Diversity and biological activity of nucleopolyhedroviruses of the leafworm *Spodoptera litura*

Ghulam Ali

Propositions

1. Molecular characterization is compulsory before proceeding to biological analysis of a (baculo)virus.
   (this thesis)

2. Geographically defined clusters of genotypic variants can be called regiotypes
   (this thesis).

3. The use of refugia to control resistance development against Bt-transgenic crops needs to be reconsidered.

4. Carbon dioxide should not be considered a noxious compound.

5. Being stuck on a glacial mountain is as terrifying as doing PhD experiments in a developing country.

6. Sustainable food systems can be best achieved by engaging the youth in agriculture.

Propositions belonging to the thesis, entitled

'*Diversity and biological activity of nucleopolyhedroviruses of the leafworm Spodoptera litura'*

Ghulam Ali

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Ghulam Ali

Thesis committee

Promotor
Prof. Dr J.M. Vlak
Personal Chair at the Laboratory of Virology
Wageningen University & Research

Co-promotor
Dr W. van der Werf
Associate Professor, Centre for Crop System Analysis
Wageningen University & Research

Other members
Prof. Dr W. Takken, Wageningen University & Research
Dr J.T. Wennmann, Julius Kühn Institute, Darmstadt, Germany
Prof. Dr J.A.G.M. de Visser, Wageningen University & Research
Dr F.J.J.A. Bianchi, Wageningen University & Research

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Diversity and biological activity of nucleopolyhedroviruses of the leafworm *Spodoptera litura*

Ghulam Ali

Thesis

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Ghulam Ali
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Abstract

Increased resistance of emerging cotton leafworm *Spodoptera litura* in Pakistan and elsewhere to chemical insecticides calls for an alternative method of control. Isolates of nucleopolyhedrovirus (NPVs) of *S. litura* (SpltNPV) were collected from infected larvae in different ecological and geographic regions of Pakistan. The genotypic diversity of these SpltNPV isolates, their relation to geographical (spatial) distribution and cropping system, and their biological activity, in particular dose response, speed of kill and effects on feeding, were explored. The first Pakistani isolate, SpltNPV-Pak-BNG, was studied and compared to a presumable SpltNPV reference isolate, SpltNPV-G1. SpltNPV-Pak-BNG killed *S. litura* larvae significantly faster than SpltNPV-G1. Genetic analysis revealed that SpltNPV-Pak-BNG and SpltNPV-G1 are variants of different virus species, SpltNPV and SpliNPV, respectively. The biological activity of SpltNPV-Pak-BNG was determined in different instars larvae of *S. litura* showing that *S. litura* second or third instar larvae are preferred targets for *S. litura* control with SpltNPV-Pak-BNG in field crops. SpltNPV-Pak-BNG infected *S. litura* larvae with final polyhedrosis showed reduced food intake and weight gain. Interestingly, the mock-infected- larvae and the larvae that survived viral exposure, exhibited the same food consumption and weight gain.

Twenty-two SpltNPV isolates were finally collected from *S. litura* from different agro-ecological regions and cropping systems in Pakistan to explore the genetic diversity of the virus on a spatial scale and explore its possible adaptation to region and crop systems. Among the SpltNPV-Pak isolates tested, isolates TAX1, SFD1, SFD2 and GRW1 were faster killing than other Pakistani isolates. All isolates were genotypic variants of a single SpltNPV 'regiotype', suggesting common recent ancestry, and distinct from the virus species type SpltNPV-G2. There was a strong correlation between geographic location and a SpltNPV genogroup, and less so between the latter and the cropping system. Sequence analysis of SpltNPV-Pak isolates BNG (slow virus) and TAX1 (fast virus) showed major differences, notably the absence of homologous repeat region 17 in TAX1 and gene 125 in BNG. There is strong purifying selection on gene 122, encoding a putative viral fibroblast growth factor known to be involved in baculovirus virulence.

The results presented in this thesis considerably enhanced our understanding of the genetic and biological diversity of SpltNPVs in conjunction with their spatial distribution and the crop system involved. In addition, the results may be used to the develop SpltNPV as an eco-rational control agent of the leafworm *S. litura* in Pakistan.

Key words: Spodoptera litura nucleopolyhedrovirus, *Spodoptera litura*, genetic diversity and biological activity, spatial distribution, cropping system, evolution and adaption, Pakistan

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List of abbreviations

AcMNPV	Autographa californica multiple nucleopolyhedrovirus
AIC	Akaike information criterion
AJK	Azad Jammu and Kashmir
Bt	*Bacillus thuringiensis*
BV	Budded virion
CABI	Centre for Agriculture and Bioscience International
Cp	*Cydia pomonella*
DNA	Deoxyribose nucleic acid
FGF	Fibroblast growth factor
GMO	Genetically modified organism
GV	Granulovirus
ICTV	International Committee on Taxonomy of Viruses
KPK	Khyber Pakhtunkhwa
LD_{50}	Median lethal dose
LdMNPV	Lymantria dispar multiple nucleopolyhedrovirus
LT_{50}	Median lethal time
MNPV	Multiple nucleopolyhedrovirus
NARC	National Agricultural Research Centre
NGS	Next-generation sequencing
NPV	Nucleopolyhedrovirus
OB	Occlusion body
ODV	Occlusion derived virus
ORF	Open reading frame
PaflNPV	Pannolis flammea nucleopolyhedrovirus
REN	Restriction endonuclease
RFLP	Restriction fragment length polymorphism
SeMNPV	Spodoptera exigua multiple nucleopolyhedrovirus
SpliNPV	Spodoptera littoralis nucleopolyhedrovirus
SpltNPV	Spodoptera litura nucleopolyhedrovirus
TTR	Transition to transversion
WNV	West Nile Virus

Chapter 1

General introduction and thesis outline

1. Baculoviruses

a. Structure

Baculoviruses are a group of arthropod-specific viruses belonging to the family *Baculoviridae* and found globally in natural habitats. They have been described from more than 600 host insect species including members of the orders Lepidoptera, Hymenoptera and Diptera. Putative members have been found from Orthoptera, Coleoptera, Neuroptera, Thysanura and Trichoptera, (Slack and Arif, 2007), but they have been provisionally classified as Nudiviruses, as the rod-shaped particles are not found embedded in occlusion bodies (OBs). Baculoviruses occur widely in Lepidoptera, and to a much lesser extent in some dipteran and hymenopteran species of agricultural and forest insects (Cory and Myers, 2003). Baculoviruses are characterized by rod-shaped enveloped virions containing a large (80-180kbp), circular double-stranded DNA genome, encoding between 90 and 180 genes (Rohrmann, 2013[a]; Van Oers and Vlak, 2007). The virions are enclosed within OBs. Each OB can contain a single virion (granuloviruses = GV) or multiple (nucleopolyhedrovirus = NPV) virions, which are called occlusion derived viruses (ODV). Each NPV virion can contain one (Single = S) or more (Multiple = M) so called nucleocapsids, each containing the full complement of DNA. The ODVs are embedded in a proteinaceous matrix that protects them against environmental factors and post-mortem decay in the host (Theilmann et al., 2005). The OB containing ODV can persist in the environment for many years (Krell, 2008; Thomas et al., 1972; Bergold, 1963).

