass a monophyletic, deeply rooted group within the poxviruses
- have a wide host range and specificity among insects
- have a unique phenotype (spheroids) protecting the virions outside the insect host
- replicate in insect cells, but not in vertebrate cells
- have no deleterious effects on vertebrate cells *in vitro* (no animals studies reported)
- can be genetically engineered

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Subfamily *Entomopoxvirinae*, Family *Poxviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	wide insect host range; low specific.
relation with vertebrate virus taxons	Y	xxx	vertebrate poxviruses (fish)
evidence for infection of vertebrate animals		x	nd
replication in vertebrate cells	N	xx	only entry in cells
monophyletic deeply-rooted clade within virus family	Y	xxx	robust
insect-specific entry/release/transmission mechanism	N	x	no, infect vertebrate cells, abortive
unique genetic traits correlating with insect-specificity	N	xx	
unique genome structure	Y	xxx	large linear dsDNA; terminal repeats
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M		often chronic
hematophagous host(s) (Yes/No)	N		
within host tropism (Broad/Restricted)	B		fat body, muscle, gut epithelia
host(s) include feed/food insects (Yes/No)	Y		likely
endemic in NL (Yes/No)	Y		possibly
persistent infections (Yes/No)	N		
horizontal transmission (Yes/No)	Y		very common, epizootics

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

On the basis of their specificity for insects, special occlusion structure, phylogenetic position, the absence of effects on vertebrate cells and restricted host specificity entomopoxviruses can be considered insect-specific (restricted hosts, hence as an ISV) viruses. The observations are summarized in Table 1.

Proposed status *Entomopoxvirinae [Poxviridae)*: Insect-Specific Viruses (ISV)

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Proposed *Entomopoxvirinae* ISV species

Species		Abbreviation
Genus ***Alphaentomopoxvirus***		
• *Anomala cuprea entomopoxvirus*		ACEV
• *Aphodius tasmaniae entomopoxvirus*		ATEV
• *Demodema bonarensis entomopoxvirus*		DBEV
• *Dermolepida albohirtum entomopoxvirus*		DAEV
• *Figulus sublaevis entomopoxvirus*		FSEV
• *Geotrupes sylvaticus entomopoxvirus*		GSEV
• *Melolontha melolontha entomopoxvirus*	type species	MMEV
Genus ***Betaentomopoxvirus***		
• *Acrobasis zelleri entomopoxvirus*		AZEV
• *Adoxophyes honmai entomopoxvirus*		AHEV
• *Amsacta moorei entomopoxvirus*	type species	AMEV
• *Arphia conspersa entomopoxvirus*		ACOEV
• *Choristoneura biennis entomopoxvirus*		CBEV
• *Choristoneura conflicta entomopoxvirus*		CCEV
• *Choristoneura diversuma entomopoxvirus*		CDEV
• *Choristoneura fumiferana entomopoxvirus*		CFEV
• *Choristoneura rosaceana entomopoxvirus*		CREV
• *Chorizagrotis auxilliaris entomopoxvirus*		CXEV
• *Heliothis armigera entomopoxvirus*		HAVE
• *Locusta migratoria entomopoxvirus*		LMEV
• *Mythimna separate entomopoxvirus*		MSEV
• *Oedaleus senegalensis entomopoxvirus*		OSEV
• *Operophtera brumata entomopoxvirus*		OBEV
• *Schistocerca gregaria entomopoxvirus*		SGEV
Genus ***Gammaentomopoxvirus***		
• *Aedes aegypti entomopoxvirus*		AAEV
• *Camptochironomos tentans entomopoxvirus*		CTEV
• *Chironomus attenuatus entomopoxvirus*		CAEV
• *Chironomus luridus entomopoxvirus*	type species	CLEV
• *Chironomus plumosus entomopoxvirus*		CPEV
• *Goeldichironomus holoprasinus entomopoxvirus*		GHEV
Entomopoxviruses to be classified		
• *Diachasmimorpha entomopoxvirus*		DIEV
• *Melanoplus sanguinipes entompoxvirus*		MSEV

13-*Flavivirus* (genus, *cISV + dISF groups*), *Flaviviridae* (family)

The *Flaviviridae* is a family of small, enveloped viruses with a non-segmented, positive stranded RNA ranging in size from 9-13 kb (Roby *et al.*, 2012). Many of these viruses infect mammals or birds (vertebrates), and represent very pathogenic viruses, causing diseases such as yellow fever, dengue, West Nile, encephalitis, zika and hepatitis. Many vertebrate flaviviruses are transmitted by mosquitoes or ticks, and are therefore called arthropod-borne viruses (arboviruses); some are even restricted to their vertebrate host. Many new flaviviruses have caused major epidemics over the last decade (zikavirus, usutuvirus) and are therefore on close watch by the WHO.

A large group of flaviviruses is restricted to their insect hosts (Fig. 1). These so-called insect-specific flaviviruses or ISFs are the topic of this report.

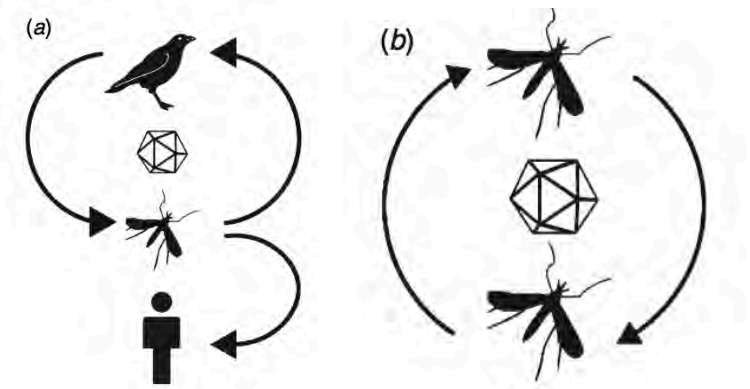

Figure 1. (a) Arbovirus transmission cycle and (b) proposed insect-specific flavivirus (ISF) transmission cycle (After Fig 1A from Hall and Hobson-Peters, 2018)

The name (flavi = yellow) is derived from the observation that yellow fever virus infection can cause jaundice in humans.

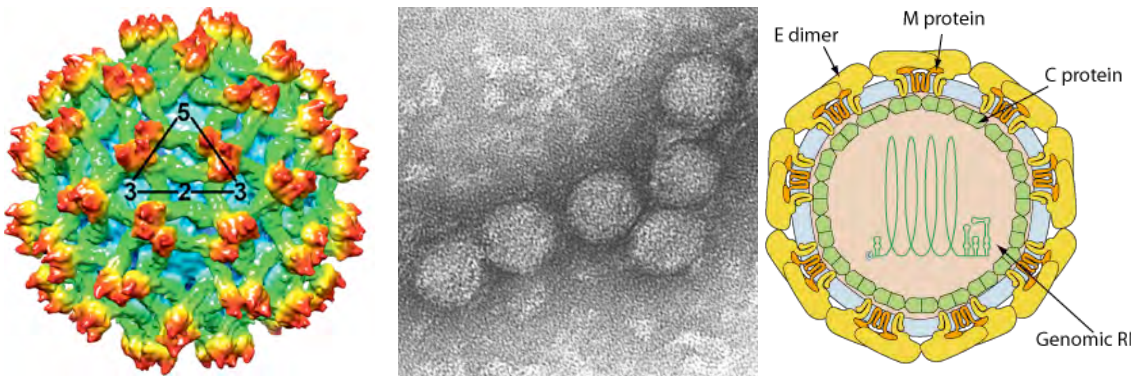

Figure 2. Left: 3-D cryo-EM of a *Flavivirus* (genus) (ICTV, 2018); Middle: TEM of West Nile Virus. Source: CDC/P.E. Rollins; Right: Schematic representation of a flavivirus. Source: ViralZone

The virions

Flavivirus virions are spherical in shape, 40-60 nm in diameter and enveloped (Fig. 2). The virions consist of a single-stranded positive RNA molecule of about 10 kb in size wrapped in

a protein capsid (C) to form a nucleocapsid. A matrix protein (M) is sandwiched between this nucleocapsid and a lipid envelope. One or more proteins extending outwards are embedded in the envelope (E) (Chambers *et al.*, 1990).

Genomics

The flavivirus RNA genome encodes a large polyprotein, which is sequentially processed into monomeric proteins by host and viral-encoded proteases. The RNA molecule has a 5'-end Cap structure for translation initiation and a loop structure at the 3'-end and no polyA (Fig. 3). Members of the *Flavivirus* genus produce 3-end subgenomic RNAs important for pathogenicity (Pijlman *et al.*, 2008). As all RNA viruses, flaviviruses have an RNA-dependent RNA polymerase (Chambers *et al.*, 1990; Simmonds *et al.*, 2017) that can be used for phylogenetic analysis.

Currently two groups of insect-specific flaviviruses have been discerned (Hall *et al.*, 2016; Blitvich and Firth, 2015). The classical insect-specific flaviviruses (cISFs) and the dual-host affiliated flaviviruses (dISFs). Both groups have an additional ORF called *fifo (*fairly interesting flavivirus ORF), overlapping with NS2A-NS2B, the function of which is unknown and which may play a role in the mosquito host. In addition, the envelope protein E of ISFs is very different from the mosquito-borne flaviviruses infecting vertebrates (Hall *et al.*, 2016) and may explain the host restriction at the virus entry level.

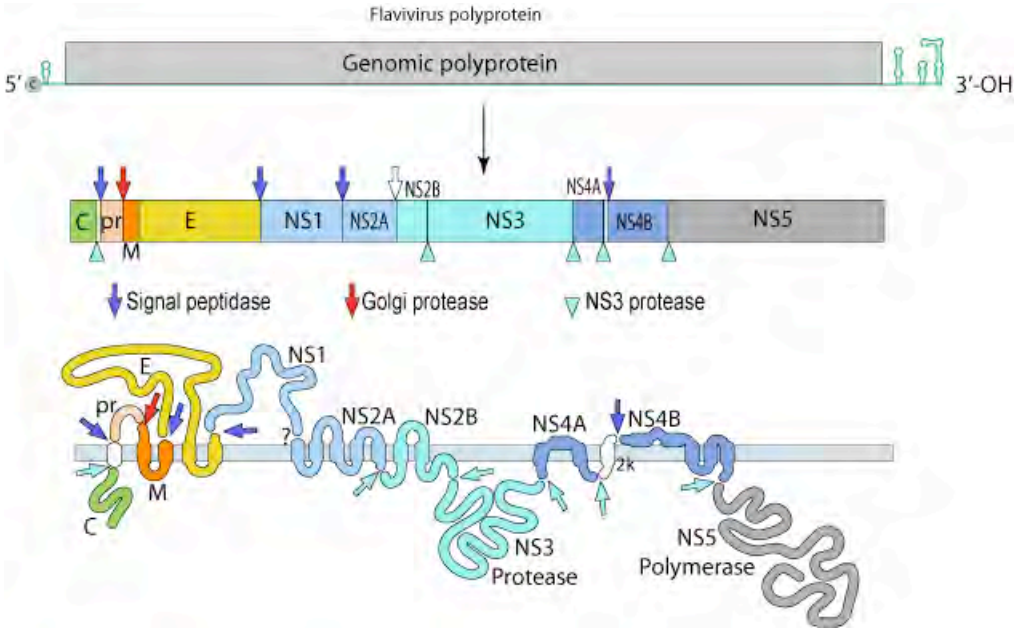

Figure 3. Genome organization and expression of members of the genus *Flavivirus*. 'Colored' protein below the genome indicate viral proteins generated by proteolytic processing by host and viral proteases. E = envelope, NS5 is the RNA-dependent RNA polymerase. The insect-specific *fifo* gene overlaps with NS2A and B. (Source figure: ViralZone

Vertebrates have a lower relative frequency of CpG nucleotides in their genome than invertebrates. This difference in CpG frequency is often mimicked by the small DNA viruses and the RNA viruses of vertebrate/invertebrate hosts (Upadhyay *et al.*, 2013). cISFs have a CpG frequency matching with their invertebrate host and not with vertebrates, whereas

arboviral (flavivirus) genomes often have an underrepresentation of CpGs, just like the vertebrate hosts (Lobo *et al.*, 2009). The dISFs have a CpG frequency intermediate to arboviruses and insect-specific viruses.

Taxonomy

The *Flaviviridae* family currently encompasses four genera: *Flavivirus, Pestivirus, Hepacivirus* and *Pegivirus*. The term 'flavivirus' is somewhat confusing, as it is sometimes used to refer to the family *Flaviviridae* as a whole, but actually should refer only to the genus *Flavivirus*. In this document flaviviruses are discussed in the context of the latter.

Flavivirus species are based on nucleotide and amino acid homology (phylogeny), host and vector associations, and geographical distribution (ICTV, 2018). At least 50 flavivirus species are identified within the genus *Flavivirus*. Members of two phylogenetically distinct groups are transmitted by mosquitoes and ticks, to vertebrates (Simmonds *et al.*, 2017). The largest group, the vertebrate-infecting flaviviruses (VIF) (mosquito-borne and tick-borne), contain important zoonotic pathogens (zikavirus, usutuvirus, denguevirus, encephalitis, etc.). These arboviruses are not discussed in this report.

There are two groups of insect-specific flaviviruses or ISFs: the cISFs (classical ISFs) and the dISFs (dual host affiliated ISFs). cISFs are insect-specific and do not replicate in vertebrates or vertebrate cells (Blitvitch and Firth, 2015, and references therein). These viruses have a world-wide distribution in mosquitoes (Bolling *et al.*, 2015; Calzolari *et al.*, 2016). cISFs are a phylogenetically distinct flavivirus subclade, with a further subclade structure representing *Culex*- and *Aedes*-associated flaviviruses (Blitvich and Firth, 2015; ICTV, 2017) and recently *Anopheles*-associated cISFs (Colmant *et al.*, 2017; Hall *et al.*, 2016). cISFs have a CpG frequency mimicking the insect genomic CpG frequency (Fig. 4). This is in contrast to VIFs (arboviruses), that have a CpG frequency matching their vertebrate host. cISFs are also highly species-specific and they may be ancestral to the *Culex*- and *Aedes*-associated ISFs (Colmant *et al.*, 2017).

The second group of ISFs is called dual host affiliated ISFs (dISFs), because they are phylogenetically sandwiched in between two VIF arbovirus clades (see Fig. 2A in Colmant *et al.*, 2017). In addition, they are slightly polyphyletic. Members of the dISF group of viruses, as does the cISF group, only replicate in mosquitoes and mosquito cells.. It is not known whether dISFs have an as yet unknown vertebrate host or whether they have lost the ability to replicate in vertebrates and have adapted to a mosquito-associated life cycle (Blitvitch and Firth, 2015). The intermediate CpG frequency of dISFs suggests that they are not typical arboviruses (Fig. 4). Antibodies against both cISF (as expected) and dISFs were not found in human or vertebrate sera (Hall & Hobson-Peters, 2018) suggesting these viruses are not infectious for vertebrates.

cISFs are deeply rooted in the phylogenetic tree (see Fig. 2A in Colmant *et al.*, 2017) and may be considered a separate genus (although not classified by ICTV yet). dISFs form a distinct clade in the phylogenetic tree, but this clade is less deeply rooted than the cISFs. As with the cISVs, the number of dISVs flaviviruses is likely to increase through NGS efforts, which will resolve the phylogenetic position of this group of viruses.

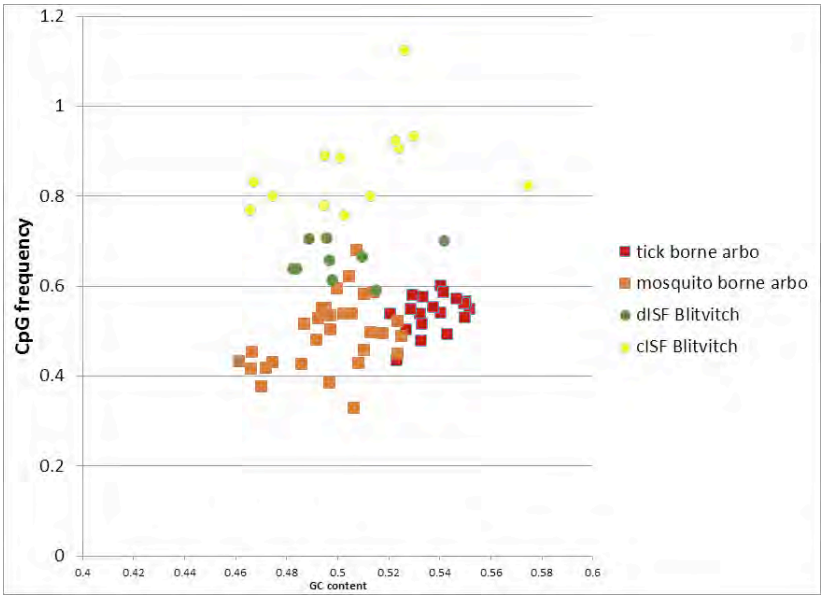

Figure 4: CpG frequency versus GC content for various flavivirus lineages (Coutesy Dr. J.J. Fros). cISFs have a significant higher CpG value, whereas dISFs are somewhat intermediate between cISFs and arboviruses.

Pathobiology

ISF members of the *Flavivirus* genus only infect insects or insect cells, more specifically mosquitoes (taxa *Aedes*, *Culex* and *Anopheles*) that are also transmitting VIFs. It is likely that they also infect other mosquitoes and other insects, but these have sampled less frequently. The cISFs are found worldwide, except for the *Anopheles*-associated ISFs, which are only found (so far) in Australia (Hall *et al.*, 2018).

ISFs are often identified by their ability to replicate in *Aedes albopictus* C6/36 or other mosquito cells, but not in vertebrate cells (Huhtamo *et al.*, 2014; Blitvich and Firth, 2015). ISFs that do not replicate in mosquito cells (*Anopheles*-associated, Hall *et al.*, 2018) escape attention. ISFs are infectious to mosquitoes *per os* and have been found in male mosquitoes, suggesting that these viruses are transmitted vertically. However, ISFs extensively replicate in all tissues of the mosquito, including salivary glands (Saiyasombat *et al.*, 2011). The latter is unexpected, because ISFs do not need to be transmitted to vertebrates.

Ecology

ISFs are found worldwide, are intimately associated with mosquitoes and are most likely vertically transmitted (Saiyasombat *et al.*, 2011; Colmant *et al.*, 2017). To what extent these viruses are a mortality factor or to cause epidemics in mosquitoes is not known. Deep sequencing surveys of mosquitoes frequently encounter novel ISFs. Very little is known about their prevalence in the field in mosquito populations. Transmission is most likely vertical

Genetic engineering

Flaviviruses in general have been extensively genetically engineered for both functional studies as well as for vaccine development (e.g. Niénokoué virus). RNA transcripts of cDNA

are used to transfect cells to retrieve infectious virus. Recently, also the insect-specific flaviviruses (cISFs) have been genetically engineered to understand the restriction of ISFs to mosquito hosts, a.o. the construction of chimaeras between cISFs and WIVs (Piyasena *et al.*, 2017; Junglen *et al.*, 2017).

Impact for human and animal health

Both cISFs and dISFs do not replicate in vertebrate (mammalian) cells (Blitvitch and Firth, 2015, and references therein). Infection of suckling mice with cISFs (AeFV and CxFV) failed and intracerebral injections were also unsuccessful (Kim *et al.*, 2009; Haddow *et al.*, 2013). The replication of ISFs in vertebrate cells is restricted at multiple levels. A switch towards an 'arbovirus-style' replication (replication in both insect and vertebrates) is unlikely to occur, as it would require a.o. adjustment of the CpG ratio.

