Diversity of Baculoviruses Isolated from Cutworms (*Agrotis* spp.)

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Content

LIST OF ABBREVIATIONS

(Baculovirus names and their abbreviations on pages 19 – 20; Table 1.1)

%	percent
°C	degree Celsius
µg	microgram
µl	microlitre
µM	micromolar
aa	amino acid
AGERI	Agricultural Genetic Engineering Research Institute
B.t.a.	*Bacillus thuringiensis* subspecies *azawai*
bp	base pair
BV	budded virus
C	cytosine
cm	centimeter
ddH_20	bidistilled water
dH_20	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ds	double stranded
e.g.	for example
et al.	and others
exp	exponential function
g	gravitational; multiple of *g*
G	guanine
Glu	glutamic acid
gran	granulin
GV	granulovirus
h	hour
ha	hectare
His	histidine
HRI	Horticulture Research International
ICTV	International Committee on Nomenclature of Viruses
JKI	Julius Kühn Institute
K-2-P	Kimura-2-parameter
kbp	kilo base pairs
LC10	10% lethal concentration
LC50	median lethal concentration
LD50	median lethal dose
log	logarithm
LSC	low speed centrifugation
M	molar

MBV	Monodon baculovirus
mM	millimolar
mm	millimetre
MNPV	multiple nucleopolyhedrovirus
MP	maximum parsimony
NCBI	National Centre for Biotechnology Information
ng	nanogram
NGS	next generation sequencing
nm	nanometre
no.	number
NPV	nucleopolyhedrovirus
nt	nucletide
NV	nudivirus
OB	occlusion body
ODV	occlusion derived virus
ORF	open reading frame
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
pH	measure of acidity of basicityof aqueous solutions
PM	peritrophic membrane
polh	polyhedrin
qPCR	quantitative polymerase chain reaction
REN	restriction endonuclease
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SCC	sucrose cushion centrifugation
SDS	sodium dodecyl sulfate
sec	second
SGHV	salivary gland hypertrophy virus
SGU	sucrose gradient ultracentrifugation
SNPV	single nucleopolyhedrovirus
spp.	*species pluralis*; multiple species
T_A	annealing temperature
TAE	tris base, acetic acid and EDTA buffer solution
Taq	DNA polymerase from *Thermus aquaticus*
TBE	tris base, boric acid and EDTA buffer solution
TBR	tree-bisection-reconnection
TE	tris base and EDTA buffer solution
U	enzyme unit
UV	ultraviolet light
V	volt
v/v	volume per volume
w/w	weight per weight
WSSV	white spot syndrome virus
Xaa	unspecific amino acid

CHAPTER 1: GENERAL INTRODUCTION

An Introduction to Baculoviruses

The description of diseased insect larvae goes back to the silk production in ancient China that based on the caterpillars of the silk moth *Bombyx mori*. In 1527, long time before any knowledge of microorganisms and viruses, an Italian poet described symptoms of diseased silk moth larvae, which are known today to be caused by baculoviruses (Benz, 1986). The craving of men for costly and fine silk let not only the silk moth and humans share a long part of history, but also baculoviruses that are our constant companion in a wide variety of fields, until today.

One outstanding characteristic of this virus group encouraged research and their investigation: the formation of occlusion bodies (OBs). These virus protective protein coats are observable under the light microscope and aroused the interest of scientists around the world. Since the middle of the 20th century, the potential of baculoviruses as prospective biological control agents of insect pests was understood. First baculovirus based biocontrol agents were developed, increasing the interest in the biology and the molecular setup of these fascinating viruses (Miller, 1997).

Since then, various baculovirus based biocontrol agents have been registered and applied for a sustainable control of insect pests in agriculture, horticulture and forestry. Stimulated by groundbreaking progress in cell culture techniques baculoviruses are further tested and applied in the fields of eukaryotic gene expression, gene therapy and vaccine production (for review van Oers, 2011).

Baculovirus Biology

In several insect virus groups, including the baculoviruses (*Baculoviridae*), cypoviruses (*Reoviridae*) and entomopoxviruses (*Poxviridae*) embedding of virions within huge crystalline protein matrixes is found (Eberle et al., 2012; King et al., 2011). These so-called occlusion bodies (OBs) (Fig. 1.1A and B) protect the virions from hazardous environmental conditions, such as UV light, drought, excess humidity as well as enzymatic degradation. OBs are able to persist outside the host for a certain period of time (Rohrmann, 2013).

Baculoviruses have been isolated from hundreds of insect species and are considered as the largest and the most intensively studied group of insect viruses (Martignoni and Iwai, 1987). The name baculovirus derived from the rod-shaped nucleocapsids (latin word *baculum*, meaning stick or staff), which contain the circular and double-stranded (ds) DNA genome (Herniou et al., 2011). The nucleocapsids of baculoviruses are enveloped (Fig. 1.1C). They are infectious to larval stages of the Lepidoptera, Hymenoptera and Diptera. Their life cycle is biphasic with two phenotypes, the occlusion derived virus (ODV) and the budded virus (BV). The ODVs are embedded in OBs and are responsible for the primary infection of insect midguts and the horizontal virus transmission from insect to insect. ODVs obtain their envelope by *de novo* assembly within the host cell before becoming packed in OBs. The BVs describe the second baculovirus phenotype that spreads the infection within the infected larvae from cell to cell and convey secondary infection of other tissues. They receive their envelope by budding from host cells. Both, the ODV and BV are genetically identical but are distinguished by their function and envelope components.

Morphologically, the baculoviruses are subdivided into two groups, the granuloviruses (GVs) and nucleopolyhedroviruses (NPVs) (Herniou et al., 2011). A granulovirus OB (granule) is comparably small in size (< 500 nm), contains only a single virion with one nucleocapsid and its OB matrix protein is granulin (Fig. 1.1A). The OB of a nucleopolyhedrovirus (polyhedron) is generally 1-2 µm (up to 15 µm) and can contain up to several hundreds of virions and is made of polyhedrin (Fig. 1.1B), the major OB matrix protein of the NPVs (Herniou et al., 2011). The virions envelope can include a single (SNPV) or multiple (MNPV) nucleocapsids (Fig. 1.1B). The differentiation between SNPVs and MNPVs has no taxonomic meaning and genetic factors leading to these phenotypes are unknown.

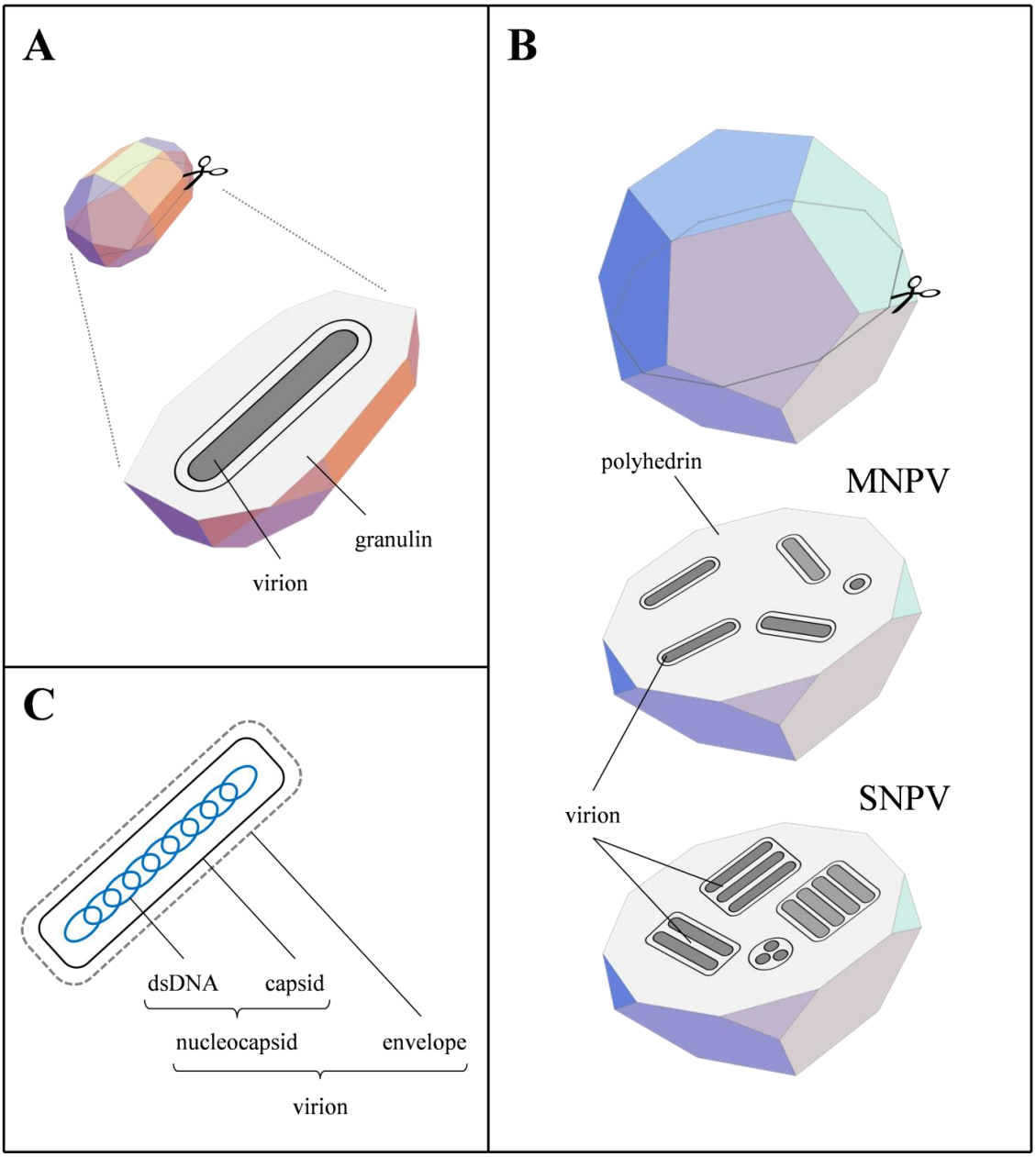

Figure 1.1. Schematic illustration of occlusion bodies (OBs) of a **(A)** granulovirus (GV) **(B)** nucleopolyhedrovirus (NPV). GV form capsule-like OBs which harbors only a single virion. The OBs of NPVs are polyhedral and embed up to many virions. In single nucleocapsid (S)NPVs a single nucleocapsid is enclosed by the virion envelope whereas in multiple nucleocapsid (M)NPV multiple nucleocapsids are enveloped. **(C)** Structure of a baculovirus virion with a single nucleocapsid. GV and NPV OB are not true to scale. Information about size of ODV-derived virions and occlusion bodies are given in the text.

Classification and Nomenclature

Naming of baculoviruses mirrors the name of the host species from which they were initially isolated. Their names consist of the scientific name of the host followed by the type of OB. The abbreviation is gained by the first two letters of the genus and species name plus OB type GV or NPV (Volkman et al., 1995). This rule for abbreviations is not followed by several historical early described baculoviruses, e.g. the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) or *Cydia pomonella* granulovirus (CpGV). These old notations have been kept also to conform to historical literature.

Not before the 1970th, the International Committee on Nomenclature of Viruses (ICTV) faced the difficulty of a rapidly increasing number of non-classified invertebrate viruses. At this time, the name and genus "Baculovirus" was first introduced. In the meantime, the baculovirus that infects caterpillars of the silk moth *B. mori* was characterized and had become the type species of this newly established invertebrate virus genus (Vago et al., 1974). Today, baculoviruses are classified as an own family, the *Baculoviridae* (Herniou et al., 2011).

Based on phylogenetic analysis the origin and diversification of baculoviruses goes back to the Paleozoic Era within the Carboniferous Period, a time at which insects also diversified into new orders (Theze et al., 2011). Viruses of the *Baculoviridae* appear to follow their host classification and are subdivided into four genera: *Alphabaculovirus, Betabaculovirus, Gammabaculovirus* and *Deltabaculoviruses* (Jehle et al., 2006a). The *Alpha-* and *Betabaculovirus* represent the largest genera and harbor NPVs and GVs that are infectious to larvae of the Lepidoptera. Within the alphabaculoviruses distinction is made between two sublineages, the group I NPVs and group II NPVs (Zanotto et al., 1993). They differ in their BV fusion protein, which is GP64 and F-protein for group I and group II NPVs, respectively (Wang et al., 2014; Miele et al., 2011).

Only two species are identified for the genus *Gammabaculovirus,* the *Neodiprion lecontei nucleopolyhedrovirus* (NeleNPV) and *Neodiprion sertifer nucleopolyhedrovirus* (NeseNPV). Their OBs contain a single nucleocapsid virions. The virus infection appears to be restricted to the midgut epithelium (Lauzon et al., 2004). *Deltabaculoviruses* are represented by *Culex nigripalpus nucleopolyhedrovirus* (CuniNPV) the only classified species of this genus (Jehle

et al., 2006a; Becnel et al., 2001; Moser et al., 2001). The virus infects midgut cells of dipteran mosquito larvae but also adults were reported to get infected (Herniou et al., 2011). CuniNPV is unique as its polyhedrin is not homologous to the granulin and polyhedrin of other baculoviruses.

Beside the *Baculoviridae* other invertebrate viruses from the genus *Nudivirus* (unclassified family) and the families *Nimaviridae* and *Hytrosaviridae* were characterized with an enveloped rod-shaped nucleocapsid, a large circular dsDNA genome, replication within the nucleus, and a shared set of genes (Herniou et al., 2011; Wang et al., 2011). Nudiviruses (*Nudivirus*), which are pathogenic to larvae and adults of various invertebrate hosts represent the closest related group to baculoviruses (Wang and Jehle, 2009). Their virions were considered to produce non-occluded virions, but the *Oryctes rhinoceros* nudivirus (OrNV), *Heliothis zea* nudivirus 1 (HzNV-1), and *Gryllus bimaculatus* nudivirus (GvNV) were found to encode for OB matrix proteins that show homology to the polyhedrin and granulin of alpha-, beta- and gammabaculoviruses (Wang et al., 2011). The transcription and functionality of these putative OB protein genes remained unknown, although OB-like structures were observed for HzNV-1 (Raina et al., 2000). An OB forming nudivirus, the so-called marine shrimp Monodon baculovirus (MBV), was discovered, which embed its virions by an unique OB matrix protein that showed no homology to any other OB protein (Yang et al., 2014; Chaivisuthangkura et al., 2008). Completely sequenced nudivirus genomes were shown to share at least 20 homologous genes that are present in all sequenced baculovirus genomes (Wang et al., 2011). Among these homologues, six *per os infectivity factor* (*pif*) genes were found to encode essential factors that are located in the ODV envelope of baculoviruses and play an important role in the primary infection process (see chapter below). Homologous *pif* genes were further identified within the genomes of the whispoviruses (white spot syndrome viruses, WSSV) (*Nimaviridae*: *Whispovirus*) and the salivary gland hypertrophy viruses (SGHV) (*Hytrosaviridae*) (Abd-Alla et al., 2008; Wang et al., 2011). WSSV infect aquatic crustaceans, whereas SGHV were characterized from dipteran adults of the tsetse fly *Glossina pallidipes* (GpSGHV), the narcissus bulb fly *Merodon equestris* (MeSGHV), and the housefly *Musca domestica* (MdSGHV) (Abd-Alla et al., 2008). SGHV were not detected to generate OB, although MdSGHV encodes for a putative OB protein similar to the polyhedrin and granulin of the *Baculoviridae* (Wang et al., 2011).

Baculovirus related genes are further detected in the genomes of bracoviruses (*Polydnaviridae*: *Bracovirus*). Their genomes are incorporated as proviruses into the genomes of parasitoid wasps (*Hymenoptera*: *Braconidae*) (Herniou et al., 2011). Production of virus-like particles is restricted to the ovaries of female wasps, where non-occluded but enveloped nucleocapsids are produced (Louis et al., 2013). The virus-like particles are injected along with the wasp's egg into the host larvae, where virus particles initiate infection of host cells and expression of immune-supressive genes packed within the virus-like particles (Bezier et al., 2009). Thereby, the virus interferes with the immune system of the host and enables the wasp larva to develop successfully. Virus replication does not take place within the lepidopteran larvae (Bezier et al., 2009). Injected nucleocapsids contain multiple segmented dsDNA circles encoding only virulence genes (Bezier et al., 2009). Genes that encode factors for virus particle structures and virus cell entry (*pif*) are missing in virus-like particles of bracoviruses and remain as provirus in the wasp genome (Louis et al., 2013; Wang et al., 2011).

Structural similarities and a large number of functional homologous genes in genomes of baculoviruses, nudiviruses, whispoviruses, SGHV, and bracoviruses let assume a common ancestor of these viruses (Bezier et al., 2009; Wang and Jehle, 2009). Their evolution and classification is not yet completely understood and recent concepts of classification are in revision.

The Baculovirus Infection Cycle

The infection cycle of baculoviruses starts with the peroral uptake of OBs by insect larvae (Fig. 1.2). Within the larval intestinal tract the OBs are transported with the food bolus to the midgut where alkaline conditions (pH > 10) lead to the disintegration of the OB matrix. Thereby, the ODVs are released from the OB. ODVs pass the peritrophic membrane (PM), which lines the midgut and functions as a natural physical barrier that protects the epithelial cells from physical damage and pathogens (Hegedus et al., 2009). It mainly consists of glycoprotein and chitin (Hegedus et al., 2009).

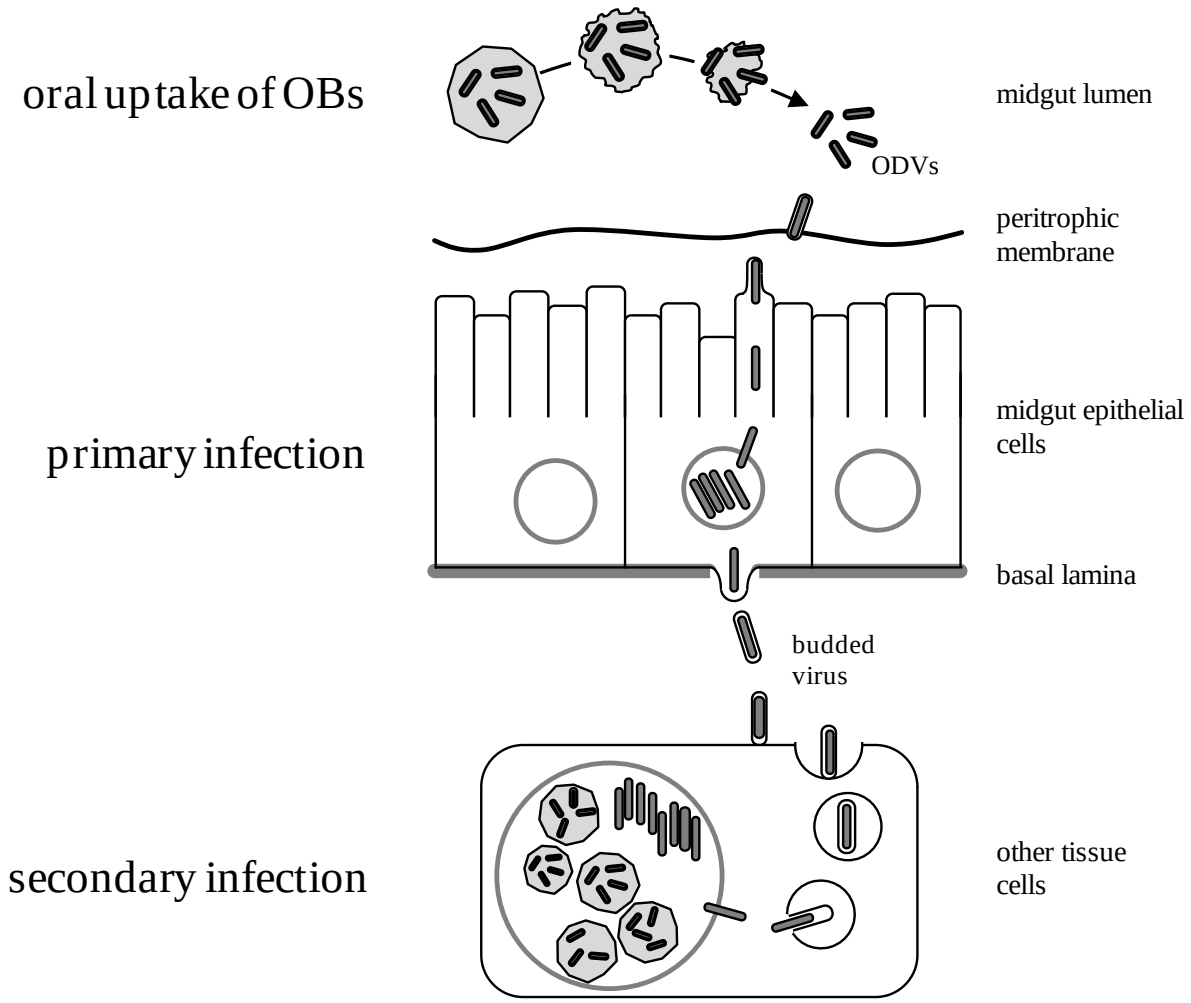

Figure 1.2. Exemplary life cycle of a SNPV. Oral uptake of OBs leads to transportation to the midgut lumen, where the OBs become dissolved by the alkaline milieu. The released ODVs pass the peritrophic membrane (PM) and fuse with the cellular membrane of the midgut epithelial cells, which initiate the release of nucleocapsids into the cell (primary infection). Upon arrival in the nucleus replication starts and new nucleocapsids are assembled. Nucleocapsids are transported to the basal membrane from where they bud into the hemolymph and spread within the whole insect. Released budded viruses (BVs) come in contact with the membrane of an uninfected cell and enter via endocytosis (secondary infection). Replication within the nucleus leads to the assembly of new nucleocapsids, which get embedded in occlusion bodies at the later stage of infection.

A variety of baculoviruses were found to encode for enhancin proteins, also referred to as enhancins, synergistic factors or viral enhancin factors (vef), which are able to disintegrate the PM membrane of the midgut. The lysis of the PM is caused by the proteinase activity of enhancins that degrade the glycoprotein mucin, the major compound of the PM (Derksen and Granados, 1988). The enzymatic degradation of the PM facilitates the access and entry of ODV to the epithelial midgut cells, where they initiate the primary infection. Enhancins were first reported for *Pseudaletia unipuncta* GV (PsunGV) for which the protein was described as a synergistic factor, increasing the susceptibility of *Pseudaletia unipuncta* NPV (PsunNPV) to larvae of the Armyworm, *Pseudaletia unipuncta*, in co-infections (Tanada and Hukuhara, 1971; Tanada, 1959). For PsunGV, enhancins were characterized to be part of the granule matrix, whereas studies on the Lymantria dispar MNPV (LdMNPV) demonstrated that enhancins are components of the ODV (Slavicek and Popham, 2005). Baculovirus enhancin proteins belong to a metalloproteinase family (Lepore et al., 1996) that have a conserved zinc-binding domain, His-Glu-Xaa-Xaa-His or HEXXH (Rawlings and Barrett, 1995). This metalloproteinase motif was first confirmed for the amino acid sequences of the two LdMNPV enhancin proteins and is found in many but not all enhancins of GVs and NPVs indicating that some may have lost their enzymatic function (Slavicek and Popham, 2005). Not all baculovirus species encode for an *enhancin* gene. The *enhancin* gene richest baculovirus to date is the *Xestia c-nigrum* GV (XecnGV) with four gene copies (Hayakawa et al., 1999). For baculoviruses that do not encode for enhancin proteins the mechanism of passing the PM is not understood. It assumed that the microvilli of the epithelial cells occasionally cut through the PM and thereby provide surface for ODV fusion with the host cell membrane.

Baculoviruses encode for several *per os infectivity factors* (*pifs*) whose products are assumed to be part of the ODV envelope, where they help to fuse the ODV with the microvilli of midgut cells and facilitate the viral entry (Fig. 1.2). Eight genes, *pif-0*/*p74* (Ac138), *pif-1* (Ac119), *pif-2* (Ac22), *pif-3* (Ac115), *pif-4* (Ac96), *pif-5* (Ac148), *pif-6* (Ac68), and *vp91*/*p95* (Ac83) have been identified to encode for factors that play an important role in the primary infection process (Zhu et al., 2013; Nie et al., 2012; Sparks et al., 2011; Fang et al., 2009). Although their deletion often leads to the loss of ODV infectivity to midgut cells, the virions remain infectious to host cells when bypassed the insect gut by injection into the hemolymph (Fang et al., 2009). PIFs are present in all sequenced baculovirus genomes, some homologues

were also found in nudiviruses (*pif-0*/*p74*, *pif-1*, *pif-2*, *pif-3*, and *pif-4*) (Wang et al., 2011), SGHV (*pif-0*/*p74*, *pif-1*, *pif-2*, *pif-3*) (Abd-Alla et al., 2008), whispoviruses (*pif-0*/*p74*, *pif-1*, *pif-2*, *pif-3*) (Wang et al., 2011) and bracoviruses (*pif-0*/*p74*, *pif-1*, *pif-2*, *pif-3*) (Bezier et al., 2009), which underlines their conserved and essential role in the infection process of these viruses. After fusion of ODVs with the membrane of midgut epithelial cells the nucleocapsids are released into the cytoplasm (Fig. 1.2). The number of simultaneously released nucleocapsids per ODVs of a MNPV can be larger than one, whereas the ODVs of SNPVs and GVs lead to a single nucleocapsid entry. From there, the nucleocapsids are transported to the nucleus of the host cell, where the viral DNA is released from the nucleocapsids and the transcription of viral genes starts (Fig. 1.2). The transcription process of baculoviruses is divided into three temporal stages: early, late and very late (Friesen, 1997; Lu and Miller, 1997). Early genes are transcribed by the host RNA polymerase II, whereas a baculovirus encoded RNA polymerase is responsible for the transcription of late and very late genes (Huh and Weaver, 1990). The nucleus of infected host cells become hypertrophied and can fill almost the entire cell (for review see Rohrmann, 2013). In alphabaculovirus induced infections the nucleus membrane remains intact, whereas in betabaculovirus infected cells the nuclear membrane is early degraded and a nuclear-cytoplasmic milieu is formed (Federici, 1997). In both cases new nucleocapsids are assembled and transported to the basal cell membrane of epithelial midgut cells (Fig. 1.2). From there they receive their viral envelope by budding from the cell membrane. These new virions are called budded viruses (BV) and represent the first virus phenotype generated during the baculovirus replication cycle (Fig. 1.2). BVs contain only a single nucleocapsid and spread through the larval body via hemolymph or the tracheal system (Engelhard et al., 1994). The infection becomes now systemic and spreads to further tissues. A second pathway of ODV-derived nucleocapsids is further assumed that a part of them bypasses the nucleus of midgut cells. In this case nucleocapsids are transported directly to the basal cell membrane where they “wait” for budding from the cell (Ohkawa et al., 2010; Granados and Lawler, 1981). In the meantime other nucleocapsids enter the nucleus, initiate the infection process, which leads to the synthesis of envelope fusion proteins that get integrated in the cytoplasmic membrane. Only then the bypassing nucleocapsids bud from the cell (Ohkawa et al., 2010).

An important prerequisite for a successful systemic infection is the modified viral envelope of BVs that differs from its components from ODVs. The BV envelope fusion protein is GP64

for group I NPVs and F-protein for group II NPVs and betabaculoviruses (Rohrmann, 2013) both facilitating the entry of nucleocapsids to new host cells. As shown for AcMNPV the inactivation of *gp64* prevented the newly assembled nucleocapsids from budding and thereby a transmission between cells within host larvae (Oomens and Blissard, 1999; Monsma et al., 1996). The functional recovery of an inactivated AcMNPV *gp64* by a *f-protein* of a group II NPV or a betabaculovirus led to recovery of AcMNPV BV infectivity (Yin et al., 2003; Lung et al., 2002). Studies on both envelop fusion proteins showed that *gp64* is likely to be idependently obtained by ancestral baculoviruses more recently leading to the evolution of group I NPVs (Wang et al., 2014). This thesis is supported by the presence of a F-like protein that is found in all goup I NPVs and appears to still play role in the viral entry process (Wang et al., 2014). The *gp64* and *f-protein* genes are both transcribed during the early and late (Washburn et al., 2003) stage of infection and both proteins are incorporated into the cytoplasm membrane where they become part of the BV envelope during the event of budding. While spreading within the infected larvae the BV progeny gets in contact with the cytoplasmic membrane of non-infected host cells (Fig. 1.2). Here, the GP64 and F-protein interact with receptors of the host cell membrane that initiate the endocytosis of BVs. The responsible host receptors of this process are unknown. First, the BV becomes enclosed within a coated vesicle, the endosome, which later releases the nucleocapsid (Fig. 1.2) (Long et al., 2006; Hefferon et al., 1999). Actin polymerizes behind the nucleocapsid whose appearance is described as a “comet tail” that transports the nucleocapsid to the cell nucleus (Ohkawa et al., 2010). Upon arrival of nucleocapsids within the nucleus the next round of nucleocapsid replication starts.

In the final phase of infection, the assembly of ODVs, the second virus phenotype generated during the baculovirus replication cycle, is initiated (Fig. 1.2). Nucleocapsids remain within the host cell and become enclosed by a *de novo* synthesized envelope that carries all ODV envelope proteins. At this time of infection the gene of the major OB matrix protein, *polh*/*gran*, reaches a very high level of transcription and expression. The POLH/GRAN protein constitutes the crystalline protein matrix of the OBs (Fig. 1.2). The OBs are released to the environment from dying or dead liquefied larvae.

Baculovirus Genomics

The technical progress in nucleotide sequence determination and the access to rapid sequencing techniques led to an increase of published baculovirus genomes since the late 1990th. Next-generation sequencing (NGS) techniques, e.g. 454 pyrosequencing and Illumina sequencing, are widely applied and allow the *de novo* sequencing of complete baculovirus genomes within a single run. To date, at least 57 baculovirus genomes were completely sequenced (Eberle et al., 2012; Garavaglia et al., 2012; Miele et al., 2011), most of them belong to *Alpha-* and *Betabaculovirus*, whereas only a few represent species of *Gamma-* and *Deltabaculovirus* (Tab. 1.1). By the high number of sequenced baculovirus genomes a comprehensive picture of the molecular setup of the *Baculoviridae* was obtained allowing the definition of common as well as distinguishing features that describe these virus genera. The circularly closed dsDNA genomes of baculoviruses range between 80 - 180 kb with a GC content of 32 – 57% (Herniou et al., 2011). The difference of more than 90 kb between the smallest genome of *Neodiprion lecontei* NPV (NeleNPV) with 81,755 bp (89 ORFs) (Lauzon et al., 2004) and the largest genome of *Xestia c-nigrum* GV (XecnGV) with 178,733 bp (181 ORFs) (Hayakawa et al., 1999) illustrates the genetic diversity of baculovirus genomes. This heterogeneity is caused by events of genetic recombination, such like duplication of genes, insertions and deletions. A total of more than 800 different genes were reported from all sequenced baculoviruses; most of them are of unknown function. Some of these genes appeared to be unique to a certain baculovirus, whereas others share orthologs with only one of a few genomes. Only 37 genes orthologs are present in all sequenced baculovirus genomes (Garavaglia et al., 2012; Miele et al., 2011; McCarthy and Theilmann, 2008). These so-called baculovirus core genes, are assumed to be the common genetic backbone of all baculoviruses. They play an essential role in primary infection of midgut cells, transcription, DNA replication, interaction with host cell, have structural or unidentified functions and are thus crucial for the baculovirus infection process (Garavaglia et al., 2012). Another common feature of many baculovirus genomes is the occurrence homologous repeat regions (*hrs*). For AcMNPV a *hr* usually consists of several imperfect palindromic repeats with a complete or incomplete *Eco*RI site in its center. More than one *hr* can be present within a baculovirus genome and studies that focused on the function of these conserved regions described them as transcriptional enhancers and putative origins of DNA replication (Kool et al., 1993; Leisy and Rohrmann, 1993).

Table 1.1. Completely sequenced genomes of the *Baculoviridae* (release 2014).

