Genomic support for speciation and specificity of baculoviruses

Agata K. Jakubowska

Thesis committee

Thesis supervisor
Prof.dr. J.M. Vlak
Personal Chair at the Laboratory of Virology
Wageningen University

Thesis co-supervisor
Dr. M.M. van Oers
Associate Professor
Laboratory of Virology
Wageningen University

Other members
Prof.dr. J.A.M. Leunissen, Wageningen University
Prof.dr. M. Dicke, Wageningen University
Dr. J.A.G.M. de Visser, Wageningen University
Prof.dr. M. López-Ferber, Industrial Environment Engineering Center, Alès, France

This research was conducted under the auspices of the Graduate School for Production Ecology and Resource Conservation (PE&RC).

Genomic support for speciation and specificity of baculoviruses

Agata K. Jakubowska

Thesis
submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 7 April 2010
at 1.30 PM in the Aula

Jakubowska, A.K.
Baculoviruses genomics and speciation
138 pages

Thesis, Wageningen University, Wageningen, NL (2010)
With references, with summaries in Dutch, English and Polish

ISBN 978-90-8585-620-7

Abstract

The *Baculoviridae* comprise a large family of double-stranded DNA viruses infecting arthropods. In this thesis two baculoviruses, *Leucoma salicis* nucleopolyhedrovirus (LesaNPV) and *Agrotis segetum* (Agse) NPV, were characterized in detail. Both viruses are potential biocontrol agents of the insects from which they were isolated. A close genetic relationship between LesaNPV and *Orgyia pseudotsugata* multiple NPV (OpMNPV) was found. *O. pseudotsugata* is known from North America and contains two baculoviruses, OpSNPV and OpMNPV. *L. salicis* is a European insect species that was accidentally introduced in the beginning of the 20th century into North America. Results from the current study suggest that LesaNPV was imported along with *L. salicis* into North America, where it infected *O. pseudotsugata* and adapted to this new host in coexistence with OpSNPV. As such, this case provides a snapshot of baculovirus evolution through speciation. The genome sequence of AgseNPV showed a striking co-linearity with *Spodoptera exigua* (Se) MNPV, although these viruses vary in biological properties such as host specificity. AgseNPV can infect *S. exigua* orally, but SeMNPV is not infectious for *A. segetum* larvae. SeMNPV causes a systemic infection in *A.segetum* only when the midgut barrier is bypassed through injection of the virus into the hemocoel. SeMNPV was able to enter *A. segetum* midgut epithelial cells and to transcribe its early genes, but was unable to replicate and produce progeny virus in these cells. The AgseNPV / SeMNPV case provides an excellent model to study baculovirus specificity by analyzing the changes in the genome sequence that lead to the differences in host range. The collected data support the view that molecular characterization is essential for proper virus classification and for assessing the phylogenetic relationships with other viruses.

Keywords: baculovirus, insects, speciation, genomics, phylogeny, host specificity

Contents

Chapter 1

General introduction

The number of viruses occupying different ecological niches is very large. Sensitivity and specificity of detection methods improve continuously and the list of reported viruses continues to expand. Due to metagenomic sequencing the number of new viral species is expected to increase by at least ten fold in the near future (Breitbart *et al.,* 2002). Currently, the International Committee on the Taxonomy of Viruses (ICTV), the official body of the Virology Division of the International Union of Microbiological Societies, lists more than 6000 viruses, classified in 1950 species, and in 391 taxa (Fauquet & Fargette*,* 2005). Insects comprise the largest group of animal species and hence are potentially the largest virus reservoir. There is already a huge variety of viruses known that infect Arthropods and these are classified in multiple taxonomic families. Despite their diversity and ubiquity a relatively limited number of research studies is focusing on insect viruses, probably because these viruses do not (directly) affect vertebrates and hence may have no direct economic impact. Only insect viruses belonging to a few insect families have relatively well been analyzed due to their utility in agriculture and biotechnology. The vast majority of these studies focused on members of the family *Baculoviridae*, as a good number of these viruses are used to control insects as biological alternatives to chemical pesticides (Szewczyk *et al.,* 2008).

Baculoviruses are large, double-stranded DNA viruses infecting only invertebrates, mainly insects from the orders Lepidoptera, Diptera and Hymenoptera. The most notable characteristic of baculoviruses is the occlusion body (OB). This appears to be a largely proteinaceous capsule, in which the virions are embedded and which provides protection to the virions in the environment and against proteolytic and chitinolytic enzymes in the decomposing larvae. Traditionally, baculoviruses have been taxonomically divided based on their OB morphology, into nucleopolyhedroviruses (NPVs) and granuloviruses (GVs), forming polyhedra and granula respectively (Ackermann & Smirnoff, 1983). The disease caused by baculoviruses has first been discovered in ancient China, where they caused collapse of silkworm populations (Miller, 1997). The first description in Western literature dates back to the XVI century (Benz, 1986). Italian bishop Marco Vida de Cremona describes the disease of silkworms in the poem “De Bombyce”. But only in the XX century it was recognized that baculoviruses are important for the natural control of insect populations and the idea of biocontrol using baculoviruses was born (Steinhaus, 1956). The first introduction of a baculovirus resulting in successful regulation of a pest insect occurred accidentally in 1930 in Canada. The European spruce sawfly *Gilpinia hercyniae* was introduced there from Europe and

became a serious defoliator of spruce. Extensive search for its natural enemies led to import of parasitoids from Scandinavia (Bird & Elgee, 1957). Soon after the discovery of an NPV probably accidentally introduced along with parasitoids, it was effectively used for control of the sawfly. Since that time baculoviruses are being used worldwide in many types of crops. Some profound examples of their use in biocontrol are *Anticarsia gemmatalis* (Ag) MNPV used on over two million hectares of soybean in Brazil (Moscardi, 1999; Moscardi & Santos, 2005) and *Helicoverpa armigera* SNPV (HearNPV) used to control cotton bollworm in China (Zhang *et al.,* 1995).

To date, many commercial products based on baculoviruses are available. SPOD-X™, based on *Spodoptera exigua* (Se) MNPV is used in Europe and USA to control *S. exigua* larvae, mainly in greenhouses (Smits & Vlak, 1994; Kolodny-Hirsch *et al.,* 1997). Mamestrin™ based on *Mamestra brassicae* (Mb) MNPV is used on cabbage, tomatoes and cotton to control cabbage moth (*M. brassicae*), American bollworm (*H. armigera*), diamondback moth (*Plutella xylostella*), potato tuber moth (*Phthorimaea operculella*) and grape berry moth (*Paralobesia viteana*). GemStar™ based on *H. zea* (Hz) SNPV provides control of pests belonging to the genera *Helicoverpa* and *Heliothis* on a wide variety of crops, for these pests are polyphagous. The best commercial example of a GV in biocontrol is *Cydia pomonella* (Cp) GV being sold under at least five commercial names, Carpovirusine™, Madex™, Granupom™, Granusal™ and VirinCyAP. Also forest insects are being controlled with baculoviruses and several formulations of *Lymantria dispar* (Ld) MNPV (Gypchek™, Virin-ENSH™), *Orgyia pseudotsugata* (Op) MNPV (TM-BioControl-1™ and Virtuss™) and *Gylpinia herciniae* NPV (Abietiv™) are currently available on the market.

Already in the 1980s, more than 600 baculovirus isolates had been described from different insect species (Martignoni & Iwai, 1981). Despite of this large number only a small proportion has been studied in detail, which means that only a small part of baculovirus diversity has been unveiled. This thesis describes the characterization of two NPVs, isolated from the satin moth *Leucoma* (*Stilpnotia) salicis* and the black cutworm *Agrotis segetum,* two insect species of economic importance in large areas of Europe.

Baculovirus research revealed other unique features which led to their wide use as expression vectors to produce large amounts of near-authentic recombinant proteins for instance for vaccines and diagnostic purposes (reviewed by Condreay & Kost, 2007 van Oers, 2006). Due to the extremely high levels of expression of the *polh* and *p10* genes (see further on) baculoviruses became one of the most popular viral vectors for production of recombinant proteins. Crucial for this success was the development of insect cell lines which enable fast replication of recombinant virus in culture flasks (Smith *et al.,* 1983) and bioreactors (Tramper *et al.,* 1993). The initial limitations in glycosylation patterns of insect cells have been overcome by supplementing the cells with genes encoding enzymes required for complex N-glycan biosynthesis (Harrison & Jarvis, 2006).

About 15 years ago the first transduction of mammalian cells (hepatocytes) by the baculovirus AcMNPV was reported (Hofmann *et al.,* 1995). This report was immediately followed by a number of others showing that AcMNPV can successfully transduce many other cell types (Shoji *et al.,* 1997). As a consequence this virus has emerged as a novel vector for *in vitro* and *in vivo* gene delivery (reviewed by Condreay & Kost, 2007; Hu, 2008). AcMNPV appeared to have low cytotoxicity towards mammalian cells and does not replicate in these cells. The increasing number of studies on the application of baculovirus for gene therapy indicates that this vector is a useful tool for gene and stem cell therapies and the development towards the first human trials continues (Räty *et al.,* 2008).

Infection cycle and pathology

Baculoviruses are released in the form of OBs from deceased insect larvae and are present in the environment, e.g. in the soil, on plant leaves or tree barks. Infection of insect larvae occurs by oral ingestion of viral OBs. OBs dissolve in the alkaline larval midgut (pH ≈ 11) releasing a number of enveloped virions (occlusion derived virion, ODV). The peritrophic membrane (PM) overlaying the midgut constitutes the first barrier for ODVs (Shapiro & Argauer, 1997; Wang & Granados, 2000; Haas-Stapleton *et al.,* 2003; Hegedus *et al.,* 2009, for review). It has been shown that the PM can block the passage of baculovirus to the underlying epithelial gut cells (Peng *et al.,* 1999). How the ODVs eventually pass the PM is not clear. It may be possible that the tips of microvilli of the columnar epithelial cells occasionally poke through the PM matrix and enable the contact with the virus (Adang & Spence, 1981). OBs from some baculovirus species contain a viral enhancing factor (VEF, enhancin), which facilitates the infection of larvae by increasing the permeability of the PM (Bischoff & Slavicek, 1997; Derksen & Granados, 1988; Wang *et al.,* 1994).

After passing the PM, the infection of the larval midgut cells occurs by direct fusion of the virion envelope with the membrane of the microvilli (Granados & Lawler, 1981) (Fig. 1-1A). It was demonstrated for LdMNPV that the entry occurs in two steps in which binding of ODVs to the cell membrane is followed by fusion, mediated by attachment and fusion factors (Horton & Burand, 1993). Four structural ODV proteins (P74, PIF1, PIF2 and PIF3) participate in this process and three of these (P74, PIF1 and PIF2) mediate ODV binding to the cells (Haas-Stapleton *et al.,* 2004; Ohkawa *et al.,* 2005; Slack & Arif, 2007, for review). However, it has not been elucidated to which host factor they bind, whether they form a complex and whether the binding of these three proteins runs in parallel or sequential (Song *et al.,* 2008). Research suggests that the PIFs are specific for each baculovirus (Song *et al.,* 2008). After the ODVs have successfully fused with a midgut cell, the nucleocapsids (NCs) are transported to the nucleus through actin-packed microvilli. The mechanism of this transport is not known, however it is possible that NCs may use myosin VI and actin-binding for transport through the microvilli (Volkman, 2007). NCs enter the nucleus through nuclear pores (Granados, 1978) and subsequently, virus gene expression, DNA replication and assembly of new NCs take place in

the nucleus of the epithelial cells. The NCs are then transported via the cytoplasm to the cell surface and 'budd' from there into the extracellular space.

This second baculovirus phenotype, called budded virus (BV), is produced by budding through the basal lamina or into the tracheal system that penetrates the basal lamina surrounding the midgut (Engelhard *et al.,* 1994). BVs spread the infection to other insect tissues (hemocytes, fat body, muscle, and trachea), causing a systemic infection (also called a secondary infection). BVs are very efficient in establishing infection and very few intrahemocoelic BV particles (possibly only one initial BV) are needed to achieve a fatal infection (Volkman, 2007).

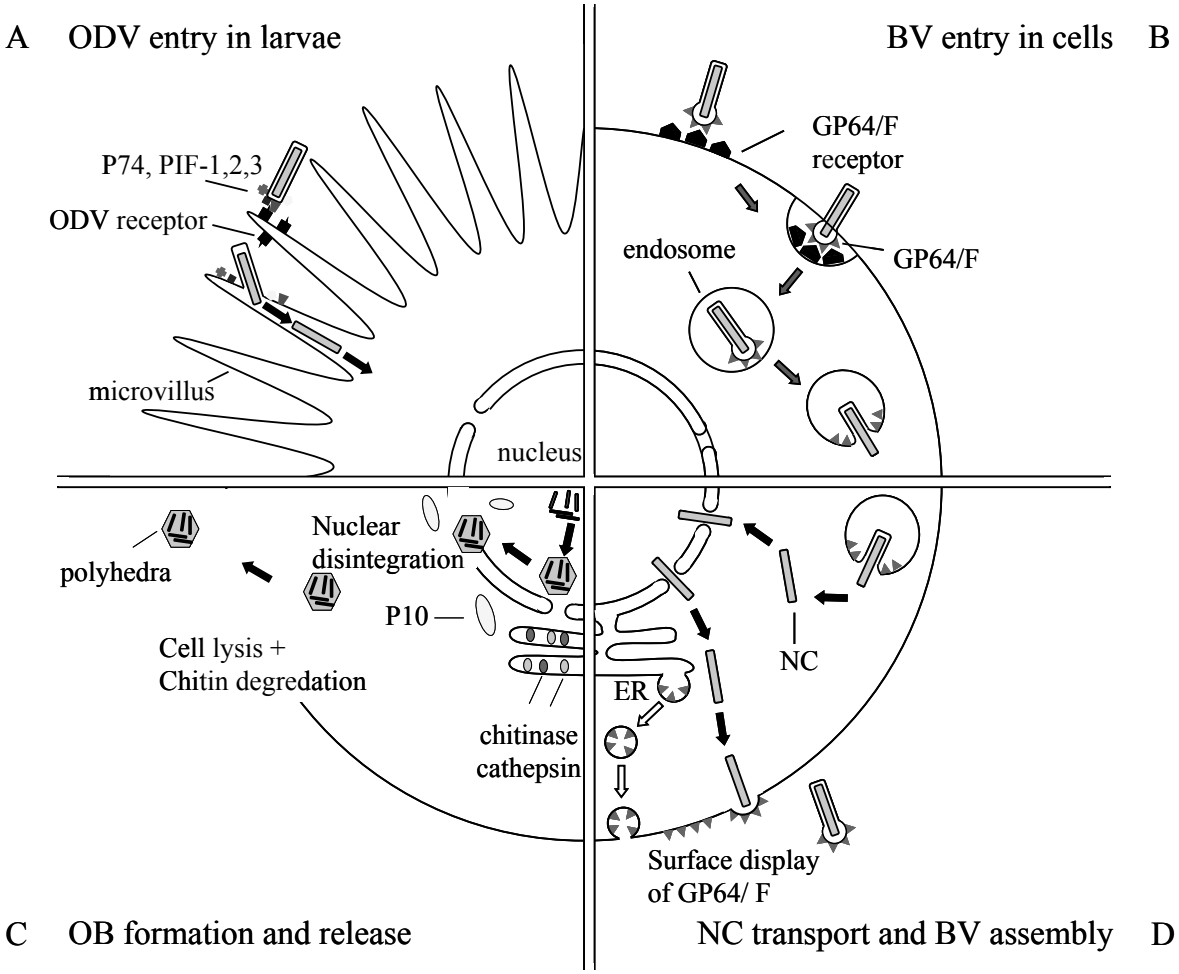


Fig. 1-1. NPV infection cycle in insect larvae. A) Infection of the midgut epithelial cells with ODVs. ODV envelope proteins mediate binding to the putative receptor on the microvilli of the epithelial cell; B) BV entry into secondary target cells by endocytosis and fusion with endosomic membrane mediated by GP64 or F protein; C) Nucleocapsid (NC) transport to the nucleus, entry to the nucleus through nuclear pores, replication and packaging into new NCs, and budding through the plasma membrane; D) NCs are enveloped in the nucleus and are embedded in polyhedrin to form OBs in the late stages of infection; cell lysis and nuclear breakdown occurs, mediated by the p10 protein structure, and OBs are released into the environment (mediated by chitinase and cathepsin) (adapted from a figure by M. Westenberg, not-published).

After binding to the cell membrane through not yet identified receptors, BVs are taken up by endosomes in a process called clathrin-mediated endocytosis (Fig. 1-1B). The acidification of the endosomes triggers fusion of the viral and endosomal membrane (Pearson *et al.,* 2000; Long *et al.,* 2006). NCs are released in the cytoplasm close to the nucleus and transported into the nucleus via nuclear pores in a cytoskeleton-dependent way, by their association with F-actin cables (Lanier & Volkman, 1998). NCs are capable of nucleating actin polymerization for intracellular and intercellular movement via the action of WASP-like proteins (Wang *et al.,* 2007). In the nucleus transcription and viral DNA replication takes place, and progeny NCs are assembled. In the early phase of baculovirus infection, newly assembled NCs move to the cytoplasm and bud through the plasma membrane (Fig. 1-1C). It is estimated that each cell in the insect body is infected with an average of 4 BVs (Bull *et al.,* 2003). In the very late state of infection NCs become packaged in the nucleus in a *de novo* synthesized envelope (Hong *et al.,* 1997). These enveloped nuclear virus particles, the new generation ODVs, are subsequently occluded in the viral matrix protein, polyhedrin or granulin, to form OBs. These OBs are released upon death and liquefaction of the infected larvae (Fig. 1-1D) and are waiting to start a new round of infection of healthy larvae.

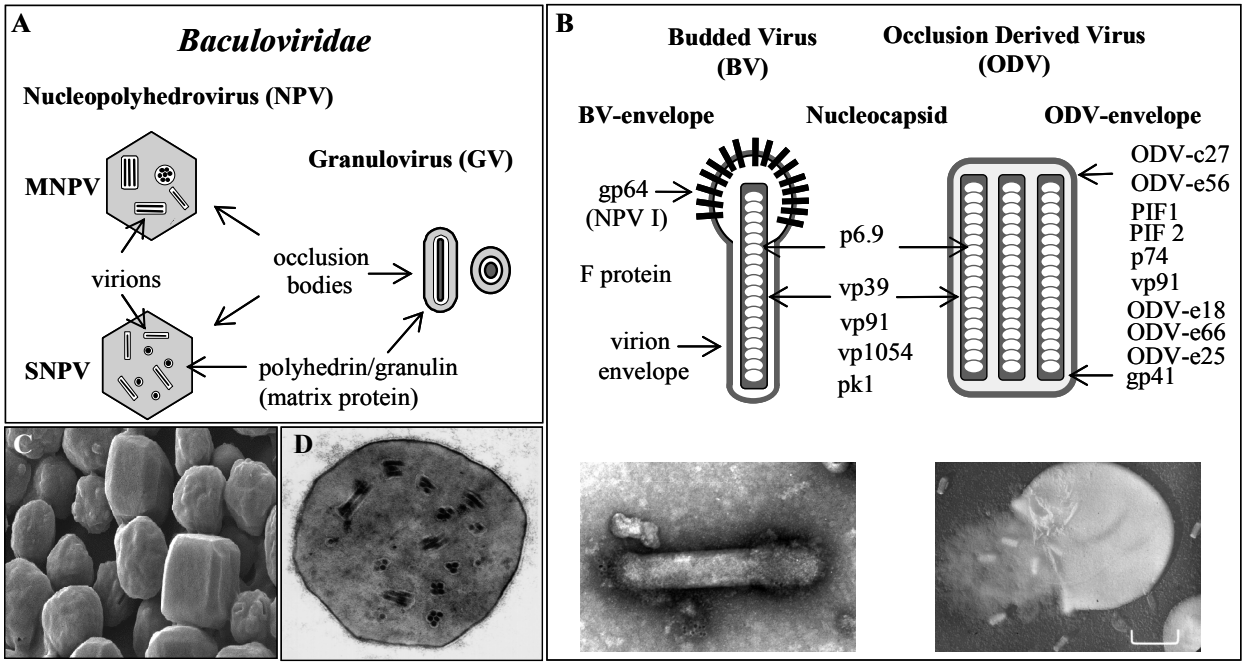


Fig. 1-2. Baculovirus morphology and phenotypes**.** A) Comparison of OB morphology in multiple (M) and single (S) nucleocapsid NPVs, and GVs; B) Schematic drawing and EM photographs of baculovirus BVs and ODVs. The location of GP64, the BV envelope fusion protein of group I NPVs, is also indicated (original idea from Funk *et al.,* 1997). C) Scanning EM image of OBs of an NPV. D) Transmission EM image of a cross-section of an OB, showing an MNPV phenotype. (Figure derived from van Oers & Vlak, 2007).

BVs contain a single nucleocapsid and their envelope is derived from the cell membrane. ODVs may contain single (S type NPVs or GVs) or multiple (M type NPVs) nucleocapsids (NCs) and receive their envelope by *de novo* assembly in the nucleus. The genetic information (ds circular DNA) within the BVs and ODVs is the same, but ODVs may have one (GV, SNPV) or more genome copies (MNPVs) per virion. In addition they differ in lipid and protein composition of the envelope as a reflection of their genesis (Fig. 1-2).

Baculoviruses infect all larval stages. The infection of adults has been reported only for the mosquito-infecting baculovirus *Culex nigripales* NPV (Becnel *et al.,* 2003). Depending on the virus species the initial symptoms of baculovirus infection appear from three to ten days after virus ingestion. Infected larvae become lethargic and discoloration of the cuticle can often be observed. One of the first microscopical infection signs are enlarged cell nuclei, which slowly become occupied by OBs. This gives a whitish/yellowish color to the hemolymph and later to whole larval bodies (Federici, 1997; Goulson, 1997), hence the old name "jaundice" for this disease (Wood & Granados, 1991). Baculovirus infection also influences the rate of larval development (O'Reilly, 1995). Lengths of larval stages increase due to expression of the viral *egt* gene. *Egt* encodes ecdysteroid UDP-glucosyltransferase, which inactivates insect ecdysteroid hormones. The lack of appropriate amounts of ecdysteroids leads to delayed molting and thus to prolonged larval development (Clarke *et al.,* 1996). This in turn enables maximal virus production within a larval stage (O'Reilly & Miller, 1991). In a later stage of the infection process larvae stop feeding. This stage is characterized by the production of copious amounts of OBs which finally fill the whole larval body (up till 1.5 x 10^{10} OBs/larva or 25% of the entire biomass, Evans *et al.,* 1981). Two enzymes encoded by baculoviruses, cathepsin and chitinase, enhance the disintegration of larval bodies, leading to their liquefaction (Hawtin *et al.,* 1995; Slack *et al.,* 1995). Infected insects die after four days to three weeks after infection, depending on the virus species and environmental factors, such as temperature.

Baculovirus taxonomy

In view of the variability of viruses, it is often difficult to decide what a virus species is in taxonomic terms, if a virus belongs to the same species or not (variants), and where the boundaries are. So this refers to the problem of identity: how different is different enough to be another species? One of the difficulties in defining species is that the term is used in many different ways and the exact criteria to set the boundaries are unfortunately not always clearly described. A virus possesses biochemical and structural properties which include the composition and sequence of the viral genome. The genome of a virus however is not represented by a single well-defined RNA or DNA molecule, but must be viewed as a dynamic population of variants that are always present in any viral isolate. This is particular true for RNA viruses, hence the term quasi-species, where the species consists of a mixture of genomes (Domingo *et al.,* 1996), but also in DNA viruses where the variability can also be high. In addition to physical and chemical properties a virus has a number of features that

become visible when it infects a host and starts replicating, such as host range, tissue tropism, pathology, ability to block apoptosis etc. All of these characteristics together with virus ecology should support species demarcation. This has lead to the polythetic concept of a virus species (van Regenmortel, 1992), where a species is defined as a set of (biochemical, biological) characters, which do not all have to be shared by all members of the species.

The naming of viruses adds to the difficulties associated with distinguishing species. Baculoviruses are traditionally named after the host from which they were first isolated. This is sometimes very confusing because a particular virus may infect more than one insect species, and one insect species may be the host for several clearly different viruses. The first may lead to the situation that a virus has more than one name, like in the case of *Autographa californica* MNPV (AcMNPV), which is also called *Anagrapha falcifera* MNPV (AfMNPV). *Orgyia pseudotsugata* is an example of an insect infected with two distinct NPVs, fortunately in this case a MNPV and an SNPV, so OpMNPV and OpSNPV. Frequent changes in the name of the insect hosts may bring additional confusion in baculovirus nomenclature. *Laspeyresia, Carpocapsa* or *Cydia pomonella*, *Stilpnotia* or *Leucoma salicis*, *Spodoptera* or *Prodenia*, *Porthetria* or *Lymantria dispar*, *Euproctis chrysorrhoea* or *Nygmia phaeorrhoea* are only few examples.

Traditionally, the classification of baculoviruses was based on the morphology of the OBs and on their pathology. Occasionally other properties like serological characteristics, host range and virion morphology were taken into account in baculovirus classification methods. None of these criteria proved satisfactory in describing baculovirus species and their evolutionary relationships. The latter only recently became possible due to the increasing amount of nucleotide sequence data (Herniou & Jehle, 2007). In the 8th report of the ICTV the family *Baculoviridae* was divided into two genera: NPV and GV (Theilmann *et al.,* 2005). NPVs form large (0.15-15 μm), polyhedral-shaped OBs called polyhedra (Fig. 1-1C and 1-1D), which contain many virions, whereas GVs form smaller (0.3 – 0.5 μm), cylindrical OBs called granules, which contain a single virion. Both types of baculoviruses are occluded in a proteinaceous matrix consisting of polyhedrin (NPVs) or granulin (GVs). NPVs have been isolated from lepidopteran and non-lepidopteran hosts; GVs were only found in lepidopteran hosts up till now. However, on the basis of nucleotide sequencing and phylogenetic analysis a further proposal for classification of baculoviruses has been made (Jehle *et al.,* 2006; see below).

Phylogenetic analyses based on polyhedrin gene sequences further subdivided lepidopteran NPVs into group I and group II NPVs (Zanotto *et al.,* 1993, see also Fig. 1-4). This division moreover appeared to correlate with the utilization of two different BV envelope fusion proteins. Group I NPVs contain the major envelope glycoprotein GP64, which mediates membrane fusion (Blissard & Wenz, 1992). Group II NPVs as well as GVs lack GP64 protein, but contain a functional homolog designated as F protein (Westenberg *et al.,* 2002). The baculovirus F protein is structurally similar to some vertebrate virus envelope fusion proteins,

like human cytomegalovirus glycoprotein B, human parainfluenza virus type 3F, Ebola virus glycoprotein GP, and avian influenza virus hemaglutinin HA (Westenberg *et al.,* 2002).
Single or multiple packaging of NCs in baculoviruses was first believed to have taxonomic value (Bulach *et al.,* 1999). Over time, it became clear that S and M type NPVs can not be categorized in separate taxonomical units, as viruses of each type are present in the phylogenetically distinct group I and group II NPVs (Herniou *et al.,* 2003; see also Fig. 1-4).

Early phylogenetic analyses were often based on the sequence of single genes. The mayor OB proteins polyhedrin and granulin are made in large amounts and for this reason large quantities can be obtained and sequenced from the N-terminus (Rohrmann *et al.,* 1981). Moreover, the genes coding for polyhedrin and granulin are highly conserved and thus easily identified in new baculovirus isolates, by DNA hybridization or, more common these days, PCR analysis with degenerate primers. Single gene phylogenies, however, may occasionally lead to misinterpretation as they not always reflect authentic relations between viruses. This was for example the case for the AcMNPV polyhedrin gene, which appeared to have a mosaic structure resulting most probably from recombination events (Lange *et al.,* 2004). While biological data as well as sequence information for other AcMNPV genes clearly show that AcMNPV belongs to group I NPVs, its polyhedrin gene is most closely related to the homologue in *Trichoplusia ni* SNPV, a group II NPV. Nevertheless, polyhedrin gene phylogenies as well as phylogenies of other conserved baculovirus genes usually reflect baculovirus relationships and evolution. In fact, polyhedrin was until recently the only choice for analysis of large numbers of baculoviruses due to the limited sequences available for other genes.

The first baculovirus genome to be completely sequenced was that of the AcMNPV C6 strain (Ayres *et al.,* 1994). Up till now genome sequences of more than 50 baculoviruses have been determined and new complete genome sequences are frequently being released in the databases (Table 1-1). The genome size varies between 80 kbp (*Neodiprion sertifer* (Nese) NPV and 180 kbp (*Xestia c-nigrum* (Xecn) GV (reviewed by van Oers & Vlak, 2007). Complete genome sequences provide information about gene sequences, gene content and genome organization (Fig. 1-3), and therefore contain several levels of phylogenetic input data. These data allow genome-wide comparisons between virus species, which can be used to analyze how viruses are related to one another and may give insight in their evolutionary path. The assumption here is that more closely related viral genomes show high nucleotide and predicted amino acid similarities. They also have similar gene content and the individual genes are positioned in a similar order along the genome (Herniou *et al.,* 2001). Such genetic co-linearity, however, does not per se correspond to biological characteristics of the viruses, such as infectivity and host range.

Table 1-1. Entirely sequenced baculovirus genomes

	Virus	Abbreviation	Genome length (kb)	Reference	GenBank accession
Lepidoptera NPV Group I	*Antheraea pernyi* MNPV – L2	AnpeMNPV	126.246	Fan *et al.,* 2007	EF207986
	Antheraea pernyi MNPV	AnpeMNPV	126.629	Nie *et al.,* 2007	DQ486030
	Anticarsia gemmatalis MNPV	AgMNPV	132.239	Oliveira *et al.,* 2006	DQ813662
	Autographa californica MNPV	AcMNPV	133.894	Ayres *et al.,* 1994	L22858
	Bombyx mori NPV – T3	BmNPV	128.413	Gomi *et al.,* 1999	L33180
	Bombyx mandarina NPV – S1	BomaNPV	126.770	Xu *et al.,* Unpubl.	FJ882854
	Choristoneura fumiferana MNPV	CfMNPV	129.609	De Jong *et al.,* 2005	AF512031
	C. fumiferana DEFMNPV	CfDEFMNPV	131.160	Lauzon *et al.,* 2005	AY327402
	Epiphyas postvittana NPV	EppoNPV	118.584	Hyink *et al.,* 2002	AY043265
	Hyphantria cunea NPV	HycuNPV	132.959	Ikeda *et al.,* 2006	AP009046
	Maruca vitrata NPV	MaviNPV	111.953	Chen *et al.,* 2008	EF125867
	Orgyia pseudotsugata MNPV	OpMNPV	131.990	Ahrens *et al.,* 1997	U75930
	Plutella xylostella NPV	PlxyNPV	134.417	Harrison & Lynn, 2007	DQ457003
	Rachiplusia ou NPV	RoNPV	131.526	Harrison & Bonning, 2003	AY145471
Lepidoptera NPV Group II	*Adoxophyes honmai* NPV	AdhoNPV	113.220	Nakai *et al.,* 2003	AP006270
	Adoxophyes orana NPV	AdorNPV	111.724	Hilton & Winstanley, 2008	EU591746
	Agrotis ipsilon NPV	AgipNPV	155.122	Harrison, 2009	EU839994
	Agrotis segetum NPV	AgseNPV	147.544	Jakubowska *et al.,* 2006	DQ123841
	Chrysodeixis chalcites NPV	ChchNPV	149.622	van Oers *et al.,* 2005	AY864330
	Clanis bilineata NPV	ClbiNPV	135.545	Zhu *et al.,* 2009	DQ504428
	Ectropis obliqua NPV	EcobNPV	131.204	Ma *et al.,* 2007	DQ837165
	Euproctis pseudoconspersa NPV	EupsNPV	141.291	Tang *et al.,* 2009	FJ227128
	Helicoverpa armigera SNPV – G4	HearSNPV	131.403	Chen *et al.,* 2001	AF271059
	Helicoverpa armigera SNPV – C1	HearSNPV	130.759	Zhang *et al.* 2005	AF303045
	Helicoverpa armigera SNPV	HearSNPV	154.196	Tang *et al.* Unpubl.	EU730893
	Helicoverpa armigera SNPV NNg1	HearSNPV	132.425	Ogembo *et al.* Unpupl.	AP010907
	Helicoverpa zea SNPV	HzSNPV	130.869	Chen *et al.,* 2002	AF334030
	Leucania separata NPV	LeseNPV	168.041	Xiao and Qi, 2007	AY394490
	Lymantria dispar MNPV	LdMNPV	161.046	Kuzio *et al.,* 1999	AF081810
	Mamestra configurata NPV-A 90/2	MacoNPV-A	155.060	Li *et al.,* 2002a	U59461
	Mamestra configurata NPV-A 90/4	MacoNPV-A	153.656	Li *et al.,* 2005	AF539999
	Mamestra configurata NPV-B	MacoNPV-B	158.482	Li *et al.,* 2002b	AY126275
	Orgyia leucostigma NPV CFS-77	OrleNPV	156.179	Eveleigh *et al.,* Unplubl.	EU309041
	Spodoptera exigua MNPV	SeMNPV	135.611	IJkel *et al.,* 1999	AF169823
	Spodoptera frugiperda	SfMNPV	135.611	Wolff *et al.,* 2008	AF169823
	Spodoptera frugiperda – 3AP2	SfMNPV	131.330	Harrison *et al.,* 2008	EF035042
	Spodoptera litura MNPV	SpltMNPV	139.342	Pang *et al.,* 2001	AF325155
	Spodoptera litura NPV II	SpltNPV II	148.634	Li *et al.,* Unpubl.	EU780426
	Trichoplusia ni SNPV	TnSNPV	134.394	Willis *et al.,* 2005	DQ017380
Lepidoptera GV	*Adoxophyes orana* GV	AdorGV	99.657	Wormleaton *et al.,* 2003	AF547984
	Agrotis segetum GV	AgseGV	131.680	Ai *et al.,* Unpubl.	AY522332
	Choristoneura occidentalis GV	ChocGV	104.710	Escasa *et al.,* 2006	DQ333351
	Cryptophlebia leucotreta GV	CrleGV	110.907	Lange & Jehle, 2003	AY229987
	Cydia pomonella GV	CpGV	123.500	Luque *et al.,* 2001	U53466
	Helicoverpa armigera GV	HearGV	169.794	Harrison & Poplam, 2008	EU255577
	Phthorimaea operculella GV	PhorGV	119.217	Croizier *et al.,* Unpubl.	AF499596
	Plutella xylostella GV – K1	PlxyGV	100.999	Hashimoto *et al.,* 2000	AF270937
	Spodoptera litura GV – K1	SpltGV	124.121	Wang *et al.,* Unpubl.	DQ288858
	Xestia c-nigrum GV	XecnGV	178.733	Hayakawa *et al.,* 1999	AF162221
Hymenoptera NPV	*Neodiprion abietis* NPV	NeabNPV	84.264	Duffy *et al.,* 2006	DQ317692
	Neodiprion lecontei NPV	NeleNPV	86.462	Lauzon *et al.,* 2004	AY349019
	Neodiprion sertifer NPV	NeseNPV	81.755	Garcia-Maruniak *et al.,* 2004	AY430810
Diptera NPV	*Culex nigripalpus* NPV	CuniNPV	108.252	Afonso *et al.,* 2001	AF403738

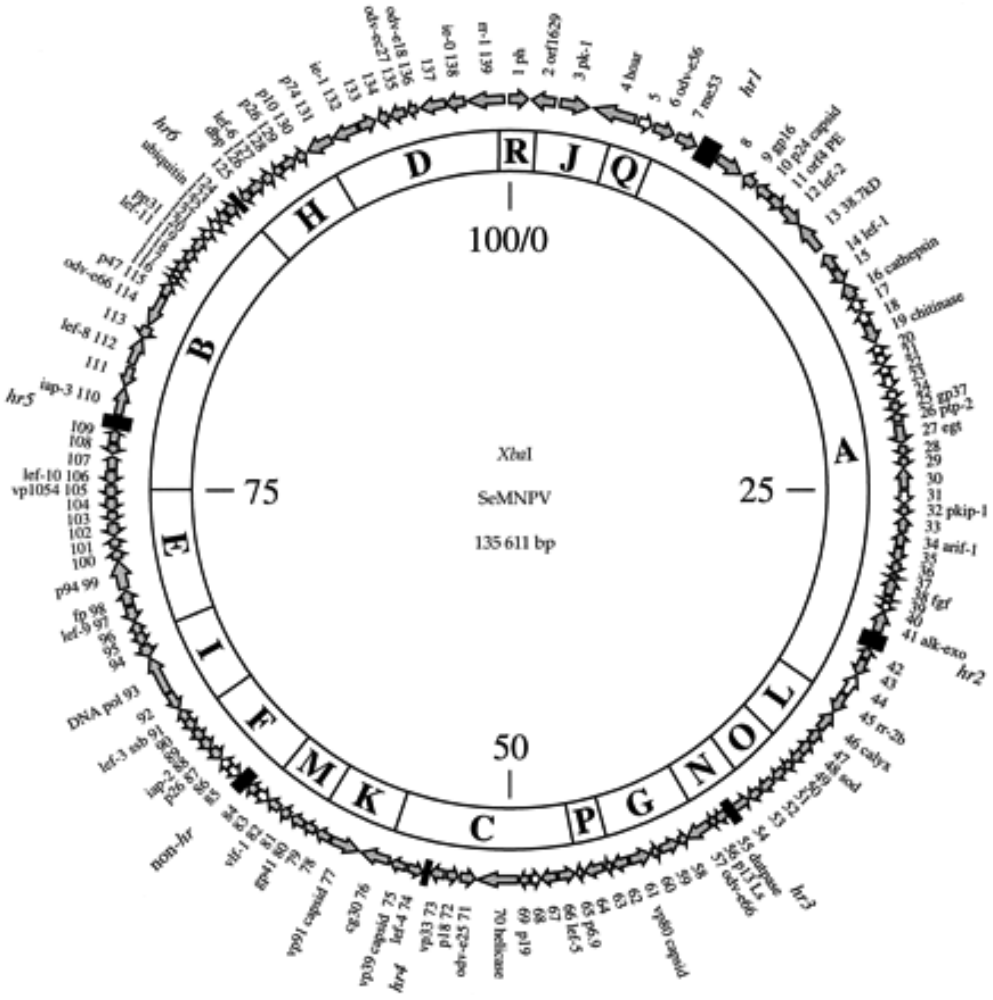


Fig. 1-3. Circular map of the genomic organization of *Spodoptera exigua* (Se) MNPV (from IJkel *et al.,* 1999).

Phylogenies based on whole genome sequences confirmed the earlier classification of lepidopteran baculoviruses into NPVs and GVs (Theilmann *et al.,* 2005), but also demonstrated, with the availability of the genome sequences of hymenopteran NPVs (*Neodiprion abietis* NPV, *N. lecontei* NPV and Nese NPV) and the dipteran CuniNPV, that NPVs infecting dipteran and hymenopteran hosts are phylogenetically separated from lepidopteran baculoviruses (Fig. 1-4). Hymenopteran NPVs have small genomes in comparison to lepidopteran NPVs, between 82-86 kbp and encode about 90 open reading frames (ORFs). Interestingly, the latter group does not contain homologues of either the F protein or GP64, which may explain why they only infect the gut and probably do not spread from cell-to-cell via BVs. Hymenopteran NPVs share 43 ORFs with lepidopteran baculoviruses. CuniNPV differs from lepidopteran NPVs in the size of the polyhedrin protein, which is 90 kDa (versus 30 kDa in other NPVs) and appears non-homologous to polyhedrin (Perera *et al.,* 2006). Although it contains an F protein, its replication is limited to the gut (Becnel *et al.,* 2003). CuniNPV shares 30 ORFs with other baculoviruses.

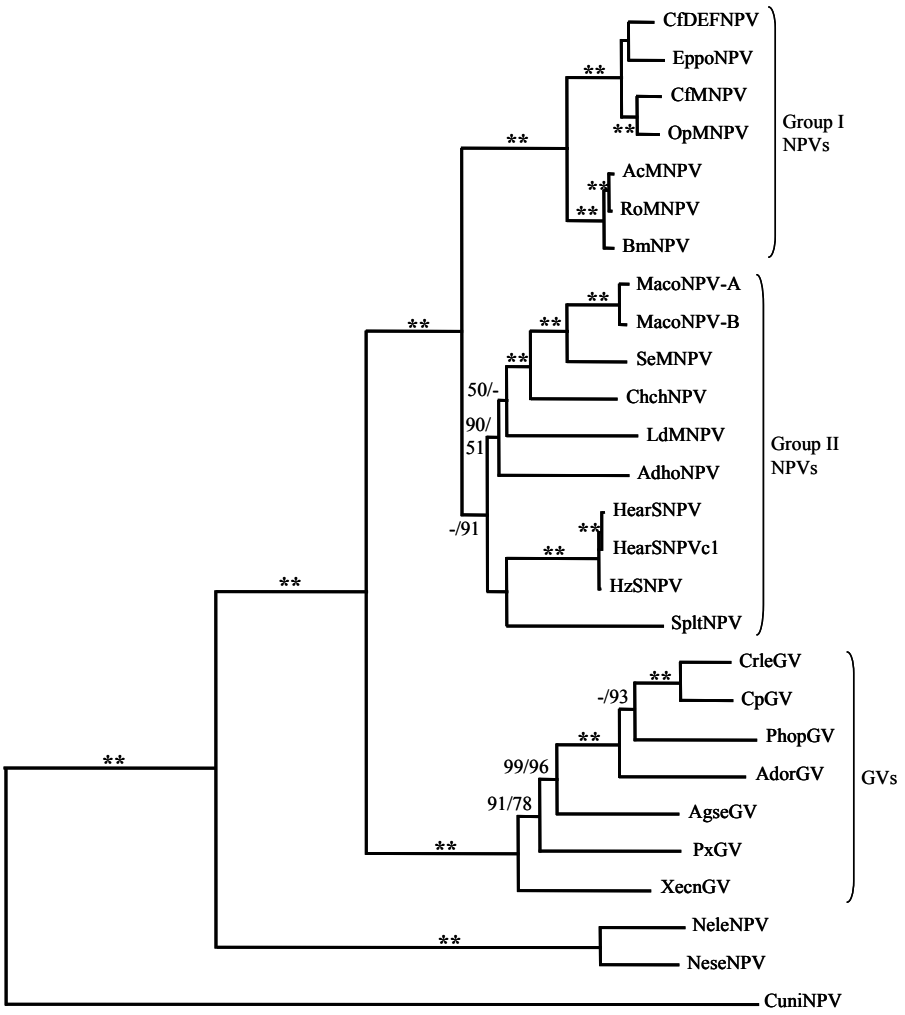


Fig. 1-4. Phylogeny of a selection of baculovirus genomes. The tree was obtained by maximum likelihood (ML) analysis using amino acid sequences of individually aligned and concatenated baculovirus shared genes. The numbers indicate bootstrap scores above 50 for ML and Maximum parsimony (MP) analysis (adapted from van Oers *et al.,* 2005).

Recently a new classification of baculoviruses based on these molecular characteristics and phylogenetics has been proposed for the 9th ICTV report (Jehle *et al.,* 2006) and was approved by the ICTV in 2008 (Carstens & Ball, 2009; www.ictvonline.org). The new classification reorganizes the family *Baculoviridae* into four genera: *Alphabaculovirus* (lepidopteran NPVs), *Betabaculovirus* (lepidopteran GVs), *Gammabaculovirus* (hymenopteran NPVs) and *Deltabaculovirus* (dipteran NPVs). Interestingly these four major groups of baculoviruses were identified already in the eighties by N-terminal sequencing of major occlusion body proteins (Rohrmann *et al.,* 1981). At this time hymenopteran baculoviruses were represented only by NeseNPV and *Tipula paludosa* (Tipa) NPV was the dipteran NPV. It has been shown that concatenated sequences of three conserved genes (*polh, lef-8* and *lef-9)* or a combined

phylogeny of the separate analyses of each of these give the same tree topologies as the analysis of complete genome sequences (review by Herniou & Jehle, 2007) and thus the approach to take multiple conserved genes is strongly advised for identification of new baculovirus isolates.

Based on all these sequencing studies, a criterion for demarcating baculovirus species has been depicted. The evolutionary distance between a pair of sequences usually is measured by the number of mutual nucleotide (or amino acid) substitutions. One of the models used to estimate the evolutionary distance between sequences is the Kimura 2-parameter. This method corrects for differences in the rates of transition and transversion, in different words allows weighing a quality of difference between transition and transversion (Kimura, 1980). The proposed criterion suggests that when the Kimura 2-parameter between single or concatenated genes is larger than 0.050, two viruses are distant enough to be considered as different virus species. As a consequence, the proposed rules to discriminate baculovirus species are as follows: two (or more) baculovirus isolates belong to the same species if the Kimura-2-parameter between single and/or concatenated *polh, lef-8* and *lef-9* nucleotide sequence is smaller than 0.015. Two viruses should be considered as different virus species if that distance is bigger than 0.050. For the pair of viruses with the distance between 0.015 and 0.050 complementary information such as biological characteristics such as host range should be provided for species demarcation (Jehle *et al.,* 2006).

Baculovirus evolution

The current view on the evolution of baculoviruses states that they have evolved from non-occluded viruses infecting midgut tissue, to occluded viruses infecting midguts (gamma- and deltabaculoviruses) and finally to occluded viruses with the ability to spread the infection to other tissues (alpha- and betabaculoviruses) (Herniou & Jehle, 2007). The most likely scenario is that over time baculoviruses have gained features to infect more cell types and become more independent from the host cell machinery.

Genotypic variation in baculoviruses appears to be very common. It occurs not only between isolates collected from the same host species at different locations, but also between isolates collected at the same site and even within virus samples collected from an individual larva. Hence, natural populations of baculoviruses comprise a number of different genotypes that differ in gene content. For example, seven genotypes were found in the original AcMNPV isolate (Stiles & Himmerich, 1998) and *Spodoptera frugiperda* MNPV (SfMNPV) appeared to be a mixture of nine genotypes (Simon *et al.,* 2004). Twenty four NPV genotypes were found in a single *Panolis flammea* larva (Cory *et al.,* 2005). This genetic variability results from the very high natural recombination rate of baculoviruses (Hajos *et al.,* 2000) and the ubiquitous presence of transposon-like elements (Jehle *et al.,* 1998), which may have lead to horizontal gene transfer, insertion/deletion (indel) mutations and transposition events. Several studies show that the genetic variation of baculoviruses is linked to hypervariable regions in the

genome rather than being evenly spread (Garcia-Maruniak *et al.,* 1996; Muñoz *et al.,* 1999, Cory *et al.,* 2005).

This genetic variability provides a large opportunity for natural selection, since small changes within the viral genome can result in significant modifications in virus characteristics. It has been demonstrated that genotypic variants differ in both pathogenicity and speed of kill, though it is not yet clear which genetic changes lead to these differences. For SfMNPV it was shown that any single genotype had much lower fitness than the mixture of genotypes (Simon *et al.,* 2008) indicating that high genetic variability is favored by selection because it maximizes the probability of successful infection of a potential host (Hodgson *et al.,* 2004; Simon *et al.,* 2005) as well as survival in the environment (Murillo *et al.,* 2007). Baculovirus genotypes can be separated using *in vivo* cloning in insect larvae or by plaque purification in insect cells (Lynn *et al.,* 1992; Ribeiro *et al.,* 1997; Smith & Crook, 1988).

Baculovirus evolution depends on genetic variation in both baculovirus and insect populations. Viruses in this co-evolutionary duet have of course more chances to adapt than the hosts, due to a shorter replication time, higher offspring numbers and a high natural heterogeneity. Since baculoviruses can persist outside their host for long times, also the environment exerts selection pressure. The first hypothesis for evolution of baculoviruses in relation to their host stated that baculoviruses have evolved first in one insect order and then colonized other groups (Rohrmann, 1986). The second postulates that the association between baculoviruses and their hosts dates back to the origin of insects and that they coevolved with their host during evolutionary time (Federici, 1997). Recently, these two hypotheses have been tested and the results lead to a new hypothesis, postulating that ancestral baculoviruses horizontally infected hosts of different orders and that later progressive specialization into different baculovirus lineages took place (Herniou *et al.,* 2004). Support for this latter hypothesis comes from the fact, that the phylogeny of baculoviruses follows the phylogeny of the different hosts within an order, hence reflects the pattern of insect families, but does not clearly reflect the evolution of insect orders.

Baculovirus genomics

Baculoviruses show a large variability in their gene content. Their genomes have between 90 and 181 open reading frames (ORFs), already standing for more than 800 different genes when 29 genomes were sequenced (Jehle *et al.,* 2006). The increasing number of virus genes and genome sequences shows that only 30 baculovirus genes are common and conserved in all baculoviruses and thus are considered as the core set of baculovirus genes (Herniou *et al.,* 2003; van Oers & Vlak, 2007; McCarthy & Theilmann, 2008) (Table 1-2). The core genes belong to various functional categories. Most serve DNA replication, transcription, virion assembly or oral infectivity. However, the function has not been elucidated for all core genes.

Table 1-2. The thirty core baculovirus genes.

Function	Name	Function	ORF in AcMNPV	ORF in SeMNPV
Transcription	*lef-4*	RNA polymerase subunit	90	74
	lef-5	Unknown	99	66
	lef-8	RNA polymerase subunit	50	112
	lef-9	DNA condensation	62	97
	p47	RNA polymerase subunit	40	115
	vlf-1	Very late genes expression	77	82
Replication	*dpol*	DNA polymerase	65	93
	helicase	Helicase	95	70
	lef-1	Primase	14	14
	lef-2	Dimer with LEF-1	6	12
Structural	*gp41*	ODV protein	80	80
	odv-e-27	Cyclin	144	135
	odv-e56	Unknown	148	6
	p6.9	DNA condensation	100	65
	p74	Binding in the midgut	138	131
	vp91	Capsid protein	83	77
	pif-1	Binding to midgut cells	119	36
	pif-2	Binding to midgut cells	22	35
	pif-3	Unknown	115	50
	vp1054	Virion assembly	54	105
	vp39	Major capsid protein	89	75
	38K	Nucleocapsid assembly	98	67
	ac142	BV production and RNA transcription	142	137
	ac143	BV production and RNA transcription	143	136
Auxiliary	*alk-exo*	5'→3' exonuclease	133	41
Unknown role	*19kda*	Unknown function	96	69
	p33	Unknown function	92	73
	ac68	Unknown function	68	90
	ac81	Unknown function	81	79
	ac109	Unknown function	109	59

Baculovirus ORFs are present on both DNA strands, with around 50% in each orientation. They are expressed in a cascade as in most large DNA viruses, starting from immediate early genes, through early, late to very late genes (see Miller, 1997). Early and late genes are not grouped, but scattered evenly over the genome and this requires complex regulation of gene expression at each time point of the replication cycle. Non-coding regions constitute less than 10% of the genome and contain gene promoter regions, untranslated regions (UTRs) and homologous regions (hrs), which are rich in sequence repeats.