Relevant observations

Insect-specific flaviviruses (ISVs in the family *Flaviridae*, genus *Flavivirus*)

- occur world-wide, in a number of insects, currently only in mosquitoes
- are species-specific, but the molecular basis of specificity largely unknown,
- specificity for insects is at various levels of the viral life cycle (entry, replication)
- are not very virulent and are not known to cause epidemics
- have not been reported to enter / infect / replicate in vertebrate(s) / - cells
- have been genetically engineered for research purposes (cISFs)
- dISFs have arbovirus characteristics (phylogeny, CpG), but only insect hosts
- cISFs have no arbovirus characteristics and replicate only in insects (or cells)

Conclusion

The conclusion reached is that, on the basis of available literature, the classical insect-specific viruses (cISFs) can be considered as true insect viruses, as they have high specificity for mosquito species (restricted host range) and no effect on vertebrate cells. An insect-like CpG bias in the RNA genome and the presence of an cISV-specific *fifo* ORF give further credit to this notion. This group (cISFs) is strongly supported by phylogenetic analysis of its members to be a well-defined clade within the *Flavivirus* genus. They can be recognized not only by phylogenetic analysis, but also their insect-specific CpG distribution of their genomes.

dISFs have a number of insect-specific characteristics, multilevel replication restrictions in vertebrate cells, a distinct but intermediate CpG bias and no antibodies against dISVs ere found in vertebrates (to date). However, the position of dISFs in the phylogenetic tree of flaviviruses is ambiguous. Therefore dISFs can be provisionally assigned as insect-specific for now.

ISFs in general also replicate in the salivary glands of mosquitoes, providing the essential conduit to vertebrates. This is a potential risk factor, in particular working with chimeras between ISFs and VIFs. The observations have been summarized in Table 1.

Table 1a: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
cISF group, Genus *Flavivirus*, Family *Flaviviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	only mosquitoes; host-specific
relation with vertebrate virus taxons	Y	x	flavivirus morphology, genome
evidence for infection of vertebrate animals		x	nd
replication in vertebrate cells	N	xxx	abortive infections
monophyletic deeply-rooted clade within virus family	Y	xxx	deeply rooted among arbo(flavi)viruses
insect-specific entry/release/transmission mechanism		x	nd
unique genetic traits correlating with insect-specificity	x/-	xxx	*fifo* gene (ISV-specific
unique genome structure	Y	xxx	insect specific CpG
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L		no specific pathology
hematophagous host(s) (Yes/No)	Y		mosquitoes
within host tropism (Broad/Restricted)	B		including salivary glands
host(s) include feed/food insects (Yes/No)	N		nd, but unlikely (no mosquitoes)
endemic in NL (Yes/No)	N		nd
persistent infections (Yes/No)	Y		most likely
horizontal transmission (Yes/No)	N	xx	vertical

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = not data available

Table 1b: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
dISF group, Genus *Flavivirus*, Family *Flaviviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	mainly mosquitoes and flies
relation with vertebrate virus taxons	Y	xxx	flavivirus morphology, genomere plication
evidence for infection of vertebrate animals		xxx	even > 30 oC
replication in vertebrate cells		xxx	abortive infections
monophyletic deeply-rooted clade within virus family		xxx	equivocal within family (arbovirus)
insect-specific entry/release/transmission mechanism			nd
unique genetic traits correlating with insect-specificity	Y	xx	lack of replication > 30 oC
unique genome structure		x	no insect specific CpG
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M		no specific pathology
hematophagous host(s) (Yes/No)	Y		mosquitoes
within host tropism (Broad/Restricted)	B		nd
host(s) include feed/food insects (Yes/No)			nd, but unlikely (no mosquitoes)
endemic in NL (Yes/No)			nd
persistent infections (Yes/No)	Y		most likely
horizontal transmission (Yes/No)	Y	xx	probably also vertically

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = not data available

Proposed status *Flaviviridae*, cISF species: Insect-Specific Virus (ISV)

Proposed status *Flaviviridae*, dISF species: *Provisionally* Insect-Specific Virus (ISV)

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Appendix *Flavivirus* (genus) ISV species

Species	Abbreviation	ICTV-approved
cISFs		
• *Aedes flavivirus*	AEFV	Y
• *Calbertado virus*	CLBOV	Y
• *Cell fusing agent flavivirus*	CFAV	Y
• *Culex flavivirus*	CxFV)	Y
• *Cuacua virus*	CuCuV	likely
• *Culex theileri flavivirus*	CTFV	Y
• *Culiseta flavivirus*	CFV	likely
• *Dairy Swamp virus*	DSwV	likely
• *Ecuador Paraiso Escondido virus*	EPEV	Y
• *Hanko virus*	HANKV	Y
• *Kamiti River virus*	KRV	Y
• *Karumba virus*	KRBV	likely
• *Haslams Creek virus*	HaCV	likely
• *MacPeak virus*	McPV	likely
• *Manghai flavivirus*	MFV	likely
• *Mercado virus*	MeV	likely
• *Mosquito flavivirus*	MoV	Y
• *Nakiwogo virus*	NAKV	Y
• *Niénokoué virus*	NIEV	Y
• *Ochlerotatus caspius flavivirus*	OcFV	likely
• *Palm Creek virus*	PCV	Y
• *Paramatta River virus*	PaRV	likely
• *Quang Bin virus*	QBV	Y
• *Xishuangbanna flavivirus*	XFV	likely
dISFs		
• *Barkedji virus*	BJV	likely
• *Chaoyang virus*	CHAOV	Y
• *Donggang virus*	DONV	likely
• *Ilomantsi virus*	ILOV	likely
• *Lammi virus*	LAMV	Y
• *Marisma mosquito virus*	MMV	likely
• *Nanay virus*	NANV	likely
• *Ngoye virus*	NGOV	Y
• *Nhumirim virus*	NHUV	likely
• *Nounane virus*	NOUV	Y
• *Tamata bat virus*	TABV	Y

likely = not yet approved as species by the ICTV
Sources: ICTV and pertinent literature up to the end of 2018

14-*Hytrosaviridae* (family)

The family *Hytrosaviridae* accommodates a small number of large, enveloped, double stranded DNA viruses that infect only some dipterans, more specifically hematophagous tsetse flies and filth-feeding houseflies. A hytrosavirus in the tsetse fly was first described by Armagier *et al.* (1979), later also in the house fly (Coler *et al.*, 1993). The most notable symptom is hypertrophy of the salivary glands of male and female flies in the acute phase of the disease, salivary gland hypertrophy (SGH). The name of the family *Hytrosaviridae* is derived from the disease: Hypertrophy salivary gland viruses. Hytrosaviruses (virus particles) are present abundantly in tsetse flies and houseflies in a covert state, but with low disease prevalence in the field (Lietze *et al.*, 2011a).

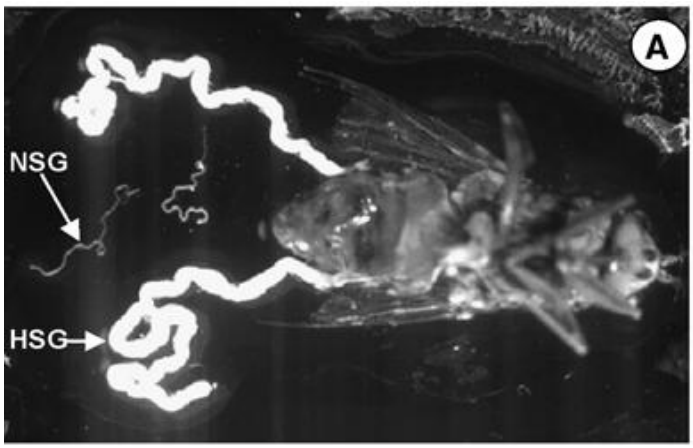

Figure 1: Normal (NSG) and hypertrophied salivary glands (HSG) of *Glossina pallidipes* (Fig. 1 in ICTV 2009, https://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/invertebrate-official/4101

The virions

Hytrosavirus virions are elongated, flexible, enveloped, rod-shaped particles measuring anywhere from about 60 nm wide and 550 – 1000 nm long. The virions consist of a proteolipid envelope, a tegument and nucleocapsids, the latter containing the double stranded circular DNA molecule ranging in size, depending on the species, from 125-180 kbp (Kariithi *et al.*, 2013).

The virions contain many virus-encoded and also host proteins reflecting their morphogenetic history in the host cell. The virions contain multiple, so called *per os* infectivity proteins (PIFs) that are also present in other taxa of large invertebrate circular double stranded DNA viruses (baculoviruses, nudiviruses, nimaviruses, polydnaviruses) (Jehle *et al.*, 2013; Boogaard *et al.*, 2018). In baculoviruses these PIFs form a complex in the envelope of virions and likely assist in the peroral entry of these viruses into their host via the gut (Kariithi *et al.*, 2013).

Genomics

The genomes of GpSGHV and MdSGHV are 180 kbp and 120 kbp in size and circular (Abd-Alla *et al.*, 2008; Garcia-Maruniak *et al.*, 2008)). The DNA of hytrosaviruses has dispersed

homologous repeat regions (putative replication origins and transcriptional enhancers) encodes 108 to 160 non-overlapping open reading frames (ORFs). Many of these coding for virion proteins, others for proteins involved in virus replication and host-interaction. Variants of hytrosavirus exist (Kariithi *et al.*, 2013; Abd-Alla *et al.*, 2016).

Taxonomy

The taxonomy of hytrosaviruses is primarily based on genome size, gene content, phylogenetic relatedness and host specificity (Abd Alla *et al.*, 2009; Jehle *et al.*, 2013, ICTV 2018). Two genera are currently recognized within the family *Hytrosaviridae*: *Glossinavirus* and *Muscavirus*, each with one species.

The genus *Glossinavirus* accommodates Glossina pallidipes salivary gland hypertrophy virus (GpSGHV) from tsetse flies (*Glossina* spp.). GpSGHV virions are typically 50-80 x 500-1000 nm in size. Two GpSGHV strains have been fully sequenced and are about 180 kbp in size coding for about 160 open reading frames (Abd-Alla *et al.*, 2008; ibid. 2016). The Uganda strain is taken as type species for this genus. Ecogeographic variant GpSGHVs are found in other *Glossina* species and are restricted to the equatorial zone of Africa (Kariithi *et al.*, 2013).

The genus *Muscavirus* contains one fully sequenced virus species: Musca domestica salivary gland hypertrophy virus (MdSGHV) from the housefly (*Musca domestica*). MdSGHV virions are typically 65 x 550 nm in size and have a genome of about 125 kbp. MdSGHV is limited (so far) to the housefly and related species. Ecogeographic variant MdSGHVs are found only in houseflies, but all over the world (Garcia-Maruniak *et al.*, 2009).

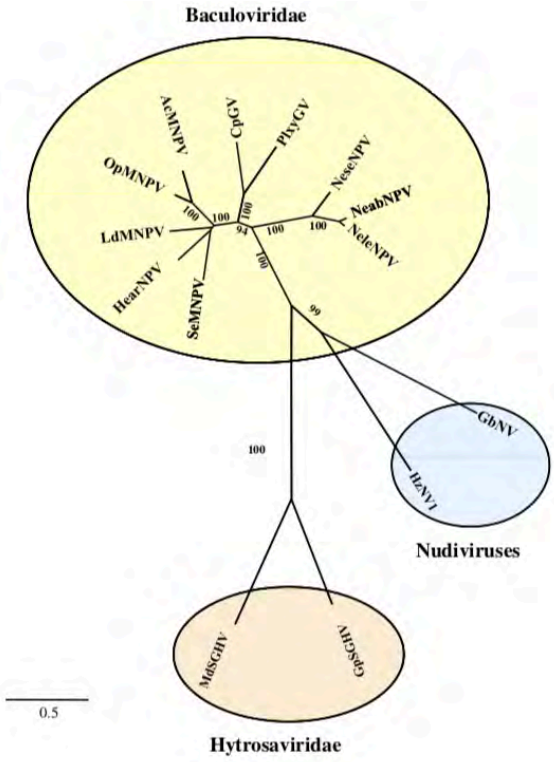

Figure 2. Neighbor-Joining (NJ) trees of concatenated predicted amino acid sequence of the four *pif* genes (*p74, pif-1, pif-2 and pif-3*) shared by all family members (Fig. 3 in ICTV, 2009, https://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/invertebrate-official/4101

Phylogenetic analysis using concatenated conserved ORFs among large invertebrate circular double stranded DNA viruses shows that hytrosaviruses form a distinct clade, well separated from other large invertebrate double stranded DNA virus families. Within the hytrosavirus clade, members of the two genera are also distinct subgroups (Garcia-Maruniak *et al.*, 2009; Jehle *et al.*, 2013). There is no (phylo)genetic relationship with any of the vertebrate viruses to date. A parity plot aligning GpSGHV and MdSGHV (Fig. 1A in Garcia-Maruniak *et al.*, 2009; https://doi.org/10.1099/vir.0.006783-0) highlights the relationship between members of these two genera.

Pathobiology

Overt SGHV infections cause hypertrophy of the salivary glands of their dipteran hosts, although other tissues can be affected. Whereas in the case of glossinavirus hypertrophy is the result of cell division and proliferation, in the case of muscavirus the hypertrophy is caused by cell enlargement (Lietze *et al.*, 2011b). SGHV infections also result in testicular degeneration and ovarian abnormalities. This in turn affects the fecundity of the fly population. Transmission of SGHV in *Glossina* spp is mainly vertical, whereas for *M. domestica* SGHV transmission is exclusively horizontal. There is evidence to suggest that GpSGHV is present in tsetse flies in a covert / latent state, but the control mechanisms remain to be elucidated. Replication of GpSGHV and MdSGHV in insect cell culture failed so far (Arif and Pavlik, 2013).

Ecology

Hytrosaviruses are very specific, occurring so far only in tsetse flies (*Glossina* spp) and houseflies (Geden *et al.*, 2008). A report claiming a hytrosavirus in the onion fly *Merodon equestris* has not (yet) been experimentally validated. SGH in *Glossina* spp. in the field is rare, but it is very common under laboratory conditions where transmission of SGHV in *Glossina* spp can also be horizontal (via blood meals). The prevalence of SGHV in tsetse and housefly in the field varies between 1-40%. The impact of SGHV on tsetse flies in the field remains to be determined. It seems that the vertical transmission mode of hytrosaviruses is selected for in the field, since *Glossina* spp. are solitary insects (Kariithi *et al.*, 2013). Houseflies are gregarious and benefit form horizontal transmission Lietze *et al.*, 2009; ibid, 2012). The hytrosaviruses are specific for tsetse flies and house flies only (so far).

Impact for human and animal health

There is no information on the effect of hytrosaviruses on humans or animals, nor on its effect on human or other vertebrate cells. Although tsetse flies suck blood and transmit trypanosomes, they also transmit hytrosavirus, but no antibodies against hytrosavirus proteins have been detected in livestock (O. Koekemoer, personal information).

Relevant information

Hytrosaviruses

- occur specifically in a restricted number of dipteran species (high host specificity).
- replication is largely restricted to dipteran salivary glands
- are very unlikely to infect vertebrates, as the unique PIF complex required for virus entry *per os* (in analogy to baculoviruses) is unique to invertebrates
- cannot readily infect cultured insect cells; human cells have not been tested but successful replication is highly unlikely due to transcription and replication constraints.
- (GpSGHV) occur only in equatorial Africa or (MdSGHV) world-wide, the latter probably also in the Netherlands (possibly *Merodon equestris*)

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Family *Hytrosaviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	only two species of flies (tsetse)
relation with vertebrate virus taxons	N	xxx	none
evidence for infection of vertebrate animals	N	x	no antibodies in wild animals
replication in vertebrate cells		xx	nd, unlikely, not in invertebr. cells
monophyletic deeply-rooted clade within virus family	Y	xxx	family highest taxon; no vert. relative
insect-specific entry/release/transmission mechanism	Y	xx	very specific replication cycle
unique genetic traits correlating with insect-specificity		xx	nd, replic. mechanism (large dsDNA)
unique genome structure	Y	xxx	large circular dsDNA; repeat regions
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L/H	xx	low in the field, high in fly rearings
hematophagous host(s) (Yes/No)	Y		important for hor. transm. in rearings
within host tropism (Broad/Restricted)	R	xxx	salivary glands; specific pathology
host(s) include feed/food insects (Yes/No)	N	x	no flies produced
endemic in NL (Yes/No)	x/-	x	possibly onion fruit fly (?)
persistent infections (Yes/No)	Y		mechanism unknown; vert. transm.
horizontal transmission (Yes/No)	Y	xxx	not in field (vertical), only in rearing

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

The conclusion reached is that, on the basis of available literature, hytrosaviruses can be considered true insect viruses. They are very host-specific and unlikely to switch hosts, and are unlikely to have any effect beyond the insect kingdom. The observations are summarized in Table 1.

Proposed status *Hytrosaviridae* species: Insect-Specific Virus (ISV)

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Proposed *Hytrosaviridae* ISV species

Genus	Species	Abbreviation
Glossinavirus		
	• *Glossina pallipides salivary gland hypertrophy virus*	GpSGHV
Muscavirus		
	• *Musca domestica salivary gland hypertrophy virus*	MdSGHV

15-*Iflaviridae* (family)

This group of viruses represents small spherical, non-enveloped, non-segmented positive-sense RNA viruses present in arthropods (insects and ticks) (Valles *et al.*, 2017; Van Oers, 2010). They infect insects of economic and ecological importance, such as honeybees (Möckel *et al.*, 2011) and silkworms, but also a plethora of lepidopteran insects.

Iflaviruses currently form a single taxon (family *Iflaviridae*) with one genus (*Iflavirus*), but are related to two other taxa in the *Picornavirales* order associated with invertebrates (*Dicistroviridae* (lemma 10) and the tentative genus *Noravirus* (lemma 18)). A number of these viruses is a clear pathogens in bees and associated with colony collapse disorder.

Virions

The iflaviruses are non-enveloped viruses, spherical in appearance with a diameter of about 30 nm. (Fig. 1). The virions are highly symmetrical (5-fold symmetry, Fig. 1). Their appearance is highly similar to polioviruses (vertebrates), and dicistroviruses (lemma 10) and noraviruses (lemma 18) (invertebrates). The virions contain four virus-encoded proteins and a single positive-sense RNA molecule.

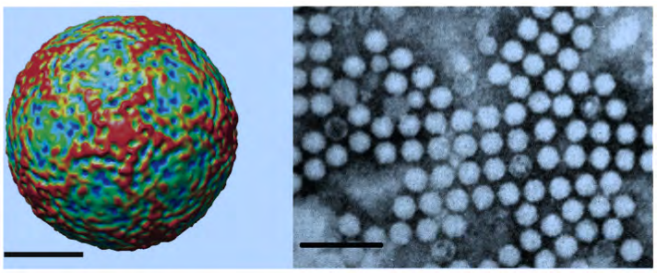

Figure 1. Surface view of an iflavirus (cryo_EM) (left) and negatively stained virus particles (EM). Hypothetical model of an iflavirus (Fig. 1 from Valles *et al.*, 2017).

Genetics

The general coding structure of an iflavirus viral RNA (about 9-10 kb, positive-sense) has a single cistron, encoding the virion proteins, VP2, VP4, VP3 and VP1, at the 5'-end and the RNA-dependent RNA polymerase (RdRp) at the 3'-end along with a helicase (Hel) and protease (Pro) (Fig. 2) (ICTV, 2018). The 5' non-translated region is highly structured and serves as a ribosomal entry site (IRES). The coding regions are flanked by non-translated regions, and the RNA contains a polyA tail at the 3'-end.

The coding structure is significantly different from the dicistroviruses, where the virion proteins are encoded in the second cistron whereas the RdRP, Hel and Pro genes are encoded in the 5' end region of the RNA genome. The genetic organization of iflaviruses is similar to vertebrate polioviruses. Noraviruses yet have another genetic structure having the RdRP, Hel and Pro genes sandwiched between the virion protein genes split to the right and left hand end of the RNA molecule.

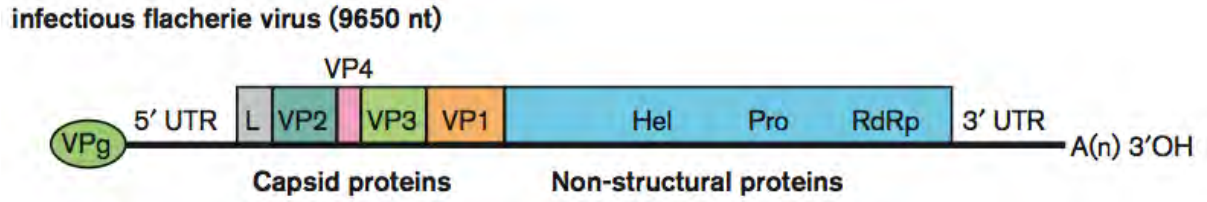

Figure 2. Genome structure of a typical iflavirus, Infectious flacherie virus, where the virion proteins are at the 5'-end and the RNA-dependent RNA polymerase gene is at the 3'-end The positive strand has a polyA tail at the 3'end and a VPg protein at the 5'-end. (Fig 2 from Valles *et al.*, 2017).