Genus	Baculovirus	Acronym	length (bp)	%GC	GenBank Accession no.
Alphabaculovirus	*Adoxophyes honmai* nucleopolyhedrovirus	AdhoNPV	113,220	35.6	NC_004690
	Adoxophyes orana nucleopolyhedrovirus	AdorNPV	111,724	35.0	NC_011423
	Agrotis ipsilon multiple nucleopolyhedrovirus	AgipNPV	155,122	48.6	NC_011345
	Agrotis segetum nucleopolyhedrovirus A	AgseNPV-A	147,544	45.7	NC_007921
	Agrotis segetum nucleopolyhedrovirus B	AgseNPV-B	148,986	45.7	KM102981
	Antheraea pernyi nucleopolyhedrovirus Z	AnpeNPV-Z	126,629	53.5	NC_008035
	Antheraea pernyi nucleopolyhedrovirus L2	AnpeNPV-L2	126246	53.5	EF207986
	Anticarsia gemmatalis nucleopolyhedrovirus 2D	AgMNPV-2D	132,239	44.5	NC_008520
	Apocheima cinerarium nucleopolyhedrovirus	ApciNPV	123876	33.4	FJ914221
	Autographa californica nucleopolyhedrovirus C6	AcMNPV-C6	133,894	40.7	NC_001623
	Bombyx mandarina nucleopolyhedrovirus	BomaNPV	126,770	40.2	NC_012672
	Bombyx mori nucleopolyhedrovirus	BmNPV	128,413	40.4	NC_001962
	Buzura suppressaria nucleopolyhedrovirus S13	BuzuNPV	120,420	36.8	KF611977
	Choristoneura fumiferana DEF multiple nucleopolyhedrovirus	CfDEFNPV	131,160	45.8	NC_005137
	Choristoneura fumiferana multiple nucleopolyhedrovirus	CfMNPV	129,593	50.1	NC_004778
	Chrysodeixis chalcites nucleopolyhedrovirus	ChchNPV	149,622	39.0	NC_007151
	Clanis bilineata nucleopolyhedrovirus	ClbiNPV	135,454	37.7	NC_008293
	Ecotropis obliqua nucleopolyhedrovirus	EcobNPV	131,204	37.6	NC_008586
	Epiphyas postvittana nucleopolyhedrovirus	EppoNPV	118,584	40.7	NC_003083
	Euproctis pseudoconspersa nucleopolyhedrovirus	EupsNPV	141,291	40.3	NC_012639
	Helicoverpa armigera multiple nucleopolyhedrovirus	HearMNPV	154,196	40.1	NC_011615
	Helicoverpa armigera nucleopolyhedrovirus NNg1	HearNPV NNg1	132,425	39.2	NC_011354
	Helicoverpa armigera nucleopolyhedrovirus C1	HearNPV-C1	130,759	38.9	NC_003094
	Helicoverpa armigera nucleopolyhedrovirus G4	HearNPV-G4	131,405	39.0	NC_002654
	Helicoverpa zea single nucleopolyhedrovirus	HearSNPV	130,869	39.1	NC_003349
	Hyphantria cunea nucleopolyhedrovirus	HycuNPV	132,959	45.5	NC_007767
	Leucania separata nucleopolyhedrovirus	LsNPV	168,041	48.6	NC_008348
	Lymantria dispar multiple nucleopolyhedrovirus	LdMNPV	161,046	57.5	NC_001973
	Lymantria xylina multiple nucleopolyhedrovirus	LyxyMNPV	156,344	53.5	NC_013953
	Mamestra brassicae multiple nucleopolyhedrovirus	MbMNPV	152,710	41.5	JQ798165
	Mamestra configurata nucleopolyhedrovirus A (90-2)	MacoNPV-A (90-2)	155,060	41.7	NC_003529
	Mamestra configurata nucleopolyhedrovirus A (90-4)	MacoNPV-A (90-4)	153,656	41.7	AF539999
	Mamestra configurata nucleopolyhedrovirus B	MacoNPV-B	158,482	40.0	NC_004117
	Maruca vitrata multiple nucleopolyhedrovirus	MaviNPV	111,953	38.6	NC_008725

Table 1.1. continued

	Orgyia leucostigma nucleopolyhedrovirus	OrleSNPV	156,179	39.9	NC_010276
	Orgyia pseudotsugata multiple nucleopolyhedrovirus	OpMNPV	131,995	55.1	NC_001875
	Plutella xylostella multiple nucleopolyhedrovirus	PlxyMNPV	134,417	40.7	NC_008349
	Rachiplusia ou multiple nucleopolyhedrovirus	RoMNPV	131,526	39.1	NC_004323
	Spodoptera exigua multiple nucleopolyhedrovirus	SeMNPV	135,611	43.8	NC_002169
	Spodoptera frugiperda multiple nucleopolyhedrovirus 3AP2	SfMNPV-3AP2	131,331	40.2	NC_009011
	Spodoptera frugiperda multiple nucleopolyhedrovirus 19	SfMNPV-19	132,565	40.3	EU258200
	Spodoptera litura nucleopolyhedrovirus G2	SpltNPV-G2	139,342	42.8	NC_003102
	Spodoptera litura nucleopolyhedrovirus II	SpltNPV II	148,634	45.0	NC_011616
	Thysanoplusia orichalcea nucleopolyhedrovirus	ThorNPV	132,978	39.5	JX467702
	Trichoplusia ni single nucleopolyhedrovirus	TnSNPV	134,394	39.0	NC_007383
Betabaculovirus	*Adoxophyes orana* granulovirus	AdorGV	99,657	34.5	NC_005038
	Agrotis segetum granulovirus XJ	AgseGV-XJ	131,680	37.3	NC_005839
	Agrotis segetum granulovirus L1	AgseGV-L1	131,422	37.3	KC994902
	Choristoneura occidentalis granulovirus	ChocGV	104,710	32.7	NC_008168
	Cryptophlebia leucotreta granulovirus	CrleGV	110,907	32.4	NC_005068
	Cydia pomonella granulovirus	CpGV	123,500	45.3	NC_002816
	Helicoverpa armigera granulovirus	HearGV	169,794	40.8	NC_010240
	Phthorimaea operculella granulovirus	PhopGV	119,217	35.7	NC_004062
	Plutella xylostella granulovirus	PlxyGV	100,999	40.7	NC_002593
	Pieris rapae granulovirus	PrGV	108,592	33.2	NC_013797
	Pseudaletia unipuncta granulovirus	PsunGV	176,677	39.8	NC_013772
	Spodoptera litura granulovirus	SpltGV	124,121	38.8	NC_009503
	Xestia c-nigrum granulovirus	XecnGV	178,733	40.7	NC_002331
Gammabaculovirus	*Neodiprion sertifer* nucleopolyhedrovirus	NeseNPV	84,264	33.4	NC_005905
	Neodiprion lecontei nucleopolyhedrovirus	NeleNPV	81,755	33.3	NC_005906
	Neodiprion abietis nucleopolyhedrovirus	NeabNPV	86,462	33.8	NC_008252
Deltabaculovirus	*Culex nigripalpus* nucleopolyhedrovirus	CuniNPV	108,252	50.9	NC_003084

Application of Baculoviruses as Biological Bontrol Agents

Baculoviruses offer great opportunities for the biological control of pests due to their high virulence against insect larvae and their very narrow host range. The very narrow host range of single baculoviruses distinguishes them from broad spectrum chemical pesticides that not only take effect against target but also non-target organisms. So far, baculoviruses were only isolated from insect species, mainly from larvae of the order Lepidoptera. They usually infect only several close related host species, often from the same family or genus. The reasons for their high specificity against few host species are found in the close co-evolutionary relationship with their hosts that resulted in adaptations to molecular, physiological, morphological, and behavioral characteristics of the host insects (Herniou et al., 2003). One of these characteristics is the alkaline milieu of midgut lumen of Lepidoptera. There, the OBs become dissolved and ODVs are released to infect the epithelial midgut cells. If such an alkaline environment is not present in the digestive tract, OBs are likely to passage the midgut without any effect, as shown for some non-lepidopteran insects and vertebrate species (Black et al., 1997; Miller and Lu, 1997). However, non-target lepidopteran organisms are also considered to uptake BVs or released ODVs that both conduct crucial steps of host cell infection by selective interaction of envelope components with cell membrane receptor site. It was shown for AcMNPV that infection of non-permissive cell lines resulted in cell entry of BVs but no BV progeny was generated (Miller and Lu, 1997). Therefore, primary factors that determine the host range are found more likely on the molecular level like gene expression and interaction of gene products that interact with molecular host functions.

The high virulence of baculoviruses is another crucial aspect for a successful application for the control of insect pests. Especially in fruit crops, any damage is not accepted by the market and insecticides have to be highly effective. An example of a highly virulent baculovirus is the GV of the codling moth, *Cydia pomonella*, a key pest in apple, pear and walnut production (Eberle et al., 2009). *Cydia pomonella* GV (CpGV) exhibits a median lethal dose (LD_{50}) of less than 2 granules per neonate larva (Huber, 1986). Nowadays, it is one of the most successful baculovirus-based biocontrol agents that is registered and commercially applied in more than 30 countries around the world; in Europe alone its application area is estimated to be more than 100,000 hectares (Gebhardt et al., 2014; Eberle and Jehle, 2006). Another success story in baculovirus pest control is the *Anticarsia gemmatalis* NPV

(AgMNPV) that is used for the control of the velvet bean caterpillar, *Anticarsia gemmatalis*, on soybeans in Brazil since the 1970s. AgMNPV has been applied on up to 2 million hectares of soybean in 2003/2004, approx. 10% of the total cultivation area (Ferreira et al., 2014; Moscardi, 1999). Interestingly, its success is not only attributed to its high virulence but also to the lack of two genes of the AgMNPV genome, *cathepsin* (*cath*) and *chitinase* (*chit*), which play role in the liquefaction and disintegration at the late stage of larval infection (Hawtin et al., 1997). As a consequence larval bodies infected with AgMNPV remain intact after death and can be used for OB recovery after collecting from the field (Oliveira et al., 2006). The development of baculovirus-based biocontrol agents does not only focus on a highest possible virulence and narrow host range. In fact, the host range of a baculovirus has to meet the requirements of a pest complex that may consist of several host species that are intended to be controlled by a single product. Furthermore, other factors like host (feeding) behavior, plant parts to be protected, area and rate of application as well as speed of kill of a virus are critical. To optimize these parameters and to develop successful pest control strategies a sound understanding of host-virus interaction is required. For example, the AcMNPV encoded *ecdysteroid glucosyltransferase* (*egt*) gene was found to intervene in the larval molting and pupation process of infected caterpillars by prolonging larval life in order to maximize baculovirus progeny production (O'Reilly et al., 1998). In some naturally occurring baculovirus genotypes this non-essential gene is missing (Simon et al., 2004) or partially deleted (Harrison, 2013) leading to changes in virulence to host larvae. Thus, the systematic and targeted screening for such Δ*egt* genotypes or a genetically engineered deletion of the *egt* gene may help to developed improved baculovirus pesticides. Other challenges of the development of baculovirus biopesticides concern the production, safety and stability of baculoviruses in the field. The production of baculovirus OBs in sufficient quantity for commercialization is often labor-intensive. Since large-scale commercial production of baculovirus biocontrol agents is mainly done *in vivo,* it requires large insect rearing facilities as well as techniques for infection of larvae, OB purification and formulation. The efforts of baculovirus *in vivo* production generally exceed the costs of chemical pesticide productions, especially when host insects are cannibalistic and had to be reared individually. For this reason new approaches focus on *in vitro* baculovirus production applying insect cell culture techniques, which are considered to be a promising alternative in baculovirus production (Nguyen et al., 2011).

The *Agrotis* Baculovirus Complex

Agrotis cutworms – serious agricultural and horticultural pests

Some polyhphagous caterpillar species of the genera *Agrotis*, *Euxoa*, *Feltia*, *Nephelodes*, *Noctua*, *Peridroma* and *Xestia* (family Noctuidae, owlet moth) that share a common habitat and feeding behavior represent some of the most important agricultural and horticultural pests and are often summarized under the non-taxonomic term "cutworm" (Hill, 1997). They are described as hazardous pests of numerous vegetables and field crops around the world (Hill, 1997; Zhang, 1994; Metcalf and Flint, 1951). Spending most of their life cycle close to the soil surface, cutworms mainly feed on plant parts that are located close to the ground. The larvae mainly feed at night and damage plants underground or close to the soil surface. There, they are difficult to detect; especially late larval stages stay within the soil, which complicates their control by spray applications of insecticides. Seedlings are particularly endangered and can get fatally damaged (Hill, 1997). The German Federal database for crop protection products in horticulture (*Deutsches Pflanzenschutzmittelverzeichnis*, https://portal.bvl.bund.de/psm/jsp/) lists five registered chemical insecticides (status 2014) based on lambda-cyhalothrin (pyrethroid) for the control of noctuid pests on tomato, paprika, potato, lettuce, cabbage, leek, root vegetables, asparagus, onions, and sugar beet. The only registered biological control agent is the bacterium *Bacillus thuringiensis* subspecies *azawai* (B.t.a.).

Two species of the genus *Agrotis* are well described in literature as worldwide distributed serious pests: the common cutworm, *Agrotis segetum* (Denis & Schiffermüller), and the black cutworm, *Agrotis ipsilon* (Hufnagel). Whereas *A. segetum* is mainly distributed in Africa, Asia and Europe, the larvae of *A. ipsilon* represent a serious pest in North America especially to corn. Both pests are known to feed on vegetables, root crops, turnips, cotton, tobacco, corn, sugar beet and onions (Zhang, 1994). In Germany, *Agrotis* cutworms endanger the production of field-grown spring onions, potatoes and asparagus. During the yearly resting period of asparagus, which starts in the middle of June, the stems of growing plants suffer feeding damage that can lead to bending of the stems, secondary infections and harvest loss in the following year. Mainly chemical insecticides, such as pyrethroids, are applied for their control (Ziegler, personal communication). Since many field crops endangered by cutworms, e. g. onions, cabbage, turnips and beets, grow in the soil and are directly supplied to the customer

for consumption the use of chemical pesticides is considered as critical. Hence, alternative, more consumer friendly and environmentally safe biocontrol agents are needed for the control of cutworms. Several studies focusing on parasitoids, entomopathogenic fungi, nematodes, and baculoviruses, all isolated from cutworms, suggested some potential of biological alternatives to chemicals for cutworm control (Gokce et al., 2013; Wraight et al., 2010; Caballero et al., 1993; Ignoffo and Garcia, 1979).

Agrotis baculoviruses and their potential for the biological control of cutworms

The history of discovery of *Agrotis* baculoviruses goes back to beginnings of baculovirus discovery itself. In 1936, André Paillot, a pioneer of baculovirus research, published a review about "*Des Maladies à Ultravirus des Insectes*" (Paillot, 1936). There, he first reported histological studies of infections of *A. segetum* larvae caused by a GV and a NPV. Thereby, the first GV and NPV disease of *A. segetum* were described. However, it remains unclear if these baculoviruses have been preserved until today and if they are identical to those *Agrotis* baculoviruses, which have been later studied. Since the first description of *A. segetum* granulovirus (AgseGV), at least five different isolates have been mentioned in literature (Tab. 1.2). Molecular analysis, however, revealed only minor differences between these AgseGV isolates. According to DNA restriction endonuclease (REN) fragment profiles the Spanish and Danish isolates were regarded as "indistinguishable" (Bourner et al., 1992). Minor differences in their nucleotide sequence were detected between two completely sequenced Chinese isolates AgseGV-XJ and AgseGV-L1 (Tab. 1.2) (Zhang et al., 2014), which both share a high pairwise nucleotide sequence identity of more than 99%. Furthermore, AgseGV DNA restriction patterns of the Danish isolate (*Eco*RI) and JKI isolate (*Hind*III) are published (Allaway and Payne, 1984; Gürlich, 1993).

Table 1.2. *Agrotis* baculoviruses isolated from larvae of the genus *Agrotis* (Noctuidae: Lepidoptera). References that name persons, their institutes, sampling location and, if available, year of sampling are underlined.

Agrotis baculovirus	strain/isolate	location	sampled by	trivia names[a]	references
AgseGV	JKI isolate	Wien, Austria	Otto Muhr, 1964[b]	AsGV	Zethner, 1980; Gürlich, 1993
	Danish isolate	Denmark	Dr. O. Zethner, Royal Veterinary and Agricultural University, Copenhagen, Denmark	AsGV	Allaway and Payne, 1984; Bourner and Cory, 1992
	Spanish isolate	Spain	unknown to the author	AsGV	Caballero et al, 1991; Bourner et al, 1992
AgseGV-XJ[c]	Xinjiang isolate	Xinjiang, China	unknown to the author	AsGV, AsGV-XJ	Zhang et al, 2014
AgseGV-L1	Shanghai isolate	Shanghai, China	Huiyin Peng, Wuhan Institute of Virology, Chinese Academy of Sciences, 1997	AsGV	Zhang et al, 2014
AgseNPV-A[d]	Polish isolate	Poland	Dr. J. J. Lipa, Institute of Plant Protection, Poznan, Poland, 1966	$AsNPV^{P}$, AgseNPV-P	Lipa et al., 1971; Jakubowska et al., 2006
AgseNPV-B[e]	English isolate, Oxford isolate	Oxford, England	Dr. P. L. Sherlock, Rothamsted Experimental Station, Harpenden, Herts., UK	$AsNPV^{E}$, AgseNPV-UK	Allaway and Payne, 1983; Jakubowska et al., 2005, 2006
AgseNPV-B[e]	French isolate	France	Dr. A. Burgerjon, INRA Biological Control Station, La Minière, France	$AsNPV^{F}$, AgseNPV-F, AgseNPV (A12-3)	Allaway and Payne, 1983; Jakubowska et al., 2005, 2006
AgexNPV	Polish isolate	Poland	Dr. J. J. Lipa, Institute of Plant Protection, Poznan, Poland, 1967		Lipa et al., 1971
AgipNPV	Illinois strain[d]	Illinois, USA	Dr. J. Maddox, Illinois Natural History Survey, Champaign, IL, USA	AgipMNPV; AgipNPV-M6-2	Boughton et al., 1999; Harrison, 2009
	Kentucky strain	Kentucky, USA	unknown to the author		Harrison, 2009

[a] trivia names should help to identify viruses in old literature; their usage is not recommended anymore

[b] person and date mentioned in entry of virus collection of the Julius Kuehn Institute, Institute for Biological Control, Federal Research Center for Cultivated Plants (former Biologische Bundesanstalt für Land- und Forstwirtschaft), Darmstadt Germany

[c] listed as putative species of the genus *Betabaculovirus* but have not been approved yet (Herniou et al., 2011)

[d] listed as distinct species of the genus *Alphabaculoviruses* (Herniou et al., 2011)

[e] isolates proved as identical and merged under the name AgseNPV-B

So far, three geographic AgseNPV were obtained and described from infected *A. segetum* larvae. One AgseNPV originated from Poland (Tab. 1.2) and was compared biochemically and by REN fragment analysis with the isolates from England and France (Tab. 1.2) (Allaway and Payne, 1983). The results indicated that the Polish isolate significantly differs from the English and French isolates, which themselves appeared to be identical. The hypothesis that the three viruses represent two different strains or species was supported phylogenetic analysis based on partial sequencing analysis of the *polyhedrin* and *late expression factor 8* (*lef-8*) (Jakubowska et al., 2005; El-Salamouny et al., 2003) and whole genome comparisons (Jakubowska et al., 2006).

Accordingly, the Polish isolate was named AgseNPV-A and later classified as a species of the genus *Alphabaculovirus*. The identical English and French isolates were summarized as AgseNPV-B. The taxonomic status of AgseNPV-B remained unclear but phylogenetic analysis that based on partial nucleotide sequences of *polyhedrin* and *late expression factor 8* (*lef-8*) genes placed AgseNPV-B in a closer relationship to a third *Agrotis* baculoviruses, which was isolated from larvae of *A. ipsilon* in Illinois, USA (Jakubowska et al., 2005). This so-called *Agrotis ipsilon* nucleopolyhedrovirus (AgipNPV) Illinois strain is identified as the second classified distinct *Agrotis* baculovirus species (Harrison, 2009; Boughton et al., 1999). It forms a monophyletic clade with AgseNPV-A (Harrison, 2009). Whether AgseNPV-B represents a third *Agrotis* baculovirus species or has to be considered as a genetic variant of AgipNPV Illinois strain remained unclear. A second AgipNPV isolate from Kentucky, USA (Tab. 1.2) is also mentioned in literature but is thought to be highly similar to the Illinois strain (Harrison, 2009). From caterpillars of *A. exclamationis* in Poland another NPV, the *Agrotis exclamationis* NPV (AgexNPV) was isolated (Tab. 1.2). Lipa et al. (1971) compared this virus with AgseNPV-A under the electron microscope, found only minor differences and assumed that both viruses were probably identical. Later on, partial sequencing results indicated nucleotide sequence identity with AgseNPV-B (Jakubowska et al., 2005). Since comprehensive nucleotide sequence analysis are missing for AgseNPV-B and AgexNPV the taxonomic status for both viruses remained unclear.

The potential of *Agrotis* baculoviruses as environmental friendly biocontrol agents of *Agrotis* cutworms was early understood. It was discovered that *Agrotis* baculoviruses were applicable for the control of not only a single cutworm species but show high pathogenicity to

A. segetum, *A. ipsilon* and several other noctuid larvae allowing a combined pest control by a single virus application (El-Salamouny et al., 2003; Allaway and Payne, 1984; Lipa et al., 1971). For a targeted virus application for cutworm control two strategies are considered and were tested in experimental case studies. The first method is the spray application that scatters evenly the aqueous OB suspension along the row of plants covering soil and ground near leaves and stems of plants (Zethner et al., 1987; Zethner, 1980; Shah et al., 1979). An alternative approach is the usage of *Agrotis* baculoviruses in bait formulations. For this application method an aqueous virus suspension is mixed with a solidifying substrate, which serves as larval food and attracts cutworms. As additive wheat bran was considered and used for bait formulation with AgseGV (Bourner et al., 1992; Caballero et al., 1991). Virus and attractant were then spread evenly on the soil surface close to the plants. In both spray application and bait formulation, the cutworms are thought to take up the OBs during their feeding and to get infected. The spray application was favored in early attempts of *Agrotis* cutworm control, when AgseGV was applied in field trials in Northern Pakistan (Shah et al., 1979). In this region, larvae of the black cutworm, *A. ipsilon*, were described as the most devastating pest for tobacco seedlings leading to about 25% loss of plants (Shah et al., 1979). In standardized field trials second instar *A. ipsilon* larvae feeding on tobacco seedlings were tried to be controlled by AgseGV sprays at a concentration of 2×10^{14} GV/ha. After three weeks the plants were monitored and AgseGV treated tobacco plants showed a damage reduction of about 78%, and 43% fewer plants were cut by cutworms in comparison with an untreated control (Shah et al., 1979). Related experiments within the same geographic region and using the same virus were conducted on tobacco, okra and sugar beet (Zethner et al., 1987). Here, larvae of *A. segetum* and *A. ipsilon* were not released since large populations of both species occurred naturally. A 64-85% reduction in damage was reported. A similar success was achieved on carrots in Denmark where the application of AgseGV reduced the damage by 50% (Zethner, 1980). The extensive testing of AgseGV as a biological control agent in the field led to the registration of a virus preparation called AGROVIR (Saturnia, Copenhagen) in Denmark as a biocontrol agent (Huber, 1995). The product contained the Danish isolate of AgseGV (Tab. 1.2), which was also tested in the field studies in Northern Pakistan and Denmark by Zethner (1980) and Zethner et al. (1987). Another attempt of cutworm control by *Agrotis* baculoviruses was done in the former Soviet Union (USSR) by producing an AgseGV based product called Virin-OS. An amount of 5×10^{12} OB/ha was

applied for cutworm control on cotton, sugar beet, vegetables and winter cereals resulting in a of 65% to 85% reduction of the in cutworm population (Lipa, 1998). Even a mixture of AgseGV and a not further classified AgseNPV was produced and used (Lipa, 1998). According to Lipa (1998) the effect was "reasonable" and numbered by an efficacy of 77.5% after application of 5 x 10^{11} to 2.5 x 10^{12} OB/ha. The registration of AgseGV products in Denmark and the former Soviet Union was terminated in the 1990s due to changes in the European policy of registration of plant protection agents (implementation of Directive 91/414/EEC) and the collapse of the USSR, respectively.

Promising alternatives to AgseGV for the control of cutworms were found in AgseNPV-A, AgseNPV-B and AgipNPV, which exhibit a broader host range and a faster efficacy than AgseGV (Bourner and Cory, 2004; El-Salamouny et al., 2003; Bourner et al., 1992; Lipa et al., 1971). In particular, this was demonstrated for AgseNPV-B and AgseGV in comparative field studies that focused on the effect of both viruses on populations of released *A. segetum* larvae on corn (Bourner et al., 1992). The larvae were allowed to feed for certain periods of time on treated seedlings and were sampled afterwards. Collected caterpillars were reared under laboratory conditions, checked for symptoms of viral infection and their time to death was recorded. Whereas former studies calculated the effect of virus application by measuring the damage reduction on plants, this study showed the effect of virus application on larvae in the field. It was proven that larvae become infected by bait and spray application methods and that under field conditions *A. segetum* larvae were killed earlier by AgseNPV-B than by AgseGV. Also a higher percentage of larvae died indicating a better protection of plants by the NPV than the GV.

Another aspect of a successful biological control of cutworms is the application of more than one virus that simultaneously infects pest larvae and may lead to an increased mortality. In natural virus populations the common occurrence of AgseGV and AgseNPV-B was observed but it remained unclear if this virus interaction is beneficial in terms of pest control. At least one attempt of a combined virus application for cutworm control is reported (Lipa, 1998) but no statement was made according to any possible virus interaction. At least the capability of *A. segetum* caterpillars to simultaneously host a GV and NPV was already described by Shvetsova and Ts'ai (1962). In general, simultaneous infections of larvae by a GV and NPV could be beneficial by increasing mortality rates or killing speed. However, their level of

interaction has to be extensively evaluated first. The classic example of a synergistic interaction of a GV and an NPV was described for PsunGV and PsunNPV that lead to an increased larval mortality when both viruses were administered together (Tanada, 1959). However, studies that investigated possible interaction of *Agrotis* baculoviruses in cutworms are missing, although they may provide new possibilities in cutworm pest control.

THESIS OUTLINE

Until today, cutworms of the genus *Agrotis* remain a serious threat to various vegetable and field crops. They are mainly controlled by chemical pesticides that present a hazard for customers and the environment. Recent attempts strengthen the efforts to control these polyphagous pests by biological control agents such as baculoviruses. However, for licensing baculoviruses as biological control agents a profound understanding of their characteristics, biology, and pathogenicity is required.

Chapter 2 presents the genome sequence analysis of AgseNPV-B. The genome of AgseNPV-B is compared with the previously published whole genome sequences of AgipNPV and AgseNPV-A. The studies put AgseNPV-B in the taxonomic context of the *Baculoviridae* and provide answer if AgseNPV-B should be considered as a new baculovirus species of the *Agrotis* baculovirus complex.

For studying *Agrotis* baculoviruses a reliable technique for the detection of AgseNPV-A, AgseNPV-B, AgipNPV, and AgseGV in larvae as well as virus samples is required. In **Chapter 3**, the development of a *multiplex* PCR based method for the simultaneous identification of these viruses is described. A refinement of the method further allows quantitation of OBs of AgseGV and AgseNPV-B within samples and will give insight into the difficulty in working with viruses that infect the same host species.

Since *Agrotis* baculoviruses were shown to share a common host range, a mixed application of more than one *Agrotis* baculovirus gained interest to increase the efficacy and to avoid the development of resistance in insect populations. In the past, AgseGV was one of the most intensively tested *Agrotis* baculoviruses for the control of cutworms beside AgseNPV-B.

Additionally, co-infections of host *A. segetum* larvae of these two viruses had been reported, but detailed studies on virus interactions were not performed. If a mixed application is beneficial in terms of pest control remained uncertain, as well. Therefore, **Chapter 4** will focus on the level of interaction of AgseGV and AgseNPV-B in single and mixed infection scenarios of *A. segetum* larvae. Besides rates of mortality, the generation of progeny of AgseGV and AgseNPV-B is studied on the individual level of singly infected and co-infected larvae by using the qPCR method described in Chapter 2.

Chapter 5 will provide a concluding discussion of the results obtained in Chapter 2 to 4.

CHAPTER 2: THE GENOME SEQUENCE OF *AGROTIS SEGETUM* NUCLEOPOLYHEDROVIRUS B (AGSENPV-B) REVEALS A NEW BACULOVIRUS SPECIES WITHIN THE *AGROTIS* BACULOVIRUS COMPLEX

Abstract

The genome of *Agrotis segetum* nucleopolyhedrovirus B (AgseNPV-B) was completely sequenced and compared with whole genome sequences of the *Agrotis segetum* nucleopolyhedrovirus A (AgseNPV-A) and *Agrotis ipsilon* nucleopolyhedrovirus (AgipNPV). The AgseNPV-B genome is 148,981 bp in length and encodes 150 putative open reading frames. AgseNPV-B contains two copies of the gene coding *viral enhancing factor* (*vef*), making the *Agrotis* nucleopolyhedroviruses and *A. segetum* granulovirus (AgseGV) very rich in *vef* in comparison to other baculoviruses. Genome alignments of AgseNPV-B, AgseNPV-A and AgipNPV showed a very high genome co-linearity interspersed with variable regions, which are considered as putative sites of genomic recombination. Phylogenetic analyses revealed that all three viruses are distinct. However, AgseNPV-B is more closely related to AgipNPV suggesting that both viruses are at an early stage of phylogenetic divergence. It is proposed that AgseNPV-B belongs to a third *Alphabaculovirus* species of the *Agrotis* baculovirus complex. The *Agrotis exclamationis* nucleopolyhedrovirus (AgexNPV) shared high nucleotide sequence identities with AgseNPV-B, suggesting it is actually an AgseNPV-B isolate.

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Introduction

The non-taxonomic term “cutworm” is comprised of various caterpillar species in the lepidopteran family Noctuidae, namely from the genera *Agrotis*, *Apamea*, *Euxoa*, *Feltia*, *Nephelodes*, *Noctua*, *Peridroma* and *Xestia* (Metcalf and Flint, 1951). Cutworms spend most of their larval stages on or below the soil surface. They feed on the roots and stems of plants. *Agrotis ipsilon* (Hufnagel), the black or greasy cutworm, and *A. segetum* (Denis & Schiffermüller), the common cutworm, are two *Agrotis* species of economic importance in Africa, Europe and North America (Zhang, 1994). The control of these pests with chemical pesticides is difficult but biological control agents, such as entomopathogenic fungi (Wraight et al., 2010), bacteria (Ruiz de Escudero et al., 2014), nematodes (Han et al., 2014; Gokce et al., 2013), parasitoids (Caballero et al., 1993, 1990) and baculoviruses (Bourner et al., 1992; Caballero et al., 1991), were successful.

Baculoviruses (family *Baculoviridae*) include a large number of dsDNA viruses, which infect the larval stage of Lepidoptera, Diptera and Hymenoptera (Herniou et al., 2011). Baculovirus phylogeny follows host phylogeny, which indicates a close co-evolution of virus and host (Jehle et al., 2006b). This co-evolutionary relationship is also reflected through the very narrow host range of most baculoviruses, making them highly specific for only a few susceptible host species. Due to their high virulence and narrow host range baculoviruses have been developed as biocontrol agents for the control of insect pests in agriculture, horticulture and forestry (Kabaluk et al., 2010; Szewczyk et al., 2009; Moscardi, 1999).