The first sequenced baculovirus was AcMNPV with the *ptp* gene as the number 1 open reading frame (ORF) (Ayres *et al.,* 1994). In all subsequently sequenced baculoviruses the polyhedrin or granulin gene was assigned number 1, for convenience of comparison. The numbering of genes based on their position relative to polyhedrin allows comparison of gene order in baculovirus genomes, using gene parity plots (Hu *et al.,* 1998) (see Fig. 1-5 for an explanation). From these graphs it becomes clear, that the arrangement of genes is not completely random. In general, genome organization is more conserved in GVs than in

lepidopteran NPVs (Herniou *et al.,* 2003; Lange & Jehle, 2003). Examination of gene order conservation revealed a core gene cluster of *helicas*e, *lef-5*, *Ac96* homologues and *38K* in all baculoviruses (Heldens *et al.,* 1998; Herniou *et al.,* 2003). These genes may be part of a single transcriptional unit or their conservation as a group reflects their essential contribution to a concerted function in virus replication, leading to preservation as a group. Baculovirus gene order is a measurable feature and as a consequence also used for phylogenetic analysis.

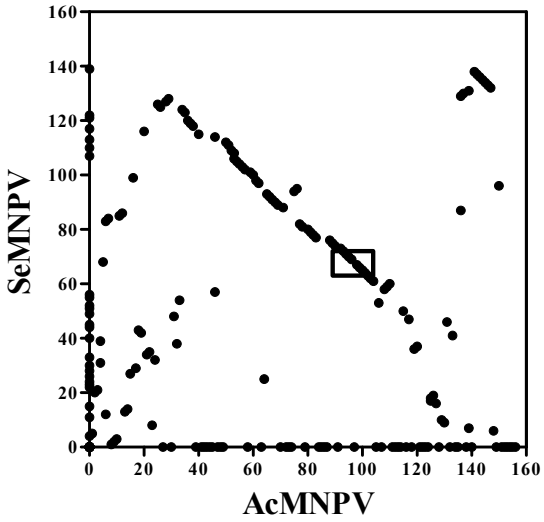


Fig. 1-5. Gene Parity Plot of the SeMNPV versus the AcMNPV genome (after IJkel *et al.,* 1999). The plots are graphic representations of the collinearity of baculovirus genomes obtained by GeneParityPlot analysis. Numbers on the axes represent the relative position of each ORF along the genome. ORFs without homologue in the other genome appear on the axes. The cluster of *helicase*, *ac96*, *38K* and *lef-5* ORFs is boxed.

Gene expression and DNA replication

Baculovirus transcription and replication both take place in the nucleus (Mikhailov, 2003). Four phases can be distinguished, immediate early, early, late and very late (see Friesen & Miller, 1986). Small DNA viruses that replicate in the nucleus use host RNA polymerases to transcribe their genes. In contrast, large DNA viruses, such as baculoviruses, use host and viral RNA polymerases to do so (Passarelli, 2007). In the early phase (before DNA replication) baculoviral genes are transcribed by host RNA polymerase II (Fuchs *et al.,* 1983). Early genes promoters contain typical eukaryotic transcription motifs as a TATA box, where polymerase binds, and a CAGT motif, where the transcript is initiated (Blissard *et al.,* 1992). Additionally other early transcription regulation motifs may be present, like GATA and CACGTG (Krappa *et al.,* 1992; Kogan *et al.,* 1995). One of the first transcribed baculovirus genes is the immediate early gene 1 (*ie-1*), responsible for activation of other early genes and involved in DNA replication. Early genes code among others for proteins necessary for DNA replication, like helicase and DNA polymerase. Many early genes function as regulators of late and very late genes transcription

(Friesen & Miller, 1986) or are involved in preventing host defense responses (like the anti-apoptotic genes *p35* and *iap*).

Late and very late genes promoters are characterized by a canonical (A/G/T)TAAG motif. Their expression is dependent on DNA replication and is not observed when replication is inhibited (for example by aphidicolin or AraC) (Friesen & Miller, 1986). Late and very late genes are transcribed by an α-amanitin resistant, virus-encoded RNA polymerase (Grula *et al.,* 1981). This polymerase is a complex of four viral proteins, P47, LEF-4, LEF-8 and LEF-9, and has both promoter recognition and catalytic activities (Guarino *et al.,* 1998). Eighteen baculovirus genes are required for late gene expression, hence the name late essential factor (*lef*) genes (Todd *et al.,* 1995). The majority of late and very late genes encode virus structural proteins or proteins involved in ODV or polyhedron assembly. Polyhedrin/granulin and *p10* belong to the very late genes, involved in OB formation and release. Expression of these genes requires very late expression factors, like very late factor 1 (VLF-1) (Yang & Miller, 1999).

Baculovirus replication and gene expression involves many *cis*- and *trans*-acting elements. Homologous repeat regions (*hrs*) identified in many baculoviruses have been demonstrated to be *cis*–acting enhancers for early gene expression (Guarino & Summers, 1986; Lu & Carstens, 1993). *Hrs* are composed of varying numbers of highly conserved repeated sequences of about 70 bp and have been shown to function as origins of DNA replication, at least in cell culture (Kool *et al.,* 1995) and enhancers of transcription (Guarino & Summers, 1986; Guarino *et al.,* 1986). In addition, other repeat elements with a putative origin function (non-*hr*) have been characterized (Pearson *et al.,* 1992; Kool *et al.,* 1993). Putative origins of replication differ in structure within the same virus genome and among baculoviruses. DNA polymerase and helicase belong to the *trans*-acting replication factors (Kornberg & Baker, 1992; Ahrens & Rohrmann, 1995). Baculovirus DNA most likely replicates according to the rolling circle model (Oppenheimer & Volkman, 1997; Wu *et al.,* 1999). This strategy is also used by other large double-stranded DNA viruses like herpes simplex virus (Kornberg & Baker, 1992).

The satin moth *Leucoma salicis* and its baculovirus

L. salicis, the white satin moth, belongs to the family Lymantriidae (Lepidoptera) and is one of the most harmful defoliators of *Populus* and *Salix* spp. trees in Europe and Asia. In the 1920s this insect was also introduced in North America, in British Columbia as well as in New England, and is established in the Northern part of the USA and in Canada. Periodic outbreaks of the satin moth are recorded in temperate areas of the Northern hemisphere where the satin moth is endemic and both parasites and microbials are effective control agents. The satin moth is susceptible to a number of entomopathogens, including viruses, bacteria, spiroplasma, fungi, and microsporidia (Reeks & Smith, 1956; Laméris *et al.,* 1985; Lipa & Ziemnicka, 1996; Ziemnicka & Sosnowska, 1996). Bauloviruses have been identified in the satin moth from

various European countries, such as Poland (Ziemnicka, 1981), former Yugoslavia (Sidor, 1967), Germany (Skatulla, 1985)

Microbial control strategies initially focused on the use of *Bacillus thuringensis*, which showed high efficacy in several countries in Europe (Schotveld & Wigbels, 1975; Donaubauer, 1976, Maksymov, 1980; Szalay-Marzso *et al.,* 1981). Recent reports based on long term observation of *L. salicis* outbreaks in Poland revealed that NPVs and cypoviruses (CPVs, *Reoviridae*) are the most important natural epizootic factors (Ziemnicka, 2008). CPVs, however, caused collapse of pest populations only in coexistence with NPVs. This clearly indicates that NPVs may be the most effective candidates for biocontrol of *L. salicis,* also as CPVs are more difficult to disperse and the disease is slightly chronic. Field tests with *Leucoma salicis* (Lesa) NPV were conducted in former Yugoslavia (Sidor, 1967), Belgium (Nef, 1975) and the Netherlands (Grijpma *et al.,* 1986), but no commercial product based on this virus has been commercialized so far. Recently an NPV was identified in the satin moth in Turkey (Yaman, 2008). *L. salicis* NPV (LesaNPV) isolates have not been characterized genetically yet, so their taxonomic position needs to be resolved.

The black cutworm *Agrotis segetum* and its baculoviruses

A. segetum belongs to the cutworms, a group of caterpillars from the family *Noctuidae* that live in the soil and feed on roots and gnaw the petioles of nearly all types of vegetables and field crops, with the greatest damage caused in cotton, tomato, maize, legumes, tobacco, sunflower and sugar beet (Sukhareva, 1999). Cutworms are difficult to control due to their behavior and often they are only detected when the plants are already damaged. A variety of pathogens have been isolated from *A. segetum* larvae, including several baculoviruses, both NPVs and GVs, (Lipa *et al.,* 1971; Lipa & Ziemnicka, 1971; Allaway & Payne, 1983; Caballero *et al.,* 1991) and these were evaluated for their potential in biocontrol (Lipa & Wiland, 1972; Lossbroek & Theunissen, 1985; Thomsen *et al.,* 2001). Initially the interest was mainly in *Agrotis segetum* (Agse) GV since several authors had reported its successful use in the field (Zethner *et al.,* 1987; Caballero *et al.,* 1991). A biocontrol product based on AgseGV, Agrovir, was commercialized in Denmark (Saturnia-Copenhagen). Nonetheless, direct comparison of AgseGV and AgseNPV indicated that AgseNPV has better potential in the field as a control agent for *A. segetum* (Bourner *et al.,* 1992). Moreover, it was observed that baculoviruses isolated from *Agrotis* spp. effectively cross-infect other *Agrotis* species, like both AgseGV and AgseNPV successfully infect *A. ipsilon* larvae (Shah *et al.,* 1979; El-Salamouny *et al.,* 2003). Cross-infectivity is not surprising as these noctuids cover the same ecological niches and pathogen exchange is therefore highly possible (Bourner & Cory, 2004). More host range studies and infectivity comparisons are needed to evaluate the best biocontrol candidate out of the range of baculoviruses isolated from cutworms. The baculovirus complex in cutworms is, however, intriguing and they constitute a perfect model for studying ecological relations between hosts and their pathogens.

Of the cutworm baculoviruses only *A. ipsilon* MNPV (AgipNPV) (*Alphabaculovirus,* group II) and AgseGV (*Betabaculovirus*) have been entirely sequenced (Harrison, 2009; Ai *et al.,* AY522332). Other AgseNPVs have been partially characterized, i.e. by restriction enzyme analysis (Allaway & Payne, 1983; El-Salamouny *et al.,* 2003)

Scope of the thesis

The starting point for the research presented in this thesis is the virus collection of the Institute of Plant Protection in Poznan, in Poland. Many of the virus samples from the collection have never been characterized at a molecular level so far, whereas they might have potential as natural bioinsecticides against common pests in agri- or silviculture. Analysis of other archival baculovirus samples have greatly aided in determining the phylogenetic relationship among the baculoviruses (Herniou *et al.,* 2003; Jehle *et al.,* 2006). Such analysis also may shed more light on the evolution of baculoviruses along with their hosts (Herniou *et al.,* 2004).

The first virus characterized in this thesis is LesaNPV, for which the pathology has been described previously (Ziemnicka, 1981), but which lacks a solid classification. Therefore LesaNPV is analyzed genetically by sequencing a few conserved baculovirus genes (polyhedrin (*polh*), late expression factor (*lef-8*)*, per os* infectivity factor (*pif-2*)) followed by phylogenetic analysis (**Chapter 2)**. Based on the observed close relationship to *Orgyia pseudotsugata* NPV (OpMNPV), an insect from North America, a study is undertaken to compare these two viruses to a second NPV isolated from *O. pseudotsugata*, OpSNPV (**Chapter 3**).

Molecular classification is also lacking for a NPV from the Polish collection pathogenic to the black cutworm *Agrotis segetum.* This information is particularly relevant as various NPVs have been isolated from *A. segetum* larvae at different geographical locations in the Northern hemisphere, but their evolutionary relationship is unknown. The sequences of four conserved baculovirus genes were determined *polh*, *lef-8, pif-2* and DNA polymerase (*dpol*), and used for phylogenetic analysis. In **Chapter 4** AgseNPV is characterized by restriction enzyme analysis and by phylogenetic studies using the genes mentioned above, and compared to two other NPVs isolated from *A. segetum*. The genome of the Polish AgseNPV isolate, which is clearly different form the two other AgseNPV isolates, is entirely sequenced and analyzed (**Chapter 5**). Since AgseNPV and *Spodoptera exigua* NPV (SeMNPV) despite being different baculovirus species show similarity in sequence and particularly genome organization, the ability of these viruses to cross-infect *S. exigua* and *A. segetum* larvae, respectively, is analyzed in infectivity assays *in vivo* and *in vitro* and by monitoring early and late gene expression at the mRNA level in homologous and reciprocal infections (**Chapter 6**).

In **Chapter 7** the results of the studies presented are discussed in the context of the need for detailed characterizations of baculovirus isolates using molecular analyses to determine baculovirus relationships and in view of the need to correlate genetic information with biological properties and functions. A future outlook will complete this chapter.

Chapter 2

European *Leucoma salicis* MNPV is closely related to North American *Orgyia pseudotsugata* MNPV

The satin moth *Leucoma salicis* L. (Lepidoptera, Lymantriidae) is a frequent defoliator of poplar trees (*Populus* spp.) in Europe and Asia (China, Japan). Around 1920 the insect was introduced into the USA and Canada. In this paper, a multicapsid nucleopolyhedrovirus isolated from *L. salicis* larvae in Poland (LesaNPV) was characterized and appeared to be a variant of *Orgyia pseudotsugata* (Op) MNPV. *O. pseudotsugata*, the Douglas fir tussock moth (Lepidoptera, Lymantriidae), occurs exclusively in North America. Sequences of three conserved baculovirus genes, *polh*, *lef-8*, and *pif-2*, were amplified in polymerase chain reactions using degenerate primer sets, and revealed a high degree of homology to OpMNPV. Restriction enzyme analysis confirmed the close relationship between LesaNPV and OpMNPV, although a number of restriction fragment length polymorphisms were observed. The *lef-7* gene, encoding late expression factor 7, and the *ctl-2* gene, encoding a conotoxin-like protein, were chosen as putative molecular determinants of the respective viruses. The *ctl-2* region appeared suitable for unequivocal identification of either virus as LesaNPV lacked a dUTPase gene in this region. Our observations may suggest that LesaNPV, along with *L. salicis*, was introduced into *O. pseudotsugata* after introduction of the former insect into North America in the 1920s.

This chapter has been published in a slightly modified form as: Jakubowska, A., van Oers, M.M., Cory, J.S., Ziemnicka J. & Vlak, J.M. (2005). European *Leucoma salicis* MNPV is closely related to North American *Orgyia pseudotsugata* MNPV. Journal of Invertebrate Pathology 88:100-107.

Introduction

The satin moth *Leucoma salicis* L. (Lepidoptera, Lymantriidae), previously known as *Stilpnotia salicis*, is a serious defoliator occurring throughout Europe and Asia (Lipa & Ziemnicka, 1996). In the 1920s, the insect was introduced into North America, where it was first detected near Boston, Massachusetts. Currently, it is distributed over New England in the northern United States and British Columbia in Canada (Langor, 1995). Satin moth larvae feed on all species of poplar and willow (*Populus* spp.), but also on oak and crabapple. On both continents they are most common on eastern cottonwood, white and black poplar, bigtooth, and trembling aspen, in both planted trees and natural stands. Usually there is only one generation of the insect per year, although up to three generations may occur in the warmer climate zones. Larvae diapause in the L2 stage, but hibernation as eggs has also been reported (Ziemnicka, 2000). In Europe, the first signs of tree damage appear in late May when larvae resume feeding. After mid-June the late instar larvae are capable of massive, complete defoliation of trees. Severe feeding damage results in reduced growth of stems and finally tree mortality (Langor, 1995).

L. salicis has been shown to be susceptible to a number of entomopathogens, including viruses, bacteria, spiroplasma, fungi, and microsporidia (Lipa & Ziemnicka, 1996). The occurrence of a baculovirus infecting satin moth larvae was first reported by Weiser *et al.* (1954) and later by Skatulla (1985). This virus, *L. salicis* (Lesa) NPV (also known as *Stilpnotia salicis* (Ss) MNPV was shown to play a major role in regulating the size of *L. salicis* populations (Ziemnicka, 1981). The biological activity of LesaNPV against satin moth larvae has been evaluated (Lameris *et al.,* 1985) and a sevenfold difference in virulence between LesaNPV from Poland and from former Yugoslavia has been noted. The genome of LesaNPV has been estimated at 128–134 kb in size, based on restriction enzyme analysis of four Polish isolates (Strokovskaya *et al.,* 1996), but its phylogenetic status has not been investigated.

Baculoviruses comprise a family of double stranded DNA viruses infecting primarily insects from the orders Lepidoptera, Diptera, and Hymenoptera. The family *Baculoviridae* is divided into four genera: *Alphabaculovirus,* with the lepidoptera-infecting NPVs, *Betabaculovirus*, encompassing the granuloviruses, *Deltabaculovirus* and *Gammabaculovirus,* infecting dipteran and hymenopteran hosts, respectively (Jehle *et al.,* 2006). The lepidopteran-specific NPVs are further divided into two subgroups, group I and group II NPV based on single gene phylogeny (Bulach *et al.,* 1999), and this division was confirmed by whole genome phylogenies (Herniou *et al.,* 2001). Up till now, more than 700 baculoviruses have been recorded, and many of these have been characterized biologically and/or biochemically (Moscardi, 1999). More than 23[1] baculovirus genomes have been fully sequenced and characterized (Lange *et al.,* 2004). Most phylogenetic analyses were based on

[1] As of December 2009, more than 50 baculovirus genomes have been entirely sequenced.

single-gene sequences from lepidopteran baculoviruses, which often led to conflicting phylogenies when different genes were used.

The analyses based on complete genome sequences enabled the selection of the most suitable genes for single gene phylogenetic studies (Herniou *et al.,* 2004): *lef-8* and *pif-2* (*Ac22*). The *polh* gene sequence is the most widely used gene for phylogenetic analyses, however, phylogenies derived for this gene are usually at variance to those of other core genes (Harrison & Bonning, 2003; Herniou *et al.,* 2001). It has recently been shown that this is in part due to the mosaic structure of the *polh* gene in *Autographa californica* MNPV (Lange *et al.,* 2004). *Polh*, *lef-8*, and *pif-2* are conserved in all lepidopteran baculoviruses analyzed thus far. The p*olh* gene encodes polyhedrin, the major protein of occlusion bodies (OBs). *Lef-8* encodes a late expression factor which is required for transcription of late baculovirus genes and forms, together with *lef-4*, *lef-9*, and *p47*, the baculovirus encoded RNA polymerase (Titterington *et al.,* 2003). *Pif-2* is essential for oral infectivity, but is not required for virus replication in cultured insect cells (Pijlman *et al.,* 2003).

The aim of the current study was to characterize LesaNPV on a molecular basis and to evaluate its taxonomic status using sequences of the conserved baculovirus genes, *lef-8*, *pif-2*, and *polh.*

Material and methods

Insects and viruses

Satin moth larvae were reared in the Department of Biocontrol and Quarantine of the Institute of Plant Protection in Poznan, Poland. Third and fourth instar larvae were collected from poplar trees in the years 1998-2002 and reared in the laboratory on fresh poplar (*Populus nigra*) leaves during the season. Second instar larvae were kept at 4°C over winter. The larvae were reared in large glass vessels at 20-25°C, 60-70% relative humidity and 18:6 hours photoperiod up to pupation. They were fed with poplar leaves changed at least every 2 days. Emerging adults were transferred to new vessels to lay eggs on paper. Egg masses were placed in plastic or glass boxes with fresh leaves.

LesaNPV was isolated in Poland (Kutno) in 1984 from a number of infected larvae feeding on poplar trees and stored at -20°C. The original virus isolate was freshly amplified in fourth instar larvae of *L. salicis* reared in the laboratory in 2004. Larvae were infected individually by feeding with poplar leaf discs contaminated with 10 µl of virus suspension (10^7 OBs/ml). OBs were purified from dead larvae as described by Mūnoz *et al.* (1997). *Orgyia pseudotsugata* (Op) MNPV (TM Biocontrol-1) was kindly obtained from Dr. Imre Otvos, Pacific Forestry Centre, Victoria, Canada.

DNA extraction and restriction enzyme analysis

DNA was isolated from OBs according to Reed *et al.* (2003) with the modification of using dialysis after phenol:chloroform:isoamyl alcohol extraction. The DNA solution was dialyzed against three changes of TE buffer (1 mM Tris.HCl, 0.1 mM EDTA pH 8.0) at 4°C for 24-48h. For restriction enzyme analyses 1 µg of DNA was digested for 3.5 h at 37°C with *Hin*dIII, *Not*I or *Pst*I, electrophoresed in 0.7% TAE (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]), separated in 0.6% agarose gels at 15 mA for 18 h and analyzed under UV light after staining the DNA with ethidium bromide.

PCR amplification and cloning

Purified DNA was used as a template for PCR. The degenerate primer set for the *polh* gene was previously described by Moraes & Maruniak (1997) and for the *lef-8* and *pif-2* genes by Herniou *et al.* (2004). Each 25 µl PCR reaction mixture contained 30-50 ng viral DNA, 400 nM of each primer (Table 2-1), 0.2 mM of each dATP, dCTP, dGTP and dTTP, 0.5 U Taq DNA polymerase (Promega), 1.5 mM $MgCl_2$ and 2.5 µl 10 x reaction buffer (Promega). Reactions were carried out in a Bio-Rad thermocycler using the following parameters: 95°C denaturation (5 min), 10 cycles of 94°C (60 s), 45°C (45 s), 72°C (60 s), followed by 25 cycles of 94°C (45 s), 50°C (30 s), 72°C (60), and finally 72°C (5 min) for *polh*, *ctl-2* and *lef-7* gene specific primer sets, and 95°C denaturation (5 min), 10 cycles of 94°C (60 s), 42°C (45 s), 72°C (60 s), followed by 25 cycles of 94°C (45 s), 60°C (30 s), 72°C (60 s), and finally 72°C (5 min) for *lef-8* and *pif-2* gene specific primer sets. The PCR products were either directly sequenced (primer sets with 5'extensions of universal M13 (-20) and M13 R primers) or after cloning into pGEM-T easy (Promega). The nucleotide sequences of the PCR products were determined by automated sequencing (BaseClear, The Netherlands). The sequences obtained for *polh, lef-8* and *pif-2* were deposited in GenBank under numbers AY729808, AY729809 and AY729810, respectively.

Table 2-1. PCR primer sequences.

Target gene	Oligonucleotide sequence*	Amino acid motif **	Product size	Reference
polh	TAYGTGTAYGAYAACAAG TTGTARAAGTTYTTCCAG	YVYDNK WENFYK	600	De Moraes & Maruniak 1997
lef-8	gtaaaacgacggccagtTTYTTYCAYGGNGARATGAC aacagctatgaccatgGGNAYRTANGGRTCYTCNGC	FFHGEMT TAEDPY(IV)P	800	Herniou *et al.* 2004
pif-2	gtaaaacgacggccagtGGWNNTGYATNSGNGARGAYC aacagctatgaccatgRTYNCCRCANTCRCANRMNCC	W(TSN)CI(AP)EDP G(EVF)C(ED)CG(DN)	400	Herniou *et al.* 2004
ctl-2/hr2	gtaaaacgacggccagtCGTGCAGCCGTTGCTGGTGT aacagctatgaccatgGCAGGTGGAGGTGTATGAG	Not relevant	1974	this study
lef-7/hr4	gtaaaacgacggccagtCACAATTCGTTACACGCG aacagctatgaccatgGAGGGGCGACTTGATTTC	Not relevant	1461	this study

* the nucleotides in lower case represent recognition sites for primers used in subsequent sequence analysis
** amino acid motifs are given for degenerate primers only

Sequence analysis and phylogeny

Baculovirus *polh*, *lef-8* and *pif-2* gene sequences were obtained from GenBank (see Table 2-2) to be compared with LesaNPV. The BLAST program (Altschul *et al.,* 1990) at the National Center for Biotechnology Information (NCBI) was used for nucleotide and predicted amino acid sequence homology searches. Alignments of 498, 390 and 315 nt long fragments for *polh*, *lef-8* and *pif-2* genes, respectively, were performed. Maximum parsimony alignments of the nucleotide sequences were performed using PAUP 4.0 (Swofford, 1998). The analysis of *lef-8* and *pif-2* was combined as their tree structures have been found to be congruent in phylogenetic analyses (Herniou *et al.,* 2004). The *polh* gene was analyzed separately. The alignment for *lef-8* + *pif-2* contained eight NPV sequences and for *polh* 18 baculovirus sequences. The alignments were bootstrapped 1000 times and the dendrograms were drawn using Tree View. Additionally, *polh, lef-8* and *pif-2* sequences were aligned in CLUSTALX (Thompson *et al.,* 1994) with those of seven closely related NPVs, to show nucleotide and protein identities (Table 2-3).

Table 2-2. Baculovirus sequences used for phylogenetic analyses.

Virus name	Abbreviation	*Polh*	*Pif-2*	*Lef-8*	References
Autographa californica MNPV	AcMNPV	L22858	L22858	L22858	Ayers *et al.,* 1994
Amsacta albistriga NPV	AmalNPV	AF118850	-	-	-
Anticarsia gemmatalis MNPV	AgMNPV	Y17753	-	-	Zanotto *et al.,* 1992
Anagrapha falcifera NPV	AnafaNPV	U64896	-	-	Federici & Hice, 1997
Archips cerasivoranus NPV	ArceNPV	U40834	-	-	-
Attacus riccini NPV	AtruNPV	S68462	-	-	Hu *et al.,* 1993
Bombyx mori MNPV	BmMNPV	U75359	-	-	Gomi *et al.,* 1999
Choristoneura fumiferana MNPV	CfMNPV	AF512031	AF512031	AF512031	Lee *et al.,* 1992
Choristoneura rosaceana MNPV	ChroMNPV	U91940	-	-	Lucarotti & Morin, 1997
Epiphyas postvittana MNPV	EppoMNPV	AY043265	AY043265	AY043265	Hyink *et al.,* 2002
Heliconius erato NPV	HeerNPV	-	AY449792	AY449771	Herniou *et al.,* 2004
Helicoverpa armigera SNPV	HaSNPV	AF271059	AF271059	AF271059	Chen *et al.,* 2001
Leucoma salicis MNPV	LesaNPV	AY729808	AY729809	AY729810	this study
*Lonomia obliqua*MNPV	LoobMNPV	AF232690	-	-	Wolff *et al.,* 2002
Lymantria dispar MNPV	LdMNPV	AF081810	AF081810	AF081810	Kuzio *et al.,* 1999
Orgyia pseudotsugata MNPV	OpMNPV	NC001875	NC001875	NC001875	Ahrens *et al.,* 1997
Perina nuda NPV	PnNPV	U22824	-	-	Chou *et al.,* 1996
Rachiplusia ou MNPV	RoMNPV	AF068270	-	-	Harrison & Bonning, 1999
Spodoptera exigua MNPV	SeMNPV	AF169823	-	-	van Strien *et al.,* 1992
Thysanoplusia orichalcea NPV	ThorNPV	AF169480	-	-	Cheng *et al.,* 1998
Xestia c-nigrum GV	XcGV	U70069	-	-	Goto *et al.,* 1998

Cross infectivity

Two separate bioassays were performed in order to check the cross infectivity of LesaNPV and OpMNPV for *O. pseudotsugata* and *L. salicis* larvae, respectively. The bioassay with *O. pseudotsugata* was performed by Andrea Schiller in the laboratory of Dr. Imre Otvos, Canadian Forest Service. *O. pseudotsugata* larvae were reared on artificial diet (Thompson & Peterson, 1978). Newly molted third instar *O. pseudotsugata* larvae were exposed to six concentrations of LesaNPV: 10^1 – 10^6 OBs/larva. Larvae were starved for 24 h and then fed with a diet plug contaminated with one of the six virus concentrations and reared separately for 21 days. After they consumed the entire contaminated diet plug they were given fresh diet and the diet was changed weekly. The larvae were incubated at 25°C with a relative humidity of 60-70% and 16:8 h day:night photoperiod. Twenty larvae were used per dose, with no replicates. Twenty larvae inoculated with distilled water served as controls. Mortality was recorded daily for 21 days post inoculation. There was no mortality in controls.

OpMNPV was tested on first instar (3 days old) *L. salicis* larvae. Six virus concentrations were used: 10^3- 10^8 OBs per 12 larvae. Larvae were starved for 6 h and then fed with leaf discs contaminated with virus suspension. After they consumed the contaminated leaves they were given fresh leaves. Tested larvae were incubated in glass vessels (Ø 10 cm) on the lab bench at 20ºC with 50% relative humidity and 14:10 h day:night photoperiod. Twenty-four larvae per dose were tested and there were three replicates. In each replicate, 24 larvae served as control. Mortality was recorded for 15 days post initial exposure. There was no mortality in controls.

Results

The purpose of this study was to determine the taxonomic position of LesaNPV. To this aim, a 600-nt fragment of the polyhedrin (*polh*) sequence, a 800-nt fragment of the *lef-8* gene, and a 450 nt fragment of the *pif-2* gene were amplified by PCR using degenerate primers. Alignment of the partial *polh*, *lef-8* and *pif-2* sequences with corresponding genes fragments of seven other baculoviruses revealed high nucleotide and amino acid identities with group I NPVs, in particular with OpMNPV and to a lesser degree with PnMNPV and CfMNPV (Table 2-3). The phylogenetic analysis of the *polh* sequence of group I baculoviruses showed a strongly supported group comprising LesaNPV, OpMNPV and *Perina nuda* MNPV (Fig. 2-1A). The combined *lef-8* and *pif-2* analysis localized LesaNPV together with OpMNPV and CfMNPV, and this was supported by high bootstrap scores (Fig. 2-1B). In both cases (*polh* and *lef8* + *pif-2*) LesaNPV is most closely related to OpMNPV. The degree of similarity between *polh*, *lef-8* and *pif-2* gene fragments of LesaNPV and OpMNPV was 99, 98, and 97%, respectively, at the nucleotide level. The sequence of the polyhedrin proteins was

identical for these two viruses. The LesaNPV and OpMNPV LEF-8 protein sequence varied in two amino acids and the PIF-2 proteins revealed differences in five amino acid positions.

Table 2-3. Nucleotide (upper, bold) and amino acid (lower) identities in percentages between various baculoviruses for *polh* (A), *lef- 8* (B) and *pif-2* (C).

A

Polh	**LesaNPV**	**OpMNPV**	**PnMNPV**	**CfMNPV**	**EppoNPV**	**AcMNPV**	**LdMNPV**	**HaSNPV**
LesaNPV		**99**	**92**	**82**	**82**	**75**	**73**	**71**
OpMNPV	98		**92**	**82**	**82**	**76**	**74**	**71**
PnMNPV	93	94		**86**	**84**	**78**	**74**	**73**
CfMNPV	92	93	98		**83**	**77**	**72**	**71**
EppoNPV	92	93	98	97		**74**	**72**	**72**
AcMNPV	83	84	89	87	88		**74**	**72**
LdMNPV	75	76	78	77	79	79		**70**
HaSNPV	79	81	83	81	82	84	80	

B

Lef-8	**LesaNPV**	**OpMNPV**	**CfMNPV**	**EppoNPV**	**AcMNPV**	**LdMNPV**	**HaSNPV**	**HeerNPV**
LesaNPV		**98**	**83**	**71**	**58**	**60**	**57**	**44**
OpMNPV	98		**83**	**71**	**58**	**60**	**57**	**43**
CfMNPV	83	83		**73**	**60**	**58**	**57**	**43**
EppoNPV	79	79	77		**61**	**52**	**59**	**40**
AcMNPV	59	58	60	63		**53**	**55**	**32**
LdMNPV	63	62	60	57	55		**62**	**35**
HaSNPV	63	63	61	65	57	64		**32**
HeerNPV	56	50	45	43	40	37	35	

C

Pif-2	**LesaNPV**	**OpMNPV**	**CfMNPV**	**EppoNPV**	**AcMNPV**	**LdMNPV**	**HaSNPV**	**HeerNPV**
LesaNPV		**97**	**84**	**78**	**78**	**74**	**62**	**82**
OpMNPV	97		**85**	**78**	**79**	**75**	**63**	**82**
CfMNPV	89	91		**79**	**77**	**74**	**65**	**82**
EppoNPV	84	86	89		**75**	**66**	**66**	**78**
AcMNPV	88	89	90	91		**70**	**65**	**77**
LdMNPV	79	70	69	70	71		**68**	**72**
HaSNPV	68	69	72	71	73	82		**65**
HeerNPV	87	88	87	86	85	66	66	

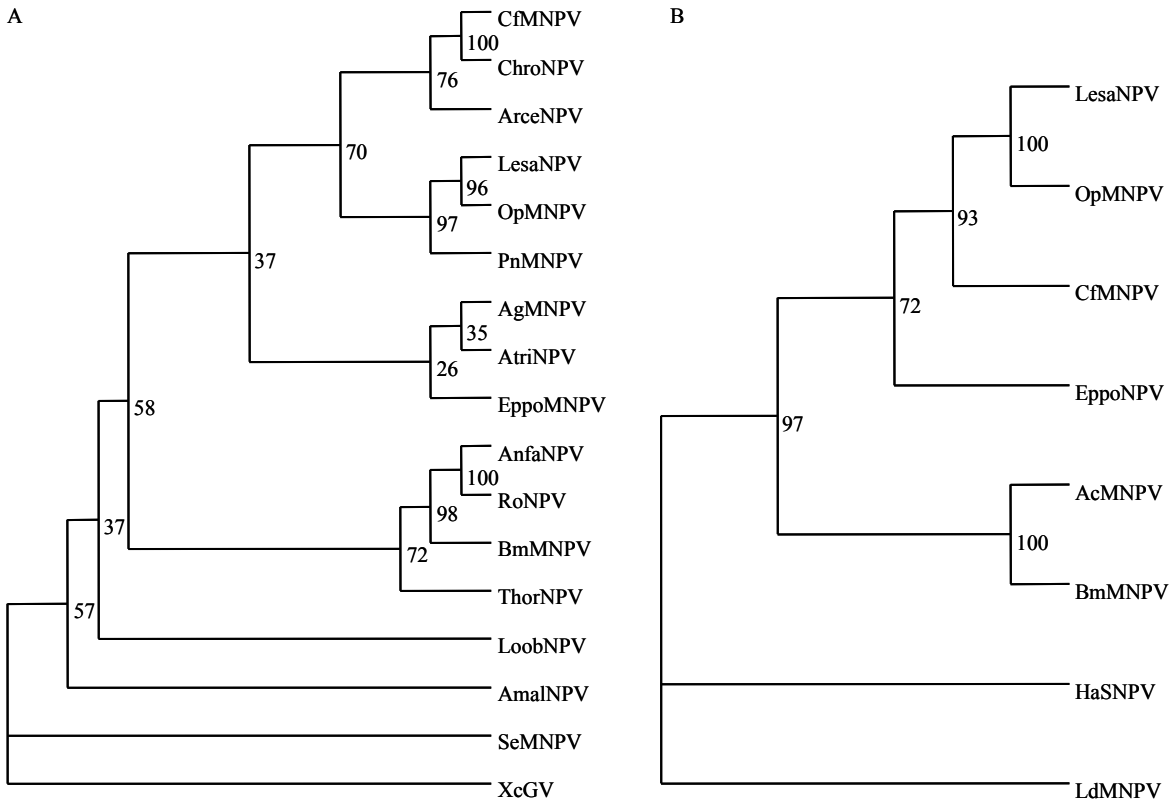


Fig. 2-1. *Alphabaculovirus* group I NPV phylogenies. A) *polh* tree (XcGV was used as outgroup), B) Combined *lef-8* and *pif-2* tree (LdMNPV and HaSNPV were used as outgroup). The phylogeny trees were obtained by maximum parsimony analyses of partial DNA sequence data, numbers indicate bootstrap scores.

Restriction profiles of LesaNPV in comparison to OpMNPV

Since sequencing of three conserved baculoviruses genes suggested a very high level of similarity between LesaNPV and OpMNPV their restriction endonuclease digestion profiles were compared (Fig. 2-2). Restriction patterns using *Hin*dIII, *Not*I or *Pst*I, indicated that LesaNPV and OpMNPV, although closely related, showed very clear restriction fragment length polymorphisms (RFLPs). The most similar were the *Pst*I digestion profiles, although clear differences were observed in the 5– 9 kbp area. Comparison of the *Not*I and *Pst*I profiles showed common digestion products as well as products specific to either virus. The presence of submolar bands in the LesaNPV digests suggests genetic heterogeneity.

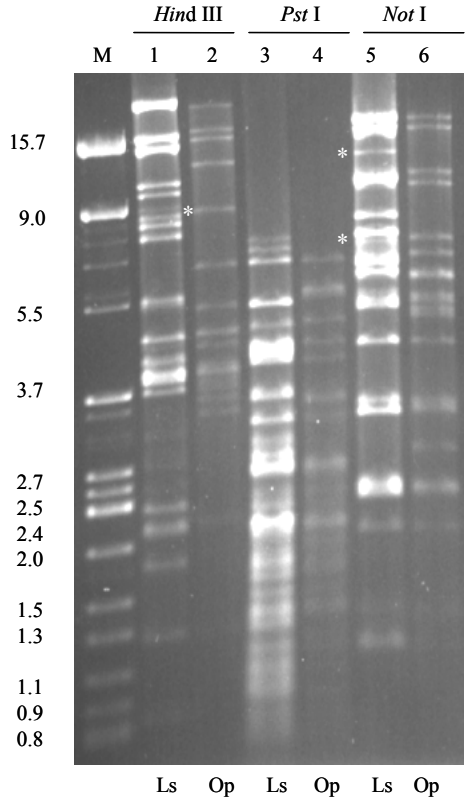


Fig. 2-2. Comparison of restriction enzyme digestion profiles of LesaNPV and OpMNPV. Viral DNA was isolated from OBs from infected satin moth larvae (LesaNPV) and from powder formulated TM Biocontrol-1 (OpMNPV). LesaNPV and OpMNPV DNA were digested with *Hin*dIII (lanes 1 and 2), *Pst*I (lanes 3 and 4) and *Not*I (lanes 5 and 6) and analysed by electrophoresis in an ethidium bromide stained 0.7% agarose gel. M-Lambda *Eco*RI/*Bam*HI/*Hin*dIII fragments. Asterisks (*) indicate minor molar bands.

Analysis of the lef-7and ctl-2 regions

To locate differences between LesaNPV and OpMNPV, two genome fragments were selected that could have the potential to serve as molecular determinants for either virus, the *lef-7* region and the conotoxin like-2 (*ctl-2*) region, both present in OpMNPV. *Lef-7* encodes a late expression factor found to be necessary for late promoter-driven reporter gene expression in a *Spodoptera frugiperda* cell line, but not in a *Trichoplusia ni* cell line. Substitution in this gene may modulate the ability of NPVs to replicate in different cell lines (Lu & Miller, 1995). *Lef-7* was also found to undergo positive selection in alternate hosts (Harrison & Bonning, 2004). *Ctl-2* gene was selected after analysis of differences between the LesaNPV and OpMNPV restriction enzyme (*Hin*dIII) profiles, using the whole genome sequence of OpMNPV (Ahrens *et al.,* 1997), to locate variable regions. A single *ctl* gene is present in several baculovirus genomes (Harrison & Bonning, 2003), whereas OpMNPV encodes two ORFs with conotoxin-like domains, *ctl-1* and *ctl-2*. As OpMNPV is so far the only virus with two *ctl-2* genes, this region had the potential to show differences between the two viruses.

Primers specific for the *lef-7* and *ctl-2* regions were designed using the published OpMNPV sequence (Ahrens *et al.,* 1997). The targeted regions also contained homologous repeats (*hrs*), sites of frequent recombination and rearrangement in baculovirus genomes: the *ctl-2* region harbors *hr2;* the *lef-7* region *hr4*.

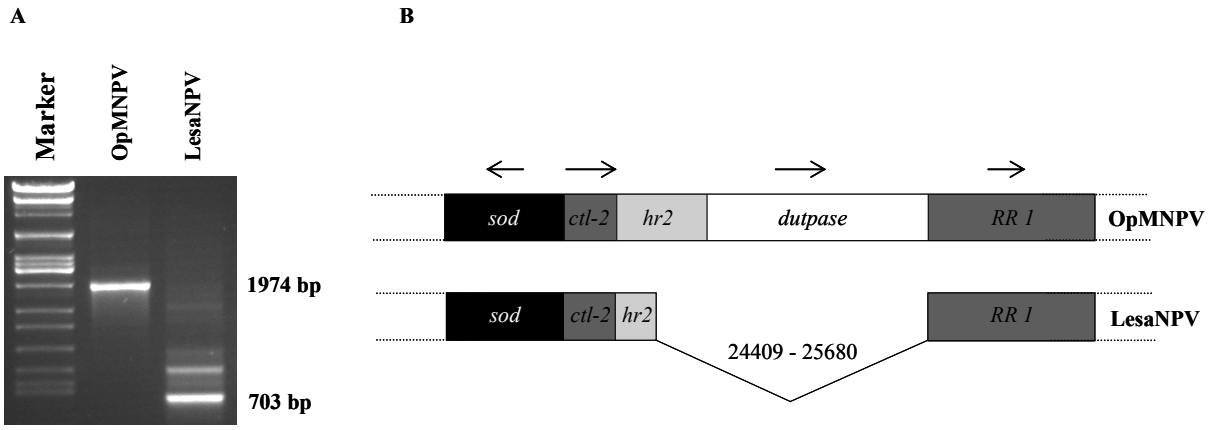


Fig. 2-3. Products obtained by PCR using primers sets specific for *ctl-2* A. The *ctl-2* region was amplified in PCR and analysed in an ethidium bromide stained 1.2 % agarose gel. Marker: Lambda *Eco*RI/*Bam*HI/*Hind*III fragments. B. Organization of the *ctl-2* region in OpMNPV and LesaNPV (numbers indicate the position of the deletion in LesaNPV according to the numbering of OpMNPV (Ahrens *et al.,* 1997). Arrows indicate the direction of transcription.

With primers specific for the *lef-7/hr4* region, PCR products of a similar, expected size were obtained for both viruses. Only minor differences (2%) were revealed at the sequence level (data not shown). PCR amplification of the *ctl-2/hr2* region resulted in a PCR product of the expected size for OpMNPV (1974 bp), whereas for LesaNPV several shorter products were obtained (Fig. 2-3A). The amplified *ctl-2* region of OpMNPV contained part of the *superoxide dismutase* (*sod*) gene (5'end), *ctl-2*, *hr2*, *dUTPase*, and part of the *ribonucleotide reductase* large subunit (*RR1*) gene (3'end). Only one of the major PCR products obtained with LesaNPV, a 703 bp product, was amplified from the region of interest. This LesaNPV product lacked nucleotides 24,409–25,680 (Fig. 2-3B) when compared to the OpMNPV genome (numbering of Ahrens *et al.,* 1997). In OpMNPV, the fragment of 1271 nt which is absent in the LesaNPV genome, harbors the *dUTPase* gene (953 nt), a large part of *hr2* (144 nt), two intragenic regions (52 and 102 nt) and 20 nt from the 3'end of the *RR1* gene (Fig. 2-3B). The nature of the minor LesaNPV PCR products was not analyzed. An additional set of primers was designed to amplify the *dUTPase* gene. These primers were used for a nested PCR with the *ctl-2/ hr2* products of both viruses as template. A product of the expected size was obtained for OpMNPV and no products were found for LesaNPV, suggesting that *dUTPase* was indeed absent from this part of the genome of the latter virus. When these *dUTPase* primers were used for a PCR with the LesaNPV genome as template no amplification product was observed either.

Cross infectivity

The close genetic relatedness between LesaNPV and OpMPNV may be reflected in their biological activity and host range. Therefore, the cross infectivity of LesaNPV and OpMNPV for *O. pseudotsugata* and *L. salicis*, respectively, was tested. Infectivity assays were conducted to obtain a "yes or no" answer for cross infectivity in both hosts. OpMNPV did not kill *L. salicis* larvae, whereas LesaNPV caused mortality at a dose as low as 10 OBs per *O. pseudotsugata* larva. Doses of 10^4, 10^5 and 10^6 LesaNPV OBs per larva caused 100% mortality in *O. pseudostugata*. The infection of *O. pseudotsugata* larvae with LesaNPV was confirmed by PCR analysis of the *ctl-2* region, showing that the progeny virus was LesaNPV-specific.

Discussion

The studies provide molecular characteristics of a multicapsid nucleopolyhedrovirus pathogenic to the satin moth *L. salicis.* Phylogenetic analyses showed that LesaNPV belongs to the group I NPVs and is closely related to OpMNPV, which infects another member of the Lymantriidae. The obtained sequences of three genes conserved among lepidopteran baculoviruses, *polh*, *lef-8*, and *pif-2*, were nearly identical to those of OpMNPV. The restriction enzyme analysis nevertheless showed differences between these two viruses (Fig. 2-2). These data suggest that LesaNPV and OpMNPV may be variants of the same virus species.

Two genomic regions were selected as putative molecular determinants: the *lef-7* region and the *ctl-2* region. Both regions contain homologous repeats, shown to be sites with a high recombination and rearrangement rate in baculovirus genomes (Harrison & Bonning, 2003). *Lef-7* has been defined as a late expression factor able to transactivate TAAG-containing promoters (Morris *et al.,* 1994). It has a large stimulatory effect but is not essential for replication in transient expression assays (Lu & Miller, 1995). *Lef-7* was chosen in this study as a putative molecular determinant as in group I NPVs this gene has been shown to undergo positive selection in alternate hosts (Harrison & Bonning, 2004). Genes under positive selection pressure may account for differences in species-specific virulence or host range among NPVs and this may be the case for LesaNPV and OpMNPV in *O. pseudotsugata* and *L. salicis*, respectively. Sequencing of the *lef-7* region of LesaNPV however, revealed only slight differences compared to OpMNPV, and therefore this region cannot be used as a simple marker to discriminate between these viruses. On the other hand, the *ctl-2* region can be used to discriminate LesaNPV from OpMNPV (Fig. 2-3) as was shown by PCR and sequence analysis. The primers used to amplify the *ctl-2* region also included the *dUTPase* gene and parts of the *sod* and RR1 genes. Both *sod* and *ctl-2* genes are present in the targeted region in LesaNPV and showed high homology to OpMNPV. The amplified part of *RR1* showed only

61% amino acids identity with OpMNPV, but amino acids shown to be essential for enzyme activity (van Strien *et al.,* 1992) were present. The LesaNPV *ctl-2* region lacked a *dUTPase*, and the primers specific for OpMNPV *dUTPase* gave no products with neither genomic LesaNPV DNA nor with the *ct l-2/hr-2* region.. We assume that this gene is not present in the genome of LesaNPV.

Baculoviruses are identified and named according to the insect host species from which they were first isolated. They are often infectious for more than one insect species or are variants of the same virus species and this may result in the double naming of identical viruses (Harrison & Bonning, 1999; Lange *et al.,* 2004). The baculovirus isolated from the satin moth for instance was tentatively renamed *L. salicis* nucleopolyhedrovirus (LesaNPV), originally LsMNPV, but is clearly a variant of OpMNPV. The satin moth, *L. salicis* occurs in Europe and Asia and was introduced around 1920 into North America. The Douglas fir tussock moth, *Orgyia pseudotsugata*, occurs exclusively in North America and has never been recorded in Europe. The insects are taxonomically related and both belong to the family Lymantriidae, subfamily Orgyiinae, but to different tribes, Lymantriini (*L. salicis*) and Orgyiini (*O. pseudotsugata*). The high level of similarity in several baculovirus core genes indicates that the NPVs isolated from both insects are closely related.

A nucleopolyhedrovirus infecting *O. pseudotsugata* was first documented by Eveden & Jost (1947). Later two different types of OBs were isolated from *O. pseudotsugata* larvae (Hughes & Addison, 1970), representing OpMNPV and OpSNPV. In the satin moth from Europe, only an MNPV type has been described, and preliminary results using OpSNPV specific primers in a PCR reaction with LesaNPV as template suggests that an OpSNPV-related virus is absent from the LesaNPV isolate. These results suggest two possible scenarios: LesaNPV may have migrated together with the host insect *L. salicis* to North America and may be the ancestor of OpMNPV, or alternatively that LesaNPV and OpMNPV have a common ancestor with a broader geographic distribution.

A correlation between baculovirus phylogenies and the taxonomic and ecological relationships of their hosts has been observed and coevolution between interacting insect and virus species has been postulated (Herniou *et al.,* 2003). Therefore, when a baculovirus invades a new host species (e.g., *O. pseudotsugata*) it is likely that this host would be closely related to its current host. Adaptation to the new host may eventually lead to the isolation of a different genotype (Herniou *et al.,* 2004). This may be the case for the satin moth and the Douglas fir tussock moth and their NPVs. Although the host plants of these two forest species are not the same, their geographical ranges overlap in the Pacific northwest. In addition, it is well established that baculoviruses can be dispersed rapidly by a variety of means, including birds (Entwistle *et al.,* 1993), which will enable the virus to spread over wide areas and encounter a variety of potential host species. Additionally, studies on the geographic distribution of *O. pseudotsugata* MNPV and SNPV show that the OpMNPV only occurs in the area around British Columbia, Washington, and Idaho, whereas the OpSNPV is

distributed throughout the range of the *Orgyia* species in different geographical locations in the North America (Hughes, 1976), providing indirect support for an introduction in that area. LesaNPV was infectious for *O. pseudostugata*, whereas OpMNPV was not infectious for *L. salicis*. This suggests that LesaNPV could have infected *O. pseudotsugata* and the resulting progeny has since evolved into OpMNPV. This would also imply that OpMNPV has acquired a *dUTPase* gene recently. This is not impossible and is in line with the phylogenetic observation that the *dUTPase* gene is lost and gained by baculoviruses (Herniou *et al.,* 2003). It would require further sequencing and analysis of the LesaNPV genome to confirm that a *dUTPase* gene is indeed absent.