Taxonomy

The *Iflaviridae* is one of the 6 families within the order *Picornavirales*, accommodating viruses from vertebrates, insect, algae or plants. e.g. *Dicistroviridae* (insects), *Picornaviridae* (vertebrates), *Marnaviridae* (aquatic), *Secoviridae* (plant) (Fig. 3; Le Gall *et al.*, 2008; Sanfacon *et al.*, 2011). A new family may be the Noraviruses (lemma 18) (not yet in the phylogeny). A main characteristic of iflaviruses is the strictly conserved order of the individual protein open reading frames along the RNA genome (Fig. 2). Currently 15 species are recognized within the iflavirus family and quite a number are not classified yet (ICTV 2018 Release) (Table 1).

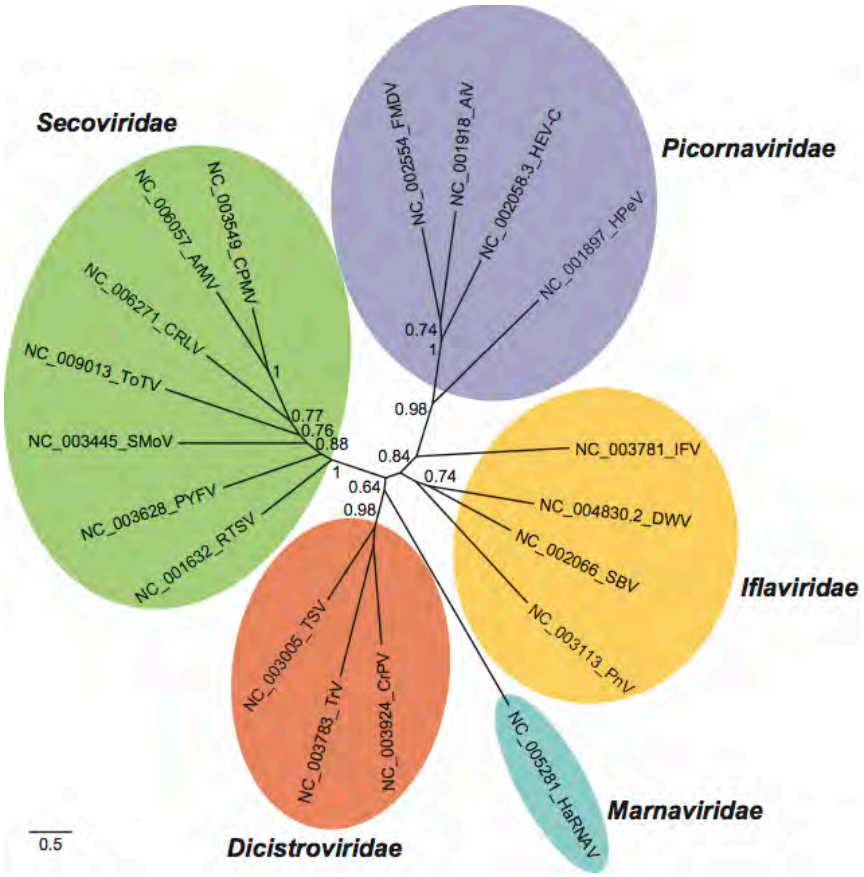

Figure 3. Phylogeny of the order *Picornavirales* using RdRP sequences (Source ICTV 10th report, 2011; https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/227/picornavirales). The noraviruses are not in here (see Lemma 18).

The current accepted taxonomy (Valles *et al.*, 2017) has one genus (*Iflavirus*) accommodating all iflaviruses to date. These viruses show >90% homology in their amino acids sequence of capsid proteins. However there is a strong subclade structure (lineages) in the phylogenetic analysis (Fig. 3a in O'Brien *et al.*, 2018, https://doi.org/10.1007/s00705-018-3868-9), suggesting these lineages (provisionally named 'Types') may become genera with time. An increasing number of iflaviruses is being identified by next-generation-sequencing of insect host RNAs, but correlation to pathology is mostly enigmatic.

Replication

Iflavirus replication occurs in the cytoplasm of infected cells. After uncoating, the viral RNA is translated into a polyprotein that is then further cleaved by viral and host-specific proteases into single proteins, such as RNA-dependent RNA polymerase, helicase and virion proteins. The viral RNA serves as a template for complementary negative-strand synthesis, which in turn serves as a template to run off viral RNAs available for encapsidation into virions. In cases of overt infections, progeny production is a matter of hours rather than days.

Pathobiology

Once the insects are infected, either *per os* or via injection of insect hosts, the iflavirus infection proceeds rapidly, and shows in almost all organs of the animal (Möckel *et al.*, 2011). Sometimes infection leads to signs of disease symptoms or changes (vomiting, *Antheraea pernyi iflavirus*). In other cases, iflavirus infections go asymptomatic (unnoticed). Infections are generally chronic. Iflaviruses are reasonably species-specific,

Despite attempts insect cell lines are not readily susceptible to iflavirus replication as they may require specific cells to replicate.

Ecology

Iflaviruses are widespread in the insect kingdom (*Diptera*, *Hymenoptera*, *Hemiptera* and *Lepidoptera*) and only in some cases give rise to disease. They are found worldwide. The virus is transmitted horizontally (= *per os*) (De Miranda and Fries, 2008) as well as vertical (Geng *et al.*, 2017). Infections are usually covert, but sometimes overt, showing malformations (deformed wings in bees by DWV), discoloration or behavioral changes (Kaguga virus = DWV variant). Many new iflavirus sequences appear from next-generation-sequencing ventures, but the ecological function of such viruses remains to be elucidated.

Genetic engineering

Infection cDNA clones have been generated and used for iflaviruses (deformed wing virus) to study gene regulation and pathology (Lamp *et al.*, 2016). In this study infectious RNA was injected into the thorax of flies and produced infectious virus and symptoms.

Impact on human and animal health

Iflaviruses are not infectious for vertebrate cells. There are no reports indicating any deleterious effects on humans or vertebrates. Iflavirus-like sequences have been found in bats (bat iflavirus), but such viruses are likely derived from the feces as bats predate on insects (Kwe Yinda *et al.*, 2017).

Relevant observations

Iflaviruses

- occur ubiquitously in a wide range of insects and mites
- encompass a monophyletic group (family) of viruses from insects
- are well separated phylogenetically from the vertebrate polioviruses and insect-specific dicistroviruses and noraviruses
- have restricted host ranges and specificities
- have not experimentally been tested in vertebrate of vertebrate cells
- have been genetically engineered

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Family *Iflaviridae*, Order *Picornavirales*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	wide range of insects; reanably specific
relation with vertebrate virus taxons	Y	xx	picornaviruses; virion structure; RNA size
evidence for infection of vertebrate animals		x	nd, unlikely
replication in vertebrate cells		x	nd
monophyletic deeply-rooted clade within virus family	Y	xxx	very separate from dicistro's and picorna's
insect-specific entry/release/transmission mechanism		x/-	common to picornavirales (cytoplasmic repl)
unique genetic traits correlating with insect-specificity	Y	x	IRES; species specific
unique genome structure	Y	xxx	similar organization as picornavirus
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M	x	no gross pathology; chronic to lethal
hematophagous host(s) (Yes/No)	Y		mosquitoes among many other insects
within host tropism (Broad/Restricted)	B		gut, fat body
host(s) include feed/food insects (Yes/No)		x/-	nd, very likely
endemic in NL (Yes/No)	Y		probably
persistent infections (Yes/No)	Y	x/-	likely
horizontal transmission (Yes/No)	Y		per os, but also vertical

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

The conclusion is reached that iflaviruses can be considered insect-specific, without any known (negative) effect on vertebrates or vertebrate cells. The observations have been summarized in Table 1.

Proposed status *Iflaviridae* species: Insect-Specific Virus (ISV)

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Proposed *Iflaviridae* (genus *Iflavirus*) ISV species

Virus name	Abbreviation	ICTV Approved
• *Antheraea pernyi iflavirus*	LnApIV	Yes
• *Armigeres iflavirus*	ArIFL	pending
• *Bat iflavirus*		pending
• *Bombyx mori iflavirus*		pending
• *Brevicoryne brassicae (picorna-like) virus*	BBV	Yes
• *Chequa iflavirus*		pending
• *Culex picorna-like virus*	CuPV-1	pending
• *Deformed wing virus*	DWV	Yes
• *Diamondback moth iflavirus*		pending
• *Dinocampus coccinellae paralysis virus*	DcPV	Yes
• *Ectropis oblique virus*	EoV	Yes
• *Euscelidius variegatus virus 1*		pending
• *Formica exsecta virus 2*		pending
• *Graminalla nigrifrons virus 1*		pending
• *Heliconius erato iflavirus 1*		pending
• *Halyomorpha halys virus*		pending
• *Hubei odonate virus 2*		pending
• *Hubei odonate virus 4*	HuAV1	pending
• *Hubei picorna-like virus 34*		pending
• *Infectious flacherie virus**	IFV	Yes
• *La Jolla virus*		pending
• *Loadelphax striatellus picornalike virus 1*		pending
• *Lygus lineolaris virus 1*	LyLV1	Yes
• *Lymantria dispar iflavirus 1*	LdIV1	Yes
• *Moku virus*		pending
• *Nilapravata lugens honeydew virus 1*	NLHV1	Yes
• *Opsiphanes inviriae iflavirus*		pending
• *Perina nuda virus*	PnV	Yes
• *Psammotettix alienus iflavirus 1*	PaIV1	pending
• *Sacbrood virus*	SBV	Yes
• *Slow bee paralysis virus*	SBPV	Yes
• *Sogatella furcifera honeydew virus*	SFHV1	pending
• *Spodoptera exigua iflavirus 1*	SEIV1	Yes
• *Spodoptera exigua iflavirus 2*	SEIV2	Yes
• *Thaumetopoea pytiocampa iflavirus 1*		pending
• *Varroa destructor virus*	VDV-1	Yes

- Type species

16-*Mesoniviridae* *(family)*, *Alphamesonivirus* (genus), *Nidovirales* (order)

Mesoniviruses are a recently identified family of enveloped viruses from insects, more specifically mosquitoes (Lauber *et al.*, 2012; Zirkel *et al.*, 2013; ICTV, 2014). They belong to the nidoviruses, and have a positive, single-stranded RNA and a characteristic expression strategy. Among the nidoviruses are also important human pathogens such as SARS and MERS (*Coronaviridae*), animal pathogens such as equine arthritis virus and porcine reproductive and respiratory syndrome virus (*Arteriviridae*) and crustacean pathogens such as yellow head in shrimp (*Roniviridae*).

Virions

The virions are enveloped and about 70-80 nm in diameter, with club-shaped projections on the surface. The nucleocapsid contains a nucleoprotein core and a matrix protein.

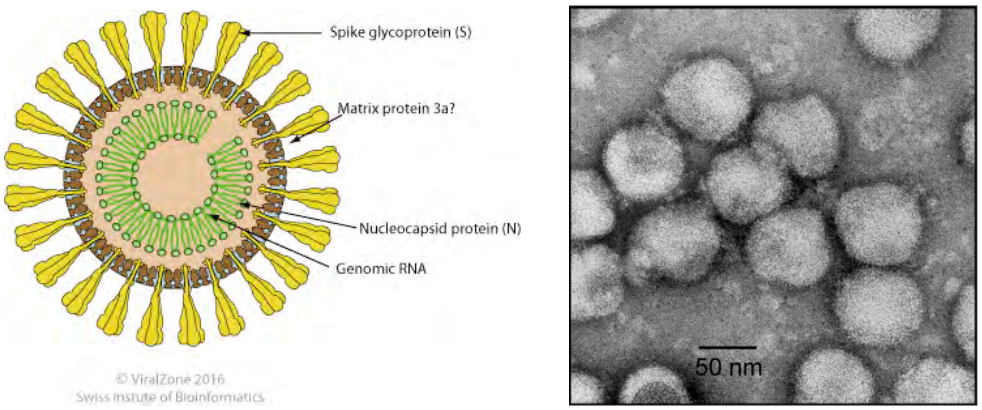

Figure 1. Left: Model of a mesonivirus (Source: ViralZone 2015). See also Fig. 3A, Nga *et al.* (2011).

Genetics

Mesoniviruses have a size of about 20 kb, which is intermediate between the arteriviruses about 12-15 kb) and coronaviruses en roniviruses with a size of about 25-31 kb. Hence, the name mesoniviruses (meso = middle). The 5' end ORF encodes a RNA-dependent RNApolymerase. ORFs downstream are expressed by frameshifting from a nested set of 3′-coterminal subgenomic mRNAs, a characteristic shared by all nidoviruses (nido = nest) (see also Fig. 3D, Nga *et al.*, 2011).

Taxonomy

The *Mesoniviridae* family belongs to the order *Nidovirales* and the virions are characterized by the presence of double-stranded, segmented RNA. Currently the order *Nidovirales* consists of four families: *Arteriviridae*, *Coronaviridae*, *Roniviridae* and *Mesoniviridae*, accommodating nidoviruses from terrestrial and aquatic vertebrates and from insects (ICTV, 2001, ibid. 2014). So, far the *Mesoniviridae* family has one subfamily (*Hexponivirinae*), one genus (*Alphamesonivirus*) and eight subgenera. The *Alphamesonivirus* genus has nine recognized species (Table 1) (ICTV 2018), but this number is currently in a positive flux.

− **Family:** *Mesoniviridae*
− **Subfamily:** *Hexponivirinae*
− **Genus:** *Alphamesonivirus*
− **Subgenus:** *Casualivirus*
Species: *Alphamesonivirus 4*
− **Subgenus:** *Enselivirus*
Species: *Alphamesonivirus 8*
− **Subgenus:** *Hanalivirus*
Species: *Alphamesonivirus 5*
− **Subgenus:** *Kadilivirus*
Species: *Alphamesonivirus 7*
− **Subgenus:** *Karsalivirus*
Species: *Alphamesonivirus 2*
Species: *Alphamesonivirus 3*
− **Subgenus:** *Menolivirus*
Species: *Alphamesonivirus 9*
− **Subgenus:** *Namcalivirus*
Species: *Alphamesonivirus 1*
− **Subgenus:** *Ofalivirus*
Species: *Alphamesonivirus 6*

Table 1. Taxonomy of *Mesoniviridae* (ICTV 2018 Release)

On the basis of genome size (Fig. 3) and phylogeny (Fig. 4) the *Mesoniviridae* is a distinct nidovirus family. Using the RNA-dependent RNA polymerase (RdRp), a robust phylogenetic tree can be constructed (Fig. 2 in Zhou *et al.* (2017) showing that members of the *Mesoniviridae* form a distinct clade in the tree that further contains the *Toroviridae*, *Coronaviridae*, *Roniviridae* and *Arteriviridae*.

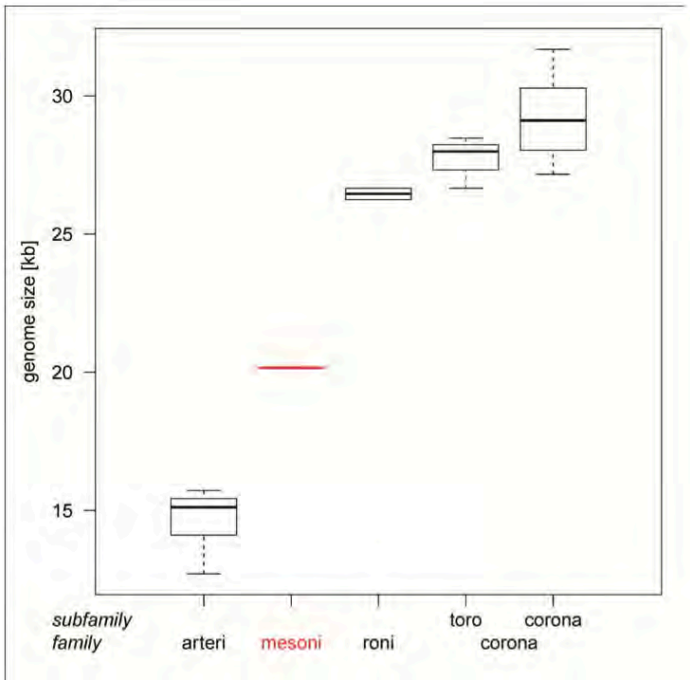

Figure 3. Genome sizes for various nidoviruses (*Nidovirales*). (Source: ICTV2012).

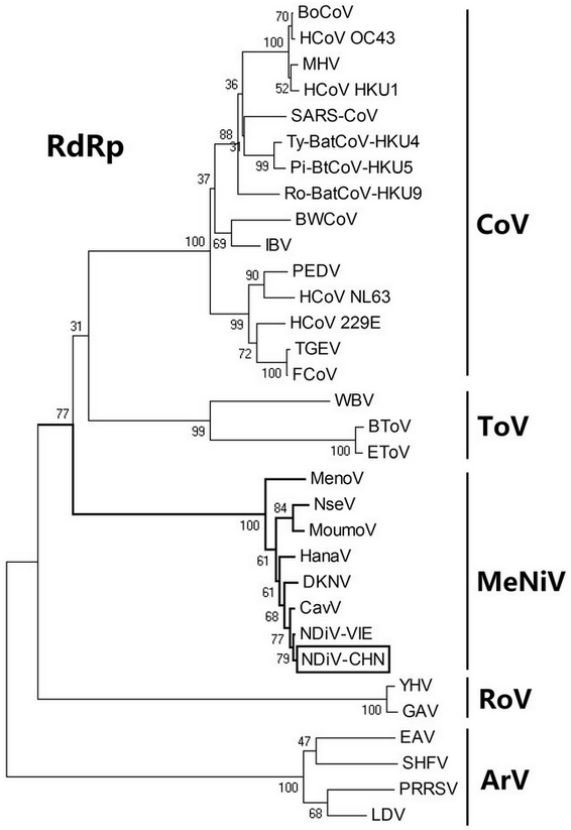

Figure 4. Phylogeny of nidoviruses (*Nidovirales*) based on RdRP. CoV = coronaviruses; ToV = togaviruses; MeNiV = mesoniviruses; RoV = roniviruses; ArV = arteriviruses. (Source: Fig 2, Zhou *et al.*, 2017).

Pathobiology

Mesoniviruses were originally found via a screen for the virome in mosquitoes. Only in females virus was found (Cavalli virus = CavV). The virus happily grows efficiently in the cytoplasm of *Aedes* mosquito cells (C6/36 and Aag2 cells) at high titers (Zirkel *et al.*, 2011; Börstler, 2016). There is no clear or specific pathology associated with mesoniviruses and the viruses do not seem to be temperature restricted.

Mesoniviruses may alter the mosquito competence for other arboviruses (Vasilakis *et al.*, 2014). Mesoniviruses (CASV) do not replicate in vertebrate cells (BHK (hamster), SW13 (human) and DF-1 (Chicken)) or produce viral RNA beyond input (Warrilow *et al.*, 2014).

Ecology

Mesoniviruses are isolated from a wide array of mosquitoes, that occur world-wide (*Aedes*, *Culex* and *Anopheles* species) and are ubiquitously found in urban areas (Wood *et al.*, 2014; Wang *et al.*, 2017)). The prevalence of mesoniviruses in German mosquitoes is about 43% (Börstler, 2016). Very little is known about the transmission of mesoniviruses (horizontal, vertical, persistence?), neither about their role in ecology (as holds for the recently discovered negeviruses (lemma 17). There is some evidence that mesoniviruses also transmit vertically (Börstler, 2016). However, their wide host range and relatively low specificity seems to indicate horizontal transmission.

Genetic engineering

No information is available about genetic engineering of mesoniviruses.