Previously the genera of the *Baculoviridae* were classified on the basis of OB morphology as granulovirus and nucleopolyhedrovirus (Rohrmann, 2013[a]). In 2008 the International Committee on Taxonomy of Viruses (ICTV) proposed these viruses to be re-classified to genus level (Jehle et al., 2006; ICTV, 2008). According to the proposal, the current classification now includes four genera: Alphabaculovirus (lepidopteran-specific NPV), Betabaculovirus (lepidopteran-specific Granuloviruses), Gammabaculovirus (hymenopteran-specific NPV) and Deltabaculovirus (dipteran-specific NPV). This division of baculoviruses does not necessarily reflect the evolutionary history of baculoviruses. The alphabaculoviruses of the genus NPV can be divided – based on phylogenetic information – into group I and II, both of which contain SNPVs and MNPVs (Herniou and Jehle, 2007).

b. Infection process

Baculoviruses can only infect the larval stages of insects. Infection occurs orally when the insect host feeds on OB contaminated plant parts or from soil. The OBs and food particles travel

through the foregut and enter the midgut of the larvae, where they initiate infection. Lepidopteran larvae have alkaline (pH10-11) midgut juices (Terra and Ferreira, 1994) and the baculoviruses have evolved to exploit this alkaline microenvironment. The alkalinity of the midgut enables dissolution of OBs, releasing the embedded occlusion derived virions (ODVs) in the midgut lumen. These ODVs are released within about 10 minutes of post entry into the midgut (Adams and McClintock, 1991). The liberated ODVs can then breach the peritrophic membrane, enter the midgut epithelial cells and subsequently establish efficient systemic infections (Passarelli, 2011; Federici, 1997).

Normally, two virion phenotypes occur in baculovirus infections: ODVs and budded virions (BVs), which spread the infection to tissues in the insect host (Erlandson, 2008). Thus the ODVs are associated with horizontal transmission between insect hosts and BVs are responsible for systemic spread of the infection within a host (Van Oers and Vlak, 2007). These ODVs and BVs differ in their origin, composition of their envelopes and their roles in viral infection, but have identical genomes (Rohrmann, 2013[b]). When a fatal infection occurs, characteristically the infected larval lepidopteran host integument is softening, followed by the dissolution of the host tissues, thereby releasing newly-made OBs in the environment to infect the next cohort of insects (Slack and Arif, 2006). Yields of 10^{10} OBs per insect cadaver have been reported for alphabaculoviruses (Sun et al., 2005), while baculoviruses are obligate host killers (Murillo et al., 2011).

The baculovirus can persist for long periods of time in the insect host populations until it is triggered by stressing the host and causes disease (polyhedrosis, granulosis) (Khurad et al., 2004; Cooper et al., 2003; Hughes et al., 1993). Insects can also transmit the virus vertically, transovarially, from the adult to the offspring (Vilaplana et al., 2010; Granados and Federici, 1986) and such an infection can be passed on vertically without becoming apparent (Burden et al., 2002). Many lepidopteran insect cultures and natural populations of insects have latent baculoviruses (Ilinykh and Ulyanova, 2005), suggesting that latent infections are important for baculovirus persistence (Burden et al., 2003). Sublethal viral infections in the host can lead to physiological disorders like lower fecundity and fertility, changes in the host development time, reduction in pupal and adult body weight and altered pre-oviposition period (Vilaplana et al., 2010; Burden et al., 2002; Myers et al., 2000) A significant increase in time for pupation and progeny mortality as well as reduced fecundity was observed for surviving larvae (Monobrullah and Shankar, 2008). These sublethal infections, that cause latency, in baculoviruses are advantageous for their prevalence in many insect generations in natural habitats for long periods

of time (Cabodevilla et al., 2011). Latent baculovirus infections are common in lepidopteran species, and it is proposed as a strategy for the virus to survive in the host, even when the host population density is low (Cory and Myers, 2003). However, little is known about the molecular mechanisms involved in the trade-off of a latent baculovirus infection versus an overt infection in the insect host.

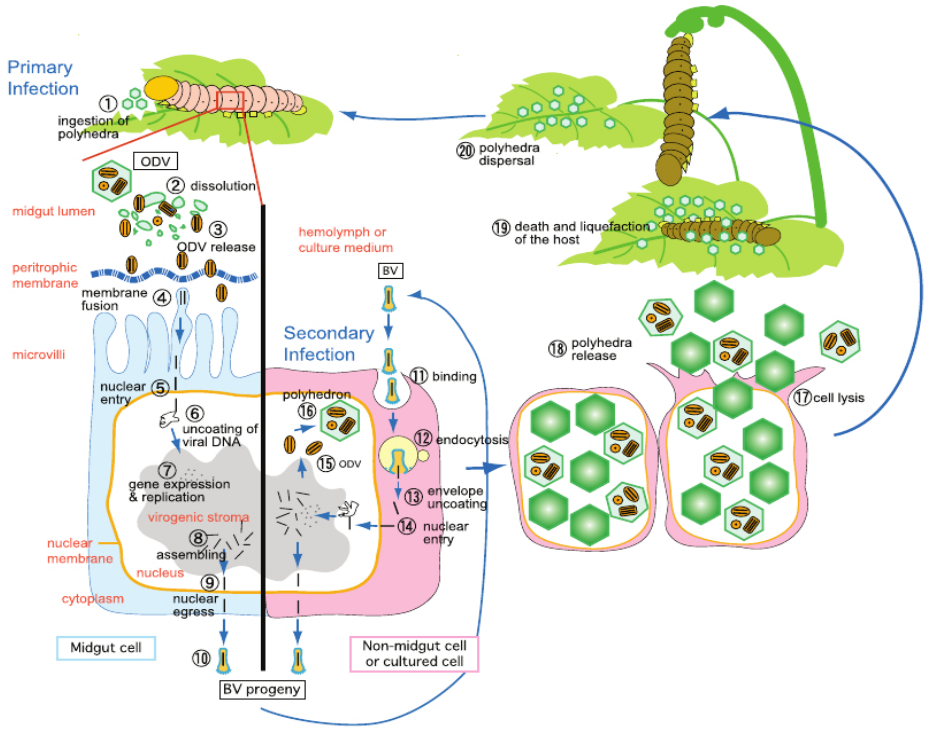

Figure 1. Multiplication cycle of lepidopteran nucleopolyhedrovirus (NPV). Sequential steps of NPV multiplication during primary and secondary infections are numbered (Adapted from Ikeda et al., 2014).

c. Host range

Most baculoviruses have a restricted host range, often a single insect species or a number of related species. For example, the MNPVs of beet army worm, *Spodoptera exigua*, and of the gypsy moth, *Lymantria dispar*, are pathogenic to only a single species, whereas the MNPV of *A. californica* has at least 70 susceptible hosts (Possee et al., 1993; Payne, 1986). The molecular basis for this striking difference in host range is not yet fully understood (Thiem, 1997), but is most likely multigenic. The susceptibility of different hosts for AcMNPV also differs

considerably. *S. exigua* for example is about 5 times less susceptible for AcMNPV than *Heliothis virescens*, whereas it takes at least 50-100 times more AcMNPV OBs to reach the same level of mortality in *Helicoverpa armigera* (Payne, 1986; Black et al., 1997).

Their host specificity ensures that non-target organisms will not be harmed (Heinz et al., 1995; Bonning and Hammock, 1996; Sun et al., 2009). Therefore, this would make baculoviruses promising candidates for biological control agents for insect pests. However, most of the baculoviruses suffer the disadvantage of having a relatively slow speed of kill as compared to chemical insecticides and a low virulence for older instar larvae (Moscardi, 1999). These disadvantages limit the use of baculoviruses on an even wider scale and have contributed to the focus of research on baculovirus engineering for improved insecticidal properties over the last couple of decades. Previous studies revealed that through genetic engineering, the envisaged insecticidal properties of baculoviruses, such as increased speed of action, enhanced virulence and extended host range, might be achieved (Georgievska et al., 2010; Zwart et al., 2010; Tang et al., 2011) but awaits public acceptance.