The so-called *Agrotis* baculovirus complex is composed of several closely related baculoviruses that were isolated from different *Agrotis* species. Three European isolates, a Polish, a French, and an English (Oxford) isolate of *Agrotis segetum* nucleopolyhedrovirus (AgseNPV), were previously described (Allaway and Payne, 1983; Jakubowska et al., 2005; Lipa et al., 1971). Biochemical and DNA restriction endonuclease analyses of the Polish isolate, which is also designated AgseNPV-A, were found to be significantly different from those of the English and French isolates (Allaway and Payne, 1983; Jakubowska et al., 2005; Jakubowska et al., 2006).The French and English isolates are actually identical and were designated as AgseNPV-B (Oxford strain) (Jakubowska et al., 2006). At least two NPVs were isolated from *A. ipsilon* (Agip) larvae: AgipNPV (Illinois strain) (Harrison, 2009; Boughton et al., 1999) and AgipNPV (Kentucky strain) (Harrison, 2009). Another NPV originated from *A.*

exclamationis (AgexNPV) and was compared with AgseNPV-A by electron microscopy (Lipa et al., 1971). However, no analyses of AgexNPV have been conducted to evaluate its taxonomic position within the *Agrotis* baculovirus complex. Furthermore, in previous publications *A. segetum* granulovirus (AgseGV) isolates were described for *A. segetum* larvae from Austria (Zethner, 1980), Spain (Caballero et al., 1991) and China (Zhang et al., 2014).

These viruses are able to cross-infect *A. segetum* and *A. ipsilon*. Additionally, simultaneous infections of larvae by more than one *Agrotis* baculovirus were observed where viruses interacted and competed for larval resources (Chapter 4). Different *Agrotis* baculoviruses were already field-tested as biocontrol agents for the control of *A. segetum* and *A. ipsilon* ((Bourner and Cory, 2004; El-Salamouny et al., 2003).

In recent years the genome sequences of various *Agrotis* baculoviruses including AgseNPV-A (Jakubowska et al., 2006), AgipNPV (Harrison, 2009) and AgseGV-XJ, Xinjiang strain (GenBank accession number NC_005839), AgseGV-L1, and the Shanghai strain (Zhang et al., 2014) have been determined, leaving the genome of AgseNPV-B uncharacterized. Due to partial sequencing of the *polyhedrin* (*polh*), *late expression factors 8* (*lef-8*) and *9* (*lef-9*) genes, AgseNPV-B was proposed to be closely related to AgipNPV (Jehle et al., 2006b). To complete the comparative whole genome analysis of the *Agrotis* baculoviruses complex, the AgseNPV-B genomic sequence was completed and described in this study. By DNA endonuclease restriction analysis and genome sequence analysis, it was demonstrated that AgseNPV-B is a distinct NPV that should be classified as a separate species. Sequence comparison with other *Agrotis* baculoviruses provides insight into the co-evolution of these viruses that adapted closely to their hosts.

Materials and Methods

Agrotis baculoviruses

Four different nucleopolyhedroviruses and one granulovirus were isolated and characterized from larvae of the genus *Agrotis* (Lepidoptera: Noctuidae) and were used in this study. The *Agrotis segetum* nucleopolyhedrovirus A (AgseNPV-A, Polish strain) and the *Agrotis exclamationis* nucleopolyhedrovirus (AgexNPV) were both obtained from Agata Jakubowska (University of Valencia, Spain). The *Agrotis segetum* nucleopolyhedrovirus B (AgseNPV-B, Oxford Strain) and the *Agrotis segetum* granulovirus (AgseGV) were both provided by Doreen Winstanley (Horticulture Research International (HRI) collection, Warwick, UK), and the *Agrotis ipsilon* nucleopolyhedrovirus (AgipNPV, Illinois Strain) was obtained from Naglaa A. Abdallah (Faculty of Agriculture, Cairo University, Giza, Egypt).

Viral genomic DNA isolation

The protein matrix of viral occlusion bodies (OB) of AgseNPV-A, AgseNPV-B, AgipNPV and AgseGV was dissolved in 0.1 M Na_2CO_3 for 1 h. The suspension containing released occlusion derived virus (ODV) was adjusted to a pH 7 by titrating it with 1 N HCl and incubated with 250 µg proteinase K for 1 h at 50 °C to hydrolyze viral proteins. RNA contaminants were treated with 250 µg of RNase A for 30 min at 37 °C. Viral genomic DNA was extracted by two phenol-chloroform extractions followed by ethanol precipitation (Sambrook and Russell, 2001). The purified DNA was finally dissolved in an appropriate volume of ddH_2O and stored at -20 °C.

Pulsed-field gel electrophoresis (PFGE) of linearized genomic DNA

About 2.5 µg viral genomic AgseNPV-B DNA were digested with 5 U *Avr*II (New England Biolabs) in a total volume of 10 µl at 37 °C for 5 h in the recommended reaction buffer. Digested and untreated samples were loaded on a 0.8% pulsed-field certified agarose gel and were analyzed by PFGE (Bio-Rad CHEF-DR III system) at 6 v/cm with switch ramped times

from 1 to 12 s for 15 h at 14 °C in 0.5x TBE buffer. DNA was stained with 0.01% ethidium bromide solution and documented by a fluorescence imager.

DNA restriction analysis

One µg DNA samples from AgseNPV-A, AgseNPV-B, AgipNPV, and AgseGV were each treated with 5 U of either *Eco*RI or *Hind*III (Thermo Fisher Scientific). Digestions were performed overnight and analyzed on a 0.8% agarose gel in 1x TAE buffer for 16 h at 25 V.

Sequencing analysis

About 15 µg of AgseNPV-B genomic DNA was sequenced by using the 454 sequencing system (Genterprise Company, Mainz, Germany) resulting in about 250,000 single-end reads with an average length of 232 nt. We assumed total genome length of AgseNPV-B was 150,000 bp, which resulted in 384-fold coverage. Assembling of reads was performed using SeqMan (Lasergene 8.0; Dnastar Inc.). Provided contigs >2000 nt were re-assembled or manually fitted together. Sequence regions that could not be resolved, such as adenine-rich regions, were determined by Sanger sequencing (StarSEQ GmbH, Germany). Oligonucleotide primer pairs located about 200 - 400 bp upstream and downstream to the regions of concern were designed and used for PCR amplification. The resulting PCR fragments were directly used for sequencing and the results were integrated into the assembly of the AgseNPV-B genome.

To identify open reading frames (ORFs), the following criteria were chosen: i. they should not overlap by more than 100 nt; ii. they should be longer than 150 nt; iii. they were not located within a homologous region (hr). ORF annotation was done using GeneQuest (Lasergene 8.0; Dnastar Inc.) and blastp (http://www.ncbi.nlm.nih.gov/BLAST/). ORFs that did not fit these criteria but were homologues of other baculoviruses ORFs were included in the analysis. ORFs were translated *in silico* and amino acid (aa) sequence identities were calculated on the basis of pairwise sequence alignments, which were generated using CLUSTAL W (Thompson et al., 1994) with GONNET matrix. Gap penalty was set to 10 and gap extension

penalty was set to 0.1 for pairwise and 0.2 for multiple alignments in Geneious v5.6.2 (Biomatters). Oligonucleotide primer pairs that were used for re-sequencing of unresolved AgseNPV-B regions were further used on AgexNPV DNA for PCR amplification. The amplicons were purified and sent for sequencing (StarSEQ GmbH, Germany); the AgexNPV sequences were compared with the corresponding AgseNPV-B sequences of this study.

Phylogenetic analysis

For phylogenetic analyses the aa sequences of 37 baculovirus core genes (Garavaglia et al., 2012; Miele et al., 2011) of selected group I NPVs and group II NPVs (both belong to the genus *Alphabaculovirus*), AgseGV-XJ and *Cydia pomonella* granulovirus (CpGV) (genus *Betabaculovirus*), *Neodiprion sertifer* NPV (NeseNPV) (genus *Gammabaculovirus*), and *Culex nigripalpus* NPV (CuniNPV) (genus *Deltabaculovirus*) were aligned and then concatenated. From the concatenated alignment a phylogram was constructed by maximum parsimony analysis and the robustness of the tree was tested by bootstrap analysis (1000 replicates) using MEGA 5.2 software (Tamura et al., 2011). The same phylogenetic approach was performed for the aa sequences of the viral enhancing factors (*vef*) of AgseNPV-A, AgseNPV-B, and AgipNPV.

Distances between AgseNPV-A, AgseNPV-B, and AgipNPV were calculated with MEGA according the Kimura-two-parameter (Kimura, 1980) for complete nucleotide sequences of *polyhedrin* (*polh*), *late expression factor 8* (*lef-8*) and *late expression factor 9* (*lef-9*) (Jehle et al., 2006b). Conservation and co-linearity of *Agrotis* baculovirus genomes were visualized by gene-parity plot analysis (Hu et al., 1998).

Results and Discussion

Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis is a powerful and reliable method for the identification and discrimination of baculoviruses at the DNA level to distinguish between baculovirus species, isolates (Eberle et al., 2009), and genotypic variants (Simon et al., 2004). The *Hind*III and *Eco*RI restriction patterns of AgseNPV-A, AgseNPV-B, and AgipNPV (Fig. 2.1) were identical to the RFLP results of previous studies (Jakubowska et al., 2005; El-Salamouny et al., 2003; Boughton et al., 1999; Allaway and Payne, 1983). The *Eco*RI restriction profile of AgseGV (Wennmann and Jehle, 2014) was identical to the deduced restriction patterns of AgseGV-XJ (GenBank accession no. NC_005839) and AgseGV-L1 (GenBank accession no. KC994902) genome sequences. The *Hind*III restriction profile of AgseGV, provided in this study, also matched sequence based simulated RFLP patterns (data not shown). To confirm the correctness of the sequence assembly of the AgseNPV-B genome, *in silico* DNA restriction patterns were computed for *Hind*III and *Eco*RI. The match between computed and observed restriction patterns (Fig. 2.1) confirmed the correctness of the genome sequence assembly.

AgseNPV-B genome characterization

The assembly of reads resulted in various numbers of contigs. The largest contigs were aligned manually to obtain the complete genome of AgseNPV-B. Eventually, the length of the circular genome of AgseNPV-B was determined to be 148,981 bp. The two DNA restriction endonucleases *Sbf*I and *Avr*II were found to cut the genome of AgseNPV-B only once at position 25,368 and 128,124, respectively.

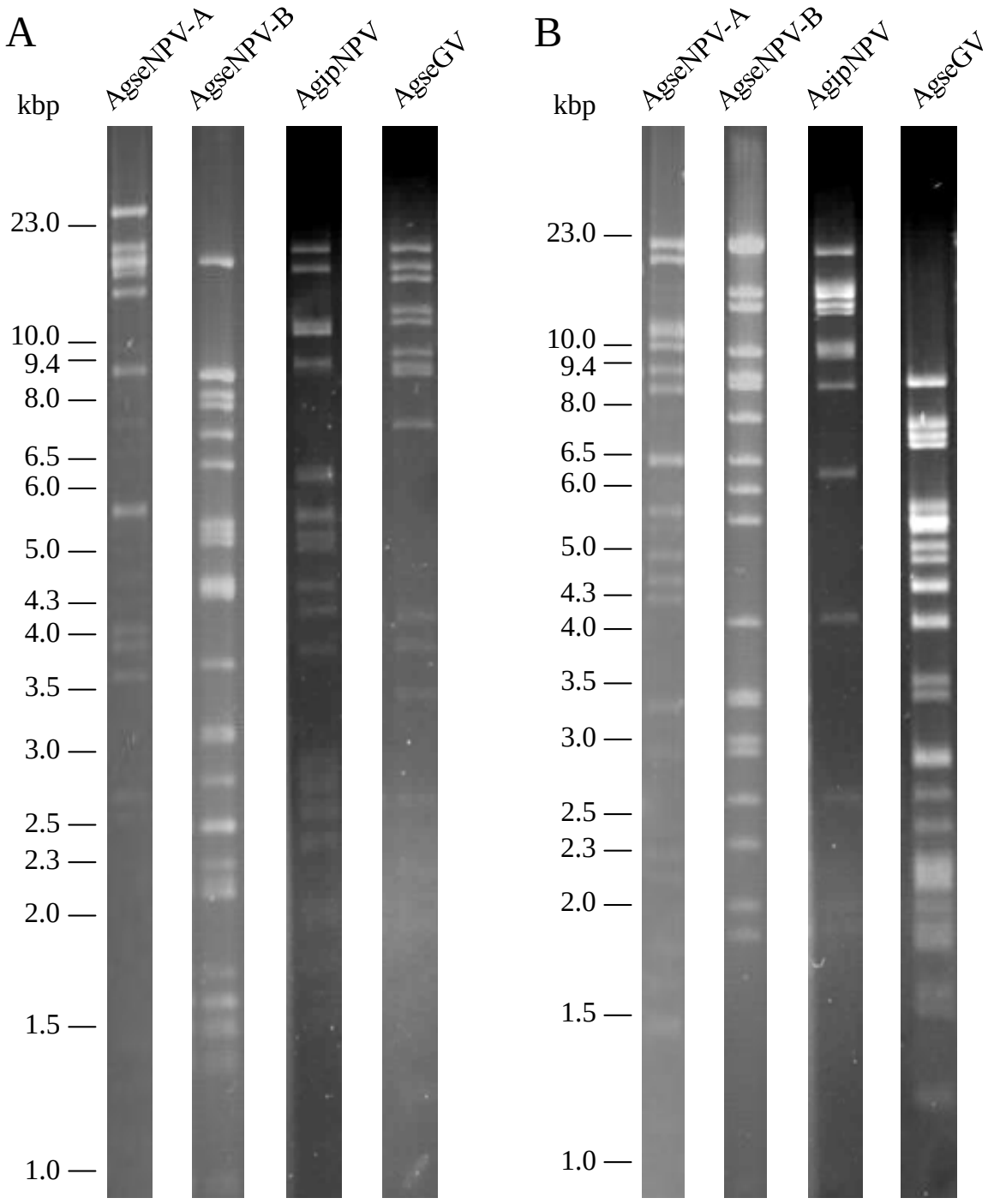

Figure. 2.1. (**A**) *Eco*RI and (**B**) *Hin*dIII restriction analysis of genomic DNA isolated from AgseNPV-A, AgseNPV-B, AgipNPV and AgseGV (HRI). Fragments were separated on 0.8% agarose gels. Size marker (kbp) is given to the left.

To confirm the length of the AgseNPV-B genome, *Avr*II treated linearized DNA was separated by PFGE and a band slightly larger than 145.5 kb (Fig. 2.2) confirmed the genome size obtained by DNA sequencing. Degraded and undigested viral genomic DNA was resolved as a broad smear between 23.1 – 97 kb (Fig. 2.2). Compared to other *Agrotis* NPVs, the genome of AgseNPV-B was 1442 bp longer than AgseNPV-A (Jakubowska et al., 2006) but 6136 bp shorter than the genome of AgipNPV (Illinois Strain) (Harrison, 2009) (Table 2.1). The G+C content of AgseNPV-B (45.69%) was higher than that in most sequenced NPVs and almost identical to that of AgseNPV-A (45.71%) but still lower than the G+C content of AgipNPV (48.57%) (Harrison, 2009) (Table 2.1).

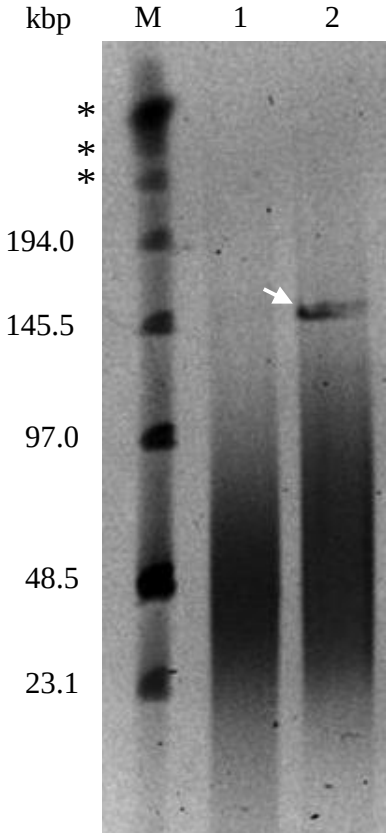

Figure. 2.2. Pulse field gel electrophoresis (PFGE) of AgseNPV-B DNA digested with *Avr*II. Lane 1 = untreated viral genomic DNA; lane 2 = *Avr*II-digested genomic DNA of AgseNPV-B, linearized AgsNPV-B DNA is marked by an arrow; lane M = low range PFGE size standard (NEB) size marker (kbp) is given to the left. Asterisks (*) mark bands of concatemers of lambda DNA that are successively larger than the 194 kb fragments.

For ORF annotation the polyhedrin (*polh*) was set as ORF number one (*asb1*) and the following ORFs were numbered consecutively in a clockwise manner. In total, 150 ORFs were identified for AgseNPV-B. Thereof 37 belong to the baculovirus core genes (Garavaglia et al., 2012; Miele et al., 2011; McCarthy and Theilmann, 2008) present in all sequenced baculoviruses to date, 60 ORFs were with a putatively known function and are present in all lepidopteran NPVs (Herniou et al., 2003), whereas 53 ORFs have unknown function or encode a hypothetical protein.

AgseNPV-A and AgipNPV were described to contain 153 ORFs and 163 ORFs, respectively (Jakubowska et al., 2005; Harrison, 2009). Thus, the three *Agrotis*-specific NPVs are highly ORF-rich, beside *Lymantria dispar* multiple NPV (LdMNPV) (Kuzio et al., 1999), *Mamestra configurata* NPV-A (MacoNPV-A), and MacoNPV-B (L. Li et al., 2002; Q. Li et al., 2002) with 163, 169, and 168 ORFs, respectively.

Table 2.1. Basic features of *Agrotis* baculovirus genomes.

Baculovirus	Length (bp)	No. ORF	GC content (%)	Reference
AgseNPV-B	148,981	150	45.69	This study (KM102981)
AgseNPV-A	147,544	153	45.71	NC_007921*
AgipNPV	155,122	163	48.57	NC_011345 **
AgseGV-XJ	131,680	132	37.31	NC_005839
AgseGV-L1	131,442	149	37.27	KC_994902 ***

* Jakubowska et al., 2005, ** Harrison, 2009, *** Zhang et al., 2014

Comparison with partial AgseNPV-B sequences from other studies

At least two different stocks of AgseNPV-B, the so-called "English" isolate, were previously used in molecular studies, though they may have the same origin. The isolate of the virus collection of the JKI (JKI stock), internally termed BBA-165/A12-3, was used for phylogenetic studies (Jehle et al., 2006b). The same isolate was also used to establish a PCR based system for simultaneous discrimination and quantitation of *Agrotis* baculoviruses (Wennmann and Jehle, 2014). A second "HRI stock", which served in this study as template for whole genome sequencing, was derived from the collection of Horticulture Research International (HRI), Warwick, England. The "HRI stock" was used in initial molecular studies of *Agrotis* baculoviruses (Jakubowska et al., 2005; El-Salamouny et al., 2003).

Partial sequences of the highly conserved baculovirus genes *polh*, *lef-8*, *lef-9* and peroral *infectivity factor* (*pif*) *2* were previously determined at least once for both stocks. The sequencing results of the present study (HRI stock was used) were identical to the partial *polh* (AY706683), *lef-8* (AY706535), and *lef-9* (AY706600) sequences of AgseNPV-B (JKI stock) (Jehle et al., 2006b). Previously published partial sequences of *polh* (AY136482), *lef-8* (AY971678), and *pif-2* (AY971679) of AgseNPV-B (HRI stock) (Jakubowska et al., 2005; El-Salamouny et al., 2003) showed minor differences with those of the present study. The differences appeared at the end of the partial sequences and may have been caused by the increasing terminal ambiguity of long Sanger sequencing runs. Thus, it can be assumed that the two stocks of AgseNPV-B do not differ or are at least highly similar to each other.

Phylogeny of Agrotis baculoviruses

Baculoviruses share a common set of core genes present in all sequenced baculovirus genomes (Herniou et al., 2003). Core genes are considered to play an essential role in the infection cycle of baculoviruses, especially in genome replication, gene transcription, packaging, assembly and release of virions, oral infectivity, cell cycle arrest, and interaction with host proteins (Miele et al., 2011). So far, 37 core genes present in all sequenced baculovirus genomes have been identified (Garavaglia et al., 2012; Ke et al., 2008; McCarthy and Theilmann, 2008; Herniou et al., 2003; Lange and Jehle, 2003).

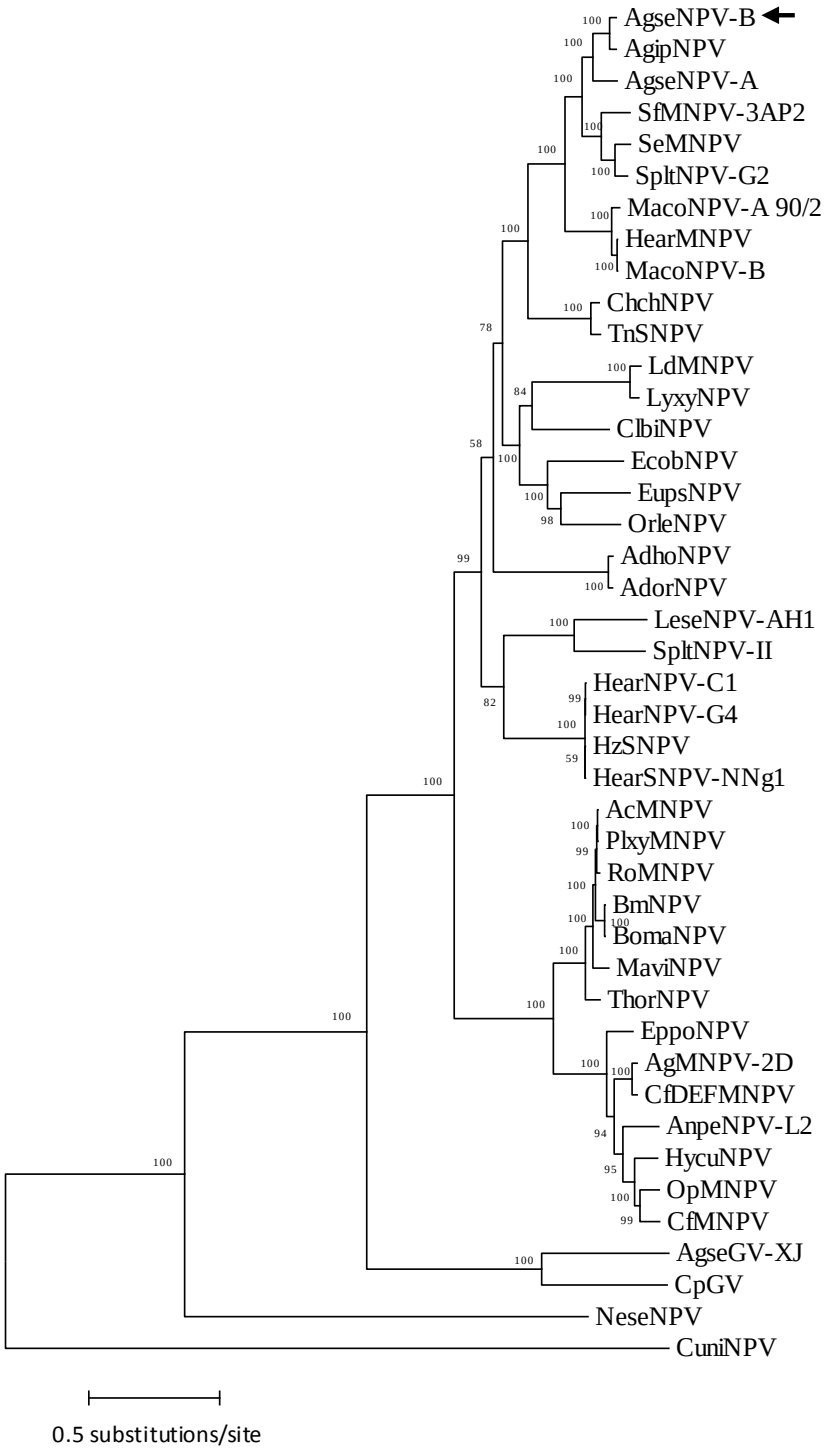

Figure 2.3. Baculovirus phylogeny based on maximum likelihood method of the predicted aa sequences of 37 core genes from 43 baculoviruses. Amino acid sequences of each core gene were separately aligned and alignments were concatenated using Geneious v5.6.2. Bootstrap consensus tree was obtained by maximum parsimony (MP) analysis heuristic search with 1000 bootstrap replicates and tree-bisection-reconnection (TBR) branch swapping option. Bootstrap values (> 50%) are shown at each node. AgseNPV-B is marked by an arrow. Baculoviruses and their abbreviations are shown as described by Miele et al. (Miele et al., 2011). The baculovirus *Thysanoplusia orichalcea* NPV (ThorNPV) (Cheng et al., 2005) was added to the analysis.

The presence of these conserved 37 orthologues in all baculoviruses allows phylogenetic analyses on a broad basis of genetic information. To propose evolutionary trends and phylogenetic patterns, concatenated alignments of predicted aa sequences of all core genes were subjected to sequence-based phylogenetic inference (Herniou et al., 2003). This approach identified AgipNPV as a closely related sister species of AgseNPV-A (Miele et al., 2011; Harrison, 2009). Due to the lack of genome sequence information, the relationship of AgseNPV-B to other *Agrotis* NPVs, however, has been only studied using the partial aa sequences of *polh*, *lef-8* and *lef-9* of AgseNPV-B (El-Salamouny et al., 2003; Jehle et al., 2006b). These studies placed AgseNPV-B in close relationship to AgipNPV within group II NPVs. Analysis that included partial aa sequences of *polh*, *lef-8* and *pif-2* from AgseNPV-A, AgseNPV-B, and AgipNPV (Jakubowska et al., 2006) suggested that AgseNPV-B is more closely related to AgipNPV than to AgseNPV-A. Based on differences in the DNA restriction patterns and sequence information, AgseNPV-A was regarded as a baculovirus species distinct from AgipNPV. However, the taxonomic relationship between AgipNPV and AgseNPV-B has remained unresolved (Jakubowska et al., 2006). The genome sequence of AgseNPV-B clearly shows its close phylogenetic relationship to AgipNPV (Fig. 2.3) based on a high degree of sequence identity (Table 2.2) and identical ORF orientation (Fig. 2.4) with AgipNPV. Thus, AgseNPV-B appears to be more closely related to AgipNPV than to AgseNPV-A. As supported by high bootstrap values, the Agrotis NPVs form a monophyletic clade that is closely related to the *Spodoptera* NPVs within group II NPVs (Fig. 2.3).

Speciation of viruses can be considered as a continuous evolutionary process without any specific point where viruses can be clearly defined as distinct. For baculoviruses the Kimura-2-parameter (K-2-P) of *polh*, *lef-8*, and *lef-9* was suggested as a species demarcation criterion (Jehle et al., 2006b). If the K-2-P distance is larger than 0.050, two baculoviruses were regarded to belong to distinct species. According to the K-2-P differences, AgseNPV-B appeared to be distinct from AgseNPV-A (K-2-P = 0.214) and from AgipNPV (K-2-P = 0.090). The closer relatedness between AgseNPV-B and AgipNPV was thereby confirmed. Thus, the three viruses are proposed to belong to three different baculovirus species, which are in an early stage of speciation.

Table 2.2. Open reading frames (ORFs) and features of the AgseNPV-B genome. ORF lengths are given in nucleotides (nt) and amino acids (aa).