Several other baculoviruses isolated from *Orgyia* species are singly enveloped NPVs (Hughes, 1976; Richards *et al.,* 1999; Sohi *et al.,* 1984), and more importantly, phylogenetic evidence has shown that both Old and New world *Orgyia* NPV species, except OpMNPV but including OpSNPV, cluster together within the group II NPVs (Herniou *et al.,* 2003). Thus there is little evidence for MNPVs being found in other *Orgyia* species and the data imply a close relation between other *Orgyia* NPVs. Further information is needed to support the hypothesis of host switching, in particular, analysis of archival samples of OpMNPV or NPVs from *L. salicis*. Both viruses and their insect hosts provide an excellent system for studying baculovirus host range and evolution.

Acknowledgements

This research was partly supported by a scholarship from the European Union (Functional Biodiversity and Crop Protection), contract *HPMT-CT-2000-00199*. The authors are grateful to Dr. Imre Otvos and Andrea Schiller for providing OpMNPV and for carrying out infectivity assays on *O. pseudotsugata*.

Chapter 3

Phylogenetic analysis of *Orgyia pseudotsugata* single nucleocapsid nucleopolyhedrovirus

The Douglas-fir tussock moth *Orgyia pseudotsugata* (Lepidoptera: Lymantriidae) is a frequent defoliator of Douglas-fir and true firs in western USA and Canada. A single nucleopolyhedrovirus (SNPV) isolated from *O. pseudotsugata* larvae in Canada (OpSNPV) was previously analyzed via its polyhedrin (*polh*) gene, but its phylogenetic status remained ambiguous due to an anomaly in the *polh* tree. Sequences of four conserved baculovirus genes, *polh*, *lef-8*, *pif-2* and *dpol*, were amplified from OpSNPV DNA in polymerase chain reactions using degenerate primer sets and their sequences were analyzed phylogenetically. The analysis revealed that OpSNPV belongs to group II NPVs and is most closely related to SNPVs that infect the closely related *O. ericae* and *O. anartoides* species. The results show the need for multiple, concatenated gene phylogenies to classify baculoviruses.

This chapter has been published in a slightly modified form as: Jakubowska, A., van Oers, M.M., Otvos, I.S., & Vlak, J.M. (2005). Phylogenetic analysis of *Orgyia pseudotsugata* single nucleopolyhedrovirus. Virologica Sinica 22:257-265.

Introduction

Baculoviruses comprise a large family of invertebrate pathogenic viruses, infecting primarily insect species of the order Lepidoptera. They are considered to be safe biological insecticides with great potential in pest control. Recently, a new classification for the family *Baculoviridae* with now four genera (*Alpha-, Beta-, Gamma-* and *Deltabaculovirus*) has been proposed (Jehle *et al.,* 2006) and this proposal was accepted by the International Committee on Taxonomy of Viruses in 2008. The current baculovirus taxonomy is based on genome sequence analyses that revealed that baculovirus phylogeny follows the classification of the host insect (Herniou *et al.,* 2004) and that morphological traits of e.g. occlusion bodies can be a misleading taxonomic character (Jehle *et al.,* 2006). The genus *Alphabaculovirus* harbors the nucleopolyhedroviruses that infect lepidopetran insects. The virus particles of these NPVs have a single (SNPV) or multiple (MNPV) nucleocapsid capsid morphology, depending on the number of nucleocapsids that is surrounded by a common envleope. On the basis of single-gene and genomic phylogenies the lepidopteran NPVs (alphabacuoviruses) have been divided into two groups, group I NPVs and group II NPVs (Zanotto *et al.,* 1993; Bulach *et al.,* 1999; Herniou *et al.,* 2001). This subdivision correlates with the presence of unique envelope fusion proteins GP64 (Group I) and F (Group II) encoded by the viruses from each group (IJkel *et al.,* 2000; Pearson *et al.,* 2000). Forty-one baculovirus genomes have been fully sequenced and characterized and new whole genome sequences are being published regularly (GenBank June 2007)[2].

At present, more than 700 baculoviruses have been reported (Moscardi, 1999) and many are considered or actually used as biocontrol agents of pest insects. Most notable are the baculoviruses of *Anticarsia gemmatalis* and *Helicoverpa armigera* for the large scale control of the soybean looper in Brazil and cotton bollworm in China respectively (Zhang *et al.,* 1995; Moscardi, 1999). Despite of their large number only a small subset of baculoviruses has been studied in detail. Most phylogenetic analyses so far have been based on single-gene sequences (Zanotto *et al.,* 1993; Bulach *et al.,* 1999), which often led to conflicting results when different genes were examined. Combined phylogenies based on more than one gene have shown to alleviate this problem and to make the phylogenetic analyses more robust (Herniou *et al.,* 2001; Lange *et al.,* 2004; Jehle *et al.,* 2006). Recently a criterion for distinguishing virus species has been proposed. The evolutionary distance between a pair of sequences usually is measured by the number of nucleotide (or amino acid) substitutions occurring between them. One of the models used to estimate the evolutionary distance between sequences is the Kimura 2-parameter, which corrects for multiple hits, taking into account transitional and transversional substitution rates and assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites. The proposed criterion suggests that when the Kimura 2-parameter distance between single or concatenated

[2] As of December 2009 more than 50 baculovirus genome sequences have been determined.

genes is larger than 0.05, two viruses may be considered as different virus species (Jehle *et al.,* 2006).

Two different nucleopolyhedroviruses naturally occur in populations of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (Lepidoptera: Lymantriidae) (Hughes & Addison, 1970), an important pest of interior Douglas-fir (*Pseudotsuga menzesii* var. *glauca* (Beissn.) and several species of true firs (*Abies* spp.) in North America (Beckwith, 1978). OpMNPV, the bioactive ingredient of the bioinsecticide TMBiocontrol-1 (registered both in the United States and Canada) is used to control the tussock moth in North America (Reed *et al.,* 2003). The genome of OpMNPV has been completely sequenced, and the function of many genes has been determined (Ahrens *et al.,* 1997). OpSNPV was also originally isolated from *O. pseudotsugata* and is less virulent than OpMNPV. Both viruses are often found intermixed in the same insect population (Hughes, 1976). The current hypothesis is that OpMNPV was introduced in the 1920s from Europe together with *Leucoma salicis* larvae and partially displaced OpSNPV (Jakubowska *et al.,* 2005). In Europe *L. salicis* NPV was probably an ancestral variant of OpMNPV, but names after its European host, *Leucoma (Stilpnotia) salicis* (LesaNPV) (Ziemnicka, 2000; Jakubowska *et al.,* 2005). Both OpSNPV and OpMNPV show distinct restriction enzymes profiles (Richards *et al.,* 1999). Phylogenetic analysis of the polyhedrin (*polh*) genes showed that OpMNPV belongs to group I NPVs, while OpSNPV was ambiguously assigned as a group I (Walsh *et al.,* 1999) or group II NPV (Zanotto *et al.,* 1993; Bulach *et al.,*1999). Relative to OpMNPV much less analysis has been done on OpSNPV, as to date only its *polh* gene has been studied (Rohrmann & Beaudreau, 1977; Leisy *et al.,* 1986a; Leisy *et al.,* 1986b). The *polh* gene encodes the matrix protein of the NPV occlusion body and is one of the most conserved baculovirus genes (Vlak & Rohrmann, 1985). Initially, baculovirus phylogenies were created based on *polh* gene sequences due to the high number of such sequences available. The subdivision of lepidopteran NPVs in group I and II was made based on this protein (Zanotto *et al.,* 1993). Although this grouping has been confirmed recently by gene content, gene order and whole genome phylogenies (Herniou, 2003, Herniou *et al.,* 2004), the *polh* gene phylogenetic analyses often gave conflicting results when compared to the phylogenies of multiple genes or genomes. *Autographa californica* MNPV is the type species of group I NPVs, while its *polh* gene falls out of group I and belongs to group II NPVs (Jehle, 2004). This suggests that phylogenies based on single genes can give misleading results and that multiple gene sequence data are preferred for baculovirus characterization. Detailed analysis using models that can detect recombination events revealed that the AcMNPV *polh* gene is in fact a mosaic of group I and group II NPV-specific *polh* sequences (Jehle, 2004). OpSNPV was included in only one of the gene phylogenies (Herniou *et al.,* 2004), since further gene sequence information of OpSNPV was lacking. In the present study we sequenced four conserved baculovirus core genes from OpSNPV and constructed a phylogenetic tree based on these four core genes sequences to resolve the phylogenetic position of OpSNPV.

Material and methods

In this study we amplified four conserved baculovirus genes from OpSNPV in PCR reactions using degenerate primer sets for *polh* and for the genes encoding late expression factor 8 (*lef-8*), *per os* infectivity factor 2 (*pif-2*) and DNA polymerase (*dpol*), respectively. The degenerate primer set for the *polh* gene was previously described by Moraes & Maruniak (1997) and for the *lef-8, pif-2* and *dpol* genes by Herniou (2003) and Herniou *et al.* (2004) (Table 3-1). Reaction products were cloned into pGEM-T easy plasmids (Promega) and automatically sequenced (BaseClear, the Netherlands). The sequences obtained were deposited in GenBank under numbers: AY895150- AY895153.

Table 3-1. PCR primer sequences.

Target gene	Oligonucleotide sequence	Expected product size in bp	Reference
polh	Forward 5'TAYGTGTAYGAYAACAAG3' Reverse 5'TTGTARAAGTTYTTCCAG3'	600	De Moraes & Maruniak 1997
lef-8	Forward 5'TTYTTYCAYGGNGARATGAC3' Reverse 5'GGNAYRTANGGRTCYTCNGC3'	800	Herniou et al. 2004
pif-2	Forward 5'GGWNNTGYATNSGNGARGAYCC3' Reverse 5'RTYNCCRCANTCRCANRMNCC3'	400	Herniou et al. 2004
dpol	Forward 5'AYRYIAAYMGIGTICAIATGC3' Reverse 5'SIGAYCCITAYWTICCICC3'	600	Herniou 2003

Other baculovirus *polh, lef-8, pif-2* and *dpol* sequences were downloaded from GenBank to be compared with OpSNPV. The BLAST program (Altschul *et al.,* 1990) at the National Center for Biotechnology Information (NCBI) was used for nucleotide and predicted amino acid sequence homology searches. Multiple sequence alignments with amino acid sequences were done in ClustalX. Phylogenies were constructed using the neighbor-joining method of Mega 3.1. (Kumar *et al.,* 2004), using the p-distance method of amino acid substitution. Gaps were treated as missing data. Tree topologies were evaluated by bootstrap analysis with 1000 replicates. The analysis of *lef-8* and *pif-2* was combined and the *polhn* and *dpol* genes were analyzed separately. The selected genes have previously been indicated as most suitable for phylogenetic analyses (Herniou, 2003). Additionally, *lef-8* and *pif-2* were found to be congruent allowing combined analysis (Herniou *et al.,* 2001). For *polh* gene analysis all completely sequenced lepidopteran NPVs (GenBank 2007) were included as well as those from partial sequencing projects that showed the highest amino acid identity with the OpSNPV *polh* gene. For *lef-8/pif-2* analysis all completely sequenced lepidopteran NPVs were included. For *dpol* sequence analysis, all known baculovirus DNA polymerase gene sequences that aligned with the OpSNPV *dpol* sequence were included. For each analysis an outgroup was chosen, the *Plutella xylostella* GV *polh*, the *Xestia c-nigrum* GV *lef-8/pif-2,* and the *Culex nigripalpus* NPV and human DNA polymerase (*Dpol*) genes, respectively.

Table 3-2. Baculovirus sequences used for phylogenetic analyses.

Virus	Abbreviation	*polh*	*lef-8*	*pif-2*	*Dpol*
***Adoxophyes honmai* NPV**	**AdhoNPV**	**NC004690**	**NC004690**	**NC004690**	**NC004690**
***Adoxophyes orana* GV**	AdorGV	-	-	-	**NC005038**
***Agrotis segetum* GV**	AgseGV	-	-	-	**AY522332**
***Agrotis segetum* NPV**	**AgseNPV**	**NC007921**	**NC007921**	**NC007921**	**NC007921**
***Antheraea pernyi* NPV**	**AnpeNPV**	-	**NC008035**	**NC008035**	**NC008035**
***Anticarsia gemmatalis* MNPV**	**AgMNPV**	-	**NC008520**	**NC008520**	**NC008520**
Apocheima cinerarium NPV	ApciNPV	AY706688	-	-	-
***Autographa californica* MNPV**	**AcMNPV**	**NC001623**	**NC001623**	**NC001623**	**NC001623**
***Bombyx mori* MNPV**	**BmMNPV**	**NC001962**	**NC001962**	**NC001962**	**NC001962**
Buzura suppressaria NPV	BusuNPV	X70844	-	-	AF068184
***Choristoneura fumiferana* MNPV**	**CfMNPV**	**NC004778**	**NC004778**	**NC004778**	**NC004778**
***Choristoneura fumiferana defective* MNPV**	**CfDEFMNPV**	**NC005137**	**NC005137**	**NC005137**	**NC005137**
***Chrysodeixis chalcites* NPV**	**ChchNPV**	**NC007151**	**NC007151**	**NC007151**	**NC007151**
***Clanis bilineata* NPV**	**ClbiNPV**	**NC008293**	**NC008293**	**NC008293**	**NC008293**
***Cryptophlebia leucotreta* GV**	**CrleGV**	-	-	-	**NC005839**
***Culex nigripalpus* NPV**	**CuniNPV**	-	-	-	**NC003084**
***Cydia pomonella* GV**	**CpGV**	-	**NC002816**	**NC002816**	**NC002816**
***Ecotropis obliqua* NPV**	**EcobNPV**	**NC008586**	**NC008586**	**NC008586**	**NC008586**
***Epiphyas postvittana* NPV**	**EppoNPV**	**NC003083**	**NC003083**	**NC003083**	**NC003083**
***Helicoverpa armigera* SNPV-C1**	**HearSNPV-C1**	**NC003094**	**NC003094**	**NC003094**	**NC003094**
***Helicoverpa armigera* SNPV-G4**	**HearSNPV-G4**	**NC002654**	**NC002654**	**NC002654**	**NC002654**
***Helicoverpa zea* SNPV**	**HzSNPV**	**NC003349**	**NC003349**	**NC003349**	**NC003349**
Hemerocampa vetusta NPV	HeveNPV	AY706699	-	-	-
***Hyphantria cunea* NPV**	**HycuNPV**	**NC007767**	**NC007767**	**NC007767**	**NC007767**
***Leucania separata* NPV**	**LeseNPV**	**NC008348**	**NC008348**	**NC008348**	**NC008348**
***Lymantria dispar* NPV**	**LdMNPV**	-	**NC001973**	**NC001973**	**NC001973**
Mamestra brassicae NPV	MbMNPV	M20927	-	-	AF068183
***Mamestra configurata* NPV-A 90-2**	**MacoNPV-A**	**NC003529**	**NC003529**	**NC003529**	**NC003529**
***Mamestra configurata* NPV-A 90-4**	**MacoNPV-A**	**AF539999**	**AF539999**	**AF539999**	**AF539999**
***Mamestra configurata* NPV-B**	**MacoNPV-B**	**NC004117**	**NC004117**	**NC004117**	**NC004117**
***Maruca vitrata* NPV**	**MaviNPV**	**NC008725**	**NC008725**	**NC008725**	**NC008725**
***Neodiprion lecontei* NPV**	**NeleNPV**	-	-	-	**NC005906**
***Neodiprion setifer* NPV**	**NeseNPV**	-	-	-	**NC005905**
Orgyia anartoides NPV	OaNPV	AF068188	-	-	AF068185
***Orgyia pseudotsugata* MNPV**	**OpMNPV**	**NC001875**	**NC001875**	**NC001875**	**NC001875**
Orgyia pseudotsugata SNPV	OpSNPV	M32433	AY895150	AY895151	AY895152
Panolis flamea NPV	PaflNPV	D00437	-	-	-
***Phthorimaea operculella* GV**	**PhopGV**	-	-	-	**NC004062**
Plusia orichalcea NPV	PlorNPV	AF019882	-	-	-
***Plutella xylostella* GV**	**PlxyGV**	-	-	-	**NC002593**
Plutella xylostella NPV	PlxyNPV	**NC008349**	**NC008349**	**NC008349**	**NC008349**
***Rachiplusia ou* MNPV**	**RoMNPV**	**NC004323**	**NC004323**	**NC004323**	**NC004323**
Spodoptera exigua MNPV	**SeMNPV**	**NC002169**	**NC002169**	**NC002169**	**NC002169**
***Spodoptera frugiperda* NPV**	**SfMNPV**	**NC009011**	**NC009011**	**NC009011**	**NC009011**
Spodoptera littoralis NPV	SpliNPV	-	-	-	AF215639
***Spodoptera litura* NPV**	**SpltNPV**	**NC003102**	**NC003102**	**NC003102**	**NC003102**
***Trichoplusia ni* NPV**	**TnNPV**	**NC007383**	**NC007383**	**NC007383**	**NC007383**
***Xestia c-nigrum* GV**	**XnGV**	-	-	-	**NC002331**

* Complete genomes in bold

Results

Polh, combined *lef-8* and *pif-2*, and *Dpol* phylogenies clearly place OpSNPV among group II NPVs (Fig. 3-1A, 3-1B and 3-1C). OpSNPV is most closely related to two other lymantrid baculoviruses, from *O. anartoides* SNPV (OranNPV) and *O. ericea* SNPV (OrerNPV) (Fig. 3-1A and 3-1C). Analyses further show that OpSNPV is only distantly related to OpMNPV, although these two are often found intermixed in insect populations. OpMNPV is a group I NPV (26). OpSNPV branched together with *Buzura suppressaria* NPV (*polh* and *Dpol* trees), *Ectropis obliqua* (Ecob) NPV (*Dpol* tree) and *Clanis bilineata* (Clbi) NPV (*Dpol* tree). *Polh* sequence analysis further groups OpSNPV with *Apochemia cinerarium* NPV, but only the *polh* sequence is available for the latter virus and analysis of the other genes is necessary to confirm the common ancestry of these two baculovirus species. Interestingly, *polh* and *Dpol* analyses show different branching for OpSNPV and EcobNPV, suggesting that, despite distant correlation between these two species they may have acquired their *Dpol* gene from a common source.

The combined analysis of *lef-8/pif-2* genes, which includes only sequences of completely sequenced lepidopteran NPVs, confirms the placement of OpSNPV in group II NPVs and its close relation to EcobNPV and ClbiNPV. In this analysis, however, OpSNPV is placed together with *Lymantria dispar* (Ld) MNPV in a common branch and this is in contrast to two other analyses. Close relationship between some open reading frames of OpSNPV and LdMNPV is not surprising as both viruses infect Lymantriidae, enabling the exchange of genetic material.

Discussion

Similarity between all known *Orgyia* NPVs has been implied by Hughes (1976) and supported by Richards *et al*. (1999) on the basis of biological characters. Both SNPVs and MNPVs isolated from the *Orgyia* genus show a high degree of cross infectivity among insect species in this genus. The restriction profiles of OpMNPV and OpSMPV, however, showed enough differences to warrant a distinct taxonomic status of these two viruses (Richards *et al.,* 1999). From the *Orgyia* NPVs only OpMNPV belongs to group I NPVs. OpMNPV, which is found only in North America is closely related to another baculovirus infecting the Lymantrid, *L. salicis* (LeseNPV), which was described from Europe (Ziemnicka, 2000). Both viruses are probably derived from a very recent ancestor (Jakubowska *et al.,* 2005). It can even be concluded that they are variants of the same virus type and speciated recently through regional separation into separate ecological niches.

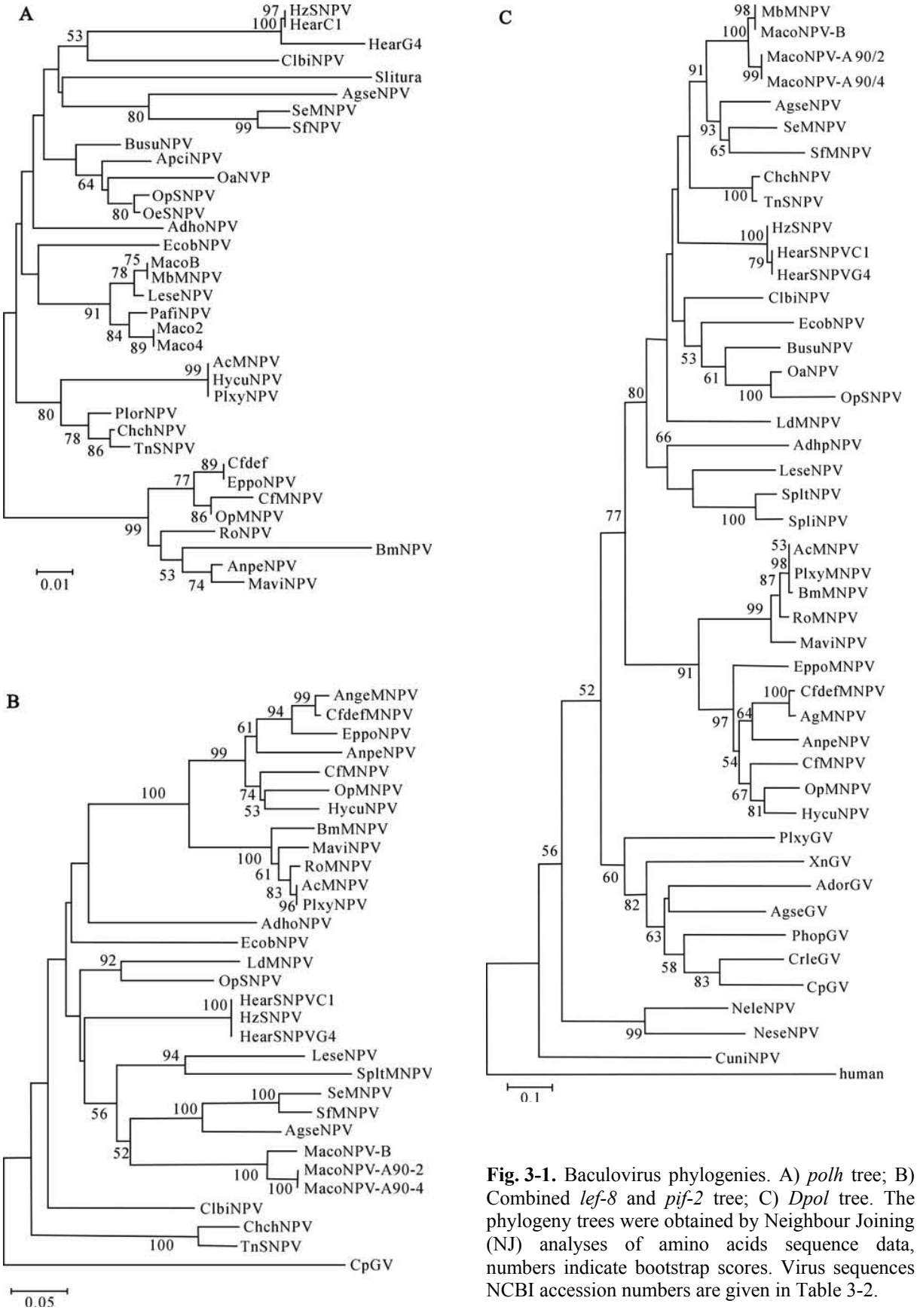


Fig. 3-1. Baculovirus phylogenies. A) *polh* tree; B) Combined *lef-8* and *pif-2* tree; C) *Dpol* tree. The phylogeny trees were obtained by Neighbour Joining (NJ) analyses of amino acids sequence data, numbers indicate bootstrap scores. Virus sequences NCBI accession numbers are given in Table 3-2.

OpSNPV has previously been found to share relatively similar restriction analysis profiles with *O. antiqua* SNPV and cross infections between both host species were documented (Richards *et al.,* 1999). It has also been suggested that OpSNPV and *O. leucostigma* (Orle) SNPV are variants of the same virus infecting both species (Hughes, 1976).

Walsh *et al*. (1999) showed that the molecular characteristics of OpSNPV are distinct from OrleSNPV and classified OpSNPV together with OpMNPV as group I NPVs, based on their polyhedrin amino acid sequences. This finding contrasted with the study of Zanotto *et al*. (1993), which positioned OpSNPV among group II NPVs according to its *polh* gene sequence. The study of Zanotto *et al*. (1993) indicated that the OpSNPV *polh* gene had an unstable position in the phylogenetic tree when its promoter sequences were included. The OpSNPV polyhedrin promoter contains elements common to the AcMNPV *polh* gene, the type species of group I NPVs (not shown). Our study, based on four conserved gene sequences, clearly supports the positioning of OpSNPV within group II NPVs. Recently, another *Orgyia* NPV has been isolated from *O. ericea* (Yang *et al.,* 2006). OpSNPV is closely related to OrerSNPV on the basis of the *polh* sequence (Fig. 3-1A), even closer than to *O. antiqua* SNPV. OrerSNPV is found in China and it would be interesting to study the cross infectivity of these *Orgyia* NPVs in *O. oricea*.

Molecular and biological information about baculoviruses is essential for understanding the relationships within this family of viruses and to promote the use of baculoviruses in insect pest control strategies. Knowledge of the taxonomic position of baculoviruses and their host range is essential in bioinsecticides registration procedures. Most baculovirus phylogenies so far are based on a single gene, usually the *polh* gene, as it is available for a large number of baculoviruses. The *polh* tree topologies do not always match with tree topologies of concatenated sequences and, although very useful, need complementation with other gene phylogenies. Here we report that the OpSNPV *polh* gene phylogeny, positioning this virus in group II NPVs, is supported by the analysis of sequences of three other genes, *lef-8, pif-2* and *Dpol*, which are conserved in all baculoviruses to date. Finally, this paper shows that it is highly recommended to use a universal set of primers for four conserved genes to generate reliable and robust information for the classification of baculoviruses.

Acknowledgments

We thank Prof. C.X. Zhang from Institute of Insect Sciences Zhejiang University for kindly providing us with the nucleotide sequence of OrerSNPV polyhedrin gene. Agata Jakubowska was supported by a Marie Curie fellowship from the European Union and a visiting scientist grant from Wageningen University made available via the Graduate School Production Ecology and Resource Conservation.

Chapter 4

Molecular characterization of *Agrotis segetum* nucleopolyhedrovirus from Poland

The turnip moth, *Agrotis segetum* (Lepidoptera, Noctuidae), is an important pest insect in Europe, Asia, and Africa. We have genetically characterized and classified a nucleopolyhedrovirus isolated from *A. segetum* larvae in Poland (AgseNPV-P). The restriction pattern of AgseNPV-P was distinct from an isolate from England/France (AgseNPV-UK and AgseNPV-F). Sequence analysis of three conserved baculovirus genes, *polh*, *lef-8* and *pif-2*, revealed that AgseNPV-P differs substantially from the already described NPVs isolated from *A. segetum* and possibly represents a new NPV species. Phylogenetic analysis placed AgseNPV-P among group II NPVs and showed the closest relationship to *Agrotis ipsilon* (Agip) NPV and *Spodoptera exigua* (Se) MNPV.

This chapter has been published in a slightly modified form as: Jakubowska, A., van Oers, M.M., Ziemnicka, J., Lipa, J.J. & Vlak, J.M. (2005). Molecular characterization of *Agrotis segetum* nucleopolyhedrovirus from Poland. Journal of Invertebrate Pathology 90:64-68.

Introduction

The turnip moth *Agrotis (Scotia) segetum* Dennis and Schiffenmüller (Lepidoptera, Noctuidae) is a serious pest occurring throughout Europe, Asia, and part of Africa. The larvae belong to cutworms (noctuids spending a large portion of their time in the soil, Bourner & Cory, 2004) and feed on many vegetable and field crops including corn, rape, beet, potatoes, cabbage, cereals, tobacco, vine, and many others (Ignoffo & Garcia, 1979). *A. segetum* is often part of a complex further consisting of *Agrotis ipsilon* and *Agrotis exclamationis*. The turnip moth is susceptible to a number of entomopathogens, including viruses, bacteria, nematodes, and fungi, which have been evaluated as possible biological control agents of cutworms (Caballero *et al.,* 1991; Lossbroek & Theunissen, 1985; Thomsen *et al.,* 1998).

Baculoviruses have high potential to be used as microbial control agenst against lepidopteran pests due to their high pathogenicity, environmental stability, and biosafety, i.e., restricted host range. They comprise a large family of insect specific DNA viruses, traditionally divided into, nucleopolyhedroviruses (NPVs) and granuloviruses (GVs) (Blissard *et al.,* 2000) based on occlusion body morphology. The lepidopteran-specific NPVs, all accomodated in the genus *Alphabaculovirus,* are further divided into two taxonomic groups, group I and group II NPV (Bulach *et al.,* 1999; Herniou *et al.,* 2001, 2003). Two baculovirus species have been isolated from *A. segetum* larvae, the granulovirus AgseGV (genus *Betabaculovirus*) and the nucleopolyedrovirus AgseNPV (Allaway & Payne, 1983; Lipa & Ziemnicka, 1971; Lipa *et al.,* 1971). Both types of viruses showed biocontrol potential in the field (Bourner *et al.,* 1992; Caballero *et al.,* 1991). Host range studies for AgseGV and AgseNPV within the cutworm complex revealed that AgseNPV had a higher potential to act as a broad insect control agent (Bourner & Cory, 2004).

The aim of this current study was the molecular characterization of an AgseNPV isolated from *A. segetum* larvae in Poland (AgseNPV-P) and the evaluation of its taxonomic status. Lipa et al. (1972) showed that AgseNPV-P infects *Agrotis segetum*, *Agrotis exclamationis*, *Heliothis armigera*, *Heliothis zea*, *Mamestra brassicae*, and *Scotogramma trifolii* (Lipa *et al.,* 1972). However, the identity of the progeny virus was not confirmed in their studies. Bourner & Cory (2004) confirmed with molecular methodologies (restriction enzyme analysis and squash blot analysis) the pathogenicity of the English strain of AgseNPV (AgseNPV-UK) for eight species of noctuids: *A. segetum*, *A exclamationis*, *A. puta*, *Noctua comes*, *Peridroma saucia*, *Xestia sexstrigata*, and *X. xanthographa*. Allaway & Payne (1983) compared the DNA restriction profiles of AgseNPV-UK, an isolate from France (AgseNPV-F) and AgseNPV-P and found that AgseNPV-P was distinct from the other two isolates (Fig. 4-1A). Recently, *Hin*dIII and *Eco*RI restriction profiles of AgseNPV-UK and *A. ipsilon* (Agip) NPV were compared and found to be considerably different from each other (El-Salamouny *et al.,* 2003).

Material and methods

Restriction analysis

AgseNPV-P was isolated in 1975 from *A. segetum* larvae and stored at 20°C. The virus was freshly amplified in second instar larvae of *A. segetum* reared in the laboratory in 2002. For genetic analysis third instar larvae were infected individually with diet discs contaminated with 10 µl of virus suspension in a concentration of 10^5 occlusion bodies (OBs) per ml. OBs were purified from dead larvae as described by Muñoz *et al.* (1997) and occluded virions were released by incubation of OBs in 0.1 M Na_2CO_3 for 10–15 min at 37°C. DNA was purified from these virions according to the method described by Reed et al. (2003). The DNA was dialyzed against 0.1 x TE (1 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) after phenol:chloroform extraction. For restriction enzymes analysis 1 µg DNA was digested for 3.5 h at 37°C with *Hin*dIII and the fragments were separated by size in 0.7% agarose gels containing 40 mM Tris–acetate,1 mM EDTA (pH 8.0) at 15 mA for 18 h and analyzed under UV light.

PCR analysis

To further determine the taxonomic status of AgseNPV-P three conserved baculovirus genes were amplified, sequenced and analyzed phylogenetically according to Herniou et al. (2004). For comparison, the AgseNPV-UK *lef-8* and *pif-2* genes were also amplified and sequenced. The *polh* gene sequence of AgseNPV-UK was obtained from GenBank (NCBI) (El-Salamouny *et al.,* 2003). Purified AgseNPV-P DNA was used as a template for PCR. The degenerate primer set for the *polh* gene was previously described by Moraes & Maruniak (1997), and for the *lef-8* and *pif-2* genes by Herniou et al. (2004). PCR products were cloned into pGEM-T easy plasmids (Promega) and the inserts were analyzed by automated sequencing (BaseClear, The Netherlands). The sequences obtained were deposited in GenBank under Accession Nos. AY971675–AY971679.

Phylogenetic analysis

The BLAST program (Altschul *et al.,* 1990) at the National Center for Biotechnology Information (NCBI) was used for nucleotide and predicted amino acid sequence homology searches. *Polh*, *lef-8* and *pif-2* gene sequences from other baculoviruses were obtained from GenBank and used for phylogenetic analysis. For AgipNPV only the *polh* and *lef-8* genes are available from GenBank. *Polh*, *lef-8* and *pif-2* nucleotide and amino acid sequences were aligned in ClustalX (Thomsen *et al.,* 1998). Phylogenetic analyses were performed using PAUP* 4.0 (Swofford, 1998). The trees were bootstrapped using maximum parsimony with 1000 replicates. The dendrograms were drawn using Tree View (Page, 1996).

Results and discussion

The *Hin*dIII restriction enzyme profile of the examined isolate (Fig. 4-1B) was identical to that described by Allaway & Payne (1983) for a Polish isolate (AgseNPV-P), but distinct from the English and French isolates (AgseNPV-UK; AgseNPV-F) described in the same study. *Hin*dIII restriction profiles of AgseNPV-P, AgseNPV-UK, and AgipNPV (El-Salamouny *et al.,* 2003) look mutually very different; we thus conclude that they may represent distinct virus species. The *Hin*dIII restriction fragment profile of AgseNPV-P was also clearly distinct from the profile of *A. ipsilon* (Agip) NPV (Boughton *et al.,* 1999) and this supports the likelihood of AgseNPV-P to be a new baculovirus species.

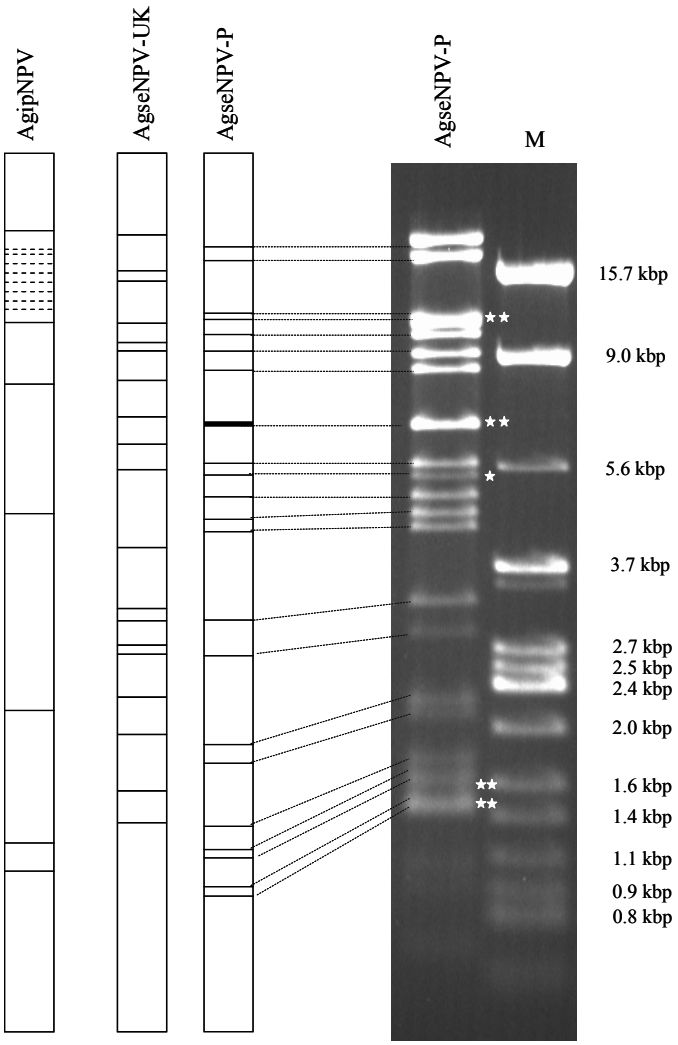
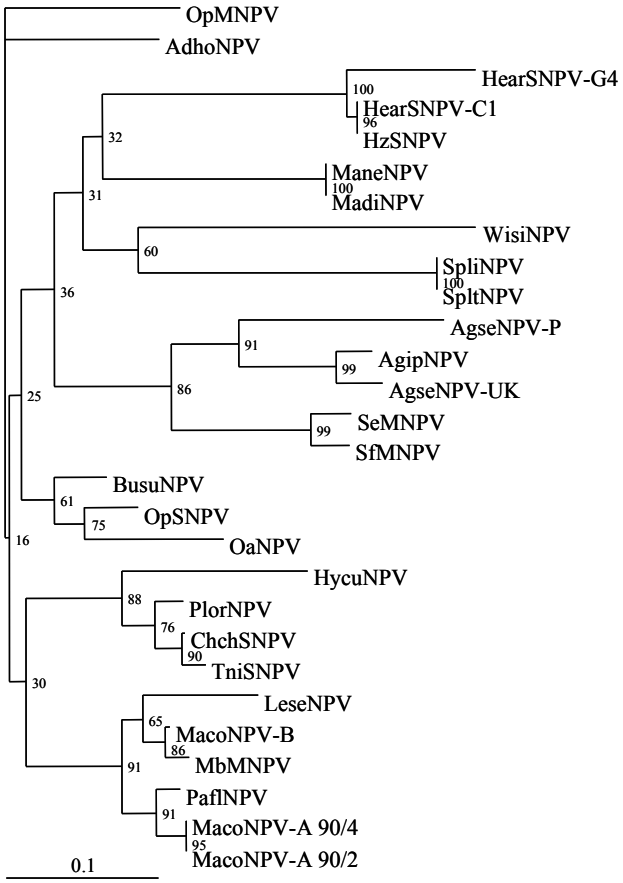


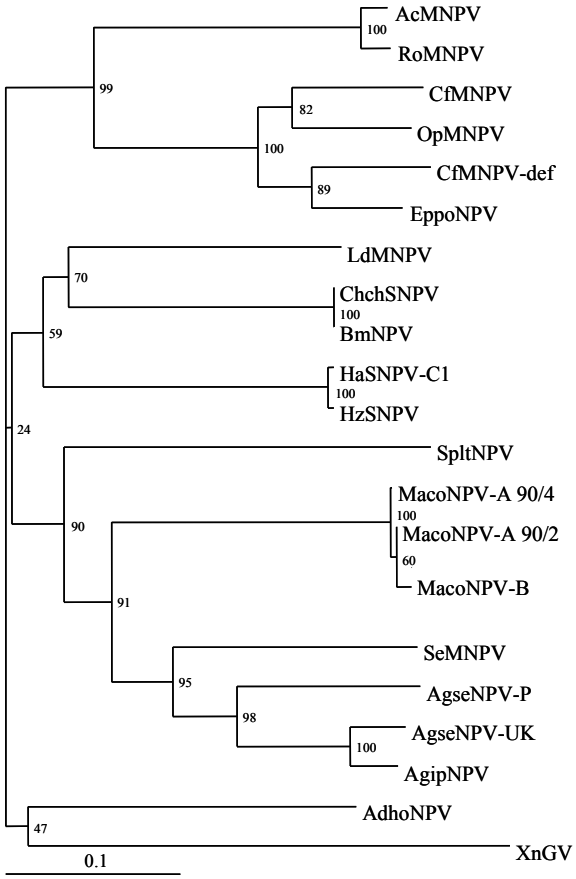
Fig. 4-1. *Hin*dIII digestion profiles of AgseNPV genomic DNA. A) Schematic representation of *Hin*dIII restriction fragments profiles of AgseNPV-P and AgseNPV-UK (after Allaway & Payne, 1983) and AgipNPV (after Boughton *et al.,* 1999); B) *Hin*dIII restriction profile of AgseNPV-P genomic DNA; M, lambda digested with *Bam*I/*Eco*RI/ *Hin*dIII; *, submolar fragments; **, double fragments.

The *polh* sequence of AgseNPV-P showed 83% nucleotide identity with the *polh s*equence of AgseNPV-UK published by El- Salamouny et al. (2003). A similarity of 86% at the nucleotide level was found with *A. ipsilon* NPV. The predicted AgseNPV-P polyhedrin protein is 91% identical and 97% similar to the AgseNPV-UK and 92% identical and 96% similar to *A. ipsilon* (Agip) NPV polyhedrin, respectively. It also has a high amino acid identity (90%) and similarity (96%) with polyhedrin of *Spodoptera exigua* (Se) MNPV (van Strien *et al.,* 1992). The highest values were found for AgseNPV-UK and AgipNPV: 90% nucleotide and 98% amino acids identity. *Lef-8* homology searches revealed the closest relation of AgseNPV-P with AgseNPV-UK and AgipNPV (75 and 77%, nucleotide identity, respectively). The *pif-2* gene was analyzed for AgseNPV-P, AgseNPV-UK, and SeMNPV, since the *pif-2* sequence of AgipNPV is not known. At the nucleotide level 85% identity was found between AgseNPV-P and AgseNPV-UK *pif-2*, at the amino acids level 91%. These comparisons revealed that AgseNPV-P differs relatively equally from the other viruses analyzed, while there is a close relation between AgseNPV-UK and AgipNPV.

Alignments and phylogenetic analyses of all three genes placed AgseNPV-P in group II of the NPVs (Fig. 4-2). Relatively high bootstrap values supported the close relation of AgseNPV spp. with *Spodoptera* spp. nucleopolyhedroviruses namely SeMNPV and *Spodoptera frugiperda* MNPV. Furthermore, phylogenetic analysis confirmed that AgseNPV-UK is more related to AgipNPV than to AgseNPV-P, which may lead to the conclusion that AgseNPV-P is a distinct NPV species, while AgseNPV-UK and AgipNPV may represent variants of the same NPV species. Despite these clear differences, AgseNPV-P and AgseNPV-UK/AgseNPV-F infect a common host, the cutworm, *A. segetum.* This is not the first report about more than one baculovirus infecting a common host. Two distinct nucleopolyhedroviruses were for example isolated from *Orgyia pseudotsugata* (OpMNPV and OpSNPV) (Hughes & Addison, 1970) and *M. configurata* (MacoNPV-A and MacoNPV-B) (Li *et al.,* 2002, 2005). For comparison the amino acid sequence identities/similarities between the *polh, lef-8* and *pif-2* genes of MacoNPV-A and MacoNPV-B, closely related but separate baculovirus species, are 98/100, 97/98, and 95/98 %, respectively. These values are in case of AgseNPV-P and AgseNPV-UK 91/97, 79/88, and 91/96 %, respectively. In many cases genetic variants of a particular virus species have been isolated from a single host species, like from *S. littoralis* (SpliNPV) (Kislev & Edelman, 1982), *H. armigera* and *H. zea* (Chen *et al.,* 2002). Recently, genetic NPV variants were found in spatially separated populations of the winter moth *Operophtera brumata* (Graham *et al.,* 2004).

To the best of our knowledge the English/French isolate of AgseNPV was not found in Poland and the isolate described in our study is the only baculovirus infecting *A. segetum* in this region of Europe. The lack of common restriction fragments and the relatively low sequence similarity between Polish and English/French isolates of AgseNPV suggest that these isolates represent distinct NPV species.





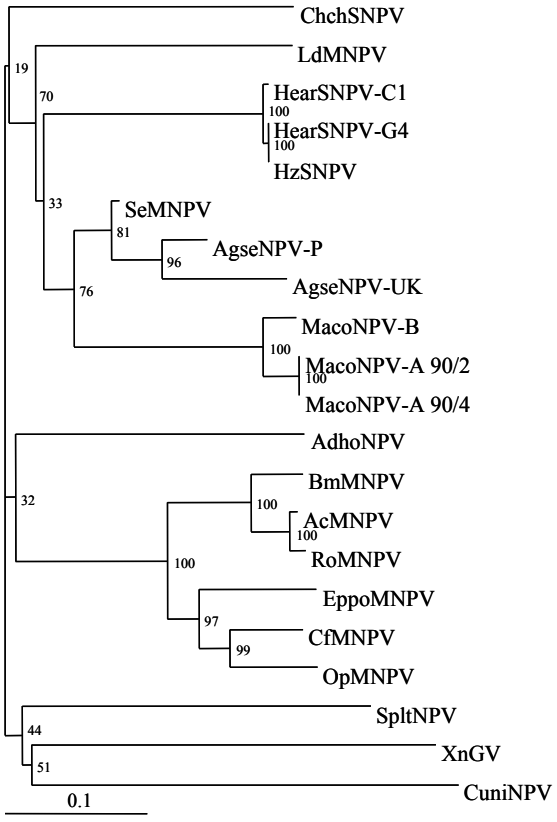


Fig. 4-2. NPV group II phylogenies based on nucleotide sequences. A) *Polh* tree; B) *lef-8*; and C) *pif-2* tree. The maximum parsimony analysis was performed in PAUPbeta10 program (Swofford, 1998); The trees Bootstrap values above 50% are indicated. NPVs outside group II were used to root the trees. GenBank accession numbers are given in brackets.

So, two distinct but related NPV species are found in two geographically separated populations of *A. segetum* and may suggest NPV speciation over geographical distance. Alternatively AgseNPV-P may have developed primarily in a different host accelerating speciation.

Agrotis spp. NPVs and their host species may constitute a useful model for investigating speciation and local adaptation. Baculoviruses are currently named after the hosts from which they were originally isolated. The cases that the same virus species be given different names and that different virus species have been isolated from one host species underscores the need for clearer criteria to designate different baculovirus species and also shows the relevance of detailed biological and molecular information for baculovirus nomenclature.

Acknowledgments

This research was partly supported by a Polish State Committee for Scientific Research, Grant No. 2P06R 73 26 and scholarship from the European Union (Functional Biodiversity and Crop Protection), contract no *HPMT-CT-2000-00199*.

Chapter 5

Genome sequence of an enhancin rich nucleopolyhedrovirus (NPV) from *Agrotis segetum*: collinearity with *Spodoptera exigua* multiple NPV

The genome sequence of a Polish isolate of *Agrotis segetum* nucleopolyhedrovirus (AgseNPV-A) was determined and analyzed. The circular genome is composed of 147,129 bp and has a G+C content of 45.7 mol %. It contains 153 putative, non-overlapping open reading frames (ORFs) encoding predicted proteins of more than 50 aa, together making up 89.8% of the genome. The remaining 10.2% of the DNA constitutes non-coding regions and homologous-repeat regions. One hundred and forty-three AgseNPV-A ORFs are homologues of previously reported baculovirus gene sequences. There are ten unique ORFs and they account for 3% of the genome in total. All 63 lepidopteran baculovirus genes, including the 30 core baculovirus genes, were found in the AgseNPV-A genome. The gene content and gene order of AgseNPV-A are most similar to those of *Spodoptera exigua* (Se) multiple NPV and their shared homologous genes are 100% collinear. Three putative enhancin genes were identified in the AgseNPV-A genome. In phylogenetic analysis, the AgseNPV-A enhancins form a cluster separated from enhancins of the *Mamestra* species NPVs.

This chapter has been published in a slightly modified form as: Jakubowska, A., Peters, S., Ziemnicka, J., Vlak, J.M. & van Oers, M.M. (2005). Genome sequence of an enhancine rich nucleopolyhedrovirus (NPV) from *Agrotis segetum*: collinearity with *Spodoptera exigua* multiple NPV. Journal of General Virology 87:537-551.

Introduction

The turnip moth, *Agrotis segetum* Dennis & Schiffenmüller (Lepidoptera, Noctuidae), is an important pest of many crops in Europe, Asia and Africa. The larvae feed on many vegetable and field crops, including corn, rape, beet, potatoes, cabbage, cereals, tobacco, vine and many others (Ignoffo & Garcia, 1979). *A. segetum* is often part of a cutworm complex, further consisting of *Agrotis ipsilon*, *Agrotis exclamationis*, *Agrotis subterranea*, *Peridroma saucia* and other noctuid species, that destroy plants near the soil surface (Bourner & Cory, 2004). Viruses with biocontrol potential in the field, belonging to the family *Baculoviridae*, have been isolated from various *Agrotis* species (Caballero *et al.,* 1991; Bourner *et al.,* 1992; Boughton *et al.,* 1999). Baculoviruses form a family of large, circular, double-stranded DNA viruses that occur widely in invertebrates, in particular insect species of the orders Lepidoptera, Diptera and Hymenoptera. To date, over 700 baculoviruses have been isolated and several of these have been investigated as bioinsecticides of phytophagous insects (Federici, 1999) and showed potential against field and forest pests all over the world.

The family *Baculoviridae* is divided into four genera: *Alphabaculovirus,* with the lepidoptera-infecting NPVs, *Betabaculovirus*, encompassing the granuloviruses, *Deltabaculovirus* and *Gammabaculovirus* infecting dipteran and hymenopteran hosts, respectively (Jehle *et al.,* 2006). Lepidopteran NPVs show a further division into group I and group II NPVs (Bulach *et al.,* 1999; Herniou *et al.,* 2001). Group I NPVs appear to be much more conserved than those of group II (Hyink *et al.,* 2002; Lange & Jehle, 2003). NPVs are designated single (S) or multiple (M) depending on the number of nucleocapsids packaged in a virion, although this feature has no taxonomic value (Volkman *et al.,* 1995). Twenty-eight completely sequenced baculovirus genomes have been released in GenBank[3] (25 from lepidopteran viruses, two from hymenopteran viruses and one from a dipteran virus), including a GV isolated from *A. segetum* (AgseGV) (GenBank accession no. NC005839). Comparison of these baculovirus genomes revealed 30 core genes, shared by all baculoviruses, and 63 genes common to lepidopteran baculoviruses (Herniou *et al.,* 2003; Lange & Jehle, 2003). Apart from those conserved genes, baculoviruses contain genes shared by a variable number of related virus species or even contain unique genes. Two different NPVs have been isolated from the turnip moth *A segetum*, an English/French and a Polish isolate, both of the multiple nucleocapsid (M) type (Allaway & Payne, 1983). Restriction-enzyme profiling and phylogenetic analysis based on three conserved baculovirus genes – polyhedrin (*polh*), late expression factor 8 (*lef-8*) and *per os* infectivity factor 2 (*pif-2*) – revealed that both isolates are relatively distant and probably represent different virus species (Jakubowska *et al.,* 2005).

[3] As of December 2009, more than 50 baculovirus genomes have been entirely sequenced.