Wild type baculovirus isolates consist of multiple genotypes, which play a role in biological performance (Cory et al., 2005; Clavijo et al., 2010). Plaque isolation procedures in cell culture or end-point cloning in vivo have been employed to identify these genotypes and to select the genotype with the best biological properties for insect control. AcMNPV has been selected as type strain of the alphabaculoviruses; the E2 genotype has been isolated and selected and characterized in much detail (Smith and Summers, 1978; Rohrmann, 2013[a]). This strain is genetically homogeneous and the nucleotide sequence (133,894 nucleotides) has been entirely determined (Ayres et al., 1994).

d. Potential for biological control

Environmental and food residue problems on the one hand and insect resistance to chemical management strategies on the other have encouraged the pursuit for more sustainable insect control measures. The obvious nature of baculovirus epizootics led to an early interest in their potential as biological control agents. Evidence to control insect populations with nucleopolyhedroviruses (NPVs) exists as early as the 1890s (Huber, 1986). Baculoviruses have the potential to act as microbial insecticides and have been successfully used for the control of insect pests in agriculture and forestry (Sun et al., 2004; Moscardi, 1999; Black et al., 1997; Fuxa, 1991). Baculoviruses are insect host specific pathogens, with a considerable success in controlling lepidopteran and hymenopteran insect pests, such as the cotton bollworm,

velvetbean caterpillar, codling moth, pine sawfly, etc. (Bonning and Nusawardani, 2007). Some outstanding examples are the control of velvetbean caterpillar (*Anticarsia gemmatalis*) in Brazil, of the codling moth (*Cydia pomonella*) in Europe, of the pine sawfly (*Gylpinia herciniae*) and gypsy moth (*L. dispar*) in North America and of cotton bollworm (*Helicoverpa armigera*) in China. These have been achieved through the use of baculoviruses (Moscardi, 1999). As there is a natural diversity in baculovirus isolates, expressive as fast and slow killing, the fast killing strains are more suited to bio-control strategies. In Spain and Portugal, HaSNPV wild-type strains have been reported, which had higher pathogenicity and virulence compared to the already known Iberian genotypic variants of HaSNPV (Figueiredo et al., 2009).

In general, naturally occurring wild-type baculoviruses have low kill speeds and this can allow the insect pests to inflict significant damage on crops before they are controlled by the baculovirus (Bonning and Hammock, 1994). Due to this limitation with wild strains, some baculoviruses have been genetically engineered to improve their insecticidal properties (Sun et *al.* 2009; Georgievska et al., 2010[b]). During the 1980s, the concept of a genetically modified baculovirus was developed to improve the insecticidal activity (Keeley and Hayes, 1987; Maeda, 1989; Borkovec, 1989; Miller et al., 1983). Carbonell et al. (1988) were the first to attempt to improve the insecticidal activity of AcMNPV by expressing biologically active scorpion toxin. Unfortunately, biological activity was not detected in insect bioassay. The first successful expression of a paralytic neurotoxin from the insect predatory straw itch mite *Pyemotes tritici* (Tomalski et al., 1988, 1989) initiated a new era in the field of recombinant baculovirus insecticides. Since then, several approaches have been used to produce fast acting baculoviruses, such as (i) insertion of foreign gene (Miller and Lu, 1997; Fan et al., 2008) that expresses insect-specific neurotoxins, such as mite toxin TxPI (Burden et al., 2000), scorpion toxins AaIT (Gershburg et al*.,* 1998) and LqhIT1/LqhIT2 (Jinn et al., 2006; Regev et al., 2003), or enzymes, such as cathepsin L-like cysteine protease and cathepsin B-like proteinase (Hong-Lian et al*.,* 2008; Sun et al., 2009), (ii) deletion of the baculovirus gene (O'Reilly and Miller, 1989; Tang et al., 2011), (iii) incorporation of a (Bt) toxin into the occlusion body (Chang et al., 2003). The recent studies on SpltNPV recombinants provide further opportunities to develop these viruses into commercially viable products to control *S. litura* populations (Tang et al., 2011).

Nevertheless, ecological impacts of using recombinant viruses must be given careful consideration. One strategy for mitigating risk might be simultaneous release of a wild-type virus, so as to facilitate rapid displacement of recombinant virus by a wild-type (Lee, et al.,

2001). The other suggested strategies for mitigating the persistence of recombinant baculoviruses are the use of co-occluded occlusion bodies containing both a wild-type virus and a recombinant baculovirus, the latter lacking the polyhedrin gene but a toxin in the same locus and therefore incapable of generating OBs autonomously (Hamblin et al., 1990). The use of only Ac-E10 virions for biological control is advocated because (i) they are highly infectious, ensuring effective biological control, and (ii) they remain infectious only for a short period of time, and therefore the virus will quickly get lost from the environment (Wood et al., 1993; Hughes and Wood, 1996).

2. Baculovirus population structure

Baculovirus isolates contain a variable number of genotypes (Rowley et al*.,* 2011; Redman et al., 2010; Figueiredo et al., 2009; Cory et al., 2005), which can be often discriminated by restriction fragment length polymorphism (RFLP). Nucleopolyhedroviruses (NPVs) and granuloviruses (GVs), isolated from a single host species in diverse agro-ecological regions, frequently show restriction fragment length polymorphisms (Christian et al., 2001; Shapiro et al., 1991). Studies at even geographically smaller scales have shown that restriction endonuclease (REN) profiles of NPVs and GVs isolated from individual caterpillars also vary both within and between populations of the same host species (Cooper et al., 2003; Parnell et al., 2002). Genotypic diversity also occurs within a baculovirus isolate, which is typically indicated by the presence of sub-molar bands in the REN analysis of viral DNA. This genotypic diversity in a baculovirus population can be the result of genetic drift from a single parent strain, due to mutations, deletions and insertion during virus replication, or it can result from mixing and recombination of different baculovirus strains (Williams and Otvos, 2005; Lauzon et al., 2005; Jakubowska et al., 2005).

Each isolate can have different biological properties and this has often been the basis for selection of isolates for practical application. It is possible that infection by more than one genotype might actually be beneficial to the virus. Coevolution theory predicts that mixed infections should be more virulent, as multiple genotypes will increase the rate of host exploitation (Frank, 1996). Genotypic variants of the same baculovirus, such as those commonly found at different geographical locations and sometimes even in the same host individual, have been reported to differ in their DNA restriction endonuclease (REN) profiles and also in their biological activities (Takatsuka et al., 2003; Cory et al*.,* 2005; Patel et al., 2010). Virulence and speed of action, as related to dose and yield of OBs from the dead larvae

are important effectiveness-determining properties of insect-pathogenic bio-control agents. Virus isolates differ in virulence and survival time (Alexandre et al., 2010). The genetic variation in NPV populations provides novel information on the distinct groups in which these NPVs occur, and contributes to the knowledge required for improved selection of baculoviruses as biological control agents (Rowley et al., 2011). These differences in pathogenicity and virulence due to natural intraspecific heterogeneity may represent an interesting tool to develop tailored baculovirus-based bio-insecticides (Moscardi, 1999).