ORF	Name	Position			nt	aa	No. ORF (% aa identities)			
							AgseNPV-B/ AgipNPV	AgseNPV-B/ AgseNPV-A	AgipNPV/ AgseNPV-A	AgseNPV-B/ AcMNPV
1	*polh*	1	>	741	741	246	1 (98.4)	1 (93.9)	(94.7)	8 (84.6)
2	*orf1629*	883	<	2373	1491	496	2 (75.5)	2 (49.4)	(50.7)	9 (19.4)
3	*pk-1*	2399	>	3196	798	265	3 (94.7)	3 (81.5)	(83.4)	10 (42.2)
4	*hoar*	3267	<	5432	2166	721	4 (52.6)	4 (39.3)	(38.6)	
5		5877	<	6329	453	150	7 (43.1)			
6		6350	>	7114	765	254	8 (21.1)			
7	*odv-e56*	7162	>	8268	1107	368	9 (85.9)	6 (71.5)	(71.5)	148 (50.5)
8	*me53*	8592	>	9653	1062	353	10 (94.4)	7 (81.1)	(82.2)	139 (24.5)
9	*F protein*	10535	>	12589	2055	684	12 (92.3)	8 (65.4)	(65.4)	23 (16.4)
10		12740	<	13699	960	319	13 (86.4)	9 (46.2)	(46.9)	
11	*gp16*	13787	<	14080	294	97	14 (88.7)	10 (77.3)	(78.4)	130 (32.7)
12	*p24*	14107	<	14817	711	236	15 (85.6)	11 (66.8)	(67.2)	129 (37.2)
13		14896	>	15255	360	119	16 (74.2)	12 (55.0)	(52.6)	
14	*lef-2*	15212	>	15856	645	214	17 (83.8)	13 (66.4)	(68.5)	6 (38.6)
15		15870	<	16043	174	57				
16		16064	>	16540	477	158				
17		16738	>	17493	756	251				
18		17650	<	18333	684	227				
hr1 repeat region		18428		18683						
19	*38.7 k*	18750	<	19856	1107	368	21 (78.0)	16 (64.0)	(64.8)	13 (25.5)
20	*lef-1*	19858	<	20517	660	219	22 (86.0)	17 (74.5)	(72.9)	14 (41.9)
21	*v-cath*	20779	<	21927	1149	382	23 (87.4)	19 (83.7)	(84.4)	127 (53.2)
22	*he65*	22022	>	23644	1623	540	25 (88.2)	20 (70.4)	(74.0)	105 (39.3)
23		23724	<	24155	432	143	26 (73.6)			
24	*chitinase*	24346	>	26091	1746	581	27 (93.3)	23 (82.8)	(83.3)	126 (63.2)
25		26149	<	26451	303	100	29 (86.1)			
26	*gp37*	26492	>	27274	783	260	30 (91.2)	26 (76.2)	(75.8)	64 (48.6)
27	*ptp-2*	27271	<	27765	495	164	31 (90.9)	27 (65.5)	(64.2)	1 (16.5)
28	*egt*	27926	>	29530	1605	534	32 (91.6)	28 (80.8)	(83.0)	15 (46.2)
29		29753	>	30289	537	178	33 (87.7)	29 (57.7)	(58.8)	
30		30195	>	30968	774	257	34 (85.0)	30 (55.9)	(58.0)	
31		31040	<	33757	2718	905	35 (77.3)	31 (49.6)	(51.9)	
32		34068	>	34610	543	180	37 (56.0)			

Table 2.2. continued

33	*pkip-1*	34718	>	35215	498	165	38	(90.4)	33	(75.4)	(74.3)	23	(22.1)
34		35409	<	35741	333	110	40	(91.9)	34	(60.2)	(60.2)		
35	*arif-1*	35746	<	36549	804	267	41	(88.2)	35	(58.7)	(57.6)	21	(18.8)
36	*pif-2*	36464	>	37699	1236	411	42	(95.9)	36	(87.5)	(86.5)	22	(58.6)
37	*pif-1*	37726	>	39369	1644	547	43	(90.5)	37	(70.2)	(71.0)	119	(45.5)
38		39425	>	39688	264	87	44	(89.8)	38	(61.7)	(63.0)	120	(25.9)
39	*fgf*	39725	<	40828	1104	367	45	(72.5)	39	(46.5)	(45.5)	32	(22.7)
40		41173	>	41901	729	242	46	(84.6)	40	(60.5)	(58.1)		
41	*alc-exo*	41956	<	43173	1218	405	47	(85.2)	41	(64.5)	(66.5)	133	(38.1)
hr2 repeat region		43213		43593									
42		44327	<	44653	327	108	49	(91.7)	44	(75.2)	(78.6)	19	(23.9)
43		44667	>	45839	1173	390	50	(89.0)	45	(72.4)	(74.7)	18	(23.8)
44		45882	<	46283	402	133	51	(95.3)	46	(73.2)	(78.3)		
45	*rr2B*	46380	>	47321	942	313	52	(95.9)	47	(88.9)	(89.2)		
46		47498	>	48892	1395	464	53	(67.2)	48	(36.2)	(37.2)		
47	*pep*	48977	<	50110	1134	377	54	(90.8)	49	(69.4)	(70.6)	131	(27.6)
48		50300	<	50608	309	102	55	(90.2)	51	(75.8)	(77.8)	117	(30.8)
49		50657	<	51007	351	116			52	(64.0)			
50		51083	<	51490	408	135	56	(71.2)	53	(17.9)	(18.7)		
51	*sod*	51530	<	51988	459	152	57	(96.7)	54	(84.2)	(82.9)	31	(71.7)
52		52050	>	52424	375	124	58	(80.8)	55	(51.1)	(53.8)		
53	*pif-3*	52463	>	53155	693	230	59	(88.3)	56	(72.3)	(79.8)	115	(45.7)
54		53046	>	53621	576	191	60	(80.6)	57	(53.2)	(58.4)		
55		53692	>	55140	1449	482	61	(90.1)	58	(72.5)	(73.1)		
56	*ac106*	55192	>	55854	663	220	62	(93.1)	59	(86.4)	(83.1)	106	(33.3)
57	*hisP*	55906	<	56985	1080	359	63	(89.7)	60	(68.6)	(68.9)	33	(26.7)
hr3 repeat region		57140		57791									
58	*dUTPase*	57849	>	58277	429	142	64	(96.5)	62	(79.7)	(79.7)		
59		58481	>	59083	603	200	69	(74.0)	63	(73.1)	(77.4)		
60	*p13*	59277	>	60107	831	276	70	(96.8)	64	(85.1)	(86.2)		
61	*odv-e66*	60225	>	62291	2067	688	71	(90.0)	125*	(30.8)	(31.6)	46	(34.9)
62		62281	<	62643	363	120	72	(86.8)	65	(58.3)	(33.0)	108	(30.2)
63	*odv-ec43*	62649	<	63719	1071	356	73	(98.9)	66	(94.7)	(95.2)	109	(44.5)
64		63703	<	63885	183	60	74	(93.4)	67	(88.5)	(90.2)	110	(32.7)
65	*p87*	63882	<	65639	1758	585	75	(79.0)	68	(59.5)	(58.3)	104	(17.6)
66	*p48*	65681	>	66814	1134	377	76	(97.4)	69	(88.1)	(88.4)	103	(52.1)

Table 2.2. continued

67	*p12*	66804	>	67127	324	107	77	(86.4)	70	(74.4)	(72.9)	102	(29.7)
68	*p40*	67151	>	68290	1140	379	78	(92.5)	71	(80.5)	(80.7)	101	(45.1)
69	*p6.9*	68379	>	68642	264	87	79	(85.6)	72	(67.4)	(69.7)	100	(42.0)
70	*lef-5*	68639	<	69469	831	276	80	(94.9)	73	(87.9)	(88.6)	99	(48.5)
71	*38k*	69371	>	70273	903	300	81	(88.0)	74	(91.4)	(85.7)	98	(43.5)
72	*vef-1*	70367	>	73003	2637	878	82	(41.1)	75	(83.2)	(40.3)		
73	*bro-a*	73059	<	74096	1038	345	83	(82.8)	77	(83.0)	(77.7)	2	(53.3)
74		74202	<	74642	441	146	84	(83.0)	78	(83.2)	(87.2)		
75	*pif-4*	74705	<	75214	510	169	85	(97.1)	79	(89.4)	(91.2)	96	(50.3)
76	*helicase*	75168	>	78854	3687	1228	86	(94.8)	80	(83.9)	(84.9)	95	(40.1)
77	*odv-e25*	78980	<	79633	654	217	87	(94.0)	81	(88.1)	(89.0)	94	(43.1)
78	*p18*	79630	<	80106	477	158	88	(97.5)	82	(92.5)	(94.3)	93	(49.1)
79	*p33*	80115	>	80873	759	252	89	(96.8)	83	(91.7)	(93.3)	92	(52.3)
80		80984	>	81577	594	197	90	(71.2)	84	(34.9)	(35.0)		
81	*lef-4*	81620	<	83059	1440	479	91	(84.2)	85	(69.4)	(69.9)	90	(45.4)
82	*vp39*	83058	>	84041	984	327	92	(97.6)	86	(88.1)	(89.0)	89	(41.4)
83	*cg30*	84200	>	85714	1515	504	93	(49.5)	87	(24.7)	(22.1)	88	(15.7)
84	*vp91*	85817	<	88282	2466	821	94	(87.8)	88	(70.7)	(71.9)	83	(39.9)
85	*ac82*	88251	>	88880	630	209	95	(80.1)	89	(68.6)	(68.7)	82	(26.3)
86	*ac81*	88639	>	89433	795	264	96	(86.8)	90	(77.3)	(77.2)	81	(46.6)
87	*gp41*	89396	>	90400	1005	334	97	(94.0)	91	(94.3)	(94.3)	80	(49.7)
88	*ac78*	90397	>	90759	363	120	98	(86.2)	92	(63.0)	(62.5)	78	(35.2)
89	*vlf-1*	90761	>	91894	1134	377	99	(93.4)	93	(93.7)	(93.0)	77	(63.2)
90	*p26*	92003	<	92743	741	246	100	(93.9)	94	(73.3)	(74.9)	136	(22.4)
91	*iap-2*	92828	<	93706	879	292	101	(84.8)	95	(66.5)	(63.1)	71	(32.6)
92		93579	<	94454	876	291	102	(84.0)	96	(67.0)	(69.1)	69	(43.9)
93	*ac68*	94429	<	94803	375	124	103	(88.1)	97	(80.5)	(82.3)	68	(45.9)
94	*lef-3*	94802	>	95995	1194	397	104	(84.1)	98	(64.6)	(62.8)	67	(26.1)
95	*ac66*	96110	<	98308	2199	732	105	(87.4)	99	(58.6)	(58.0)	66	(18.5)
96	*dnapol*	98310	>	101417	3108	1035	106	(91.1)	100	(80.3)	(81.0)	65	(43.5)
97	*ac75*	101471	<	101860	390	129	107	(98.5)	101	(92.3)	(93.1)	75	(24.2)
98	*ac76*	101867	<	102124	258	85	108	(96.5)	102	(86.0)	(89.5)	76	(42.5)
99		102252	>	102779	528	175	109	(53.8)	103	(29.7)	(25.9)		
100		102844	>	103512	669	222	111	(85.0)	104	(69.2)	(71.1)		
101	*lef-9*	103582	<	105120	1539	512	113	(94.9)	105	(89.7)	(88.8)	62	(63.3)
102	*fp25k*	105197	>	105802	606	201	114	(97.5)	106	(91.6)	(91.7)	61	(60.7)

Table 2.2. continued

103	*p94*	105955	>	108552	2598	865	115	(66.9)					134	(40.4)
104	*bro-b*	108639	>	109121	483	160	116	(90.7)	107	(71.0)	(71.0)			
105		109161	>	109460	300	99	117	(88.5)	108	(75.8)	(73.5)		60	(39.1)
106		109476	>	110060	585	194	118	(75.6)	109	(48.7)	(49.7)		59	(30.8)
107		110069	<	110551	483	160	119	(87.0)	110	(68.9)	(68.5)		57	(43.3)
108		110621	<	110779	159	52								
109		110955	<	111287	333	110	120	(90.4)	111	(45.1)	(46.3)		56	(26.4)
110		111178	<	111546	369	122	121	(65.0)	112	(75.7)	(74.3)			
111	*vp1054*	111500	<	112537	1038	345	122	(94.2)	113	(78.4)	(77.0)		54	(40.1)
112	*lef-10*	112398	<	112634	237	78	123	(94.9)	114	(74.7)	(74.4)		53	(43.8)
113		112612	>	112821	210	69	124	(89.9)	115	(72.9)	(69.6)			
114		112832	>	113872	1041	346	125	(81.0)	116	(61.8)	(61.4)			
115	*ac53*	113883	<	114296	414	137	126	(87.7)	117	(71.3)	(71.3)		53	(47.5)
116		114369	>	114932	564	187	127	(71.7)	118	(46.8)	(46.5)		52	(13.4)
hr4 repeat region		114968		115370										
117	*iap-3*	115566	>	116369	804	267	128	(81.8)	119	(61.9)	(63.0)		27	(30.1)
118	*bjdp*	116424	<	117635	1212	403	129	(88.1)	120	(55.0)	(56.3)		51	(15.1)
119	*lef-8*	117656	>	120313	2658	885	130	(95.5)	121	(87.1)	(87.4)		50	(62.0)
120		120482	<	120889	408	135	131	(72.1)	122	(36.0)	(33.9)			
121		121032	<	121238	207	68	132	(61.1)	124	(48.6)	(70.6)		43	(28.9)
122	*odv-e66b*	121378	<	123489	2112	703	133	(81.8)	125	(62.8)	(64.7)		46	(25.0)
123	*p47*	123533	>	124726	1194	397	134	(92.5)	126	(82.6)	(83.3)		40	(52.9)
124		124765	<	125772	1008	335	135	(75.9)	127	(48.5)	(54.7)			
hr5 repeat region		125889		125988										
125	*vef-3*	126054	>	128687	2634	877	82	(31.9)	128	(76.1)				
126		128744	>	129265	522	173	136	(62.9)	129	(84.0)	(63.5)			
127	*ac38*	129359	>	130057	699	232	137	(99.1)	130	(92.5)	(91.6)		38	(60.5)
128	*lef-11*	129988	>	130359	372	123	138	(95.2)	131	(72.0)	(72.8)		37	(31.7)
129	*39k/pp31*	130325	>	131227	903	300	139	(79.2)	132	(69.9)	(65.9)		36	(34.1)
130		131301	>	131600	300	99	140	(81.0)	133	(46.4)	(45.8)			
131		131678	<	131872	195	64	141	(85.9)	134	(57.6)	(58.5)			
132	*v-ubi*	131866	<	132123	258	85	142	(90.7)	135	(82.6)	(88.0)		35	(75.6)
133		132197	>	132730	534	177	143	(90.4)	136	(79.8)	(82.6)		34	(33.3)
134		132949	<	133119	171	56								
hr6 repeat region		133359		133902										
135		133926	<	134300	375	124	145	(83.1)	138	(58.4)	(63.7)		26	(34.2)

Table 2.2. continued

136	*dbp1*	134421	>	135401	981	326	146	(91.7)	139	(69.9)	(70.2)	25	(24.3)
137	*lef-6*	135430	>	135948	519	172	147	(78.7)	140	(55.4)	(58.5)	28	(23.6)
138	*ac29*	136016	<	136321	306	101	148	(83.3)	141	(74.4)	(80.7)	29	(26.1)
139	*p26b*	136372	>	137220	849	282	149	(63.5)	142	(53.5)	(71.9)	136	(25.2)
140	*p10*	137252	>	137554	303	100	150	(81.6)	143	(61.4)	(58.6)	137	(21.6)
141	*p74*	137677	<	139617	1941	646	151	(93.5)	144	(78.7)	(78.5)	138	(54.7)
142		139751	>	140017	267	88	152	(83.1)	145	(52.9)	(54.1)		
143	*ie-1*	140117	<	142129	2013	670	153	(72.4)	146	(59.1)	(57.4)	147	(23.7)
144	*ac146*	142156	>	142740	585	194	154	(86.0)	147	(64.6)	(65.3)	146	(29.6)
145	*ac145*	142766	<	143044	279	92	155	(87.1)	148	(82.8)	(87.1)	145	(39.7)
146	*odv-ec27*	143074	<	143907	834	277	156	(98.6)	149	(87.8)	(88.2)	144	(53.6)
147	*odv-e18*	143977	<	144258	282	93	158	(85.3)	150	(63.8)	(62.8)	143	(57.7)
148	*49k*	144273	<	145655	1383	460	160	(99.1)	151	(92.6)	(92.8)	142	(45.5)
149	*ie-0/exon0*	145670	<	146392	723	240	161	(87.1)	152	(69.5)	(72.7)	141	(9.5)
150	*rr1*	146445	<	148784	2340	779	163	(92.1)	153	(76.7)	(78.5)		

* AgseNPV-A is missing a complete copy of odv-e66a (Jakubowska et al., 2006). The *odv-e66a* of AgseNPV-B and AgipNPV were compared with the AgseNPV-A *odv-e66b* instead.

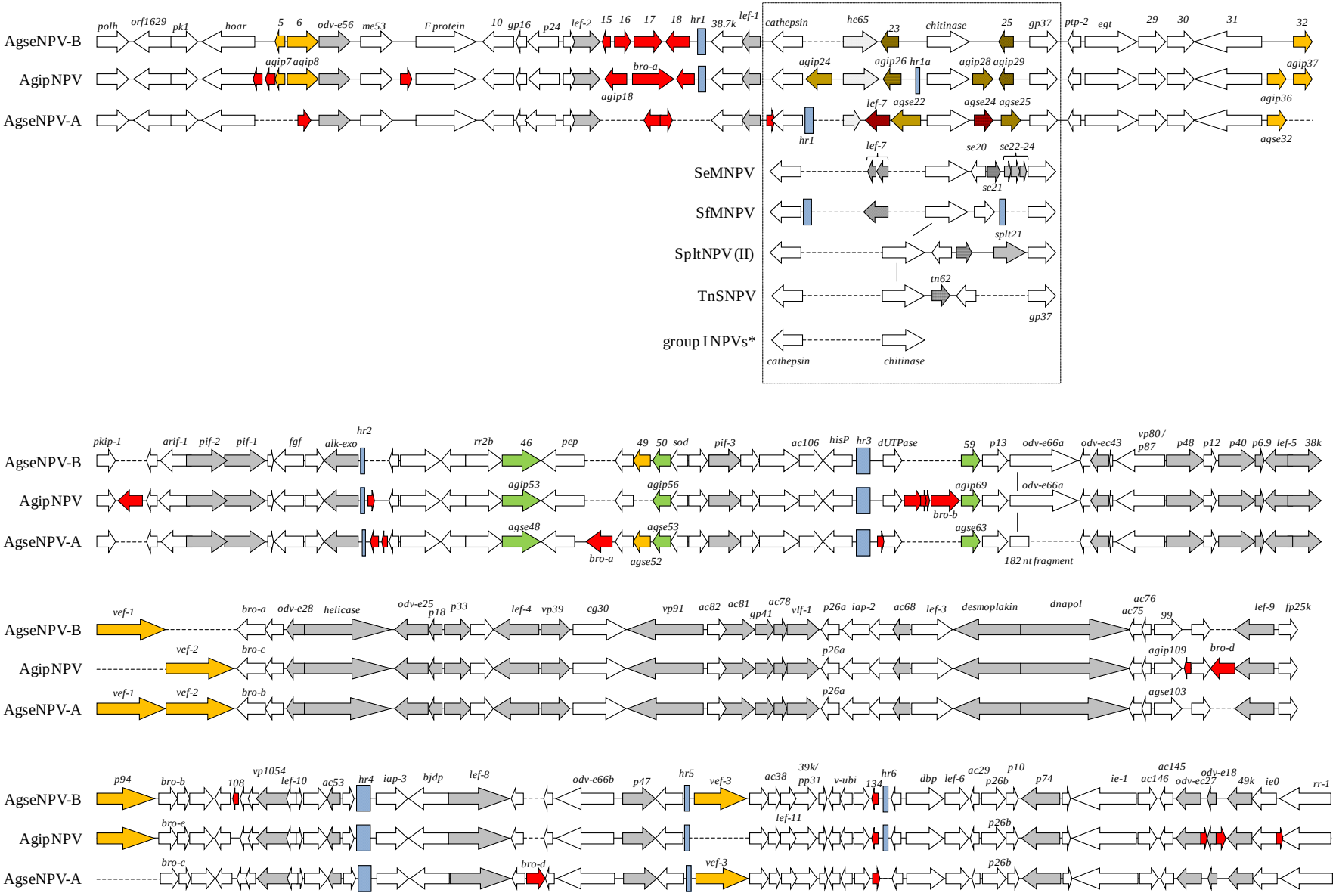
AgseNPV-B
AgipNPV
AgseNPV-A
SeMNPV
SfMNPV
SpltNPV (II)
TnSNPV
group I NPVs*
polh orf1629 pk1 hoar 5 6 odv-e56 me53 F protein 10 gp16 p24 lef-2 15 16 17 18 hr1 38.7k lef-1
cathepsin he65 23 chitinase 25 gp37 ptp-2 egt 29 30 31 32
agip7 agip8 bro-a agip18
agip24 agip26 hr1a agip28 agip29 agip37 agip36
lef-7 agse22 agse24 agse25 agse32
hr1
lef-7 se20 se22-24 se21
splt21
tn62 gp37
cathepsin chitinase
pkip-1 arif-1 pif-2 pif-1 fgf alk-exo hr2 rr2b 46 pep 49 50 sod pif-3 ac106 hisP hr3 dUTPase 59 p13 odv-e66a odv-ec43 vp80 / p87 p48 p12 p40 p6.9 lef-5 38k
agip53 agip56 agip69 odv-e66a bro-b
agse48 bro-a agse53 agse52 agse63 182 nt fragment
vef-1 bro-a odv-e28 helicase odv-e25 p18 p33 lef-4 vp39 cg30 vp91 ac82 ac81 gp41 ac78 vlf-1 p26a iap-2 ac68 lef-3 desmoplakin dnapol ac76 ac75 99 lef-9 fp25k
vef-2 bro-c p26a agip109 bro-d
vef-1 vef-2 bro-b p26a agse103
p94 bro-b 108 vp1054 lef-10 ac53 hr4 iap-3 bjdp lef-8 odv-e66b p47 hr5 vef-3 ac38 39k/ pp31 v-ubi 134 hr6 dbp lef-6 ac29 p10 p26b p74 ie-1 ac145 ac146 odv-e18 odv-ec27 49k ie0 rr-1
bro-e lef-11 p26b
bro-c bro-d vef-3 p26b

Figure 2.4. Graphical genome alignment of AgseNPV-A, AgseNPV-B and AgipNPV. Relative length, orientation and position of ORFs are represented by arrows. Colour code: grey = baculovirus core genes, green = ORFs that are found in all three *Agrotis* NPVs analyzed in this study, but not found in any other fully sequenced baculovirus to date, orange = ORFs that are shared between two of the three *Agrotis* NPVs compared in this study, red = ORFs that are found in only one of the three *Agrotis* NPVs compared in this study. Box marks the *cathepsin*/*chitinase* cluster between *asb21* (*cathepsin*) and *asb26* (*gp37*). In this region selected group II NPVs (*Spodoptera exigua* (Se) MNPV, NC_002169, *Spodoptera frugiperda* (Sf) MNPV, NC_009011, *Spodoptera litura* (Splt) NPV (II), NC_011616 and *Trichoplusia ni* (Tn) SNPV, NC_007383) were added to the alignment. *In group I NPVs the genomic region between *cathepsin* und *chitinase* is conserved except in *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (NC_008520). ORFs at the same position and the same color or labeling represent homologues.

Co-linearity of Agrotis nucleopolyhedrovirus genomes

Gene alignment of all three Agrotis NPVs genomes illustrated a close relationship among AgseNPV-A, AgseNPV-B and AgipNPV based on a high number of shared ORFs that were not only located at the same relative position in the genome, but also shared the same orientations and are of similar lengths (Fig. 2.4). In general, the first half of *Agrotis* NPV genomes, from *polh* to *helicase* gene (Fig. 2.4), seemed to be less conserved than the second half, where the genomic setup in all three viruses exhibited lower variation. This observation is also supported by the gene-parity plot analyses (Fig. 2.5) where, in direct comparisons of two genomes, more unique ORFs were found in the first half of the genomes than in the second half, as represented by black dots on the x and y axis of the plot. Similar observations were made for closely related viruses of the *Mamestra* NPV and *Spodoptera* NPV group, where unique ORFs appeared only (MacoNPV-A/MacoNPV-B; Q. Li et al., 2002) or mainly (*Spodoptera exigua* MNPV (SeMNPV)/*Spodoptera frugiperda* MNPV (SfMNPV); Harrison et al., 2008) within the first genomic half downstream of *polh*. The most striking heterogeneity in genomic organization was at the *cathepsin*/*chitinase* gene cluster, which spanned from cathepsin (*asb21*) to *gp37* (*asb26*) (Harrison, 2009; Jakubowska et al., 2006). *Cathepsin* and *chitinase* are not essential for viral replication, but play a cooperative role in liquefaction and disintegration of infected larvae (Hawtin et al., 1995). In all group I NPVs, except in *Anticarsia gemmatalis* MNPV (AgMNPV) (Oliveira et al., 2006) where both *cathepsin* and *chitinase* are missing, the two ORFs are in a consecutive order with reversed orientation. This orientation and order are also widely conserved in group II NPVs, though this gene cluster is enlarged and penetrated by recombination events even among closely related species (Fig. 2.4). Among the *Agrotis* NPVs, this cluster was found to be the only genomic region where orthologous genes appeared to be translocated (*agip24* and *agse22* are

located upstream or downstream of *he65*, respectively) (Fig. 2.4). An insertion and a deletion were marked by the presence of *lef-7* and truncation of *he65* in AgseNPV-A (Fig. 2.4). An inversion of ORFs se22-24 appeared to be a truncated homologue of *agse22* (Harrison, 2009) and located on the complementary strand (Fig. 2.4). In *Chrysodeixis chalcites* NPV (ChchNPV) a proliferating cell nuclear antigen (*pcna*) (*chch66*) was found downstream and upstream of the *chitinase* and *gp37*, respectively (van Oers et al., 2005). Remarkably, *pcna* was previously known to be present in three group I NPVs only, where it is not located adjacent or in close proximity to the *cathepsin*/*chitinase* cluster. Differences in this genomic region are observed on the level of baculovirus species and isolates. In field and plaque isolates of SfMNPV a remarkably high degree of variation was described. As demonstrated by Harrison (2009) SfMNPV isolates showed partial deletions of varying sizes upstream and downstream of the *ecdysteroid UDP-glucosyltransferase* (*egt*) ORF, that is in close proximity to *cathepsin*, *chitinase*, and *gp37*. In some SfMNPV isolates, partial deletions included the *chitinase* and in one case parts of the *cathepsin* ORF. These findings underline the specific nature of the *cathepsin*/*chitinase* region as a hotspot of recombination in baculovirus genomes.

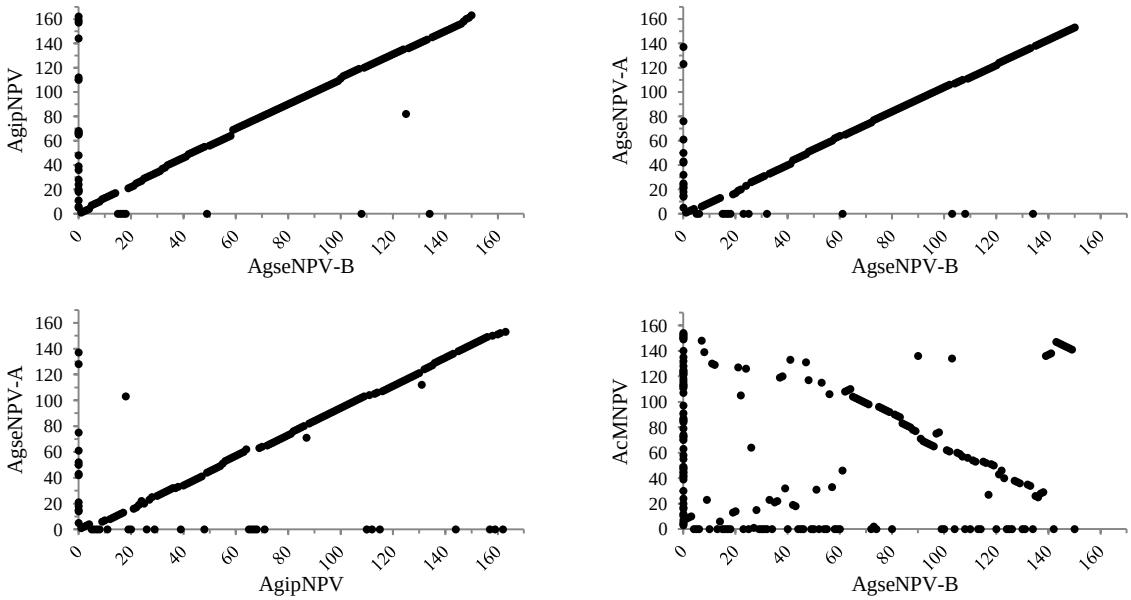

Figure 2.5. Gene parity plot analysis of *Agrotis* NPVs as well as AgseNPV-B and AcMNPV show the presence and relative position of genes (black dots) in pairwise comparisons. Black dots on the x and y axis represent ORFs that are absent in one of the two viruses.

Sequence identity with AgexNPV

The previously published *polh* sequence of AgexNPV (GenBank accession no. GQ475265) showed only four single nucleotide differences in pairwise comparison with the polh sequence of AgseNPV-B. The first two substitutions of AgseNPV-B genome in positions 192 and 195 were synonymous whereas positions at 280 and 417 were non-synonymous. This suggested a very close relationship of AgexNPV and AgseNPV-B. When DNA of AgexNPV, which originated from the same stock as the sample used to obtain the sequence GQ475265, was re-sequenced for the positions 229 to 420, none of the sequence ambiguities at the positions 280 and 417 could be confirmed (Table 2.3). In addition, nine pairs of oligonucleotide primers that were used for sequencing and verifying genomic sequence ambiguities of AgseNPV-B were applied on AgexNPV DNA for PCR amplification and subsequent sequencing. As shown in Table 2.3, the sequenced fragments of AgexNPV were identical to those of AgseNPV-B indicating a very close relationship or even identity of AgexNPV and AgseNPV-B.

Table 2.3. Sequencing of selected PCR fragments of AgexNPV.

Location in AgseNPV-B genome	Feature (ORF no.) of sequenced AgseNPV-B region	Number of sequenced nucleotides (bp)	Nucleotide identity (%)	Source	GenBank accession no.
187- 649	*polyhedrin* gene (asb1)	463	99.1	GenBank	GQ475265
223 - 422	*polyhedrin* gene (asb1)	200	100	this study	KJ995661
4,375 – 4,894	unknown function (asb4)	520	100	this study	KJ953919
5,453 – 5,870	intergenic region (asb4-5)	418	100	this study	KJ953920
6,285 – 6,513	unknown functions and intergenic region (asb5-6)	229	100	this study	KJ953917
6,617 – 6,845	unknown function (asb6)	229	100	this study	KJ953918
7,098 – 7,315	unknown function (asb6) to *odv-e56* (asb7) and intergenic region (asb6-7)	218	100	this study	KJ953916
12,917 – 13,304	unknown function (asb10)	388	100	this study	KJ953914
13,708 – 13,917	intergenic region (asb10-11) and *gp16* (asb11)	210	100	this study	KJ953915
141,255 – 141,734	*ie-1* gene (asb143)	480	100	this study	KJ995662

Egt gene of Agrotis baculoviruses

Ecdysteroids are insect hormones that trigger the cuticula molting (ecdysis) and thereby play a regulatory role in larval growth. This larval development is interfered through the expression of a baculovirus encoded ecdysteroid uridine 5'-diphosphate (UDP)-glucosyltransferase (EGT) enzyme, which catalyzes glucose transfer to ecdysteroid hormones and render them inactive (O'Reilly, 1995; O'Reilly and Miller, 1989). Once these molting hormones are blocked, infected larvae continue feeding and larval development is prolonged, thus providing the virus time to propagate in the host (O'Reilly, 1995).

The *egt* gene is not essential for baculovirus replication and, as shown for *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), its deletion can result in an increased speed of kill, resulting in an improvement of their efficacy as biocontrol agent (O'Reilly and Miller, 1991). As *Agrotis* NPVs are considered potential biocontrol agents against cutworms, the presence or absence of *egt* may play an important role in field efficacy. In AgipNPV, several isolates were distinguished by a 128 bp deletion within the *egt* ORF, which led to a premature stop codon that resulted in enhanced virulence of these AgipNPV isolates (Harrison, 2013). Deletion of the *egt* gene was also observed in various SfMNPV genotypes (Harrison et al., 2008; Simon et al., 2004). In contrast to AgipNPV egt^- genotypes, the deletion is not restricted to the *egt* gene, but neighboring ORFs are affected, too, encompassing *cathepsin* to *pif-2* (Harrison et al., 2008). For SfMNPV-NIC (Nicaraguan isolate), a population of nine genotypes was detected from which eight were egt^- (Simon et al., 2004). Whereas some genotypes lost their infectivity to *S. frugiperda* larvae others had an increased speed of kill. However, the complete mixture of genotypes showed a higher efficacy to *S. frugiperda* larvae than a mixture of the egt^+ genotype population (Simon et al., 2004). Despite the close relatedness of AgipNPV and AgseNPV-B, no such deletions were observed in AgseNPV-B and a functional *egt* gene may be assumed based on the nucleotide sequence. Instead, an insertion of additional 15 bp (5 aa) was found at 28,137 – 28,151 bp that is not present in AgseNPV-A or AgipNPV.

Table 2.4 ORFs of AgseNPV-A, AgseNPV-B and AgipNPV that are unique or have homologues within the *Agrotis* NPVs only.

Virus	ORF	Feature
AgseNPV-A	*agse5, agse14, agse15, agse42, agse43, agse61, agse137*	unique to AgseNPV-A; no homologues in other baculoviruses detected*
	agse18	similar to SeMNPV ORF-15 and MacoNPV-B ORF-29*
	agse21	*lef-7*, missing in AgseNPV-B and AgipNPV
	agse24	low similarity to TnSNPV ORF-62
	agse50	*bro-a*, AgseNPV-A*
	agse123	*bro-d*, AgseNPV-A*
AgseNPV-B	*asb15, asb16, asb108, asb134*	unique to AgseNPV-B; no homologues in other baculoviruses detected
	asb17	similar to ChchNPV ORF-24
	asb18	*caspase* like protein
AgipNPV	*agip5, agip6, agip11, agip20, agip39, agip48, agip66, agip67, agip110, agip144, agip157, agip159, agip162*	unique to AgipNPV; no homologues in other baculoviruses detected**
	agip19	*bro-a*, AgipNPV**
	agip65	similar to CpGV ORF-109, ORF-64 and HearSNPV ORF-134**
	agip68	*bro-b*, AgipNPV**
	agip112	*bro-d*, AgipNPV**
AgseNPV-A/ AgseNPV-B	*agse52/asb49*	similar to MacoNPV-B ORF-63*
AgseNPV-B/ AgipNPV	*asb5/agip7*	similar to *Ac152*
	asb6/agip8	AgseNPV-B and AgipNPV specific ORFs (aa identity 21.1%); no orthologues found in other sequenced baculoviruses
	asb23/agip26	similar to SeMNPV ORF20
	asb25/agip29	similar to AcMNPV ORF-79
	asb32/agip37	similar to SeMNPV ORF-31 and SfMNPV ORF-30
	asb103/agip115	*p94*, missing in AgseNPV-A
AgipNPV/ AgseNPV-A	*agip24/agse22*	similar to SeMNPV ORF-22(-24)** and SpltNPV(II) ORF-21
	agip28/agse25	similar to MabrNPV ORF-16 and MacoNPV-B ORF-16**
	agip36/agse32	similar to SeMNPV ORF-31** and MacoNPV-B ORF-39*
AgseNPV-A/ AgseNPV-B/ AgipNPV	*agse48/asb46/agip53*	no homologues in other baculoviruses detected
	agse53/asb50/agip56	no homologues in other baculoviruses detected
	agse63/asb59/agip69	similar to LsNPV ORF-67 and ThorMNPV ORF-108

* (Jakubowska et al., 2006), ** (Harrison, 2009)

Unique Agrotis nucleopolyhedrovirus genes

Following the sequencing of more than 50 baculovirus genomes, a more and more comprehensive picture of a huge genetic diversity has emerged. Although the number of ORFs in a single baculovirus is restricted to 180 or less, approximately 600 ORFs appear to be present in members of the genus *Alphabaculovirus* (Miele et al., 2011). This includes core genes, as well as genus or species specific ORFs with known or unknown function. Regarding the host specificity of baculoviruses that exhibit a narrow host range, genomic variations such as additional or unique ORFs, as well as mutations at the molecular level were assumed to play an important role in co-evolution with their hosts. Therefore, the search for such unique characteristics is important and may help to understand which genomic features led viruses adapt to their hosts, and how the host range may be coded within the viral genome. The NPVs of the *Agrotis* complex were found to contain only two pairs of homologues *asb46*/*agip53*/*agse48* and *asb50*/*agip56*/*agse53* that were shared by AgseNPV-B, AgipNPV and AgseNPV-A only and which were not found in other sequenced baculoviruses. Blastp searches did not reveal any significant hits with other sequences and their function is unknown.