In this paper, the genome sequence of the Polish isolate of *A. segetum* NPV is analysed. To avoid nomenclatural confusion, we propose to name this Polish isolate AgseNPV-A, as it is the first AgseNPV isolate whose genome is sequenced, and hence propose the name AgseNPV-B for the English/French isolate. The Polish isolate was indicated before as AgseNPV-P (Jakubowska *et al.,* 2005). AgseNPV-A is compared in genome sequence and organization with *Spodoptera exigua* (Se) MNPV (IJkel *et al.,* 1999), *Autographa californica* (Ac) MNPV (Ayres *et al.,* 1994), *Mamestra configurata* (Maco) NPV-B (Li *et al.,* 2002) and the recently sequenced GV from *A. segetum* (AgseGV) (GenBank accession no. NC005839). The gene order of AgseNPV-A was found to be highly collinear with that of SeMNPV, despite relatively low amino acid identity values (60% on average).

Material and methods

Virus and insects

AgseNPV-A was isolated in 1975 from *A. segetum* larvae and stored at -20°C. The virus was freshly amplified in the laboratory in second-instar larvae of *A. segetum*. Larvae were infected individually with diet discs contaminated with 10 µl virus suspension at a concentration of 10^5 occlusion bodies (OBs) ml^{-1}.

DNA isolation

Polyhedra were isolated from infected insects. Dead larvae were homogenized by using a glass homogenizer and filtered through four layers of cheesecloth. The filtrate was layered onto a 30% (w/w) sucrose cushion and centrifuged for 15 min at 5300 r.p.m. at 4°C. The pellet containing polyhedra was resuspended and the sucrose purification was repeated two times. The final pellet was washed three times in distilled water and finally resuspended in 1 ml water. Polyhedra were dissolved by incubation for 30 min at 37°C in 0.1 M sodium carbonate (final pH, approx. 11). Large debris was removed by 5 min centrifugation at 1000 r.p.m. and the supernatant was centrifuged for 30 min at 14,000 r.p.m. to pellet the occluded virions. DNA was isolated according to Reed et al. (2003) and dialysed for 24 h at 4°C against 0.1 x TE buffer (10 mM Tris/HCl; 1 mM EDTA; pH 7.5). Quantity and quality of isolated DNA were determined spectrophotometrically and by electrophoresis in 0.7% agarose.

Nucleotide sequence determination

The full genome sequence was determined by shotgun cloning of 10 mg sheared DNA of 1–1.5 kbp. The DNA fragments were cloned into pBluescript II SK(+) (Stratagene) by using Escherichia coli XL2 Blue ultracompetent cells (Stratagene). End-in sequencing was performed on a 3730xL DNA analyzer (Applied Biosystems) and a 3100 Genetic Analyzer

(Applied Biosystems); sequences were assembled with Gap4 from the Staden-Solaris-1-5-3 software package and then checked in detail manually (van Oers *et al.,* 2005). In total, 1 517,059 nt were determined, with a mean redundancy of 9.88.

Sequence analysis

Genes were located with GeneMark software (Borodovsky & McIninch, 1993) and ORF Finder (NCBI). All open reading frames (ORFs) with a minimal size of 150 nt (50 aa), which did not overlap for major parts with other ORFs, were analysed. In addition, the genome was checked in detail for the presence of any ORFs identified for SeMNPV (IJkel *et al.,* 1999) or any other baculovirus in GenBank. Homology searches were performed by using BLAST (Altschul *et al.,* 1990). To easily compare sequence information from different baculovirus genomes, the GECCO program was exploited. GECCO is a gene content comparison tool able to align large numbers of sequences quickly by using the standard NCBI BLAST (van Oers *et al.,* 2005). The percentages of identity of all AgseNPV-A ORFs with their homologues in selected genomes were calculated for complete ORFs by using CLUSTAL-X (Thompson *et al.,* 1997). To detect homologous regions, DOTPLOT analysis (DNAstar) and the EMBOSS program (http://bioweb.pasteur.fr/seqanal/EMBOSS/) were applied under various stringency conditions. Pairwise gene-order analysis was performed by making gene-parity plots as described previously (Hu *et al.,* 1998). In this analysis, both shared and non-shared genes were included.

Phylogenetic analysis

For phylogenetic analysis of enhancing genes, protein sequences were aligned in CLUSTAL-X. Maximum parsimony (MP) analysis was performed by using PAUP* (Swofford, 1998). Bootstrap analyses were performed to evaluate the robustness of the phylogenies using 1000 replicates. Branch lengths were calculated by using the neighbour-joining (NJ) method. The enhancing sequences used for this analysis were found by using the AgseNPV-A enhancin sequences as queries for the BLAST link at NCBI.

Results and discussion

Genome features

The AgseNPV-A genome is a circular, double-stranded DNA molecule containing 147,129 bp, in line with the predicted size of 148 kbp based on restriction-enzyme analysis (Jakubowska *et al.,* 2005). The computationally derived *Hin*dIII restriction map is in agreement with the experimentally constructed map from restriction-enzyme analysis, with the exception of an additional submolar band in the latter, indicating that more than one genetic variant is present in the isolate (Jakubowska *et al.,* 2005). This is confirmed by the

presence of several single-base polymorphisms in the sequence. The G+C content of the AgseNPV-A genome is 45.7 mol%, which is slightly higher than that of most sequenced baculoviruses. Only *Choristoneura fumiferana* (Cf) MNPV, *Orgyia pseudotsugata* (Op) MNPV, *Lymantria dispar* (Ld) MNPV and *Culex nigripalpus* (Cuni) NPV have G+C contents higher than that of AgseNPV-A (Ahrens *et al.,* 1997; Kuzio *et al.,* 1999; Afonso *et al.,* 2001; de Jong *et al.,* 2005).

Gene content and genome organization

Using computational analysis, 419 methionine-initiated ORFs of more than 50 aa were initially identified. The maximal acceptable overlap was set at 20 aa, with the exception of overlapping genes showing significant similarity to ORFs known in other baculoviruses. From those ORFs, 153 ORFs were assigned as AgseNPV-A ORFs, after elimination of ORFs located within larger ORFs and without similarity to baculovirus ORFs (Fig. 5-1, Table 5-1). The ORF density is comparable to that of other NPVs, when calculated as no. ORFs/genome size. According to the adopted convention (Vlak & Smith, 1982), polyhedrin was designated gene number 1 (Agse1) and the adenine of the start codon of the polyhedrin gene was assigned as the first nucleotide of the AgseNPV-A circular genome. Of the 153 AgseNPV-A ORFs, 143 have an assigned function or homologues in other baculoviruses.

Ten AgseNPV-A ORFs have no homologues in baculoviruses and thus are considered unique to AgseNPV-A until homologues in other baculoviruses are found. The total length of those unique ORFs is 4379 nt (3% of the genome). Four of these have no consensus promoter and may be non-functional. Predicted proteins are encoded by 89.8%of the genome; the rest forms non-coding regions and homologous regions (hrs). Five hrs were identified in the AgseNPV-A genome dispersed around the genome (see below); the maximum distance between two hrs is 57 kbp.

All 30 core baculovirus genes and all 63 lepidopteran baculovirus genes (Herniou *et al.,* 2003) were found in the AgseNPV-A genome. There are 21 pairs of overlapping genes along the genome, eight of which have overlaps longer than 20 nt. All of them showed significant similarity to known baculovirus ORFs and thus were assigned as AgseNPV-A ORFs. The maximal overlap of 139 nt exists between *vp1054* and *lef-10* (Agse113/114). Both genes are oriented anticlockwise and both have homologues in SeMNPV. However, a promoter motif was not found upstream of the AgseNPVA *lef-10* gene. In general, the AgseNPV-A genome is densely packed, with minimal intergenic distances. There are three gene clusters packed very tightly: ORFs 66–70, 95–100 and 109–115. Fifty-five per cent of the ORFs are directed clockwise and 45% anticlockwise, with respect to the orientation of transcription of the polyhedrin gene (Vlak & Smith, 1982).

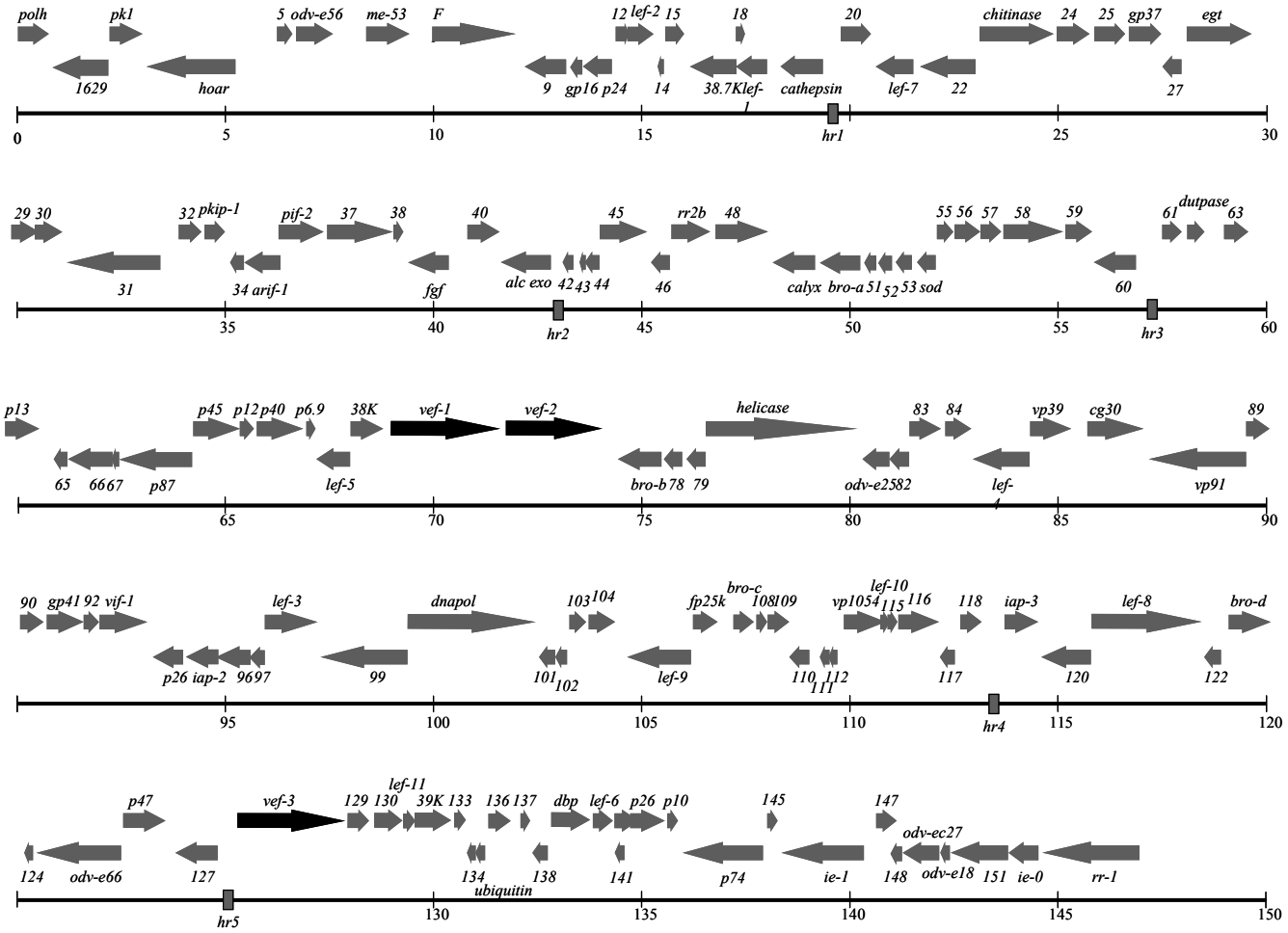


Fig. 5-1. Linear presentation of the circular AgseNPV-A genome with annotated genes. ORFs are numbered 1–153 relative to polyhedrin (ORF1). Arrows indicate the orientation of the predicted genes. Numbers refer to the nucleotide position in kbp relative to the start codon of polyhedrin. Arrows representing enhancin genes are in black.

Table 5-1. Potentially expressed ORFs in the AgseNPV-A in comparison to other baculovirus genomes.

ORF	+/-[1]	Position		Name[2]	aa	E / L[3]	Homologues				% ID / range[4]			
							SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]	SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]
1	+	1	741	*polh*	246	L	Se1	Ac8	Mc1	As1	92	84	89	52
2	-	2179	833	*orf 1629*	448		Se2	Ac9	Mc2	-	44	14	30	-
3	+	2202	2996	*pk1*	264	L	Se3	Ac10	Mc3	As3	67	36	56	24
4	-	5211	3097	*hoar*	704		Se4	-	Mc4	-	33	-	20	-
5	+	6226	6600		124	E,L	-	-	-	-	-	-	-	-
6	+	6687	7796	*odv-e56*	369		Se6	Ac148	Mc6	As15	61	48	58	39
7	+	8037	9086	*me 53*	349	L	Se7	Ac139	Mc7	As131	53	21	46	17
8	+	9959	11971	F protein	670	E,L	Se8	Ac23	Mc8	As25	60	14	40	20
9	-	13181	12174		335	E	-	-	Mc9	-	-	-	44	-
10	-	13568	13278	*gp16*	96	L	Se9	Ac130	Mc10	-	62	31	63	-
11	-	14283	13585	*p24*	232	L	Se10	Ac129	Mc11	As62	63	33	62	18
12	+	14365	14685		106	L	Se11	-	Mc12	-	43	-	36	-
13	+	14645	15277	*lef-2*	210		Se12	Ac6	Mc13	As35	62	37	53	19
14	-	15530	15378		50		-	-	-	-	-	-	-	-
15	+	15551	16012		153	E	-	-	-	-	-	-	-	-
16	-	17251	16148	*38.7K*	367	L	Se13	Ac13	Mc31	As63	58	25	59	10
17	-	17900	17253	*lef-1*	215	L	Se14	Ac14	Mc30	As64	65	33	62	30
18	+	18100	18327		75		Se15	-	Mc29	-	38	-	47	-
19	-	19355	18324	*cath*	343		Se16	Ac127	Mc28	As31	84	51	80	44
hr1							-	-	-	-	-	-	-	-
20	+	19775	20512		245		-	Ac105	Mc27	As132	-	13	22	19
21	-	21541	20615	*lef-7*	308	E	17+18	Ac125	-	-	12	14	-	-
22	-	23022	21673		449	E	22-24	-	-	As39	36	-	-	46
23	+	23142	24890	chitinase	582	L	Se19	Ac126	Mc19	As32	79	62	79	53
24	+	24968	25705		246	E	-	-	-	-	-	-	-	-
25	+	25863	26606		247	E	-	-	Mc16	-	-	-	25	-
26	+	26698	27480	*gp37*	260	L	Se25	Ac64	Mc32	-	72	47	70	-

ORF	+/-[1]	Position		Name[2]	aa	E / L[3]	Homologues				% ID / range[4]			
							SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]	SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]
27	-	28008	27496	*ptp-2*	170		Se26	-	Mc33	-	56	-	44	-
28	+	28088	29659	*egt*	523	L	Se27	Ac15	Mc34	As129	77	44	73	34
29	+	29843	30403		186	E	Se28	-	Mc35	-	47	-	50	-
30	+	30405	31049		214		Se29	Ac17	Mc36	-	44	13	40	-
31	-	33740	31179		853		Se30	-	Mc37	-	45	-	40	-
32	+	33862	34422		186	L	Se31	Ac4	Mc39	As58	32	15	33	12
33	+	34485	34985	*pkip-1*	166	L	Se32	Ac24	Mc40	-	61	19	52	-
34	-	35440	35102		112		Se33	-	Mc41	-	48	-	51	-
35	-	36314	35442	*arif-1*	290		Se34	Ac21	Mc42	-	42	15	35	-
36	+	36205	37407	*pif-2*	400		Se35	Ac22	Mc43	As43	80	56	69	42
37	+	37436	39019	*pif-1*	527		Se36	Ac119	Mc44	As65	56	46	60	30
38	+	39024	39266		80		Se37	Ac120	Mc45	-	53	28	43	-
39	-	40494	39373	*fgf*	373	E,L	Se38	Ac32	Mc46	As113	38	11	34	8
40	+	40802	41575		257		Se 40	-	Mc 47	-	45	-	33	-
41	-	42799	41591	*alc exo*	402	L	Se41	Ac133	Mc48	As115	54	36	47	30
hr2							-	-	-	-	-	-	-	-
42	-	43348	43079		89		-	-	-	-	-	-	-	-
43	-	43641	43483		52	L	-	-	-	-	-	-	-	-
44	-	43978	43622		118	L	Se42	Ac19	Mc49	-	41	15	57	-
45	+	43980	45122		380	L	Se43	Ac18	Mc50	-	64	61	22	-
46	-	45673	45215		152	L	Se44	-	Mc51	-	35	-	40	-
47	+	45706	46647	*rr2b*	313		Se45	-	Mc52	-	77	-	71	-
48	+	46761	48026		421	L	-	-	-	-	-	-	-	-
49	-	49162	48137	*calyx/pep*	341	L	Se46	Ac131	Mc61	-	68	23	63	-
50	-	50244	49270	*bro-a*	324	E,L	-	-	-	-	-	-	-	-
51	-	50629	50336		97	L	Se47	Ac117	Mc62	-	58	28	56	-
52	-	51012	50674		112		-	-	Mc63	-	-	-	48	-
53	-	51488	51090		132	E,L	-	-	-	-	-	-	-	-
54	-	52058	51603	*sod*	151	L	Se48	Ac31	Mc65	As54	80	71	80	53

ORF	+/-[1]	Position		Name[2]	aa	E / L[3]	Homologues				% ID / range[4]			
							SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]	SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]
55	+	52080	52484		134		Se49	-	Mc66	-	15	-	39	-
56	+	52509	53132	*pif-3*	207	L	Se50	Ac115	Mc67	As30	66	44	68	30
57	+	53137	53643		168	L	Se51	-	Mc68	-	39	-	43	-
58	+	53689	55131		480	L	Se52	-	Mc69	-	54	-	34	-
59	+	55162	55812	*ac106*	216	L	Se53	Ac106	Mc70	As46	77	15	77	38
60	-	56940	55852		362	E	Se54	Ac33	Mc71	-	57	13	58	-
hr3							-	-	-	-	-	-	-	-
61	+	57494	57964		156		-	-	-	-	-	-	-	-
62	+	58093	58521	*dutpase*	142	L	Se55	-	Mc73	-	72	-	69	-
63	+	58987	59574		195	E,L	-	-	-	-	-	-	-	-
64	+	59694	60518	*p13*	274	L	Se56	-	Mc75	As42	70	-	67	44
65	-	61188	60859	*hp Se 58*	109	L	Se58	Ac108	Mc78	-	60	24	55	-
66	-	62271	61201	*ac109*	356		Se59	Ac109	Mc79	As48	89	43	77	31
67	-	62434	62255	*ac110*	59		Se60	Ac110	Mc80	As92	74	29	71	20
68	-	64179	62431	*p87*	582	L	Se61	Ac104	Mc81	-	56	13	34	-
69	+	64212	65345	*p45*	377	L	Se62	Ac103	Mc82	As72	79	52	79	33
70	+	65335	65676	*p12*	113	L	Se63	Ac102	Mc83	As73	63	26	47	18
71	+	65739	66848	*p40*	369	L	Se64	Ac101	Mc84	As74	75	40	67	19
72	+	66932	67174	*p6.9*	80	L	Se65	Ac100	Mc85	As75	54	41	64	28
73	-	67989	67171	*lef-5*	272	L	Se66	Ac99	Mc86	As76	77	48	77	36
74	+	67996	68790	*38K*	264	L	Se67	Ac98	Mc87	As77	65	38	63	36
75	+	68965	71598	*vef-1*	877	L	-	-	Mc88	As55	-	-	36	14
76	+	71721	74372	*vef-2*	883	L	-	-	Mc88	As55	-	-	37	15
77	-	75470	74418	*bro-b*	350		-	-	-	-	-	-	-	-
78	-	75969	75526		147		-	-	Mc90	-	-	-	68	-
79	-	76582	76060	*ac 96*	173	L	Se69	Ac96	Mc91	As78	78	48	79	30
80	+	76538	80179	helicase	1213	L	Se70	Ac95	Mc92	As79	73	40	78	25
81	-	80943	80293	*odv-e25*	216	L	Se71	Ac94	Mc93	As81	84	43	83	46
82	-	81416	80940	*ac 93*	158	L	Se72	Ac93	Mc94	As82	77	49	86	32

ORF	+/-[1]	Position		Name[2]	aa	E / L[3]	Homologues				% ID / range[4]			
							SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]	SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]
83	+	81424	82182	*ac 92*	252	L	Se73	Ac92	Mc95	As83	87	53	82	32
84	+	82279	82902		207		-	-	Mc96	-	-	-	28	-
85	-	84319	82946	*lef-4*	457		Se74	Ac90	Mc97	As85	64	46	66	29
86	+	84318	85310	*vp39*	330	L	Se75	Ac89	Mc98	As85	77	40	54	10
87	+	85692	87053	*cg30*	453		Se76	Ac88	Mc99	-	17	10	14	-
88	-	89514	87178	*vp91*	778	L	Se77	Ac83	Mc100	As91	58	37	56	16
89	+	89519	90082	*ac 82*	187	L	Se78	Ac82	Mc101	As93	62	25	60	19
90	+	90054	90629	*ac 81*	191	L	Se79	Ac81	Mc102	As94	58	42	52	44
91	+	90688	91581	*gp41*	297	L	Se80	Ac80	Mc103	As95	81	40	71	27
92	+	91581	91955	*ac 78*	124	L	Se81	Ac78	Mc104	As96	58	24	41	16
93	+	91957	93105	*vlf-1*	382	L	Se82	Ac77	Mc105	As97	88	63	90	31
94	-	93973	93239	*p26*	244	E	Se87	Ac136	Mc108	-	62	19	65	-
95	-	94807	94028	*iap-2*	259	L	Se88	Ac71	Mc109	-	50	30	55	-
96	-	95597	94755	*hp Se 89*	280	L	Se89	Ac69	Mc110	-	62	40	60	-
97	-	95933	95566	*ac 68*	122		Se90	Ac68	Mc111	As104	69	31	74	28
98	+	95933	97201	*lef-3*	422	L	Se91	Ac67	Mc112	As103	52	21	47	11
99	-	99363	97276	*ac 66*	695	L	Se92	Ac66	Mc113	As102	45	12	26	10
100	+	99362	102439	*dpol*	1025		Se93	Ac65	Mc114	As101	74	42	72	29
101	-	102909	102520	*ac 75*	129	L	Se94	Ac75	Mc115	As100	79	21	84	15
102	-	103173	102916	*ac 76*	85	L	Se95	Ac76	Mc116	As99	85	40	87	32
103	+	103250	103651		133	L	Se96	Ac150	-	-	25	16	-	-
104	+	103691	104355		221		-	-	Mc120	As89	-	-	46	7
105	-	106171	104627	*lef-9*	514		Se97	Ac62	Mc123	As107	85	61	82	50
106	+	106225	106812	*fp25K*	195	L	Se98	Ac61	Mc124	As108	90	55	88	27
107	+	107188	107679	*bro-c*	163		-	-	-	-	-	-	-	-
108	+	107738	107998		86	E,L	Se100	Ac60	Mc127	As84	77	37	59	26
109	+	108012	108538		174	L	Se101	Ac59	Mc128	-	48	16	41	-
110	-	109013	108531		160		Se102	Ac57	Mc129	-	61	37	57	-
111	-	109500	109267		77	L	Se103	Ac56	Mc130	-	39	20	41	-
112	-	109687	109478		69		Se104	Ac55	Mc131	-	66	30	70	-

ORF	+/-[1]	Position		Name[2]	aa	E / L[3]	Homologues				% ID / range[4]			
							SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]	SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]
113	-	110856	109843	*vp1054*	337	L	Se105	Ac54	Mc132	As127	72	39	67	30
114	-	110717	110947	*lef-10*	76		Se106	Ac53a	Mc133	As126	70	42	57	27
115	+	110892	111134		80	L	-	-	Mc134	-	-	-	45	-
116	+	111150	112130		326	L	Se107	-	Mc135	-	49	-	30	-
117	-	112517	112152	*ac 53*	121	E,L	Se108	Ac53	Mc136	As122	59	43	51	18
118	+	112642	113151		169	E	Se109	Ac52	Mc137	-	26	13	46	-
hr4							-	-	-	-	-	-	-	-
119	+	113702	114517	*iap-3*	271		Se110		Mc138	As53	42	-	40	30
120	-	115789	114596		397	L	Se111	Ac51	Mc139	-	42	9	32	-
121	+	115795	118443	*lef-8*	882		Se112	Ac50	Mc140	As118	77	61	78	48
122	-	118907	118506		133	E	-	-	Mc141	-	-	-	23	-
123	+	119095	120105	*bro-d*	336	E	-	-	-	-	-	-	-	-
124	-	120360	120154		68	L	Se113	Ac43	Mc142	-	47	30	50	-
125	-	122479	120443	*odv-e66*	678	L	Se114	Ac46	Mc143	As33	51	22	47	20
126	+	122528	123733	*p47*	401		Se115	Ac40	Mc144	As60	77	50	75	40
127	-	124794	123778		338	E	-	-	Mc145	-	-	-	31	-
hr5							-	-	-	-	-	-	-	-
128	+	125278	127866	*vef-3*	862	L	-	-	Mc88	As55	-	-	35	15
129	+	127923	128444		173		Se117	-	Mc146	As60	35	-	27	6
130	+	128559	129236	*ac 38*	225	E,L	Se118	Ac38	Mc147	As61	69	51	82	33
131	+	129168	129544	*lef-11*	125		Se119	Ac37	Mc148	As52	52	30	60	26
132	+	129534	130412	*39K/pp31*	292	E,L	Se120	Ac36	Mc149	As51	50	27	45	11
133	+	130485	130769		94		Se121	-	-	-	28	-	-	-
134	-	131004	130795		69		Se122	-	Mc150	-	42	-	32	-
135	-	131234	130998	ubiquitin	78	L	Se123	Ac35	Mc151	As47	91	73	74	59
136	+	131300	131836		178	L	Se124	Ac34	Mc152	-	70	27	60	-
137	+	132071	132316		81		-	-	-	-	-	-	-	-
138	-	132733	132362		123	L	Se125	Ac26	Mc153	-	51	25	56	-
139	+	132816	133754	*dbp*	312	L	Se126	Ac25	Mc154	As69	58	23	59	12
140	+	133790	134295	*lef-6*	168	L	Se127	Ac28	Mc155	As68	40	23	47	6

ORF	+/-[1]	Position		Name[2]	aa	E / L[3]	Homologues				% ID / range[4]			
							SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]	SeMNPV	AcMNPV	MacoNPV[5]	AgseGV[6]
141	-	134578	134345	*ac 29*	77	E,L	Se128	Ac29	Mc156	As16	42	32	75	16
142	+	134714	135541	*p26*	275	E,L	Se129	Ac136	Mc157	-	58	30	62	-
143	+	135601	135864	*p10*	87	L	Se130	Ac137	Mc158	As114	72	20	60	19
144	-	137902	135968	*p74*	644	E,L	Se131	Ac138	Mc159	As56	72	53	64	38
145	+	138006	138257		83	E,L	-	-	Mc160	-	-	-	50	-
146	-	140327	138342	*ie1*	661	L	Se132	Ac147	Mc161	As8	46	21	43	8
147	+	140336	140911	*ac 146*	191	L	Se133	Ac146	Mc162	As9	54	31	63	19
148	-	141239	140961	*ac 145*	92	L	Se134	Ac145	Mc163	As10	81	33	70	36
149	-	142103	141264	*odv-ec27*	279	L	Se135	Ac144	Mc164	As87	87	53	90	25
150	-	142399	142166	*odv-e18*	77	L	Se136	Ac143	Mc165	As11	67	35	62	36
151	-	143790	142408	*ac 142*	460	L	Se137	Ac142	Mc166	As12	88	48	77	33
152	-	144524	143805	*ie-0*	239	L	Se138	Ac141	Mc167	-	64	24	61	-
153	-	146949	144610	*rr-1*	779	E	Se139	-	Mc168	As37	58	-	60	16

[1]ORF directed clockwise (+) and anticlockwise (-),with respect to the orientation of transcription of the polyhedrin gene.
[2]Genes present in all lepidopteran NPVs and GVs are underlined.
[3]E-early promoter, L-late promoter.
[4]Percentage identity values were calculated for complete ORFs by using CLUSTAL-X.
[5]MacoNPV isolate 96B (Li *et al.,* 2002a).
[6]AgseGV (GenBank accession no. NC005839).

Table 5-2. Characteristics of some baculovirus genomes.

Characteristics	AgseNPV-A	SeMNPV	MacoNPV-B	AcMNPV	AgseGV
Size (bp)	**147,544**	135,611	158,482	133,894	131,680
G+C content (%)	**46**	44	40	41	37
ORFs total	**153**	139	168	156	132
Number of hrs	**5**	6	4	8	-
Mean % aa ID with *AgseNPV-A*	-	60.0	55.9	33.4	27.0
Number of homologues in *AgseNPV-A*	-	127	134	103	81
References	**This paper**	IJkel *et al.* 1999	Li *et al.,* 2002	Ayres *et al.,* 1994	Ai *et al.,* AY522332

The AgseNPV-A genome content and organization were compared with those of four other baculoviruses: SeMNPV, AcMNPV, MacoNPV-B and AgseGV (Table 5-2). AgseNPV-A shares 127 ORFs with SeMNPV, 134 with MacoNPV-B, 103 with AcMNPV and 81 with AgseGV, with mean amino acid identities of 60.0, 55.9, 34.2 and 26.9%, respectively. The highest mean amino acid identity was found between AgseNPV-A and SeMNPV, but the largest number of ORFs is shared with MacoNPV-B (134), underscoring a close relationship with MacoNPV-B as well. This is further evidenced by phylogenetic (Jakubowska *et al.,* 2005) and gene parity plot (Fig. 5-2) analyses. Seventy-eight ORFs show the highest percentage of identity with SeMNPV, 53 with MacoNPV-B, one with AcMNPV (Agse21) and one with AgseGV (Agse22).

The most-conserved genes between AgseNPV-A, SeMNPV, AcMNPV and MacoNPV-B are polyhedrin (*polh*), superoxide dismutase (*sod*), ubiquitin, chitinase, *vfl-1*, *lef-8*, *lef-9*, *ac92*, *fp25K* and *ac38*. One hundred AgseNPV-A ORFs have homologues in all three NPVs with which they were compared. The presence of an *F* gene (Agse8) and the absence of *gp64* classify AgseNPV-A as a group II NPV and this is in agreement with previous phylogenetic analyses based on *polh*, *lef-8* and *pif-2* sequences (Jakubowska *et al.,* 2005). The identity of the 81 genes shared by AsgeNPV-A and AgseGV, despite being isolated from the same host (*A. segetum*), is very low. None of the 19 shared genes that do not belong to the 63 common lepidopteran baculovirus genes (Herniou *et al.,* 2003; McCarthy & Theilmann, 2008) are exclusively present in AgseNPV and AgseGV.

To examine the genome organization, the order of homologous ORFs of AgseNPV-A, SeMNPV, MacoNPV-B and AcMNPV was compared by using gene-parity plot analysis as described previously (Hu *et al.,* 1998) (Fig. 5-2). Genes of AcMNPV were renumbered manually, starting with the *polh* gene as number 1. The gene arrangement of AgseNPV-A was completely collinear with that of SeMNPV. A high degree of collinearity was also observed with MacoNPV-B, with exception of a short gene cluster including *38.7K*, *lef-1* and *v-cath.*.

When compared with AcMNPV, a major part of the AgseNPV-A genome (around 70,000–140,000 bp) is inversely oriented relative to the orientation of the polyhedrin gene, but the gene order in this region is similar in both viruses. The other parts of the genome differ considerably. Parity analysis of AgseNPV-A and AgseGV ORFs only provided a scattered, noninformative distribution (not shown).

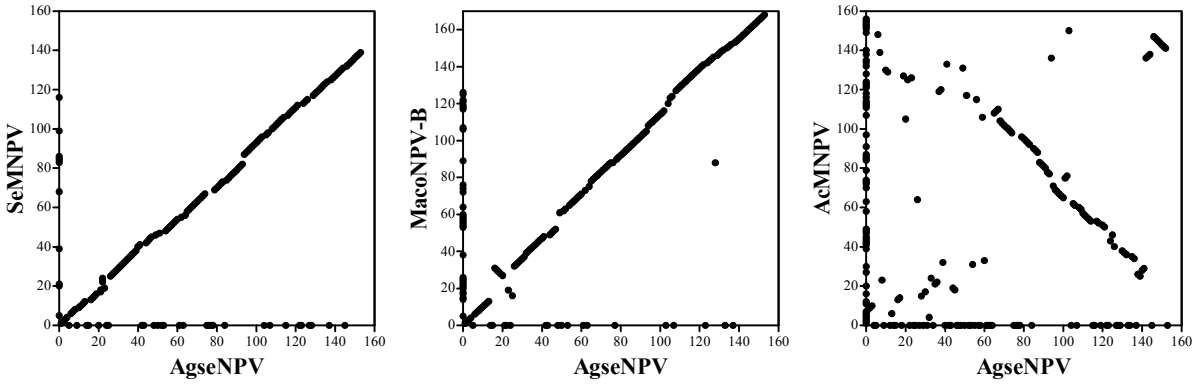


Fig. 5-2. Pairwise comparison of gene content and position of AgseNPV-A with SeMNPV, AcMNPV and MacoNPV-B using gene-parity plot analysis. Genes present in only one of the two viruses in the pairwise comparison appear on the x or y axis.

Comparison of AgseNPV-A and SeMNPV ORFs

Having observed the high level of genome collinearity of AgseNPV-A and SeMNPV, we compared the gene content of both NPVs. Among the 127 homologues between AgseNPV-A and SeMNPV, the most conserved genes are *polh*, with amino acid identity of 92 %, and *sod*, *ubiquitin, chiA, vfl-1, lef-8* and *lef-9*, with >60%amino acid identity. Two putative SeMNPV ORFs, Se17 and Se18, constitute one ORF in AgseNPV-A (Agse21), which was assigned as *lef-7* according to homology with Ac125. The best BLAST match for Agse21 was found with *Xestia c-nigrum* (Xecn) GV ORF129 (38% identity) (Hayakawa *et al.,* 1999). Agse22 was homologous to three SeMNPV ORFs (Se22, Se23 and Se24) and assigned as a single ORF in HearNPV (Chen *et al.,* 2002). The best BLAST match for Agse22 was found with AgseGV ORF39 (46% identity), but its function is unknown. There are 12 ORFs present in SeMNPV that are absent in AgseNPV-A: Se5, Se20, Se21, Se39, Se57, Se68, Se83, Se84, Se85, Se86, Se99 and Se116. All except Se99, which was assigned as *p94*, are genes with unknown functions (IJkel *et al.,* 1999). The *p94* gene is not essential for virus replication in cell culture, but may be involved in inhibition of apoptosis (Friesen & Miller, 1987; Clem & Miller, 1994). Having seen the different location of *p94* in AcMNPV compared with AgseNPV, MacoNPV-A and B and SeMNPV, it may have been acquired by the ancestor of these viruses in an independent insertion and from a source different from the *p94* of AcMNPV (as

suggested for SeMNPV p94; IJkel *et al.,* 2001). No homologue was found in AgseGV. Between Agse64 and Agse65, a 182 nt fragment was detected with nucleotide similarity to *odv-e66*. This gene encodes an occlusion-derived virion (ODV) protein and is present in SeMNPV in two copies, Se57 and Se114, which are both >2 kbp long. The identity between the two SeMNPV ORFs is only 32% (IJkel *et al.,* 2001). An intact *odv-e66* gene appears to be present in the AgseNPV-A genome in another position (Agse125) and has a length of 2036 nt, which is comparable in size with the complete Se114 ORF (2057 nt). In AgseNPV-A, the odv-e66 gene is located next to the p47 gene, as is the case in SeMNPV. The identity between Agse125 and Se114 is 52%, which is higher than that between the two SeMNPV *odv-e66* genes. It is probable that Se57 was acquired independently of Se114 from a source related more closely to LdMNPV (Kuzio *et al.,* 1999) or Leucania separata (Ls) NPV (Wang *et al.,* 1995). In AgseNPV-A, an Se57 homologue may have been lost during evolution, as there is a small part of this gene left in the AgseNPV-A genome between Agse64 and Agse65. There are also two copies of *odv-e66* in AcMNPV and MacoNPV-B. All sequenced GVs, except for *Adoxophyes orana* (Ador) GV (Wormleaton *et al.,* 2003), contain a single copy of an *odv-e66* gene. With an increasing number of complete baculovirus genome sequences, the number of unique ORFs decreases. Twenty unique ORFs were assigned to SeMNPV (IJkel *et al.,* 1999). A homologue of Se121 (Agse133) is now identified in the AgseNPV-A genome, thus reducing the number of unique SeMNPV ORFs to six. The function of Agse133 is so far unknown. Similarly, a homologue of *Trichoplusia ni* SNPV ORF62 (Willis *et al.,* 2005) (Tn62) was identified in the AgseNPV-A genome. This ORF is so far present exclusively in TnSNPV and AgseNPV-A.

Unique genes in AgseNPV-A

There are ten unique ORFs in AgseNPV-A (Agse5, Agse14, Agse15, Agse42, Agse43, Agse48, Agse53, Agse61, Agse63 and Agse137) and they account for 3% of the genome in total. Their length ranges from 50 to 421 aa. For six unique ORFs, baculovirus early or late promoter motifs were found upstream of the ATG codons, as well as putative polyadenylation signals, indicating that these may represent expressed genes. Five of these (Agse5, Agse15, Agse48, Agse53 and Agse63) are larger than 100 aa. Agse5 exhibits homology to an *Arabidopsis thaliana* helicase domain-containing protein, with a BLAST e value of 0.049.

Bro genes

A common feature of baculovirus genomes is the presence of repeated ORFs, named bro (baculovirus repeated ORFs) genes by Kuzio et al. (1999). The highest number of *bro* genes was identified in LdMNPV (Kuzio *et al.,* 1999), which contains 16 ORFs related to the AcMNPV *bro* gene Ac2. The role of the *bro* gene family has not yet been defined. The bro genes were subdivided into four groups, depending on the percentage of amino acid identity in both termini and in the central region, as well as their length (Kuzio *et al.,* 1999). We have

identified four bro genes dispersed along the genome of AgseNPV-A (Agse50, Agse77, Agse107 and Agse123) and named them *bro-a* to *bro-d*, according to the order of appearance on the linearized genome. Bro-a shows the highest identity at the amino acid level (53%) to bro-c of *Bombyx mori* NPV, bro-b to bro-d of MacoNPV-B (63 %), bro-c to bro-h of MacoNPV-A (51 %) and bro-d to Ac2 (58 %). The size of each *bro* gene corresponds well to that of their homologues in other baculoviruses. *bro-a*, *bro-b* and *bro-d* belong to group I *bro* genes, whilst *bro-c* shows the highest similarity to group II *bro* genes according to the classification of Kuzio et al. (1999). AgseNPV-A *bro-b* is located directly downstream of the second enhancin gene (Agse76), like bro-d in the MacoNPV-B genome. We have not found any relatedness of the location of *bro* genes to that of hrs, which was suggested for the LdMNPV genome (Kuzio *et al.,* 1999). Alignment of all AgseNPV-A *bro* genes revealed similarities ranging from 7% (*bro-b/bro-d* against *bro-c*) to 53% between *bro-b* and *bro-d* (analysis not shown).

Hrs

Hrs were first identified in AcMNPV (Cochran & Faulkner, 1983; Pearson *et al.,* 1992). *Hrs* are cis-acting putative origins of DNA replication (ori) (Ahrens & Rohrmann, 1995; Kool *et al.,* 1995) and may also act as enhancers of transcription (Guarino & Summers, 1986; Guarino *et al.,* 1986). AcMNPV hrs have two to eight repeats of a 72 bp long sequence, with an internal imperfect palindrome with an *EcoR*I restriction site in the centre. In five of the 28 baculoviruses sequenced until now (*Chrysodeixis chalcites* (Chch) NPV (van Oers *et al.,* 2005), TnSNPV, AdorNPV, AgseGV and *Cydia pomonella* (Cp) GV (Luque *et al.,* 2001)), canonical hrs were not identified. The remaining fully sequenced baculoviruses contain between three (*Cryptophlebia leucotreta* (Crle) GV) (Lange & Jehle, 2003) and 17 (*Spodoptera litura* (Splt) NPV) (Pang *et al.,* 2001) hrs. Five hrs have been identified in the AgseNPV-A genome (Fig. 5-3). They are dispersed along the genome in intergenic regions, around map positions 19.5 (hr1), 42.8 (hr2), 57.0 (hr3), 113.2 (hr4) and 124.8 (hr5) (see Fig. 5-1, Table 5-1). The conserved repeat size is around 40 nt. All AgseNPV-A hrs contain from one to four imperfect palindromic repeats. Thirteen nucleotides are absolutely conserved in all 16 repeats identified. The striking feature of AgseNPV-A hrs is a very high consensus sequence similarity to SeMNPV hrs. Two motifs also characteristic for SeMNPV hrs, TTTGCTTT and GAAAGCAAAC, are present in almost all AgseNPV-A hr repeats. A notable feature of the AgseNPV-A hrs also is a low G+C content of around 30 mol%, as in SeMNPV.

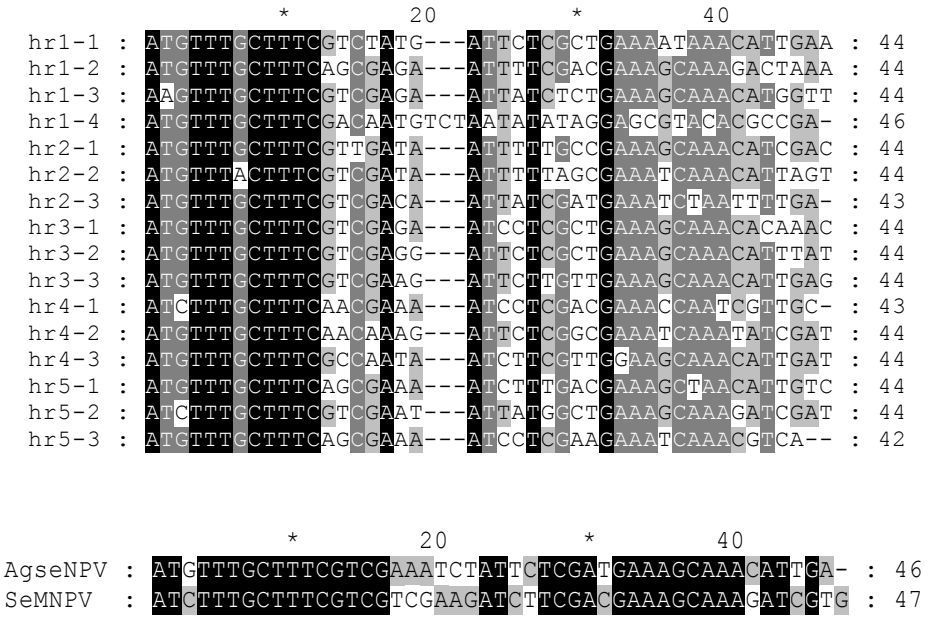


Fig. 5-3. AgseNPV-A homologous regions in comparison with SeMNPV. The nucleotide sequences were aligned in CLUSTAL-X and displayed in GeneDoc. Black shading indicates 100% identity, dark-grey shading indicates 80–100% identity and light-grey shading indicates 60–80% identity. The last two lines show the AgseNPV-A and SeMNPV hr consensus sequence.

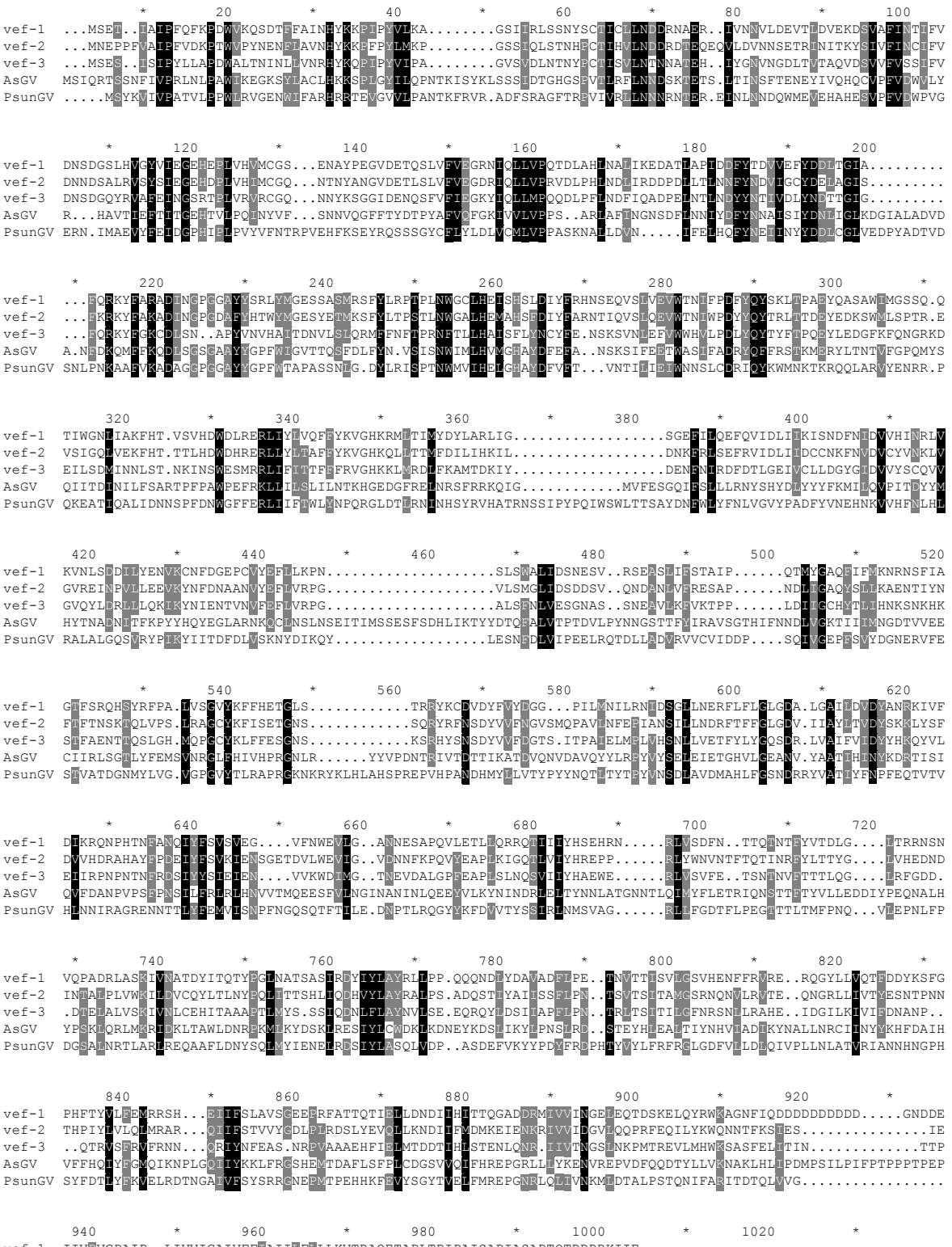
Enhancin genes

A most significant feature of AgseNPV-A, in particular relative to SeMNPV, is the presence of three enhancin genes (vef) in AgseNPV-A. Enhancins, or synergistic or viral enhancing factors (VEF), have been found to dramatically increase the infectivity of baculoviruses in non-natural lepidopteran hosts (Derksen & Granados, 1988). They function by enzymic hydrolysis of the peritrophic membrane, the barrier for pathogens in the insect midgut, or by increasing the fusion efficiency with midgut cells through interaction between the viral envelope and the cell plasma membrane (Wang *et al.,* 1994; Bischoff & Slavicek, 1997). Enhancins are metalloproteases, which degrade mucins, the major proteins of the peritrophic membrane (Lepore *et al.,* 1996; Wang *et al.,* 1997). The first enhancin described originated from *Pseudaletia unipuncta* (Psun) GV and increased the infectivity of PsunNPV in NPV/GV mixed infections (Tanada & Hukuhara, 1971; Yamamoto & Tanada, 1980; Zhu *et al.,* 1989). Enhancin protein constitutes up to 5% of the total protein content in the granules of GVs and is localized in the granule matrix (Tanada, 1985). Recently, it was shown that LdMNPV enhancins are present in ODV envelopes in association with nucleocapsids (Slavicek & Popham, 2005). To date, enhancins have been described in many GVs, including PsunGV, T. ni (Tni) GV, *Helicoverpa armigera* (Hear) GV (Roelvink *et al.,* 1995), AgseGV (GenBank accession no. NC005839), *C. fumiferana* (Cf) GV (GenBank accession no. AF319939) and

XecnGV (Hayakawa *et al.,* 1999), and in four group II NPVs, CfMNPV (de Jong *et al.,* 2005), LdMNPV (Bischoff & Slavicek, 1997), MacoNPV-A isolates 90/2 and 90/4 (Li *et al.,* 2002a, 2005) and MacoNPVB (Li *et al.,* 2002b). So far, enhancin has not been found in any group I NPV. Most of the group II NPVs and GVs carry a single copy of an enhancin gene. LdMNPV has two enhancing copies, whilst in the XecnGV genome, four enhancins were found. AgseNPV-A encodes three enhancin genes, *vef-1*, *vef-2* and *vef-3* (Fig. 5-4), as Agse75, Agse76 and Agse128. For all three AgseNPV-A *vefs*, potential baculovirus consensus late promoter motifs were found, suggesting expression in the late stage of infection. This is compatible with their association with ODVs (Slavicek & Popham, 2005). The first two enhancin genes, *vef-1* and *vef-2*, are located in tandem downstream of the *38K* gene (Agse74). This location corresponds to the position of Se68 in the SeMNPV genome (IJkel *et al.,* 1999). AgseNPV-A lacks a Se68 homologue (function unknown) at this position. Compared with SeMNPV, *vef-3* (Agse128) is in place of Se116, also an ORF with unknown function. All three AgseNPV-A *vef* genes contain large ORFs of 2633, 2652 and 2588 nt, respectively. These sizes are comparable to those of other baculovirus enhancin genes, which range from 2352 nt in LdMNPV to 3015 nt in AgseGV. The identity between the predicted AgseNPV-A VEF proteins ranges from 30 to 40% and the similarity oscillates around 60% (Fig. 5-4A). Overall, AgseNPV-A enhancins have only about 20% amino acid identity with other baculovirus enhancins. Identities of up to 40% were only found with the MacoNPV species enhancins.