The genotypes within an isolate can be separated by end-point cloning in vivo (Makela et al., 2010) or by plaque purification in cell culture (Stiles and Himmerich, 1998; Toprak et al., 2007). Co-occlusion of genotypically distinct virions in OBs is an adaptive mechanism that favors the maintenance of virus diversity during insect-to-insect transmission (Clavijo et al., 2010). These strains often have differential biological properties and are of practical use. Analysis of *S. exigua* NPV revealed that mutants with large deletions occur naturally in wild-type isolates (Muñoz et al., 1998), but these variants were not capable of initiating oral infection alone. They were abundant in wild-type virus and more importantly reduced the pathogenicity of virus population, indicating that they may act as parasitic genotypes (Muñoz and Caballero, 2000). The absence of oral infectivity was related to a specific gene or genes), named 'per os infectivity factor(s)' (*pif*). The product of this gene appears to be a structural protein associated with the envelopes of occlusion-derived virus that is essential for the initial stages of infection of the midgut by a baculovirus (Kikhno et al., 2002; Peng et al., 2010).

It is clear that multiple genotypes of viruses occur even within individual infected larvae (Erlandson, 2009; Chateigner et al., 2015). However, the significance of the genetic variation and how this variation has arisen over time and under certain selection pressure is not clear. The variation has also been important for selection of more virulent strains for alternate hosts (Kolodny-Hirsch et al., 1997). The pool of genotypes also may provide a reservoir for a quick response to environmental changes, such as parasitization (Escribano et al., 2001). The genetic diversity within many DNA virus populations in nature is unknown, but for those that have been studied it has been found to be relatively high. The proportion of polymorphic loci in *Pannolis flammea* (Pafl) NPV changed after passage in different insect species and the extent of this change varied among species, suggesting a role for host selection of pathogen genotypes in the field as a mechanism for maintaining genetic diversity (Hitchman et al., 2007).

Even within a single isolate there is genetic variation (1 in 4000 nucleotides = 320 per baculovirus genome on average) as evidenced from deep sequencing AcMNPV (Chateigner et

al., 2015). Most of this variation is in the form of single nucleotide polymorphic sites (SNPs) and larger deletions in the homologous regions of the genome (Van Oers and Vlak, 2007). Despite rigorous in vivo cloning or plaque purification, the final isolate cannot be easily proven to be genetically homogeneous, as variation can occur even with a replication cycle in insect larvae or cell culture. In particular cell culture can contribute to the generation of deletion mutants and defective interfering viruses (Pijlman et al*.,* 2001) or exert a bias in selecting genotypes.

3. Bio-fitness

Fitness means the ability of an organism (or pathogen) to produce progeny that survive to contribute to the following generation, and is often described using a term known as basic reproductive ratio, R_o (Stearns, 1992; Begon et al., 1996). The parasites provoke host immune responses, infect host tissues, and control the flow of host resources through mechanisms of pathogenesis, that determine the relations between virulence and components of parasite fitness, such as transmission to new hosts and survival within host (Frank and Schmid-Hempel, 2008). For parasites like a baculovirus, fitness is usually described in terms of epidemic infections, so in this case baculovirus fitness is the number of infected hosts resulting from a single baculovirus-infected host (Anderson and May, 1981). The recombinant baculoviruses havereduced within-host fitness (likely to be less fit) compared to its parent wild-type (Cory, 2000; Zwart et al., 2009; Georgievska, 2010[a]).

It is quite common that baculovirus isolates consist of a mixture of genotypes (Figueiredo et al., 2009; Redman et al., 2010; Rowley et al*.,* 2011). Hence, competition of virus genotypes in an insect host is a key element of virus fitness, contributing to their persistence in agro-ecosystems. The genetic diversity in West Nile Virus (WNV) is directly related to virus' success in new and changing environments in the laboratory and that difference in a virus' ability to produce and maintain heterogeneous populations in nature (Ciota et al., 2007).

a. Speed of kill and virus yield (or productivity)

A limitation of wild-type baculoviruses as a bio-control agent is, among others, their slow speed of kill. To eliminate this drawback, baculoviruses have been genetically modified to improve their insecticidal property and reduce crop losses (Sun et al., 2009; Inceoglu et al., 2006; Szewczyk et al., 2006). A number of approaches have been used to produce fast acting baculoviruses i.e. (i) insertion of a foreign gene e.g. specific toxin (Tang et al., 2011; Gramkow

et al., 2010), hormone or enzyme (ii) deletion of a baculovirus gene e.g. the ecdysteroid glucosyltransferase or *egt* gene (Georgievska et al., 2010; Zwart et al., 2010; Erlandson, 2009). The occlusion body (OBs) production ability of a virus within a caterpillar host is an important parameter to predict its sustainability in the agro-ecosystem. Competition of two genotypes in mixed infection may modify the survival of the co-infecting genotypes, but may also affect the within-host parasite growth, host survival and reproduction (Vizoso and Ebert, 2005).

b. Influence of baculovirus on larval behavior

Nature is full of examples of parasites and pathogens that induce behavioral changes of their hosts upon infection (Libersat et al., 2009; Lefevre and Thomas, 2008; Van Houte et al., 2013). These changes vary from slight alterations of existing behavioral traits to completely new behavior. Well-known examples are: toxoplasma-infected rodents losing their innate aversion to cats (Berdoy et al., 2000), lancet liver fluke infected ants climbing into grass leaves (Hohorst and Graefe, 1961; Moore, 1995; Libersat et al., 2009) and the suicidal behavior of crickets and grasshoppers that have been infected with a Gordian worm (Thomas et al., 2002; Biron et al., 2005). This parasitic worm manipulates its host in such a way that the infected grasshopper is driven towards water. It will then drown itself by jumping into the water, thus releasing the parasitic worm in the water, where the latter lives and reproduces. These changes are generally assumed to be advantageous for the parasite by increasing the likelihood of transmission and survival.

Some parasites use their host to protect themselves from hyper-parasitism (Grosman et al., 2008; Harvey et al., 2008; Maure et al., 2011). *Thyrinteina leucocerae* larvae protect pupae of the braconid parasitoid *Glyptapanteles sp*. (Grosman et al., 2008). Once the parasitoid pupate after leaving the host, the host larvae defend the pupae by knocking off the hyper-parasitoids with violent head swings, resulting in reduced mortality of the parasitoids' pupae. Bracoviruses and ichnoviruses (Polydnaviridae) play vital roles in the development of some braconid and ichneumonid parasitoids (Burke and Strand, 2012)

Vertebrate viruses, such as rabies virus and hantavirus, are known to induce enhanced aggressive behavior in the host, leading to increased biting rates and, as a consequence, increased virus transmission (Lefevre and Thomas, 2008). Being obligate intracellular parasites, viruses are also known to modify host behavior and induce behavioral changes in the infected larvae. An extraordinary example of altered behavior is 'Wipfelkrankheit' or 'tree-top' disease (Raymond et al., 2005; Hernandez-Crespo et al., 2001; Vasconcelos, 1996). This

phenomenon is characterized by baculovirus-induced hypermobile behavior of the insect in the first stage of infection. In a later stage of infection the insect climbs to the canopy of the plant or tree, where it waits until it dies. This behavior is assumed to enhance the spread of progeny virus upon larval death and disintegration, by the spraying of virus particles on lower foliage (short distance transmission) and by increasing the visibility of the diseased insect to predators such as birds (longer distance transmission). Larval behavior is an important parameter, which influences transmission of baculoviruses in the field, in terms of where infected insects die and the possibility of healthy insects to acquire the virus (Cory and Evans, 2007).

c. Transmission

The transmission process from infected to healthy populations, plays a central role in the ecology of baculoviruses and the population dynamics of their hosts. Baculoviruses are usually transmitted horizontally from an infected cadaver to healthy susceptible caterpillars. Horizontal transmission occurs primarily when a baculovirus-infected larva dies and lyses, releasing a massive number of occlusion bodies (OBs) onto foliage and soil. Susceptible hosts become infected when they ingest OBs while feeding. Defecation and regurgitation by the infected larvae have been found as additional phenomenon of contamination of host plants with virus (Young, 1998; Vasconcelos et al., 1996). Moreover, some studies suggest that cannibalism and predation may also be a route of transmission (Dhandapani et al., 1993). Environmental factors such as rainfall, wind (Fuxa and Richter, 1991) and contaminated ovipositor of parasitic hymenopterans (Hamm et al., 1988) could contribute to NPV transmission as well.