Table 2: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Proposed Family *Mesoniviridae,* Order *Nidovirales*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	mosquitoes; selection on cells
relation with vertebrate virus taxons	Y	x	nidovirales / arboviruses
evidence for infection of vertebrate animals		x	nd, unlikely
replication in vertebrate cells	N	xxx	various lines
monophyletic deeply-rooted clade within virus family	Y	xx	close to arboviruses, but very distinct
insect-specific entry/release/transmission mechanism	x/-	x	intercytoplasmic vacuoles
unique genetic traits correlating with insect-specificity		x	nd
unique genome structure	N	x	specific size range, gene organization
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L		no obvious pathology, infectious
hematophagous host(s) (Yes/No)	Y		mosquitoes only
within host tropism (Broad/Restricted)			nd; likely salivary glands
host(s) include feed/food insects (Yes/No)	N		
endemic in NL (Yes/No)			nd, possible
persistent infections (Yes/No)			nd, probable
horizontal transmission (Yes/No)			nd, possible

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Relevant observations

Insect-specific mesoniviruses (ISVs, family *Mesoniviridae*, order *Nidovirales*)

- occur ubiquitously world-wide in mosquito species
- have wide host range in mosquitoes
- are very infectious in insect cell culture
- are not associated with disease in mosquitoes and are not known to cause epidemics
- transmit both horizontally as well as vertically
- do not replicate in vertebrate cells: the effect on vertebrate animals is unknown
- have not (yet) been genetically engineered

Conclusion

On the basis of available literature, members of the family *Mesoniviridae* (Order *Nidovirales*) can be considered classical insect-specific viruses (ISVs), without any documented effect on vertebrates and with high specificity for dipteran hosts. The grouping of mesoniviruses is strongly supported by phylogenetic analysis of its members as a well-defined clade within the *Nidovirales* Order. The observations are summarized in Table 2.

Proposed status *Mesoniviridae* species: Insect-Specific Virus (ISV)

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Proposed *Alphamesonivirus, Mesoniviridae* ISV species

Species	Virus name*	Abbreviation
• *Alphamesonivirus 1*	Nam Dinh virus	NDiV
•	Cavally virus	CavV
• *Alphamesonivirus 2*	Karang Sari virus	KSaV
•		BTaV
• *Alphamesonivirus 3*	Dak Nong Virus	DKNV
•	Kamphaeng Phet virus	KPhV
• *Alphamesonivirus 4*	Casuarina virus	CASV
• *Alphamesonivirus 5*	Hana virus	HanaV
• *Alphamesonivirus 6*		
• *Alphamesonivirus 7*	Kadilivirus	
• *Alphamesonivirus 8*	Enseli virus	
• *Alphamesonivirus 9*	Menolivirus	
•	Bontag Baru virus	BBaV
•	Hanalivirus	
•	Karsalivirus	
•	Menolivirus	
•	Namcalivirus	
•	Ofalivirus	

Possible ISV Species outside the *Alphamesonivirus* genus

• *Mesonivirus 1*	Méno virus	MenoV
• *Mesonivirus 2*	Nsé virus	NseV
•	Moumo virus	MoumoV
• *Mesonivirus 3*	Yichang virus	YcV

* Some virus names may be variants of the species

17-*Negevirus* (floating taxon)

Negevirus is a new taxon (Vasilakis *et al.*, 2013) of insect-specific, enveloped, positive-sense single-stranded RNA viruses of mosquitoes and phlebotomine sandflies, all biting dipterans, from around the world. This taxon is not yet considered and recognized by the ICTV, but the number of species in this taxon is increasing rapidly, even beyond the diptera (Nunes *et al.*, 2017).

Virions

The virions are enveloped and about 45-55 nm in diameter, with club-shaped projections on the surface (Fig. 4 in O'Brien *et al.*, 2017). The nucleocapsid contains a nucleoprotein core and a matrix protein. Virions are often isolated from macerated mosquito pools and the ability of the virus to replicate in C6/36 cells.

Genetics

The virus contains a single-stranded RNA genome of about 9500 nucleotides and of positive polarity. The virus has three, sometimes overlapping (*Negev virus*), Open Reading Frames (ORFs): ORF1 (7000 kb) encodes an RNA-dependent RNA polymerase, helicase and two methyltransferases; ORF2 (1400 bp) and a putative ORF3 (600 bp). ORF 2 possibly encodes a structural or regulatory protein and ORF3 possibly encodes an envelope protein (see also Fig. 1 in Vasilakis *et al.*, 2013).

Taxonomy

Currently about 100+ putative negeviruses have been reported and proposed to form a new floating taxon: *Negevirus* (Vasilakis *et al.*, 2013; Kallies *et al.*, 2014; Nabeshima *et al.*, 2014; Nunes *et al.*, 2017). This taxon is distantly related to the genus *Cilevirus*, encompassing plant viruses (see Fig. 2 in Fujita *et al.*, 2017). New negeviruses are reported regularly (O'Brien *et al.*, 2017; Charles *et al.*, 2018)

Within this taxon two provisional groups are recognized. Group I negeviruses are accommodated in subclade provisionally named *Nelorpivirus*, group II negeviruses in a subclade provisionally named *Sandewavirus*. The *Nelorpivirus* group includes among others Negev virus (NEGV), Loreto virus (LORV), Piura virus (PIUV) and Castlerea virus (CsV). Representatives of the *Sandewavirus* group are Santana virus (SANV), Dezidougou virus (DEZV) and Wallerfield virus WALV (Nunes *et al.*, 2017; Fujita *et al.*, 2017). There are indications of further clade structure (see Fig. 2 in Fujita *et al.*, 2017).

It is of interest that the negeviruses are related to *Vigaviridae* (plant viruses transmitted by insects) and few other plant virus genera (Kallies *et al.*, 2014).

Pathobiology

Negeviruses replicate in various insect cells, but not in vertebrate cells (see Fig. 9 in Vasilakis *et al.*, 2013). Infected insect cells show clear and extensive pathology after 24 hours as evidenced by cell rounding and detachment. They produce virus to high titers (Vasilakis *et al.*, 2013; Nunes *et al.*, 2017). Negeviruses show host range specificity for insect cell lines, underscoring the specificity for specific insects.

The pathology of negeviruses in insects is not much investigated. When infected *per os*, the gut is heavily infected suggesting that the virus is horizontally transmitted.

Ecology

Negeviruses are isolated from a wide array of dipteran from around the world and they have high natural infection rates. Their specificity seems limited to dipterans and not extremely high. Very little is known about the transmission of negeviruses, but since they have been found in larvae this is likely to be vertical (Kawakami *et al.*, 2016).

Very little is known about the impact of negeviruses on their hosts in ecology, but the prevalence in the field is quite high. The observation that negeviruses do not infect vertebrate cells nor vertebrate animals suggests, that they have no vertebrate host.

Genetic engineering

From one member of the *Negevirus* taxon (Okishiri negevirus) an infectious cDNA clones has been derived (Kurnia, 2016).

Impact on human and animal health

Negeviruses (UXMV, BUSV, NEGV) are unable to infect and produce virus in mammalian (avian, human, monkey, hamster, murine) cells (Vasilakis *et al.*, 2013; Fujita *et al.*, 2017). Negeviruses (NEGV) do not infect rodents (hamsters, newborn mice) (Vasilakis *et al.*, 2013; Auguste *et al.*, 2014).

Relevant information

Insect-specific negeviruses

- occur ubiquitously world-wide, primarily in hematophagous (biting) insects
- are dipteran-specific (as far as literature goes), but the (molecular) basis of host specificity is unknown
- are very virulent in insect cell culture
- are not associated with disease (epidemics) in dipterans
- do not enter / infect / replicate in vertebrate cells; no effects on vertebrate animals
- have been genetically engineered (OKV)

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Genus *Negevirus* (pending taxon)

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	only insects and sandflies; specific
relation with vertebrate virus taxons	N	xx	
evidence for infection of vertebrate animals	N	xx	newborn mice, hamster
replication in vertebrate cells	N	xxx	no pathology
monophyletic deeply-rooted clade within virus family	Y	xxx	very separate from other -ssRNA viruses
insect-specific entry/release/transmission mechanism		x	nd
unique genetic traits correlating with insect-specificity			nd
unique genome structure	Y	xxx	three ORFS
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L	xx	no gross pathology; chronic to lethal
hematophagous host(s) (Yes/No)	Y	xx	mosquitoes and many other insect hosts
within host tropism (Broad/Restricted)			nd
host(s) include feed/food insects (Yes/No)	N		nd, unlikely
endemic in NL (Yes/No)			nd, probably
persistent infections (Yes/No)	Y	x	likely
horizontal transmission (Yes/No)	N	xx	most likely vertical

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

On the basis of available literature, members of the proposed taxon *Negevirus* can be considered classical insect-specific viruses (ISVs), without any documented effect on vertebrates and with specificity for hematophagous dipteran hosts (mosquito and sandflies). This group of viruses is strongly supported by phylogenetic analysis of its members. The observations are summarized in Table 1.

Proposed status *Negevirus* species: Insect-Specific Viruses (ISV)

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Proposed *Negevirus* ISV species (not yet approved by the ICTV)

Species name	Abbreviation
Group I *Negevirus* (Nelorpivirus subgoup)	
• *Brejeira virus*	BCPV
• *Big Cypress virus*	BREJV
• *Castlerea virus*	CsV
• *Daeseongdong virus*	
• *Cordoba virus*	CDBV
• *Loreto virus*	LORV
• *Hubei negev-like virus*	
• *Manglie virus*	MAV
• *Negev virus*	NEGF
• *Negev-like virus*	
• *Negevirus Nona 1*	
• *Ngewotan virus*	NWTV
• *Okushiri virus*	OKV
• *Piura virus*	PIUV
• *San Bernardo virus*	SBDV
Group II *Negevirus* (Sandewavirus subgoup)	
• *Biggievirus*	
• *Biratnagar virus*	BIRV
• *Bustos virus*	BUSV
• *Culex negev-like virus*	CNLV1
• *Dezidougou virus*	DEZV
• *Goutanap virus*	GANV
• *Hubei virga-like virus*	
• *Santana virus*	SANV
• *Tanay virus*	TANAV
• *Uxmal virus*	UXMV
• *Wallerfield virus*	WALV

18-*Noravirus* (floating genus, tentative), *Picornavirales* *(family)*

Noraviruses (provisional name) are a new group of small, unenveloped isometric positive-sense RNA viruses causing persistent infections in insects. The name is derived from 'Nora' (= 'new' in Armenian language). They form a clear taxon within the *Picornavirales* Order, but have to be further assigned (nomenclature) and approved (taxonomy) by the ICTV. They were first found in *Drosophila melanogaster* (Habayeb *et al.*, 2009a), but more recently also in lepidopterans and parasitoid wasps (Yang *et al.*, 2019).

Virions

The noraviruses are spherical and non-enveloped viruses, spherical and with diameter of approximately 25 nm (Habayeb *et al.*, 2006), similar to other invertebrate members of the *Picornavirales* Order (*Iflaviridae*, *Dicistroviridae*). The capsid consists of two proteins with a 'jelly roll' and a-helix fold, respectively, a structural characteristic shared by all picornavirales capsid proteins.

Genetics

The noravirus RNA is a polycistronic, positive-sense molecule of about 12-kb in size. It encodes at least four non-overlapping ORFs (ORF1-4). ORF2 is the largest and has an RNA-dependent RNA polymerase (RdRp) signature. ORF4 is located at the 3' end and encodes a capsid polyprotein. The latter is very distinct from that of other picorna-like viruses (Ekstrom *et al.*, 2011). The VP1 protein encoded by ORF1 suppresses the antiviral RNAi machinery of the host. The function of VP3, encoded by ORF3, is unknown, although it has been found in the virions. These properties are distinct from other picorna-like viruses. VP4 encodes the capsid proteins VP4A-C, with a unique alphahelical confirmation (Ekström *et al.*, 2011).

The virion structure, RNA genome size, polycistronic nature and coding structure is reminiscent to picornaviruses, but the presence of four ORFs that are also overlapping is a distinct feature of these viruses (Fig. 1A in van Mierlo *et al.*, 2014, https://doi.org/10.1371/journal.ppat.1004256). The RNA-dependent RNA polymerase (RdRp) of noraviruses is distantly related to that of iflaviruses (*Iflaviridae*) (Habayeb *et al.*, 2006), with low bootstrap values.

Taxonomy

Two species of noraviruses have been identified: *Drosophila Nora Virus* (DmelNV) and *Nasonia vitripennis noravirus-3* (NviNV) (both proposed) (Ekström *et al.*, 2011). Three more tentative species have been identified in *D. immigrans*, *D. subobscura*, and *Haematobia irritans*, respectively, through multiple NGS efforts. Genetically they form a distinct taxon as compared to other taxa within the *Picornavirales* order ((Fig. 1A in van Mierlo *et al.*, 2014, https://doi.org/10.1371/journal.ppat.1004256). The nucleotide identity amongst the two recognized noravirus species is about 27%, suggesting the two viruses are distinct species.

Phylogenetically, based on the RdRp and helicase, noraviruses are well separated from the dicistroviruses (high bootstrap value), but also from the iflaviruses and vertebrate

picornaviruses (Fig. 1b in Yang et al., 2019, https://doi.org/10.1016/j.jip.2018.11.003). However, the other viral proteins of noraviruses, notably the capsid proteins are very distinct from the sister families in the *Picornavirales* Order.

Noraviruses are as of yet unassigned (ICTV 9th Report, 2011; ICTV Online 2018). They differ substantially from ifla-, dicistro- and picornavirus families, both genetically as well as in genome structure. Whereas ifla- and picornaviruses are monocistronic, dicistroviruses have two non-overlapping cistrons, whereas noraviruses have multiple overlapping cistrons.

Replication

Nothing has been reported on the mechanism of RNA replication of noraviruses, except that the RNAi suppressor machinery is noravirus- and insect species-specific (Van Mierlo *et al.*, 2014; Lopez *et al.*, 2018). Immune-related genes of the host (*Drosophila melanogaster*) are upregulated after Nora virus infection. It is likely though that noraviruses replicate like other picornaviruses, generating a negative-strand copy, which in turn serves as a template for positive strand viral RNA synthesis.

Pathobiology

Drosophila melanogaster Nora Virus (DmNV) and *Helicoverpa armigera Nora Virus* (= HaNV) infection is found primarily in the intestinal tract (gut-restricted) of adult flies and larvae (Habayeb *et al.*, 2009a; Yang *et al.*, 2019). Upon infection, larvae become fragile and the cells vacuolizes (Habayeb *et al.*, 2009a). When injected into the fly, variable titers of noravirus were obtained, but no clear or significant biological or behavioral effects have been observed. Indeed, many laboratory stocks of *Drosophila melanogaster* are persistently infected with noravirus, and these flies do not show apparent pathology. Absence of pathology suggests adaptation of the virus to the host (Habayeb *et al.*, 2009a).

Ecology

Noraviruses are found in a number of related *Drosophila* spp, other dipterans and lepidopterans (*Spodoptera exigua Nora virus* and *Helicoverpa armigera Nora virus*), as persistent infections (Ekstrom *et al.*, 2011; Yang *et al.*, 2019). Most likely noraviruses are horizontally transmitted (*per os*) via the oral-fecal route (Habayeb *et al.*, 2009), while vertical transmission is highly efficient (100%) (Yang *et al.*, 2019). The role of noraviruses in host ecology is unexplored.

Genetic engineering

An infectious cDNA clone has been genetically engineered for *Drosophila Nora Virus*, which generates infectious virus when microinjected into *Drosophila* embryos (Ekstrom *et al.*, 2011; Sadanandan *et al.*, 2016). Thus far, no cell line has been found that supports replication of Nora virus.

Impact for human and animal health

Reports on effects of noraviruses on humans or vertebrates or cells derived from these taxa were not found. The fact that noraviruses express a suppressor of the RNAi machinery that directly interact with insect Argonaute-2 is a strong indication that these viruses are insect restricted and do not infect humans. Also, there is evidence for Nora Virus-like sequences in metagenomic data sets of vertebrates.

Relevant observations

Insect-specific noraviruses

- encompass a monophyletic group (distinct clade) of viruses
- are provisionally assigned to the *Picornavirales* order, as a putative invertebrate sister family to the insect-specific ifla- and dicistroviruses
- are species-specific, mostly in dipterans
- have a distinct genomic structure (RdRP + Helicase sandwiched between virion protein genes)
- have a virus species-specific insect host response
- are most likely horizontally transmitted (enteric viruses)
- have not (yet) been genetically engineered (DmNV)
- effects on vertebrates (or vertebrate cells) have not been studied so far

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Genus *Noravirus* (pending taxon); *Picornavirales*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	diptera mosttly; reasonably specific
relation with vertebrate virus taxons	N	xx	picornaviruses; virion + RNA structure
evidence for infection of vertebrate animals	N	x	nd, unlikely infectious
replication in vertebrate cells		x	nd
monophyletic deeply-rooted clade within virus family	Y	xxx	very separate from dicistros and picronas
insect-specific entry/release/transmission mechanism		x/-	nd, common to picornavirales (cytopl. replic.)
unique genetic traits correlating with insect-specificity		xx	nd
unique genome structure	Y	xxx	position VPs (5' and 3' terminal)
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M	xx	no gross pathology; chronic to lethal
hematophagous host(s) (Yes/No)	Y		mosquitoes among many other insect hosts
within host tropism (Broad/Restricted)	R		gut-restricted
host(s) include feed/food insects (Yes/No)			nd, unlikely (leps), but not tested
endemic in NL (Yes/No)			nd, probably
persistent infections (Yes/No)	Y	x	likely
horizontal transmission (Yes/No)	Y	xx	predominantly per os, but also vertical

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

On the basis of the species-specificity, monophylicity and genome structure the unclassified noravirus group can be considered insect-specific (restricted hosts, hence as an ISV). The insect-specific noravirus clade is strongly supported by phylogenetic analysis and is unlikely to infect and cause disease in vertebrates. The observations are summarized in Table 1.

Proposed status *Noravirus* species: **Insect-Specific Viruses (ISV)**

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Proposed *Noravirus* (genus) ISV species

Species	Abbreviation
• *Drosophila Nora virus*	DmNV
• *Helicoverpa armigera Nora virus*	HaNV
• *Hornfly H. irritants Nora-like virus*	
• *Nasonia vitripennis Nora virus*	NvNV
• *Spodoptera exigua Nora virus*	SeNV

Possible *Noravirus* species

- *Lacewing C. Pallens Nora-like sequence*
- *Moth S. exigua Nora-like sequence*

19-*Nudiviridae* (family)

The family *Nudiviridae* was established in 2013 to accommodate large enveloped double-stranded DNA (dsDNA) viruses that have similar structure as baculoviruses, but have facultative occlusion bodies (OBs) and slow disease progression (Huger, 2005; ICTV, 2013; Williams *et al.*, 2017). The name (nudi = naked) is derived from the observation that nudiviruses are occasionally found occluded in occlusion bodies (OBs), as all baculovirus do. In contrast to baculoviruses, nudiviruses are likely to have a very wide host range, including crustaceans.