Proteins in the metalloprotease family are characterized by an HEXXH motif (Bischoff & Slavicek, 1997). AgseNPV-A vef-1 and vef-2 encode this conserved zinc-binding domain as HEISH and HEMAH, respectively. AgseNPV-A vef-3 encodes an HAISF motif at a comparable position, which does not meet the requirement of an HEXXH motif. A similar case was described for two XecnGV enhancin genes (ORF150 and ORF166), with HQIGH and QKIGD motifs aligning with the HEXXH motif of other enhancins (Hayakawa *et al.,* 1999). It is not known whether the aberrant XecnGV enhancin is active. Only a part of known metalloproteases contains this conserved motif, which can be even better defined as abXHEbbHbc, where ‘a’ is most often valine or threonine, ‘b’ an uncharged residue and ‘c’ a hydrophobic residue (Rawlings & Barrett, 1995). Phylogenetic analysis of enhancins was performed (Fig. 5-4B) to address the question as to whether the origin of the AgseNPV-A enhancing genes could be found in the granulovirus AgseGV, a virus with the same host. In addition to previously presented phylogenies (Popham *et al.,* 2001; Li *et al.,* 2003), we also included in our analysis the CfMNPV, MacoNPV-A 90/4, MacoNPV-B and AgseGV enhancins, as well as several bacterial enhancin sequences from *Bacillus* spp. and *Yersinia* spp.

A



B

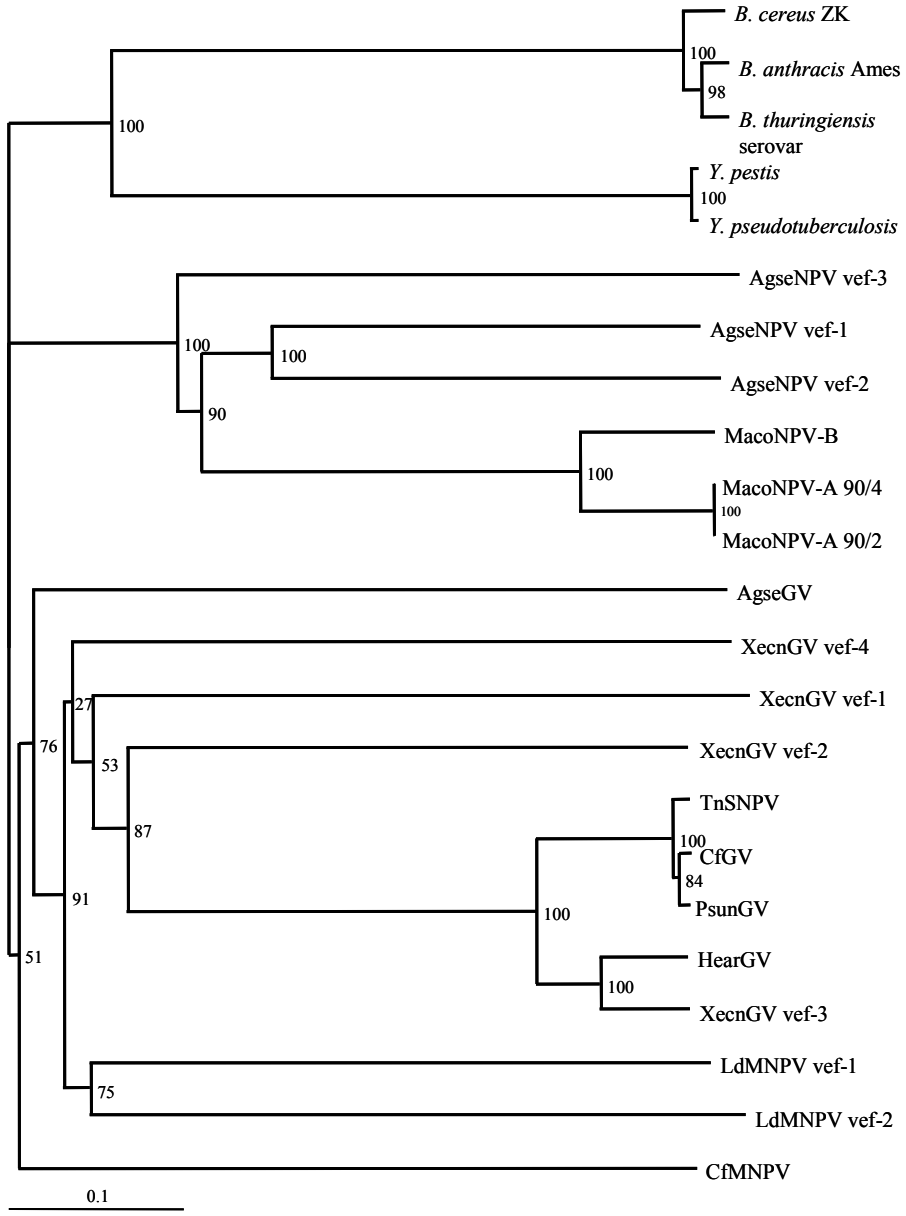


Fig. 5-4. Alignment and phylogenies of enhancin genes. A) Alignment of enhancin sequences of AgseNPV-A (vef1–vef3), AgseGV and PsunGV. The amino acid sequences were aligned in CLUSTAL-X and displayed in GeneDoc. Black shading indicates 100% identity, dark-grey shading indicates 80–100% identity and light-grey shading indicates 60–80% identity. Conserved motif is underlined; B) Phylogenies of enhancin amino acid sequences obtained by MP analysis. Branch lengths were determined by NJ. Numbers indicate MP bootstrap scores using 1000 replicates. Bacterial enhancin sequences were used as an outgroup. GenBank accession numbers: AgseGV, NC_005839; AgseNPV-A, DQ123841; Bacillus anthracis Ames, AE017034; Bacillus cereus ZK, NC_006274; Bacillus thuringiensis serovar konkukian str. 97-27, AE017355; ChfuGV, AF319939; CfMNPV, AF512031; HearGV, D28558; LdMNPV, AF081810; MacoNPV-A 90/2, AF467808; MacoNPV-A 90/4, AF539999; MacoNPV-B, AY126275; PsunGV, D14871; TnSNPV, D12617; XecnGV, AF162221; Yersinia pestis biovar Medievalis, AE017128; Yersinia pseudotuberculosis IP32953, BX936398.

This resulted in a slightly different clustering of baculovirus enhancin genes, but the previously observed tendency that enhancins of similar size fall in the same clade (Li *et al.,* 2003) is supported by our analysis. Also in this case, AgseNPV and MacoNPVs are closely related. We obtained two baculovirus enhancin clusters, one consisting of MacoNPVs and AgseNPV-A and a second one including all GV, LdMNPV and CfMNPV enhancins. In the second cluster, AgseGV groups with CfMNPV and LdMNPV enhancins and is apart from other GV enhancins. The bacterial enhancins are grouped together and are separated from the baculovirus enhancins. The analysis is supported by relatively high (mostly >80 %) bootstrap values for all branches. Phylogenetic analysis also showed that CfMNPV and ChfuGV, as well as AgseNPV-A and AgseGV, enhancins are not closely related, rejecting thehypothesis, at least for these viruses, that baculoviruses infecting a common host have gained enhancin genes from each other.

Conclusion

In conclusion, the genome of AgseNPV-A was found to be highly collinear with that of SeMNPV in organization. So far, ten ORFs were found to be unique to AgseNPV-A and one ORF, Agse133, is so far only shared with SeMNPV (Se121). The most prominent difference between AgseNPV-A and SeMNPV genomes is the presence of three enhancing gene copies (*vef*) in AgseNPV-A. Sequence information of the AgseNPV genome adds to the knowledge of baculovirus genomes and, in comparison to SeMNPV and AgseGV, may lead to further insight into baculovirus–host interactions.

Acknowledgments

This research was supported by the Polish State Committee for Scientific Research, grant no. 2P06R 073 26, and a scholarship from the European Union (Functional Biodiversity and Crop Protection), contract no. HPMT-CT-2000-00199.

Chapter 6

Host range expansion of *Spodoptera exigua* nucleopolyhedrovirus to *Agrotis segetum* larvae when the midgut is bypassed

Given the high similarity in genome content and organization between *Spodoptera exigua* nucleopolyhedrovirus (SeMNPV) and *Agrotis segetum* (Agse) NPV, as well as the high percentages of similarity found between their thirty core genes, the specificity of these *NPVs* was analyzed for the respective insect host *S. exigua* and *A. segetum*. The LD_{50} for AgseNPV in L2 *A. segetum* larvae was 83 OBs/larva and the LT_{50} was 8.1 days. AgseNPV was orally infectious for *S. exigua*, but the LD_{50} was 10,000-fold higher than for SeMNPV. The SeMNPV virus was not infectious for *A. segetum* larvae when administered orally, but an infection was established by injection into the hemocoel. Bypassing midgut entry by intrahemocoelic inoculations suggested that the midgut is the major barrier in *A. segetum* larvae for infection by SeMNPV. Delayed-early genes of SeMNPV are expressed in the midgut of *A. segetum* larvae after oral infections, indicating that the virus is able to enter midgut epithelial cells and that it proceeds through the first phases of the infection process. The possible mechanisms of *A. segetum* resistance to SeMNPV in *per os* infections are discussed.

This chapter has been published in a slightly modified form as: Jakubowska, A., Lynn, D.E., Herrero, S., Vlak, J.M. & van Oers, M.M. (2010). Host range expansion of *Spodoptera exigua* nucleopolyhedrovirus to *Agrotis segetum* larvae when the midgut is bypassed. Journal of General Virology 91:898-906.

Introduction

Baculoviruses are large double-stranded DNA viruses that infect invertebrates, primarily insects. They are a promising alternative to chemical pesticides for control of insect pests because they are able to kill insect larvae within a few days. Baculoviruses are safe since they are restricted to arthropods, and are non-pathogenic to vertebrates or plants (Burges *et al.,* 1980). Some baculoviruses, including the prototype *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Mamestra brassicae* MNPV, have broad host ranges and can cause mortality in larvae of an array of insect species belonging to different families. Other baculoviruses have a host range restricted to a few or even one insect species, such as *Spodoptera exigua* MNPV (SeMNPV) (Federici, 1997; Goulson, 2003). High host specificity is advantageous from a safety perspective, but can be problematic for commercialization of baculoviruses as bioinsecticides. Production of a baculovirus with a wide host range is economically more attractive than production of a very specific baculovirus able to control only one or a few closely related insect species.

Virus host range is determined by the ability of a virus to enter cells, replicate and produce infectious progeny in particular species. Baculovirus infection initiates by oral ingestion of occlusion bodies (OBs) by an insect larvae. In the alkaline environment of the insect midgut, OBs dissolve and release occlusion derived virions (ODVs), which are responsible for initiating a primary infection (Granados & Lawler, 1981). The ODV envelope binds to and fuses with the membrane of columnar cells of the midgut epithelium (Granados, 1978). The ability of the virus to enter these cells is mediated by *per os* infectivity factors (reviewed by Slack & Arif, 2007). Once the virus has entered the midgut cells, replicated and budded through the basal lamina of the midgut cells, it must successfully enter cells of other tissues, replicate and spread the infection within the insect body to produce appropriate amounts of progeny virus to infect other insects. A second viral phenotype, budded virus (BV), is responsible for the movement of the virus from the basal lamina of the midgut cells into the hemocoel or trachea (Granados & Lawler, 1981; Washburn *et al.,* 1995) and for further spreading the disease in insect tissues to cause a systemic infection. BVs enter secondary target cells by absorptive endocytosis (Hefferon *et al.,* 1999; Lung *et al.,* 2002).

Baculovirus host specificity may be determined at multiple levels and depends on many events due to the complex infection cycle of these viruses. The first barrier for virus infection is the peritrophic membrane (PM) lining the insect midgut. Baculoviruses use the enzymatic activity of chitinases and enhancins to disrupt PMs during invasion of insect midgut cells (Hegedus *et al.,* 2009; Peng *et al.,* 1999; Wang & Granados, 2001). Sloughing of the PM and midgut cells constitute an important lepidopteran defense against baculovirus infection (Shapiro & Argauer, 1997; Wang & Granados, 2000; Haas-Stapleton *et al.,* 2003). Once the virus passes the PM it attaches to microvilli of columnar epithelial cells. The nucleocapsids (NCs) enter the cytoplasm by direct membrane fusion (Granados & Lawler, 1981; Horton &

Burand, 1993). The NCs are transported to the nucleus in a cytoskeleton-dependent manner and enter the nucleus through nuclear pores (Ohkawa *et al.,* 2002) after which transcription and replication can occur.

Host range data are often lacking when a new virus species is described. In general, baculoviruses are said to have a narrow host range, though in-depth studies on the specificity of these viruses are very limited. Moreover, verification that the progeny virions produced belong to the same virus species as those used for the cross-infections does not always occur (Cory, 2003). In fact, several studies have shown that the progeny virus obtained may have resulted from contamination of the virus preparation or the induction of a latent virus already present in the insect (Doyle *et al.,* 1990; Cory *et al.,* 2000; Bourner & Cory, 2004; Takatsuka *et al.,* 2007). Hence, it is important to determine the identity of the progeny virus in cross-infection studies.

The complete genome sequences of *Agrotis segetum* MNPV (AgseNPV) and SeMNPV have been determined (Jakubowska *et al.,* 2006; IJkel *et al.,* 1999). These viruses show a less than 10% difference in genome size (147 130 bp and 135 611 bp, respectively) and a striking colinearity of the genes shared between these viruses. In addition, a high level of sequence similarity between common genes is observed. This prompts the questions, is SeMNPV infectious for *A. segetum* larvae and conversely, is AgseNPV able to orally infect *S. exigua* larvae. SeMNPV is highly specific for *S. exigua* larvae and the ability to infect other insect species has not previously been demonstrated (Gelernter & Federici, 1986). AgseNPV has a wider host range including *A. ipsilon*, *A. exclamationis*, *A. puta*, *Noctua comes*, *Peridroma saucia*, *Xestia sexstrigata*, and *X. xanthographa* (Bourner & Cory, 2004). However, the virus used to determine the host range of AgseNPV is different from the sequenced Polish AgseNPV isolate (Jakubowska *et al.,* 2006) used in the present work and may represent a different virus species (Jakubowska *et al.,* 2005).

In the current study the infectivity of AgseNPV and SeMNPV for the insects *A. segetum* and *S exigua* was examined.The results show that despite the high level of genome similarity and colinearity between AgseNPV and SeMNPV, the latter cannot infect *A. segetum* larvae by the oral route but can establish a systemic infection when the midgut barrier is bypassed.

Methods

Insects, cells and viruses

A. segetum and *S. exigua* larvae were reared on artificial diet at 25°C, 70% humidity and a 16:8 h photoperiod as described (Hinks & Byers, 1976; Smits *et al.* 1986). Insect cells used in this study included Se301 (Hara *et al.,* 1993) and SeUCR (Gelernter & Federici, 1986a) from *S. exigua* and AiE-1611T and AiE-d6T from *A. ipsilon* (Harrison & Lynn, 2008). Se301 and SeUCR cells were cultured in Grace's medium (GIBCO, Invitrogen Inc., Grand Island, NY,

USA) supplemented with 10% fetal bovine serum. AiE-1611T and AiE-6dT cells were cultured in Excell-420 medium (SAFC Biosciences, Kansas City, MO, USA) supplemented with 5% fetal bovine serum. All cells were grown at 27°C. The AgseNPV used in this study was isolated in 1975 from *A. segetum* larvae collected in cabbage crops in Poland (Jakubowska *et al.,* 2005). The virus was freshly amplified in second instar larvae of a current laboratory culture of *A. segetum*. The *S. exigua* MNPV isolate used in this study for oral infections and intrahemocoelic injections (IH inoculations) was isolate SeMNPV-US1 (Gelernter & Federici, 1986). Both virus stocks are non-cloned, field isolates.

AgseNPV infections in S. exigua and A.segetum larvae

To determine the oral infectivity of AgseNPV for *S. exigua* second instar (L2) larvae were infected by the droplet feeding method (Hunter-Fujita *et al.,* 1998) with a virus suspension of 10^8 AgseNPV OBs/ml. The consumed volume was 1-2 µl. Phenol red was added to monitor the ingestion of the suspension. Larvae that ingested the virus were provided fresh diet and reared individually until death or pupation. The mortality was checked daily until pupation.

For determining the median lethal dose (LD_{50}) of AgseNPV for both *S. exigua* and *A. segutum*, L2 larvae of both species were infected individually by feeding diet disks contaminated with 5 different doses of AgseNPV ranging from 10^1 – 10^5 OBs/larva for *A. segetum* and 10^3 – 10^8 OBs/larva for *S.exigua*. Control larvae were given diet plugs with water. Larvae were given fresh diet after they consumed the entire disk. Thirty larvae were used per treatment and the same number of larvae served as a mock infected group. The bioassay was conducted twice and the LD_{50} values for both viruses were calculated by probit analysis (Finney, 1952).

SeMNPV in A. segetum larvae

To determine the oral infectivity of SeMNPV for *A. segetum* larvae, L2 larvae were infected by the droplet feeding method as described above for *S. exigua* larvae. A concentration of 10^8 SeMNPV OBs/ml was used. According to the fact that no mortality was observed after oral administration of SeMNPV to *A. segetum* larvae, the infectivity of SeMNPV by IH inoculation was analyzed. *A segetum* third instar (L3) larvae were injected intrahemocoelically with 10 µl of SeMNPV BV-containing hemolymph mixed with phenol red solution to control the injections. To this aim, BV-containing hemolymph was collected at 6 days post infection (p.i.) from fourth instar (L4) *S. exigua* larvae infected orally with SeMNPV at a dose of 10^3 OBs/larvae by cutting the prolegs. The hemolymph was mixed with an equal volume of Grace's medium (GIBCO, Invitrogen Inc., Grand Island, NY, USA), centrifuged at 1000 g to remove hemocytes and filtered through a 0.45 µm non-pyrogenic filter. The virus titer was determined in an end point dilution assay (Vlak, 1979) in Se301 (Hara *et al.,* 1993) and a virus stock with a titer of 10^8 $TCID_{50}$ units/ml was used for IH

inoculation. Injected larvae were reared individually until death or pupation, and the mortality was recorded daily.

Infections for quantitative RT-PCR

To determine the possible barriers in *A. segetum* larvae for oral infection by SeMNPV, the infection process was monitored by measuring the presence and accumulation of viral transcripts by using quantitative RT-PCR (qRT-PCR). For comparison, the development of the infection was monitored also in *A. segetum* larvae infected with SeMNPV by IH inoculation.

For monitoring the oral infections second instar *A. segetum* larvae were infected by droplet-feeding with 10^8 OBs/ml of SeMNPV. The consumed volume was 1-2 µl. Additionally a group of *A. segetum* larvae was infected with a mixture of 10^8 OBs/ml of SeMNPV and 10^3 OBs/ml of AgseNPV to test complementation of SeMNPV by AgseNPV. Three to five larvae were collected at 0, 12, 24, 48, 72, 96 and 168 h post infection (p.i.) for total RNA isolation. The experiment was performed in threefold and the data analyzed by one-way ANOVA and Tukey test for multiple pair analysis. Additionally, at 24, 72 and 96 h p.i. midguts and hemolymph were collected from five larvae individually at each time point, for total RNA isolation.

For monitoring the IH inoculation third instar *A. segetum* larvae were injected with 10 µl SeMNPV BVs (10^8 $TCID_{50}$/ml) and with a mixture of BVs, consisting of 10^8 $TCID_{50}$/ml SeMNPV and 10^6 $TCID_{50}$/ml AgseNPV (100:1). SeMNPV-containing hemolymph was prepared as described above. AgseNPV-containing hemolymph was collected and prepared as described for *S. exigua* larvae, from *A. segetum* larvae infected with AgseNPV at a dose of 10^3 OBs/larvae. The virus titer was determined in AiE1611T cells (Harrison & Lynn, 2008). Injected larvae were reared individually and 3-5 larvae were collected per treatment at 0, 12, 24, 48, 72, 96 and 168 h p.i. for RNA isolation. The experiment was performed three times and the data analyzed as described above.

Infection of cells in the culture

Cells were seeded at 70% confluency either in T25 flasks or in 6-well plates. AiE1611T and AiEd6T cells were infected with hemolymph-derived AgseNPV BVs. Also cell lines from *S. exigua* tissues, Se301 and SeUCR, were subjected to AgseNPV inoculation. SeMNPV BVs were applied to AiE1611T and AiEd6T cells, and as a control on Se301 cells. Cross-infections were carried out at high multiplicities of infection (MOI) of 10 TCID50 units/cell. The presence of cytopathic effects and the formation of polyhedra were monitored daily by phase-contrast microscopy.

DNA extraction and restriction enzyme analysis (REN analysis)

DNA was extracted from infected larvae that died from virus infections. OBs were purified as described by Muñoz *et al.* (1997). Virions were released from OBs by incubation in 0.1 M Na_2CO_3 for 15 min at 37°C. DNA was extracted according to the method described by Reed *et al.* (2003). After phenol:chloroform extraction the DNA was dialyzed for 48 h against 1 mM Tris.HCl, 0.1 mM EDTA pH 8.0. For REN analysis 1-1.5 µg of DNA was incubated with 10 units of enzyme for 3.5 h at 37°C. Reactions were stopped at 65°C for 10 min. The resulting DNA fragments were separated by electrophoresis in 0.7% agarose gels containing 40 mM Tris-acetate, 1 mM EDTA (pH 8.0). After electrophoresis the gels were stained with the 0.5 µg/ml solution of ethidium bromide for 30 min and analyzed under UV light.

RNA extraction from insect larvae and detection of transcripts by qRT-PCR

Total RNA was extracted from insect larvae using TRIpure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The concentration and integrity of RNA samples was determined spectrophotometrically at 260 nm as well as by agarose gel electrophoresis.

qRT-PCR was employed to determine the presence or absence of SeMNPV and AgseNPV transcripts in cross-infectivity studies. One µg of RNA was subjected to DNase I (Invitrogen, Carlsbad, CA, USA) treatment for 15 min at room temperature in a volume of 10 µl. DNase I activity was inhibited by incubation in 2.5 mM EDTA at 65°C for 10 min. cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The absence of contaminating DNA was verified by performing PCR without an RT step with all sets of primers. Five µl cDNA at 1/5 or 1/10 dilutions were used for qRT-PCR. All reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total reaction volume of 25 µl. Forward and reverse primers were added to a final concentration of 300 pM. All primers used were designed on the basis of the reported genome sequences of SeMNPV (IJkel *et al.,* 1999) and AgseNPV (Jakubowska *et al.,* 2006) and are listed in Table S1. Non-template controls were performed for each set of primers in order to verify the absence of non-specific background signals. RNA isolated from mock-infected larvae served as negative control. All reactions were performed in duplets. Dilutions (10^{-1} – 10^{-8}) of purified SeMNPV and AgseNPV DNA were used for standard curves. This DNA was quantified with a BioPhotometer (Eppendorf, Hamburg, Germany) and the number of target gene copies was calculated based on the DNA concentration and the molecular mass.

PCR reactions were performed in an ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA). The program used for all primer sets was: 15 min. at 95°C, followed by 50 cycles of 94°C for 15 s and 60°C for 30 s. The fluorescence signal was acquired at 60°C. At the end of the PCR reactions dissociation curves were obtained by monitoring fluorescence data while slowly heating DNA samples from 60 to 90°C at intervals of 1°C. Primer sets were

selected based on the presence of a single melting peak, a measure for specific amplification. Moreover cross-reactions of the SeMNPV and AgseNPV specific primer sets were excluded by performing reactions with both AgseNPV and SeMNPV DNA as templates.

qRT-PCR data were visualized and analyzed using the 7000 System Sequence Detection Software version 1.2.3 (Applied Biosystems, Foster City, CA, USA). Relative quantification of gene copies was performed by applying Ct values from each reaction based on the appropriate standard curves. Note that standard curves were prepared with genomic DNA and thus the calculated number of gene copies in cDNA cannot be considered as an absolute value, since reverse transcription efficacy is not included in the calculations.

Early and late gene specific primer sets were used to determine how far the infection proceeds. Initially three sets of primers were designed for the detection of AgseNPV and SeMNPV transcripts, specific for the immediate early gene *ie-1*, the early DNA polymerase gene (*dpol*) or the very late *p10* gene. IE-1 is the principal transregulator of early gene expression. DNA polymerase is essential for baculovirus DNA replication and P10 is involved in releasing OBs from cell nuclei in the very late phase of virus infection. Due to the high similarities in the sequence of the AgseNPV and SeMNPV primer sets, the AgseNPV *dpol* primers also amplified the SeMNPV *dpol* gene, and the primer set designed to amplify SeMNPV *ie-1* gene, also amplified the AgseNPV *ie-1* gene. These two primer sets have therefore been discarded and only primer sets that amplified specifically one virus template were used in the study. Primer sets amplifying *ie-1* and *p10* were used for detection of AgseNPV specific transcripts, and for detection of SeMNPV transcripts, primer sets for *dpol* and *p10* were used.

Results

Per os infectivity of SeMNPV and AgseNPV against S. exigua and A. segetum larvae

In the first experiment, the cross-infectivity of SeMNPV and AgseNPV for *A. segetum* and *S. exigua* larvae was explored. Larvae of both species were fed high doses of suspended OBs. AgseNPV fed at the titer of 10^8 OBs/ml, caused a fatal infection in 30% of the second instar *S. exigua* larvae. OBs were present in various tissues including the fat body of infected and dead insects (data not shown). The identity of the virus recovered from *S. exigua* larvae was determined by REN analysis of DNA extracted from OBs isolated from the AgseNPV-infected larvae. The *Pst*I REN pattern of OB DNA from cross-infected *S.exigua* larvae was identical to the pattern of the input virus (Fig. 6-1A) and therefore activation of a latent virus or contamination in the original isolate with other NPVs was excluded.

Table 6-1. LD_{50} values of AgseNPV infection in *A. segetum* and *S. exigua* larvae.

Virus	Insect species	LD_{50} values	95% confidence interval	Regression line
AgseNPV	*A. segetum*	8.3×10^{1}	$2.2 \times 10^{1} – 3.2 \times 10^{2}$	y = 0.4x + 1.2
AgseNPV	*S. exigua*	8.3×10^{5}	$3.7 \times 10^{5} – 1.9 \times 10^{6}$	y = 0.7x + 0.9

A bioassay was conducted to compare the infectivity of AgseNPV for both *A. segetum* and *S. exigua*. Five doses of AgseNPV were applied on diet plugs and offered to larvae. A higher dose range of AgseNPV was used for *S. exigua* since the experiment described above had shown that these larvae are less susceptible to oral infection by AgseNPV than *A. segetum*. The LD_{50} values obtained for AgseNPV in L2 larvae were 83 OBs for *A. segetum* versus 8.3×10^{5} OBs for *S. exigua* (Table 6-1) and hence these species showed a 10,000 fold difference in susceptibility for AgseNPV. Oral inoculation of *A. segetum with* SeMNPV did not lead to fatal infection. None of the newly molted *A. segetum* L2 larvae fed with 10^{8} SeMNPV OBs/ml died from virus infection. Microscopic inspection showed that no OBs were present in the tissues of these larvae.

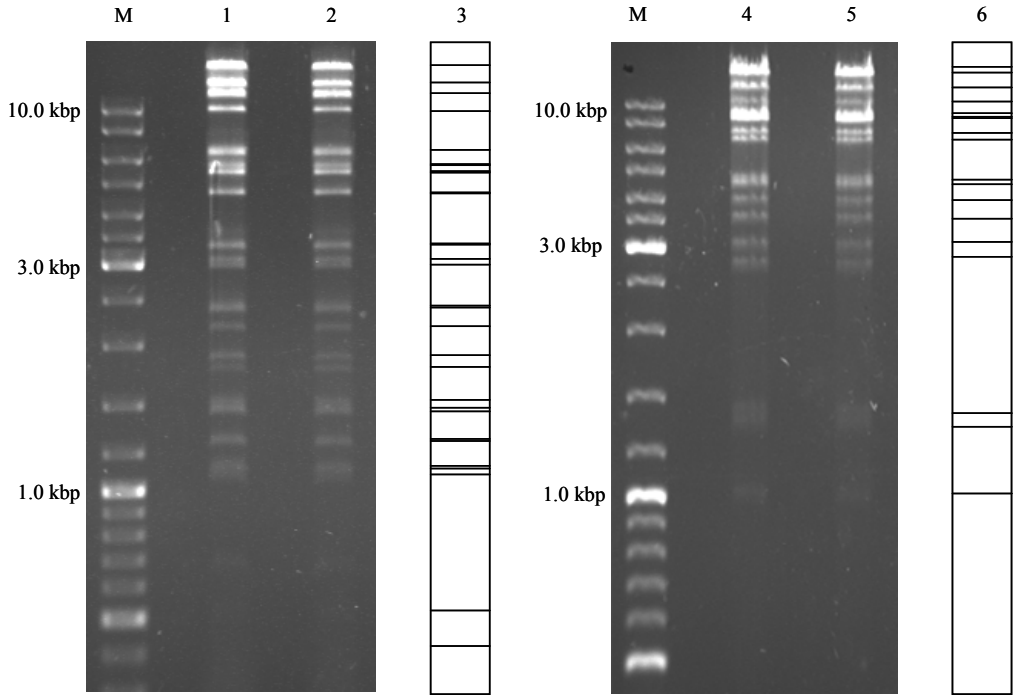


Fig. 6-1. REN analysis. *Pst*I DNA REN profiles for AgseNPV OBs used for infecting *S. exigua* larvae (lane 1); OBs resulting from *S. exigua* infection with AgseNPV (lane 2); schematic of the *Pst*I REN profile of AgseNPV (lane 3); *Pst*I digestion of SeMNPV viral DNA derived from BVs used for injecting *A. segetum* larvae (lane 4) and OBs resulting from injecting *A. segetum* larvae with SeMNPV (lane 5); Diagram of *Pst*I REN profiles of SeMNPV (lane 6); M = DNA molecular marker pUC19 DNA/ digested with *Msp*I (Fermentas). Schematics of REN profiles were generated from the genomic sequences with NEBcutter V 2.0 (Vincze *et al.,* 2003).

IH inoculation of A. segetum larvae with SeMNPV BVs

Given the observation that SeMNPV is not orally infectious for *A. segetum* larvae, IH injections were performed with SeMNPV BVs in third instar *A. segetum* larvae. More than 70% of the larvae injected with SeMNPV BVs at a dose of 10^6 $TCID_{50}$ units/larva died from virus infection, resulting in the production of large numbers of OBs. The DNA of the input and the recovered virus showed identical REN patterns (Fig. 6-1B) confirming that SeMNPV had replicated in *A. segetum* larvae and produced OBs. This result suggests that the midgut of *A. segetum* larvae is the main barrier for successful infection with SeMNPV.

Detection of SeMNPV and AgseNPV specific transcripts in cross infected larvae

qRT-PCR was performed with total RNA isolated at different times post infection from *A. segetum* larvae infected *per os* (L2) or injected IH (L3) with SeMNPV alone or with a mixture of SeMNPV and AgseNPV. No early (*dpol*) and very late (*p10*) viral transcripts were detected at any time after *per os* infection with SeMNPV alone. The larvae continued to grow regularly and finally pupated (not shown).

In larvae infected *per os* with a mixture of AgseNPV and SeMNPV, AgseNPV early (*ie-1*) and very late (*p10*) transcripts were first detected at 12 and 48 h p.i., respectively (Fig. 6-2A and 6-2B), but again no SeMNPV transcripts were found.

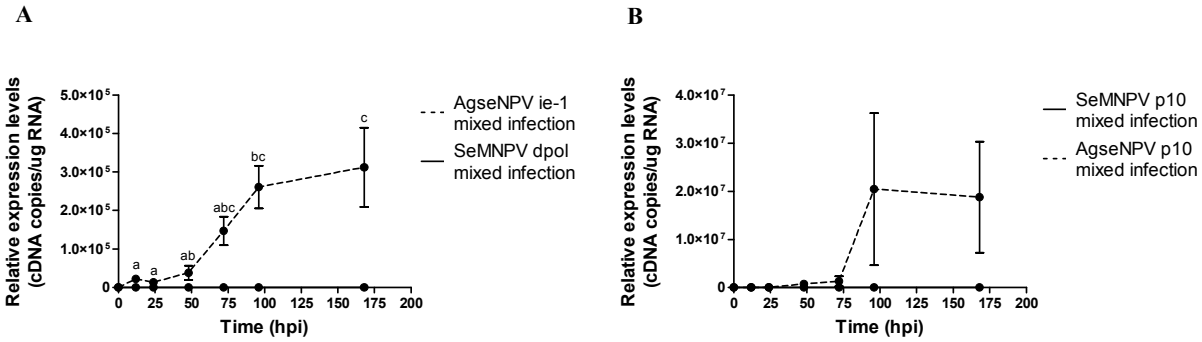


Fig. 6-2. Viral early (A) and late (B) genes expression in *A. segetum* larvae orally inoculated with SeMNPV OBs in a mixture with AgseNPV OBs. Total RNA from infected larvae was analyzed with qRT-PCR using primer sets specific to SeMNPV DNA polymerase gene and AgseNPV *ie-1* gene. Target gene copy numbers were calculated based on SeMNPV and AgseNPV genome molecular weight and standard curves. Data show the mean value ± SD (n=3). Values with different superscripts (a,b,c) are significantly different ($P \leq 0.05$).

The prevalence of AgseNPV *ie-1* transcripts increased approximately 20 times between 24 and 96 h p.i. The levels of AgseNPV *p10* transcripts increased strongly between 72 and 96 h p.i, but a large variation was seen between individual larvae (Fig. 6-2B).

Since the midgut is the first tissue affected in oral NPV infections, isolated *A. segetum* midguts were analysed for the expression of SeMNPV genes in single and mixed infections.

Low expression levels of the SeMNPV *dpol* gene were detected in one out of five midgut samples collected from L4 larvae infected with SeMNPV at 72 h p.i. (4.8 x 10^3 cDNA copies/µg RNA) and at 96 h p.i. (1.1 x 10^4 cDNA copies/µg RNA). Two out of five midguts collected at 72 h p.i. from mixed-infected larvae showed SeMNPV *dpol* transcripts (5.8 x 10^3 and 7.9 x 10^3 cDNA copies/µg RNA) (Fig. 6-3). None of these samples showed expression of the SeMNPV *p10* gene.

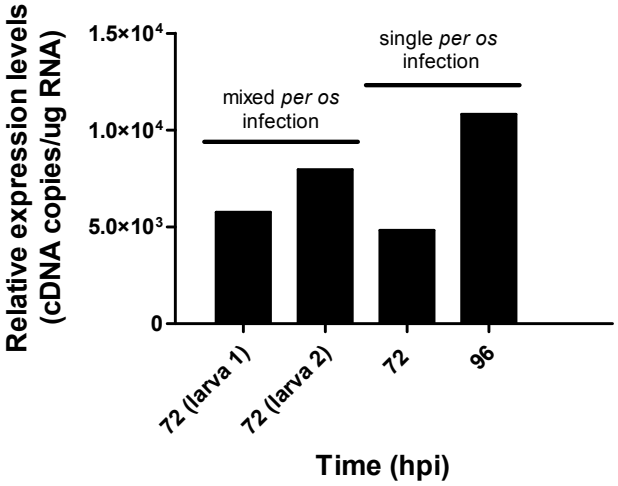


Fig. 6-3. Viral early gene (*dpol*) expression in the midguts of *A. segetum* larvae orally inoculated with SeMNPV OBs alone and with a mixture of SeMNPV and AgseNPV OBs. Midguts were isolated from infected larvae at different times post infection. Total midgut RNA was analyzed by qRT-PCR using primers specific for SeMNPV *dpol*. Target gene copy numbers were calculated based on SeMNPV genome molecular mass and standard curves.

After IH inoculation of *A. segetum* L3 larvae with SeMNPV, early gene transcripts of this virus (*dpol*) were first detected at 48 h p.i. and the levels of these transcripts increased 3.9 times between 72 and 96 h p.i. (Fig. 6-4A). Very late SeMNPV transcripts (*p10*) appeared also at 48 h p.i. and their level increased (50 times) between 72 and 96 h p.i. (Fig. 6-4B).

In mixed IH inoculations with AgseNPV and SeMNPV BVs, early SeMNPV transcripts (*dpol*) were detected at 24 h p.i. – earlier than with SeMNPV alone – and between 24 and 48 h p.i. they increased approximately 75 times (Fig. 6-4A). Between 48 and 96 h p.i SeMNPV early transcripts strongly decreased (25 times) and at 168 h p.i. they were not detectable anymore. SeMNPV *p10* transcripts were detected in mixed IH inoculations at 48 h p.i., and were maintained at the same level throughout the recording time.

AgseNPV early transcripts (*ie*-1) were detected as early as 12 h p.i. in mixed IH inoculations and their level continued to increase until 168 h p.i. (Fig. 6-4C). The transcript number increased significantly (6.9 times) between 48 and 96 h p.i. AgseNPV very late transcripts (*p10*) were detected already at 24 h p.i. and increased 2.6 times between 48 and 72 h p.i. (Fig. 6-4D). These data suggest that the SeMNPV gene expression in mixed infections is enhanced at early times p.i., but suppressed at later times p.i.

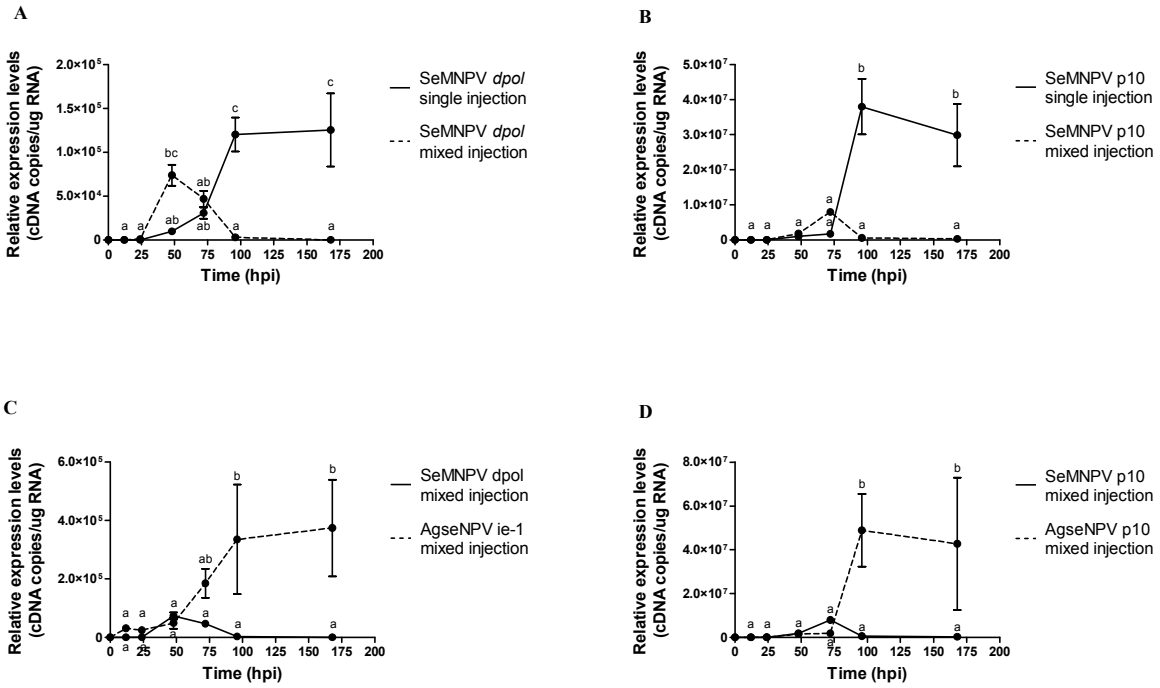


Fig. 6-4. Viral early and late gene expression in *A. segetum* larvae infected with SeMNPV alone or in a mixture with AgseNPV by IH inoculations. A) SeMNPV *dpol* expression; B) SeMNPV *p10* expression; C) SeMNPV *dpol* expression and AgseNPV *ie-1* expression in mixed IH inoculations; D) SeMNPV *p10* expression in single IH inoculations and AgseNPV *p10* expression in mixed IH inoculations. Total larval RNA was analyzed by qRT-PCR using primers specific for the SeMNPV *Dpol* and *p10* genes and the AgseNPV *ie-1* and *p10* genes. Target gene copy numbers were calculated based on SeMNPV and AgseNPV genome molecular mass and standard curves. Data show mean ±SD (n=3). Values with different superscripts (a,b,c) are significantly different ($P \leq 0.05$).

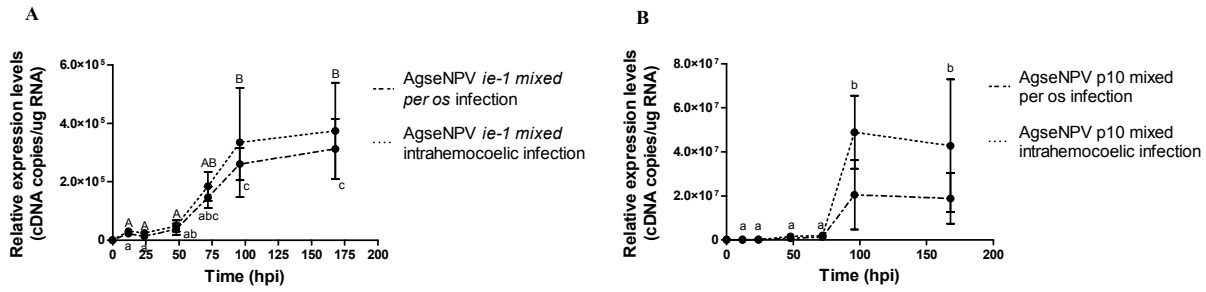


Fig. 6-5. AgseNPV early and late genes expression in *A. segetum* larvae. *A. segetum* larvae were infected *per os* and by IH inoculation with a mixture of SeMNPV and AgseNPV. A) AgseNPV *ie-1* and B) *p10* expression was analysed in infected and injected larvae. Total larval RNA was analyzed by qRT-PCR using primers specific for AgseNPV *ie-1* and *p10* genes. Target gene copy numbers were calculated based on SeMNPV and AgseNPV genome molecular weight and standard curves. Data show mean ±SD (n=3). Values with different superscripts (a,b,c; A,B,C) are significantly different ($P \leq 0.05$). *Per os* and IH inoculation were analyzed separately.

AgseNPV early (*ie*-1) and very late (*p10*) gene expression in larvae infected *per os* was almost identical in level and timing to the expression in larvae infected by IH inoculation (Fig. 6-5A and 6-5B).

Infection of cells in culture

Several *S. exigua*-derived cell lines allow permissive replication of SeMNPV, such as Se301 (Fig. 6-6C) and SeUCR cells (not shown). Since AgseNPV is orally infectious for *S. exigua* larvae, cell lines derived form this species may be susceptible to AgseNPV. However, no cytopathic effects or polyhedra were observed over a period of 15 days p.i. neither in Se301 (Fig. 6-5B) nor in SeUCR cells (not shown) inoculated with AgseNPV BVs at a MOI of 10 $TCID_{50}$ units/cell. Two recently developed cell lines from *A. ipsilon*, AiE1611T and AiEd6T, allow permissive replication of *A. ipsilon* MNPV (AgipNPV) (Harrison & Lynn, 2008). Since SeMNPV is orally infectious for *A. segetum*, the two AiE cell lines may be able to support SeMNPV replication. However, SeMNPV BVs did not cause infection in either *A. ipsilon*-derived cell line (shown for AiE611T, Fig. 6-6F). No signs of apoptosis were observed either in these cells and they continued to develop normally. In contrast both AiE cell lines did support the replication of the closely related AgseNPV as shown for AiE611T (Fig 6-5F). In this infection, typical cytopathic effects including the formation of polyhedra were observed as early as 48 h p.i. using hemolymph-derived AgseNPV for inoculation. AiE cells, remained more strongly attached to the bottom of the culture flask than other cell types infected with NPVs.

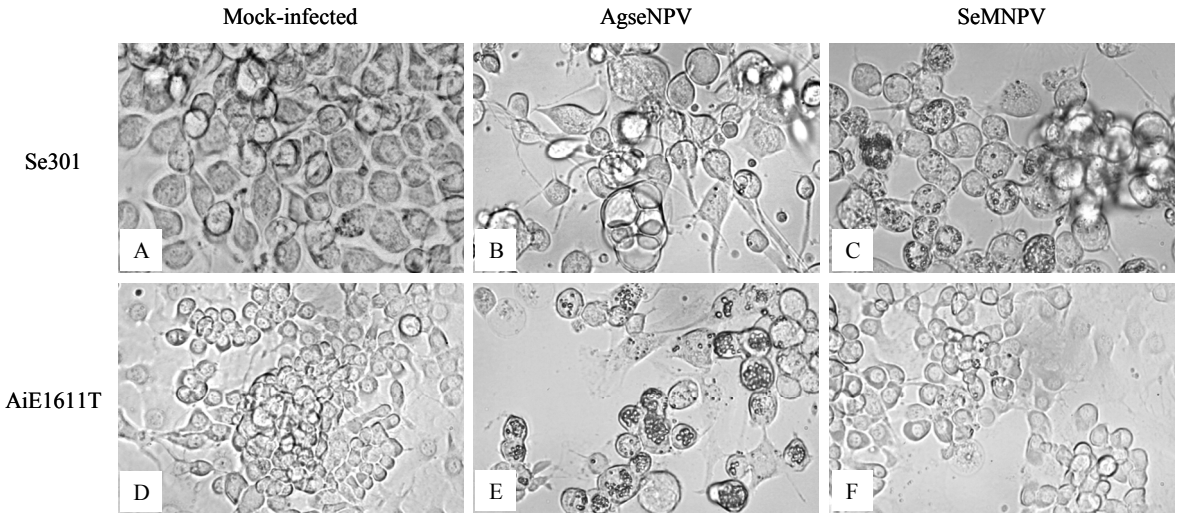


Fig. 6-6 Infection studies in cultured cells. *S. exigua* Se301 cells (A) were inoculated with AgseNPV (B) and SeMNPV (C) and *A. segetum* AiE1611T cells (D) were also inoculated with AgseNPV (E) and SeMNPV (F). Cells were seeded at 70% confluency, and inoculated with AgseNPV and SeMNPV BVs at high MOI=10 and observed for polyhedra formation.

Discussion

Host specificity studies of two closely related NPVs, SeMNPV and AgseNPV in *S. exigua* and *A. segetum,* revealed that despite a high level of sequence similarity and a remarkably similar genome organization and gene content (Jakubowska *et al.,* 2006) these viruses differ significantly in their capacity to cross-infect the alternate insect species *per os*. While AgseNPV caused lethal infections in *S. exigua* larvae when administered orally, SeMNPV was able to kill *A. segetum* larvae only when injected into the hemocoel (Table 1). Although *S. exigua* larvae were susceptible to oral infection with AgseNPV, *S. exigua* larvae are, based on LD_{50} values, four orders of magnitude less susceptible to AgseNPV than *A.segetum*. Hence, *S. exigua* appears to be a semi-permissive host for AgseNPV.

The monitoring of the SeMNPV infection process in *A. segetum* larvae by following the level of early and late viral transcripts by qRT-PCR revealed that the main barrier for infection in this virus/host system is the midgut. After oral infection of *A. segetum* larvae with SeMNPV no viral transcripts were detected when RNA was isolated from the total larval body, however in a few isolated midguts we detected early SeMNPV transcripts. P10 transcripts were never found. Thus the entry of SeMNPV into midgut epithelial cells of *A. segetum* is possible. The presence of delayed-early transcripts (*dpol*) and the lack of late viral transcripts (*p10*) suggest that the infection is blocked at the transition from early to late transcription. The fact that we only find these early transcripts in a small number of isolated midguts may indicate that the efficiency of entry is low or that the infected cells are rapidly sloughed off. The results also suggest that SeMNPV may be able to replicate in cells of more insect species than previously thought, once this midgut barrier is passed.

Successful baculovirus infection depends on many factors and may be blocked at any point in the infection cycle. Many virus/host systems have been studied to shed light on insect susceptibility to baculovirus infections and apparently many mechanisms exist that lead to a block in infection. Studies with AcMNPV carrying a *lacZ* reporter gene in non-permissive *Heliothis zea* and fully permissive *Heliothis virescens* larvae showed that immune responses may alsodefine baculovirus host range (Washburn *et al.,* 1996). For AcMNPV in *S. frugiperda* larvae, the main barrier for fatal infection was the inability to efficiently infect midgut epithelial cells and the loss of infected tracheal cells (Haas-Stapleton *et al.,* 2003). AcMNPV in *S. frugiperda* larvae shows the same pattern of infection as SeMNPV in *A. segetum*. *S. frugiperda* is extremely resistant to oral infection with AcMNPV, but is highly susceptible to systemic infection by IH inoculation with BVs. These studies suggested that the ability of ODVs to bind to epithelial midgut cells plays a crucial role in the ability to orally infect insect larvae. The binding of ODVs to midgut cells is mediated by three *per os* infectivity factors (PIFs) (P74, PIF-1 and PIF-2), associated with the ODV envelope (Haas-Stapleton *et al.,* 2004; Ohkawa *et al.,* 2005; Song *et al.,* 2008). In line with this, an AcMNPV *p74* mutant could only infect *Trichoplusia ni* and *H. virescens* larvae (Faulkner *et al.,* 1997;

Haas-Stapleton *et al.,* 2004) when injected IH. So far, the detailed role of PIFs in ODVs midgut entry remains enigmatic, but a reason for the resistance to oral infections could be inefficient binding to and fusion with primary target midgut cells. The level of amino acids similarity between SeMNPV and AgseNPV PIFs ranges from 74 to 88%. Sequence similarity however, seems not to be very informative in this case, as the similarity between PIFs of SeMNPV that does not infect orally *A. segetum* larvae and *A. ipsilon* NPV (AgipNPV) that infects *A. segetum* larvae is at the same level, between 74 and 89%. Since SeMNPV can enter *A. segetum* midgut cells, the *per os* infectivity factors (PIFs) are in this case clearly not as specific as originally thought (Song *et al.,* 2008).