It was observed, that AgseNPV-B shared almost all annotated ORFs with AgseNPV-A and AgipNPV. Only four ORFs (*asb15*, *asb16*, *asb108*, and *asb134*) had no homologues in other sequenced baculovirus genomes, and are, therefore, unique to AgseNPV-B. *Asb15* and *asb16* belonged to a genomic section located close to the first homologous region (*hr1*) of AgseNPV-B and AgipNPV (Fig. 2.4). A third (*asb17*) and fourth (*asb18*) ORF of this section were similar to ORF-24 of the ChchNPV and showed a low blastp similarity to a caspase-like protein (Table 2.4). With these six ORFs (*asb15-18*, *asb108*, and *asb134*) present in AgseNPV-B but not shared by AgseNPV-A and AgipNPV, the genome of AgseNPV-B had the lowest number of unique *Agrotis* baculovirus ORFs (Table 2.4). Out of 12 ORFs, also present in AgseNPV-A, but not in AgseNPV-B and AgipNPV, *agse21* is the only one with a known function. It is annotated as a *late expression factor 7* (*lef-7*) (Jakubowska et al., 2006) (Fig. 2.4, Table 2.4).

AgseNPV-B and AgseNPV-A shared three ORFs that were not present in AgipNPV; *asb49*/*agse52*, *asb72* (*vef-1*)/*agse75* (*vef-1*), and *asb125* (*vef-2*)/*agse128* (*vef-3*), respectively (Table 2.4). Both *asb49* and *agse52* are similar to MacoNPV-B ORF-63, whereas viral

enhancing genes (*vef*) were found in many sequenced baculoviruses (see section below). AgipNPV and AgseNPV-A also share three homologous ORFs that were not present in the genome of AgseNPV-B: *agip24*/*agse22*, *agip28*/*agse25*, and *agip36*/*agse32*. These ORFs have unknown functions and show similarities to ORFs of baculoviruses outside the *Agrotis* group (Table 2.4). Except for *agip36*/*agse32* these ORFs are located in the vicinity of the *cathepsin*/*chitinase* cluster and are most likely the result of recombination events. An additional noticeable genomic feature is the apparent homology of *asb32*/*agip37* and *agip36*/*agse32* (Table 2.4). These ORFs are all located in the same relative genomic region but are not shared by all three *Agrotis* NPVs.

AgseNPV-A is missing six ORFs that are shared by AgseNPV-B and AgipNPV (Table 2.4). One of these ORFs (*asb103*/*agip115*) is annotated as a homologue to *p94* (AcMNPV), a gene apparently not essential for the replication in cell culture but may play a role in inhibition of apoptosis (Friesen and Miller, 1987).

Homologous regions

Baculovirus genomes are characterized by the occurrence of repetitive homologous regions (*hrs*) located within intergenic regions and are considered to act as enhancers of gene transcription and possibly as origins of replication (Pearson and Rohrmann, 1995; Kool et al., 1993). Six *hrs* were detected in AgseNPV-B, one less than in AgipNPV (Harrison, 2009), and one more than in AgseNPV-A (Jakubowska et al., 2006) (Fig. 2.4, Table 2.5A). Remarkably, all six *hrs* (*hr1* to *hr6*) of AgseNPV-B are located at the same genomic position as in AgipNPV (Harrison, 2009), and even the number of palindromic repeats are similar (*hr1* and *hr3*) or identical (*hr4* with seven and *hr5* with two palindromic repeats). Compared to AgipNPV, about twice as many palindromic repeats were detected in *hr2* and *hr6* within the genome of AgseNPV-B. The additional seventh *hr* (*hr1a*) of AgipNPV, consisting of a single imperfect palindrome within the *cathepsin*/*chitinase* gene cluster (Harrison, 2009), could not be confirmed for AgseNPV-B (Fig. 2.4). Four *hrs* (*hr2* to *hr5*) in AgseNPV-A are located between the same ORFs as the hrs of AgseNPV-B and AgipNPV (Fig. 2.4), reflecting a similar basic structure of all three *Agrotis* baculovirus genomes.

Table 2.5 (**A**) Alignment of AgseNPV-B palindromic repeats of homologous regions (*hrs*). (**B**) Comparison of consensus sequences obtained from palindromic repeat alignments of SeMNPV, SfMNPV, and *Agrotis* NPVs. Nucleotide similarities are shaded in black (100%), dark grey (80-100%), light grey (60-80%) and white (<60%). Letters in consensus sequence represent majorities of nucleotides. The *Eco*RI restriction site within the palindromic repeat consensus sequence is printed in italics.

hr	nucleotide sequence
A	
hr1-1	ATGTTTGCTTTCGTCGAGAATCCTTGGCGAAAGCCAACAT
hr1-2	ATGTTTGCTTTCGACAAAGATTCTCGACGAAAGCAAACAT
hr1-3	GGGTTAGCTTTTGACAAGAATTTTCGACGAAAGCAAACAT
hr1-4	AAGTTTGCTTTCGACGAGAATTCTTTTCGAAAGCAAAGAT
hr2-1	ATCTTAGCTTTCGTCCAAAATCCTCGACGAAATCCAAGAT
hr2-2	ATCTTTGCTTTCGTCCAAAATTCTCGACGAAATCCAAGAT
hr2-3	ATCTTTGCTTTCGTCTAAAATCCTCGACGAAATCCAAGAT
hr2-4	ATCTTTGCTTTCGTCTAAAATTCTCGACGAAATCCAAGAT
hr2-5	ATCTTTACTTTCCGCTAAAATTCTCGATGAAATCCAAGAT
hr2-6	ATCTTTGCTTTCGTTCAAAATTTTCGACGAAATCCAAGAT
hr2-7	ATCTTTGCTTTCGGCAAAAATTCTCGATGAAATCCAAGAT
hr3-1	CAGTTTGCTTTCGTCGAGGATTTTGGACGAAAGCAAAGAT
hr3-2	AAGTTGGATTTCGTCAAGAATTTTAGTTGAAAGCAAAGAT
hr3-3	GTCTTGGATTTCATCGAAAATTCTTGCCGAAAGCAAAGAT
hr3-4	ATTTTGGATTTCGTCGAGAATTCTTGCCGAAAGCAAAGAT
hr3-5	AACTTGGATTTCGTCGAGAATTCTTGCCGAAAGCAAAGAT
hr3-6	ATATTGGATTTCATCGAAAATTTTGGACGAAAGCAAAGAT
hr3-7	AAGTTGGATTTCATCGAGGATTTTGGACGAAAGCAAAGAT
hr3-8	AAGTTGGATTTCGTCGAGGATTTTGGACGAAAGCAAAGAT
hr3-9	AAGTTGGATTTCGTCGAGGATTTTGGACGAAAGCAAATTT
hr4-1	TAATTTGCTTTCGACGAGAATCCTCGACGAAATCCATGAT
hr4-2	ATGTTTGCTTTCATCCAAAATCCTCGACGAAATCCAACTT
hr4-3	ATCTTTGCTTTCGTCCAAAATCCTCGACGAAATCCAACTT
hr4-4	ATCTTTGCTTTCGTCCAAAATCCTCGACGAAATCCAACTT
hr4-5	ATCTTTGCTTTCGACGAGAATCCTCGACGAAATCCATGAT
hr4-6	ATGTTTGCTTTCATCCAAAATTCTCGACGAAATCAATGAT
hr4-7	ATGTTTGCTTTCATCGAGAATTTTGGATGAAAGCAAACAG
hr5-1	CTGTTTGAGTCTGTTTAGAATTCTTGCCGAAAGCAAACAT
hr5-2	ATGTTTGCTTTTATCGAAAATTCTTGCCGAAAGCAAACAT
hr6-1	ATCTTGGATTTCGTCGAGAATTCTTGTCGAAATCCAAGAT
hr6-2	GACTTAGATTTCGTCAAGAATTCTTGCTGAAAGCAAAGAT
hr6-3	ATCTTGGATTTCGTCGAAAATTCTTGCCGAAATCAAAGAT
hr6-4	AAGTTGGATTTCGTCGAAAATTCTTGCTGAAAGCAAAGAT
hr6-5	ATCATGGATTTCGTCGAGAATTCTTGCCGAAAGCAAAGAT
hr6-6	TTGATGAATTTCGCCGAGGATTTTGGACGAAAGCAAAGAT
hr6-7	ATCTTGGATTTCGTCAAGAATTCTTGCCGAAAGCAAAGAT
hr6-8	AAGTTGGATTTCACCGAGGATTTTGGACAAAAGCAAAGAT
hr6-9	ATCTTGGATTTCGTCAAAAATTCTTTCTGAAAGCAAAGAT
consensus	ATCTTTGCTTTCGTCGAGAA*TTC*TCGACGAAAGCAAAGAT

B

AgseNPV-B	.....ATSTTTGCTTTCGTCGAGA...ATTCTYGACGAAAGCAAAGAT.....
AgipNPV	.....ATGTTTGCTTTCGTCGAAA...ATTTTCGACGAAAGCAAACAT.....
AgseNPV-A	.....ATGTTTGCTTTCGTCGAAATCTATTCTCGMTGAAAGCAAACATTGA.....
SeMNPV	TTAGTACACGATCTTTGCTTTCGTCSAAG...ATCTTCGACGAAAGCAAAGATCRTGTACTAAAA
SfMNPV	.....CAATGTTTGCTTTCGGCGAAA...TAYTTCGCYGAAAGCAAAGATYG.....
consensus	.....ATGTTTGCTTTCGTCGAAA... TTTTCGACGAAAGCAAAGATyg.....

The alignment of all imperfect palindromic repeats of AgseNPV-B led to a consensus sequence of 40 bp that exhibited a perfect *Eco*RI cleavage site in its centre (Table 2.5A). By aligning the AgseNPV-B consensus sequence with those from AgseNPV-A, AgipNPV, SeMNPV, and SfMNPV (Harrison, 2009) a pattern of highly conserved nucleotides was obtained for the *Spodoptera* and *Agrotis* baculovirus group but was disrupted by some variability in its centre (Table 2.5B).

The potential role of *hrs* as hotspots of recombination was particularly observed for *hr1*, where unique *Agrotis* ORFs (*agse15-18* and *agip18-20*) appeared in the immediate anti-clockwise vicinity of *hr1* in AgseNPV-B and AgipNPV. In clockwise orientation of *hr5* the presence of additional copies of the viral enhancing factor gene, *vef-2* and *vef-3* was noted for AgseNPV-B and AgseNPV-A, respectively. This copy is completely missing in AgipNPV (Fig. 2.4). Further variability in ORF content was found within the vicinity of baculovirus repeated ORFs (*bro*) genes that were also considered to be linked to baculovirus recombination (Kuzio et al., 1999).

Viral enhancing genes

After peroral ingestion of OBs and their alkaline lysis within the lepidopteran midgut, ODVs have to negotiate the peritrophic membrane (PM) to initiate primary infection of midgut cells. This natural barrier that separates the midgut lumen from the epithelial cells was found to be altered by a class of baculovirus metalloproteases, the so-called enhancins (Lepore et al., 1996). The PM is rich in mucin and is degraded by the enhancins (Rohrmann, 2013). The disintegration of the PM is assumed to facilitate the primary infection of midgut epithelial cells by ODVs.

The first enhancin was described for *Pseudaletia unipuncta* GV (PsunGV) as part of the OB matrix of the GV and had a synergistic effect on the infectivity of the *P. unipuncta* NPV (PsunNPV) in simultaneous infections with the GV (Tanada and Hukuhara, 1971). In *Lymantria dispar* NPV (LdMNPV) the enhancins are located in the ODV envelope (Slavicek and Popham, 2005).

Most baculovirus genomes possess only a single copy of *vef*, but there are exceptions. The genome of *Xestia c-nigrum* GV (XecnGV) contains four enhancin genes (Hayakawa et al., 1999), and the genome of LdMNPV harbors two *vef* copies (Kuzio et al., 1999). All completely sequenced *Agrotis* baculovirus genomes to date, now including AgseNPV-B, belong to the VEF-encoding baculoviruses (Fig. 2.6). Containing three *vef* copies the genome of AgseNPV-A represents one of the highly enhancin-rich NPVs (Jakubowska et al., 2006). Within the genome of AgseNPV-A, two *vef* copies (*vef-1, vef-2*) were consecutively ordered downstream of ORF *bro-b*, whereas the third copy (*vef-3*) was located in a clockwise orientation of *hr5* (Jakubowska et al., 2006) (Fig. 2.4). A single enhancin copy (*vef-2*) was found in the genome of AgipNPV at the same relative genomic location as *vef-1* and *vef-2* of AgseNPV-A (Fig. 2.4). The aa sequence of AgipNPV *vef-2* was more similar to *vef-2* (53.3% identity) than to *vef-1* (40.3%) of AgseNPV-A. Therefore, AgipNPV *vef-1* and AgseNPV-A *vef-2* are considered as homologous (Harrison, 2009). The *vef-1* and *vef-3* of AgseNPV-B were homologous to AgseNPV-A *vef-1* and *vef-3* with 83.2% and 76.1% aa sequence identity, respectively (Table 2.2; Fig. 2.4). Thus, three different vef ORFs are shared by AgseNPV-A (*vef-1*, *vef-2* and *vef-3*), AgseNPV-B (*vef-1* and *vef-3*) and AgipNPV (*vef-2*) in total (Fig. 2.4). According to the close phylogenetic relationship and the consecutive order, *vef-1* and *vef-2* are hypothesized to be the result of ancestral gene duplication. In AgipNPV and AgseNPV-B, either *vef-1* or *vef-2* was deleted. In contrast, *vef-3* of AgseNPV-A and AgseNPV-B is located at a genome locus different from *vef-1*/*vef-2*; its phylogenetic distance to *vef-1*/*vef-2* inferred that *vef-3* evolved independently from *vef-1*/*vef-2*.

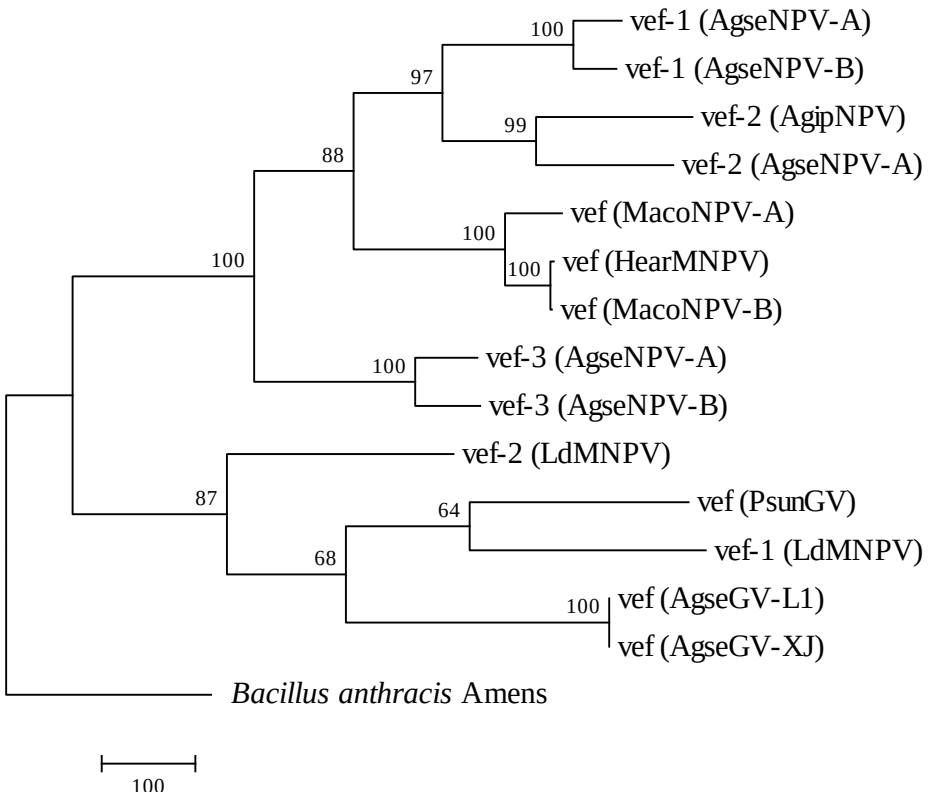

Figure 2.6. Phylogenetic analysis based on aligned enhancin aa sequences of *Agrotis* baculoviruses. Maximum Parsimony tree was calculated by using the Tree-Bisection-reconnection (TBR) algorithm. Percentage bootstrap values (1000 replicates) are given at each node. The bacterial enhancin sequence of *Bacillus anthracis* Amens (GenBank accession no. AE017034) was used as outgroup.

Both AgseGV-L1 and l-XJ contain a single *vef*, which showed high genetic distances to the *vef* of the Agrotis NPVs (Fig. 2.6). Thus, there is no evidence for a recent horizontal transfer of *vef* genes between *Agrotis* NPVs and GVs.

Another aspect of enhancin proteins concerns a conserved metalloprotease zinc-binding domain HEXXH (Rawlings and Barrett, 1995), which is also found in baculovirus enhancins (Bischoff and Slavicek, 1997). Neither the *vef-3* of AgseNPV-B, AgseNPV-A (Jakubowska et al., 2006) nor the *vefs* of AgseGV-XJ and AgseGV-L1 strains contain the consensus functional zinc-binding domain, whereas the other *vef* copies of AgseNPV-A, AgseNPV-B and AgipNPV fulfill this criterion (data not shown). In conclusion, in *Agrotis* NPVs, the presence of *vef* and their aa sequence identities highlight the close relatedness of these three virus species. The closely related *Spodoptera* NPVs (Fig. 2.3) were not found to contain *vef* and the presence or absence of *vef* within taxonomic groups may underline the specific adaption to host larvae.

Conclusions

The total genome sequence of AgseNPV-B revealed a new baculovirus species within the *Agrotis* baculovirus complex, which is proposed to comprise four characterized species; AgseNPV-A, AgseNPV-B, AgipNPV, and AgseGV. In contrast, AgexNPV was found to be highly identical to AgseNPV-B and is not considered to belong to fifth species of the *Agrotis* baculovirus complex. A close relationship of AgseNPV-B and AgipNPV was shown by highly co-linear genomes and phylogenetic analyses based on the predicted amino acid sequences of baculovirus core genes. The co-linearity of *Agrotis* NPVs is only disrupted by the presence of unique genes and certain variable genomic regions such as the *cathepsin*/*chitinase* gene cluster. These genomic regions most likely represent spots of increased recombination. Very few homologous genes of AgseNPV-A, AgseNPV-B, and AgipNPV were found to be unique to these three NPVs. These genes and regions of recombination could represent factors of host specialization by *Agrotis* NPVs.

CHAPTER 3: DETECTION AND QUANTITATION OF *AGROTIS* BACULOVIRUSES IN MIXED INFECTIONS

Abstract

At least four distinct baculoviruses, namely the *Agrotis segetum* nucleopolyhedrovirus A (AgseNPV-A), the *Agrotis segetum* nucleopolyhedrovirus B (AgseNPV-B), the *Agrotis ipsilon* nucleopolyhedrovirus (AgipNPV) and the *Agrotis segetum* granulovirus (AgseGV) have been isolated from larval stages (cutworms) of the species *Agrotis segetum* and *A. ipsilon* (Lepidoptera: Noctuidae), which are serious soil pests in agriculture. Cutworms can become infected by at least one of these four baculoviruses and also co-infections of *A. segetum* larvae with AgseNPV-B and AgseGV are observed under laboratory conditions. Because of their adaption to common hosts and the occurrence in mixed infections, these viruses have a considerable potential as biological control agents of cutworms and are suitable objects to decipher the co-evolution and population dynamics of baculoviruses in mixed infections. However, to facilitate studies on these viruses a reliable tool for detection and identification is essential. A method based on highly specific oligonucleotide primers for multiplex polymerase chain reaction (PCR) that led to the amplification of discriminating fragments of the *polyhedrin (polh)* and *granulin (gran)* gene of AgseNPV-A, AgseNPV-B, AgipNPV and AgseGV, was established. Furthermore, the AgseNPV-B and AgseGV specific pairs of primers were applied in real-time PCR (qPCR) for AgseNPV-B/AgseGV ratio determination in samples of mixed infections. It is demonstrated further that for quantifying NPVs and GVs in mixed infections, the method of occlusion body isolation is most crucial and significantly influences the results.

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Introduction

Baculoviruses (family *Baculoviridae*) are a large and highly diverse group of double-stranded DNA viruses that are specific for arthropods and infect species of the orders Lepidoptera, Diptera and Hymenoptera (Jehle et al., 2006b). A common feature of the baculoviruses is a rod-shaped enveloped nucleocapsid that contains a circular dsDNA genome of 80-180 kbp (Herniou et al., 2011). Two different types of virions occur during the infection cycle of the baculoviruses. Virions that received their membrane from the plasma membrane of the host cell during budding from the cell are called budded viruses (BV) and contain only a single nucleocapsid. Occlusion derived virions (ODV) are assembled within the host cell and are embedded within a crystalline protein matrix that forms occlusion bodies (OB). Furthermore, baculoviruses are divided in two morphological groups according to their OB morphology. The OBs of granuloviruses (GV) (genus *Betabaculovirus*) contain only one virion and are smaller than those of the nucleopolyhedroviruses (NPV) (genera *Alpha-*, *Gamma-* and *Deltabaculovirus*), which contain several to numerous ODVs (Herniou et al., 2011).

More than 600 different baculovirus species have been described (Martignoni and Iwai, 1987); in several cases different baculoviruses were initially isolated and described from same host species. The *Agrotis* baculoviruses complex describes such an example of baculoviruses that co-infect and interact in host larvae. At least four different baculoviruses were characterized from larvae of the common cutworm, *Agrotis segetum* (Agse) (Dennis & Schiffermüller), and the black cutworm, *A. ipsilon* (Agip) (Hufnagel) (both Lepidoptera: Noctuidae): the AgseNPV-A (Jakubowska et al., 2005), AgseNPV-B and AgseGV (Allaway and Payne, 1983; Jakubowska et al., 2006) and AgipNPV (Boughton et al., 1999). From the latter, different genotypes were recently isolated and characterized (Harrison, 2013).

These two cutworm species are serious agricultural soil pests that feed on roots of numerous crops and vegetables and that are difficult to be controlled. A promising approach for the biological control of *Agrotis* spp. in the field is the application of baculoviruses that are able to infect these pests (Caballero et al., 1991; Bourner et al., 1992). Both *A. segetum* and *A. ipsilon* larvae can become cross-infected by these four viruses and show a different susceptibility to the different *Agrotis* baculoviruses (Bourner and Cory, 2004; El-Salamouny et al., 2003; Caballero et al., 1991; Ignoffo and Garcia, 1979). *In vivo* interaction in co-infected larvae are described for *Pseudaletia unipuncta* GV (PsunGV) and NPV (PsunNPV)

(Tanada, 1959), *Helicoverpa armigera* GV (HearGV) and NPV (HearSNPV) (Whitlock, 1977), HearGV and *Helicoverpa zea* NPV (HzNPV) (Hackett et al., 2000), as well as *Cydia pomonella* granulovirus and *Cryptophlebia leucotreta* granulovirus (Jehle et al., 2003). Other examples of baculovirus interactions within a single host are described by various studies *in vivo* (Lauzon, 2005; L. Li et al., 2002) and *in vitro* (reviewed by Cheng and Lynn, 2009).

The occurrence of co-infected larvae poses a problem to experiments when single baculovirus infections are assumed, e.g. in bioassays to determine virus activity or in quality control measures in commercial baculovirus production systems. On the other hand, population dynamic studies aiming to investigate possible baculovirus interactions demand a reliable method for baculovirus quantification, especially in situations of mixed infections. In these cases, a rapid tool for identification and detection of different baculoviruses within a single sample is required. In addition, physical factors, such as different efficiencies of different purification protocols for OB from GVs and NPVs are assumed to have an effect on the obtained OB suspension, and hence, on the virus quantitation.

In this study, an identification and quantitation method based on qualitative and quantitative polymerase chain reaction (PCR) for AgseNPV-A, AgseNPV-B, AgipNPV and AgseGV was established. This method was applied on mixed infections with AgseNPV-B and AgseGV propagated in *A. segetum* larvae. Different OB purification protocols were applied and compared for their efficiency to isolated GV and NPV OBs from infected cadavers. This newly established PCR assay also allowed the quantitation of both AgseNPV-B and AgseGV and will provide an essential tool for studies on these baculoviruses.

Material and Methods

Insects

A population of the common cutworm *Agrotis segetum* has been maintained at the insect rearing facilities of the Institute for Biological Control in Darmstadt (Julius Kühn-Institut), where it was established at least 10 years ago. Adult moths were kept for two weeks in groups of about 30 individuals in transparent plastic cylinders (20 cm diameter, 25 cm height) that were faced inside with rough surfaced paper tissues. Insect eggs were collected three times a week by replacing the paper tissues; they were incubated at 25 °C for several days until hatching. About fifty neonates were transferred in plastic boxes that contained a thin layer of semi-synthetic diet (Ivaldi-Sender, 1974). According to personal observations *A. segetum* larvae were sensitive to excessive humidity and wet diet. For that reason only 66% of the recommended water volume was used for diet preparation. After autoclaving, the diet was poured in storage plastic boxes and was kept open over night for cooling and evaporation.

Larvae were kept at 22 °C with a 16/8 h light/dark photoperiod and were fed with pieces of diet until they reached the fourth larval stage. Then, they were transferred to boxes containing a 3 cm thick layer of vermiculite (<0.5 mm grain size) for pupation. Additional semi-artificial diet was provided shortly before pupation. Pupae were collected and incubated at 25 °C until the imagoes hatched.

Viruses

Virus stocks of *Agrotis segetum* nucleopolyhedrovirus B (AgseNPV-B) (also termed Oxford Strain) and *Agrotis segetum* granulovirus (AgseGV) were provided by Doreen Winstanley, Horticulture Research International (HRI) collection, Warwick (UK). They will be referred to as AgseNPV-B (HRI) and AgseGV (HRI) in this paper. Both viruses were propagated in *A. segetum* larvae, their purity was confirmed by DNA restriction endonuclease (REN) digestion and comparison of the fragment patterns with previously published REN patterns of AgseNPV-B (Bourner and Cory, 2004; El-Salamouny et al., 2003) or REN patterns of AgseGV that were simulated from published genome sequence (GeneBank accession no. NC_005839). An AgseNPV-B stock obtained from the virus collection of JKI in Darmstadt,

referred to as AgseNPV-B (JKI) was identified by REN analysis (see below) to be a mixture of AgseNPV-B and AgseGV. This stock was originally obtained from Burgerjon in 1978 and is designated as AgseNPV (A12-3) in the JKI virus collection. *Agrotis segetum* nucleopolyhedrovirus A (AgseNPV-A) was obtained from Agata Jakubowska, Spain, and *Agrotis ipsilon* nucleopolyhedrovirus (AgipNPV) (Illinois isolate) was provided by Prof. Naglaa A. Abdallah from the Agricultural Genetic Engineering Research Institute (AGERI) University of Cairo, Egypt. AgseNPV-B (JKI), AgseNPV-A and AgipNPV were propagated in *A. segetum* larvae as described below. Virus occlusion bodies (OBs) were enumerated by hemocytometer counting techniques (Eberle et al., 2012). NPV and GV OBs were counted in a Neubauer improved counting chamber (depth 0.1 mm) and Petroff-Hausser counting chamber (depth 0.02 mm), respectively.

Virus propagation

All *Agrotis* baculovirus were propagated in second to third instars of *A. segetum*. For infection, each larva was starved over night and provided with a small cube (3 x 3 x 3 mm) of artificial diet (Ivaldi-Sender, 1974) supplied with 1 µl OB suspension of 1,000 OBs. Larvae that had consumed the entire diet cube within 12 h were transferred to normal diet and were then incubated individually under normal rearing conditions (see above). For the next fourteen days, dead larvae of each treatment were daily collected and stored at -20 °C for OB purification.

Purification of OBs

The frozen cadavers were thoroughly homogenized in 0.5% sodium dodecyl sulfate (SDS) (about 1 – 2 ml per larva) using an Ultra-Turrax cell homogenizer. The resulting suspension was filtered through three layers of gauze that were washed with additional volumes of 0.5% SDS. Three different protocols were applied to isolate OBs from the suspensions: (i) low speed centrifugation (LSC) as described by Harrison (2009), (ii) sucrose gradient ultracentrifugation (SGU) according to O'Reilly et al. (1994) and (iii) sucrose cushion centrifugation (SCC) described in this study.

For LSC purification, the OBs were pelleted at 750 *g* for 10 min and then washed three times by re-suspending the pellet twice in 0.1% SDS and once in 0.5 M NaCl. The OBs were then centrifuged again at 750 *g* for 10 min and resuspended in an appropriate volume of deionized water (dH_2O).

For SGU purification, the homogenate was centrifuged at 5000 *g* for 10 min and the pellet was re-suspended in 0.5% SDS. The OB suspension was pelleted, then washed in 0.5 M NaCl, pelleted again and finally re-suspended in a smaller volume of dH_2O. Two ml of this suspension were carefully loaded on top of a sucrose step gradient consisting of 70% (bottom), 65%, 60%, 55%, 50%, 45%, 40% (top) (w/w) (each 1.5 ml) sucrose solution in a Open-Top Polyclear™ (Seton, Petaluma, CA, USA) (14 x 95 mm) centrifuge tube. Step gradients were loaded into a P40ST swing-out rotor and centrifuged at 96.000 *g* for 3 h at room temperature in a Hitachi 65P-7 ultracentrifuge. OBs were visible at ~55% sucrose concentration by forming a white band. The band was collected, diluted and mixed thoroughly in dH_2O. OBs were pelleted by centrifugation at 5.000 *g* for 10 min and re-suspended in a smaller volume of dH_2O.

For SCC purification, the OBs were washed three times by pelleting at 18.500 *g* for 20 min, discarding the supernatant and re-suspending the pellet in 0.5% SDS. One volume of this OB suspension was carefully loaded on top of 6 volumes of 50% (w/w) sucrose cushion layer. Samples were centrifuged in a swing-out rotor at 3200 *g* for 20 min. The supernatant was removed and sucrose removed from the pellet by performing additional washing steps, as described above but dissolving the pellet in dH_2O. Finally, the pellet was dissolved in a smaller volume of dH_2O.

For mixed infected larvae, the LSC, SSC and SGU purification protocols were applied and threefold replicated. Stocks of dead larvae that were infected by only AgseNPV-A and AgipNPV were purified after homogenization by applying the LSC protocol. AgseGV (HRI) was purified from dead larvae following the SCC method. All OB stocks and purification replicates were stored at -20 °C.

DNA extraction

For isolating viral genomic DNA the occlusion body matrix protein was solubilized by incubating the OBs in 100 mM Na_2CO_3 for 1 h. The obtained virion suspension was adjusted to pH 8 by adding 1 M HCl and incubated with RNaseA (90 µg/ml) (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at 37 °C. SDS was added to a final concentration of 1% followed by an incubation with Proteinase K (250 µg/ml) (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at 50 °C. DNA was extracted by phenol-chloroform treatment and ethanol precipitation. The pellet was washed and dissolved in an appropriate volume of dH_2O.