The vertical transmission includes transovum transmission on the egg surface, transovarian transmission, within the eggs or passage as a latent infection (Corry and Meyers, 2003; Fuxa, 2004). The transmission process therefore combines both the susceptibility of an insect to the pathogen with the chance that it is going to be infected which in turn will be influenced by behavior, host plant and other environmental conditions. Parasites with increased opportunity for vertical transmission and reduced opportunities for horizontal transmission often become less virulent (Messenger et al., 1999; Agnew and Koella, 1997; Bull et al., 1991). Recombinant baculoviruses are transmitted, in the case of *H. armigera* populations, at lower rates than the wild type virus (Zhou et al., 2005). The mechanism of vertical transmission, i.e. where and how the virus is maintained, is highly enigmatic.

d. Persistence

Soil is considered as a natural reservoir for the baculoviruses where they can persist for decades and serve as a potential source of infection for the subsequent generations of insects. From the soil baculoviruses can be translocated abiotically, by wind and rain, to new insect host plants to initiate epizootics (Fuxa et al., 2007; Ilinykh, 2007). The longest study of virus persistence in soil was the example of virus of the Douglas-fir tussock moth *Orgyia pseudotsugata*, which persisted in the soil up to 41 years (Thompson et al., 1981). A number of factors contributes to the virus' ability to accumulate and persist in the soil compartments (Young, 1998). The baculoviruses are sensitive to ultraviolet light and exposure to direct solar radiation can decrease virus infectivity (Villamizar et al., 2009). *Cydia pomonella* (Cp) GV larvicidal activity declined after 40 weeks at 35ºC, compared to 3 years at 2ºC (Lacey et al., 2008).

In conclusion, it is important to analyze and exploit the genetic variation of each individual baculovirus species as a prerequisite for selection of the best suited isolate or variant as a biological control agent. Exploitation of indigenous baculovirus isolates, which are already adapted to regional and local agro-ecological conditions, is to be preferred over isolates from elsewhere.

4. Experimental evolution

Evolution is change in the heritable characteristics of biological populations over successive generations (Hall and Grimson, 2013). Evolution of animals and plants is a slow process of mutation, selection and adaptation over long time scales. Microorganisms generally evolve much quicker and viruses are the 'champions'. The mutation rate for DNA viruses is in the order of 10E8-9, but for RNA viruses this is even faster (10E4-5), because the former has a proof reading function in the (DNA) polymerase. Viruses can replicate in a variable number of cycles into large numbers per cell and per individual and hence produce mutants relatively frequent. However, there is selection against mutations when essential genes are involved (unless in the wobbling position of a triplet encoding an amino acid), because virus isolates, even if they are clonal, contain genetic variation due to the methodology employed to get them.

The fundamental nature of experimental evolution is conceptually quite simple. For many generations, a series of replicated populations is exposed to a novel environment, while a parallel series of populations is maintained within the ancestral environment, thereby serving as experimental controls. Experimental studies can produce entirely new types of organisms for biological study, literally 'building a better mouse' (Bennet, 2003; Garland, 2003), a

complementary approach to transgenics for producing new organisms for biological study (Garland, 2003). By virtue, the experimental evolution produces populations with traits that enhance function and overall fitness in the new selective environment. In the past two decades evolutionary biology has assumed broad relevance in several applied fields, ranging from the design of biotechnology protocols to create new drugs and enzymes, to disease tracking and identification, and the avoidance of resistant pests and microbes (Bull and Wichman, 2001). Microbial organisms have been extensively used in experimental evolutionary studies because they possess a variety of valuable traits that facilitate large-scale experimentation. Many growing populations can be cultured in the laboratory simultaneously along with an appropriate control (Bennett and Hughes, 2009).

Experimental evolution has been applied successfully in viruses (Wichman and Brown, 2010), bacteria (Sanahuja et al., 2011) and fungi (Schoustra et al., 2005). The serial passage of any virus results in the accumulation of genotypic variants among any virus population, including that of baculoviruses (Vlak et al., 2002). The genetic diversity is directly related to the viruses' success in new and changing environments and differences in a virus' ability to produce and maintain heterogeneous populations in nature, may in some instances explain the variable levels of success seen among arboviruses (Ciota et al., 2007). In experimental evolution it is the carefully designed environment, which automatically selects for the desired phenotypes because the experimental set-up induces competition between different variants that originate by spontaneous mutation and/or recombination. However, an experimental setup combining artificial selection and experimental evolution is also possible (Schoustra et al., 2005). Analysis of the patterns of gene acquisition and loss among different viruses highlights the very fluid nature of baculovirus genomes (Herniou et al., 2003). The most common adaptive changes are due to the loss or modification of a pre-existing molecular function (Behe, 2010).

Experimental evolution using baculoviruses could provide information on the mutation rate and on the location of genetic information. However, this approach is compromised by the intrinsic heterogeneity of baculovirus isolates in vivo (Chateigner et al., 2015) and in vitro (Pijlman et al., 2001).

5. *Spodoptera litura*

The cotton leafworm, *S. litura*, is a polyphagous pest insect feeding on more than 300 plant species and widely distributed in Asia, Africa, North America and Oceania islands (Tuan et al., 2013). It is a very destructive insect causing economic yield losses up to 100% to some of the

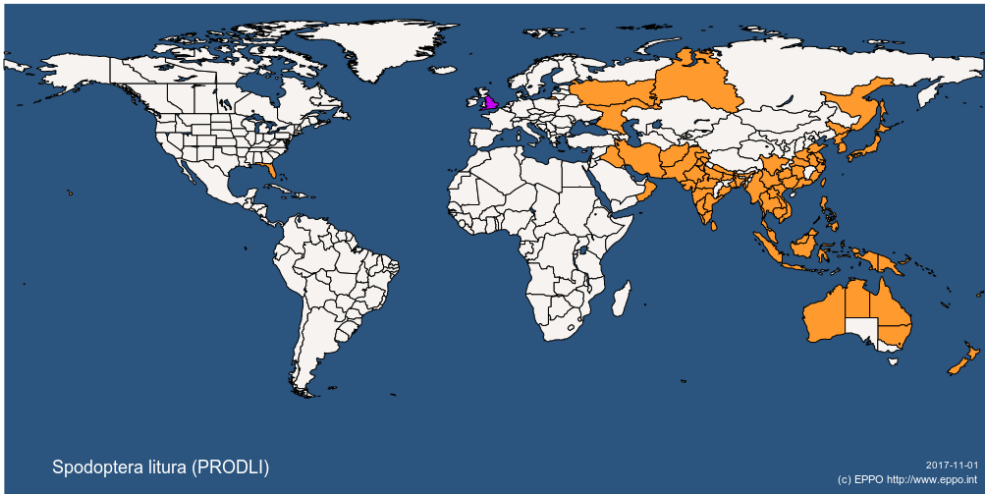

Figure 2. Geographic distribution of *S. litura* (highlighted) in the world. (Adapted from http://www.eppo.int).