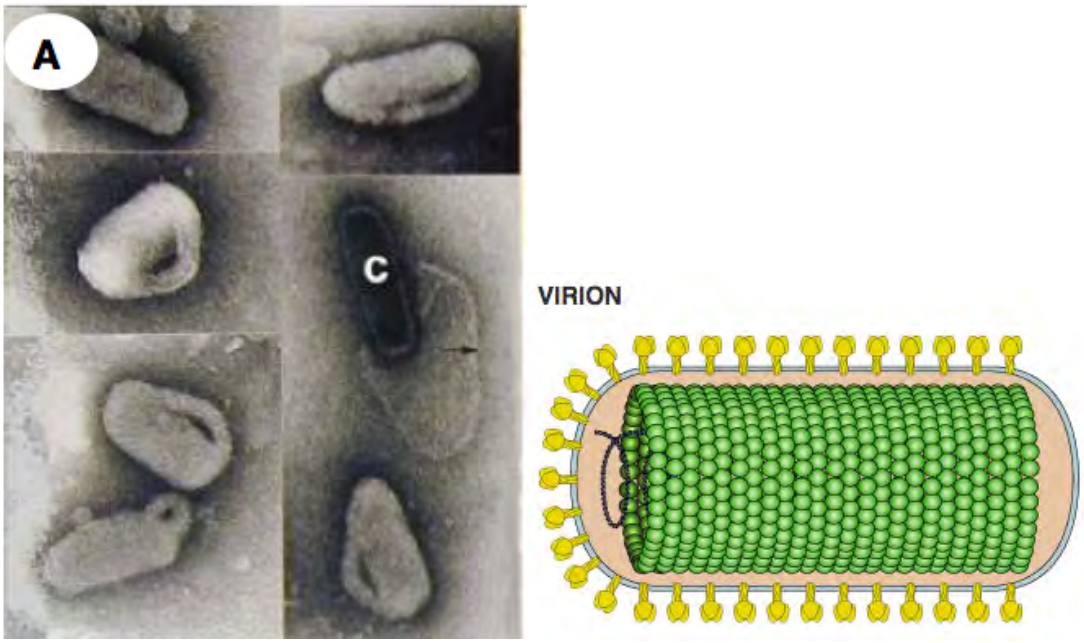

Figure 1. Left: Electron micrographs with structural details of Oryctes virus rods, negatively stained with phosphotungstic acid. (A) Virions unpenetrated by stain, often being artificially mug-shaped; middle right: the virus membrane (arrow) is shed off from the capsid (c). (Fig. 1A in https://talk.ictvonline.org/ictv/proposals/2013.003a-kI.A.v1.Nudiviridae.pdf). Right: ViralZone

The virions

Nudivirus virions are rod-to-ellipsoid-shaped particles measuring about 100 nm wide and 180-400 nm long, depending on the species. The virions consist of a lipid envelope with embedded proteins, a tegument and a single nucleocapsid, the latter containing a double-stranded circular DNA molecule ranging in size from 100-230 kbp, depending on the species. The virions (ToV) contain a considerable number (>50) of virus-encoded proteins (Bezier *et al.*, 2017).

Due to their phenotypic and genomic similarity to baculoviruses, nudiviruses have been previously assigned to the *Baculoviridae* as ‘non-occluded baculoviruses’, but since 2013 the *Nudiviridae* family has received family status (Fig. 1) (ICTV, 2014; Williams et al., 2017).

Genomics

The nudivirus genome is a large circular double-stranded DNA molecule, varying in size between 97-230 kbp and encoding about 100 to 155 non-overlapping open reading frames

(ORFs), depending on the species. The ORFs are equally distributed on both strands of the DNA molecule. Currently (2018) seven nudiviruses are completely sequenced. Phylogenetic analysis supports the existing two genera (alpha- and betanudiviruses) (ICTV, 2013) and implies to propose additional nudivirus genera (gamma- and deltanudiviruses).

All currently completely sequenced nudiviruses share 20 baculovirus 'core genes'. Among these core genes there are some of the previously mentioned PIF genes, some genes that code for virion proteins and genes for DNA replication and RNA transcription. These core genes are used to infer nudivirus relatedness and assist in nudivirus taxonomy. PIF genes are also shared with hytrosaviruses, polydnaviruses and even nimaviruses (crustacean) and are specific for invertebrates. As with the baculoviruses and hytrosaviruses, the nudivirus genome is characterized by the presence of a variable number of intergenic regions each with a variable number of homologous repeats (hrs) shared by all hrs (putative enhancers of transcription and origins of DNA replication).

There is little information about nudivirus transcription, but sequence analysis and comparison with baculoviruses also suggest several transcriptional classes of genes and phases of transcription (Wang *et al.*, 2011).

In addition nudivirus-like sequences have been found in metagenomic endeavors. Sometimes, nudivirus-like sequences are found integrated into the host genome of dipterans (*Nilaparvata lugens*) (Cheng *et al.*, 2013) and wasps. The latter cases represent polydnaviruses (see section on *Polydnaviridae*, lemma 20).

Taxonomy

The taxonomy of nudiviruses is primarily based on gene content, phylogenetic relatedness and monophylogeny versus other large invertebrate dsDNA viruses, e.g. baculoviruses, hytrosaviruses (Fig. 3), and host range (ICTV, 2013).

Two genera are currently recognized within the family *Nudiviridae* (Table 1). The genus *Alphanudivirus* accommodates two nudivirus species, one from a beetle (*Oryctes rhinoceros nudivirus*) (OrNV) (Huger, 2005; Wang *et al.*, 2001) and one from a cricket (*Gryllus bimaculatus nudivirus*) (GbNV) Wang *et al.*, 2007), whereas the genus *Betanudivirus* includes two viruses from the bollworm *Heliothis zea* (HzNV1 and HzNV2). Two other nudiviruses have been sequenced, but not yet classified (Fig. 2; ICTV, 2013), the *Penaeus monodon nudivirus* (PmNV) from shrimp (proposed genus *Gammanudivirus*) (Yang *et al.*, 2014) and *Tipula oleracea nudivirus* (ToNV) (proposed genus *Deltanudivirus*) from the dipteran crane fly (Bezier *et al.*, 2015). The recently sequenced *Drosophila innubila nudivirus* clusters with OrNV (Hill and Unckless, 2018) (see also Fig. 1B from Hill and Unckless, 2017).

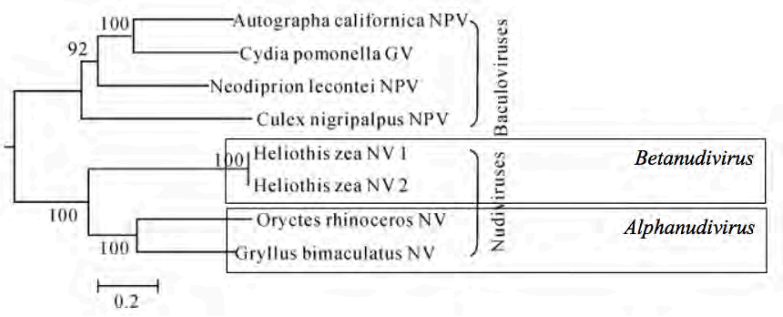

Figure 2. The midpoint rooted neighbour-joining (NJ) phylogenetic tree based on 1789 sites of concatenated amino acid sequences of the *lef-4*, *lef-5*, *dnapol* and *ac81* genes from GbNV, OrNV, HzNV-1, HzNV-2 and 4 selected baculoviruses (Fig. 8 in https://talk.ictvonline.org/ictv/proposals/2013.003a-kI.A.v1.Nudiviridae.pdf).

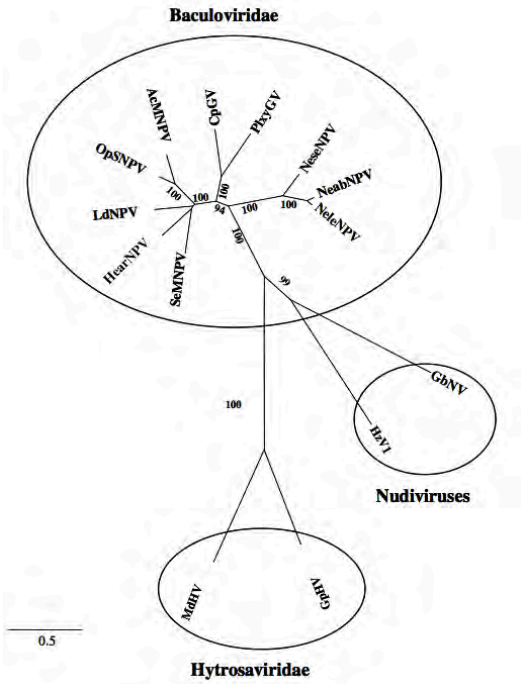

Figure 3: Neighbor-Joining (NJ) trees of concatenated predicted amino acid sequence of four *pif* genes (*p74, pif-1, pif, 2 and pif-3*) (Fig. 3 in ICTV, 2009, https://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/invertebrate-official/4101

There is no (phylo)genetic relationship to any of the vertebrate viruses to date.

Pathology

Nudiviruses infect both larvae and adults *per os* and usually target the fat body. Nudivirus infection is often chronic, but with fitness costs (fecundity, crippling). HzNV-1 and HzNV-2 replicate in cell culture. In terms of pathology the best-studied example over the years has been OrNV. After oral entry of the virions, most likely via a PIF complex since quite a few PIF genes are present in the genome, nudiviruses replicate in the midgut epithelial cells to produce virions, which move to the interior of the insect to cause a systemic infection. The major target is the fat body, where virions are produced. At the end of the infection virions are released by fecal deposition, often at breeding sites of *O. rhinoceros*. Within insect cells nudivirus replication most likely occurs, much like the baculoviruses (See Fig. 3 in Huger, 2005 and references therein). However, this is all by inference, as very little is known about nudivirus gene regulation, including transcription.

Ecology

Very little is known about the role of nudiviruses in the environment, primarily due to the fact that these viruses have not a major impact on insects and crustacean populations. Nudiviruses are found in a wide but eclectic range of insects (and even beyond, such as in crustaceans). It is not clear how widespread the nudiviruses are in nature, since most hosts do not have agronomic or ecological impact (except *O. rhinoceros*).

In the field nudivirus infections are usually chronic, but sometimes acute (OrV). HzNV-1 is a cell culture artifact, and HzNV-2 can obstruct mating (*L. dispar*).

Genetic engineering

Nudiviruses have not (yet) been engineered.

Impact for human and animal health

No negative effects of nudiviruses on humans (practitioners) or animals (adult pigs) are reported, also not in the case of OrNV, which is widely used to control the rhinoceros beetle in palm trees in Southeast Asia and the Pacific (Bedford, 1986; Gourreau *et al.*, 1979).

Relevant information

Nudiviruses

- occur in a number of very distant insect and crustacean families
- are highly specific, but the molecular basis for the specificity is unknown,
- are not very virulent and can cause epidemics
- most likely exploit a unique insect-specific mechanisms for oral entry (PIF)
- have not been reported to enter / infect / replicate in vertebrates / cells
- have not (yet) been genetically engineered for any purpose
- have been used safely to control the rhinoceros beetle for over 5 decades

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Family *Nudiviridae,* genera *Alphanudivirus, Betanudivirus and Deltanudivirus* (proposed)

		importance	**specific comments**
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	wide range of families
relation with vertebrate virus taxons	N	xxx	none
evidence for infection of vertebrate animals	N	xxx	extens. safety testing biocontr. agent
replication in vertebrate cells	N	x	nd, illogical to assume
monophyletic deeply-rooted clade within virus family	Y	xxx	family highest taxon; no vert. relative
insect-specific entry/release/transmission mechanism	Y	xxx	specific entry via PIFs
unique genetic traits correlating with insect-specificity	Y	xxx	replic. mechanism (large dsDNA)
unique genome structure	Y	xxx	large circular dsDNA; repeat regions
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M/H		for target insects; age-depend larvae
hematophagous host(s) (Yes/No)	N		
within host tropism (Broad/Restricted)	R		species-specific; fatbody as target
host(s) include feed/food insects (Yes/No)	Y		*Gryllus*
endemic in NL (Yes/No)	N		
persistent infections (Yes/No)	Y		mechanism unknown
horizontal transmission (Yes/No)	Y		very common; basis for biocontrol

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Conclusion

The conclusion is that, on the basis of available literature, members of the alpha-, beta- and deltanudiviruses can be considered true insect viruses, without any effect on vertebrates. The observations are summarized in Table 1. Gammanudiviruses are crustacean-specific and not considered.

Proposed status *Nudiviridae* species

- **genera *Alpha-*, *Beta-* and *Deltanudivirus*:** **Insect-Specific Virus (ISV)**
- **genus *Gammanudivirus*:** **Not Insect-Specific Virus (ISV)**

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Species list of the Genera *Alpha-, Beta- and deltanudivirus* (*Nudiviridae*)

Species	Abbreviations
Genus ***Alphanudivirus***	
• *Drosophila innubilis nudivirus*	DiNV
• *Gryllus bimaculatus nudivirus*	GbNV
• *Kallithea nudivirus*	
• *Oryctes rhinoceros nudivirus*	OrNV
Genus ***Betanudivirus***	
• *Heliothis zea nudivirus-1*	HzNV-1
• *Heliothis zea nudivirus-2*	HzNV-2
• *Nilaparvata lugens endogenous nudivirus*	NIENV
Genus ***Deltanudivirus*** **(proposed)**	
• *Tipula oleracea nudivirus*	ToNV

20-*Polydnaviridae*

Polydnaviruses are an unconventional group of double-stranded DNA containing 'viruses' exclusively occurring in well-defined groups of insect parasitic wasps (*Hymenoptera*) (Strand and Burke, 2014). These viruses are very specific, obligate but mutualistic agents. Each polydnavirus is intrinsically associated with single wasp species. Their unique 'life style' is a result of a longtime (100M years) association with hymenopteran wasps and their lepidopteran host. The name of the taxon ('polydna') is derived from the property that the polydnavirus virions encase multiple circular DNA molecules.

Parasitic female wasps depose their eggs along with calyx fluid and polydnaviruses into their lepidopteran larval host. The eggs develop into juvenile wasps at the expense of their lepidopteran host, in which the injected polydnavirus shuts down the immune defense (Fig. 1).

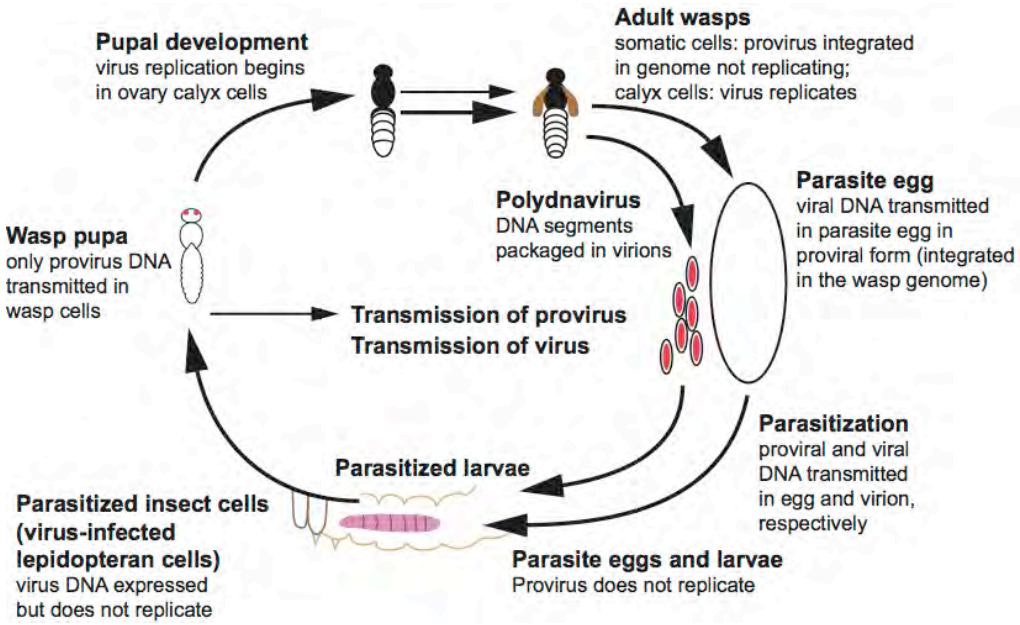

Figure 1. 'Life cycle' of polydnavirus (ICTV, 2011 (Fig. 3)).

Virions

The polydnavirions are single (*Bracovirus*) or double (*Ichnovirus*) enveloped particles, cylindrical (30-40 to 150-300 nm) in size (Strand and Drezen, 2011; ICTV, 2018) (Fig. 2). The virions occlude multiple circular double-stranded DNA molecules of various sizes and in different proportions. These DNA assemblies are characteristic for each polydnavirus species and can amount to 500 kilobase pairs of genetic information as part of the wasp genome. The encased DNA molecules encode proteins active in shutting down the defense of the host.

The polydnavirions contain multiple proteins, including peroral infectivity (PIF) proteins, which are encoded by genes present in the wasp genome (see Genetics and Pathobiology section). A characteristic feature is that polydnavirus virions are only produced in tissues of the reproductive tract of female wasps.

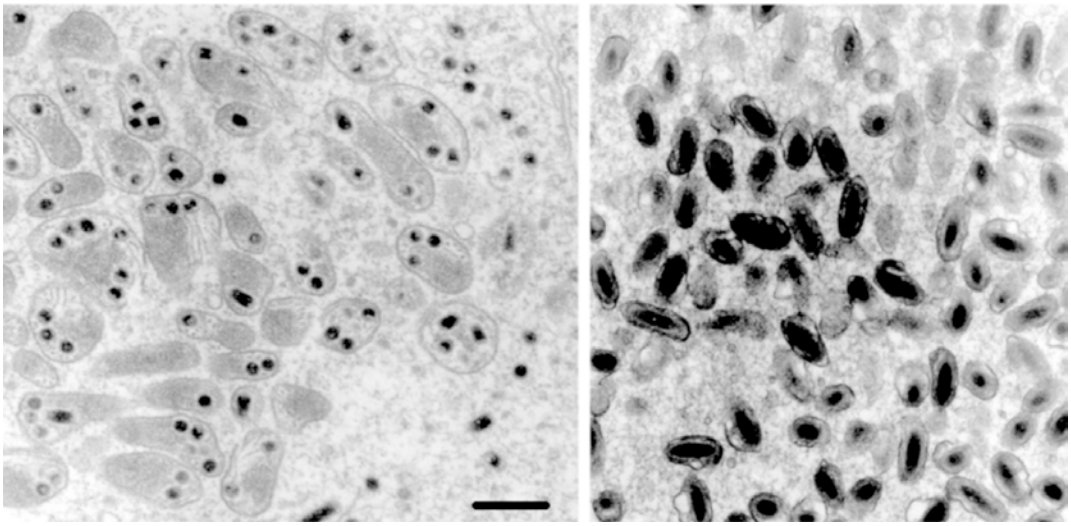

Figure 2. Cotesia melaniscela bracovirus (left) and Campoletis sonoresnsis ichnovirus (right) virions. Source: ICTV, 2011 (Fig. 1)

Taxonomy

The *Polydnaviridae* have been recognized by the ICTV in 1991 and encompass two genera: *Bracovirus* and *Ichnovirus*. They are specific for braconid and ichnumonid wasps and may ubiquitously present in nature. The taxonomy is based on the morphology of the virions (genera), the composition of the host DNA content and phylogenetic information of the viral genes present in the wasp host genome (Drezen *et al.*, 2017).

Polydnaviruses have a chimaeric appearance (Strand and Burke, 2014). On the one hand they have viral characteristics (virions, DNA cargo, infectious), on the other hand they do not replicate in host cells as viruses do. They can also be considered as virus-like particles (VLPs) carrying unrelated genetic information as cargo, with infection characteristics. Polydnavirus DNA is stably integrated in the wasp genome located on various wasp chromosomes (See Fig. 1 in Strand and Burke, 2014). The polydnavirus DNA is vertically transmitted.

Braconid polydnaviruses are clearly derived from a nudivirus ancestor, about 100 MY ago, the ancestor of ichnumonid polydnaviruses have not been identified yet. Since the bracoviruses share quite a few genes with nudiviruses (55), a phylogenetic tree can be constructed indicating the relationship of polydnaviruses with nudiviruses (Fig. 4). For ichnoviruses, this is not (yet) possible, since their ancestor is not (yet) known.

It is interesting to note that some parasitic wasps produce VLPs in the calyx without DNA cargo, but contain proteins related to (ancestral) alphabaculovirus proteins (Fig. 3), suggesting a similar evolutionary history as bracoviruses (Drezen *et al.*, 2012; Herniou *et al.*, 2013)).