A salient observation was that AgseNPV clearly out competed SeMNPV in a single passage after mixed IH inoculations (Figs 6-2 and 6-3). AgseNPV probably possesses some features in comparison to SeMNPV that enable a faster or more efficient infection process, leading to a higher mortality and greater yield of progeny virus. Alternatively, AgseNPV may activate insect responses that result in the clearance of SeMNPV. The competition between viruses may happen at different stages in the infection process, including the release of BVs from midgut cells, the entry into secondary target cells, the efficacy of replication and transcription, or the assembly of a new generation of BVs and OBs. We also find the observation of interest that at 48 and 72 h p.i. the abundance of both early and late SeMNPV transcripts was higher in mixed infections than in single infections with SeMNPV. This suggests that AgseNPV assists in the replication of SeMNPV in *A. segetum* larvae in the first few days after co-injection. At 96 h p.i. the situation changed dramatically in favor of AgseNPV transcripts, indicating that AgseNPV, despite serving as a helper for SeMNPV in the beginning, finally takes over completely. These data are in agreement with co-infection studies in various *Spodoptera* species with SeMNPV, *S. frugiperda* MNPV (SfMNPV) and *S. littoralis* MNPV (SpliMNPV) (Simon *et al.,* 2004), with the difference that when *S. frugiperda* and *S. littoralis* larvae where co-infected with SeMNPV and their natural viruses all classes of SeMNPV transcripts were found in both *per os* infections and IH inoculation. In our study, SeMNPV transcripts were not detected in oral infections except for early transcripts in a few midgut samples. Simon *et al.* (2004) also reported that SeMNPV transcript levels increased in *S. frugiperda* and *S. littoralis* in co-infections with respect to SeMNPV infection alone, similar to what was seen here in *A. segetum*. However, in REN analysis they detected only the homologous virus in co-infected larvae and concluded that REN was not sufficiently sensitive to detect the heterologous virus. The lack of SeMNPV detection in REN analysis might also have been due to the time point of OB collection. OBs were probably collected from dead larvae when the homologous virus might had already competed out the heterologous one, like in the current study. SeMNPV infection in cultured *S. frugiperda* cells is improved by co-infection with AcMNPV virus (Yanase *et al.,* 1998) and those authors suggest that AcMNPV may provide transcripts or their products that are used by SeMNPV for replication. Also, in our study, the replication of SeMNPV may increase at the

early stages of infection in co-injected insects in respect to insect IH inoculated with SeMNPV alone due to the presence of AgseNPV gene products.

The attempts to replicate AgseNPV in *S. exigua* cell lines were unsuccessful, although AgseNPV was able to cause fatal infection in *S. exigua* larvae *in vivo*. Also SeMNPV was not able to replicate in any of the *A. ipsilon* cells, in contrast to AgSeNPV. However, SeMNPV BVs injected into *A. segetum* larvae caused a full blown infection. The susceptibility of cell lines can not be used as an absolute indicator for the ability of a virus to infect larvae of the same species (Lynn, 2003) and NPVs may not affect all cell lines derived from a certain species. For example AcMNPV, replicates in some *Lymantria dispar* cell lines (LdEG, LdEI), but not in Ld652Y and does not infect *L. dispar* larvae (Chen *et al.,* 1998). Baculovirus replication may be restricted to certain tissues in such cases. *Bombyx mori* NPV (BmNPV) causes a symptomless infection in *Spodoptera frugiperda* Sf21 cells and only a small number of cells become infected (Martin & Croizier, 1997). In the latter case, the budding may be affected or budded viruses may be unable to infect neighboring cells via GP64-mediated fusion (Monsma *et al.,* 1996). We do not know, however, where the block in replication occurs in our study. Baculovirus particles can enter a wide variety of cells, but the levels of gene expression, and DNA replication vary strongly between different cell lines, making some of them permissive and others non-permissive (Morris & Miller, 1992, 1993). In non-permissive cells, no DNA replication and late gene expression occurs and virus replication is most likely blocked at the stage subsequent to early genes expression (Wilson *et al.,* 2005). A number of baculovirus genes, including helicase and host cell factor-1 (*hcf-1*), affect infection in specific cell lines and a role as host range determinant was proposed (Hefferon, 2003). The ability to infect cells successfully may also depend on external factors, for example components in the cell culture medium (Wang *et al.,* 1997). In the case of AgseNPV and SeMNPV, and the cell lines from *S. exigua* and *A. ipsilon,* varying infection conditions and determining gene expression patterns, may help to determine whether these cells could be (semi)-permissive.

A. segetum and *S. exigua*, and their respective viruses now represent an attractive model for studying the baculovirus infection process and to reveal the barriers in specificity and the identification of host range factors. It is clear that in the case of SeMNPV this barrier is beyond the initial entry and the involvement of PIFs. Furthermore, the high genomic co-linearity and gene sequence homology makes the SeMNPV/AgseNPV system highly suitable to study baculovirus adaptation, evolution and speciation.

Acknowledgments

This research was supported in part by a Marie Curie fellowship awarded to AKJ (Project N° MRTN-CT-2006-035850). S. Herrero was supported by the Spanish Ministry of Science and Innovation (Project Nº AGL2008-05456-C03-03). M.M. van Oers was sponsored by a MEERVOUD grant from the Dutch Organisation for Scientific Research (NWO).

Chapter 7

General discussion

The presented thesis describes two nucleopolyhedroviruses (NPVs) infecting economically important insect species *Leucoma salicis* and *Agrotis segetum*. Both viruses were isolated from their natural ecosystems, forest and field crop, respectively. In Northern Europe *L. salicis* larvae affect poplar trees most commonly planted in highly populated areas, in parks and housing estates (Ziemnicka, 2008), where chemical control is discouraged. *L. salicis* NPV (LesaNPV) has been evaluated for biocontrol of *L. salicis* and holds promise as a good alternative to chemical control. *A. segetum* is a field pest in Europe and outbreaks take place every 3-5 years. The larvae cause severe damage to the crop and its control is additionally complex due to the ecology of the species. *A. segetum* larvae spend most of their time in the soil and damage particularly the roots and petioles (Zethner *et al.,* 1987). Despite the fact that both, LesaNPV and *A. segetum* NPV (AgseNPV) show potential to control their insect hosts, detailed characterization, as needed for instance to allow registration as a commercial biopesticide, was lacking. The characterisation of these viruses showed that they exemplify different aspects of baculovirus evolution, divergence and speciation.

Why do we sequence virus genomes?

The family *Baculoviridae* forms a large and diverse group of dsDNA viruses. The exact number of baculovirus isolates mentioned in the literature is hard to estimate, but surely exceeds 600 (Martignioni & Iwai, 1981; Herniou & Jehle, 2007). This however is probably only a small proportion of what exists in nature awaiting discovery, as insects are the largest group of animals on earth and only a few of these have been specifically tested for the presence of viruses. The recent knowledge of the great diversity of baculoviruses was obtained from the completion of genome sequences of both NPVs and granuloviruses (GVs), from three insect orders, Lepidoptera, Diptera and Hymenoptera. Over the past fifteen years fifty-three complete genomic sequences have become available (Chapter 1, Table 1-1) and the number still increases due to novel DNA sequencing technologies, like 454 sequencing (Marguiles *et al.,* 2005). We sequence baculovirus genomes, to allow comparison of gene homology, gene content and gene order and reveal genomic features shared by all baculoviruses (Herniou *et al.,* 2001; 2003; van Oers & Vlak, 2007). Genomic information also reveals similarities and differences with other large DNA viruses (Iyer *et al.,* 2001, 2006). The large scale virus genome sequencing led to hypotheses about the origin of viruses, placing them in the earliest phases of the evolution of life and associating them with the

precursors of cellular systems (Alstein, 1992). The opposite theory states that viruses are derivatives of cellular systems that underwent degeneration as a consequence of extreme parasitism (Gibbs *et al.,* 1995). While genome sequences not necessarily solve the debate of the origin of life and the role of viruses in the evolution of living organisms, they certainly add to the recognition of the enormous diversity in viral coding capacity and the knowledge about the relationships between different virus groups and homologies between virus genes. Sequence information also allows comparison between genes of viruses and cellular organisms.

Baculovirus core genes

Baculovirus genomes carry 80-180 genes. Collectiveley, baculoviruses encode more than 1,000 genes, but a limited number has been investigated for their function. Part of these are common to all baculoviruses and these genes are called baculovirus core genes. With the increasing number of baculovirus genome sequences, the number of common (core) genes has been reduced. Thirty genes are present in all baculoviruses including dipteran and hymenopteran NPVs (van Oers & Vlak, 2007; McCarthy & Theilmann, 2008) and hence represent the baculovirus core genes. This number may still change when more dipteran and hymenopteran NPV sequences will become available, since so far there are only four of these sequenced (NeseNPV, NeleNPV, NeabNPV and CuniNPV, Chapter 1, Table 1 for references). Sixty three genes are common to lepidopteran NPVs and GVs.

Conserved (core) baculovirus genes play essential roles in DNA replication, gene expression, virus structure, virion assembly and entry into midgut epithelial cells. A number of core genes still have unassigned functions (Cohen *et al.,* 2009) and this needs further investigation. The number of baculovirus core genes is similar to the number of genes conserved in other families of large DNA viruses. For instance herpesviruses share 43 genes responsible for basic viral replication processes and building the virus particle (Davison *et al.,* 2002). Core genes represent the basic signature of a group of viruses and they were most likely inherited from a common ancestor and form an ancient system fundamental for virus replication (Zanotto & Krakauer, 2008). Since all baculoviruses have these core genes, these genes are perfectly suitable for phylogenetic analysis.

Baculovirus evolutionary relationships

Sequencing baculovirus genomes not only provides information on genetic diversity in this group of viruses, but also allows depicting their evolutionary relationships. Sequence and phylogenetic analysis showed that *Agrotis* spp. NPVs form a well defined clade of group II NPVs and cluster with viruses of *Spodoptera* spp. (SeMNPV, SfMNPV, SpltMNPV) and *Mamestra* spp. (MbMNPV and MacoNPV-A and B) (Chapter 4 , Fig. 4-2).

Analyses of single or concatenated core genes are used for assessing phylogenetic and evolutionary relationships between genes and genomes. Whole genome sequences analyses

are however more robust in comparison to single gene or genes analyses for determining the evolutionary relation ships between organisms or viruses, due to horizontal gene transfer, gene duplications or capture from host or other viruses, all mechanisms which have been frequent in large DNA viruses (Shackelton & Holmes, 2004; Herniou *et al.,* 2001).

Sequence based methods, however, have several limitations. Apart from the mechanisms mentioned above which interfere with revealing distant evolutionary traits, virus genes have enormous mutation rates. While mammalian mutation rate was calculated to be 2.2 x 10^{-9} / bp / year (Kumar & Subramanian, 2002), the mutation rate of human influenza virus was estimated 2.6 x 10^{-3} / bp / year (Nobusawa & Sato, 2006)! For large DNA viruses the substitution rate is 10^{-8} / bp / year, which places them somewhere in the middle of the mutation rate scale. This high mutation rate for viruses on one hand permits high genetic variability and hence greater adaptation abilities, but on the other hand makes it extremely difficult to follow evolution of viruses. A number of sequence-free methods are under development that do not require sequence alignment for deriving species phylogeny but are based on overall similarities of the complete genome data (Gao & Qi, 2007).

Baculovirus nomenclature

In this research, a Polish virus isolate from *A. segetum*, AgseNPV, was entirely sequenced (Chapter 5). As expected, all core baculovirus genes are present in the AgseNPV-A genome. This virus was found to be closely related to NPVs from other *Agrotis* spp. as well as to other NPVs isolated from *A. segetum* in England (isolate A12-3) and France (Allaway & Payne, 1983, El Salamouny *et al,*. 2003). We proposed to name the sequenced isolate AgseNPV-A, to be consistent with naming of MacoNPVs (MacoNPV-A and MacoNPV-B), viruses isolated from *M. configurata*, of which whole genomes have been sequenced and compared (Li *et al.,* 2002; Li *et al.,* 2002; Li *et al.,* 2005). Consequently the other AgseNPV isolates, when proven to be different species, should be named alphabetically.

From the *Agrotis* spp. NPVs only AgseNPV-A and AgipNPV-Illinois have been entirely sequenced, and these viruses are notably different (Harrison, 2009). Partial sequences are also available for AgseNPV isolate A12-3, AgipNPV-Kentucky and AgipNPV isolate M6-2 (Lange *et al.,* 2004; Harrison, 2009). In this disussion, I like to add to the list of *Agrotis* spp. NPVs a Polish NPV isolate from *A. exclamationis* (AgexNPV). Restriction analysis shows that *Pst*I profiles of AgexNPV and AgseNPV A12-3 are extremily similar to each other, but differ significantly from the AgseNPV-A profile (Fig. 7-1A). We partially sequenced the polyhedrin (*polh*) gene of AgexNPV (GenBank accession number GQ475265), which confirmed the close relationship of AgexNPV with AgseNPV A12-3 (98% nucleotide (nt) identity). The *polh* gene sequence also showed a relatively close relation to AgipNPV (Illinois) (90% nt identity), and only moderate relationship with AgseNPV-A (83% nt identity). Phylogenetic analysis (Fig. 7-1B) clearly showed that isolate A12-3 of AgseNPV

and AgexNPV may actually represent variants of the same virus species, although they were isolated from different insect species.

This brings up the issue of virus classification and nomenclature. The various NPVs from *Agrotis* spp. clearly show that naming after insect species can be very misleading and that molecular characterization of the virus isolate by means of restriction profile analysis as well as conserved genes sequence analysis is crucial but fast way to characterize and classify baculoviruses.

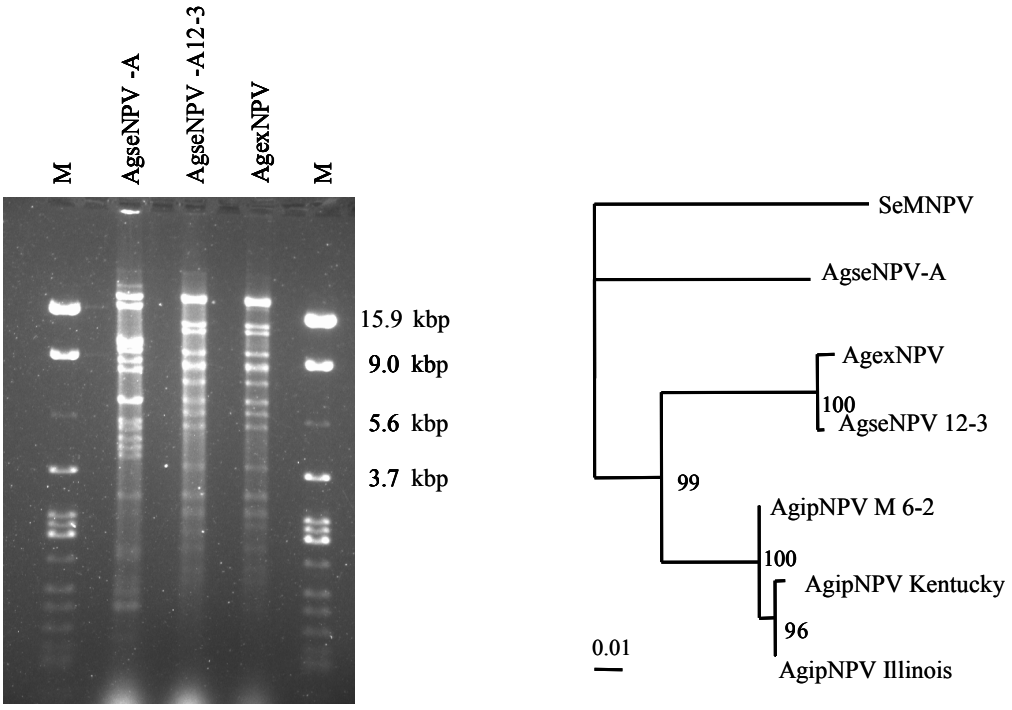


Fig. 7-1. Restriction profile and phylogeny of AgexNPV. A) *Pst*I restriction profile of AgexNPV in comparison to AgseNPV A12-3 and AgseNPV-A. M – lambda *Eco*RI/*Bam*HI/*Hin*dIII fragments; B) N-J tree obtained by analysis of partial *polh* gene sequences of *Agrotis* spp. NPVs and SeMNPV, numbers indicate bootstrap scores, accession numbers: SeMNPV (AF169823), AgseNPV-A (DQ123841), AgexNPV (GQ475265), AgseNPV A12-3 (AY706683), AgipNPV M6-2 (AY519204), AgipNPV Kentucky (DQ014542) and AgipNPV Illinois (EU839994).

New virus isolates should be described by their pathogenicity against a range of insect species and the molecular analysis should complement the characteristics before assigning a new virus species. Sequence information allows the classification of new isolates into species according to the recently proposed criterion on species demarcation based on the Kimura-2 parameter (see: Introduction, p.12). According to this method, for instance, AgseNPV-A and AgseNPV A12-3 constitute two different NPV species, while AgseNPV A12-3 and AgexNPV represent the same virus species based on the Kimura-2 parameters of the their *polh* gene sequences, which are 0.187 and 0.012, respectively (Table 1).

Table 7-1. Kimura-2 parameters for *Agrotis* spp. *polh* gene (partial sequence, 330 nt) derived in MEGA-3 software (Kumar *et al.*, 2004).

	AgseNPV-A	AgseNPV A12-3	AgipNPV M6-2	AgipNPV Illinois	AgipNPV Kentucky
AgseNPVA12-3	**0.187**				
AgipNPV M6-2	0.127	0.099			
AgipNPV Illinois	0.127	0.099	0.000		
AgipNPV Kentucky	0.127	0.099	0.000	0.000	
AgexNPV	0.198	**0.012**	0.110	0.110	0.110

AgseNPV and AgipNPV genome comparison

Availability of complete genome sequences of AgseNPV-A (Chapter 4) and AgipNPV (Harrison, 2009) allows the direct comparison of these two closely related NPVs. Both genomes belong to the larger baculovirus genomes (147,130 bp for AgseNPV-A and 155,122 bp for AgipNPV), which feature is obviously reflected in the genome content. Both contain all 30 core baculovirus genes as well as all the 63 genes common for lepidopteran baculoviruses (van Oers & Vlak, 2007). The most pronounced feature that differentiates them from most other baculoviruses is the presence of viral enhancing factors (VEFs), also called enhancins. While AgipNPV codes for one VEF, AgseNPV-A codes for three. VEFs were first identified in GVs, but later also in NPVs, like MacoNPV, LdMNPV and CfMNPV (Derksen & Granados, 1988; Li *et al.,* 2003; Bischoff & Slavicek, 1997; De Jong *et al.,* 2005). AgseNPV-A, AgipNPV and MacoNPVs VEFs seem to have a common origin, which is different from the VEFs from LdMNPV and CfMNPV. Since enhancins are also present in soil bacteria (Parkhil *et al.,* 2001; Read *et al.,* 2003; Galloway *et al.,* 2005), it can be speculated that their presence in baculoviruses may have been the result of two or three independent gene capture events from such organisms. The two enhancins from LdMNPV contribute to viral potency (Popham *et al.,* 2001) and are components of ODVs (Slavicek & Popham, 2005). Li *et al.* (2003) showed that MacoNPV VEF enhances the infectivity of this virus and concluded that enhancin increases the number of initial infections in the insect midgut, most likely due to its action on the peritrophic membrane (PM) of the insect larvae. These authors also speculate that encoding active enhancins may be beneficial to NPVs with large genomes resulting in bigger virus particle size, as a strategy to overcome host barriers, like PM.

AgseNPV-A and AgipNPV have similar gene content, however notable differences in individual ORFs are also observed. AgipNPV is missing 15 ORFs present in AgseNPV-A. Among them is *lef*-7, believed to encode a late expression factor required for optimal late gene expression and replication, at least in cell cultures. In group II NPVs, *lef-7* is present

only in AgseNPV-A, SeMNPV, SfMNPV and MacoNPV-A, whearas it is present in all group I NPVs.

Baculovirus homologous regions (*hrs*) in both AgseNPV-A and AgipNPV contain very similar palindromic repeats. Five *hrs* were identified in AgseNPV-A and seven in AgipNPV. The position of four of them is conserved in both genomes. Gene order is in general conserved between both viruses, with the only exception in the *he65*, *agse22/agip24*, *chitinase* region, where rearrangements can be observed. Rearrangements in this area are also observed in comparison to SeMNPV and MacoNPVs, viruses closely related to AgseNPV. Interestingly this region overlaps with *hr1* and *hr1a* in AgipNPV (in AgseNPV-A only *hr1*), and the *lef-7* gene is also juxtaposed with this region. It is known that baculoviruses possess several hypervariable regions (Garcia-Maruniak *et al.,* 1996; Muñoz *et al.,* 1999; Cory *et al.,* 2005). There has been speculation that variable regions may be linked to *hr* regions in baculoviruses (Majima *et al.,* 1993) and that variable regions are 'hot spots' for recombination. For instance, a significant number of indel mutations in HearGV and XecnGV was shown to occur next to *hr* sequences or *bro* genes, strongly suggesting that these regions may play an important role in recombination events leading to acquisition/deletion (indel mutations) of genes (Harrison, 2009).

A feature that distinguishes AgseNPV-A and AgipNPV from other baculoviruses is the arrangement of genes in the cluster of *lef-5*, *38K*, *odv*-e28 and *helicase*. While in most baculoviruses this cluster is highly conserved (Heldens *et al.,* 1998; Herniou *et al.,* 2003) and it was proposed that all four genes may be transcribed as a unit (Herniou *et al.,* 2003), in AgseNPV-A and AgipNPV this cluster is interrupted by the presence of enhancin(s) gene(s), one in AgipNPV and two in AgseNPV-A.

Baculovirus evolution and host specificity

Analysis of sequence data showed that the gene content of AgseNPV and *Spodoptera exigua* NPV (SeMNPV) is strikingly similar and that the order of shared genes shows 100% collinearity (Chapter 4). This high genomic collinearity as well as relatively high sequence similarity (average 70% identity of 30 core genes) prompted the question as to whether the two NPVs cross-infect their native hosts. Some baculoviruses, like the type species, AcMNPV, have a very broad host range comprising at least 43 insect species from 11 families (Payne, 1986). Others, like SeMNPV, cause fatal disease in only one insect species as far as we know.

Due to its broad host range AcMNPV-like viruses have been isolated from many different insect species and obviously given different names according to the accepted convention of naming virus isolates after the host in which the virus was first found. Sequencing the entire genomes of those isolates gave the possibility to shed some light on the virus specificity issue. The genomes of RoMNPV, AfMNPV and PlxyMNPV, three AcMNPV-like viruses, show only minor variations in size, gene content and nucleotide and

amino acids identities. Still their host range differs considerably and moreover the infectivity against certain insect species differs even more (Harrison, 2009).

The role of geographical separation in baculovirus evolution

AgseNPVs have been isolated only in Europe (Allaway & Payne, 1983; Lipa *et al.,* 1971) as its host, *A. segetum* is found only in Europe, while AgipNPV has been identified only in the United States (Boughton *et al.,* 1999; Prater *et al.,* 2006), but its host, *A. ipsilon* is found in both, the United States and Europe. This distribution of hosts and viruses provokes the hypothesis that both viruses are descendants from a common ancestor, which is supported by their phylogenies, and then separated into two species, by adapting to different hosts. *A. segetum* and *A. ipsilon*, are found in the same ecological niches. Since AgseNPV performs well in both hosts, AgseNPV may be the NPV naturally occurring in European populations of both insect species. However, it may be that the European AgipNPV has not yet been discovered. Interestingly it was reported that AgipNPV is equally effective against both *Agrotis* spp. while AgseNPV is about 40 times more effective on *A. segetum* than on *A. ipsilon* (El-Salamouny *et al.,* 2003). It is hard to find an explanation at this point for the equal AgipNPV performance in both insect species, given the fact that in natural conditions it never encounters *A. segetum* larvae. With the genetic information of both AgseNPV and AgipNPV, and the observation that both viruses replicate in a cell culture derived from the tissues of *A. ipsilon* the possibility to study this aspect further is now open.

Geographical heterogeneity in host-pathogen interactions is thought to play a major role in evolutionary processes (Cory & Myers, 2003). The pathogen likely adapts to the local host population, which is possible due to genetic variability in host and virus (Ebert, 1994). Many mechanisms for generating this variability in baculoviruses have been proposed in the literature, but none is entirely satisfactory.

L. salicis is a species native for Europe, however, at the beginning of 20th century it was introduced into North America (Langor, 1995). *O. pseudotsugata* is a native species for North America, and is believed not to occur in Europe. Since sequence analysis of a few core genes of two viruses isolated from these hosts, LesaNPV and OpMNPV, showed their very close relationship (98% nucleotide identity), it was hypothesized that these viruses originated from one virus species that evolved by adaptation to different host and environmental conditions and hence show considerable variation at the genetic level (Chapter 2). The Kimura-2 parameter proposed as a criterion for virus species demarcation is 0.010 for polyhedrin gene sequence of LesaNPV and OpMNPV, indicating that these two viruses represent actually the same virus species. Geographical separation might have led to speciation resulting in two viruses, from which one still is able to infect both insect species (LesaNPV), while the other is not (OpMNPV). LesaNPV and OpMNPV may exemplify different evolutional phenomena than AgseNPV and AgipNPV.

Specialists versus broad-host range viruses in relation to ecological parameters

Many forests insects belonging to the order Lepidoptera (often from the family Lymantriidae) and Hymenoptera are known for their population cycles (Myers, 1988). Populations of these species rise and fall over a predictable period of time. Baculoviruses isolated from these species appear to be specialists. Specialization may result from constant and predictable host availability, in annual cycles. Selection pressure is then switched to improved infectivity against one insect host. *Leucoma salicis* and *Orgyia pseudotsuagata* are examples of forest lepidopterans showing cyclic population outbreaks.

Inversely, crop pest species are often polyphagous and show population outbreaks, which are not necessarily cyclic, as this depends on crop management. Shortly after a population outbreak viral epizootics are likely to occur. High population densities provide the best conditions for the virus transmisson. In addition, different polyphagous pest species often co-habit the same environmental niche and co-infections with various virus species are very likely. Logically virus genotypes that are able to infect more insect species are fitter on these crops, as they have a bigger chance for survival.

Intuitively there should be a trade-off associated with a broad host range. Generalist viruses, which obviously are less specialized, would be expected to be less infective against their multiple hosts than the specialist viruses. But this is not the case. Generalist viruses, in most susceptible hosts, are also highly infectious (Goulson, 2003).

The use of molecular techniques which allows to distinguish virus genotypes revealed that the host is usually infected with a mixture of genotypes and that maintaining this mixture is evolutionary favorable because it enables fast adaptation to changing host and environment conditions (Simon *et al.,* 2004). In a given condition, especially in cyclic insect populations with regular outbreaks with a predictable time frame, a pathogen adapts to its host and some genotypes are more prevalent than others. A drastic change in genotype ratios can be seen when the virus is replicating in cell culture, where the number of defective genotypes almost immediately increases after just one passage (Heldens *et al.,* 1996, Pijlman *et al.,* 2001). Defective genotypes are also present in natural baculovirus populations and are critical for high infectivity (Muñoz *et al.,* 1998). Expanding the host range will be reflected in a shift in virus genotype ratios and may even lead to disappearance of some genotypes. Other mechanisms, like mutations (Pijlman *et al.,* 2001), homologous recombination (Arends & Jehle, 2002), transposon insertions (Jehle *et al.,* 1995) and horizontal gene transfer, including the introduction of host genes (Hughes & Friedman, 2003) allow optimal adaptation of the baculovirus to the local host and environmental conditions, which eventually may result in new virus species.

Host range determinants

Successful (fatal) infection of an insect involves a number of stages. However, the ability/inability of the virus to establish infection may depend on only a single mutation.

Croizier *et al.* (1994) reported that a change in only two amino acids of the AcMNPV helicase gene extends its host range to *Bombyx mori* larvae. Thus we can expect that the susceptibility of the host may often be a matter of chance of a single mutation!

SeMNPV is believed do be monospecific, infecting only *S. exigua*, however, it should be mentioned that host range studies for this virus are very limited and so far cover only six insect species (Gelernter & Federici, 1986; Smits & Vlak, 1988, Simon *et al.,* 2004). In this study (Chapter 6) it was shown that SeMNPV does not cause infection in *A. segetum* larvae *per os*, but only by intrahemocoelic injection this can be achieved. Thus in this case the midgut is the barrier preventing infection. Other examples of overcoming infection barriers by bypassing the midgut are given in literature. AcMNPV is known for its low infectivity in *Spodoptera frugiperda* fifth instar larvae *per os*, but when administered in the hemocoel, causes a full infection (Haas-Stapleton *et al.,* 2003). Oral infection starts when Occlusion Derived Virions (ODVs) are released from occlusion bodies (OBs) in the alkaline environment of the midgut after ingestion by the insect larvae. ODVs pass through the peritrophic membrane (PM) and fuse to midgut epithelial cells. These first events are crucial for establishing initial infection. While passing the PM is unlikely to be specific to the virus species (whatever the mechanism is, it has to be dependent only on the size of the ODV), ODV binding to the cell membrane and subsequent fusion is believed to be specific and hence determine the virus entry in the midgut. It has been confirmed that five proteins (P74, PIF1, PIF2, PIF-3 and PIF4) associated with the ODV envelope are indispensable for oral infectivity and deletion of any of those eradicates oral infectivity (Song *et al.,* 2008; Fang *et al.*, 2009). Thus *per os* infectivity factors (PIFs) are believed to be the main determinants of oral infectivity. In this study, SeMNPV was found non-infective for *A. segetum* larvae; however, transcription of the delayed early genes was detected in the midgut cells. This brings into question how specific PIFs are and it at least indicates that PIFs may be not the only determinants of oral infection. Apparently the virus infection can be blocked at any following stage and the entry to midgut cells does not necessarily result in a successful infection. Some events in the midgut cells obviously prevent development of secondary infection. Is this at the level of virus gene expression, DNA replication, assembly of new NCs or re-packaging of NCs into BVs (assuming that this scenario is possible), still remains unknown for SeMNPV infection in *A. segetum*.

Future aspects/perspectives

The elucidation of each genome sequence reveals new features. In the case of AgseNPV-A it is undoubtedly the presence of three enhancin genes. Investigation of the function of these genes is needed to unravel their contribution to the virulence of the virus. Transcriptional analysis showed that all AgseNPV enhancin genes are transcribed after infection of AiE1611T cell cultures. So far we do not know of all of them are actually expressed during the infection process and what the nature is of their putative interactions with the gut as well

as between them. Do these enhancin proteins work together in a synergistic interaction or do they performance independently of each other? The expression of all *vef* genes together or each gene separately can be silenced using RNA interference strategies (for instance dsRNA), which should result in occlusion bodies lacking VEFs. The biological activity of these deletion mutants may be assessed in bioassays to reveal the contribution of VEFs to viral activity.

There is a group of AgseNPV-A unique genes, as well as a group of common baculovirus genes which functions still remain unknown (Cohen *et al.,* 2009). Proteomic approaches may help to assess the timing of synthesis of these proteins during the course of infection and reveal possible interactions between viral proteins. A few AgseNPV-A ORFs are found only in AgseNPV-A and AgipNPV (*agse48, agse53* and *agse63*). These genes may be responsible for the effective cross-infectivity observed in *A. segetum* and *A. ipsilon*. Gene swapping approaches could shed light on virus specificity in the group of *Agrotis* spp. infecting viruses, which is now possible due to the availability of a cell line derived from *A. ipsilon* (AiE1611, Harrison & Lynn, 2008).

Detailed studies on specificity of *Agrotis* spp. viruses and SeMNPV in their native hosts will give further insight in virus interactions with the host and in which factors are responsible for virus host range. SeMNPV performance in *A. segetum* is of special interest, since although it was found not infectious against *A. segetum* larvae, its delayed-ealry transcripts were detected in midgut cells. Further analysis of the first moments of infection, the binding to midgut epithelial cells, may bring additional knowledge about the action of PIF proteins and their specificity.

LesaNPV and OpMNPV constitute another ideal model for studying virus specificity. While both viruses apparently represent the same virus species or are at least very closely related, LesaNPV causes a fatal infection in *O. pseudotsugata* larvae, but OpMNPV does not produce any level of infection in *L. salicis* larvae. Which factors (genes) determine the specificity of these viruses? Would one virus serve as a helper for the other in co-infection studies in the heterologous host? These questions remain so far open. Injecting OpMNPV in the hemocoel of *L. salicis* would give an answer if the midgut is the barrier for infection or, like in the case of SeMNPV in *A. segetum*, blocking of infection is beyond the midgut. Sequencing of LesaNPV using easily available sequencing techniques, and genome sequence comparison with OpMNPV which sequence is already available (Ahrens *et al.,* 1997) would be a good starting point for studying the relationship between these two NPVs.

Conclusion

The main outcome of the presented research is the gain of genetic information of two NPVs, AgseNPV and LesaNPV. AgseNPV and LesaNPV evolutionary traits indicate that these viruses are probably examples of two different aspects of baculovirus evolution. AgseNPV

and a group of viruses closely related to AgseNPV, like AgipNPV, are most likely descendants from a common ancestor, which then separated into two species, by adapting to different hosts. LesaNPV also evolved by adaptation to a new host, *Orgya pseudotsugata,* and is now better known as OpMNPV. Taxonomically speaking LeseNPV and OpMNPV are variants of the same species at this point in time, and they may evolve into separate species in the future. In the case of the *Agrotis* NPV species this event could have taken place much earlier in time, since LesaNPV was imported into North America at the beginning of the 20th century. The finding that the AgseNPV genome is collinear with SeMNPV is quite striking and suggests that this collinearity is a remnant of the evolutionary past of both viruses. The SeMNPV / AgseNPV system is now quite suitable for studying the underlying principles of host range specificity and baculovirus speciation and adaptation

The results obtained for AgseNPV and LesaNPV show once more the complications that may arise with the currrent baculovirus nomenclature. Present naming after the insect species was criticized here as very misleading; therefore I feel the need to propose an alternative, better option. New virus isolates may be named after the insect from which they were isolated but only after basic molecular characterization (for example of polyhedrin gene) and a thorough check in the database for similarity to any already described virus. This should be sufficient to prevent naming the same virus with two or more names. Of course it can still lead to the situation that the virus is given a name of one insect species and appears to be more pathogenic against another insect species. This however cannot be avoided unless a second option is followed and that is the establishmment of a whole new system of baculovirus nomenclature as has been the case for iridoviruses and adenoviruses (ICTV, 2008). Since sequence data are more trustful than laboratory bioassay data or field observations, the sequence information should be leading to drive the taxonomy and nomenclature.

References

Ackermann, H. W. & Smirnoff, W. A. (1983). A morphological investigation of 23 baculoviruses. *Journal of Invertebrate Pathology* **41**, 269-280.

Adang, M. J. & Spence, K. D. (1981). Surface morphology of peritrophic membrane formation in the cabbage looper, *Trichoplusia ni*. *Cell and Tissue Research* **218**, 141-147.

Afonso, C. L., Tulman, E. R., Lu, Z., Balinsky, C. A., Moser, B. A., Becnel, J. J., Rock, D. L. & Kutish, G. F. (2001). Genome sequence of a baculovirus pathogenic for *Culex nigripalpus*. *Journal of Virology* **75**, 11157-11165.

Ahrens, C. & Rohrmann, G. (1995). Identification of essential trans-acting regions required for DNA replication of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus: *lef-1* is an essential replication gene. *Virology* **407**, 417-428.

Ahrens, C. H. & Rohrmann, G. F. (1995). Replication of *Orgyia pseudotsugata* baculovirus DNA: *lef-2* and *ie-1* are essential and *ie-2*, *p34*, and Op-*iap* are stimulatory genes. *Virology* **212**, 650-662.

Ahrens, C. H., Russell, R. L., Funk, C. J., Evans, J. T., Harwood, S. H. & Rohrmann, G. F. (1997). The sequence of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. *Virology* **229**, 381-399.

Allaway, G. P. & Payne, C. C. (1983). A biochemical and biological comparison of three European isolates of nuclear polyhedrosis viruses from *Agrotis segetum*. *Archives of Virology* **75**, 43-54.

Alstein, A. D. (1992). The protocellular concept of the origin of viruses. *Seminars in Virology* **3**, 409-417.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Arends, H. M. & Jehle, J. A. (2002). Homologous recombination between the inverted terminal repeats of defective transposon TCp3.2 causes an inversion in the genome of *Cydia pomonella* granulovirus. *Journal of General Virology* **83**, 1573-1578.

Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M. & Possee, R. D. (1994). The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* **202**, 586-605.

Beckwith, R. (1978). Biology of the insect. In *The Douglas-fir Tussock Moth: A Synthesis United States Department of Agriculture Forest Service Technical Bulletin 1585*, pp. 25-30. Edited by M. H. Brookes, R. W. Stark & R. W. Campbell. Washington DC.

Becnel, J. J., White, S. E. & Shapiro, A. M. (2003). *Culex nigripalpus* nucleopolyhedrovirus (CuniNPV) infections in adult mosquitoes and possible mechanisms for dispersal. *Journal of Invertebrate Pathology* **83**, 181-183.

Benz, G. A. (1986). Introduction: historical perspectives. In *Biology of Baculoviruses*, pp. 1-35. Edited by R. R. Granados & B. Federici. Boca Raton, Florida: CRC Press.

Bird, F. T. & Elgee, D. E. (1957). A virus disease and introduced parasites as factors controlling the European spruce sawfly, *Diprion hercyniae* (Htg.), in central New Brunswick. *Canadian Entomologist* **89**, 371-378.

Bischoff, D. S. & Slavicek, J. M. (1997). Molecular analysis of an enhancin gene in the *Lymantria dispar* nuclear polyhedrosis virus. *Journal of Virology* **71**, 8133-8140.

Blissard, G. W., Black, B., Crook, N., Keddie, B.A., Possee, R., Rohrman, G. F., Theilman, D. A. & Volkman, L. (2000). Family *Baculoviridae*. In *Virus taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses*. Edited by M. H. V. van Regenmortel. California: Academic Press.

Blissard, G. W., Kogan, P. H., Wei, R. & Rohrmann, G. F. (1992). A synthetic early promoter from a baculovirus: roles of the TATA box and conserved start site CAGT sequence in basal levels of transcription. *Virology* **190**, 783-793.

Blissard, G. W. & Wenz, J. R. (1992). Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *Journal of Virology* **66**, 6829-6835.

Borodovsky, M. & McIninch, J. (1993). GeneMark: Parallel gene recognition for both DNA strands. *Computers & Chemistry* **17**, 123-133.

Boughton, A. J., Harrison, R. L., Lewis, L. C. & Bonning, B. C. (1999). Characterization of a nucleopolyhedrovirus from the black cutworm, *Agrotis ipsilon* (Lepidoptera: Noctuidae). *Journal of Invertebrate Pathology* **74**, 289-294.

Bourner, T. C. & Cory, J. S. (2004). Host range of an NPV and a GV isolated from the common cutworm, *Agrotis segetum*: pathogenicity within the cutworm complex. *Biological Control* **31**, 372.

Bourner, T. C., Vargas-Osuna, E., Williams, T., Santiago-Alvarez, C. & Cory, J. S. (1992). A comparison of the efficacy of nuclear polyhedrosis and granulosis viruses in spray and bait formulations for the control of *Agrotis segetum* (Lepidoptera: Noctuidae) in maize. *Biocontrol Science & Technology* **2**, 315-326.

Breitbart, M., Salamon, P., Andresen, B., Mahaffy, J. M., Segall, A. M., Mead, D., Azam, F. & Rohwer, F. (2002). Genomic analysis of uncultured marine viral communities. *Proceedings of the National Academy of Sciences USA* **99**, 14250–14255.

Bulach, D. M., Kumar, C. A., Zaia, A., Liang, B. & Tribe, D. E. (1999). Group II nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedrin and DNA polymerase gene sequences. *Journal of Invertebrate Pathology* **73**, 59-73.

Bull, J. C., Godfray, H. C. & O'Reilly, D. R. (2003). A few-polyhedra mutant and wild-type nucleopolyhedrovirus remain as a stable polymorphism during serial coinfection in *Trichoplusia ni*. *Applied and Environmental Microbiology* **69**, 2052-2057.

Burges, H. D., Croizer, G. & Huber, L. (1980). A review of safety tests on baculoviruses. *Entomophaga* **25**, 329-340.

Caballero, P., Vargas-Osuna, E. & Santiago-Alvarez, C. (1991). Efficacy of a Spanish strain of *Agrotis segetum* granulosis virus (*Baculoviridae*) against *Agrotis segetum* Schiff (Lep., Noctuidae) on corn. *Journal of Applied Entomology* **112**, 59-64.

Carstens, E. B. & Ball, L. A. (2009). Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2008). *Archives of Virology* **154**, 1181-1188.

Chen, C. J., Quentin, M. E., Brennan, L. A., Kukel, C. & Thiem, S. M. (1998). *Lymantria dispar* nucleopolyhedrovirus hrf-1 expands the larval host range of *Autographa californica* nucleopolyhedrovirus. *Journal of Virology* **72**, 2526-2531.

Chen, X., WF, I. J., Tarchini, R., Sun, X., Sandbrink, H., Wang, H., Peters, S., Zuidema, D., Lankhorst, R. K., Vlak, J. M. & Hu, Z. (2001). The sequence of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus genome. *Journal of General Virology* **82**, 241-257.

Chen, X., Zhang, W. J., Wong, J., Chun, G., Lu, A., McCutchen, B. F., Presnail, J. K., Herrmann, R., Dolan, M., Tingey, S., Hu, Z. H. & Vlak, J. M. (2002). Comparative analysis of the complete genome sequences of *Helicoverpa zea* and *Helicoverpa armigera* single-nucleocapsid nucleopolyhedroviruses. *Journal of General Virology* **83**, 673-684.

Chen, Y. R., Wu, C. Y., Lee, S. T., Wu, Y. J., Lo, C. F., Tsai, M. F. & Wang, C. H. (2008). Genomic and host range studies of *Maruca vitrata* nucleopolyhedrovirus. *Journal of General Virology* **89**, 2315-2330.

Cheng, X. W., Carner, G. R. & Fescemyer, H. W. (1998). Polyhedrin sequence determines the tetrahedral shape of occlusion bodies in *Thysanoplusia orichalcea* single-nucleocapsid nucleopolyhedrovirus. *Journal of General Virology* **79**, 2549-2556.

Chou, C. M., Huang, C. J., Lo, C. F., Kou, G. H. & Wang, C. H. (1996). Characterization of *Perina nuda* nucleopolyhedrovirus (PenuNPV) polyhedrin gene. *Journal of Invertebrate Pathology* **67**, 259-266.

Clarke, E. E., Tristem, M., Cory, J. S. & O'Reilly, D. R. (1996). Characterization of the ecdysteroid UDP-glucosyltransferase gene from *Mamestra brassicae* nucleopolyhedrovirus. *Journal of General Virology* **77** 2865-2871.

Clem, R. J. & Miller, L. K. (1994). Control of programmed cell death by the baculovirus genes *p35* and *iap*. *Molecular Cell Biology* **14**, 5212-5222.

Cochran, M. A. & Faulkner, P. (1983). Location of homologous DNA sequences interspersed at five regions in the baculovirus AcMNPV genome. *Journal of Virology* **45**, 965-970.

Cohen, D. P. A., Marek, M., Davies, B. G., Vlak, J. M. & van Oers, M. M. (2009). Encyclopedia of *Autographa californica* nucleopolyhedrovirus genes. *Virologica Sinica* **24**, 359-414.

Condreay, J. P. & Kost, T. A. (2007). Baculovirus expression vectors for insect and mammalian cells. *Current Drug Targets* **8**, 1126-1131.

Cory, J. S. (2003). Ecological impacts of virus insecticides: host range and non-target organisms. In *Environmental Impacts of Microbial Insecticides*, pp. 73-91. Edited by H. M. T. Hokkanen & A. E. Hajek: BIOS.

Cory, J. S., Green, B. M., Paul, R. K. & Hunter-Fujita, F. (2005). Genotypic and phenotypic diversity of a baculovirus population within an individual insect host. *Journal of Invertebrate Pathology* **89**, 101-111.

Cory, J. S., Hirst, M. L., Sterling, P. H. & Speight, M. R. (2000). Narrow host range nucleopolyhedrovirus for control of the brown-tail moth, *Euproctis chrysorrhoea* (L) (Lepidoptera: Lymantriidae). *Environmental Entomology* **29**, 661–667.

Cory, J. S. & Myers, J. H. (2003). The ecology and evolution of insect baculoviruses. *Annual Review of Ecology, Evolution and Systemics* **34**, 239-272.

Croizier, G., Croizier, L., Argaud, O. & Poudevigne, D. (1994). Extension of *Autographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the p143 helicase gene. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 48-52.

Davison, A. J., Dargan, D. J. & Stow, N. D. (2002). Fundamental and accessory systems in herpesviruses. *Antiviral Research* **56**, 1-11.

de Castro Oliveira, J. V., Wolff, J. L. C., Garcia-Maruniak, A., Ribeiro, B. M., de Castro, M. E. B., de Souza, M. L., Moscardi, F., Maruniak, J. E. & de Andrade Zanotto, P. M. (2006). Genome of the most widely used viral biopesticide: *Anticarsia gemmatalis* multiple nucleopolyhedrovirus. *Journal of General Virology* **87**, 3233-3250.

de Jong, J. G., Lauzon, H. A. M., Dominy, C., Poloumienko, A., Carstens, E. B., Arif, B. M. & Krell, P. J. (2005). Analysis of the *Choristoneura fumiferana* nucleopolyhedrovirus genome. *Journal of General Virology* **86**, 929-943.

De Moraes, R. R. & Maruniak, J. E. (1997). Detection and identification of multiple baculoviruses using the polymerase chain reaction (PCR) and restriction endonuclease analysis. *Journal of Virological Methods* **63**, 209-217.

Derksen, A. C. & Granados, R. R. (1988). Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. *Virology* **167**, 242-250.

Domingo, E., Escarmis, C., Sevilla, N., Moya, A., Elena, S. F., Quer, J., Novella, I. S. & Holland, J. J. (1996). Basic concepts in RNA virus evolution. *FASEB Journal* **10**, 859-864.

Donaubauer, E. (1976). The use of bacteria and viruses in the control of forest pests. *Allgemeine-Forstzeitung* **87**, 112-114.

Doyle, C. J., Hirst, M. L., Cory, J. S. & Entwistle, P. F. (1990). Risk assessment studies: detailed host range testing of wild-type cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae), nuclear polyhedrosis virus. *Applied Environmental Microbiology* **56**, 2704–2710.

Duffy, S. P., Young, A. M., Morin, B., Lucarotti, C. J., Koop, B. F. & Levin, D. B. (2006). Sequence analysis and organization of the *Neodiprion abietis* nucleopolyhedrovirus genome. *Journal of Virology* **80**, 6952-6963.

Ebert, D. (1994). Virulence and local adaptation of a horizontally transmitted parasite. *Science* **265**, 1084-1086.

El Salamouny, S., Lange, M., Jutzi, M., Huber, J. & Jehle, J. (2003). Comparative study on the susceptibility of cutworms (Lepidoptera: Noctuidae) to *Agrotis segetum* nucleopolyhedrovirus and *Agrotis ipsilon* nucleopolyhedrovirus *Journal of Invertebrate Pathology* **84**, 75-82.

Engelhard, E. K., Kam-Morgan, L. N., Washburn, J. O. & Volkman, L. E. (1994). The insect tracheal system: a conduit for the systemic spread of *Autographa californica* M nuclear polyhedrosis virus. *Proceedings of the National Academy of Sciences USA* **91**, 3224-3227.

Entwistle, P. F., Forkner, A. C., Green, B. M. & Cory, J. S. (1993). Avian dispersal of nuclear polyhedrosis viruses after induced epizootics in the pine beauty moth, *Panolis flammea* (Lepidoptera: Noctuidae). *Biological Control* **3**, 61-69.

Escasa, S. R., Lauzon, H. A. M., Mathur, A. C., Krell, P. J. & Arif, B. M. (2006). Sequence analysis of the *Choristoneura occidentalis* granulovirus genome. *Journal of General Virology* **87**, 1917-1933.

Evans, H. F., Lomer, C. J. & Kelly, D. C. (1981). Growth of nuclear polyhedrosis virus in larvae of the cabbage moth, *Mamestra brassicae* L. *Archives of Virology* **70**, 207-214.

Eveden, J. & Jost, E. J. (1947). A report of the tussock moth control, North Idaho, p. 55: U.S. Department of Agriculture, Forest Service, Idaho State Forest.

Fan, Q., Li, S., Wang, L., Zhang, B., Ye, B., Zhao, Z. & Cui, L. (2007). The genome sequence of the multinucleocapsid nucleopolyhedrovirus of the Chinese oak silkworm *Antheraea pernyi*. *Virology* **366**, 304-315.

Fang, M., Nie, Y., Harris, S., Erlandson, M.A., Theilmann, D.A. (2009).*Autographa californica* multiple nucleopolyhedrovirus core gene ac96 encodes a *per os* infectivity factor (PIF-4). *Journal of Virology* **83**:12569-12578.

Faulkner, P., Kuzio, J., Williams, G. V. & Wilson, J. A. (1997). Analysis of p74, a PDV envelope protein of *Autographa californica* nucleopolyhedrovirus required for occlusion body infectivity in vivo. *Journal of General Virology* **78**, 3091–3100.

Fauquet, C. M. & Fargette, D. (2005). International Committee on Taxonomy of Viruses and the 3,142 unassigned species. *Virology Journal* **2**, 64.

Federici, B. (1997). Baculovirus pathogenesis. In *The Baculoviruses*, pp. 33-59. Edited by L. K. Miller. New York: Plenum Press.

Federici, B. A. (1999). Naturally occuring baculoviruses for insect pest control. *Methods in Biotechnology* **5**, 301-320.

Federici, B. A. & Hice, R. H. (1997). Organization and molecular characterization of genes in the polyhedrin region of the *Anagrapha falcifera* multinucleocapsid NPV. *Archives of Virology* **142**, 333-348.

Finney, D. J. (1952). *Probit analysis: a statistical treatment of a sigmoid response curve*. London and NY: Cambridge University Press.

Friesen, P. D. & Miller, L. K. (1986). The regulation of baculovirus gene expression. *Current Topics in Microbiology and Immunology* **131**, 31-49.

Friesen, P. D. & Miller, L. K. (1987). Divergent transcription of early 35- and 94-kilodalton protein genes encoded by the HindIII K genome fragment of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* **61**, 2264-2272.

Fuchs, L. Y., Woods, M. S. & Weaver, R. F. (1983). Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: A novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *Journal of Virology* **48**, 641-646.

Funk, C. J., Braunagel, S. C. & Rohrmann, G. (1997). Baculovirus structure. In *The Baculoviruses*, pp. 7-32. Edited by L. K. Miller. New York: Plenum Press.