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cultivated field crops like cotton, groundnut, soybean, tobacco and vegetables (Qin et al., 2004). The insect is also known as common or tobacco cutworm or armyworm.

S. litura is an emerging pest in Pakistan and the problem is increasing as it fills the void of *H. armigera* upon successful introduction of Bt-cotton crops. *S. litura* is not sensitive to the Bt-toxin in the transgenic cotton. The application of synthetic insecticides is considered the most commonly used method for controlling insect pests. This irrational use of insecticides has created an ideal environment for the development of resistance in many pest insects, also in *S. litura* (Hirose, 1995; Tong et al., 2004), and also in Pakistan (Ahmad et al., 2007; Ahmad et al., 2008; Abbas et al., 2012; Shad et al., 2012; Saleem et al., 2016). Public awareness to high toxicity, residues in food, contamination of water and the environment, and concerns of human health effects are increasing due to pesticides (Regnault-Roger et al., 2005). There is thus need to develop and implement more eco-friendly methods to manage insect pests that are biologically based and therefore more sustainable (O’Callaghan and Brownbridge, 2009). Microbial pathogens could be an alternative due to their pathogenicity, persistence and ecological safety. In addition, the successful application of Bt-cotton to control the cotton bollworm *H. armigera* left an opportunity for *S. litura* to move into this crop. The Bt-toxin in transgenic cotton is ineffective against *S. litura*. So in this case there is a need to control this insect and baculoviruses can be an attractive alternative for chemical insecticides.

6. Spodoptera litura nucleopolyhedrovirus (SpltNPV)

Entomopathogenic viruses, fungi and bacteria exhibit a significant potential as bio-pesticides and have been developed and used across the globe (Nicholson, 2007; Erlandson, 2008; Islam and Omar, 2012). The alphabaculoviruses (Baculoviridae), lepidopteran specific nucleopolyhedrosis viruses or NPVs have an established potential of effective bio-insecticide due to their high virulence, host specificity and compatibility with other bio-control agents (Arrizubieta et al., 2013). Spodoptera litura nucleopolyhedrovirus (SpltNPV) is host specific and infects only *S. litura* (Pang, 1994). It has been tested and recognized as a potential candidate to control *S. litura* (Okada, 1977) and has been successfully used in China as a biological insecticide against cotton leaf-worm (Chen et al., 1998). A total of 189 nucleopolyhedrovirus clones were isolated from NPV infected larvae of *S. litura* collected from Japan. Restriction endonuclease (REN) analysis of these clones demonstrated that these clones represented three distinct NPV types designated as type A, type B and type C SpltNPVs based on the similarity of REN patterns (Kamyia et al., 2004). Type A SpltNPV coincide with SINPV-D (Kislev and Edelman, 1982) or SINPV-B (Cherry and Summers, 1985). Type B SpltNPV coincide with NPV identified widely in *S. litura* in Japan (Laviana et al., 2001; Lavina-Caoili et al., 2001; Pang et al., 2001; Takatsuka et al., 2003). Type C isolate was unique in its REN pattern as compared to any of the previously isolated NPVs in *S. litura* or *S. littoralis* larvae, including SINPV-A (Cherry and Summer, 1985), SpltNPV isolated in India (Das and Prasad, 1996) and AcMNPV isolated in Japan (Maeda et al., 1990). Similarly, nine NPV isolates from Japan, Vietnam, Malaysia and India were studied for genetic and biological comparison to select a novel isolate to control *S. litura*. In REN analysis, submolar bands were detected demonstrating a mixture of genotypes (Takatsuka et al., 2003). The isolates were devided in two groups: *S. litura* NPV-type and *S. littoralis* NPV-type based on the equimolar bands (Takatsuka et al., 2003). A unique isolate of S. litura nucleopolyhedrovirus (SltMNPV) was isolated in the Philippines (Lavina et al., 2001), having a potential of microbial insecticide. NPVs infecting *S. litura* have been isolated from several Asian countries (Hunter-Fujita et al., 1998).

Spodoptera littoralis nucleopolyhedrovirus (SpliNPV) has been isolated from *S. littoralis* populations in the Azores islands, Egypt, France, Israel, Morocco, Tunisia and Turkey (Cherry and Summers, 1985; Croizier et al., 1989; Kislev and Edelman, 1982; Laarif et al., 2011; Martins et al., 2005; Topark and Gurkan, 2004). The host range of SpliNPV is shown to be narrow and likely to be infecting *Spodoptera sp.* (Takatsuka et al., 2007; Toprak et al., 2006). SpliNPV was isolated from NPV infected larvae of *S. litura* (Kamiya et al., 2004). SpliNPV

Figure 3. (a) Emergence of *S. litura* neonates from the egg batch; (b) *S. litura* larva feeding on *Abelmoschus esculentus*; (c) *S. litura* larva infected with SpltNPV; (d) *S. litura* larva killed by SpltNPV (Photo credit: Ghulam Ali)

has extensive similarity with SpltNPV but is in fact a different species (Breitenbach et al, 2013), and exhibited similar biological activity to *S. litura* (Takatsuka et al., 2007).

Despite of the commercial status of SpltNPV as bio-insecticide in many countries, little information is available on its genetic and biological diversity. Regardless of frequent variation in natural population of baculoviruses, there has been inadequate systematic study of SpltNPV diversity and its importance to insect-pathogen interactions. We need to explore the associated SpltNPV wild-type isolates and study their relevant biological and genetic diversity. Howmany genotypes of SpltNPV are available in Pakistan? and what is the genetic diversity in reference to geographical distribution and cropping system? No clue of what maintains this heterogeneity and what are the genetic changes that contribute to virulence? These are the fundamental questions that need to be addressed before using SpltNPV as a bio-control agent.

7. Outline of this thesis

Wild-type baculovirus isolates exhibit natural diversity and are composed of mixed genotypes. The genotypic composition plays an important role in the biological performance of the virus in controlling phytophagous insect populations in vegetable and fibre crops such as cotton. In-depth understanding of the genetic and biological diversity within and among the wild-type baculovirus populations can assist in the selection of potentially superior virus isolates for their development as a commercial biological insecticide. Though it may explain a number of questions raised above, the main objective of this thesis is to investigate the functional diversity of SpltNPV over spatial and temporal scales and in different crops. *S. litura* baculovirus, more specifically SpltNPV from Pakistan, is taken as an example, because there is no recorded artificial introduction of the virus in Pakistan and the viruses are considered endemic. In addition, there is an urgent need for controlling *S. litura* (cotton leafworm or tobacco cutworm), as it is filling the void as result of a successful reduction of the cotton bollworm *Helicoverpa armigera* in Bt-crops, notably cotton. **Chapter 1** contains a general introduction to the theme of the thesis and contains a literature review focussing on baculovirus diversity, more specifically in *S. litura* and *S. litoralis*. The chapter also contains a scope of the investigation, a problem statement, and research hypotheses and objectives.

Chapter 2 describes the biological and genetic properties of a native, wild-type SpltNPV-BNG-Pak isolate from Pakistan and compares this to a Chinese isolate (SpltNPV-G1) considered to be a reference isolate against the insect host *S. litura*. Key biological properties, such as dose-mortality and time-to-death relationships, are determined and compared for the two baculovirus isolates. Since the SpltNPV-BNG-Pak showed a faster speed of kill than the reference isolate, while the dose-response relationships were very similar, the DNA of the two isolates was compared by restriction enzyme analysis and nucleotide sequencing. In **Chapter 3** the susceptibility of different larval stages of *S. litura* against SpltNPV-BNG-Pak was studied to assist in the selection as a microbial insecticide. The information leads to the need to explore the potential of SpltNPV isolates from different ecological regions of Pakistan.