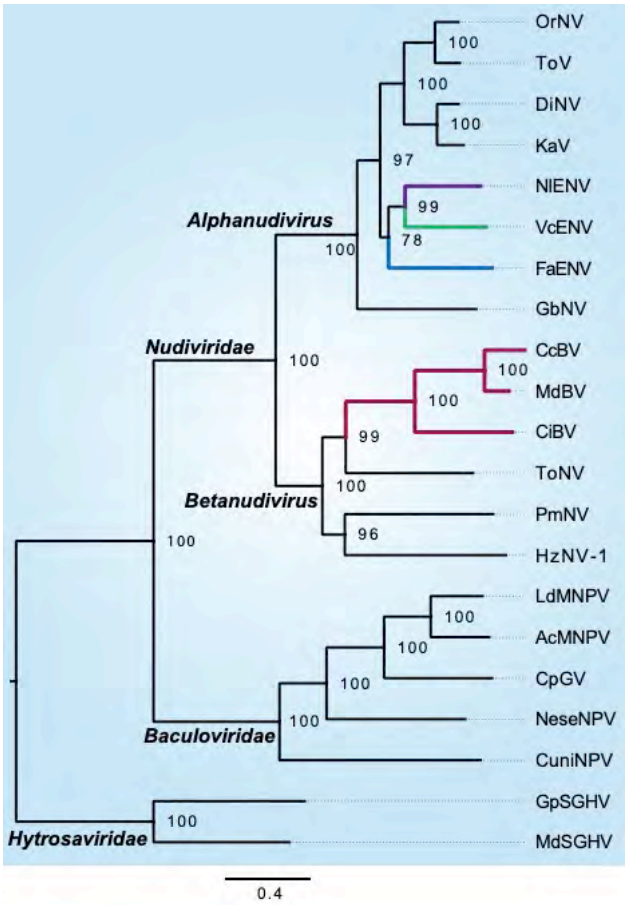

Figure 3. Phylogenetic relationship between polydnaviruses (bracovirus: light purple; VLP: dark purple, green and blue), nudiviruses, baculoviruses and hytrosaviruses, using 19 shared genes. (After Fig. 2 in Burke *et al.*, 2018)

Genetics

The DNA encased in the wasp virion is derived from the wasp host genome and encodes genes whose products interfere with the lepidopteran larval host physiology (interfere with defense responses). The DNA molecules from the virions do not replicate in the wasp or lepidopteran larva. They have a characteristic pattern when analyzed by agarose gel electrophoresis (Fig. 4). The viral genes are scattered over the wasp chromosome as endogenous virus elements (EVEs). They are only transcribed by the lepidopteran host transcription machinery and not by the wasp's machinery.

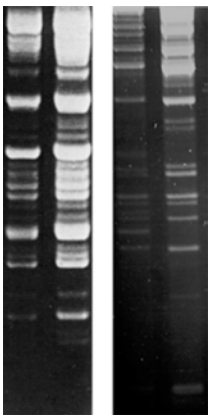

Figure 4. Profile of DNA genomic segments (non-equimolar) of an ichnovirus (left) and bracovirus (right) genome Source: ICTV, 2011 (Fig. 2)

Replication

Polydnaviruses do not replicate as such, but virions in the form of VLPs are produced in cells of the reproductive tract of the wasp. The DNA ending up in these VLPs is the result of multiplication of wasp genomic segments of lepidopteran origin (multiple circles, Fig. 4). Polydnaviral proteins are expressed from integrated polydnaviral DNA in the wasp genome.

Pathobiology

Polydnavirus virions are only produced in the reproductive tract (calyx) of parasitic female wasps, although their viral genes as well as lepidopteran host genes are present in every cell of the hymenopteran wasp (male and female). The virions (VLPs) are injected into their lepidopteran larval host along with the parasite egg and calyx fluid (Fig. 3).

The virions infect a plethora of host larval tissues including hemocytes involved in innate immunity responses. The lepidopteran genes encased into VLPs are released to express genes, whose products interfere (compromise) with larval host defense responses (innate immunity responses, melanization and endocrinology), all to the benefit of parasite development in the host. Finally, the larval host dies and juvenile parasites emerge.

Ecology

Polydnaviruses are very ubiquitous in nature. They occur only in parasitic wasps of the braconid (estimates 50,000 species) and ichneumonid (estimates 16,000 species) wasp family and are dependent on the polydnavirus cargo to develop in their lepidopteran host into progeny wasps. Polydnaviruses are species-specific, but the majority of polydnaviruses remain unidentified. Polydnavirus-infected lepidopteran larvae also suppress, via their saliva, the plant defense against larval attack (Tan *et al.*, 2018).

In nature, parasitic wasps cause high mortality in herbivorous hosts and regulate the size of insect populations (Hawkins *et al.*, 1997). Parasitic wasps are frequently used as biocontrol agents of pest insects and in many (but not all) polydnaviruses play a key role.

Genetic engineering

Polydnaviruses can infect lepidopteran insect cells and express their cargo DNA, but do not replicate. As such these viruses cannot be genetically engineered, except possibly when the wasp host is genetically modified, e.g. by CRISPR-Cas methodology (Drezen *et al.*, 2017).

Impact on human and animal health

Reports on effects of polydnaviruses on humans or vertebrates or cells derived from these taxa were not found. Effects though are very unlikely as the virion cargo (DNA) cannot replicate (no specific DNA polymerase) in vertebrate cells and can only be expressed by the lepidopteran, host-specific transcription machinery. Therefore, there is no impact on vertebrate health expected.

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Family *Polydnaviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	only parasitic wasps
relation with vertebrate virus taxons	N	xxx	none
evidence for infection of vertebrate animals			nd
replication in vertebrate cells	N	x	virions contain host and no viral DNA
monophyletic deeply-rooted clade within virus family	Y	xxx	family highest taxon; no vert. relative
insect-specific entry/release/transmission mechanism	Y	xxx	very specific cycle
unique genetic traits correlating with insect-specificity	Y	xxx	viral genes integrated in host genome
unique genome structure	Y	xxx	multisegmented DNA in virions
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L		not a real pathogen
hematophagous host(s) (Yes/No)	N		
within host tropism (Broad/Restricted)	R		calyx female wasps
host(s) include feed/food insects (Yes/No)	N		
endemic in NL (Yes/No)	Y		
persistent infections (Yes/No)	Y		obligate parasite
horizontal transmission (Yes/No)	N		only vertical

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Relevant observations

Polydnaviruses

- encompass a polyphyletic group of ‘viruses’
- are hymenopteran and species-specific
- have a unique, very specific ‘lifecycle’
- viral genomes are part of the wasp genome
- have functional lepidopteran host DNA (from the wasp) in multiple circles as cargo of virions
- genes are vertically transmitted via the wasp genome
- do not replicate in insect cells
- effects on vertebrates have not been determined so far

Conclusion

On the basis of the species-specificity, intricate relation with the wasp genome and mutualistic lifestyle, polydnaviruses can be considered insect-specific (restricted hosts, hence as an ISV) viruses. Although tangible data on effects of vertebrates are lacking, it is very unlikely that there is any effect on vertebrates. The observations are summarized in Table 1

Proposed status species *Polydnaviridae*: Insect-specific virus (ISV)

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Proposed *Polydnaviridae* ISV Species

Species	Abbreviation
Genus ***Bracovirus***	
• *Apanteles crassicornis bracovirus*	AcBV
• *Apanteles fumiferana bracovirus*	AfBV
• *Ascogaster argentifrons bracovirus*	AaBV
• *Ascogaster quadridentata bracovirus*	AqBV
• *Cardiochiles nigriceps bracovirus*	TnBV
• *Chelonus altudinis bracovirus*	CalBV
• *Chelonus blackburni bracovirus*	CbBV
• *Chelonus nr curvimaculatus bracovirus*	CcvBV
• *Chelonus inanitus bracovirus*	CiBV
• *Chelonus insularis bracovirus*	CinsBV
• *Chelonus texanus bracovirus*	CtBV
• *Cotesia congregata bracovirus*	CcBV
• *Cotesia flavipes bracovirus*	CfBV
• *Cotesia glomerata bracovirus*	CgBV
• *Cotesia hyphantriae bracovirus*	ChBV
• *Cotesia kaiyai bracovirus*	CkBV
• *Cotesia margiventris bracovirus*	CmaBV
• *Cotesia melaniscela bracovirus* (type species)	CmeBV
• *Cotesia rubecula bracovirus*	CrBV
• *Cotesia schaeferi bracovirus*	CsBV
• *Diolcogaster facetosa bracovirus*	DfBV
• *Glyptapateles flavicoxis bracovirus*	GfBV
• *Glyptapanteles indiensis bracovirus*	GiBV
• *Glyptapanteles liparidis bracovirus*	GlBV
• *Hypomicrogaster canadensis bracovirus*	HcBV
• *Hypomicrogaster ectdytolophae bracovirus*	HecBV
• *Microplitis croceipes bracovirus*	McBV
• *Microplitis demolitor bracovirus*	MdBV
• *Phanerotoma flavitestacea bracovirus*	PfBV
• *Pholetesor ornigis bracovirus*	PoBV
• *Protapanteles paleacritae bracovirus*	PpBV
• *Tranosema rostrale bracovirus*	TrBV
Genus *Ichnovirus*	
• *Campoletis aprilis ichnovirus*	CaIV
• *Campoletis flavicincta ichnovirus*	CaIV
• *Campoletis sonorensis ichnovirus* (type species)	CsIV
• *Casinaria arjuna ichnovirus*	CarIV
• *Casinaria forcipata ichnovirus*	CfoIV

- *Casinaria infesta ichnovirus* CarIV
- *Diadegma acronyctae ichnovirus* DaIV
- *Diadegma interruptum ichnovirus* DiIV
- *Diadegma terebrans ichnovirus* DtIV
- *Enytus montanus ichnovirus* EmIV
- *Eriborus terebrans ichnovirus* EtIV
- *Glypta fumiferanae ichnovirus* GfIV
- *Hyposoter annulipes ichnovirus* HaIV
- *Hyposoter exiguae ichnovirus* HeIV
- *Hyposoter fugitivus ichnovirus* HfIV
- *Hyposoter lymantriae ichnovirus* HlIV
- *Hyposoter pilosulus ichnovirus* HpIV
- *Hyposoter rivalis ichnovirus* HrIV
- *Olesicampe benefactor ichnovirus* ObIV
- *Olesicampe geniculatae ichnovirus* OgIV
- *Synetaeris tenuifemur ichnovirus* StIV

21-*Reoviridae* (family), *Spinareovirinae* and *Sedovirinae* (subfamilies)

Reoviruses are a large group, of non-enveloped isometric viruses, with a multi-segmented RNA genome (up to 12 segments), with members isolated from insects, plants and vertebrates (King *et al.*, 2011; ICTV, 2018). A notable example is rotavirus causing disease in e.g. humans (diarrhea) and sheep (bluetongue). The name of the group is derived from '*r*espiratory *e*nteric *o*rphan' virus. Some reoviruses are transmitted by contact or by insect vectors to vertebrates (e.g. *Culicoides* and bluetongue virus). Only the potential insect-specific viruses are discussed here. Insect reoviruses are predominantly found in Lepidoptera, Diptera and Hymenoptera. Infection is usually chronic, but sometimes acute.

Virions

Reovirus particles (virions) have an icosahedral symmetry and a spherical appearance, 60-80 nm in diameter (Fig. 1). They have a multilayered proteinaceous capsid occluding multiple (9-12) linear multi-segmented double-stranded RNA molecules ranging in size from 0.2 to 3 kbp. The virion proteins range in size from 15-155 kDa.

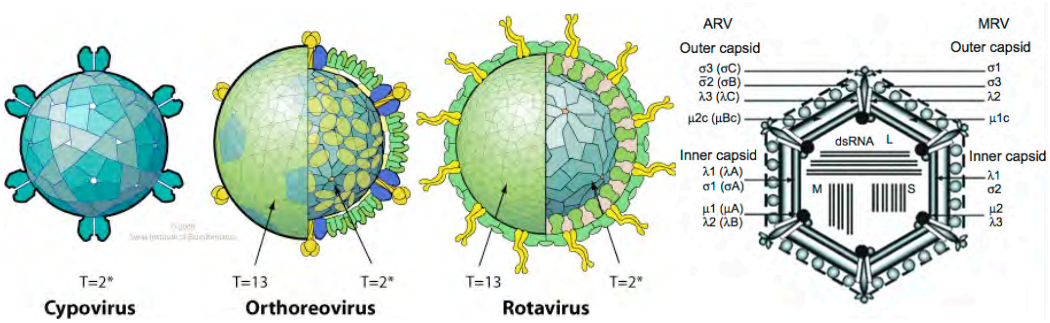

Figure 1a. Virion structure of a rotavirus. Source: ViralZone (left) and ICTV Reovirus Taxonomy Release 2018 (right).

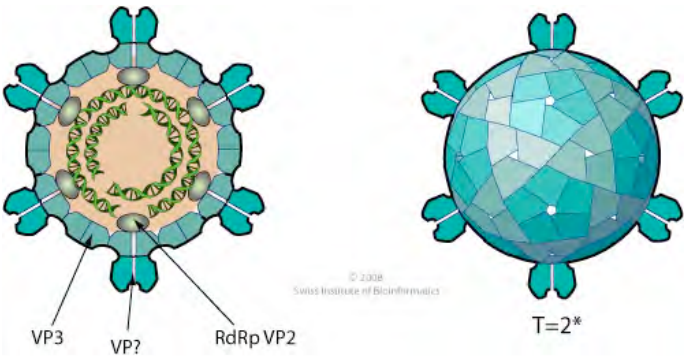

Figure 1b. Virion structure (inside and outside) of a dinovernavirus. Source: ViralZone (left) and ICTV Reovirus Taxonomy Release 2018 (right).

Taxonomy

Reoviruses form a unique taxon, with no relation to other virus families. The family *Reoviridae* (ICTV 2018 Release) consists of two subfamilies, the *Sedoreovirinae* and

Spinareovirinae. The difference between these two families is the absence (*Sedoreovirinae*) (sedo = smooth in Latin) or presence (*Spinareovirinae*) (*spina* = spike in Latin) of surface projections on the vertices of the virions. The *Sedovirinae* (*S*outh-*E*astern *A*sia *do*deca *RNA* viruses) compass six genera, with species from plants as well as from vertebrates.

Nine genera are accommodated in the *Spinoreovirinae* subfamily, but only three are potentially restricted to insects (*Cypovirus*, *Dinovernavirus* and *Idnoreovirus*). 'Cypo' is derived from cytoplasmic polyhedrosis; 'Dinoverna' from double stranded '*i*nsect *novem*' (= nine); 'Idno' = *i*nsect *d*erived *n*on-*o*ccluded). Phylogenetically cypoviruses, dinovernaviruses and idnoviruses form distinct clades in the phylogenetic tree of reoviruses (Fig. 2), within a branch also containing the genus *Oryzavirus* (plant reovirus transmitted by aphids in rice).
Seadornaviruses encompass six genera, but only one (genus *Seadornavirus*) is potentially insect-specific. This genus branches off relatively late from vertebrate ancestors (rotaviruses) (Fig. 2).

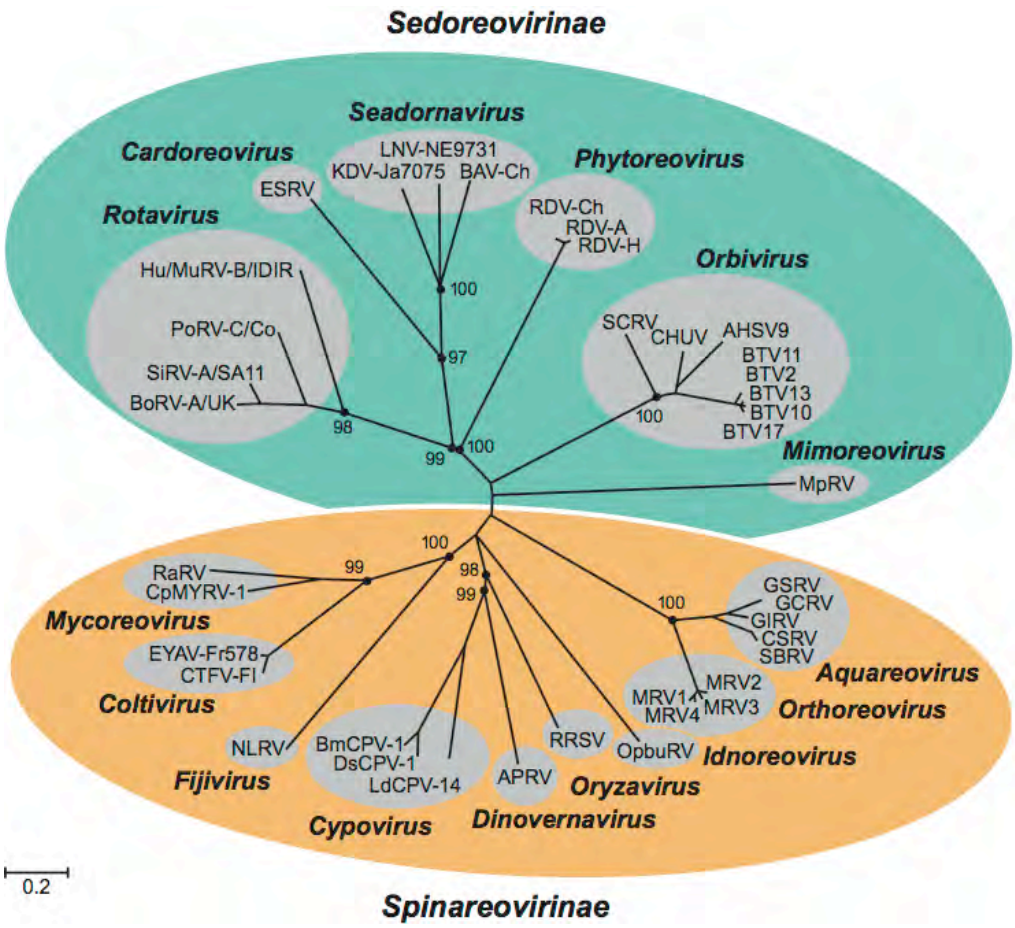

Figure 2. Neighbor joining tree constructed with the amino acid sequences of putative RNA-dependent RNA polymerase of representative viruses from the genera of the family *Reoviridae* (Fig. 35 in ICTV Reovirus Release 2018).

The genus *Cypovirus* has 16 recognized species mainly from a wide variety of lepidopteran and dipteran insects (and some pending members). They are characterized by the occlusion of virions in large proteinacious bodies (just as the baculoviruses and entomopoxviruses), but the constituent proteins of baculovirus, entomopoxvirus and cypovirus occlusion bodies are very different (but functionally the same). *Aedes pseudoscutellaris reovirus* (APRV), the only recognized member of the genus *Dinovernavirus*, was found in persistently infected insect cells (Attoui *et al.*, 2005), but related viruses have been identified in NGS ventures from wild

mosquitoes as well (Auguste *et al.*, 2015). Five species are recognized in the *Idnoreovirus* genus. *Drosophila S virus* may be a member of this genus.

Genetics

The reovirus genome is a linear multi-segmented, double-stranded RNA molecule. Each RNA segment encodes a protein. Their numbers range, dependent on the genus, from 9-12 segments, with 5' and 3' conserved terminal sequences. The largest segment encodes an RNA-dependent RNA polymerase, which is associated in the virion to each RNA segment to initiate transcription in infected cells. Multiple fragments are encoding virion capsid proteins and enzymes involved in virus entry (King *et al.*, 2011).

Cypoviruses form occlusion bodies, just like the baculoviruses, entomopoxviruses and sometimes nudiviruses, which contain the virions. However, the occlusion body protein of cypoviruses is very different from that of the other, unrelated taxa, a case of convergent evolution. Dinovernaviruses do not make polyhedral (King *et al.*, 2011).

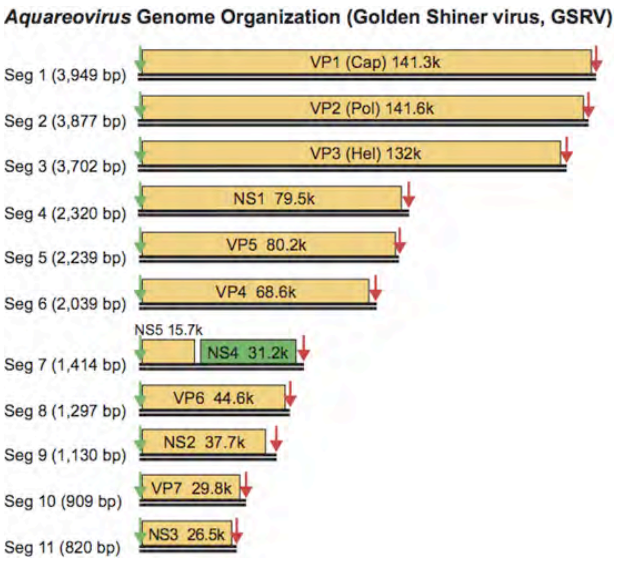

Figure 3. Genome organization of the 11 dsRNA segments of golden shiner virus (species *Aquareovirus C*). (Fig. 7 in ICTV Reovirus Release 2018).