Galloway, C. S., Wang, P., Winstanley, D. & Jones, I. M. (2005). Comparison of the bacterial enhancin-like proteins from *Yersinia* and *Bacillus* spp. with a baculovirus enhancin. *Journal of Invertebrate Pathology* **90**, 134-137.

Gao, L. & Qi, J. (2007). Whole genome molecular phylogeny of large dsDNA viruses using composition vector method. *BMC Evolutionary Biology* **7**.

Garcia-Maruniak, A., Maruniak, J. E., Zanotto, P. M. A., Doumbouya, A. E., Liu, J.-C., Merritt, T. M. & Lanoie, J. S. (2004). Sequence analysis of the genome of the *Neodiprion sertifer* nucleopolyhedrovirus. *Journal of Virology* **78**, 7036-7051.

Garcia-Maruniak, A., Pavan, O. H. & Maruniak, J. E. (1996). A variable region of *Anticarsia gemmatalis* nuclear polyhedrosis virus contains tandemly repeated DNA sequences. *Virus Research* **41**, 123-132.

Gelernter, W. D. & Federici, B. A. (1986). Continuous cell line from *Spodoptera exigua* (Lepidoptera: Noctuidae) that supports replication of nuclear polyhedrosis viruses from *Spodoptera exigua* and *Autographa californica*. *Journal of Invertebrate Pathology* **48**, 199-207.

Gelernter, W. D. & Federici, B. A. (1986). Isolation, identification and determination of virulence of a nuclear polyhedrosis virus from the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environmental Entomology* **15**, 240-245.

Gibbs, A., Calisher, C. H. & Garcia-Arenal, F. (1995). *Molecular basis of virus evolution*. Cambridge, UK.

Gomi, S., Majima, K. & Maeda, S. (1999). Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. *Journal of General Virology* **80**, 1323-1337.

Goto, C., Hayakawa, T. & Maeda, S. (1998). Genome organization of *Xestia c-nigrum* granulovirus. *Virus Genes* **16**, 199-210.

Goulson, D. (1997). Wipfelkrankheit modification of host behaviour during baculoviral infection. *Oecologia (Berl)* **109**, 219-228.

Goulson, D. (2003). Can host susceptibility to baculovirus infection be predicted from host taxonomy or life history? *Environmental Entomology* **32**, 61-70.

Graham, R. I., Tyne, W. I., Possee, R. D., Sait, S. M. & Halis, R. S. (2004). Genetically variable nucleopolyhedroviruses isolated from spatially separate populations of the winter moth *Operophtera brumata* (Lepidoptera: Geometridae) in Orkney. *Journal of Invertebrate Pathology* **87**, 29-38.

Granados, R. R. (1978). Early events in the infection of *Heliothis zea* midgut cells by a baculovirus. *Virology* **90**, 170-174.

Granados, R. R. & Lawler, K. A. (1981). *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. *Virology* **108**, 297-308.

Grijpma, P., Persoons, C. J., Peters, D. & Vlak, J. M. (1986). Biological control of the satin moth with pheromones and baculoviruses. In *Proceedings of the European seminar on wood production and harvesting* pp. 37-42. Bologna, Italy.

Grula, M. A., Buller, P. L. & Weaver, R. F. (1981). α – Amanitin-resistant viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus-infected *Heliothis zea* larvae and *Spodoptera frugiperda* cells. *Journal of Virology* **38**, 916-921.

Guarino, L. A., Gonzales, M. A. & Summers, M. D. (1986). Complete sequence and enhancer function of the homologous DNA regions of Autographa californica nuclear polyhedrosis virus. *Journal of General Virology* **60**, 224-229.

Guarino, L. A., Jin, J. & Dong, W. (1998). Guanylyltransferase activity of the LEF-4 subunit of baculovirus RNA polymerase. *Journal of Virology* **72**, 10003-10010.

Guarino, L. A. & Summers, M. D. (1986). Functional mapping of a trans-activating gene required for expression of a baculovirus delayed-early gene. *Journal of Virology* **57**, 563-571.

Haas-Stapleton, E. J., Washburn, J. O. & Volkman, L. E. (2003). Pathogenesis of *Autographa californica* M nucleopolyhedrovirus in fifth instar *Spodoptera frugiperda*. *Journal of General Virology* **84**, 2033-2040.

Haas-Stapleton, E. J., Washburn, J. O. & Volkman, L. E. (2004). P74 mediates specific binding of *Autographa californica* M nucleopolyhedrovirus occlusion-derived virus to primary cellular

targets in the midgut epithelia of *Heliothis virescens* larvae. *Journal of Virology* **78**, 6786-6791.

Hajos, J. P., Pijnenburg, J., Usmany, M., Zuidema, D., Zavodszky, P. & Vlak, J. M. (2000). High frequency recombination between homologous baculoviruses in cell culture. *Archives of Virology* **145**, 159-164.

Hara, K., Funakoshi, M., Tsuda, K. & Kawarabata, T. (1993). New *Spodoptera exigua* cell lines susceptible to *Spodoptera exigua* nuclear polyhedrosis virus. *In Vitro Cellular and Developmental Biology* **29**, 904-907.

Harrison, R. L. (2009). Genomic sequence analysis of the Illinois strain of the *Agrotis ipsilon* multiple nucleopolyhedrovirus. *Virus Genes* **38**, 155-170.

Harrison, R. L. & Bonning, B. C. (1999). The nucleopolyhedroviruses of *Rachiplusia ou* and *Anagrapha falcifera* are isolates of the same virus. *Journal of General Virology* **80**, 2793-2798.

Harrison, R. L. & Bonning, B. C. (2003). Comparative analysis of the genomes of *Rachiplusia ou* and *Autographa californica* multiple nucleopolyhedroviruses. *Journal of General Virology* **84**, 1827-1842.

Harrison, R. L. & Bonning, B. C. (2004). Application of maximum-likelihood models to selection pressure analysis of group I nucleopolyhedrovirus genes. *Journal of General Virology* **85**, 197-210.

Harrison, R. L. & Jarvis, D. L. (2006). Protein N-glycosylation in the baculovirus-insect cell expression system and engineering of insect cells to produce 'mammalianized' recombinant glycoprotein. *Advances in Virus Research* **68**, 159-191.

Harrison, R. L. & Jarvis, D. L. (2007). Transforming lepidopteran insect cells for improved protein processing. In *Baculovirus and Insect Cell Expression Protocols*, Second edn., pp. 341-356. Edited by D.W. Murhammer: Humana Press.

Harrison, R. L. & Lynn, D. E. (2007). Genomic sequence analysis of a nucleopolyhedrovirus isolated from the diamondback moth, *Plutella xylostella*. *Virus Genes* **35**, 857-873.

Harrison, R. L. & Lynn, D. E. (2008). New cell lines derived from the black cutworm, *Agrotis ipsilon*, that support replication of the *A. ipsilon* multiple nucleopolyhedrovirus and several group I nucleopolyhedroviruses. *Journal of Invertebrate Pathology* **99**, 28-34.

Harrison, R. L. & Popham, H. J. R. (2008). Genomic sequence analysis of a granulovirus isolated from the Old World bollworm, *Helicoverpa armigera*. *Virus Genes* **36**, 565-581.

Harrison, R. L., Puttler, B. & Popham, H. J. R. (2008). Genomic sequence analysis of a fast-killing isolate of *Spodoptera frugiperda* multiple nucleopolyhedrovirus. *Journal of General Virology* **89**, 775-790.

Hashimoto, Y., Hayakawa, T., Ueno, Y., Fujita, T., Sano, Y. & Matsumoto, T. (2000). Sequence analysis of the *Plutella xylostella* granulovirus genome. *Virology* **275**, 358-372.

Hawtin, R. E., Arnold, K., Ayres, M. D., Zanotto, P. M., Howard, S. C., Gooday, G. W., Chappell, L. H., Kitts, P. A., King, L. A. & Possee, R. D. (1995). Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. *Virology* **212**, 673-685.

Hayakawa, T., Ko, R., Okano, K., Seong, S. I., Goto, C. & Maeda, S. (1999). Sequence analysis of the *Xestia c-nigrum* granulovirus genome. *Virology* **262**, 277-297.

Hefferon, K. L. (2003). Characterization of HCF-1, a determinant of *Autographa californica* multiple nucleopolyhedrovirus host specificity. *Insect Molecular Biology* **12**, 651-658.

Hefferon, K. L., Oomens, A. G. P., Monsma, S. A., Finnerty, C. M. & Blissard, G. W. (1999). Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. *Virology* **258**, 455-468.

Hegedus, D. D., Erlandson, M. A., Gillott, C. & Toprak, U. (2009). New insights into peritrophic matrix synthesis, architecture, and function. *Annual Review of Entomology* **54**, 285-302.

Heldens, J. G., Liu, Y., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1998). A highly conserved genomic region in baculoviruses: sequence analysis of an 11.3 kbp DNA fragment (46.5-55.1 m.u.) of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus. *Virus Research* **55**, 187-198.

Heldens, J. G. M., Van Strien, E. A., Feldmann, A. M., Kulcsar, P., Muñoz, D., Leisy, D. J., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1996). *Spodoptero exigua* multicapsid nucleopolyhedrovirus deletion mutants generated in cell culture lack virulence in vivo. *Journal of General Virology* **77**, 3127-3134.

Herniou, E. A. (2003). Use of comparative genomics and phylogenetics to study the evolution of the *Baculoviridae*. In *Centre for Ecology & Hydrology*, p. 211. London: Thesis. Imperial College.

Herniou, E. A. & Jehle, J. A. (2007). Baculovirus phylogeny and evolution. *Current Drug Targets* **8**, 1043-1050.

Herniou, E. A., Luque, T., Chen, X., Vlak, J. M., Winstanley, D., Cory, J. S. & O'Reilly, D. R. (2001). Use of whole genome sequence data to infer baculovirus phylogeny. *Journal of Virology* **75**, 8117-8126.

Herniou, E. A., Olszewski, J. A., Cory, J. S. & O'Reilly, D. R. (2003). The genome sequence and evolution of baculoviruses. *Annual Review of Entomology* **48**, 211-234.

Herniou, E. A., Olszewski, J. A., O'Reilly, D. R. & Cory, J. S. (2004). Ancient coevolution of baculoviruses and their insect hosts. *Journal of Virology* **78**, 3244-3251.

Hilton, S. & Winstanley, D. (2008). Genomic sequence and biological characterization of a nucleopolyhedrovirus isolated from the summer fruit tortrix, *Adoxophyes orana*. *Journal of General Virology* **89**, 2898-2908.

Hinks, C. F. & Byers, J. R. (1976). Biosystematics of the genus Euoxa (Lepidoptera: Noctuidae). V. Rearing procedures and life cycles of 36 species. *Canadian Entomologist* **108**, 1345 –1357.

Hodgson, D. J., Hitchman, R. B., Vanbergen, A. J., Hails, R. S., R.D., P. & Cory, J. S. (2004). Host ecology determines the relative fitness of virus genotypes in mixed-genotype nucleopolyhedrovirus infections. *Journal of Evolutionary Biology* **17**, 1018-1025.

Hofmann, C., Sandig, V., Jennings, G., Rudolph, M., Schlag, P. & Strauss, M. (1995). Efficient gene transfer into human hepatocytes by baculovirus vectors. *Proceedings of the National Academy of Sciences USA* **92**, 10099-10103.

Hong, T., Summers, M. D. & Braunagel, S. C. (1997). N-terminal sequences from *Autographa californica* nuclear polyhedrosis virus envelope proteins ODV-E66 and ODV-E25 are sufficient to direct reporter proteins to the nuclear envelope, intranuclear microvesicles and the envelope of occlusion derived virus. *Proceedings of the National Academy of Sciences USA* **94**, 4050-4055.

Horton, H. M. & Burand, J. P. (1993). Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. *Journal of Virology* **67**, 1860-1868.

Hu, J., Ding, H. & Wu, X. (1993). Cloning and sequencing of *Attacus ricini* nuclear polyhedrosis virus polyhedrin gene. *Acta Genetica Sinica* **20**, 300-304.

Hu, Y. C. (2008). Baculoviral vectors for gene delivery: A review. *Current Gene Therapy* **8**, 54-65.

Hu, Z., Arif, B., Jin, F., Martens, J., Chen, X., Sun, J., Zuidema, D., Goldbach, R. & Vlak, J. (1998). Distinct gene arrangement in the *Buzura suppressaria* single nucleocapsid nucleopolyhedrovirus genome. *Journal of General Virology* **79**, 2841-2851.

Hughes, A. L. & Friedman, R. (2003). Genome-wide survey for genes horizontally transferred from cellular organisms to baculoviruses. *Molecular Biology and Evolution* **20**, 979-987.

Hughes, K. M. (1976). Notes on the nuclear polyhedrosis viruses of tussock moths of the genus *Orgyia* (Lepidoptera). *Canadian Entomologist* **108**, 479-484.

Hughes, K. M. & Addison, R. B. (1970). Two nuclear polyhedrosis viruses of the Douglas-fir tussock moth. *Journal of Invertebrate Pathology* **16**, 196-204.

Hunter-Fujita, F. R., Entwistle, P. F., Evans, H. F. & Crook, N. E. (1998). *Insect Viruses and Pest Management*. New York: John Wiley & Sons..

Hyink, O., Dellow, R. A., Olsen, M. J., Caradoc-Davies, K. M., Drake, K., Herniou, E. A., Cory, J. S., O'Reilly, D. R. & Ward, V. K. (2002). Whole genome analysis of the *Epiphyas postvittana* nucleopolyhedrovirus. *Journal of General Virology* **83**, 957-971.

Ignoffo, C. M. & Garcia, C. (1979). Susceptibility of larvae of the black cutworm to species of entomopathogenic bacteria, fungi, protozoa and viruses. *Journal of Economical Entomology* **72**, 767-769.

IJkel, W., Lebbink, R. J., Op den Brouw, M. L., Goldbach, R. W., Vlak, J. M. & Zuidema, D. (2001). Identification of a novel occlusion derived virus-specific protein in *Spodoptera exigua* multicapsid nucleopolyhedrovirus. *Virology* **284**, 170-181.

IJkel, W., van Strien, E. A., Heldens, J. G., Broer, R., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1999). Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. *Journal of General Virology* **80**, 3289-3304.

IJkel, W., Westenberg, M., Goldbach, R. W., Blissard, G. W., Vlak, J. M. & Zuidema, D. (2000). A novel baculovirus envelope fusion protein with a proprotein convertase cleavage site. *Virology* **275**, 30-41.

Ikeda, M., Shikata, M., Shirata, N., Chaeychomsri, S. & Kobayashi, M. (2006). Gene organization and complete sequence of the *Hyphantria cunea* nucleopolyhedrovirus genome. *Journal of General Virology* **87**, 2549-2562.

Iyer, L. M., Aravind, L. & Koonin, E. V. (2001). Common origin of four diverse families of large eukaryotic DNA viruses. *Journal of Virology* **75**, 11720-11734.

Iyer, L. M., Balaji, S., Koonin, E. V. & Aravind, L. (2006). Evolutionary genomics of nucleo-cytoplasmic large DNA viruses. *Virus Research* **117**, 156-184.

Jakubowska, A., van Oers, M. M., Cory, J. S., Ziemnicka, J. & Vlak, J. M. (2005). European *Leucoma salicis* NPV is closely related to North American *Orgyia pseudotsugata* MNPV. *Journal of Invertebrate Pathology* **88**, 100-107.

Jakubowska, A. K., Peters, S. A., Ziemnicka, J., Vlak, J. M. & van Oers, M. M. (2006). Genome sequence of an enhancin gene-rich nucleopolyhedovirus (NPV) from *Agrotis segetum*: Collinearity with *Spodoptera exigua* multiple NPV. *Journal of General Virology* **87**, 537-551.

Jakubowska, A. K., van Oers, M. M., Ziemnicka, J., Lipa, J. J. & Vlak, J. M. (2005). Molecular characterization of *Agrotis segetum* nucleopolyhedrovirus from Poland. *Journal of Invertebrate Pathology* **90**, 64-68.

Jehle, J. A. (2004). The mosaic structure of the polyhedrin gene of the *Autographa californica* nucleopolyhedrovirus (AcMNPV). *Virus Genes* **29**, 5-8.

Jehle, J. A., Blissard, G. W., Bonning, B. C., Cory, J. S., Herniou, E. A., Rohrmann, G. F., Theilmann, D. A., Thiem, S. M. & Vlak, J. M. (2006). On the classification and nomenclature of baculoviruses: A proposal for revision. *Archives of Virology* **151**, 1257-1266.

Jehle, J. A., Fritsch, E., Nickel, A., Huber, J. & Backhaus, H. (1995). TCl4.7: A novel lipidopteran transposon found in *Cydia pomonella* granulosis virus. *Virology* **207**, 369-379.

Jehle, J. A., Lange, M., Wang, H., Hu, Z., Wang, Y. & Hauschild, R. (2006). Molecular identification and phylogenetic analysis of baculoviruses from Lepidoptera. *Virology* **346**, 180-193.

Jehle, J. A., Nickel, A., Vlak, J. M. & Backhaus, H. (1998). Horizontal escape of the novel Tc1-like Lepidopteran transposon TCp3.2 into *Cydia pomonella* granulovirus *Journal of Molecular Evolution* **46**, 215-224.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111-120.

Kislev, N. & Edelman, M. (1982). DNA restriction pattern differences from geographic isolates of *Spodoptera littoralis* nuclear polyhedrosis virus. *Virology* **119**, 219-222.

Kogan, P. H., Chen, X. & Blissard, G. W. (1995). Overlapping TATA-dependent and TATA-independent early promoter activities in the baculovirus gp64 envelope fusion protein gene. *Journal of Virology* **69**, 1452-1461.

Kolodny-Hirsch, D. M., Sitchawat, T., Jansiri, T., Chenrchaivachirakul, A. & Ketunuti, U. (1997). Field evaluation of a commercial formulation of the *Spodoptera exigua* (Lepidoptera: Noctuidae) nuclear polyhedrosis virus for control of beet armyworm on vegetable crops in Thailand. *Biocontrol Science and Technology* **7**, 475-458.

Kool, M., Ahrens, C. H., Vlak, J. M. & Rohrmann, G. F. (1995). Replication of baculovirus DNA. *Journal of General Virology* **76**, 2103-2118.

Kool, M., Voeten, J. T., Goldbach, R. W., Tramper, J. & Vlak, J. M. (1993). Identification of seven putative origins of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus DNA replication. *Journal of General Virology* **74**, 2661-2668.

Kornberg, A. & Baker, T. A. (1992). *DNA replication*. New York: Plenum Press.

Krappa, R., Behn-Krappa, A., Jahnel, F., Doerfler, W. & Knebel-Morsdorf, D. (1992). Differential factor binding at the promoter of early baculovirus gene PE38 during viral infection: GATA motif is recognized by an insect protein. *Journal of Virology* **66**, 3494-3503.

Kumar, S. & Subramanian, S. (2002). Mutation rates in mammalian genomes. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 803-808.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in bioinformatics* **5**, 150-163.

Kuzio, J., Pearson, M. N., Harwood, S. H., Funk, C. J., Evans, J. T., Slavicek, J. M. & Rohrmann, G. F. (1999). Sequence and analysis of the genome of a baculovirus pathogenic for *Lymantria dispar*. *Virology* **253**, 17-34.

Lameris, A. M. C., Ziemnicka, J., Peters, D., Grijpma, P. & Vlak, J. M. (1985). Potential of baculoviruses for control of the satin moth, *Leucoma salicis* L. (Lepidoptera: Lymantriidae). *Mededelingen Faculteit Landbouwwetenschappen, Rijksuniversiteit Gent (Belgium)* **50**, 431-439.

Lange, M. & Jehle, J. A. (2003). The genome of the *Cryptophlebia leucotreta* granulovirus. *Virology* **317**, 220-236.

Lange, M., Wang, H., Zhihong, H. & Jehle, J. A. (2004). Towards a molecular identification and classification system of lepidopteran-specific baculoviruses. *Virology* **325**, 36-47.

Langor, D. W. (1995). Satin moth. *Forestry Leaflet 35, Edmonton, Alberta: Natural Resources Canada, Canadian Forest Service, Northern Forest Center.*

Lanier, L. M. & Volkman, L. E. (1998). Actin binding and nucleation by *Autographa california* M nucleopolyhedrovirus. *Virology* **243**, 167-177.

Lauzon, H. A. M., Jamieson, P. B., Krell, P. J. & Arif, B. M. (2005). Gene organization and sequencing of the *Choristoneura fumiferana* defective nucleopolyhedrovirus genome. *Journal of General Virology* **86**, 945-961.

Lauzon, H. A. M., Lucarotti, C. J., Krell, P. J., Feng, Q., Retnakaran, A. & Arif, B. M. (2004). Sequence and organization of the *Neodiprion lecontei* nucleopolyhedrovirus genome. *Journal of Virology* **78**, 7023-7035.

Lee, H. Y., Arif, B., Dobos, P. & Krell, P. (1992). Identification of bent DNA and ARS fragment in the genome of *Choristoneura fumiferana* nuclear polyhedrosis virus. *Virus Research* **24**, 249-264.

Leisy, D., Nesson, M., Pearson, M., Rohrmann, G. F. & Beaudreau, G. (1986). Location and nucleotide sequence of the *Orgyia pseudotsugata* single nucleocapsid nuclear polyhedrosis virus polyhedrin gene. *Journal of General Virology* **67**, 1073-1079.

Leisy, D., Rohrmann, G. & Beaudreau, G. (1986). The nucleotide sequence of the polyhedrin gene region from the multicapsid baculovirus of *Orgyia pseudotsugata*. *Virology* **153**, 280-288.

Lepore, L. S., Roelvink, P. R. & Granados, R. R. (1996). Enhancin, the granulosis virus protein that facilitates nucleopolyhedrovirus (NPV) infections, is a metalloprotease. *Journal of Invertebrate Pathology* **68**, 131-140.

Li, L., Donly, C., Li, Q., Willis, L. G., Keddie, B. A., Erlandson, M. A. & Theilmann, D. A. (2002). Identification and genomic analysis of a second species of nucleopolyhedrovirus isolated from *Mamestra configurata*. *Virology* **297**, 226-244.

Li, L., Li, Q., Willis, L. G., Erlandson, M., Theilmann, D. A. & Donly, C. (2005). Complete comparative genomic analysis of two field isolates of *Mamestra configurata* nucleopolyhedrovirus-A. *Journal of General Virology* **86**, 91-105.

Li, Q., Donly, C., Li, L., Willis, L. G., Theilmann, D. A. & Erlandson, M. (2002). Sequence and organization of the *Mamestra configurata* nucleopolyhedrovirus genome. *Virology* **294**, 106-121.

Li, Q., Li, L., Moore, K., Donly, C., Theilmann, D. A. & Erlandson, M. (2003). Characterization of *Mamestra configurata* nucleopolyhedrovirus enhancin and its functional analysis via expression in an *Autographa californica* M nucleopolyhedrovirus recombinant. *Journal of General Virology* **84**, 123-132.

Lipa, J. & Ziemnicka, J. (1971). Studies on the granulosis virus of cutworms *Agrotis* spp. (Lepidoptera, Noctuidae). *Acta Microbiologica Polonica* **3**, 155-162.

Lipa, J., Ziemnicka, J. & Gudz-Gorban, A. (1971). Electron microscopy of nuclear polyhedrosis virus from *Agrotis segetum* Schiff. and *A. exclamationis* L. (Lepidoptera, Noctuidae). *Acta Microbiologica Polonica* **3**, 55-61.

Lipa, J. J., J., Z. & Lipa, I. (1972). Studies on the susceptibility of nine noctuids (Noctuidae) to infection with the nuclear polyhedrosis virus *Borrelinavirus agrotidis*. *Prace Naukowe Instytutu Ochrony Roslin* **14**, 47-64.

Lipa, J. J. & Wiland, E. (1972). Bacteria isolated from cutworms and their activity to *Agrotis* spp. (Lepidoptera: Noctuidae). *Acta Microbiologica Polonica* **4**, 127-140.

Lipa, J. J. & Ziemnicka, J. (1996). Spiroplasma and microsporidian infections in populations of *Leucoma salicis* L. (Lepidoptera: Lymantriidae) in Poland. In *Integrated Management of Forest Lymantriidae*, pp. 27-29. Warsaw-Sekocin, Poland.

Long, G., Pan, X., Kormelink, R. & Vlak, J. M. (2006). Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. *Journal of Virology* **80**, 8830-8833.

Lossbroek, T. G. & Theunissen, J. (1985). The entomogenous nematode *Neoplectana bibionis* as a biological control agent of *Agrotis segetum* in lettuce. *Applied and Experimental Nematology* **39**, 261-264.

Lu, A. & Carstens, E. B. (1993). Immediate-early baculovirus genes transactivate the *p143* gene promoter of *Autographa californica* nuclear polyhedrosis virus. *Virology* **195**, 710-718.

Lu, A. & Miller, L. K. (1995). The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication. *Journal of Virology* **69**, 975-982.

Lucarotti, C. J. & Morin, B. (1997). A nuclear polyhedrosis virus from the obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae). *Journal of Invertebrate Pathology* **70**, 121-126.

Lung, O., Westenberg, M., Vlak, J. M., Zuidema, D. & Blissard, G. W. (2002). Pseudotyping *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV): F proteins from group II NPVs are functionally analogous to AcMNPV GP64. *Jounal of Virology* **76**, 5729-5736.

Luque, T., Finch, R., Crook, N., O'Reilly, D. R. & Winstanley, D. (2001). The complete sequence of the *Cydia pomonella* granulovirus genome. *Journal of General Virology* **82**, 2531-2547.

Lynn, D. E. (2003). Comparative susceptibilities of twelve insect cell lines to infection by three baculoviruses. *Journal of Invertebrate Pathology* **82**, 129-131.

Lynn, D. E., Shapiro, M. & Dougherty, E. M. (1992). Selection and screening of clonal isolates of the abington strain of gypsy moth nuclear polyhedrosis virus. *Journal of Invertebrate Pathology* **62**, 191-195.

Ma, X. C., Shang, J. Y., Yang, Z. N., Bao, Y. Y., Xiao, Q. & Zhang, C. X. (2007). Genome sequence and organization of a nucleopolyhedrovirus that infects the tea looper caterpillar, *Ectropis obliqua*. *Virology* **360**, 235-246.

Majima, K., Kobara, R. & Maeda, S. (1993). Divergence and evolution of homologous regions of Bombyx mori nuclear polyhedrosis virus. *Journal of Virology* **67**, 7513-7521.

Maksymov, J. K. (1980). Biological control of the satin moth *Stilpnotia salicis* L. with *Bacillus thuringiensis* Berliner *Anzeiger fuer Schaedlingskunde Pflanzenschutz* **53**, 52-56.

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., Berka, J., Braverman, M. S., Chen, Y. J., Chen, Z., Dewell, S. B., Du, L., Fierro, J. M., Gomes, X. W., Godwin, B. C., He, W., Helgesen, S., Ho, C. H., Irzyk, G. P., Jando, S. C., Alenquer, M. L. I., Jarvie, T. P., Jirage, K. B., Kim, J. B., Knight, J. R., Lanza, J. R., Leamon, J. H., Lefkowitz, S. M., Lei, M., Li, J., Lohman, K. L., Lu, H., Makhijani, V. B., McDade, K. E., McKenna, M. P., Myers, E. W., Nickerson, E., Nobile, J. R., Plant, R., Puc, B., Ronan, M. T., Roth, G. T., Sarkis, G. J., Simons, J. F., Simpson, J. W., Srinivasan, M., Tartaro, K. R., Tomasz, A., Vogt, K. A., Volkmer, G. A., Wang, S. H., Wang, Y., Weiner, M. P., Yu, P., Begley, R. F. & Rothberg, J. M. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437** 376-380.

Martignoni, M. E. & Iwai, P. J. (1981). A catalogue of viral diseases of insects, mites and ticks. In *Microbial Control of Pests and Plant Diseases 1970-1980*, pp. 897-911. Edited by H. D. Burges. London, NY: Academic Press.

Martin, O. & Croizier, G. (1997). Infection of a *Spodoptera frugiperda* cell line with *Bombyx mori* nucleopolyhedrovirus. *Virus Research* **47**, 179–185.

McCarthy, C. B. & Theilmann, D. A. (2008). AcMNPV *ac143 (odv-e18)* is essential for mediating budded virus production and is the 30th baculovirus core gene. *Virology* **375**, 277-291.

Mikhailov, V. S. (2003). Replication of the baculovirus genome *Molecular Biology* **37**, 250-259.

Miller, L. K. (1997). Baculovirus interaction with host apoptotic pathways. *Journal of Cellular Physiology* **173**, 178-182.

Monsma, S. A., Oomens, A. G. & Blissard, G. W. (1996). The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. *Journal of Virology* **70**, 4607–4616.

Moraes, R. R. & Maruniak, J. E. (1997). Detection and identification of multiple baculoviruses using the polymerase chain reaction (PCR) and restriction endonuclease analysis. *Journal of Virological Methods* **63**, 209-217.

Morris, T. D. & Miller, L. K. (1992). Promoter influence on baculovirus-mediated gene expression in permissive and nonpermissive insect cell lines. *Journal of Virology* **66**, 7397–7405.

Morris, T. D. & Miller, L. K. (1993). Characterization of productive and non-productive AcMNPV infection in selected insect cell lines. *Virology* **197**, 339–348.

Morris, T. D., Todd, J. W., Fisher, B. & Miller, L. K. (1994). Identification of *lef-7*: a baculovirus gene affecting late gene expression. *Virology* **200**, 360-369.

Moscardi, F. (1999). Assessment of the application of baculoviruses for control of lepidoptera. *Annual Review of Entomology* **44**, 257-289.

Moscardi, F. & Santos, B. (2005). Producao comercial de nucleopoliedrosis de Anticarsia gemmatalis HUBNER (Lep.: Noctuidae) em laboratorio. In *IX Simposio de Controle Biologico* p. 42. Recife, Brazil.

Muñoz, D., Castillejo, J. I. & Caballero, P. (1998). Naturally occurring deletion mutants are parasitic genotypes in a wild- type nucleopolyhedrovirus population of *Spodoptera exigua*. *Applied and Environmental Microbiology* **64**, 4372-4377.

Muñoz, D., Murillo, R., Krell, P. J., Vlak, J. M. & Caballero, P. (1999). Four genotypic variants of a *Spodoptera exigua* nucleopolyhedrovirus (Se-SP2) are distinguishable by a hypervariable genomic region. *Virus Research* **59**, 61-74.

Muñoz, D., Vlak, J. M. & Caballero, P. (1997). *In vivo* recombination between two strains of the genus nucleopolyhedrovirus in its natural host, *Spodoptera exigua*. *Applied Environmental Microbiology* **63**, 3025–3031.

Murillo, R., Muñoz, D., Ruiz-Portero, M. C., Alcazar, M. D., Belda, J. E., Williams, T. & Caballero, P. (2007). Abundance and genetic structure of nucleopolyhedrovirus populations in greenhouse substrate reservoirs. *Biological Control* **42**, 216-225.

Myers, J. H. (1988). Can a general hypothesis explain population cycles of forest Lepidoptera? *Advances in Ecological Research* **18**, 179–242.

Nakai, M., Goto, C., Kang, W., Shikata, M., Luque, T. & Kunimi, Y. (2003). Genome sequence and organization of a nucleopolyhedrovirus isolated from the smaller tea tortrix, *Adoxophyes honmai*. *Virology* **316**, 171-183.

Nef, L. (1975). Pathogenic microorganisms in the control of defoliating Lymantriidae. In *Semaine d'etude Agriculture et hygiene des plantes*, pp. 325-330.

Nie, Z. M., Zhang, Z. F., Wang, D., He, P. A., Jiang, C. Y., Song, L., Chen, F., Xu, J., Yang, L., Yu, L. L., Chen, J., Lv, Z. B., Lu, J. J., Wu, X. F. & Zhang, Y. Z. (2007). Complete sequence and organization of *Antheraea pernyi* nucleopolyhedrovirus, a dr-rich baculovirus. *BMC Genomics* **8**.

Nobusawa, E. & Sato, K. (2006). Comparison of the mutation rates of human influenza A and B viruses. *Journal of Virology* **80**, 3675-3678.

Ohkawa, T., Rowe, A. R. & Volkman, L. E. (2002). Identification of six *Autographa californica* multicapsid nucleopolyhedrovirus early genes that mediate nuclear localization of G-actin. *Jounal of Virology* **76**, 12281-12287.

Ohkawa, T., Washburn, J. O., Sitapara, R., Sid, E. & Volkman, L. E. (2005). Specific binding of *Autographa californica* M nucleopolyhedrovirus occlusion-derived virus to midgut cells of *Heliothis virescens* larvae is mediated by products of pif genes *ac119* and *ac022* but not by *ac115*. *Journal of Virology* **79**, 15258-15264.

Oppenheimer, D. I. & Volkman, L. E. (1997). Evidence for rolling circle replication of *Autographa californica* M nucleopolyhedrovirus genomic DNA. *Archives of Virology* **142**, 2107-2113.

O'Reilly, D. R. (1995). Baculovirus-encoded ecdysteroid UDP-glucosyltransferases. *Insect Biochemistry and Molecular Biology* **25**, 541-550.

O'Reilly, D. R. & Miller, L. K. (1991). Improvement of a baculovirus pesticide by deletion of the EGT gene. *Nature Biotechnology* **9**, 1086-1089.

Page, R. D. M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers *Computer Applications in the Biosciences* **12**, 357-358.

Pang, Y., Yu, J., Wang, L., Hu, X., Bao, W., Li, G., Chen, C., Han, H., Hu, S. & Yang, H. (2001). Sequence analysis of the *Spodoptera litura* multicapsid nucleopolyhedrovirus genome. *Virology* **287**, 391-404.

Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T. G., Prentice, M. B., Sebaihia, M., James, K. D., Churcher, C., Mungall, K. L., Baker, S., Basham, D., Bentley, S. D., Brooks, K., Cerdáş˝o-TĂˇrraga, A. M., Chillingworth, T., Cronin, A., Davies, R. M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Karlyshev, A. V., Leather, S., Moule, S., Oyston, P. C. F., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S. & Barrell, B. G. (2001). Genome sequence of Yersinia pestis, the causative agent of plague. *Nature* **413**, 523-527.

Passarelli, A. L. (2007). Baculovirus RNA polymerase: Activities, composition, and evolution. *Virologica Sinica* **22**, 94-107.

Payne, C. C. (1986). Insect pathogenic viruses as pest control agents. In *Biological Plant and Health Protection*, pp. 183-200. Edited by J. M. Franz. Stuttgart: Fisher Verlag.

Pearson, M., Bjornson, R., Pearson, G. & Rohrmann, G. F. (1992). The *Autographa californica* baculovirus genome: evidence for multiple replication origins. *Science* **257**, 1382-1384.

Pearson, M. N., Groten, C. & Rohrmann, G. F. (2000). Identification of the *Lymantria dispar* nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the *Baculoviridae*. *Journal of Virology* **74**, 6126-6131.

Peng, J., Zhong, J. & Granados, R. R. (1999). A baculovirus enhancin alters the permeability of a mucosal midgut peritrophic matrix from lepidopteran larvae. *Journal of Insect Physiology* **45**, 159-166.

Perera, O. P., Valles, S. M., Green, T. B., White, S., Strong, C. A. & Becnel, J. J. (2006). Molecular analysis of an occlusion body protein from *Culex nigripalpus* nucleopolyhedrovirus (CuniNPV). *Journal of Invertebrate Pathology* **91**, 35-42.

Pijlman, G. P., Pruijssers, A. J. P. & Vlak, J. M. (2003). Identification of *pif-2*, a third conserved baculovirus gene required for per os infection of insects. *Journal of General Virology* **84**, 2041-2049.

Pijlman, G. P., van den Born, E., Martens, D. E. & Vlak, J. M. (2001). *Autographa californica* baculoviruses with large genomic deletions are rapidly generated in infected insect cells. *Virology* **283**, 132-138.

Popham, H. J., Bischoff, D. S. & Slavicek, J. M. (2001). Both *Lymantria dispar* nucleopolyhedrovirus enhancin genes contribute to viral potency. *Journal of Virology* **75**, 8639-8648.

Prater, C. A., Redmond, C. T., Barney, W., Bonning, B. C. & Potter, D. A. (2006). Microbial control of black cutworm (Lepidoptera: Noctuidae) in turfgrass using *Agrotis ipsilon* multiple nucleopolyhedrovirus. *Journal of Economic Entomology* **99**, 1129-1137.

Räty, J. K., Lesch, H. P., Wirth, T. & Ylä-Herttuala, S. (2008). Improving safety of gene therapy. *Current Drug Safety* **3**, 46-53.

Rawlings, N. D. & Barrett, A. J. (1995). Evolutionary families of metallopeptidases. *Methods in Enzymology* **248**, 183-228.

Read, T. D., Peterson, S. N., Tourasse, N., Baillie, L. W., Paulsen, I. T., Nelson, K. E., Tettelin, H., Fouts, D. E., Eisen, J. A., Gill, S. R., Holtzapple, E. K., Ă kstad, O. A., Helgason, E., Rilstone, J., Wu, M., Kolonay, J. F., Beanan, M. J., Dodson, R. J., Brinkac, L. M., Gwinn, M., DeBoy, R. T., Madpu, R., Daugherty, S. C., Durkin, A. S., Haft, D. H., Nelson, W. C., Peterson, J. D., Pop, M., Khouri, H. M., Radune, D., Benton, J. L., Mahamoud, Y., Jiang, L., Hance, I. R., Weidman, J. F., Berry, K. J., Plaut, R. D., Wolf, A. M., Watkins, K. L., Nierman, W. C., Hazen, A., Cline, R., Redmond, C., Thwaite, J. E., White, O., Salzberg, S. L., Thomason, B., Friedlander, A. M., Koehler, T. M., Hanna, P. C., KolstĂ¸, A. B. & Fraser, C. M. (2003). The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* **423**, 81-86.

Reed, C., Otvos, I. S., Reardon, R., Ragenovivh, I. & Williams, H. L. (2003). Effects of long-term storage on the stability of OpMNPV DNA contained in TM Biocontrol-1. *Journal of Invertebrate Pathology* **84**, 104-113.

Reeks, W. A. & Smith, C. C. (1956). The satin moth, *Stilpnotia salicis* (L.), in the Maritime Provinces and observations on its control by parasites and spraying. *Canadian Entomologist* **88**, 565-579.

Ribeiro, H. C. T., Pavan, O. H. O. & Muotri, A. R. (1997). Comparative susceptibility of two different hosts to genotypic variants of the *Anticarsia gemmatalis* nuclear polyhedrosis virus. *Entomologia Experimentalis et Applicata* **83**, 233-237.

Richards, A., Speight, M. & Cory, J. (1999). Characterization of a nucleopolyhedrovirus from the vapourer moth, *Orgyia antiqua* (Lepidoptera Lymantriidae). *Journal of Invertebrate Pathology* **74**, 137-142.

Roelvink, P. W., Corsaro, B. G. & Granados, R. R. (1995). Characterization of the *Helicoverpa armigera* and *Pseudaletia unipuncta* granulovirus enhancin genes. *Journal of General Virology* **76**, 2693-2705.

Rohrmann, G. F. (1986). Polyhedrin structure. *Journal of General Virology* **67** 1499-1513.

Rohrmann, G. F. & Beaudreau, G. S. (1977). Characterization of DNA from polyhedral inclusion bodies of the nucleopolyhedrosis single-rod virus pathogenic for *Orgyia pseudotsugata*. *Virology* **83**, 474-478.

Rohrmann, G. F., Pearson, M. N., Bailey, T. J., Becker, R. R. & Beaudreau, G. S. (1981). N-terminal polyhedrin sequences and occluded baculovirus evolution. *Journal of Molecular Evolution* **17**, 329-333.

Schotveld, A. & Wigbels, V. (1975). Control of *Leucoma salicis* in East Flevoland. *Populier* **12**, 77-78.

Shackelton, L. A. & Holmes, E. C. (2004). The evolution of large DNA viruses: Combining genomic information of viruses and their hosts. *Trends in Microbiology* **12**, 458-465.

Shah, B. H., Zethner, O., Gul, H. & Chaudhry, M. I. (1979). Control experiments using *Agrotis segetum* granulosis virus against *Agrotis ipsilon* [Lep.: Noctuidae] on tobacco seedlings in northern Pakistan. *Entomophaga* **24**, 393-401.

Shapiro, M. & Argauer, R. (1997). Components of the stilbene optical brightener tinopal LPW as enhancers for the gypsy moth (Lepidoptera: Lymantriidae) baculovirus. *Journal of Economic Entomology* **90**, 899-904.

Shoji, I., Aizaki, H. & Tani, H. (1997). Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. *Jornal of General Virology* **78**, 2657–2664.

Sidor, C. (1967). Virus diseases as limiting factors of some lepidopterous defoliators on poplar trees in Vojvodina. In *Insect Pathology and Microbial Control*, pp. 168-173. Amsterdam: North Holland.

Simon, O., Williams, T., Asensio, A. C., Ros, S., Gaya, A., Caballero, P. & Possee, R. D. (2008). Sf29 gene of *Spodoptera frugiperda* multiple nucleopolyhedrovirus is a viral factor that determines the number of virions in occlusion bodies. *Journal of Virology* **82**, 7897-7904.

Simon, O., Williams, T., Lopez-Ferber, M. & Caballero, P. (2004). Virus entry or the primary infection cycle are not the principal determinants of host specificity of *Spodoptera* spp. nucleopolyhedroviruses. *Journal of General Virology* **85**, 2845-2855.

Simon, O., Williams, T., Lopez-Ferber, M. & Caballero, P. (2005). Functional importance of deletion mutant genotypes in an insect nucleopolyhedrovirus population. *Applied and Environment Microbiology* **71**, 4254-4262.

Skatulla, U. (1985). Untersuchungen zur Wirkung eines Kernpolyedervirus aus *Leucoma salicis* (Lep., Lymantriidae) auf einige Lymantriiden-Arten. *Anz für Schändlings Pflanzenschutz Umweltschutz* **58**, 41-47.

Slack, J. & Arif, B. M. (2007). The baculoviruses occlusion derived virus: virion structure and function. *Advances in Virus Research* **69**, 99-165.

Slack, J. M., Kuzio, J. & Faulkner, P. (1995). Characterization of *v-cath*, a cathepsin L-like proteinase expressed by the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus. *Journal of General Virology* **76**, 1091-1098.

Slavicek, J. M. & Popham, H. J. R. (2005). The *Lymantria dispar* nucleopolyhedrovirus enhancins are components of occlusion-derived virus. *Journal of Virology* **79**, 10578-10588.

Smith, G. E., Summers, M. D. & Fraser, M. J. (1983). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Molecular and Cellular Biology* **3**, 2156-2165.

Smith, I. R. L. & Crook, N. E. (1988). *In vivo* isolation of baculovirus genotypes. *Virology* **166**, 240-244.

Smits, P. H., van de Vrie, M. & Vlak, J. M. (1986). Oviposition of beet armyworm (Lepidoptera: Noctuidae) on greenhouse crops. *Environmental Entomology* **15**, 1189-1191.

Smits, P. H. & Vlak, J. M. (1988). Biological activity of *Spodoptera exigua* nuclear polyhedrosis virus against *S. exigua* larvae. *Journal of Invertebrate Pathology* **51**, 107-114.

Smits, P. H. & Vlak, J. M. (1988). Selection of nuclear polyhedrosis viruses as biological control agents of *Spodoptera exigua* [Lep.: Noctuidae]. *Entomophaga* **33**, 299-308.

Smits, P. H. & Vlak, J. M. (1994). Registration of the first viral insecticide in The Netherlands the development of Spod-X, based on *Spodoptera exigua* nuclear polyhedrosis virus. *Mededelingen Faculteit Landbouwwetenschappen, Universiteit Gent (Belgium)* **59**, 385-392.

Sohi, S. S., Percy, M. & Cunningham, J. C. (1984). Replication and serial passage of a singly enveloped baculovirus from *Orgyia leucostigma* in homologous cell lines. *Intervirology* **21**, 50–60.

Song, J., Wang, R., Deng, F., Wang, H. & Hu, Z. (2008). Functional studies of per os infectivity factors of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus. *Journal of General Virology* **89**, 2331-2338.

Steinhaus, E. A. (1956). Microbial control. The emergence of an idea. *Hilgardia* **26**, 107-160.

Stiles, S. & Himmerich, B. (1998). *Autographa californica* NPV isolates: restriction endonuclease analysis and comparative biological activity. *Journal of Invertebrate Pathology* **72**, 174-177.

Strokovskaya, L., Ziemnicka, J. & Michalik, J. (1996). Genetic variability of four natural isolates of the *Stilpnotia salicis* multiple-enveloped nuclear polyhedrosis virus. *Acta Biochmica Polonica* **43**, 633-638.

Sukhareva, I. L. (1999). Noctuidae. In *Insects and mites - agricultural pests*, pp. 332-378. Edited by V. I. Kuznetsov. St. Petersburg: Nauka.

Swofford, D. L. (1998). PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, MA.

Szalay-Marzso, L., Halmagyi, L. & Fodor, S. (1981). Microbial control experiment against *Stilpnotia salicis* L., pest of poplar stands in northwest Hungary. *Acta Phytopathologica et Entomologica Hungarica* **16**, 189-197.

Szewczyk, B., Barski, P., Sihler, W., Rabalski, L., Skrzecz, I., Hoyos-Carvajal, L. & De Souza, M. L. (2008). Detection and identification of baculovirus pesticides by multitemperature single-strand conformational polymorphism. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes* **43**, 539-545.

Takatsuka, J., Okuno, S., Ishii, T., Nakai, M. & Kunimi, Y. (2007). Host range of two multiple nucleopolyhedroviruses isolated from *Spodoptera litura*. *Biological Control* **41**, 264-271.

Tanada, Y. (1985). A synopsis of studies on the synergistic properties of an insect baculovirus: a tribute to Edward A. Steinhaus. *Journal of Invertebrate Pathology* **45**, 125-138.

Tanada, Y. & Hukuhara, T. (1971). Enhanced infection of nuclear polyhedrosis virus in larvae of the armyworm, *Pseudaletia unipuncta*, by a factor in the capsule of a granulosis virus. *Journal of Invertebrate Pathology* **17**, 116-126.

Tang, X. D., Xiao, Q., Ma, X. C., Zhu, Z. R. & Zhang, C. X. (2009). Morphology and genome of *Euproctis pseudoconspersa* nucleopolyhedrovirus. *Virus Genes* **38**, 495-506.

Theilmann, D. A., Blissard, G. W., Bonning, B., Jehle, J., O'Reilly, D. R., Rohrman, G. F., Thiem, S. & Vlak, J. M. (2005). *Baculoviridae*. In *Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses*, pp. 177-185. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. London, UK: Elsevier/Academic Press

Thompson, C. G. & Peterson, L. J. (1978). Rearing the douglas-fur tussock moth. *USDA Agriculture Handbook No 520, Combined Forestry Pest Research and Development Program*, 20.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**, 4876-4882.

Thomsen, L., Damgaard, P. H. & Eilenberg, J. (1998). Screening of selected *Bacillus thuringiensis* strains against *Agrotis segetum* larvae. *IOBC/WPRS Bulletin* **21**, 235-239.

Thomsen, L., Eilenberg, J. & Damgaard, P. H. (2001). The potential of *Bacillus thuringiensis* for control of turnip moths (*Agrotis segetum*) and their outdoor pest insects. In *Plant Production*, pp. 45-50: DIAS Report. Plant Production.

Titterington, J. S., Nun, T. K. & Passarelli, A. L. (2003). Functional dissection of the baculovirus late expression factor-8 gene: Sequence requirements for late gene promoter activation. *Journal of General Virology* **84**, 1817-1826.

Todd, J. W., Passarelli, A. L. & Miller, L. K. (1995). Eighteen baculovirus genes, including *lef-11*, *p35*, *39K*, and *p47*, support late gene expression. *Journal of Virology* **69**, 968-974.

Tramper, J., de Gooijer, K. D. & Vlak, J. M. (1993). Scale-up considerations and bioreactor development for animal cell cultivation. *Bioprocess Technology* **17**, 139-177.

van Oers, M. M. (2006). Vaccines for viral and parasitic diseases produced with baculovirus vectors. *Advanced Virus Research* **68**, 193-253.

van Oers, M. M., Abma-Henkens, M. H. C., Herniou, E. A., de Groot, J. C. W., Peters, S. & Vlak, J. M. (2005). Genome sequence of *Chrysodeixis chalcites* nucleopolyhedrovirus, a baculovirus with two DNA photolyase genes. *Journal of General Virology* **86**, 2069-2080.

van Oers, M. M. & Vlak, J. M. (2007). Baculovirus genomics. *Current Drug Targets* **8**, 1051-1068.

van Regenmortel, M. H. V. (1992). Concept of virus species. *Biodiversity and Conservation* **1**, 263-266.

van Strien, E. A., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1992). Nucleotide sequence and transcriptional analysis of the polyhedrin gene of *Spodoptera exigua* nuclear polyhedrosis virus. *Journal of General Virology* **73**, 2813-2821.

Vincze, T., Posfai, J. & Roberts, R. J. (2003). NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acid Research* **31**, 3688-3691.

Vlak, J. M. (1979). The proteins of nonoccluded nuclear polyhedrosis virus produced in an established cell line of *Spodoptera frugiperda*. *Journal of Invertebrate Pathology* **34**, 110-118.

Vlak, J. M. & Rohrmann, G. F. (1985). The nature of polyhedrin. In *Viral insecticides for biological control*, pp. 489-544. Edited by K. Maramorosh & K. E. Sherman. Orlando: Academic Press.

Vlak, J. M. & Smith, G. E. (1982). Orientation of the genome of *Autographa californica* nuclear polyhedrosis virus: a proposal. *Journal of Virology* **41**, 1118-1121.

Volkman, L. E. (2007). Baculovirus infectivity and the actin cytoskeleton. *Current Drug Targets* **8**, 1075-1083.

Volkman, L. E., Blissard, G. W., Friesen, P. D., Keddie, B. A., Possee, R. D. & Theilman, D. A. (1995). Family *Baculoviridae*. In *Virus Taxonomy: Sixth Report of the International Committee on Taxonomy of Viruses*, pp. 104-113. Edited by F. A. Murphy, C. M. Fauquet, S. A. Bishop, A. W. Ghabrial, G. P. Jarvis, M. A. Martelli, M. A. Mayo & M. D. Summers. Vienna: Springer.