A batch of SpltNPV isolates (22) from different ecological regions of Pakistan and from different crops was obtained from the field to characterize the diversity of baculoviruses found in *S. litura* and potentially identify baculovirus isolates that may be developed into a biological product to manage *S. litura* populations in various crops. **Chapter 4** describes the survey approach and the biological and genetic diversity among the virus isolates. The biological diversity of the collected virus isolates is characterized in terms of survival time distribution

after exposure to a lethal virus dose. Genetic diversity among the collected isolates is characterized by restriction enzyme analysis. The hypothesis is that there is a spatiotemporal pattern in the (micro)evolution of baculoviruses after a natural introduction via insect larvae. This can be studied by comparing restriction enzyme profiles of the various isolates and ultimately by sequencing.

The extent of crop damage by baculovirus infected larvae as compared to uninfected larvae is a most important aspect in deciding whether to use baculovirus as a viral insecticide, or not. The feeding behaviour of the infected larvae contributes to the estimation of crop damage at field level. The infected larvae are often impaired relative to healthy larvae. However, there is not enough information on the virus induced impaired feeding behaviour, in particular of *S. litura,* and a precise quantitative measure of food consumption by infected larvae has not been reported. In **Chapter 5** the feeding behaviour of infected larvae versus uninfected larvae was studied. Virus-induced modifications in feeding behaviour and weight gain, as a measure of food intake of *S. litura* under laboratory conditions, were described.

On the basis of the outcome of Chapter 4, the genomes of a selection of SpltNPV isolates (SpltNPV-Pak -BNG and SpltNPV-Pak -TAX1) were sequenced and compared to the reference isolate SpltNPV-G2 (**Chapter 6**). Physical maps will be constructed and compared. This comparison should reveal the micro-differences between these isolates. It will also aid in determining the taxonomic position of the Pakistan SpltNPV isolates within the group of viruses, infecting larvae of the *S. litura* / *S. littoralis* complex worldwide. Furthermore, two SpltNPV isolates will be subjected to deep sequencing to observe genetic variation within these isolates. The extent of genetic variation may give indications on the adaptive potential of SpltNPV to other hosts or (micro)environments.

In **Chapter 7** the results of the experimental endeavors are discussed in the light of the practical use of SpltNPV in the control of the cotton leaf worm *S. litura* in Pakistan and the possible role of genetic variation in successful pathogenesis in this insect. Particular emphasis will be put on the hypothesis that genetic variation is important to various spatio-temporal scales. Finally, a potential outlook is given on the impact of genetic variation in baculoviruses and the information required to explain the process of genetic information and evolution of baculoviruses in general.

Chapter 2

Biological and genetic characterization of a Pakistani isolate of Spodoptera litura nucleopolyhedrovirus

Adapted from:
Ghulam Ali, Wopke van der Werf and Just M. Vlak. 2017. Biological and genetic characterization of a Pakistani isolate of Spodoptera litura nucleopolyhedrovirus, *Biocontrol Science and Technology*, in press. https://doi.org/10.1080/09583157.2017.1409339

Abstract

Spodoptera litura is an emerging insect pest in a wide range of crops worldwide. The insect is difficult to control because of resistance development to synthetic insecticides and emerging resistance to *Bacillus thuringiensis* toxins. Therefore there is a need to develop biological control agents, preferably from an indigenous source to avoid risks associated with importation of exotic natural antagonists. A Pakistani isolate of S. litura nucleopolyhedrovirus (SpltNPV, *Baculoviridae*), SpltNPV-Pak-BNG, was obtained from the field and characterized biologically and genetically, and compared to a SpltNPV reference isolate, SpltNPV-G1, thought to be of Chinese origin. The dose mortality response (LD_{50}) of SpltNPV-Pak-BNG was not significantly different from that of the reference isolate SpltNPV-G1, but the time-to-death (LT_{50}) was significantly shorter for SpltNPV-Pak-BNG than for SpltNPV-G1. DNA restriction enzyme profiling indicated that SpltNPV-G1 and SpltNPV-Pak-BNG are different viruses. Sequence analysis of 'ORF24', specific for SpltNPV (and S. littoralis NPV as ORF21), and the conserved baculovirus core genes *polyhedrin*, *DNApol*, *pif-2* and *lef-8* confirmed that this was indeed the case and that SpltNPV-Pak-BNG is a genuine SpltNPV variant whereas the SpltNPV-G1 isolate we used is in fact a SpliNPV variant, renamed to SpliNPV-G1. The newly isolated SpltNPV-Pak-BNG has potential for development as a biocontrol agent of *S. litura* in Pakistan.

Keywords: *Spodoptera litura* nucleopolyhedroviruses; biological activity; genetic characterization

1. Introduction

Insect-pathogenic baculoviruses offer great potential for biological insect pest control in agriculture because they are host specific and do not harm non-target organisms, including pest natural enemies or pollinators. They are therefore highly compatible with integrated control strategies. They can be easily produced at both the industrial as well as the farm scale (Inceoglu et al., 2006).

A limited number of baculoviruses is used around the globe (Moscardi et al., 2011; Sun, 2015; Haase et al., 2015) because practical limitations hamper their commercial development. These include low speed of kill as compared to chemical insecticides, narrow host range and unavailability of suitable isolates for commercialization (Szewczyk et al., 2006; Erlandson, 2008). Baculoviruses have been genetically engineered to improve the insecticidal properties (Inceoglu et al., 2006). However, to date, no genetically modified baculovirus based insecticide is commercially available (Beas-Catena et al. 2014). The search for wild-type isolates with good insecticidal properties remains therefore necessary to unlock the potential of baculoviruses for biological pest control.

The oriental leafworm *Spodoptera litura* is an important pest in vegetable crops in Asia and the Indian subcontinent. It is also an emerging pest in cotton occupying the niche left following the effective control of *Helicoverpa armigera* by Bt cotton (Wan et al., 2008; Naik et al., 2015). A Chinese isolate of S. litura nucleopolyhedrovirus (SpltNPV ZHU strain) was originally collected from dead larvae of *S. litura* in a cotton field near Guangzhou in China in 1976 (Pang, 1994) and its genome was completely sequenced (Pang et al., 2001). This isolate is considered the prototype SpltNPV (ICTV, 2011) and was called SpltNPV-G2. Later, this isolate was developed into a commercial bioinsecticide and registered under the trademark "Chongwen No. 1" in 1996 (Pang et al., 2001, Sun, 2015). A number of SpltNPV isolates has been isolated in Japan (Takatsuka et al., 2003) and one of them is being used as microbial insecticide (Hasumon Tenteki, Nippon Kayaku Group; Mitsuhashi, 2009). Similarly, in Taiwan and India SpltNPV has been developed as insecticide against *S. litura* in vegetables, peanut, cotton and rice (Das and Durga, 1996; Moscardi, 1999). There are no reports that SpltNPV has been used in Pakistan and the virus-based insecticides available in other parts of the world would be a possible option of controlling *S. litura*. However, the use of exotic nucleopolyhedroviruses may have an adverse impact on naturally occurring SpltNPVs (Munoz and Caballero, 2000). Indigenous isolates are hence preferred, provided they have sufficient insecticidal properties. In this paper we describe the first indigenous SpltNPV isolated from

Pakistan (SpltNPV-Pak-BNG) and determine some of its basic molecular and biological properties.