Replication

Reoviruses have a complex replication cycle (Fig. 3). The virus replicates in the cytoplasm of infected cells and produce an abundant number of messenger RNAs to express, among others, copious amounts of capsid proteins for virion assembly. The latter process is highly specific to secure correct insertion of each of the segments into one virion. The mRNA's are capped, but not polyadenylated. Transcription occurs within the icosahedral core. A characteristic feature of cypoviruses is, that the virions are occluded into a large proteinaceous polyhedral body. These so-called polyhedra protect the virions, when released into the environment awaiting another encounter with a susceptible insect.

When the four major capsid proteins are expressed in a protein expression system they form 'empty' virus-like particles that can be used as vaccines.

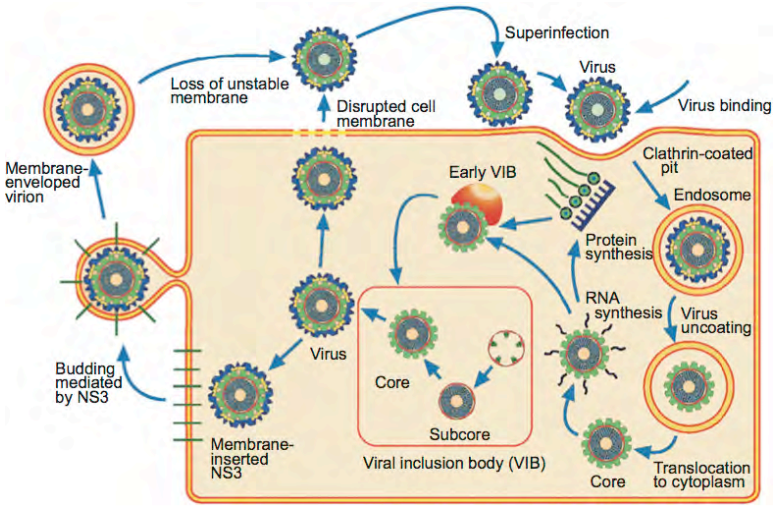

Figure 3. Typical virus replication cycle of a reovirus (presented for an orbivirus) (Fig. 3 in ICTV Reovirus Release 2018).

Pathobiology

Cypoviruses infect insects *per os* and initially affect midgut columnar cells of the insect. Infection is characterized by loss of appetite and coloration (white appearance). Later (a few days) they catch diarrhea and surviving insects show reduced fecundity. Infected cells (CPV) show virus replication and virion assembly in the cytoplasm.

Whereas cypoviruses, divernaviruses and idnoviruses (spinareoviruses) are clearly insect-specific (no effect on vertebrates or vertebrate cells), the status of the sedoreoviruses is currently equivocal. Attoui *et al.* (2006) reported replication of sedoreoviruses (Lam ning virus) in vertebrate cells and animals, compatible with their taxonomic position close to rotaviruses. In contrast, Prow *et al.* (2018), for an Australian lineage of this virus, did not find replication in a range of vertebrate cells.

Ecology

Cypoviruses are ubiquitously found in insects (250 species) as a chronic infection, which can become acute. They are predominantly found in Diptera, Lepidoptera and Hymenoptera (King *et al.*, 2011). Transmission is predominantly *per os*, but transovarial transmission has been reported (Chen *et al.*, 2012). Cypoviruses are used as biocontrol agents of insect pests, to reduce insect populations.

Dinovernaviruses were not only found from persistently infected dipteran cells, but also in wild mosquitoes (Fako virus) (Auguste *et al.*, 2015). Idnoreoviruses are only found in parasitic wasps (*Hymenoptera*).

Genetic engineering

A reverse genetics system has been developed for the reoviruses, blue tongue virus and rotavirus (Kobayashi *et al.*, 2007; Trask *et al.*, 2010 and Trask *et al.*, 2013) using the intracellular expression of reovirus cDNA plasmids. *Bombyx mori cypovirus* (Guo *et al.*, 2018) has been genetically engineered by transfection of insect cells with *in vitro* transcribed transcripts of BmCPV to express a fluorescent marker for pathology studies.

Impact for human and animal health

Cypoviruses fail to replicate in vertebrate cells. Replication in insect cells as well as in insects is inhibited at 25 ^{o}C, well below the ambient temperature of (higher) vertebrates. Cypoviruses only dissolve under alkaline conditions (in the insect gut) and are thus likely to pass the vertebrate intestine unaffected. Cypoviruses are considered 'safe' for humans and other vertebrates and are used as biocontrol agents.

No studies are available for dinovernaviruses and idnoviruses, but there is no reason to believe that these viruses affect vertebrates.

Of note is the Lia Ning virus (LNV) (subfam. *Sedoreovirina*e, genus *Seadornavirus*) from a mosquito, for which it is not yet clear whether it replicates in vertebrate cells / animals (Attoui *et al.*, 2000) or not (Prow *et al.*, 2018). The latter authors tested a range of human cell lines (BHK, Vero, MDCK, etc.) negative for reovirus replication. Other seadornaviruses (*Bannavirus* and *Kadipiravirus*) have not been tested in vertebrate cells.

Relevant observations

Insect-specific reoviruses

- encompass members of three genera of the *Reoviridae*: *Cypovirus*, *Idnoreovirus* and *Dinovernavirus*
- are lepidopteran, dipteran and hymenopteran-specific
- possibly include *Seadornavirus* members *(Sedoreovirinae)*
- have a distinct genomic structure (based on electropherotype and sequence analysis
- are predominantly horizontally and possibly vertically transmitted
- have been genetically engineered
- are not able to replicate in vertebrate cells and not beyond 25 ^{o}C in insect cells

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Genera *Cypovirus*, *Dinovernavirus* and *Idnoreovirus*, Subfamily *Spinareovirinae*, Family *Reoviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	leps (majority), dipt and wasps; specific
relation with vertebrate virus taxons	Y	xx	plant + vert viruses
evidence for infection of vertebrate animals	N	xxx	safe as biol. contr. agent
replication in vertebrate cells	N	xxx	
monophyletic deeply-rooted clade within virus family	Y	xx	sandwiched between plant/aquatic reoviruses
insect-specific entry/release/transmission mechanism	Y	xx	occlusion bodies (*Cypovirus*)
unique genetic traits correlating with insect-specificity			nd
unique genome structure	N	x	common multisegmented RNA
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	M/H	xx	chronic to lethal
hematophagous host(s) (Yes/No)	Y/N		one exception (*Dinovernavirus*)
within host tropism (Broad/Restricted)	R	xx	gut, fat body
host(s) include feed/food insects (Yes/No)	Y		very likely
endemic in NL (Yes/No)	Y	x	probably
persistent infections (Yes/No)	Y	x	likely
horizontal transmission (Yes/No)	Y	**x**	per os

x/- = inconclusive evidence
x = little evidence
xx = moderate evidence
xxx = strong evidence
nd = data not available

Conclusion

On the basis of their specificity for insects and at the specie level, phylogenetic rooting, absence of replication in vertebrate cells, safety profile (cypoviruses) the cypo-, dinoverna- and idnoviruses can be considered insect-specific. The specificity of seadornaviruses for insects is equivocal at the moment. The observations are summarized in Table 1 (*Cypovirus, Diavernavirus, Idnoreovirus*).

Proposed status species *Reoviridae* (genera *Cypovirus*, *Idnovirus* and *Diavernavirus*): Insect-specific virus (ISV)

Proposed status species *Reoviridae* (genus *Seadornavirus*): No insect-specific virus (ISV)

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Species listing ISV

Subfamily ***Spinareovirinae***

Genus **Cypovirus**

Species ***Cypovirus 1*** (type species) Abbreviation

Bombyx mori cypovirus 1		BmCPV-1
Dendrolimus punctatus cypovirus 1		DpCPV-1
Dendrolimus spectabilis cypovirus 1		DSCPV-1
Lymantria dispar cypovirus 1		LdCPV-1

Species ***Cypovirus 2***

Aglais urticae cypovirus 2		AuCPV-2
Agraulis vanillae cypovirus 2		AvaCPV-2
Arctia caja cypovirus 2		AcCPV-2
Arctia villica cypovirus 2		AviCPV-2
Boloria dia cypovirus 2		BdCPV-2
Dasychira pudibunda cypovirus 2		DpCPV-2
Eriogaster lanestris cypovirus 2		ElCPV-2
Hyloicus pinastri cypovirus 2		HpCPV-2
Inachis io cypovirus 2		IiCPV-2
Lacanobia oleracea cypovirus 2		LoCPV-2
Malacosoma neustria cypovirus 2		MnCPV-2
Mamestra brassicae cypovirus 2		MbCPV-2
Operophtera brumata cypovirus 2		ObCPV-2
Papilio machaon cypovirus 2		PmCPV-2
Phalera bucephala cypovirus 2		PbCPV-2
Pieris rapae cypovirus 2		PrCPV-2

Species ***Cypovirus 3***

Anaitis plagiata cypovirus 3		ApCPV-3
Arctia caja cypovirus 3		AcCPV-3
Danaus plexippus cypovirus 3		DpCPV-3
Gonometa rufibrunnea cypovirus 3		GrCPV-3
Malacosoma neustria cypovirus 3		MnCPV-3
Operophtera brumata cypovirus 3		ObCPV-3
Phlogophera meticulosa cypovirus 3		PmCPV-3
Pieris rapae cypovirus 3		PrCPV-3
Spodoptera exempta cypovirus 3		SexmCPV-3

Species ***Cypovirus 4***

Actias selene cypovirus 4		AsCPV-4
Antheraea assamensis cypovirus 4		AaCPV-4
Antheraea mylitta cypovirus 4		AmCPV-4
Antheraea pernyi cypovirus 4		ApCPV-4
Antheraea proylei cypovirus 4		AprCPV-4

Species ***Cypovirus 5***

Euxoa scandens cypovirus 5		EsCPV-5
Heliothis armigera cypovirus 5		HaCPV-5
Orgyia pseudosugata cypovirus 5		OpCPV-5
Spodoptera exempta cypovirus 5		SexmCPV-5
Tichoplusia ni cypovirus 5		TnCPV-5

Species ***Cypovirus 6***

Aglais urticae cypovirus 6		AuCPV-6
Agrochola helvolva cypovirus 6		AhCPV-6
Agrochola lychnidis cypovirus 6		AlCPV-6
Anaitis plagiata cypovirus 6		ApCPV-6
Anti xanthomista cypovirus 6		AxCPV-6
Biston betularia cypovirus 6		BbCPV-6
Eriogaster lanestris cypovirus 6		E1CPV-6
Lasiocampa quercus cypovirus 6		LqCPV-6

Species ***Cypovirus 7***

Mamestra brassicae cypovirus 7		MbCPV-7
Noctua pronuba cypovirus 7		NpCPV-7

Species ***Cypovirus 8***

Abraxas grossulariata cypovirus 8		AgCPV-8
Heliothis armigera cypovirus 8		HaCPV-8
Malacosoma disstria cypovirus 8		MdCPV-8
Nudaurelia cytherea cypovirus 8		NcCPV-8
Phlogophora meticulosa cypovirus 8		PmCPV-8
Spodoptera exempta cypovirus 8		SexmCPV-8

Species ***Cypovirus 9***

Agrotis segetum cypovirus 9		AsCPV-9

Species ***Cypovirus 10***

Aporophyla lutulenta cypovirus 10		AlCPV-10

Species ***Cypovirus 11***

Heliothis armigera cypovirus 11		HaCPV-11
Heliothis zea cypovirus 11		HzCPV-11
Lymantria dispar cypovirus 11		LdCPV-11
Mamestra brassicae cypovirus 11		MbCPV-11
Pectinophora gossypiella cypovirus 11		PgCPV-11
Pseudaletia unipuncta cypovirus 11		PuCPV-11
Spodoptera exempta cypovirus 11		SexmCPV-11
Spodoptera exigua cypovirus 11		SexgCPV-11

Species ***Cypovirus 12***

Autographa gamma cypovirus 12		AgCPV-12
Mamestra brassicae cypovirus 12		MbCPV-12
Pieris rapae cypovirus 12		PrCPV-12
Spodoptera exempta cypovirus 12		SexmCPV-12

Species ***Cypovirus 13*** Abbreviation

Polistes hebraeus cypovirus 13		PhCPV-13

Species ***Cypovirus 14***

Heliothis armigera cypovirus 14		HaCPV-14
Lymantria dispar cypovirus 14		LdCPV-14

Species ***Cypovirus 15***

Trichoplusia ni cypovirus 15		TnCPV-15

Species ***Cypovirus 16***

Choristoneura fumiferana cypovirus 16		CfCPV-16

Genus ***Dinovernavirus***

Species		Abbreviation
Aedes pseudoscutellaris	type species	ApRV

Genus ***Idnoreovirus***

Species ***Idnoreovirus 1***

		Abbreviation
Diadromus pulchellus idnoreovirus-1	Diadromus pulchellus reovirus	DpIRV-1

Species ***Idnoreovirus 2***

Hyposoter exiguae idnoreovirus-2	Hyposoter exiguae reovirus-2	HeIRV-2

Species ***Idnoreovirus 3***

Musca domestica idnoreovirus-3	Musca domestica reovirus	MdIRV-3

Species ***Idnoreovirus 4***

Dacus oleaeidnoreovirus-4	Dacus oleae reovirus	DoIRV-4

Species ***Idnoreovirus 5***

Ceratitis capitata idnoreovirus-5	Ceratitis capitata I virus	CcIRV-5
Drosophila melanogaster idnoreovirus-5	Drosophila F virus	DmIRV-5

22-*Sigmavirus* (genus), *Rhabdoviridae* (family), *Mononegavirales* (order)

The *Rhabdoviridae* are a diverse group of viruses occurring in vertebrates, invertebrates and plants, with a bullet-shaped morphology and containing a negative-sense RNA as genetic material (Walker *et al.*, 2018). Among the rhabdoviruses are notable vertebrate pathogens, e.g. Lyssavirus, Vesicular stomatitis virus (VSV) and rabies virus. Many rhabdoviruses are transmitted to vertebrates by insects. Hence these viruses are called arboviruses and not discussed here.

A few rhabdoviruses, more specifically members of the genus *Sigmavirus* and *Almendravirus* only occur in insects and cause disease in mainly *Drosophila*. Sigmaviruses and almendraviruses are highly monophyletic groups among the rhabdoviruses and are species-specific (Walker *et al.*, 2018). This lemma is about sigmaviruses. The name 'sigmavirus' was coined in 1958 (L'Heritier, 1958).

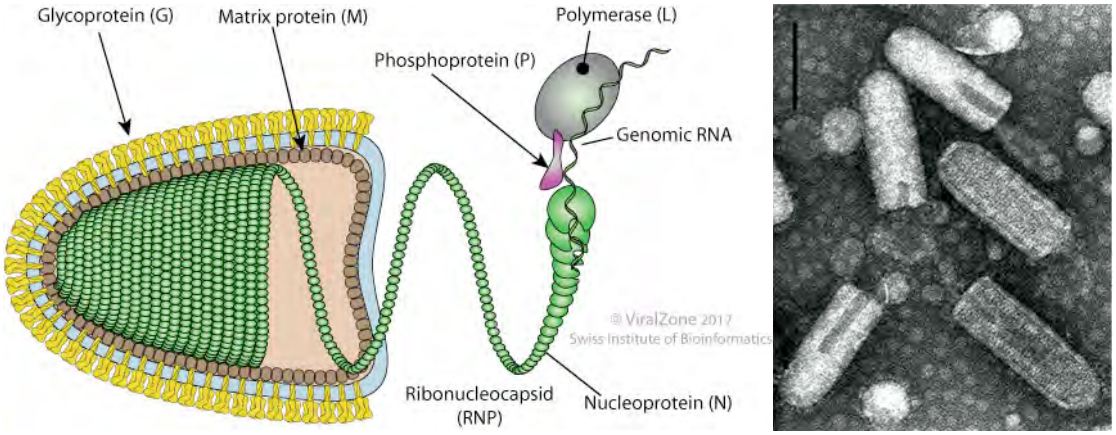

Figure 1. (Left) Diagram illustrating a rhabdovirus virion and the nucleocapsid structure (ViralZone 2017); (right) negative contrast electron micrograph of virions of an isolate of vesicular stomatitis Indiana virus https://talk.ictvonline.org/ictv-reports/ictv_online_report/negative-sense-rna-viruses/mononegavirales/w/rhabdoviridae, ICTV, 2011. The bar represents 100 nm.

Virions

Sigmavirus virions are bullet-shaped, approximately 50-140nm long particles. They have an envelope with surface projections (G-protein) wrapped around a nucleocapsid containing a singe negative-stranded RNA molecule.

Genomics

The single negative-sense RNA is about 12.5 kb in size (Longdon *et al.*, 2010; King *et al.*, 2011)) with the general structure 3'-N-P-M-G-L-5 (N = nucleoprotein, P = phosphoprotein, M = matrix protein, G = glycoprotein and L = RNA polymerase) and share sequence homology with cognate proteins from other rhabdoviruses. At the 3'-end the RNA is coupled to the L protein in a ribonucleoprotein complex (= RNP) in the virion in order to initiate transcription of the positive strand upon infection. A common character of sigmaviruses is the presence of an X gene (unknown function), sandwiched between the phosphoprotein P and the matrix protein M on the linear genome (Fig. 2).

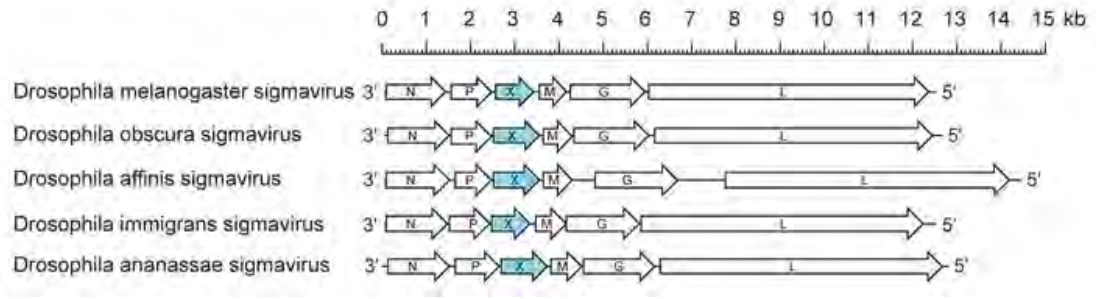

Figure 2. *Sigmavirus.* Schematic representation of sigmavirus genome organisations. N, P, M, G and L represent ORFs encoding the structural proteins. ORF X (U1) encoding a protein of unknown function is highlighted (turquoise). (Fig 1 in https://talk.ictvonline.org/ictv-reports/ictv_online_report/negative-sense-rna-viruses/mononegavirales/w/rhabdoviridae/799/genus-sigmavirus

Taxonomy

Rhabdoviruses are member of the *Mononegavirales* superfamily (Amarasinghe *et al.*, 2017; Maes *et al.*, 2019). Currently there are eighteen genera recognized within the *Rhabdoviridae* family, but only two genera, *Sigmavirus* and *Almendravirus* are restricted to insects only (Fig. 3 in Bolling *et al.*, 2015, https://doi.org/10.3390/v7092851). Most sigmavirus-like viruses have been described for drosophilids and a few other flies, but recently also in lepidopteran insects and parasitic wasps. Seven sigmavirus species are recognized by the ICTV (2018) and another fifteen are tentative sigmaviruses (ICTV 2018). *Drosophila melanogaster sigmavirus* (DMelSV) is the type species for the genus *Sigmavirus*.