Walsh, P. J., Glare, T. R., Nelson, T. L., Sadler, T. J. & Ward, V. K. (1999). Virulence of nucleopolyhedroviruses from *Orgyia pseudotsugata* and *Orgyia leucostigma* (Lep. Lymantriidae) for early instars of the white-spotted tussock moth *Orgyia thyellina*. *Journal of Applied Entomology* **123**, 375-379.

Wang, J. W., Qi, Y. P., Huang, Y. X. & Li, S. D. (1995). Nucleotide sequence of a 1446 base pair *Sal*I fragment and structure of a novel early gene of *Leucania separata* nuclear polyhedrosis virus. *Archives of Virology* **140**, 2283-2291.

Wang, P. & Granados, R. R. (2000). Calcofluor disrupts the midgut defense system in insects. *Insect Biochemistry and Molecular Biology* **30**, 135-143.

Wang, P. & Granados, R. R. (2001). Molecular structure of peritrophic membrane (PM): Identification of potential PM target sites for insect control. *Archives of Insect Biochemistry and Physiology* **47**, 110-118.

Wang, P., Hammer, D. A. & Granados, R. R. (1994). Interaction of *Trichoplusia ni* granulosis virus-encoded enhancin with the midgut epithelium and peritrophic membrane of four lepidopteran insects. *Journal of General Virology* **75** 1961-1967.

Wang, P., Hammer, D. A. & Granados, R. R. (1997). Binding and fusion of *Autographa californica* nuclear polyhedrosis virus. *Journal of General Virology* **63**, 1393-1399.

Wang, P., Hammer, D. A. & Granados, R. R. (1997). Binding and fusion of *Autographa californica* nucleopolyhedrovirus to cultured insect cells. *Journal of General Virology* **78**, 3081-3089.

Wang, Q., Liang, C., Song, J. & Chen, X. (2007). HA2 from the *Helicoverpa armigera* nucleopolyhedrovirus: a WASP-related protein that activates Arp2/3-induced actin filament formation. *Virus Research* **127**, 81-87.

Washburn, J. O., Kirkpatrick, B. A. & Volkman, L. E. (1995). Comparative pathogenesis of *Autographa californica* M nuclear polyhedrosis virus in larvae of *Trichoplusia ni* and *Heliothis virescens*. *Virology* **209**, 561–568.

Washburn, J. O., Kirkpatrick, B. A. & Volkman, L. E. (1996). Insect protection against viruses. *Nature* **383,** 767.

Weiser, J., Ludvik, J. & Veber, J. (1954). Polyedrie bekyne vbrove (*Stilpnotia salicis* L., Lepidoptera) *Zoologicke a Entomologicke Listy* **4**, 238-242.

Westenberg, M., Wang, H., WF, I. J., Goldbach, R. W., Vlak, J. M. & Zuidema, D. (2002). Furin is involved in baculovirus envelope fusion protein activation. *Journal of Virology* **76**, 178-184.

Willis, L. G., Siepp, R., Stewart, T. M., Erlandson, M. A. & Theilmann, D. A. (2005). Sequence analysis of the complete genome of *Trichoplusia ni* single nucleopolyhedrovirus and the identification of a baculoviral photolyase gene. *Virology* **338**, 209-226.

Wilson, J. A., Forney, S. D., Ricci, A. M., Allen, E. G., Hefferon, K. L. & Miller, L. K. (2005). Expression and mutational analysis of *Autographa californica* nucleopolyhedrovirus HCF-1: funcional requirements for cysteine residues. *Journal of Virology* **79**, 13900-13914.

Wolff, J. L. C., Moraes, R. H. P., Kitajima, E., De Souza Leal, E. & Zanotto, P. M. D. A. (2002). Identification and characterization of a baculovirus from *Lonomia obliqua* (Lepidoptera: Saturniidae). *Journal of Invertebrate Pathology* **79**, 137-145.

Wolff, J. L. C., Valicente, F. H., Martins, R., Oliveira, J. V. d. C. & Zanotto, P. M. d. A. (2008). Analysis of the genome of *Spodoptera frugiperda* nucleopolyhedrovirus (SfMNPV-19) and of the high genomic heterogeneity in group II nucleopolyhedroviruses. *Journal of General Virology* **89**, 1202-1211.

Wood, H. A. & Granados, R. R. (1991). Genetically engineered baculoviruses as agents for pest control. *Annual Review of Microbiology* **45**, 69-87.

Wormleaton, S., Kuzio, J. & Winstanley, D. (2003). The complete sequence of the *Adoxophyes orana* granulovirus genome. *Virology* **311**, 350-365.

Wu, Y., Liu, G. & Carstens, E. B. (1999). Replication, integration, and packaging of plasmid DNA following cotransfection with baculovirus viral DNA. *Journal of Virology* **73**, 5473-5480.

Xiao, H. & Qi, Y. (2007). Genome sequence of *Leucania separata* nucleopolyhedrovirus. *Virus Genes* **35**, 845-856.

Yamamoto, T. & Tanada, Y. (1980). Physiochemical properties and location of capsule components, in particular the synergistic factor, in the occlusion body of a granulosis virus of the armyworm, *Pseudaletia unipuncta*. *Virology* **107**, 434-440.

Yaman, M. (2008). The first record of nucleopolyhedrovirus isolated from the satin moth *Leucoma salicis* L. (Lepidoptera, Lymantriidae) in Turkey. *Folia Biologica (Krakow)* **56**, 273-276.

Yanase, T., Yasunaga, C., Hara, T. & Kawarabata, T. (1998). Coinfection of *Spodoptera exigua* and *Spodoptera frugiperda* cell lines with the nuclear polyhedrosis viruses of *Autographa californica* and *Spodoptera exigua*. *Intervirology* **41**, 244-252.

Yang, L. R., Wang, D., Duan, L. Q. & Zhang, C. X. (2006). Polyhedrin gene sequence and phylogenetic analysis of a nucleopolyhedrovirus isolated from *Orgyia ericae* Germar. *Mitochondrial DNA* **17**, 215-222.

Yang, S. & Miller, L. K. (1999). Activation of baculovirus very late promoters by interaction with very late factor 1. *Journal of Virology* **73**, 3404-3409.

Zanotto, P. M., Kessing, B. D. & Maruniak, J. E. (1993). Phylogenetic interrelationships among baculoviruses: evolutionary rates and host associations. *Journal of Invertebrate Pathology* **62**, 147-164.

Zanotto, P. M. & Krakauer, D. C. (2008). Complete genome viral phylogenies suggests the concerted evolution of regulatory cores and accessory satellites. *PLoS ONE* **3**.

Zanotto, P. M., Sampaio, M. J., Johnson, D. W., Rocha, T. L. & Maruniak, J. E. (1992). The *Anticarsia gemmatalis* nuclear polyhedrosis virus polyhedrin gene region: sequence analysis, gene product and structural comparisons. *Journal of General Virology* **73 (Pt 5)**, 1049-1056.

Zethner, O., Khan, B. M., Chaudhry, M. I., Bolet, B., Khan, S. K., H., Gul, H., Ogaard, L., Zaman, M. & Nawaz, G. (1987). *Agrotis segetum* granulosis virus as a control agent against field populations of *Agrotis ipsilon* and *A. segetum* (Lep: Noctuidae) on tobacco, okra, potato and sugar beet in northern Pakistan. *Entomophaga* **32**, 449-455.

Zhang, C. X., Ma, X. C. & Guo, Z. J. (2005). Comparison of the complete genome sequence between C1 and G4 isolates of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus. *Virology* **333**, 190-199.

Zhang, G., Sun, X., Zhang, Z. X., Z.F., Z. & Wan, F. (1995). Production and effectiveness of the new formulation of *Helicoverpa* virus pesticide-emulsifiable suspension. *Virologica Sinica* **10**, 242-247.

Zhu, S. Y., Yi, J. P., Shen, W. D., Wang, L. Q., He, H. G., Wang, Y., Li, B. & Wang, W. B. (2009). Genomic sequence, organization and characteristics of a new nucleopolyhedrovirus isolated from *Clanis bilineata* larva. *BMC Genomics* **10**, 91.

Zhu, Y., Hukuhara, T. & Tamura, K. (1989). Location of a synergistic factor in the capsule of a granulosis virus of the armyworm, *Pseudaletia unipuncta*. *Journal of Invertebrate Pathology* **54**, 49-56.

Ziemnicka, J. (1981). Studies on nuclear and cytoplasmic polyhedrosis viruses of the satin moth (*Stilpnotia salicis*) (Lepidoptera: Lymantriidae). *Prace Naukowe Instytutu Ochrony Roslin* **23**, 75-142.

Ziemnicka, J. (2000). Rola bakulowirusa SsMNPV w regulacji liczebnosci populacji bialki wierzbowki *Stilpnotia salicis* L. (Lepidoptera: Lymantriidae), p. 85: Institut of Plant Protection.

Ziemnicka, J. (2008). Outbreaks and natural viral epizootics of the satin moth *Leucoma salicis* L. (Lepidoptera: Lymantriidae). *Journal of Plant Protection Research* **48**, 499-516.

Ziemnicka, J. & Sosnowska, D. (1996). Entomopathogenic fungi in natural populations of satin moth *Stilpnotia salicis* L. *Journal of Plant Protection Research* **37**, 128-137.

List of Abbreviations

aa	Amino acid
Bp	Basepair
BV	Budded virus
CPV	Cypovirus
DNA	Deoxyribonucleic acid
egt	Ecdysteroid UDP-glucosyltransferase
F protein	Baculovirus fusion protein
GP	Glycoprotein
GV	Granulovirus
h	Hour
hrs	Homologous repeats
ICTV	International Committee on Taxonomy of Viruses
Kbp	Kilo basepair
kDa	Kilodalton
LD_{50}	Lethal dose of 50%
LEF	Late expression factor
LT_{50}	Lethal time of 50%
MNPV	Multiple nucleopolyhedrovirus
MOI	Multiplicity of infection
NC	Nucleocapsid
NCBI	National Centem for Biotechnology Information
NPV	Nucleopolyhedrovirus
nt	Nucleotide
OB	Occlusion body
ODV	Occlusion derived virus
ORF	Open reading frame
p.i.	Post infection
PCR	Polymerase chain reaction
PIF	*Per os* infectivity factor
PM	Peritrophic membrane
RNA	Ribonucleic acid
SNPV	Single nucleopolyhedrovirus
$TCID_{50}$	Tissue culture infectious dose of 50%
UTR	Untranslated region
VEF	Viral enhancing factor

Summary

Insect viruses are probably the most numerous group of viruses simply because insects comprise the largest group of animal species, with over 1 million described so far. One family of insect viruses, the *Baculoviridae*, received special interest of researchers as they are widely used as bioinsecticides, as gene expression vectors and recently also as gene therapy vectors. Baculoviruses are large dsDNA viruses and several hundreds have been described from different insect species, mainly belonging to the orders Lepidoptera, Hymenoptera and Diptera. Often however the description concerns only pathology or morphology, or source of isolation. The International Committee on Taxonomy of Viruses (ICTV) now assigns virus isolates to species based on full description of their morphology, pathology, source of isolation and entire genome sequence. Nowadays providing the complete sequence of a baculovirus isolate is also required for its registration as bioinsecticide (EU guidelines). However, partial sequence information of selected genes can serve as a good starting point to decide whether baculovirus isolates are significantly different or closely-related.

The starting point for the research presented in this thesis was a Polish virus collection present at the Institute of Plant Protection in Poznan and collected from the field in over 50 years. Many of the virus samples from the collection have never been characterized at a molecular level, while they may have potential as natural bioinsecticides against common pests in agri- or silviculture. In addition, the analysis of archival baculovirus samples has been shown valuable for the ex post identification of baculoviruses and the establishment of their phylogenetic relationships with other baculoviruses. In the presented thesis two nucleopolyhedroviruses (NPVs) from this Polish collection, *Leucoma salicis* (Lesa) NPV and *Agrotis segetum* (Agse) NPV, are characterized by molecular means, including the sequencing of the AgseNPV genome. The study confirmed that molecular data are necessary for proper virus classification and for assessing the phylogenetic relationships with other viruses. The data obtained further allowed the postulation of new hypotheses, concerning the evolution of the viruses under study.

LesaNPV was isolated from the satin moth *Leucoma salicis* L. (Lepidoptera, Lymantriidae), a frequent defoliator of poplar trees (*Populus* spp.) in Europe and Asia. Around 1920 the insect was introduced into North America. LesaNPV isolated from *L. salicis* larvae in Poland appeared to be a variant of *Orgyia pseudotsugata* (Op) MNPV. The host for this virus, *O. pseudotsugata* or the Douglas fir tussock moth (Lepidoptera, Lymantriidae), occurs exclusively in North America. Sequences of three conserved baculovirus genes, *polh*, *lef-8*, and *pif-2,* revealed a high degree of similarity to OpMNPV. Restriction enzyme analysis confirmed the close relationship between LesaNPV and OpMNPV, although a number of restriction fragment length polymorphisms was observed. The *lef-7* gene, encoding late expression factor 7, and the *ctl-2* gene, coding for a conotoxin-like protein, were tested for

there ability to serve as molecular determinants for the respective viruses. The *ctl-2* region appeared suitable for the clear identification of either virus as LesaNPV lacked a dUTPase gene in this region. The observations suggest that LesaNPV has found a susceptible host, *O. pseudotsugata*, after introduction into North America of *L salicis* larvae which carried this virus

Since LesaNPV showed a high similarity to OpMNPV its relation to another NPV from *O. pseudotsugata*, OpSNPV, was investigated. OpSNPV was previously analyzed by sequencing its *polh* gene, encoding the major occlusion body protein polyhedrin. The phylogenetic status of OpSNPV however remained ambiguous. The example of the AcMNPV *polh* gene in literature shows that phylogenies based on the *polh* gene may be misleading. The AcMNPV *polh* gene for instance shows a mosaic structure and thus complicates phylogenetic analyses. In this thesis sequences of four conserved baculovirus genes, *polh*, *lef-8*, *pif-2* and *dpol*, were amplified from OpSNPV and analyzed phylogenetically. The analysis revealed that OpSNPV belongs to group II NPVs and is most closely related to the SNPVs that infect *Orgyia ericae* and *Orgyia anartoides*. This study underscores the need for multiple, concatenated gene phylogenies to classify baculoviruses. The two viruses, OpSNPV and OpMNPV, coexist in the same host, but the consequences for fitness of either virus in time and space remains largely unexplored.

An NPV isolated from *A. segetum* and present in the same virus collection in Poland (AgseNPV-P) was first compared to AgseNPV isolates from England and France (AgseNPV-UK and AgseNPV-F), revealing clear restriction fragment length polymorphisms. Sequence analysis of three conserved baculovirus genes, *polh*, *lef-8* and *pif-2*, revealed that AgseNPV-P differs substantially from AgseNPV-UK and AgseNPV-F and possibly represents a new NPV species. Phylogenetic analysis placed AgseNPV-P among group II NPVs and showed that this virus had the closest relationship with *Agrotis ipsilon* (Agip) NPV and *Spodoptera exigua* (Se) MNPV. The hypothesis that AgseNPV-P represents a new virus species led to the sequencing of its genome and to renaming of the isolate to AgseNPV-A (for the first sequenced AgseNPV isolate). The circular genome is composed of 147,129 bp and contains 153 putative open reading frames (ORFs), of which 143 are homologues of previously reported baculovirus gene sequences. There are ten so far unique ORFs in AgseNPV-A. Three putative enhancin genes were identified in the AgseNPV-A genome. In phylogenetic analysis, the AgseNPV-A enhancins formed a cluster separated from enhancins of the NPVs infecting *Mamestra* species.

The genes in the AgseNPV genome appeared in the same order as in SeMNPV, but this co-linearity did not correspond to the infectivity properties of these viruses. AgseNPV was orally infectious for *S. exigua*, although the dose needed to kill 50% of the larvae was 10,000-fold higher than for *A. segetum*. The SeMNPV virus was not infectious for *A. segetum* larvae when administered orally, but an infection was established by injection of the budded form of the virus into the hemocoel. Bypassing the midgut entry by intrahemocoelic inoculations

suggested that the midgut is the major barrier in *A. segetum* larvae for infection by SeMNPV. However, delayed-early genes of SeMNPV are expressed in the midgut of *A. segetum* larvae after oral infections, which suggests that SeMNPV may be able to enter the midgut epithelial cells of many more hosts than previously thought.

The genomic and phylogenetic data obtained for AgseNPV and LesaNPV indicate that these viruses are probably examples of two different aspects of baculovirus evolution. AgseNPV and a group of closely related viruses, like AgipNPV, are most likely descendants from a common ancestor, which then separated into two species, by adapting to different hosts. In addition, SeMNPV and the group of *Mamestra* infecting NPVs most likely have a common ancestor with NPVs infecting *Agrotis* species. OpMNPV evolved from LesaNPV by adaptation to *O. pseudotsugata*, however this event took place much later than the speciation of the NPVs infecting different *Agrotis* species, namely when LesaNPV was imported to the North America at the beginning of 20th century. Even in such a short time frame (less than 100 years) remarkable differences in genome sequence were observed.

These two examples once again underscore that naming baculoviruses after the insect species from which they are isolated can be very misleading and may not reflect the phylogenetic position of these viruses within the baculovirus family. Therefore it is proposed to name new baculovirus isolates only after molecular characterization (at least the *polh* gene, and one other core genes, such as *lef-8* and *pif-2*) and a thorough check in the database for similarity to any already described viruses.

Samenvatting

Insectenvirussen zijn waarschijnlijk, wat betreft aantal verschillende soorten, de grootste groep virussen, simpelweg omdat insecten de grootste groep dierlijke organismen vormen met meer dan één miljoen beschreven soorten. Eén familie van insectenvirussen, de *Baculoviridae,* heeft speciale aandacht van onderzoekers gekregen. Virussen, behorend tot deze familie worden op uitgebreide schaal gebruikt als bio-insecticiden en gen-expressievectoren. Baculovirussen kunnen ook voor gentherapie ingezet worden. Baculovirusen zijn grote virussen met circulair, dubbelstrengs DNA, waarvan er honderden zijn beschreven, vooral bij insecten behorend tot de orden Lepidoptera, Hymenoptera en Diptera. De beschrijving van deze virussen betreft vaak alleen de pathologie, de morfologie van het virusdeeltje en uit welk insect het virus geïsoleerd is, wat geen eenduidige identificatie oplevert.

De toekenning van de status van virussoort aan een virusisolaat door de "International Committee on Taxonomy of Viruses (ICTV)" is tegenwoordig gebaseerd op een volledige beschrijving van de morfologie van het virus, het ziektebeeld, de herkomst én de complete nucleotidenvolgorde (sequentie) van het genoom. Tegenwoordig is het ook nodig om deze laatste informatie te verstrekken alvorens een baculovirusisolaat geregistreerd kan worden als bio-insecticide (EU regelgeving). Toch kan sequentie-informatie van een aantal geselecteerde baculovirusgenen een goed aanknopingspunt vormen om te beslissen in welke mate twee baculovirussen van elkaar verschillen of nauw verwant zijn.

De basis voor het onderzoek dat in dit proefschrift gepresenteerd wordt was een collectie baculovirussen, in het veld verzameld in Polen gedurende een periode van meer dan 50 jaar. Deze collectie wordt bewaard in het "Institute of Plant Protection" in Poznan, Polen. Een groot aantal virussen in deze verzameling is nooit gekarakteriseerd op moleculair niveau, hoewel er mogelijk goede bio-insecticiden tegen algemene plaaginsecten in akker- en bosbouw tussen zitten. Daarnaast blijkt onderzoek aan opgeslagen baculovirussen belangrijk voor de latere identificatie en voor het bepalen van de onderlinge, fylogenetische verwantschap. In dit proefschrift worden twee kernpolyhedrovirussen (NPVs), afkomstig uit bovengenoemde Poolse collectie, gekarakteriseerd met behulp van moleculaire analysemethodes: *Leucoma salicis* (Lesa) NPV en *Agrotis segetum* (Agse) NPV. De verrichte studie bevestigt dat moleculaire gegevens noodzakelijk zijn voor een correcte classificatie van het virus en om de fylogenetische relatie met andere baculovirussen te kunnen bepalen. De verkregen informatie heeft geleid tot nieuwe hypotheses betreffende de evolutie van deze virussen.

LesaNPV is geïsoleerd uit rupsen van de satijnvlinder *Leucoma salicis* L. (Lepidoptera, Lymantriidae). Deze rupsen vormen een regelmatig terugkerende plaag in Europa en Azië, welke leidt tot de ontbladering van populieren (*Populus* spp.). De satijnvlinder werd rond 1920 geïntroduceerd in Noord-Amerika. LesaNPV, afkomstig uit Poolse *L. salicis* larven,

bleek een variant te zijn van het al eerder gekarakteriseerde *Orgyia pseudotsugata* (Op) MNPV. De gastheer van dit virus is the "Douglas fir tussock moth", *O. pseudotsugata* (Lepidoptera, Lymantriidae), die alleen in Noord-Amerika voorkomt en daarom geen Nederlandse naam heeft. Analyse van de basenvolgorde van drie geconserveerde baculovirus genen, *polh*, *lef-8*, and *pif-2,* liet een hoge mate van overeenkomst zien met OpMNPV. Analyse met behulp van restrictie-enzymen bevestigde deze nauwe verwantschap tussen LesaNPV and OpMNPV, hoewel ook een aantal verschillen in het restrictiepatroon werd gevonden. Voor het *lef-7* gen, dat codeert voor een factor nodig voor late gen-expressie, en voor het *ctl-2* gen, dat voor een conotoxine-achtig eiwit codeert, werd bekeken of ze zouden kunnen dienen als moleculaire determinant of merker om snel onderscheid te kunnen maken tussen deze twee virussen. The *ctl-2* locus bleek hiervoor zeer geschikt te zijn, doordat LesaNPV geen dUTPase gen bezit in dit deel van het genoom in tegenstelling tot OpMNPV. De waarnemingen beschreven in dit proefschrift suggereren dat LesaNPV een ontvankelijke, locale gastheer heeft gevonden in *O. pseudotsugata* na de introductie van *L salicis* via motten, eieren of larven in Noord-Amerika, die dit virus bij zich droegen.

Omdat LesaNPV veel overeenkomst vertoont met OpMNPV werd de relatie van deze virussen tot een ander NPV in *O. pseudotsugata*, OpSNPV, ook onderzocht. OpSNPV was eerder geanalyseerd door de sequentie van het *polh* gen te bepalen dat codeert voor het eiwit polyhedrine. De fylogenetische positie van OpSNPV was hier echter niet afdoende mee opgehelderd. Het voorbeeld van *Autographa californica* (Ac) MNPV in de literatuur laat zien dat fylogenetische stambomen gebaseerd op het *polh* gen misleidend kunnen zijn. Het AcMNPV *polh* gen vertoont namelijk een mozaïekstructuur en dat maakt fylogenetische analyses erg ingewikkeld. In dit proefschrift zijn vier geconserveerde baculovirusgenen van OpSNPV geanalyseerd, nl. *polh*, *lef-8*, *pif-2* en *dnapol*, en gebruikt voor fylogenetische studies. Daaruit bleek dat OpSNPV tot de groep II NPVs behoort en dat het de meeste verwantschap vertoont met SNPVs die *Orgyia ericae* en *Orgyia anartoides* infecteren. Het laat geen nauwe verwantschap zien met OpMNPV, dat een groep I NPV is. Deze studie laat nadrukkelijk zien dat voor een goede classificatie van baculovirussen een fylogenetische analyse, gebaseerd op handmatig aan elkaar gekoppelde (geconcateneerde) sequenties van een aantal geconserveerde genen, noodzakelijk is. De twee virussen OpSNPV en OpMNPV komen naast elkaar voor in dezelfde gastheer, maar de gevolgen voor de 'fitness' van elk individueel virus in tijd en ruimte zijn nog onbekend.

Een NPV, geïsoleerd uit *A. segetum* larven en aanwezig in dezelfde Poolse virusverzameling (AgseNPV-P), werd eerst vergeleken met AgseNPV isolaten uit Engeland en Frankrijk (AgseNPV-UK and AgseNPV-F) en deze analyse liet duidelijke verschillen in de lengte van restrictiefragmenten zien. Sequentieanalyse van drie geconserveerde baculovirusgenen, *polh*, *lef-8* en *pif-2,* toonde aan dat AgseNPV-P aanzienlijk verschilt van AgseNPV-UK en AgseNPV-F en mogelijk een nieuwe NPV soort is. Fylogenetische studies plaatsten AgseNPV-P in groep II NPVs en maakten duidelijk dat dit virus nauw verwant is

aan *Agrotis ipsilon* (Agip) NPV en *Spodoptera exigua* (Se) MNPV. De hypothese dat AgseNPV-P een nieuwe virussoort is, maakte het bepalen van de gehele genoomsequentie en een nieuwe naam voor het virus: AgseNPV-A (voor AgseNPV isolaat, waarvan de genoomsequentie het eerste bepaald is) noodzakelijk. Het circulaire genoom bestaat uit 147,129 bp en bevat 153 voorspelde coderende regio's of "open reading frames"(ORFs), waarvan er 143 homoloog zijn aan eerder gerapporteerde baculovirusgenen. Tien ORFs zijn tot dusverre uniek voor AgseNPV-A. Verder werden drie mogelijke enhancin genen geïdentificeerd, die niet nauw verwant bleken aan de enhancins, gevonden in NPVs die *Mamestra* soorten infecteren.

Hoewel de genensamenstelling verschillend is, is de volgorde van de overeenkomende genen in het genoom van AgseNPV en SeMNPV hetzelfde. Ondanks deze collineariteit hebben deze virussen andere infectiekenmerken. AgseNPV was infectieus via de orale route voor *S. exigua* larven maar de dosis die nodig is om 50% van de larven te doden was 10,000-maal hoger dan voor *A. segetum* larven. SeMNPV was niet infectieus voor *A. segetum* rupsen, wanneer het oraal werd toegediend, maar injectie van de "budded" vorm van dit virus in het hemocoel leidde wel tot succesvolle infectie. Omdat bij inoculatie in het hemocoel de middendarm gepasseerd wordt, is het aannemelijk dat de belangrijkste blokkade in *A. segetum* voor succesvolle infectie met SeMNPV gevormd wordt door de middendarm. SeMNPV bereikte zijn tweede genexpressiefase, die van de latere vroege genen, in middendarmcellen van AgseNPV na orale infectie. Daaruit werd geconcludeerd dat SeMNPV wel eens in staat zou kunnen zijn middendarmepitheelcellen van meer gastheren binnen te dringen dan tot nu toe werd aangenomen.

De genomische en fylogenetische gegevens, die zijn verkregen voor AgseNPV en LesaNPV, laten zien dat deze virussen waarschijnlijk een voorbeeld zijn van verschillende aspecten, die een rol spelen bij de evolutie van baculovirussen. AgseNPV en een groep van nauwverwante virussen zoals AgipNPV, zijn hoogst waarschijnlijk afstammelingen van een gezamenlijke voorouder, die op een gegeven moment twee afzonderlijke virussoorten voortbracht door zich aan te passen aan verschillende gastheren. Op soortgelijke wijze hebben SeMNPV en de groep van *Mamestra* infecterende NPVs in een verder verleden een gezamenlijke voorouder met de stamvader van de NPVs, die nu de verschillende *Agrotis* soorten infecteren. Aan de andere kant evolueerde OpMNPV uit LesaNPV door aanpassing aan *O. pseudotsugata*, maar deze gebeurtenis vond veel recenter plaats dan de soortsvorming van de NPVs in de verschillende *Agrotis* soorten; om precies te zijn, nadat LesaNPV werd ingevoerd in Noord-Amerika aan het begin van de 20ste eeuw. Voor een dergelijk kort tijdsbestek (minder dan 100 jaar) werden opmerkelijke verschillen in genoomsequentie waargenomen.

Deze twee voorbeelden laten eens te meer zien dat de naamgeving van baculovirussen naar de insectensoort, waar ze uit geïsoleerd werden, zeer misleidend kan zijn en lang niet altijd de fylogenetische positie van deze virussen in de baculovirusfamilie reflecteert. Daarom

wordt voorgesteld om nieuwe baculovirusisolaten pas een naam te geven na moleculaire karakterisering van op zijn minst het *polh* gen en één ander geconserveerd gen, bijvoorbeeld *lef-8* of *pif* en na vergelijking met bestaande gegevens in bio-informatische databanken.

Streszczenie

Wirusy owadzie są prawdopodobnie najliczniejszą grupą wirusową, ponieważ owady stanowią najliczniejszą gatunkowo grupę zwierząt, obejmującą ponad milion gatunków. Rodzina wirusów *Baculoviridae* wzbudza szczególne zainteresowanie badaczy ze względu na fakt powszechnego wykorzystywania bakulowirusów w kontroli biologicznej owadów jako wektorów ekspresji białek, a ostatnio również jako wektorów w terapii genowej. Bakulowirusy są dużymi wirusami z podwójnoniciowym DNA. Jak dotąd, opisano kilkaset bakulowirusów wyizolowanych z różnych gatunków owadzich, należących do rzędów Lepidoptera, Hymenoptera i Diptera. Opisy często ograniczają się jednak do ich patologii, morfologii i źródła izolacji. Międzynarodowa Komisja Taksonomii Wirusów [The International Committee on Taxonomy of Viruses (ICTV)] przypisuje izolatowi wirusowemu rangę gatunku na podstawie pełnego opisu jego morfologii, patologii, źródła izolacji, ale również pełnej sekwencji jego genomu. Obecnie pełna sekwencja genomu bakulowirusowego jest wymagana również do rejestracji izolatu wirusowego jako bioinsektycydu (rozporządzenia Komisji Europejskiej). Częściowa sekwencja wybranych genów może służyć jako solidny punkt wyjściowy do charakteryzacji izolatów wirusowych.

Punktem wyjściowym badań prezentowanych w niniejszej pracy była kolekcja wirusów Instytutu Ochrony Roślin w Poznaniu, zawierająca izolaty polowe wirusów, zbierane na przestrzeni ostatnich 50 lat. Większość izolatów pochodzących z tej kolekcji nie była nigdy charakteryzowana na poziomie molekularnym, choć wiele z nich może służyć jako naturalne bioinsektycydy w walce z powszechnymi szkodnikami upraw polowych i zasobów leśnych. Ponadto, molekularna analiza archiwalnych izolatów okazała się być bardzo cenna w ich identyfikacji oraz rekonstrukcji ich powiązań filogenetycznych z innymi bakulowirusami. W prezentowanej pracy dwa wirusy nuklearnej poliedrozy (nucleopoliedrovirus, NPV) z polskiej kolekcji wirusów, *Leucoma salicis* (Lesa) NPV i *Agrotis segetum* (Agse) NPV, zostały scharakteryzowane na poziomie molekularnym, a genom AgseNPV zsekwencjonowany w całości. Przedstawione badania potwierdziły, że dane molekularne są niezbędne do prawidłowej klasyfikacji wirusów oraz do ustalenia ich powiązań filogenetycznych z innymi wirusami. Uzyskane rezultaty pozwoliły również na wysunięcie nowych hipotez dotyczących ewolucji badanych wirusów.

LesaNPV był wyizolowany z białki wierzbówki, *Leucoma salicis* L. (Lepidoptera, Lymantriidae), popularnego szkodnika topoli (*Populus* spp.) w Europie i Azji. Około 1920 roku wirus ten został zawleczony do Ameryki Północnej. LesaNPV wyizolowany z *L. salicis* w Polsce okazał się być wariantem wirusa *Orgyia pseudotsugata* (Op) MNPV. Owad gospodarz dla tego wirusa, *O. pseudotsugata* lub znamionówka (Lepidoptera, Lymantriidae), występuje wyłącznie w Ameryce Północnej. Sekwencje trzech konserwatywnych genów bakulowirusowych, *polh*, *lef-8* i *pif-2,* ujawniły wysoki stopień podobieństwa LesaNPV do

OpMNPV, co zostało potwierdzone również w analizie restrykcyjnej ich gnomów. Analiza restrykcyjna wykazała jednak także znaczny polimorfizm fragmentów restrykcyjnych. Gen *lef-7*, kodujący późny czynnik ekspresyjny 7, oraz gen *ctl-2*, kodujący białko konotoksyno-podobne, były testowane jako markery molekularne badanych wirusów. Region zawierający gen *ctl-2* okazał się użyteczny w identyfikacji tych wirusów, ponieważ LesaNPV nie koduje UTPazy występującej w tym regionie w OpMNPV. Prezentowane badania sugerują, że wirus LesaNPV znalazł podatnego gospodarza *O. pseudotsugata* po zawleczeniu *L. salicis* do Ameryki Północnej.

Ze względu na duże podobieństwo LesaNPV do OpMNPV zbadano również jego powiązania filogenetyczne z innym wirusem jądrowej poliedrozy infekujcym *O. pseudotsugata,* OpSNPV. OpSNPV był uprzednio analizowany na podstawie sekwencji genu *polh*, kodującego białko okluzyjne, poliedrynę. Status filogenetyczny tego wirusa pozostawał jednakże nie do końca jasny. Przykład genu *polh* wirusa AcMNPV pokazuje, że rekonstrukcje filogenetyczne wykonywane na podstawie sekwencji genu *polh* mogą być bardzo mylące. Gen *polh* wirusa AcMNPV okazał się wykazywać strukturę mozaikową, co znacznie utrudnia analizy filogenetyczne. W prezentowanej pracy użyto sekwencji czterech konserwatywnych genów bakulowirusowych, *polh, lef-8, pif-2* i *dpol*, do rekonstrukcji filogenetycznych wirusa OpSNPV. Analiza wykazała, że OpSNPV należy do grupy II NPV i jest spokrewniony z wirusami SNPV infekującymi *Orgyia ericae* i *Orgyia anartoides*. Badania te podkreślają konieczność użycia sekwencji więcej niż jednego konserwatywnego genu do analiz filogenetycznych, mających na celu klasyfikację bakulowirusa. Dwa bakulowirusy, OpMNPV i OpSNPV, koegzystują w jednym gospodarzu, jednakże konsekwencje tej koegzystencji dla ich kondycji pozostają jak dotąd niewyjaśnione.

Wirus jądrowej poliedrozy wyizolowany z *A. segetum* i pochodzący z kolekcji wirusowej w Polsce (AgseNPV-P) został porównany do izolatów AgseNPV z Anglii i Francji (AgseNPV-UK i AgseNPV-F). Porównania te ujawniły wyraźny polimorfizm fragmentów restrykcyjnych. Analiza sekwencji trzech konserwatywnych genów bakulowirusowych, *polh, lef-8* i *pif-2*, pokazała, że AgseNPV-P różni się istotnie od AgseNPV-UK i AgseNPV-F i, prawdopodobnie, stanowi odrębny gatunek NPV. Analiza filogenetyczna umiejscowiła AgseNPV-P wśród grupy II NPV i wykazała, że AgseNPV-P jest najbardziej spokrewniony z *Agrotis ipsilon* (Agip) NPV i *Spodoptera exigua* (Se) MNPV. Hipoteza, że AgseNPV-P stanowi odrębny gatunek wirusa jądrowej poliedrozy doprowadziła do całkowitego zsekwencjonowania jego genomu oraz nadania mu nazwy AgseNPV-A (ze względu na pierwszy zsekwencjonowany genom izolatu AgseNPV). Kolisty genom składa się z 147129 par zasad i zawiera 153 domniemane otwarte ramki odczytu, z których 143 są homologami uprzednio opisanych genów bakulowirusowych. Jak dotąd, 10 otwartych ramek odczytu jest unikatowych dla wirusa AgseNPV-A. W genomie wirusa AgseNPV-A odkryto trzy geny kodujące enhancyny. Analiza filogenetyczna wykazała, że geny enhancyn AgseNPV-A są

spokrewnione z genami enhancyn wirusa infekujących *Maestra* spp., jednakże tworzą odrębną grupę.

Geny wirusa AgseNPV-A są ułożone w identycznej kolejności jak geny wirusa SeMNPV, jednakże to podobieństwo nie idzie w parze z właściwościami infekcyjnymi obu wirusów. AgseNPV-A infekuje oralnie *S. exigua,* jednakże dawka potrzebna do osiągnięcia 50% śmiertelności wśród gąsienic *S. exigua* jest 10000 razy wyższa niż dawka potrzebna do zabicia 50% gąsienic *A. segeyum*. SeMNPV jest nieinfekcyjny dla gąsienic *A. segetum* w infekcji oralnej, jednakże jest w pełni infekcyjny po dostarczeniu form pączkujących wirusa (budded wirus, BV) do hemocelu. Ominięcie bariery, jaką jest jelito środkowe, poprzez bezpośrednie wstrzyknięcie wirusa do hemocelu sugeruje, że jelito jest główną barierą dla infekcji wirusa SeMNPV w gąsienicach *A. segetum*. Zaobserwowano jednakże ekspresję wczesnych opóźnionych genów SeMNPV w jelicie gąsienic *A. segetum* po infekcji oralnej wirusem SeMNPV, co z kolei sugeruje, że SeMNPV może wnikać do komórek epitelium jelita środkowego gąsienic większej liczby gatunków niż dotychczas przypuszczano.

Genetyczne i filogenetyczne dane dla wirusów AgseNPV i LesaNPV, otrzymane w prezentowanej pracy, wskazują, że te dwa gatunki prawdopodobnie reprezentują dwa odmienne aspekty ewolucji bakulowirusów. AgseNPV i grupa ściśle spokrewnionych z nim wirusów jak AgipNPV są najprawdopodobniej potomkami jednego przodka wirusowego, z którego poprzez adaptacje do różnych gospodarzy powstały dwa odrębne gatunki wirusów. Ponadto, SeMNPV oraz grupa wirusów infekująca gatunki *Maestra* najprawdopodobniej również dzielą wspólnego przodka z wirusami infekującymi gatunki *Agrotis*. Z kolei, OpMNPV wyewoluował z LesaNPV poprzez adaptację do *O. pseudotsugata*, jednakże zdarzenie to miało miejsce dużo później niż specjacja wirusów infekujących różne gatunki *Agrotis* i najprawdopodobniej po zawleczeniu LesaNPV do Ameryki Północnej na początku XX wieku. Już po tak krótkim ewolucyjnie okresie (100 lat) obserwuje się wyraźne różnice na poziomie genetycznym omawianych wirusów.

Te dwa przykłady raz jeszcze podkreślają fakt, że nazywanie izolatów wirusowych nazwami gatunków owadów, z których zostały one wyizolowane, jest błędne i często nie odzwierciedla prawdziwej pozycji filogenetycznej wirusa w rodzinie bakulowirusów. Z tego względu proponuje się nadawać nazwę nowym izolatom wirusowym po uprzedniej analizie molekularnej (przynajmniej na podstawie genu *polh* i jednego z konserwatywnych genów takich jak *lef-8* lub *pif-2*) oraz starannemu sprawdzeniu w genetycznej bazie danych podobieństwa badanego izolatu do uprzednio opisanych wirusów.

Acknowledgments

The acknowledgment paragraph is the last section to be written while preparing the PhD thesis and one may think that it would be the easiest part. But that is actually not the case, as the PhD means not only the thesis book that you hold in your hands but also all the people, places and events that one comes across during the period of often more than 4 years, years so important in our formation as scientists.

My PhD story started in 2004, when I was awarded a EU fellowship to spend 9 months in the Laboratory of Virology in Wageningen. At that time I was working in the Institute of Plant Protection in Poznan, Poland, on my Polish thesis. Having only little experience in research I was overwhelmed with the possibilities and the way of doing research in Virology. This relatively short 9-month period allowed me to develop many useful skills, which I owed to many people.

First of all I want to acknowledge my promotor Prof. Just Vlak, for offering me the opportunity to join his virology group and for constantly believing in the success of this thesis. Thank you for your great supervision, fruitful discussions and inspiration for further research. I will always admire your unusual memory to recall what was written in which paper, and sometimes these were papers from the 70' that I talk about!

I want to thank Monique van Oers for all the help and support during all the years of my studies. From the very beginning she was the person whose door was always open for me, and the person who I was never ashamed to ask even silly questions. Thank you for so much help at the final preparation of the thesis. You were so fast that sometimes I could not imagine that it was one person doing that job!

I am grateful to my Polish supervisor, Jadwiga Ziemnicka, for introducing me into the world of baculoviruses, and for all support during the years that I was working in Poznan. I want to acknowledge Prof. Jerzy Lipa for enabling my first stay in Wageningen. I also want to thank my Polish colleagues, for enjoyable moments that we had in our lab, and all these even nicer moments that we share now during our yearly meetings. Bożenka, Renata, Lidka, Żaneta, Danusia, Jola and Ania, you are the perfect team to work with!

In the Laboratory of Virology I met many people, who contributed to my scientific and natural life. I was first introduced and later led through the lab by Remziye Nalcacioglu, who was a wonderful teacher and is and will always be a good friend. We do not see each other so often as we live far apart, but when we do, it is just like the time that we were together.

In Wageningen I also met Jenny Cory, who added a lot in my understanding of baculovirus biology and was also a person that I liked a lot to spend time with, though often I did not understand half of her monologues, due to my limited English. I hope to meet you again, Jenny!

I want to thank all my colleagues and staff in the Laboratory of Virology. Liljana, Mark, Marcel Westenberg, Gang, Els, Magda, Dick, Hanke, Angela, Nina, Jan, Juliette, Fang, Marcel Prins, Richard, Simone, Cristiano, Etienne, Agah, Afshin, Hans, Esther.

I am sorry to those that I have not mentioned. It was a pleasure to meet you all and work with you. My special thanks go to Thea who helped with organizing my stay in Wageningen. Wout, I will never forget the way you controlled the lab functioning.

My special thanks go to Salva Herrero, with whom I am working now in Spain. For your enthusiasm in science and for being a friend, though you keep saying we are just working mates. I wish you a lot of success with your career!

I met also so many people outside the lab. Jochem was one of them, and this friendship turned into a wonderful relation! You somehow manage to stand my impulsive behaviors. You were also the one to take care of all the formalities concerning the finishing of the thesis. Thank you for all your love and support!

I have to acknowledge the whole army of babysitters that we met during these years. Danusia Czechowska was so committed to our family. She died from cancer in 2005 and we miss her a lot. Ania Wiśniewska was the funniest babysitter ever. Monika Sujka has been with us already for three years and became a full member of our family. Maria, Elvira, Milagros and many others, thank you a lot for your help!

I want to thank my parents and my brother Kuba, for all the support they always give me. Though we live so far away from each other, and do not see each other so often, I know I can always count on you. Thank you! And last but not least I want to thank my children, Misia and Franek for being so good!! They are always traveling with me, adapting rapidly to new situations, new languages, schools and people. I could never dream of better kids.

List of Publications

Jakubowska, A.K., Gordon, K.H., Ferre, J., Herrero, S., **2010**. Down-regulation of a chitin deacetylase-like protein in response to baculovirus infection and its application in improving baculovirus infectivity. Journal of Virology, 84: 2547-2555.

Jakubowska, A.K., Lynn, D., Herrero., S., Vlak, J., van Oers, M., **2010**. Host range expansion of *Spodoptera exigua* nucleopolyhedrovirus to *Agrotis segetum* larvae when the midgut is bypassed. Journal of General Virology, 91: 898-906.

Filipiak, A., **Jakubowska**, A.K., Tomalak, M., **2010**. Species-specific polymerase chain reaction primers for a simple detection of *Bursaphelenchus fraudulentus* (Nematoda: Parasitaphelenchidae). Nematology, 12: 157-160.

Jakubowska, A.K., Ferre, J., Herrero, S., **2009**. Enhancing the multiplication of nucleopolyhedrovirus in vitro by manipulation of the pH. Journal of Virological Methods, 161: 254-258.

Jakubowska, A.K, Ince, I.A., Herrero, S., Vlak, J.M., van Oers, M.M., **2009**. *Spodoptera exigua* nucleopolyhedrovirus is not infectious for *Agrotis segetum* larvae *per os*, but only after intrahemocoelic injection. Bulletin IOBC/wprs 45: 99-102.

Jakubowska, A.K., Oers, M.M., Otvos, I.S., Vlak, J.M., **2007**. Phylogenetic analysis of *Orgyia pseudotsugata* single-nucleocapsid nucleopolyhedrovirus. Virologica Sinica 22: 257-265.

Filipiak, A., **Jakubowska**, A., Tomalak, M., **2007**. Utility of individual specimens for taxonomic identification of a quarantine nematode, *Bursaphelenhus xylophilus*. Progress in Plant Protection 47: 222-227.

Jakubowska, A.K., Peters, S., Ziemnicka, J., Vlak, J.M., Oers, M.M., **2006**. Genome sequence of an enhancine rich nucleopolyhedrovirus (NPV) from *Agrotis segetum*: collinearity with *Spodoptera exigua* multiple NPV. Journal of General Virology 87: 537-551.

Jakubowska, A., Ziemnicka, J., **2006**. Sublethal effects of baclovirus infection in *Spodoptera exigua.* Progress in Plant Protection 46: 441-445.

Jakubowska, A., Vlak, J.M., Ziemnicka, J., **2005**. Characterization of a nucleopolyhedrovirus isolated form the laboratory rearing of the beet armyworm *Spodoptera exigua* (Hbn.) in Poland. Journal of Plant Protection Research 45: 279-286.

Jakubowska, A., Oers, M.M., Ziemnicka, J., Vlak, J.M., **2005**. Molecular characterization of *Agrotis segetum* nucleopolyhedrovirus from Poland. Journal of Invertebrate Pathology, 90: 64-68.

Jakubowska, A., Oers, M.M., Cory, J.S., Ziemnicka, J., Vlak, J.M., **2005**. European *Leucoma salicis* MNPV is closely related to *Orgyia pseudotsugata* MNPV of North-America. Journal of Invertebrate Pathology 88: 100-107.

Jakubowska, A., Ziemnicka, J., **2004**. Effects of joint application of the cations with *Spodoptera exigua* (Hbn.) nuclear polyhedrosis virus SeMNPV. Progress in Plant Protection 44: 741-744.

Jakubowska, A., Ziemnicka, J., **2003**. Biological and biochemical characteristics of *Spodoptera exigua* nuclear polyhedrosis virus (SeMNPV) (*Baculoviridae*) stored for long time at different formulations. Journal of Plant Protection Research, 43: 297-304.

Jakubowska, A., **2002**. Integument – insect protection barrier. Progress in Plant Protection/ 42: 456-459.

Tarka, A., Kędzia W., **Jakubowska** A., Kwaśniewska A., Goździcka-Józefiak A., Kędzia H., **2000**. Perinatal Transmission of HPV Infection from mother to fetus, Archives in Perinatal Medicine, 6: 43-45.

Curriculum vitae

Agata Jakubowska was born on the 14th of September, 1975, in Poznan, Poland. In the years 1994-1999 she was studying biotechnology at Adam Mickiewicz University in Poznan, where she obtained her Master degree in the Laboratory of Molecular Virology, with the thesis entitled "Cell factors involved in human papilloma virus (HPV) infection development and transmission".

In September 1999, Agata started to work in the Department of Biological Control and Quarantine, in the Institute of Plant Protection in Poznan, as a technical assistant, and in 2000 began her PhD thesis on baculoviruses in the same Institute. In the years 2001-2002, she was working in the Department of Zoology of the University of Ulm, in Germany, as a young researcher on the project "Insect Chemical Ecology" financed by the European Commission under the 5th Frame Program. In 2004 she was awarded a Marie Curie fellowship for young researchers and spent nine months in the Laboratory of Virology at Wageningen University. In 2005 she obtained her doctor degree in Poland. In 2006 Agata was awarded a Junior Research Fellowship of Wageningen Graduate School "Production Ecology and Resource Conservation", and spent an additional 6 months at the Laboratory of Virology, yet working on her PhD research on baculovirus genomics. The results of this research are described in this thesis. In 2007 Agata started her Marie Curie individual fellowship in the Department of Genetics in Valencia, Spain. She was working for two years on the response of insects to baculovirus and bacteria infection. Now she is working in Valencia on a project aiming to improve the insecticidal properties of the baculovirus specific to the beet armyworm. Agata has two children, Misia (12 years old) and Franek (7 years old).

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of Literature (5.6 ECTS)

- Baculovirus genetics (2004/5)

Writing of Project Proposal (5ECTS)

- Characterization of two nucleopolyhedroviruses, *Agrotis segetum* (Agse) NPV and *Leucoma salicis* (Lesa) NPV (2005)

Laboratory Training and Working Visits (3 ECTS)

- Biology of baculoviruses; Institute of Plant Protection, Poznan, Poland (2004-2006)
-

Post-Graduate Courses (2.1 ECTS)

- Bioassays in insect pathology; SIP (2005)
- Bioinformatics; EPS (2007)
- Cell cultures; SIP (2009)

Deficiency, Refresh, Brush-up Courses (3.4 ECTS)

- Frontiers in veterinary and medical biology; Entomology, WUR (2007)
- Basic statistics; PE&RC (2007)
- Charles Darwin bicentenary; UV (2009)

Competence Strengthening / Skills Courses (1.2 ECTS)

- Competence assessment; PE&RC (2007)
- Writing grant proposals; CENTA (2007)

Discussion Groups / Local Seminars and Other Scientific Meetings (6.3 ECTS)

- Conference; Institute of Plant Protection, Poznan, Poland (2002-2009)
- Seminars; Laboratory of Virology, WUR (2004-2009)
- Group discussion on Ecology (2007)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (1.8 ECTS)

- PE&RC Day (2004)
- PE&RC Day (2006)
- Current themes in Ecology (2007)
- Cutting edge Ecology (2007)

International Symposia, Workshops and Conferences (15.6 ECTS)

- 37th Annual Meeting of Society for Invertebrate Pathology (2004)
- 38th Annual Meeting of Society for Invertebrate Pathology (2005)
- IOBC/EPRS Conference (2005)
- IOBC/EPRS Conference (2006)
- International Symposium in Insect Midgut Biology (2008)
- IOBC/WPRS Conference (2009)
- 43th Annual Meeting of Society for Invertebrate Pathology (2009)

The study presented in this thesis was carried out in the Laboratory of Viroloogy of Wageningen University, The Netherlands, and was financially supported by a Polish State Committee for Scientific Research grant n^{o} 2P06R 073 26, a scholarship from the European Union (Functional Biodiversity and Crop Protection), contract n^{o} HPMT-CT-2000-00199, Junior Researcher fellowship, Wageningen Graduate School 'Production Ecology and Resource Conservation' and a Marie-Curie Intra-European fellowship, project n^{o} 040691.

Financial support from Wageningen University for printing this thesis is greatefully acknowledged.

Printed by: GVO drukkers & vormgevers B.V./Ponsen & Looijen
Cover page drawing "Evolution": Misia Halwa