For reasons of standardization in qPCR assays and isolation of viral genomic DNA from small amounts of OBs at highly diluted qPCR standard samples, genomic DNA was extracted from OB suspensions using Ron's tissue DNA Mini Kit (Bioron, Ludwigshafen, Germany). After RNase A and Proteinase K treatment 3 µg Herring sperm DNA were added to each DNA sample in order to saturate the kit's silica membrane. DNA was eluted from the membrane in 400 µl of 10 mM TE buffer. In all comparative virus purification experiments the DNAs were extracted from 100 µl of each virus stock suspension by following the same DNA purification protocol. DNA of AgseNPV-B (HRI) and AgseGV (HRI) were used for all control and optimization reactions as well as qPCR standard samples, whenever a pure AgseNPV-B or AgseGV DNA was required.

PCR primer design

Based on the *polyhedrin* (*polh*) and *granulin* (*gran*) gene sequences of AgseNPV-A (Jakubowska et al. 2005, NC_007921), AgseNPV-B (Chapter 2), AgipNPV (Harrison 2009, NC_011345) and AgseGV (NC_005839) one pair of specific oligonucleotide primers was designed for each of the four *Agrotis* baculoviruses using Geneious Software (Biomatters, Auckland, New Zealand) (Table 3.1). The specificity of the different primers was determined *in silico* by NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The oligonucleotide primers were synthesized by Eurofins MWG Operon, Ebersberg Germany.

Optimization of the annealing temperature (T_A)

All PCR experiments were carried out in a gradient thermocycler (Eppendorf, Hamburg, Germany) and PCR reagents were supplied by Axon Labortechnik (Kaiserslautern, Germany). To test primers for their optimal and acceptable range of the annealing temperature T_A, each pair of primer was tested with its corresponding DNA at twelve different temperatures ranging from 48.5 – 63.0 °C in a gradient PCR. A single reaction was assembled in 50 µl volume consisting of 5 µl of 10x reaction buffer (Mg^{2+} and Tween20 free), 2 µl of 50 mM $MgCl_2$, 1 µl of dNTPs mix, each at a concentration of 10 mM, 0.5 µl of 5 U/µl *Taq* polymerase, 1 µl of 5 ng/µl template DNA, 1 µl of 10 µM forward and 1 µl of 10 µM reverse primer. The final volume was reached by adding 38.5 µl double deionized (dd) H_2O. In order to minimize the pipetting error and ensure constant reaction conditions, a PCR master mix was prepared. The master mix contained all components, except for the primers and template DNA, and was stored in small aliquots at –20 °C. For a standard PCR 47 µl master mix was combined with 1 µl of each primer and 1 µl template DNA (see above). PCR reactions were initiated with a denaturation step at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 45 sec, primer annealing at 48.5 – 63.0 °C for 45 sec and elongation at 72 °C for 1 min. Final elongation was performed at 72 °C for 5 min.

Multiplex PCR for detection and identification

For a simultaneous detection and identification of AgseNPV-A, -B, AgipNPV and AgseGV from DNA samples, a single multiplex PCR was applied in a total volume of 50 µl containing the eight primers each at the final standard concentration of 0.2 µM. A primer pre-mix was set up by equally combining one volume of each of the eight primers (10 µM). Eight µl of this oligonucleotide pre-mix was added to 5 µl of 10x reaction buffer (Mg^{2+} and Tween20 free), 2 µl of 50 mM $MgCl_2$, 1 µl of dNTPs mix, each at a concentration of 10 mM, 0.5 µl of 5 U/µl *Taq* polymerase and 1 µl template DNA. In addition, 5 µl of 100% dimethyl sulfoxide (DMSO) were added for reasons of PCR optimization and the samples were adjusted with ddH_2O to a final volume of 50 µl. According to the experimental results of T_A optimization, the T_A for multiplex PCR was also set to 49.4 °C for 45 sec. Temperatures and times for

denaturation and elongation, as well as the number of cycles were equal to standard PCR settings.

Multiplex PCR optimization and control reactions

To avoid mispriming and to reduce any unintended primer DNA and primer primer interaction between all eight different primers and four types of viral genomic DNAs in the multiplex PCR, DMSO was added to a final concentrations of 0%, 2%, 4%, 6%, 8%, 10%, 12%, and 14% (v/v), respectively. For this, the protocol described above was adjusted.

PCR based quantitation of Agrotis baculoviruses

The ratio of AgseNPV-B and AgseGV was determined by qPCR analysis using a CFX90™ real-time system (Bio-Rad, Hercules, CA, USA). For the preparation of qPCR standards, serial dilutions of OBs were set up for both viruses. OBs of AgseNPV-B (HRI) were serially diluted from 1.0 x 10^9 to 1.0 x 10^4 OBs/ml and AgseGV (HRI) from 1.0 x 10^{11} to 1.0 x 10^6 OBs/ml. DNA was extracted from 100 µl of each dilution step by the protocol as described above. The standard DNA samples were subjected to PCR with their corresponding pair of specific primers (Table 3.1), whereas DNA samples from all three LSC, SGU and SCC purified OB stocks were tested using AgseNPV-B and AgseGV specific primers in separate treatments. Each qPCR was performed in a 25 µl reaction volume containing 0.4 pM of each primer, 1x Maxima® SYBR Green/Rox qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 2 µl DNA sample. Negative controls contained 2 µl ddH_2O instead of DNA. All qPCR reactions were started with a denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation (94 °C for 1 min), primer annealing (60 °C for 30 sec) and elongation (72 °C for 30 sec) and a final elongation step (72 °C for 5 min). Melting curve analysis was performed from 40 to 95 °C with an increment of 0.5 °C each 5 sec. Each quantitation experiment included the purification experiment samples, all qPCR standard samples and non-target controls, respectively.

Data were analyzed using Bio-Rad CFX Manager 2.0 (Bio-Rad, Hercules, CA, USA) and Cq (quantitation cycle) values were determined by single threshold analysis. For determination of the AgseNPV-B/AgseGV ratio, the coefficient of both virus concentrations was calculated for each purification method and its replicates. To obtain the average NPV/GV ratio between replicates the geometric mean was calculated.

DNA restriction analysis

For DNA restriction endonuclease (REN) analysis 1 µg viral DNA was digested with 5 U *Eco*RI (Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 20 µl for 16 h at 37 °C. Restriction fragments were separated in 0.8% agarose gel in 1x TAE buffer for 16 h at 25 V. Agarose gels were stained in 0.01% ethidium bromide solution after electrophoresis and documented under UV light.

Table 3.1. Oligonucleotide primers specific for AgseNPV-A, AgseNPV-B, AgipNPV and AgseGV. Given are their positions in the genome, their names, sequences, and expected fragment size in the respective baculovirus. (ORF = open reading frame).

Target genome	Position in *polh/gran* ORF	Primer name	Primer sequence (5'-3')	Size (bp)
AgseNPV-A	165 - 186	prAsApolh-f	AGTAGCTGAAGACCCCTTCTTG	199
	342-363	prAsApolh-r	CAGGAACACGTCCATAATTTCT	
AgseNPV-B	169-187	prAsBpolh-f	GCCGAGGATCCATTTTTTG	260
	408-428	prAsBpolh-r	CGCAGAGCGTGTTGAGCTAAA	
AgipNPV	169-185	prAiIpolh-f	GCCGAGGATCCCTTCCT	527
	672-695	prAiIpolh-r	TTGATCTTAAAGACGAGTGAGAGC	
AgseGV	112-131	prAsGVgran-f	GACAGGCGTATATCGGAAGC	347
	439-458	prAsGVgran-r	TGAGCGACGTAATCTGGATG	

Results

Primer design and PCR optimization

Based on the nucleotide sequences of the *polh/gran* gene of AgseNPV-A, AgseNPV-B, AgipNPV and AgseGV, respectively, four oligonucleotide primer pairs were designed, each pair specific for one of the four viruses (Table 3.1). The primers were chosen according to their sequence specificity, similar annealing temperatures and with the aim that the resulting PCR products differ in their size (ranging from 199 to 527 bp), so that they could be applied in multiplex PCR reactions for multiple *Agrotis* baculovirus detection. In order to establish a multiplex PCR that allows the simultaneous amplification of several PCR fragments, all primer sequences were adjusted to each other in their T_A and other PCR conditions. First, each specific primer pair was tested with its corresponding template for its binding behavior in single PCR reactions at a T_A ranging from 48.5 – 63 °C (Fig. 3.1). In these control experiments all primer pairs resulted in the amplification of specific PCR fragments of predicted size. At a T_A > 56.1 °C the signal from AgseNPV-A became unpredictable and a similar slight decrease of fragment intensity was also visible for AgseNPV-B primers (Fig. 3.1A and B). The AgipNPV specific primers showed a sufficient generation of PCR fragments at all applied T_A (Fig. 3.1C). The weak signal of the AgseGV reaction at T_A = 49.4 °C (Fig. 3.1D) was considered as a failure of this single reaction because PCRs at lower and higher T_A showed sufficient amplification of AgseGV specific products. According to these results, the T_A was set to 49.4 °C for all further standard and multiplex PCR. Additional faint bands of about 400 bp (AgseNPV-A primers), 450 bp (AgseNPV-B primers) and 700 bp (AgseGV primers) were detected. These unspecific signals did not overlap and could not be confused with any specific PCR fragment of the four *Agrotis* baculoviruses.

Optimization of multiplex PCR

When the different primer pairs were applied to multiplex PCR reaction a further unspecific fragment of 0.9-1 kb was observed in addition to the expected and specific AgseNPV-A, AgseNPV-B, AgseGV and AgipNPV fragments (Fig. 3.2, lane 1). To increase the specificity of the reaction, the PCR enhancing DMSO was tested at different concentrations (Fig. 3.2).

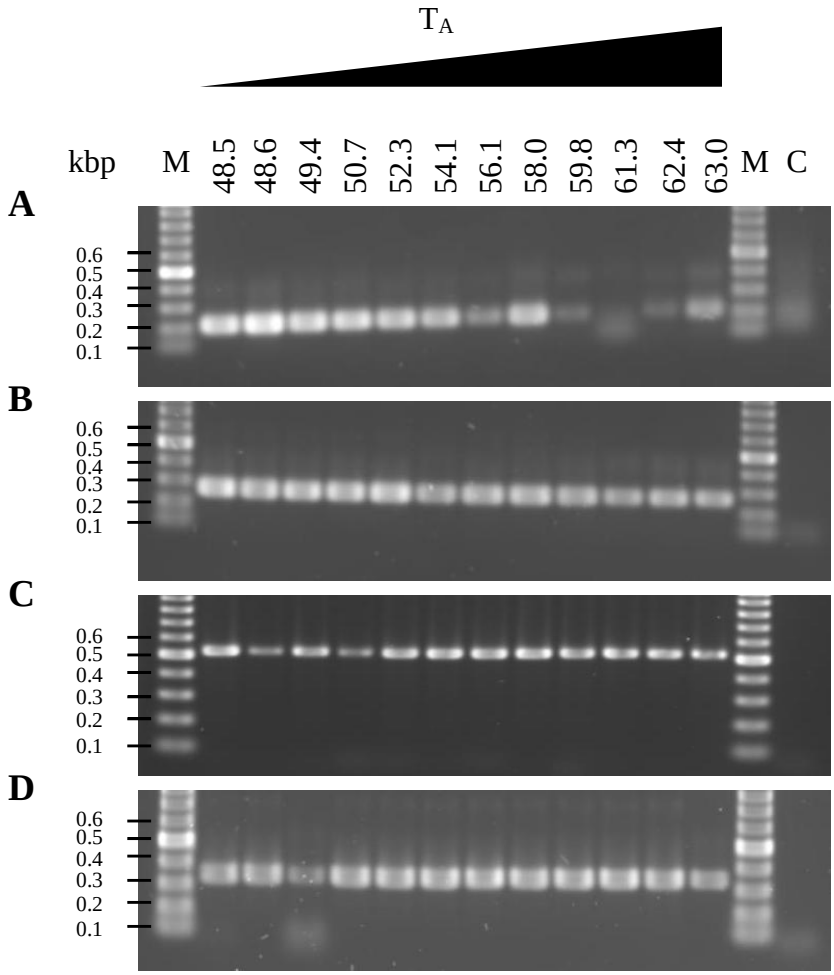

Figure 3.1. Temperature gradient PCR (1.5% agarose gel) for optimizing the annealing temperature (T_A) using oligonucleotide primers specific for AgseNPV-A (panel A), AgseNPV-B (panel B), AgipNPV (panel C) and AgseGV (panel D). The negative control (C) without DNA is given in the most right lane of each panel. M = marker, size standard to the left.

Increasing DMSO concentrations led to a successive reduction of the unspecific fragment and at about 10% DMSO the fragment almost vanished. The intensity of the specific *Agrotis* baculovirus fragments started to decrease from DMSO concentrations above 6% indicating the inhibitive effect of this PCR compound on the *Taq* polymerase. According to these observations, a DMSO concentration of 10% was applied in all further multiplex PCR applications.

To confirm the specificity of the multiplex PCR and to test potential mispriming, control reactions were performed with applying all four primer pairs but omitting one out of four *Agrotis* baculovirus DNAs. When this experiment was performed with different combinations of DNAs (Fig. 3.3), the three respective fragments of correct size were generated. Thus, the fourth pair of oligonucleotides in the reaction which was not provided a specific template DNA, did not bind to any of the three remaining unspecific types of viral genomic *Agrotis* baculovirus DNA present in the reaction mixture. Only a faint fourth fragment of about 400 bp occurred in three of four reactions (Fig. 3.3, lanes 1-3) but did not overlap with any specific fragment.

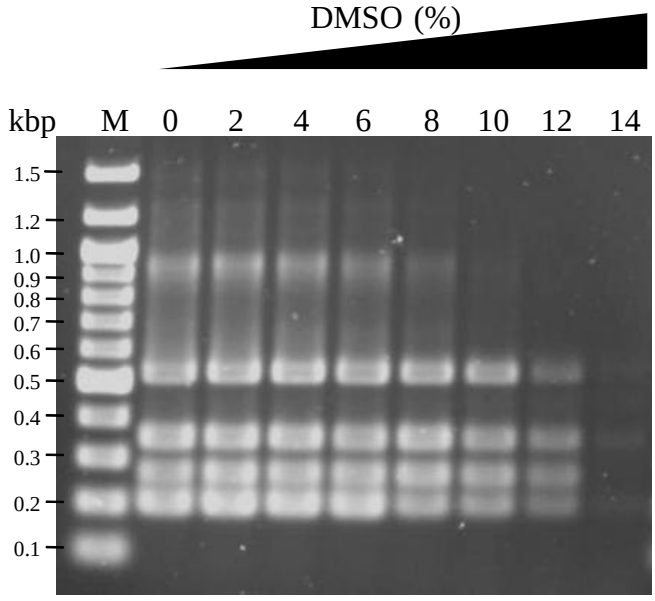

Figure 3.2. Multiplex PCR using the oligonucleotide primers given in Table 3.1 and viral genomic DNA of four *Agrotis* baculoviruses AgseNPV-A, AgseNPV-B, AgipNPV and AgseGV, respectively. The increasing concentration (%) of dimethyl sulfoxide (DMSO) is given on the top. DNA size standard is given to the left.

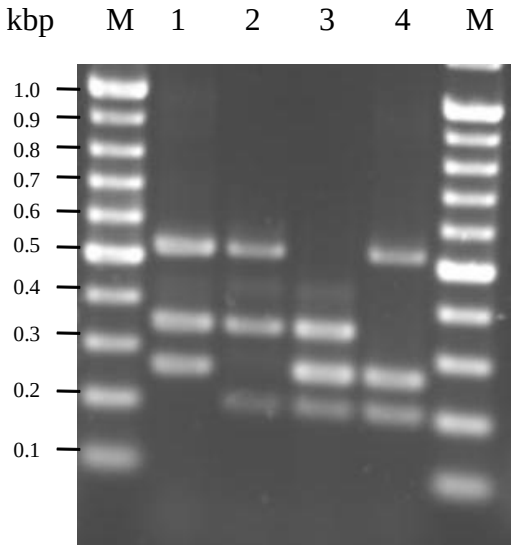

Figure 3.3. Multiplex PCR reactions (1.5% agarose gel) using all four primer pairs (Table 3.1) but only three target DNAs, lane 1 = AgseNPV-B/AgseGV/AgipNPV, lane 2 =AgseNPV-A/AgseGV/AgipNPV, lane 3 = AgseNPV-A/AgseNPV-B/AgseGV, lane 4 = AgseNPV-A/AgseNPV-B/AgipNPV, M = size standard. DNA size standards are given to the left and right.

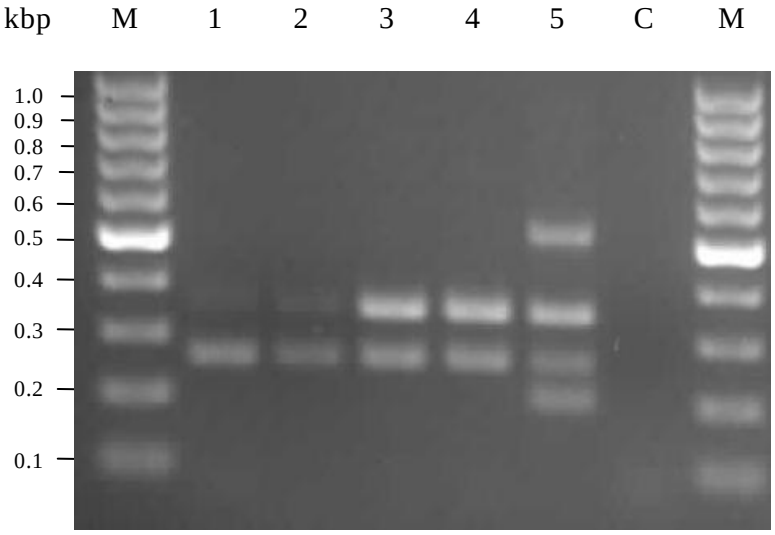

Figure 3.4. Multiplex PCR reactions using all four primers for the detection of *Agrotis* baculoviruses (Table 3.1) in DNA samples from purification experiments. Lane 1 = AgseNPV-B (JKI) inoculum used for virus propagation, lane 2 = propagation using LSC protocol, lane 3 = propagation using SCC protocol, lane 4 = propagation using SGU protocol, lane 5 = positive control with all four *Agrotis* baculovirus DNAs, C = negative control without DNAs. DNA size standards are given to the left and right.

Multiplex PCR based detection of Agrotis baculoviruses in purification experiments

When AgseNPV-B (JKI) was propagated in larvae of *A. segetum*, virus OBs were isolated from infected cadavers using three different purification methods LSC, SGU, and SCC. The application of these three protocols was intended to investigate the most efficient protocol to obtain AgseNPV OBs that were completely free of larval debris. After applying all purification methods, light microscopy of the resulting OB suspension showed small refracting particles, most presumably AgseGV granules, besides the AgseNPV-B polyhedra (data not shown). To test this hypothesis, multiplex PCR was applied on DNA samples that originated from the inoculum AgseNPV-B (JKI), and the three LSC, SCC and SGU purifications. AgseNPV-B and AgseGV were detected in all four samples (Fig. 3.4, lane 1-4). The generated PCR fragments agreed in their size with those specific for AgseNPV-B with 260 bp and for AgseGV with 347 bp. No signal was detected for AgseNPV-A and AgipNPV, indicating that the AgseNPV-B (JKI) inoculum and its progeny were a mixture of AgseNPV-B and AgseGV.

Interestingly, the intensity of the AgseNPV-B and AgseGV specific fragments varied among the inoculums sample and the three purification methods. The inoculums and the LSC sample resulted in a predominant AgseNPV-B fragment (Fig. 3.4, lane 1 and 2), whereas SCC and SGU purification showed a more intense AgseGV specific band (Fig. 3.4, lanes 3 and 4). These results corresponded with DNA restriction analyses of the LSC and SCC OB samples (Fig. 3.5). DNA prepared from LSC purified OBs (Fig. 3.5, lane 2) showed a predominant AgseNPV-B profile (Fig. 3.5, lane 1) and only faint AgseGV bands (Fig. 3.5, lane 4 and 5), whereas REN analysis of DNA from SCC purified OB (Fig. 3.5, lane 3) clearly showed AgseGV specific restriction fragments. This suggested that different purification methods had a different efficiency on the purification of OBs of AgseNPV-B and AgseGV. The restriction profile of SGU purified OBs was not generated because not enough DNA was available.

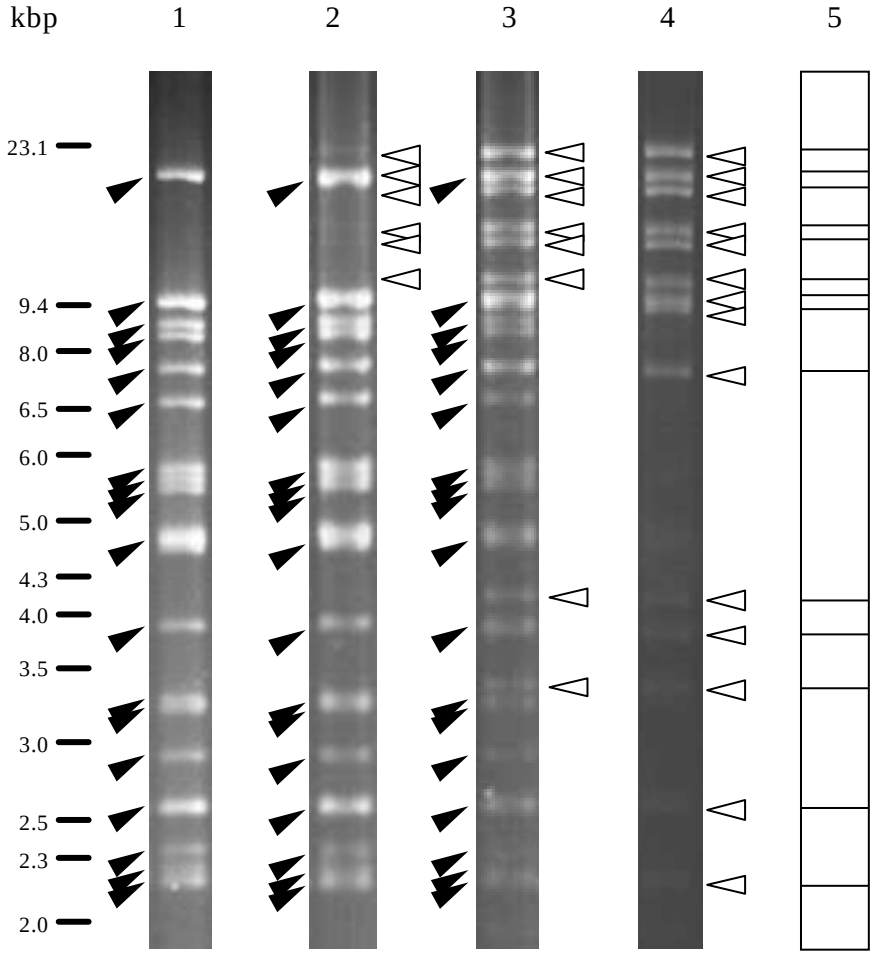

Figure 3.5. Agarose gel (0.8%) of *Eco*RI digests of viral genomic DNA purified from AgseNPV-B (JKI) infected *A. segetum* larvae using LSC protocol (lane 2) and SCC protocol (lane 3). AgseNPV-B and AgseGV characteristic fragments are marked with bold and hollow arrows, respectively. AgseNPV-B (HRI) and AgseGV (HRI) specific bands are given in lane 1 and lane 4, respectively. Simulated restriction profile of AgseGV is shown in lane 5. Size marker is given to the left.

PCR based AgseNPV-B and AgseGV quantitation

In order to support the assumption that the purification methods have a selective effect on the AgseNPV-B and AgseGV ratio, the OBs isolated from insect cadavers were quantified by applying a qPCR that used the AgseNPV-B and AgseGV specific primer pairs. Melting curve analyses of AgseNPV-B specific fragment gave a maximum at 82.4 °C, whereas the AgseGV specific fragment had its maximum at 88.5 °C, allowing to distinguish the DNAs of both viruses by their melting curves.

No secondary amplification products were observed by analyzing the melting curve. DNA standards were prepared from OB dilutions of AgseNPV-B and AgseGV and subjected to qPCR. When the starting quantity signals of the viral OB standard samples were plotted against the threshold amplification cycle (Cq value) a linear regression line was obtained over a range of six orders of magnitudes for both AgseNPV-B and AgseGV (Fig. 3.6). Both standard curves differed in their slope indicating slightly different standard amplification rates (10exp(-1/slope)) of 1.8 for AgseGV and 2.19 for AgseNPV-B. This deviation from the optimum duplication rate of 2.0/cycle did not affect the AgseNPV-B/AgseGV ratio calculation, as each virus was quantified according to its own standard. Furthermore, the regression coefficients of the standard curves of R^2 = 0.971 (AgseNPV-B) and R^2 = 0.987 (AgseGV) reflected a low variance of the protocol for OB DNA isolation that based on a commercial tissue DNA isolation kit.

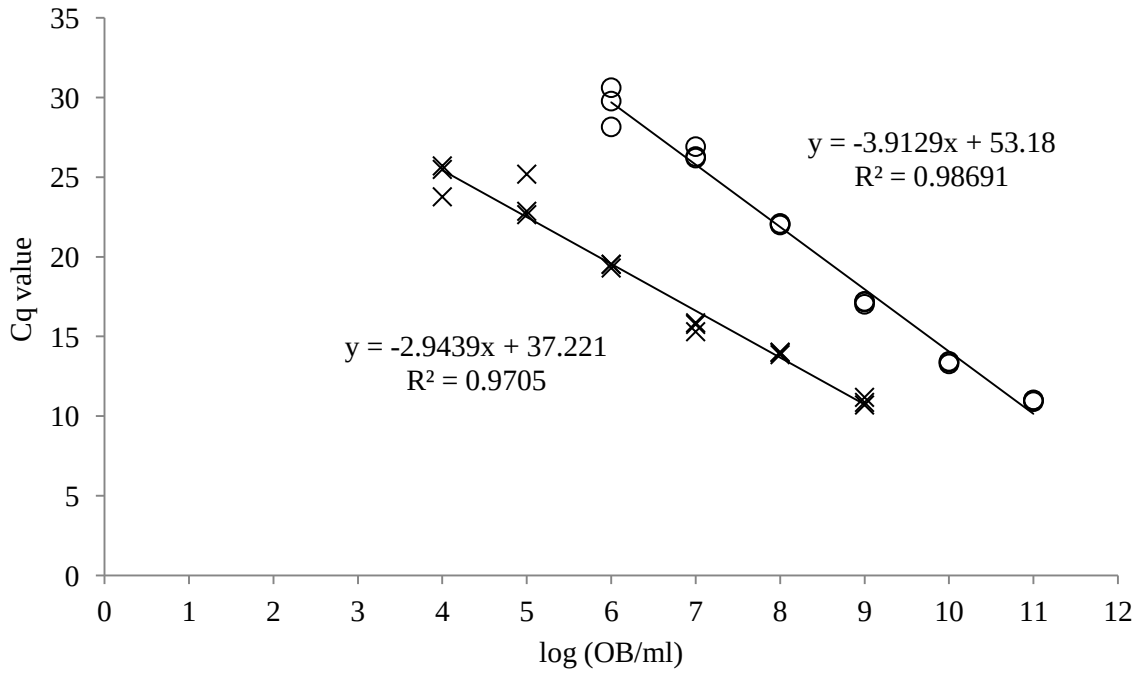

Figure 3.6. Regression lines of qPCR standards prepared for OBs of AgseNPV-B (crosses) and AgseGV (circles) using AgseNPV-B and AgseGV specific primers. Each sample was prepared in triplicate.

When the OB preparation using LSC, SCC and SGU purification protocols were subjected to qPCR, each sample was separately analyzed for its quantity of AgseNPV-B and AgseGV OBs and the GV/NPV OB ratio was calculated for each sample. The LSC, SCC and SGU purifications resulted for AgseNPV-B in median concentrations of 0.3 x 10^8, 0.2 x 10^8 and 0.05 x 10^8 OB/ml, respectively, and for AgseGV in concentrations of 3.45 x 10^8, 129.6 x 10^8 and 35.96 x 10^8 OB/ml (Fig. 3.7). Thus LSC treatment had the lowest geometric mean of the three times replicated GV/NPV OB ratios (7.7), followed by the SCC (368.0) and SGU (1055.6). The different protocols showed an internal variation in the GV/NPV OB ratio of replicates that was probably influenced by the protocols and processes of OB purification. The ratios of the LSC samples varied between replicates by a factor up to 24 times, whereas the SCC and SGU differed by a factor 2.4 (SGU) to 3.2 (SCC).

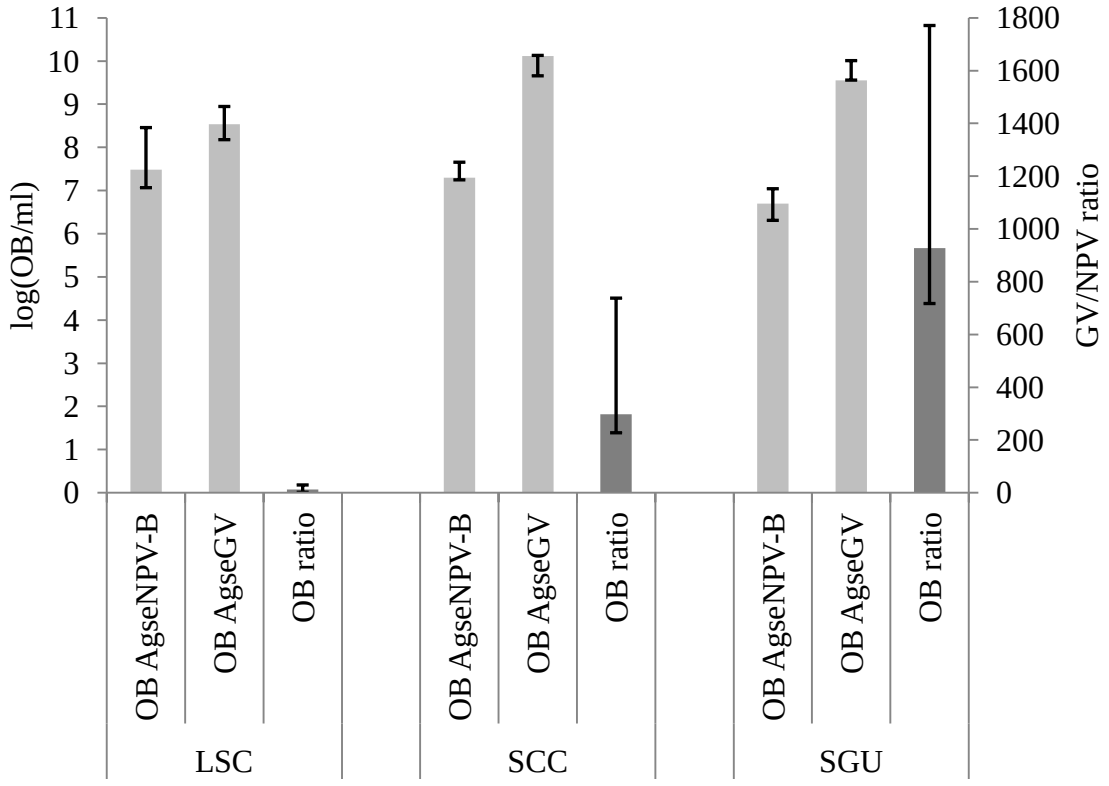

Figure 3.7. qPCR based median OB concentrations (OB/ml) (light grey bars) and median AgseNPV-B/AgseGV ratios (dark grey bars) of the LSC, SCC and SGU protocol. Each protocol was performed in triplicate, indicated by the error bars that show the minimum and maximum value.