Members of the genus *Sigmavirus* form a monophyletic group within the *Rhabdoviridae family* Bollong et al., 2015) (See Fig.2 in Longdon *et al.*, 2015). A plethora of rhabdovirus sequences becomes increasingly available, via NGS ventures and is available in databases. Some of these have characteristics of sigmaviruses, but are still unassigned awaiting acceptance by the ICTV. Assignment in the sigmavirus genus by molecular phylogeny implies that these e-viruses are also insect specific.

Pathobiology

As most of the rhabdoviruses, sigmaviruses replicate in the cytoplasm of infected insect cells and are released from the host cell by budding. Immune responses upon infection by sigmaviruses through the IMD (immunodeficiency) and Toll pathways have been recorded, but they are moderate. Sigmaviruses have been found in the central nervous system of *Drosophila* (Hogenhout *et al.*, 2003). Infection of insect cells is symptomless (Ohanessian and Echalier, 1967).

The symptoms of sigmaviruses are relatively mild and benign, sometimes reflected in lower fecundity. Under experimental conditions infected insects become typically paralyzed when treated with CO2.

Although sigmaviruses readily replicate in insect cells, they do not in vertebrate cells (Printz, 1973)

Ecology

Sigmavirus are found worldwide in drosophelids. Infections are restricted to dipterans, more specifically *Drosophila*. They can infect related species, which becomes more efficient the

more related these species are to *D. melanogaster*. This suggests a high, but not absolute host specificity. There is phylogenetic evidence to suggest that sigmaviruses are host-restricted and sometimes jump to related *Drosophila* species (Longdon *et al.*, 2015). The prevalence in the population of *D. melanogaster* in the wild is about 0-30%.

Transmission of sigmaviruses among insects is vertical and both males and females carry the virus via eggs and sperm (Carpenter *et al.*, 2007; Longdon *et al.*, 2017). This results in rapid spread of the virus in the offspring of the host and in the host population, but at certain fitness costs (Brun and Plus, 1980, Carpenter *et al.*, 2007). Considering this mode of transmission (vertical), it is not clear how sigmaviruses would switch hosts. Recombination is a very rare event with rhabdoviruses and likewise with sigmaviruses. A resistance gene (Ref(2)P) against sigmavirus infection has been identified in the *Drosophila* host genome (Carré-Mlouka *et al.*, 2007). The specificity of this resistance gene for sigmavirus, but not other RNA viruses, suggests that it has emerged and been fixed in the population due to a long co-evolution of virus and *Drosophila* host.

Impact for human and animal health

Sigmaviruses are unlikely to have impact on human or veterinary health, due to their host specificity to insects (e.g. *Drosophila* spp), very low probability to recombine with resident virus in the insect or mammalian host and their exclusively vertical transmission.

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Genus *Sigmavirus,* Family *Rhabdoviridae,* Order *Mononegavirales*

		importance	**specific comments**
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	Drosophila
relation with vertebrate virus taxons	Y	xx	rhabdovirus structure
evidence for infection of vertebrate animals			nd
replication in vertebrate cells	N	x	even at lower temperstures
monophyletic deeply-rooted clade within virus family	Y	xx	sandwiched between rhabdoviruses
insect-specific entry/release/transmission mechanism			nd
unique genetic traits correlating with insect-specificity	Y	xx	gene X, spec for Sigmavirus
unique genome structure	N	xx	rhabdovirus-like, gene X unique
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L	xx	no obvious pathol; in lab reared egss
hematophagous host(s) (Yes/No)	N		
within host tropism (Broad/Restricted)	R		
host(s) include feed/food insects (Yes/No)	N		
endemic in NL (Yes/No)	N		
persistent infections Yes/No	Y		without symptoms
horizontal transmission (Yes/No)	Y		clearly

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Relevant information

Sigmaviruses

- occur world-wide, primarily in *Drosophila* species
- are highly host-specific
- are pathogenic with variable degrees of virulence
- do not infect vertebrate cells
- have an unknown role in fly ecology
- have not (yet) been genetically engineered

Conclusion

On the basis of the available literature, sigmaviruses have a restricted host range and form a monophyletic clade within the *Rhabdoviridae*. Although sigmaviruses are known for a long time, it is only recently that many more sigmavirus sequences are being found, awaiting biological relevance to be discovered. The observations have been assembled in Table 1.

Proposed status *Sigmavirus, Rhabdoviridae,* species: Insect-Specific Virus

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Proposed genus *Sigmavirus* ISV species

Species	Virus name	Abbreviation
• *Drosophila affinis sigmavirus*	Drosophila affinis sigmavirus	DAffSV
• *Drosophila ananassae sigmavirus*	Dros. ananassae sigmavirus	DnAnaSV
• *Drosophila immigrans sigmavirus*	Dros. immigrans sigmavirus	DimmSV
• *Drosophila melanogaster sigmavirus* *	Dros. melanogaster sigmavirus	DMelSV
• *Drosophila obscura sigmavirus*	Drosophila obscura sigmavirus	DObsSV
• *Drosophila tristis sigmavirus*	Drosophila tristis sigmavirus	DTriSV
• *Muscina stabulans sigmavirus*	Muscina stabulans sigmavirus	MStaSV

* type species

23-*Alphatetraviridae*, *Carmotetraviridae* and *Permuto-tetraviridae* (families), ('*Tetravirales* order')

This group of icosahedral non-enveloped viruses represents very small spherical viruses. They occur in the intestinal tract of moth and butterfly larvae, causing larval stunting. Tetraviruses are very host-specific and the transmission is most likely both horizontal, as suggested from the occurrence in the midgut, and vertical, as evidenced from the persistence of tetraviruses in laboratory colonies (Hanzlik and Gordon, 1997).

Virions

The tetraviruses (all families) are non-enveloped icosahedral viruses of about 40 nm in diameter and contain a single-stranded RNA of about 6-7kb and of positive polarity, segmented or non-segmented. They have a T=4 icosahedral symmetry, hence the name tetraviruses.

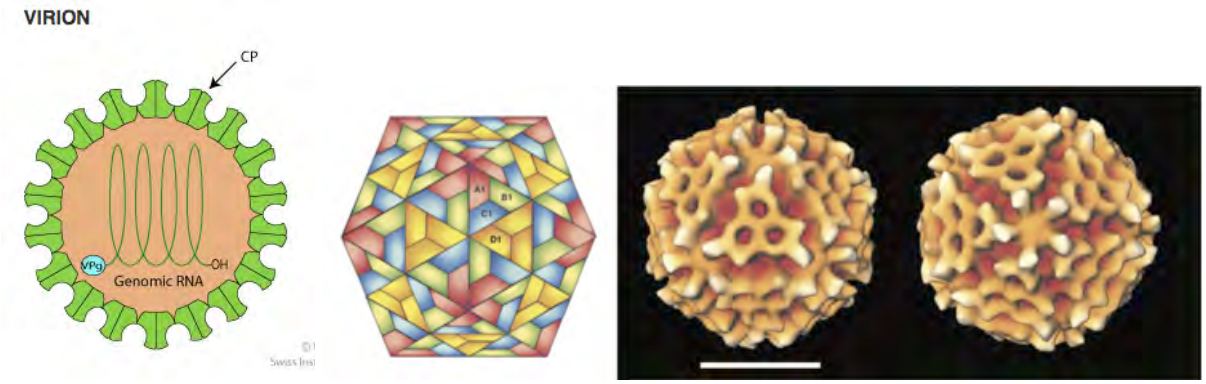

Figure 1. Left: model of an alphatertravirus. Source: ViralZone; Right: Betatetravirus capsid structure (T=4) (Fig. 2 from ICTV, 2018).

Genetics

The general structure of tetravirus RNA is a segmented/non-segmented genome of about 6-7kb. The 5'-end is not capped and the 3'-end does not contain a polyA, but a higher order structure. The genome is translated into a polyprotein that is subsequently cleaved into individual proteins, such as an RNA-dependent RNA polymerase and a coat protein (forming the capsid).

All tetraviruses have a nonsegmented genome and produce a subgenomic RNA (2.5kb), similar to alphaviruses (lemma 13), in this case encoding the coat protein (Fig. 2). The only exception is the omegatetraviruses (*Alphatetraviridae*) have two RNA segments, of which the smaller encodes the viral coat protein (L, Fig. 2).

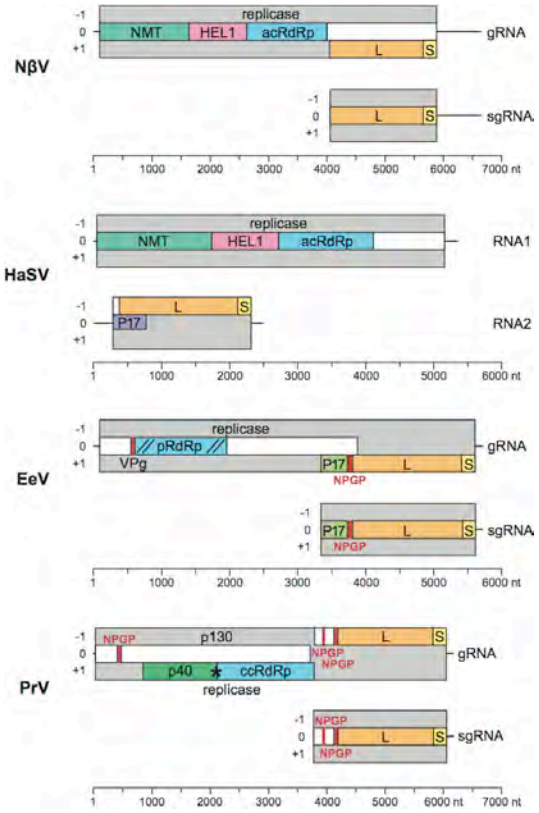

Figure 2. Tetravirus genome comparisons. The genome organization, including genome segments, ORFs and selected domains, is depicted for four tetraviruses, EeV (*Permutotetraviridae*), NβV, HaSV (*Alphatetraviridae*) and PrV (*Carmotetraviridae*), using virus-specific scales. EeV, NβV and PrV have monopartite genomes that (are predicted to) yield sgRNAs, while HaSV has a bipartite genome (RNA1 and RNA2). The selected proteins and domains are labelled, pattern-coded and coloured to indicate homology. nmT: N7-methyltransferase; HEL1, superfamily 1 helicase; acRdRp: canonical RNA dependent RNA polymerase typical of alpha-like supergroup; ccRdRp: canonical RNA dependent RNA polymerase typical of carmo-like supergroup; pRdRp: non-canonical permuted RNA-dependent RNA polymerase most related to picorna-like supergroup; NPGP: 2A-like processing site. The sequence of the major (L or β) and minor (S or γ) capsid proteins are indicated as "L" and "S", respectively. (Modified from Figure 1 of Zeddam *et al.* (2010) (Fig. 3 from ICTV, 2018).

Taxonomy

The tetraviruses have been subdivided into three families based on the structure of the nucleic acid (Fig. 2), the RdRp structure and the capsid protein phylogeny (Fig. 3) (Adams and Carstens, 2012). The common characteristic among the three families is their virion morphology (tetrahedral) and pathology in insect larvae (stunting). The *Alphatetraviridae*, with the genera *Betatetravirus* (type species: *Nudaurelia capensis beta virus* = NbV) and *Omegatetravirus* (type species: *Nudaurelia capensis omegavirus*) have one RNA segment and produce subgenomic RNAs. *Helicoverpa armigera stunt virus* (= HaSV) with a segmented genome belongs to the latter genus (Christian *et al.*, 2001). The alphatetraviruses have an 'alpha-like' RdRp. Members of the family *Permutotetraviridae* have a picorna-like RdRP, whereas the *Carmotetraviridae* (type species *Providence virus* = PrV) have a carmo-like RdRp. Members of the latter family are related to plant viruses (*Tombusviridae* and *Umbraviridae*) (Fig. 2). The *Permutotetraviridae* have one genus (*Permutotetravirus*) with the type species *Thosea asigna virus*.

An order ('*Tetravirales*') for these is not considered (yet) by the ICTV, but used here for convenience. The permutotetraviruses (with bi-segmented RNA) (Fig. 2) are distantly related to the birnaviruses (bi-segmented RNA of fish, but deeply rooted (Fig. 4).

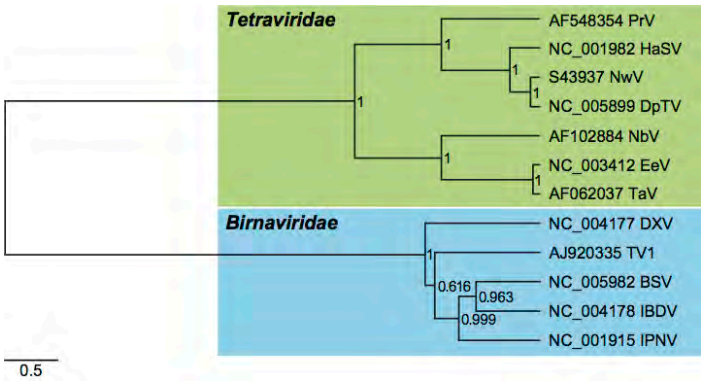

Figure 3. Phylogeny of tetraviruses on the basis of tetravirus capsid protein following the family structure. *Alphatetraviridae* (NbV, Eev and TaV), *Carmotetraviridae* (PrV) and *Permutotetraviridae* (HaSV, NxV, DpTV). *Permutatetraviridae* have a bisegmented genome; the other two families have a single RNA segment, but subgenomic RNAs (sgRNA). (Fig. 4 from ICTV, 2018).

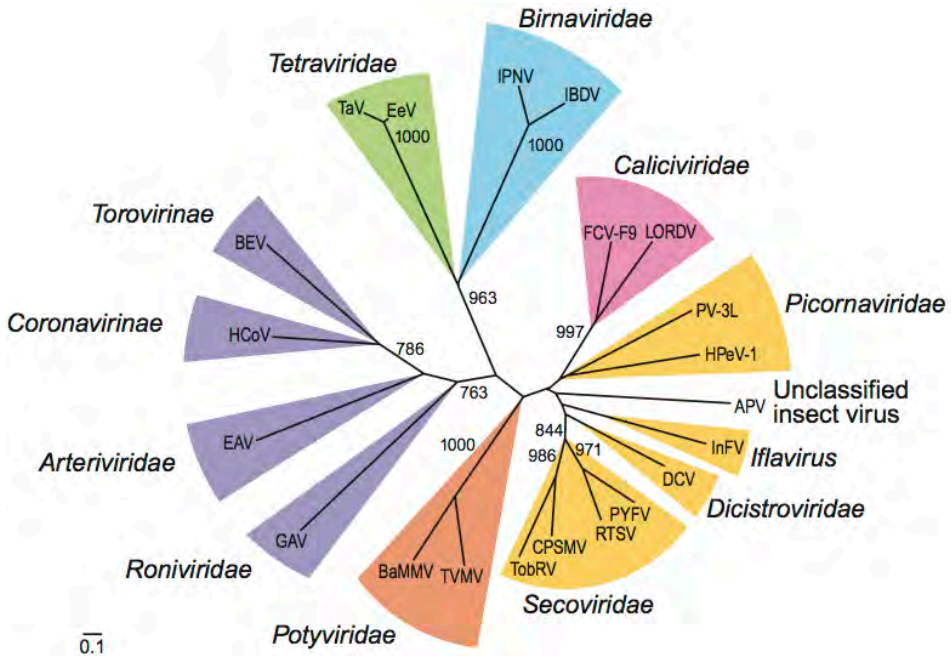

Figure 4. Unrooted phylogenetic tree on the basis of RNA-dependent RNApolymerases (Fig. 7 from ICTV, 2018). Two permutotetraviruses *Euprosterna elaea virus* (EeV) and *Thosea asigna virus* (TaV) are used.

Pathobiology

The tetraviruses infect and replicate in the midgut (goblet cells) of moth and butterfly larvae, causing stunting (Christian *et al.*, 2001). They replicate in the cytoplasm of infected cells. Only the carmotertraviruses replicate also in insect cell lines (Walter *et al.*, 2010; Dorrington and Short, 2010).

A most striking observation has been the replication of a carmotetravirus (Providence Virus = PrV) in human cells (Jiwaji *et al.*, 2016). Recently, this virus (PrV) was isolated from bats, although replication in this vertebrate was not investigated (Kemenesi *et al.*, 2016).

Ecology

Tetraviruses have a very restricted host specificity (HaSV) (Bawden *et al.*, 1999). Stunted insect larvae as a result of an infection are a 'dead end' and do not produce progeny. The role of tetraviruses in nature is enigmatic.

Genetic engineering

Tetraviruses (*Helicoverpa armigera stunt virus*) have been genetically engineered to produce infectious virus in plants and yeasts to for insect control (cotton bollworm) (Gordon *et al.*, 1995).

Impact for human and animal health

For the *Alphatetra-* and *Permutotetraviridae* there are no reports indicating any deleterious effects on humans or vertebrates. No replication in vertebrate cells has been reported so far. Carmotetraviruses (PrV) were reported to replicate in Hela cells (Jiwaji *et al.*, 2016). PrV also replicated in insect, plant and mammalian cell free systems (Jiwaji *et al.*, 2016).

Relevant observations

Insect-specific tetraviruses

- encompass two families: *Alphatetraviridae* and *Permutotetraviridae*
- form distinct clades in a RNA virus tree (+RNA)
- may have distant relatedness to birnaviruses (fish)
- are species-specific (*Lepidoptera*)
- have not been reported to enter / infect / replicate in human cells.
- have been genetically engineered into transgenic plants to control pest insects (alphatetraviruses)

Conclusion

The conclusion is reached that, on the basis of the available literature, the alpha- and permutotetraviruses can be considered as ISVs, without any known effect on vertebrates and with a high host specificity for lepidopteran species (restricted host range). The tetravirus clade structure (three families) is strongly supported by phylogenetic analysis. The carmotetraviruses replicate in vertebrate cells (Hela) efficiently suggesting a host range beyond the insect kingdom. The observations are summarized in Table 1.

Table 1: Criteria for insect-specific (ISV) status and elements for pathogenicity classification
Family *Alphatetraviridae, Carmotetraviridae* and *Permutotetraviridae*

		importance	specific comments
Insect-specificity		x, xx or xxx	
only isolated from insects	Y	xxx	predominant Lepidopt., species-specific
relation with vertebrate virus taxons	N	xx	
evidence for infection of vertebrate animals			nd
replication in vertebrate cells	N	xx	except for carmotetraviruses
monophyletic deeply-rooted clade within virus family	Y	xx	no close relatives
insect-specific entry/release/transmission mechanism			nd, gut restricted replication
unique genetic traits correlating with insect-specificity	Y	xx	virion structure
unique genome structure	Y	xx	
Pathogenicity			
pathogenicity estimate (Low/Moderate/High)	L	xx	stunting
hematophagous host(s) (Yes/No)	N		
within host tropism (Broad/Restricted)	R		gut cells
host(s) include feed/food insects (Yes/No)	Y		possibly
endemic in NL (Yes/No)	N		not reported
persistent infections Yes/No	Y		
horizontal transmission (Yes/No)	Y		clearly

x/- = inconclusive evidence

x = little evidence

xx = moderate evidence

xxx = strong evidence

nd = data not available

Proposed status *Alphatetraviridae* and *Permutotetraviridae* species: Insect-Specific Virus (ISV)

Proposed status *Carmotetraviridae* species: No Insect-Specific Virus (ISV)

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Proposed 'tetravirus' ISV species

Alphatetraviridae (family)

genus ***Betatetravirus***

Species	Abbreviation
• *Antherea eucalypti virus*	AeV
• *Darna trima virus*	DtV
• *Dasychira pudibunda virus*	DpV
• *Nudaurelia capensis beta virus**	NβV
• *Philosamia cynthia x ricini*	PxV
• *Pseudoplusia includens virus*	PiV
• *Thosea asigna virus*	TaV
• *Trichoplusia ni virus*	TnV

genus ***Omegatetravirus***

Species	
• *Dendrolimus punctatus*	DpTV
• *Helicoverpa armigera stunt virus*	HaSV
• *Nudaurelia capensis omega virus**	NωV

Permutoteraviridae (family)

genus ***Alphapermutoteravirus***

Species	
• *Pseudoplusia includens virus**	PiV
• *Euprosterna elaeasa virus*	EeV