Discussion

There are numerous reports that given Lepidopteran larvae are susceptible to different baculoviruses and that they may become cross-infected and co-infected by more than one baculovirus species (Lauzon, 2005; Jehle et al., 2003, 1992; Tanada, 1959). The *Agrotis* baculoviruses belong to one of the most complex examples of this observation as at least four different baculoviruses, i.e. AgseNPV-A, AgseNPV-B, AgipNPV and AgseGV, belonging to different baculovirus species, can infect and co-infected larvae of *A. segetum* (Herniou et al., 2011; Bourner and Cory, 2004; El-Salamouny et al., 2003; Ignoffo and Garcia, 1979). To improve the identification and quantitation of these viruses a PCR method that is based on four different primer pairs, each specific for one of these *Agrotis* baculoviruses, was established. In case that one of these four viruses is present within a DNA sample a PCR fragment of diagnostic size is produced that can directly be visualized by agarose gel electrophoresis. The analysis can be applied in single or standard PCR reactions with one primer pair as well as in multiplex PCR conditions, simplifying the whole detection process.

The amplification of multiple sequences in a single reaction runs the risk of unforeseeable primer primer and primer DNA interactions that can cause the generation of unspecific PCR products. Addition of DMSO to the PCR reaction may reduce the background of non-specific fragments (Chakrabarti and Schutt, 2001; Hung et al., 1990; Winship, 1989). Besides its beneficial ability in reduction of non-specific fragments, DMSO is also described as an inhibitor in higher concentrations by reducing the *Taq* polymerase activity (Gelfand and White, 1990). For this reason, the use of DMSO was balanced between avoiding interfering reactions and keeping the PCR sensitive enough for the detection of DNA molecules that are present within the sample by only a few DNA molecules.

The developed multiplex PCR analysis was robust and reliable for the simultaneous detection and identification of AgseNPV-A, AgseNPV-B, AgipNPV and AgseGV. It allowed detecting AgseGV in the prepared AgseNPV-B (JKI) samples as well as in the originally used inoculum. The multiplex PCR analysis will therefore facilitate the identification of and distinguishing between different *Agrotis* baculoviruses that are known to show different infectivity to *A. segetum* and *A. ipsilon* (Bourner and Cory, 2004; El-Salamouny et al., 2003). In addition, it will be useful to detect infected larvae before typical infection symptoms become visible.

Approaches to quantify baculoviruses from mixed infections have been reported by quantifying the band intensity of DNA restriction profiles in agarose gels (Arends et al., 2005) and by semi-quantitative PCR (Lopez-Ferber et al., 2003). A qPCR based method is developed in this study, which is advantageous to REN based methods because they are less time consuming and cover a wide quantitative range. This range is achieved by the qPCR standards of several orders of magnitude, which in case of an overlapping range of the standard curves allow an immediate ratio determination.

However, quantitation of NPV and GV does not only depend on the ratio of viruses in the insect but also on the purification method applied. When three different purification methods were compared, different efficacies for polyhedra and for granules were observed. The LSC and SCC protocol, which processed the pellet after centrifugation, led to a similar average amount of NPV. Other components were assumed to remain in the supernatant due to low centrifugation speed (LSC) or liquid resistance of the sucrose cushion layer (SCC). The latter assumption was supported by a turbid appearance of the upper phase. The SGU method resulted in the lowest amount of purified polyhedra, which were harvested from the grayish band in the sucrose gradient. Here, a defined band at a certain sucrose concentration was taken from the purification sample that probably consisted mainly of aggregated granules. The centrifugation parameters for polyhedral aggregation were either not optimal, e.g. too short centrifugation time, or they were spread more widely around the OB band that was taken further for purification. Contrary effects were observed for granule isolation. Here, the SGU and SCC showed the similar high efficacy, whereas the LSC protocol purified fewer granules. It can be concluded that the SGU and LSC protocols appeared to be counter-selective for polyhedra and granules, respectively, and the SCC method was the most robust purification method for both NPV and GV. Such robust methods are particularly important for studies that require a correct estimation of ratios in mixed infected insects, such as population dynamics. In initial OB suspensions with extremely shifted NPV/GV ratios, such as a covert contamination, the selective effect of a protocol may critically reduce the type of OB that has the lowest concentration. Its disappearance or slight visibility on agarose gels of a REN could lead to a misinterpretation of the result and significantly influence the outcome of a quality control or an experiment based on baculovirus OB suspensions such a bioassay analysis.

Interestingly, the qPCR analyses also allowed some estimations of the number of virions of an AgseNPV-B OB. The standard curves for the qPCR (Fig.6) showed a similar slope but differed in their y-axis increment. If the OB of AgseGV and AgseNPV-B contain the same amount of DNA (= same amount of genomes and nucleocapsids, respectively) the y-axis increment should be similar, i.e. the same amount of OB should result in the same Cq value. Thus, the difference in the Cq value for a given OB concentration indicates the difference in genomes (= nucleocapsids) per OB. A difference of one PCR cycle (Cq value on the y-axis) at the same starting quantity (OB/ml) (value on the x axis) between two samples would mean a double DNA concentration, if one PCR cycle ideally duplicates the amount of assembled PCR fragments. In contrary to the polyhedra of the NPVs, granules contain only one nucleocapsid per OB. Therefore, the qPCR based amount of AgseGV OB in a sample should approximate the number of GV nucleocapsids of this sample. The number of nucleocapsids per AgseNPV-B OB is unknown but can be estimated by average distance of the two regression lines, which was 8.69 Cq values. According to the theoretical quadratic increase of DNA concentration per Cq distance, one AgseNPV-B OB therefore contained about $2^{8.69}$ = 413 nucleocapsids. The obtained value is theoretical. It must be carefully evaluated because different efficiencies of both PCR reactions, which are based on two different slopes of the two standard curves, were taken for calculation. Even a slight variation in PCR amplification may have a considerable impact on the estimation. Ideally, the two slopes of both regression lines should be the same, which would lead to a more accurate estimation of nucleocapsids per AgseNPV-B OB.

In conclusion, the *Agrotis* baculovirus complex clearly shows that identification and quantitation methods are essential to describe and analyze infection processes in mixed infection situations with different NPVs and GVs. These methods are also highly important for the quality control in the production process of baculovirus biocontrol products and will assist in the formulation process of virus mixtures as well as in the detection of contaminations. Especially for mixed infections an appropriate and robust purification protocol for the OBs of NPVs and GVs is essential to maximize the OB recovery and to avoid the risk of biased NPV/GV identifications caused by the physical nature of the purification procedure.

CHAPTER 4: COMPETITIVE INTERACTION OF THE *AGROTIS SEGETUM* GRANULOVIRUS (AGSEGV) AND *AGROTIS SEGETUM* NUCLEOPOLYHEDROVIRUS B (AGSENPV-B) IN SIMULTANEOUSLY INFECTED CUTWORM LARVAE

Abstract

Mixed infections of insect larvae with different baculoviruses are occasionally found. They are of interest from an evolutionary point of view as well as from a practical prospect when baculoviruses are applied as biocontrol agents. Here, we report mixed infection studies of neonate larvae of the common cutworm, *Agrotis segetum*, with two baculoviruses, *Agrotis segetum* nucleopolyhedrovirus B (AgseNPV-B) and *A. segetum* granulovirus (AgseGV). By applying qPCR analysis, co-infections of individual larvae were proved and occlusion body (OB) production within single infected and co-infected larvae was determined in individual larvae. Mixtures of viruses did not lead to changes in mortality rates if compared with rates of single virus treatments, indicating an independent action within host larvae under our experimental conditions.

Whereas AgseGV showed a constant OB production during two weeks of infection, an increase in OB production was observed for AgseNPV-B infected larvae that died within the first and second week post infection. In comparison with single infected larvae, less OBs of both viruses were produced in co-infections suggesting a competitive interaction of the two viruses for larval resources. Hence, no functional or economic advantage could be deduced from larval mortality and OB production from mixed infections of *A. segetum* larvae with AgseNPV-B and AgseGV.

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Introduction

A large number of baculoviruses has been isolated and described from larvae of species of the insect orders Lepidoptera, Diptera and Hymenoptera (Martignoni and Iwai, 1987). According to the occlusion body (OB) morphology, baculoviruses are distinguished into two morphological groups; the granuloviruses (GV) with a single virion in an ovo-cylindrical OB (granule) and the nucleopolyhedroviruses (NPV) with a few to many virions in a polyhedral OB (polyheder) (Herniou et al., 2011). Taxonomically, the family *Baculoviridae* is subdivided into four genera, the *Alpha-*, *Beta-*, *Gamma-* and *Deltabaculovirus* (Herniou et al., 2011; Jehle et al., 2006). Alpha- and betabaculoviruses comprise lepidopteran-specific NPVs and GVs, respectively. Many lepidopteran species are susceptible to baculoviruses form different species. Even simultaneous infections, so-called co-infections or mixed infections, of two NPVs (Cheng et al., 2005), two GVs (Lange and Jehle, 2003), and NPVs and GVs (Hackett et al., 2000; Tanada, 1959) have been observed.

Larvae of the turnip moth, *Agrotis segetum* (Dennis & Schiffermüller) (Lepidoptera: Noctuidae), so-called common cutworms, are important agricultural pests (Hill, 1997). Two different alphabaculoviruses, *Agrotis segetum* nucleopolyhedrovirus A (AgseNPV-A, also called Polish isolate) and *A. segetum* nucleopolyhedrovirus B (AgseNPV-B, also termed Oxford isolate) (Chapter 2; Allaway and Payne, 1983; Jakubowska et al., 2005), as well as the betabaculovirus *A. seg*etum granulovirus (AgseGV) (Lipa and Ziemnicka, J., 1971), were isolated and characterized from *A. segetum* larvae. AgseGV was tested extensively as a biocontrol agent for the control of *A. segetum* in the field (Bourner et al., 1992; Caballero et al., 1991; Zethner et al., 1987; Shah et al., 1979) and AgseNPV-B has shown its potential as biocontrol agent under laboratory conditions (Bourner et al., 1992).

Both viruses were found to infect *A. segetum* larvae simultaneously (Shvetsova and Ts'ai, 1962) but little is known about possible interaction in co-infections. Shvetsova and Ts'ai (1962) reported an increased mortality when AgseGV and an *Agrotis* nucleopolyhedrovirus were simultaneously provided to larvae of *Apamea anceps* and it was concluded from mortality data that these viruses did not show antagonistic behavior. In addition, both viruses were shown histologically to infect the same insect tissues of *A. segetum* larvae (Shvetsova and Ts'ai, 1962). However, in this study it was not distinguished whether the used AgseNPV was AgseNPV-A or AgseNPV-B, since this nomenclature was later introduced by

Jakubowska et al. (2005). Infection experiments, which were conducted in *A. segetum* larvae with AgseGV and AgseNPV-B did not reveal any increase in mortality when provided to larvae at the same time (Gürlich, 1993). The ability of AgseGV and AgseNPV-B to co-infect *A. segetum* larvae raised the questions, whether there is an interaction of both viruses, and if so, which kind of interaction mechanism occurs, and if interactions between these viruses are of a functional and economic benefit when used as pest control agent. Both enhancing (Tanada and Hukuhara, 1971) and inhibiting (Whitlock, 1977) effects within common host larvae were described for GV and NPV co-infections. More recently, an AgseNPV-B virus stock containing AgseGV revealed covered co-infections of AgseGV in virus propagations in *A. segetum* larvae (Wennmann and Jehle, 2014). Within the same study, a PCR-based method for the identification and quantitation of *Agrotis* baculoviruses was established. Since a mixture of AgseGV and AgseNPV-B could be both, beneficial and disadvantageous for one or both viruses, information about their interaction mechanism are considered as important for a possible improvement of their application in the field.

This study was carried out to investigate the potential interaction of AgseGV and AgseNPV-B in mixed infections of *A. segetum* larvae. Quantitative PCR (qPCR) was applied to estimate the OB production of AgseGV and AgseNPV-B on the level of individual co-infected larvae and provided insight into a competing mechanism of interaction.

Materials and Methods

Insects and viruses

Rearing of *A. segetum* was performed at the Institute for Biological Control in Darmstadt (Julius Kühn Institute, JKI) as described in Wennmann and Jehle (2014). Neonate *A. segetum* larvae were used in all infection experiments. Stocks of *A. segetum* nucleopolyhedrovirus B (AgseNPV-B) and *A. segetum* granulovirus (AgseGV), which both originated from the virus collection of Horticulture Research International (HRI) in Warwick (UK), were propagated in fourth instar of *A. segetum* and tested for their purity by PCR (Wennmann and Jehle, 2014).

Infection studies

Bioassay analyses were performed for AgseNPV-B and AgseGV in neonate *A. segetum* larvae feeding on different virus concentrations. For AgseNPV-B the virus concentrations were 10^3, 3.1 x 10^3, 10^4, 3.1 x 10^4, 10^5, 3.1 x 10^5, and 10^6 OB/ml. The bioassay for AgseGV was performed at 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 OB/ml. Enumeration of virus occlusion bodies (OBs) was performed by hemocytometer counting as described in Eberle et al. (2012). For each treatment, 1 ml of a 50-fold of the above mentioned OB concentrations was thoroughly mixed with 49 ml 40 °C, semifluid and semiartificial diet (modified according to Wennmann and Jehle, 2014; Ivaldi-Sender, 1974) and thereby diluted to its final concentration. For the untreated control, 1 ml of water was used instead of virus suspension. Prior to solidification of the diet, 1 ml diet was filled in every well of a 50-well bioassay tray (LICEFA, Bad Salzuflen, Germany). Bioassay trays were kept open overnight to allow evaporation of excess humidity. On the following day, 50 neonate larvae per treatment were individually placed in each well of a bioassay tray. 100 larvae were used for the untreated control. After the bioassay trays were closed, they were kept at 22 °C with a 16/8 h light/dark photoperiod. Mortality was scored 1, 7 and 14 days post infection (dpi). Larvae that died within the first 24 h of the assay were assumed to have died from handling and were therefore not included in the analyses. Mortality data were corrected according to Abbott (1925). Median and 10% lethal concentrations (LC_{50}, and LC_{10}, respectively) were calculated for day 7 and day 14 for each virus by probit analysis using ToxRat software (ToxRat Solutions, Alsdorf, Germany). Parallelism of probit lines of 7 and 14 dpi was tested with potency estimation as provided by ToxRat software (ToxRat Solutions, Alsdorf, Germany).

Setup of mixed infection experiments

Neonate *A. segetum* larvae were individually exposed to AgseGV and AgseNPV-B in different concentrations and combinations. The concentration of AgseNPV-B was provided in a low (NPV_L = 0.8 x 10^3 OB/ml) and a high (NPV_H = 37 x 10^3 OB/ml) concentration, whereas the concentration of AgseGV was kept constantly (GV = 900 x 10^3 OB/ml). The treatments were set up as follows: (i) GV, (ii) NPV_L, (iii) NPV_H, (iv) GV+NPV_L, (v) GV+ NPV_H, and

(vi) untreated control. Preparation of bioassay trays and diet containing the different combinations of virus concentrations was done as described above. Twenty-five neonate *A. segetum* larvae were added to each virus and mixed virus treatment, whereas the control contained 50 neonate larvae. Larvae mortality was scored and analyzed separately for the first (days 2-7) and second (days 8-14) week. Cadavers that had not completely liquefied from viral infection, were collected individually and stored at -20 °C for further analysis. Each infection experiment was six times repeated independently. The larval mortality of each infection experiment was corrected according to Abbott (1925). Test for significant variation in mean mortality of treatments was performed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test for pairwise comparisons between treatments in RStudio (version 0.97.551) statistical software.

Viral DNA preparation

Frozen larval cadavers of mixed infection experiments were prepared for OB purification by thawing and centrifugation at 13,000 × *g* for 30 sec. By this the integrity of infected larval bodies was broken up. The supernatant was discarded and the pellet containing the OBs was subjected to DNA isolation using Ron's tissue DNA Mini Kit (Bioron, Ludwigshafen, Germany). The viral DNA was extracted separately from each individual larval sample. In short, disintegrated larvae were resuspended in 250 µl tissue lysis buffer (provided by the Ron´s tissue kit) and treated with proteinase K (250 µg/ml) (Thermo Fisher Scientific, Waltham, MA, USA). Samples were incubated overnight at 52 °C. Subsequently, 30 µl of 1 M Na_2CO_3 were added to the sample to dissolve OBs under alkaline conditions for 30 min. Samples were neutralized by adding the same volume of 1 M HCl. Larval debris was pelleted at 12.000 × *g* for 1 min and the brownish, cloudy supernatant was transferred to a fresh centrifugation tube. 3 µg of Hering sperm DNA were supplied to the samples in order to saturate the kit's silica membrane in the later course of the DNA isolation protocol. DNA solutions were processed according the kit's protocol and eventually eluted from the membrane by adding 400 µl of 10 mM TE buffer. For reasons of standardization and reproducibility, all steps of viral DNA extraction and isolation were adjusted to previously described protocols for qPCR standard sample generation (Wennmann and Jehle, 2014).

Quantitation of larval OB production

Quantitation of AgseNPV-B and AgseGV OBs of single larvae was performed by qPCR (Wennmann and Jehle, 2014). For the preparation of quantitation standards for qPCR, serial ten-fold dilutions of OBs of AgseNPV-B (in the range of 10^9 to 10^4 OB/ml) and AgseGV (10^{11} to 10^6 OB/ml) were prepared. Each standard was set up in triplicates. DNA was isolated from all dilution steps by using Ron´s tissue DNA Mini kit following standardized protocols (Wennmann and Jehle, 2014). According to the protocol, the lower limit of determination (LOD) for AgseGV and AgseNPV-B quantitation was 10^5 OBs of AgseGV and 10^3 OBs of AgseNPV-B. For a single qPCR, 2 µl of standard or sample DNA were mixed with 1 µl 0.2 pM of each forward and reverse primer (prAsBpolh-f/prAsBpolh-r and prAsGVgran-f/prAsGVgran-f, respectively) 12.5 µl 1 x Maxima® SYBR Green/Rox qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 8.5 µl ddH_2O to a total reaction volume of 25 µl (Wennmann and Jehle, 2014). The non-target controls contained 2 µl ddH_2O instead of DNA sample. Each larval DNA sample was tested twice for AgseNPV-B and AgseGV in separate qPCR runs along with their corresponding standard samples. Each run included non-target controls. QPCR parameters and melting curve settings were adjusted as previously described (Wennmann and Jehle, 2014). Data were processed by using Bio-Rad CFX Manager 2.0 (Bio-Rad, Hercules, CA, USA). Cq values were obtained by single threshold analysis. Statistical differences in OB production between treatments in single and co-infected larvae were revealed by Kruskal-Wallis test. Comparisons between groups were shown by pairwise Wilcoxon rank sum test and p value adjustment according to Holm-Bonferroni method (Holm, 1979). Statistical analyses were performed by using RStudio (version 0.97.551) software.

Results

Biological activity

Bioassay analyses were performed to compare the virulence of AgseGV and AgseNPV-B to neonate *A. segetum* larvae. At 7 dpi the LC_{10} and LC_{50} of AgseGV could not be calculated due to low mortality rates (Table 4.1). After 14 days, the LC_{10} of AgseGV was 2.60×10^3 OB/ml and the LC_{50} was 8.33×10^5 OB/ml. The 14-day LC_{50} of AgseGV was 246 times higher than the LC_{50} of AgseNPV-B with 3.38×10^3 OB/ml. The higher virulence of AgseNPV-B was also reflected by the LC_{10} (7.7×10^2 OB/ml) and LC_{50} (3.70×10^4 OB/ml) at 7 dpi and LC_{10} (3.4×10^2 OB/ml) at 14 dpi (Table 4.1).

Mortality of mixed infections

Single and mixed infections of *A. segetum* larvae were performed with a single concentration of 9.0×10^5 OB/ml for AgseGV, which equaled a LC_{50} at 14 dpi, and two concentrations of AgseNPV-B, $NPV_L = 8.0 \times 10^2$ OB/ml (LC_{10} at 7 dpi) and $NPV_H = 3{,}7 \times 10^4$ OB/ml (LC_{50} at 7 dpi) (Table 4.1). Differences between mortality results of mixed virus experiments were analyzed separately for 7 and 14 dpi by one-way ANOVA analysis. Differences between treatments were scored by post hoc Tukey's test (level of significance $p < 0.05$). At 7 dpi, significant differences between treatments were observed (One-way ANOVA, $F(4,25) = 11.13$, $p < 0.001$). The lowest mortality was observed for the GV treatment (6.17% ± 4.97%) and NPV_L (3.11% ± 3.97%) treatment. Within the first week (days 2-7) only a few larvae died and no significant difference between GV and NPV_L treatment was observed ($p = 0.934$) (Fig. 4.1A). The highest mean mortality of single virus treatments was found for NPV_H (22.7% ± 9.07%). Here, the mortality was statistically different from GV ($p = 0.002$) and NPV_L treatment ($p < 0.001$).

In all single virus treatments the mortality increased from the first to the second week (Fig. 4.1B), where significant differences were detected (One-way ANOVA, $F(4,25) = 14.21$, $p < 0.001$). At 14 dpi NPV_L treatment exhibited the lowest increase in mortality (5.87% ± 5.37%) and was statistically different from GV (36.8% ± 11.3%) ($p = 0.029$) and NPV_H (60.1% ± 20.6%) ($p < 0.001$) (Fig. 4.1B).

Table 4.1. Lethal concentration (LC_{50}) for AgseNPV-B, AgseGV and AgipNPV determined for neonate *A. segetum* larvae. Mortality rates were scored at 7 and 14 days post infection (dpi) (n = total number of individuals per bioassay; CL = confiducial limits calculated by normal approximation; df = degrees of freedom).

Virus	n	7 dpi					14 dpi				
		LC_{10} (95 % CL) [$x10^3$ OBs/ml]	LC_{50} (95% CL) [$x10^3$ OBs/ml]	Slope[a]	χ^2	df	LC_{10} (95 % CL) [$x10^3$ OBs/ml]	LC_{50} (95% CL) [$x10^3$ OBs/ml]	Slope[a]	χ^2	df
AgseGV	3,054	n.d.[b]	n.d.[b]	0.30 a	111.7	5	2.63 (0.05 – 128.0)	832.7 (80.9 – 8571.0)	0.51 a	212.5	5
AgseNPV-B	1,256	0.77 (0.20 – 4,212)	37.0 (19.4 – 70.6)	0.76 b	18.39	5	0.34 (0.22 – 0.52)	3.28 (2.66 – 4.04)	1.30 b	9.56	5

[a] Same letters indicate parallelism of probit lines at either day 7 or 14. The criteria of parallelism was rejected when $p < 0.05$.
[b] n.d. = not defined due to low mortality rates of AgseGV within the first week

The mortality of the GV+NPV_L treatment was 9.50% ± 8.97% and 50.1% ± 22.6% after the first and second week, respectively, but did not differ statistically from the mortality found in the GV treatment of the first ($p = 0.913$) and second week ($p = 0.648$). The GV+NPV_H treatment caused a mortality of 22.6% ± 5.25% and 73.7% ± 17.8% after the first and second week, respectively, which were similar to the results of the NPV_H treatments ($p = 0.999$ and p 0.632) (Fig. 4.1A and B). Thus, both mixed virus treatments equaled either the mortality caused by AgseGV (GV) or AgseNPV-B (NPV_H/NPV_L) treatment that induced the higher mortality, when provided to larvae alone.

Based on the observed mortality of mixed virus treatments the hypothesis was tested that AgseGV and AgseNPV-B exhibit independent response in *A. segetum* larvae. For testing, the survival rates (1 - mortality rate) of the GV and NPV_L, as well as the GV and NPV_H treatments of each replicate were multiplied. The products were retransformed to mortality rates (expected mortality = 1 - survival rate) and compared with their corresponding mixed virus treatments (= observed mortality) by Student's t-test. This was performed separately for the mortality observed in the first (7 dpi) (Fig. 4.2A) and second week (14 dpi) (Fig. 4.2B). At 7 dpi, observed and expected values did not significantly differ for GV+NPV_L ($t = 0.084$, $df = 10$, $p = 0.0935$) and GV+NPVH ($t = 1.115$, $df = 10$, $p = 0.291$) treatments (Fig. 4.2A). At 14 dpi, no significant differences were found for GV+NPV_L ($t = 0.941$, $df = 10$, $p = 0.369$) and GV+NPV_H ($t = 0.244$, $df = 10$, $p = 0.812$) (Fig. 4.2B). This indicated that AgseGV and AgseNPV-B acted independently within *A. segetum* larvae.

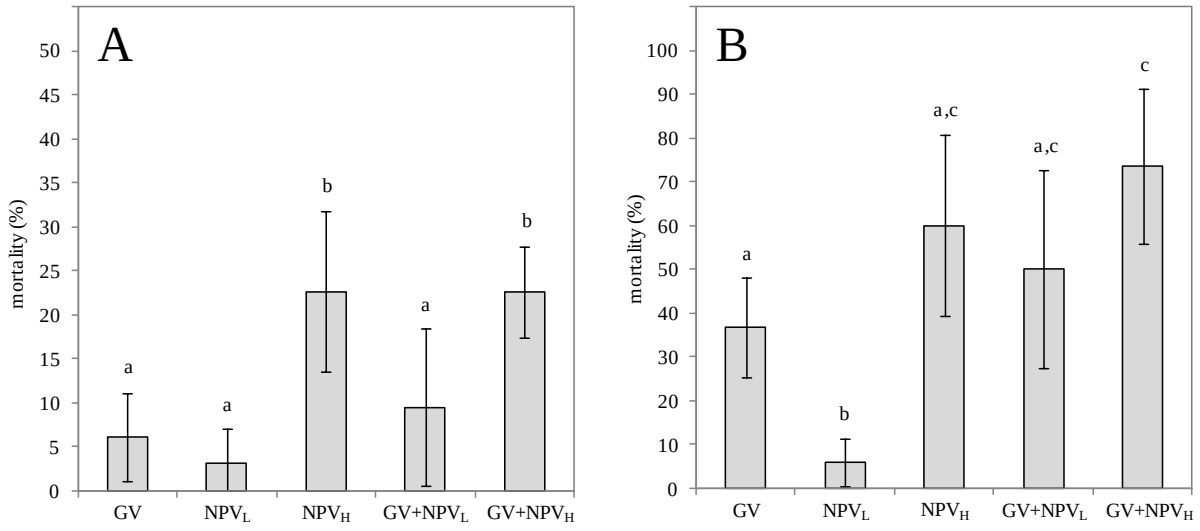

Figure 4.1. Mean mortality rates of the (**A**) first (day 2-7) and (**B**) second (day 8-14) week of single and mixed virus treatments. Different letters indicate significant differences between treatments (One-way analysis of variance (ANOVA) followed by Tukey's HSD test for pairwise comparisons between treatments, significance at $p < 0.05$). Vertical lines represent standard deviations.

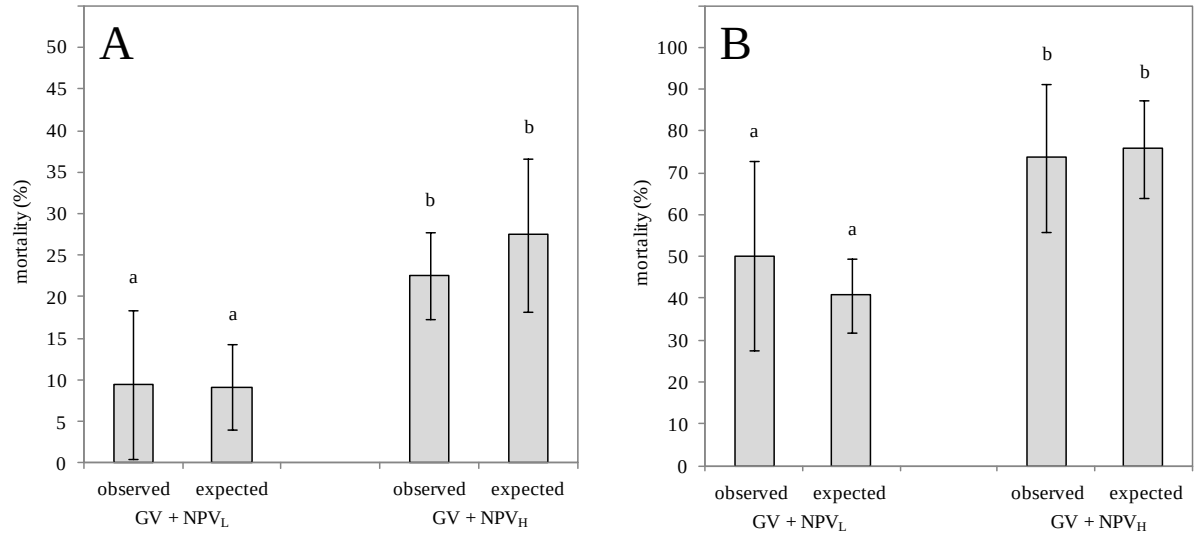

Figure 4.2. Comparison of the observed and expected mortality rates of GV+NPV$_L$ and GV+NPV$_H$ treatments observed in the (**A**) first and (**B**) second week post infection. Expected mortality values were calculated from survival rates of replicates of GV, NPV$_L$ and NPV$_H$ treatments assuming independent action (for details see Results). Statistical analyses were conducted separately for the observed and expected mortality of each treatment. Different letters indicate significant differences (Student's t-test, significance at $p < 0.05$). Vertical lines represent standard deviations.

Ratio of GV and NPV infected larvae

Though no interaction between AgseGV and AgseNPV-B was observed in terms of mortality, possible interaction on basis of virus offspring production (= OB production) was further tested. Larvae exposed to AgseGV only were found to produce only AgseGV proving the purity of the AgseGV stock (Table 4.2).

In about 50% of the larvae exposed to NPV_L only and infection by AgseNPV-B could be determined by qPCR (Table 4.2); in the remaining 50% of larvae no virus could be detected at all in the first week. A single larval cadaver contained both AgseNPV-B and AgseGV when collected from the NPV_L treatment between 8-14 days (second week) (Table 4.2). The latter finding was unexpected as only AgseNPV-B was used as an inoculum. In NPV_H only treatments the occurrence of co-infections with both AgseNPV-B and AgseGV was also confirmed. Four (14.3%) larval cadavers collected in the first week (day 2-7) and two (20%) larval cadavers obtained in the second week (day 8-14) contained both AgseNPV-B and AgseGV (Table 4.2). The remaining larvae that were collected from NPV_H and died by viral infection contained AgseNPV-B only (Table 4.2). Larvae with only AgseGV were not observed in NPV_L and NPV_H treatments (Table 4.2).

Table 4.2. Ratios of single and co-infected larvae of single and mixed virus treatments after first (day 2-7) and second week (day 8-14).

treatment	period of death (days)	number of larvae analyzed by qPCR	ratio of infection			
			GV (%)	NPV (%)	both (%)	no virus (%)
GV	2-7	9	9 (100.0)			
	8-14	10	10 (100.0)			
NPV_L	2-7	4		2 (50.0)		2 (50.0)
	8-14	3		2 (66.7)	1 (33.3)	
NPV_H	2-7	28		22 (78.6)	4 (14.3)	2 (7.14)
	8-14	10		8 (80.0)	2 (20.0)	
$GV+NPV_L$	2-7	16	12 (75.0)		3 (18.8)	1 (6.25)
	8-14	28	24 (85.7)		4 (14.3)	
$GV+NPV_H$	2-7	34	2 (5.88)	12 (35.3)	18 (52.9)	2 (5.88)
	8-14	21		5 (23.8)	16 (76.2)	

Co-infected larvae were found in both GV+NPV_L and GV+NPV_H mixed virus treatments. The ratio of co-infected larvae in GV+NPV_L reached 18.8% and 14.3% within the first and second week, respectively (Table 4.2). By this, the ratio of co-infections was similar to those of NPV_H treatment. In the GV+NPV_H treatment the ratio of co-infected larvae reached 52.9% for larvae that died within the first week. Here, the main portion of remaining larvae contained solely AgseNPV-B (35.3%), whereas in 5.9% of the succumbed larvae only AgseNPV-B was found (Table 4.2). Within the second week of the GV+NPV_H treatment a mixed infection had occurred in 76.2% of dead larvae, whereas the remaining 23.8% were infected by AgseNPV-B only (Table 4.2). According to the qPCR based ratios, all larvae of the GV and GV+NPV_L treatments that died by viral infection were infected by AgseGV, in single as well as co-infections. The contrary was observed for GV+NPV_H treatment, where majority of 88.2% larvae were found to contain AgseNPV-B (Table 4.2).

Larval occlusion body production

The aim of this experiment was to compare the capacity of virus OB formation in single and co-infections. To quantify OBs by qPCR each larva was tested twice with primers specific for AgseGV and AgseNPV-B, respectively. To assure the reliability of the experiment a minimum of five larvae (Table 4.2) was required to quantify median OB production in single and co-infected larvae per treatment. Therefore, quantitative analyses on single infected larvae were performed for the GV and GV+NPV_L treatments for AgseGV as well as the NPV_H and GV+NPV_H treatments for AgseNPV-B. OB production in co-infections was analyzed from larvae of the GV+NPV_H treatment.

The larval OB production in single and co-infections per treatment was separately analyzed for AgseGV and AgseNPV-B. Since the logarithmically transformed amounts of larval OB production in single and co-infections were not normally distributed (data not shown), non-parametric Kruskal-Wallis analysis was conducted. Significant differences in single and co-infected larvae were detected for AgseGV ($H(5) = 35.80$, $p < 0.001$) and AgseNPV-B ($H(5) = 31.35$, $p < 0.001$).

In AgseNPV-B single infections, the OB productions differed between the first (day 2-7) and second (day 8-14) week (Fig. 4.3A). Larvae of the NPV_H treatment contained significantly fewer OBs when found dead within the first week (2.3 x 10^6 OB/larva) than within the second week (5.44 x 10^7 OB/larva) ($W = 3$, $p < 0.001$) (Fig. 4.3A). A further significant increase was observed for single AgseNPV-B infected larvae of the GV+NPV_H treatment, where 5.5 x 10^6 OB/larva (day 2-7) and 89.7 x 10^6 OB/larva (day 8-14) were produced ($W = 4$, $p = 0.004$) (Fig. 4.3A). The median OB production rates of the NPV_H and GV+NPV_H treatment did not differ significantly in the first week ($W = 94$, $p = 0.896$). At a higher level, the same observation was made for OB production in the second week ($W = 17$, $p = 1.000$). An increase in median AgseNPV-B OB formation from 1.6 x 10^6 (first week) to 1.09 x 10^7 OB/larva (second week) was also measured for co-infected larvae of the GV+NPV_H treatment (Fig. 4.3A), however, this difference was not significant ($W = 79$, $p = 0.148$).

Larvae of the GV treatment produced 2.89 x 10^9 OB/larva (first week) and 1.47 x 10^9 OB/larva (second week) (Fig. 4.3B), which did not differ significantly ($W = 46$, $p = 1.000$). The same observation was made for single infected larvae of the GV+NPV_L treatment, when 4.66 x 10^8 OB/larva (first week) and 1.64 x 10^9 OB/larva (second wee) were measured ($W = 85$, $p = 0.438$) (Fig. 4.3B). A constant production of AgseGV OBs was further detected in co-infected larvae of the GV+NPV_H treatment, but at a lower level. Here, larvae that were sampled from the first and second week produced 2.36 x 10^8 OB/larva and 1.61 x 10^8 OB/larva, respectively, which were not statistically different ($W = 150$, $p = 1.000$) (Fig. 4.3B). In pairwise comparison, the produced amounts of AgseGV OBs in co-infected larvae were significantly lower than in single infections of the GV+NPV_L (second week) and GV treatment (Fig. 4.3B) ($p < 0.05$).

Ratio of infection on the individual level

As AgseNPV-B infection appeared to cause an adverse effect on the AgseGV production in co-infected larvae (Fig. 4.3B), it was tested whether this effect was visible on an individual larval level. For this reason, the individual production of AgseNPV-B and AgseGV OBs was correlated in co-infections (Fig. 4.4A and B). When applying Spearman's rank correlation coefficient (r_s), a negative but not significant correlation was observed in OB production for

larvae that died within the first ($r_s(18) = -0.329$; $p = 0.182$) and second week ($r_s(16) = -0.244$; $p = 0.361$).

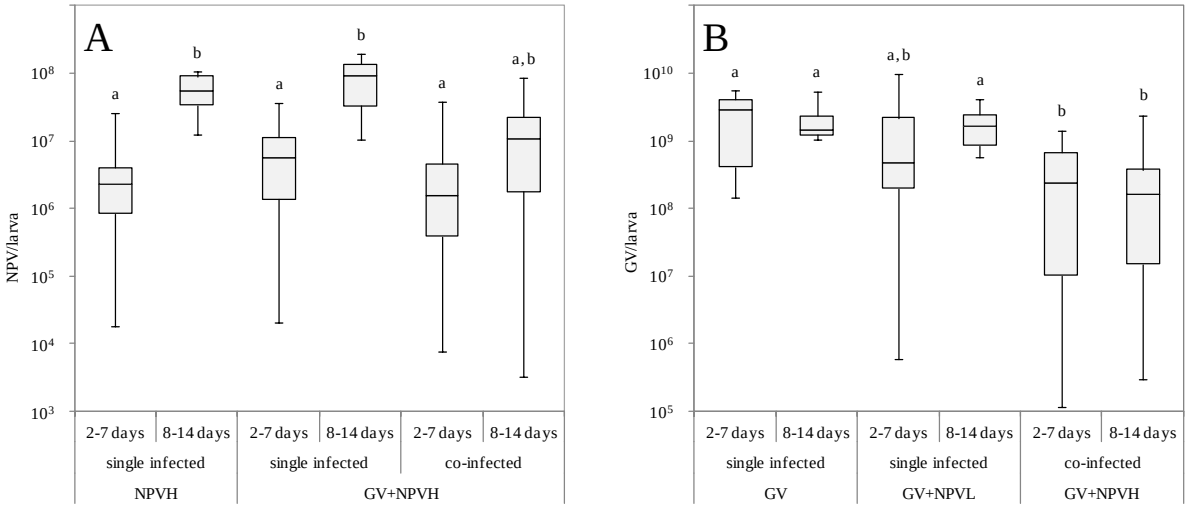

Figure 4.3. Median OB production of (**A**) AgseNPV-B and (**B**) AgseGV within single and co-infected larvae, which succumbed during the first (2-7 days) and second (8-14 days) week post infection. Minimum of y-axes represent the lower limits of detection (LOD) for AgseGV (10^5 GV/larva) and AgseNPV-B (10^3 GV/larva). Different letters indicate significant differences (Wilcox rank-sum test, significance at $p < 0.05$) between treatments.

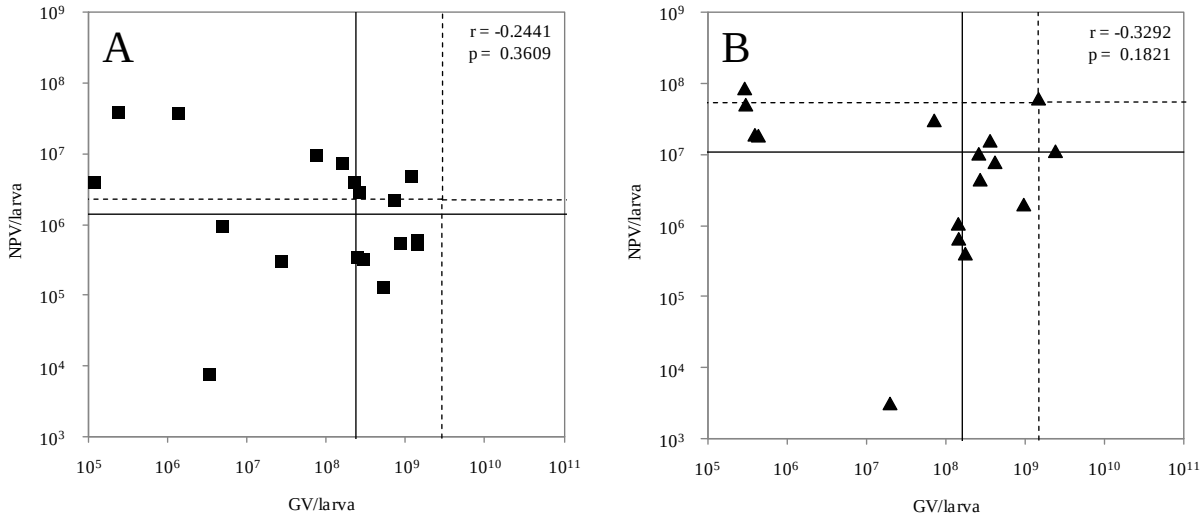

Figure 4.4. Correlation analysis (Spearman's rank correlation coefficient = r_s) of larval AgseGV and AgseNPV-B OB production within co-infected *A. segetum* larvae. Only larvae of the GV+NPV_H treatment that died within the (**A**) first and (**B**) second week were considered. Vertical lines = median AgseGV OB production; horizontal lines = median AgseNPV-B production; solid lines = mixed GV+NPV_H treatment; dashed lines = single GV and NPV_H treatment are drawn as reference.

Discussion

Personal observations in our laboratory and literature reports on simultaneous infections of *A. segetum* larvae by AgseGV and AgseNPV (Gürlich, 1993; Shvetsova and Ts'ai, 1962) provided little information about the mechanism of interaction. In the present study, not only mortality data were evaluated from single and mixed virus treatments but also qPCR analyses were performed to identify and to quantify the production of AgseGV and AgseNPV-B OB progeny in infected larvae on an individual level.

The mortality rates of AgseNPV-B bioassay experiments were consistent with previously published bioassay studies that were obtained in 10-days bioassays (El-Salamouny et al., 2003). With a LC_{50} (10 dpi) = 10 x 10^3 OB/ml the result lay between the newly determined LC_{50} (7 and 14 dpi). The AgseGV isolate used in the present study showed the same slow activity than previously described (Bourner et al., 1992; Allaway and Payne, 1984).

The present study focused primarily on co-infections with defined concentrations of AgseGV and AgseNPV-B OBs. The observed mortality data indicate that the virus, which led to the highest mortality in its corresponding single virus treatment, also dominated the overall mortality in an AgseGV and AgseNPV-B co-infection. Neither a significant increase nor a decrease in mortality was observed in mixed virus treatments that could give hints for a co-operative or inhibiting interaction of both viruses. The assumption of an independent virus interaction was underlined by the comparison of the observed and expected mortality of mixed virus treatments. The qPCR based identification and quantitation of produced viruses of single and co-infected larvae revealed that larvae mainly died from the virus, which was applied in the higher lethal concentration (LC). When the 14-day LC_{50} of AgseGV was mixed with the high concentration of AgseNPV-B (7-day LC_{50}), as done in the GV+NPV_H treatment, the ratio of co-infected larvae was at maximum. Furthermore, it could be concluded that with an increasing concentration of AgseNPV-B (GV > GV+NPV_L > GV+NPV_H) the ratio of co-infections increased. The same effect was visible for an increasing initial concentration of AgseGV (NPV_H > GV+NPV_H).

The amounts of produced AgseGV and AgseNPV-B OBs per larva were regarded as realistic of what an *A. segetum* larva could be capable to produce. With a range from 4.66 x 10^8 to 2.89 x 10^9 AgseGV OBs per single infected larva the median amounts were within the

expected range. In a study on different *Cydia pomonella* GV (CpGV) mutants replicating in *Cydia pomonella*, the virus offspring production was calculated to vary between 2.0 - 3.6 x 10^{10} CpGV/larva (Arends et al., 2005). A comparison with the present results is difficult because fifth instar *C. pomonella* larvae, thus larger caterpillars were infected with CpGV (Eberle et al., 2008). For AgseNPV-B, the median OB offspring production fitted with data from Tanada and Hukuhara (1971) for *Pseudaletia unipuncta* nucleopolyhedrovirus (PsunNPV). There, fifth instar larvae of the armyworm, *P. unipuncta*, which were infected with PsunNPV produced about 10^4 - 10^7 OB/larva and was in a similar range (2.1 x 10^6 - 8.97 x 10^7 OB/larva) as observed in our study for *A. segetum* larvae infected with AgseNPV-B. Our results on the production of AgseGV and AgseNPV-B OB progeny suggest the potential capacity OB production, which neonate *A. segetum* larvae are able to produce during the infection period. If later larval stages were able to produce higher amounts of OBs when exposed to other virus concentrations, cannot be concluded from the analysis. The co-infection experiments clearly indicate that both viruses should be propagated separately and contaminations should be avoided, since mixtures led to a decrease in yields of OBs.

In our case, a prolonged larval life induced by viral infection could not be confirmed as described for *Helicoverpa armigera* granulovirus (HearGV) infected *Heliothis armigera* larvae (Whitlock, 1974). Due to the extended survival time larvae were found to double their size and mass by a longer feeding time providing the virus more host resources for replication. For AgseGV and AgseNPV-B, this assumption could be excluded because of similar mortality in single and mixed virus treatments. The baculovirus encoded *ecdysteroid glucosyltransferase* (egt) gene that was shown to play a major role in extending larval life (O'Reilly et al., 1998) is encoded by AgseGV (Zhang et al., 2014), AgseNPV-B (Chapter 2), AgseNPV-A (Jakubowska et al., 2006) and AgipNPV (Harrison, 2009). If deleted in naturally occurring Δ*egt* genotypes of AgipNPV infected *A. ipsilon* larvae died significantly faster (Harrison, 2013).

Since infected larvae were not able to produce and hold infinite amounts of AgseGV and AgseNPV-B, the maximum number of OBs is limited (Arnott and Smith, 1968). In co-infected larvae, this limit could either be shifted in favor for AgseGV or AgseNPV-B, but both viruses could also replicate equally. AgseNPV-B exhibited a steady and significant increase in median OB formation in single infected larvae between the first and second week,

whereas AgseGV appeared constant over time. In co-infections, however, AgseNPV-B production appeared to be less influenced by the presence of AgseGV than vice versa. This was clearly observable by a significantly lower median OB production of AgseGV in co-infections than in single infections. An increasing variation of larval production of AgseGV and AgseNPV-B OB offspring substantiated the assumption of a mutual interference in co-infections. When the larval AgseGV and AgseNPV-B OB production was correlated with each other, no significant correlation on the individual level was found. But it can be concluded that co-infected larvae contained less AgseGV and AgseNPV-B than larvae that became infected by one of these viruses alone. A possible explanation for a missing correlation but a negative mutual interference could lay in the nature of the less pathogenic AgseGV and the more virulent AgseNPV-B to *A. segetum* larvae (Bourner et al., 1992), and in independent infection processes that start in different cells for both viruses. In this case, AgseNPV-B could replicate regularly within co-infected larvae within the first week, but interferes with the less virulent AgseGV in the later state of larval co-infection.

Cells, which became infected by a baculovirus, lose their susceptibility for a secondary infection, as it was demonstrated for two genotype variants of AcMNPV, as well as AcMNPV and SfMNPV (Beperet et al., 2014). So called superinfections of a single cell were shown to be temporarily possible shortly after the first viral infection (Beperet et al., 2014). If AgseGV and AgseNPV-B were able to superinfect *A. segetum* cells is unknown, but a reciprocal exclusion can be an explanation for reduced progeny generation.

In conclusion, an active interaction of both viruses within co-infected larvae was not observed. In their entirety, the results suggest a certain competition of AgseGV and AgseNPV-B for larval resources.

CHAPTER 5: GENERAL DISCUSSION

The registration of a baculovirus as a biological control agent requires an elaborate characterization of the active agent, which is represented by the baculovirus strain. Strains usually derive from field samples of different geographic locations (=isolates) and can represent populations of different genotype composition. Isolates and genotypes can exhibit different virulence to host species, which can be of economic importance, as it was shown for CpGV (Eberle et al., 2009, 2008), a commercially applied baculovirus for the biological control of codling moth, *C. pomonella* (Crook et al., 1997; Huber, 1995). After resistance to the commercially used Mexican isolate CpGV-M was observed in Europe (Asser-Kaiser et al., 2007; Fritsch et al., 2005), CpGV isolates overcoming the resistance to CpGV-M were identified and eventually commercialized to control resistant codling moth populations (Gebhardt et al., 2014; Eberle et al., 2009, 2008; Zingg, 2008).

Different geographic isolates were also found for AgseGV (Europe and Asia), AgseNPV-B (Europe), and AgipNPV (North America) (Tab. 1.2), but few efforts were made to investigate their genotype composition so far, although, genotype variation is likely to occur. For AgseGV, two isolates from China were fully sequenced showing 99% nucleotide sequence identity (Zhang et al., 2014). The newly sequenced genome of AgseNPV-B showed no genotype variation during the assembly process indicating a predominance of a certain genotype. In contrast, genetic variation was initially detected in the field isolate of AgipNPV (Illinois strain) within the *egt* gene (Harrison, 2009), which later led to the characterization of naturally occurring *egt* mutant genotypes with reduced pathogenicity, but an increased speed of kill (Harrison, 2013). Such features are of special interest in terms of pest control by baculoviruses, since manufacturers and users are interested in fast acting agents.

When speaking about geographic isolates of *Agrotis* baculoviruses, one has to focus on the distribution of pests and virus isolates, as well. To date, European and Asian isolates of AgipNPV were not identified, although *A. ipsilon* is worldwide distributed. AgseNPV-B and AgseGV isolates from North America are also absent. This is remarkable because both viruses are able to infect both host species, although AgseNPV-B was found to be about 40 times less effective against *A. ipsilon* than to *A. segetum* (El-Salamouny et al., 2003). On the

contrary, AgipNPV exhibited the same virulence to both host larvae (El-Salamouny et al., 2003). Whether the different geographic distribution of these two viruses is linked to host adaption or to missing *Agrotis* baculovirus sampling is unknown. It could be hypothesized that from *A. segetum* characterized viruses, AgseNPV-A, AgseNPV-B, and AgseGV, and from *A. ipsilon* isolated AgipNPV occupy the same ecological niche, but adapted geographically separate to *Agrotis* hosts.

AgseNPV-B was determined by whole genome sequencing as a new baculovirus species, albeit closely related to AgipNPV, but distinct enough to be classified as the third species in the *Agrotis* baculovirus complex (Chapter 2). AgexNPV, the putative forth *Agrotis* NPV, was shown to be a variant of AgseNPV-B and, henceforward, should be designated as such. Besides the phylogenetic classification of *Agrotis* baculoviruses the genome alignment revealed a high co-linearity of AgseNPV-A, AgseNPV-B, AgipNPV showing common and distinguishing features that may play role in virulence and host range determination. A striking difference was observed in the number of *vef* homologs, which were found to be present in all *Agrotis* baculoviruses and are assumed to play a role in virus infectivity and presumably their host range. As the most divergent genomic region, the *cathepsin*/*chitinase* cluster was determined in which close proximity also the *egt* gene is located. This region was found to underlie naturally occurring deletion events of different length for AgipNPV and *Spodoptera* NPV making it a suitable location for the differentiation of genotypes and geographic variants and within the *Agrotis* spp. NPV.

As *A. segetum* and *A. ipsilon* are susceptible to many different baculoviruses in single and potential co-infections, the unambiguous identification of the infective agent is essential. For this reason, a PCR based method was established (Chapter 3) to allow the simultaneous identification of *Agrotis* baculoviruses by multiplex PCR. The difficulty in working with *Agrotis* baculoviruses that can co-infect host larvae was demonstrated for AgseGV and AgseNPV-B in OB purification experiments, where the ratio of both viruses was affected significantly by the applied purification protocols. For producers, especially in quality control management, the results of Chapter 3 provide a key technique for working with *Agrotis* baculoviruses that will also facilitate future infection studies on baculoviruses of the *Agrotis* complex.

Reports on naturally occurring co-infections of *A. segetum* larvae by AgseNPV-B and AgseGV raised the question, whether these infections were the consequence of two overlapping baculovirus populations or the result of advantageous interaction, which became manifested within the host population. According to the results provided in Chapter 4, both viruses appear to share or compete for the larval host, but do not inhibit or support each other in replication. An example for inhibition of replication was demonstrated for CpGV-M, the commercially applied CpGV, and CpGV mutants, which harbored transposable elements (Arends et al., 2005). Codling moth larvae were inoculated at the same time with a constant dose of CpGV-M and one of the CpGV mutants in various doses. Even at a ratio of 90% in inoculum of the mutant, more than 60% of the generated progeny belonged to the CpGV-M wildtype. A study on a NPV suppressing GV was performed for *Helicoverpa zea* NPV (HzSNPV) and HearGV, where co-infected *Helicoverpa zea* larvae contained only HearGV, even when HearGV was provided to larvae 36 h after HzSNPV infection (Hackett et al., 2000). AgseGV and AgseNPV-B appeared to follow two different strategies of infection, which makes their common appearance even more interesting. According to general observations, AgseNPV-B exhibits a typical NPV pathogenesis, which starts in the midgut and spreads to major larval tissues, like fat body, tracheal matrix, and epidermis, followed by liquefaction of the larval body (Federici, 1997). The conducted studies of this thesis showed, that AgseNPV-B is moderate in its virulence, whereas AgseGV shows a slow pathology (Chapter 4). The slow speed of kill is characteristic for many, but not all, betabaculoviruses infecting larvae of the *Noctuidae*, e.g. AgseGV, HearGV, TnGV, and XecnGV, but also others, e.g. AdorGV (Harrison and Popham, 2008; Jehle et al., 2006b; Wormleaton and Winstanley, 2001; Federici, 1997). Phylogenetic analysis showed that the speed of kill in the genus *Betabaculovirus* is not a monophyletic trait but, more likely, developed independently (Jehle et al., 2006b). One reason for the slow killing behavior is their restricted infection to midgut epithelium and fat body cells (Federici, 1997). Infected larvae continue feeding and further develop until the last instar and usually do not liquefy from viral infection because epidermis cells are not affected. This is in contrast to NPV and fast killing GVs, like CpGV, which are able to infect all larval tissues, cause a fast endemic spread of the infection, resulting in a relatively quick death and complete disintegration of the larval body (Federici, 1997). If AgseGV and AgseNPV-B together infect the same larval tissues, like the fat body, or whether they infect separate tissues in co-infected larvae is not investigated so far. A

spatial proximity of AgseGV and AgseNPV-B infections within larval tissues would raise the question, whether multiple infections of cells occur.

The infection of a cell by a second virus is called superinfection and is often prevented by the first virus by excluding the second virus to establish an infection (Folimonova, 2012). A mechanism of active superinfection exclusion is studied for the bacteriophage lambda (λ), which represses the incorporation of other λ phages into the host genome by producing a repressor protein (Fogg et al., 2010). This phenomenon is called superinfection immunity (Carter and Saunders, 2007) and ensures that only a single phage becomes replicates by the host. By this the share of cellular resources is prohibited and the propagation efficacy maximized. On the other hand, the possibility of horizontal transmission of genetic information by recombination between viruses is possibly reduced.

Superinfections of invertebrate cells by two alphabaculoviruses were observed for AcMNPV and LdMNPV in cells of *L. dispar* leading to simultaneous viral DNA replication, but also to viral interference and reduced progeny generation (McClintock and Dougherty, 1987). Even heterologous superinfections of the Tipula iridescent virus (TIV) (*Iridoviridae*) and *Galleria mellonella* NPV (GmNPV), now designated as AcMNPV (Rohrmann, 2013), were characterized (Kurstak and Garzon, 1975). It was demonstrated by electron microscopy that TIV progeny was synthesized in the cytoplasm, whereas AcMNPV OB assembly took place in the nucleus (Kurstak and Garzon, 1975). In both combinations of superinfection, AcMNPV with LdMNPV or TIV, the progeny generation appeared to be disturbed by the presence of the second virus. This was determined by a reduction in AcMNPV/LdMNPV OB and BV production (McClintock and Dougherty, 1987) or a deformed AcMNPV OB production with no or fewer nucleocapsids (Kurstak and Garzon, 1975). From these results a competition for the cellular metabolism was concluded.

As demonstrated exemplarily for two AcMNPV genotypes, as well as AcMNPV and SfMNPV superinfections only a “temporal window of susceptibility” (Beperet et al., 2014) existed, which allows a secondary infection only during a certain period of time. This may prevent the excessive formation of superinfected cells. As a possible mechanism of superinfection exclusion the inactivation of host RNA polymerase II is discussed (Beperet et al., 2014). Host RNA polymerase II is required for the transcription of early genes, becomes replaced by the baculovirus RNA polymerase, which execute the transcription of late and very

late genes. Therefore, the absence of host RNA polymerase II is likely to prevent the initiation of transcription of early genes of a secondary infection.

If AgseGV and AgseNPV-B would be capable to superinfect *A. segetum* cells to replicate simultaneously within a nuclear-cytoplasmic milieu (AgseGV) and/or the nucleus (AgseNPV-B) at the same time, or get co-occluded in one OB, remains unknown and can only be hypothesized. Sequence comparisons of the recently sequenced AgseGV (Zhang et al., 2014) and AgseNPV-B (Chapter 2) did not reveal any evidence for recent recombination events. However, this observation does not exclude ultimately the event of recombination. Recombined genotypes could appear in co-infections and contribute genetic diversity to the cellular generated baculovirus progeny population, but at a very low level. Furthermore, they could be defective and thereby non-infectious, making them non-stable within the population. On the other hand recombination could rather appear in superinfections of more closely related *Agrotis* baculoviruses such as AgipNPV and AgseNPV-B, whose genome sequences are co-linear and exhibit a high degree of nucleotide sequence similarity. As a hotspot of recombination the *cathepsin*/*chitinase* cluster could come into consideration (Chapter 2).

With three NPVs and AgseGV, the *Agrotis* baculovirus complex provides a variety of possible combinations of these baculoviruses from which only one was investigated for advantageous interaction in respect to a potential usage as a combined biocontrol agent (Chapter 4). Although, other co-infections of *Agrotis* baculoviruses were not reported in literature, their ability to infect *A. segetum* and *A. ipsilon* larvae makes them likely to occur. Even multiple simultaneous infection could be possible and of interest in a scientific and application-oriented way. The interest of a combined baculovirus application lays not only on an increase in mortality factors by advantageous interactions, but also in preventing the occurrence of pest populations, which are resistant to a solely applied baculovirus in the field. A broader range in genetic variation, e.g. by genotypes, different virus isolates or species, may reduce the selection of resistant field population to a single baculovirus. However, unfavorable effects of combined *Agrotis* baculoviruses application like prolonged larval life or reduced mortality rates have to be excluded in advance.

In conclusion, *Agrotis* NPVs have a great potential as biological agents for the control of *A. segetum* and *A. ipsilon* in the field. The similar virulence of AgseNPV-B and AgipNPV to A. segetum and *A. ipsilon* predestine both viruses to be used as biological control agents of these pests. AgseGV may be useful for population control but its virulence is to low and its speed of kill may be too slow for being used to control damage caused by cutworms. AgseNPV-A has not been tested elaborately for its usage and future studies should focus on bioassay studies of this virus. Besides being used as biocontrol agents, the *Agrotis* baculovirus complex provides the unique model to investigate the interaction and micro-evolution of baculoviruses.

For the time being, the AgseNPV-B was characterized as a further *Agrotis* NPV species by whole genome sequencing providing comprehensive genome comparisons and allowing the development of a molecular method for identification and quantitation of *Agrotis* baculoviruses. With the new method a first quantitative insight into AgseGV and AgseNPV-B co-infected larvae was enabled. By this, this thesis prepares the ground for further studies deciphering interactions of baculoviruses in larval hosts and on the cellular and molecular level.

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SUMMARY

Baculoviruses (*Baculoviridae*) are double-stranded DNA viruses which infect the larval stages of insects belonging to the orders Lepidoptera, Hymenoptera and Diptera. Due to their narrow host range and high virulence to target insects, different baculoviruses have been used as biological control agents in pest control. Severe soil pests of many agricultural and horticultural crops, which are difficult to be controlled, are larvae of the genus *Agrotis* (Lepidoptera: Noctuidae), also called cutworms. Their habitat is the soil or soil surface where they feed on seedlings, stems, and other parts of plants. So far, cutworms are mainly control by chemical pesticides, but biological control agents would be highly desirable for environmental reasons. Two of the most important cutworm pests are the common cutworm, *Agrotis segetum* (Denis & Schiffermüller), and the black cutworm, *A. ipsilon* (Hufnagel). At least four distinct baculovirus species, three of the genus *Alphabaculovirus* and one of the genus *Betabaculovirus*, were isolated from these two cutworm species. The alphabaculoviruses are the *Agrotis segetum* nucleopolyhedrovirus A (AgseNPV-A), *Agrotis segetum* nucleopolyhedrovirus B (AgseNPV-B), and *Agrotis ipsilon* nucleopolyhedrovirus (AgipNPV). The *Agrotis segetum* granulovirus (AgseGV) represents the betabaculovirus. Together with their two host species, from which they were first isolated and characterized, they form the so-called *Agrotis* baculovirus complex. *Agrotis* baculoviruses have the potential to be used as biological control agents for the control of *Agrotis* cutworms. However, in-depth knowledge and a full characterization of their biology, molecular setup, and virulence parameters are required for a successful registration and application in the field.

In this study the genome sequence of AgseNPV-B was fully sequenced. Its genome is 148,981 bp in length and codes for 150 putative open reading frames. Whole genome comparisons with AgseNPV-A and AgipNPV, whose genome sequences have been published previously, suggested that AgseNPV-B belongs to a new species of the *Agrotis* baculovirus complex. Phylogenetic analysis indicated a very close relationship to AgipNPV and it could be concluded that both viruses are two distinct species at an early stage of separation. Whole genome alignments revealed a different number of *viral enhancing factor* (*vef*) gene copies in AgseNPV-A, AgseNPV-B and AgipNPV as one of the most striking distinguishing features

between their genomes. VEFs are known to affect the virulence of baculoviruses. A putative site of genomic recombination was found in the region of the *cathepsin* and *chitinase* genes where the high co-linearity of the genomes of all three *Agrotis* nucleopolyhedroviruses was interrupted by inversions, deletions, or insertions. A putative fifth *Agrotis* baculovirus, the *Agrotis exclamationis* nucleopolyhedrovirus (AgexNPV), was suggested to be an isolate of AgseNPV-B due to high nucleotide sequence similarities of partial genomic regions.

Based on the genome sequences of AgseNPV-A, AgseNPV-B, AgipNPV and AgseGV a multiplex PCR based method for the identification of these *Agrotis* baculoviruses was established. Highly specific oligonucleotide primers specific for the *polyhedrin* (*polh*) or *granulin* (*gran*) genes of the four viruses were developed and resulted in discriminating PCR fragments. Furthermore, this method allowed the quantitation of AgseGV and AgseNPV-B by quantitative PCR (qPCR).

Since co-infections of AgseGV and AgseNPV-B have been observed and a combination of both viruses was considered as a combined biological control agent, the potential interaction of both viruses in mixed infections was investigated. Potential interactions between AgseNPV-B and AgseGV were examined in activity studies using single virus as well as combined virus infections of neonate *A. segetum* larvae. Mortality rates were determined and the virus progeny produced in individual larvae was quantified by using the newly established qPCR method of quantitation of AgseNPV-B and AgseGV. As a result, combinations of AgseNPV-B and AgseGV did not exhibit an advantageous effect in terms of pest control. Neither an increase in mortality rates in mixed virus treatments in comparison with single virus treatments, nor an increase in production of AgseNPV-B or AgseGV progeny in co-infected larvae was observed. On the contrary, a competitive behavior of both viruses in mixed infections could be concluded.

The present thesis contribute to the biological control of *Agrotis* cutworms by providing extensive insight into the molecular setup of these viruses and the characterization of AgseNPV-B as a new *Alphabaculovirus* species. The use and registration of baculoviruses as biocontrol agents rely on such virus characterizations as well as on virus activity studies that were performed for AgseNPV-B and AgseGV. The new technique in *Agrotis* baculovirus detection and quantitation will facilitate future single and mixed infection studies of AgseNPV-B and AgseGV, as well as other combinations of *Agrotis* baculoviruses.

DANKSAGUNG

CURRICULUM VITAE