We compare the biological activity of SpltNPV-Pak-BNG with an in vivo cloned isolate of a Chinese strain of SpltNPV. This isolate is called here SpltNPV-G1. The viral DNA of both SpltNPV isolates was characterized by restriction enzyme analysis and by specific fragment sequencing to confirm their identity. The biological activity of SpltNPV-Pak-BNG and SpltNPV-G1 was characterized by comparing the dose-mortality (LD_{50}) and time-to-death (LD_{50}) responses in 3rd instar larvae of *S. litura*. We conclude that SpltNPV-Pak-BNG is a superior candidate as a biocontrol agent of *S. litura* in Pakistan and that SpltNPV-Pak-BNG and SpltNPV-G1 are variants of different NPV species.

2. Materials and methods

2.1. Insects

An *S. litura* colony, further referred to as the NARC colony, was maintained in the insectary of the National Agricultural Research Centre (NARC), Islamabad, Pakistan, at 26±2°C, 50-60% relative humidity and 14h:10h day-night photoperiod on a semi-synthetic diet. The colony was started using *S. litura* pupae from the Entomology Department, University of Arid Agriculture, Rawalpindi, Pakistan. The insect colony was cultured in the insectary for up to five generations without symptoms of infection before the insects were used in experiments. The culturing was done aseptically to the extent possible to avoid contamination by viruses or other pathogens.

2.2. Virus isolates

Two isolates of SpltNPV were used in experiments: SpltNPV-Pak-BNG and SpltNPV-G1. The SpltNPV-Pak-BNG isolate was collected from cotton in the Bahawalnagar district of Punjab, Pakistan, on 18 October 2011. A Chinese reference isolate, SpltNPV-G1, was kindly provided by Dr J. A. Jehle (Darmstadt, Germany) as occlusion body (OB) suspension. The name SpltNPV-G1 is used here for the first time.

2.3. Virus amplification and purification

The two virus isolates were both amplified in 3rd instar larvae of *S. litura.* OBs were isolated from the cadavers as described by Cory et al. (2005) with slight modifications. The cadavers were macerated in 500 µl milli-Q water using 0.1% SDS and filtered through three layers of muslin-cloth to clear large insect debris. The sample was subsequently centrifuged for 5 min at

400 g in a table-top centrifuge to remove smaller insect debris. Supernatant containing OBs was saved and the debris extracted once more using 0.1% SDS. Supernatants of the two steps were combined and centrifuged for 20 min at 3500 g to sediment the OBs. The OBs were re-suspended in 200 µl milli-Q water and stored at -20° C until required. OBs were counted in a Neubauer hemocytometer in a Leitz microscope.

2.4. Dose-mortality response and time to death of SpltNPV-Pak-BNG and SpltNPV-G1 in the third larval instar of *S. litura*

Viral doses of 1×10^3, 3×10^3, 1×10^4, 3×10^4, 1×10^5 and 1×10^8 OBs/ml/larva were used to determine dose mortality responses using *S. litura*. Viral stock concentrations of 3×10^5, 1×10^6, 3×10^6, 1×10^7, 3×10^7 and 3×10^{10} OBs/ml were prepared by counting the OBs in the virus suspension using a Neubauer haemocytometer in a phase contrast light microscope. Three replicates were counted.

Biological activity of SpltNPV-Pak-BNG and SpltNPV-G1 in *S. litura* L3

Dose response

Biological activity was characterized by measuring the dose-mortality response (LD50) and time to death (LT50) of SpltNPV-Pak-BNG and SpltNPV-G1 in *S. litura* 3rd instar larvae (L3). For each replicate, thirty newly moulted 3rd instar larvae were placed in Petri dishes, and starved for 15 h (overnight). The larvae were then fed a leaf disc with the virus suspension. Fresh tender leaves of *Ricinus communis* were cut into 3 mm^2 pieces and placed on 1% plant agar in 24 well tissue culture plates. Viral doses of 1×10^3, 3×10^3, 1×10^4, 3×10^4, 1×10^5 and 1×10^8 OBs/ml/larva were applied to the leaf disks by pipetting 3 or 3.33 µl of the appropriate stock solution on the leaf disk. The starved larvae were exposed to the inoculated leaf discs for 24 h at 26±2°C in a climate chamber. Inoculated larvae that had eaten the whole leaf disc were transferred individually to 6-well tissue culture plates with artificial diet plugs. The wells were covered with Parafilm, tissue paper and the original lid to prevent escape. Larvae were incubated at 26±2°C and a 14h:10h day-night photoperiod in a climate chamber, and mortality was observed after 24 h in first two days and subsequently every 12 h until death or pupation. Three replicates were conducted. Each replicate included a control treatment in which the insects had been mock-inoculated.

Statistical analysis: dose response

The lethal concentration (LD_{50}) causing 50% larval mortality in each of the three replicates was calculated with Probit analysis (Finny, 1971) using the software program PoloPlus (Robinson et al., 2003). LD_{50} values of the two virus isolates were compared using t-tests at P = 0.05 for each replicate. An analysis of the data with PoloPlus using the pooled data of the three replicates was also conducted. Logistic regression (Zuur et al., 2007; Crawley, 2013) was used to conduct a stratified analysis of the dose-mortality data of the three replicates, accounting for both virus genotype and replicate effects as categorical factors with two and three levels, respectively. Six logistic regression models were fitted to the data: (1) a null model with only dose effect (model 1; 2 d.f.), (2) a model with dose plus virus genotype effects (model 2; 3 d.f.), (3) a model with dose plus replicate effects (model 3; 4 d.f.), (4) a model with dose plus virus plus replicate effects, assuming no interactions (model 4; 5 d.f.), (5) a model with dose effect plus the full interaction between virus genotypes and replicates on the intercept (model 5; 7 d.f.), (6) a model allowing different intercept and slope for each virus genotype in each replicate (model 6; 12 d.f.; Table 1). The logistic regression was conducted using the function glm in R (R core team, 2015).

Table 1: overview of logistic regression models fitted to the dose-mortality data of SpltNPV-Pak-BNG and SpltNPV-G1 in *S. litura* L3

Model name	Effects	D.f.	Partitioning of d.f.
1	Dose	2	Intercept (1), slope (1)
2	Dose + Virus	3	Intercept (1), virus effect on intercept (1), slope (1)
3	Dose + Rep	4	Intercept (1), replicate effect on intercept (2), slope (1)
4	Dose + Virus + Rep	5	Intercept (1), virus effect on intercept (1), replicate effect on intercept (2), slope (1)
5	Dose + Virus * Rep	7	Intercept (1), virus * replicate interaction effect on intercept (5), slope (1)
6	Dose * Virus * Rep	12	Intercept (1), slope (1), virus * replicate interaction effect on intercept (5), virus * replicate effect on slope (5)

The model formula for logistic regression is: $y = \text{logit}^{-1}(\sum \beta_i x_i)$ where β_i values are regression coefficients, and x_i are independent variables. Virus genotype and replicate were entered as categorical variables, while dose was entered as a transformed variable: $x = {}^{e}\log(\text{dose}+1)$.

The goodness of fit of different logistic models was assessed using Akaike's information criterion. Significance of parameters in nested models was assessed using the Chi squared test (Crawley, 2013).

Statistical analysis: survival data

Survival was measured at a viral dose of 1×10^8 OBs/ml/larva, which caused 100% mortality in all replicates. Data of each replicate were analysed using the survival analysis procedure in SPSS 21.0 (IBM Corp., 2012). The time required for the virus isolates to cause 50% larval mortality (ST_{50}) was determined using the Kaplan-Meier estimator. The log-rank test was used to test for difference in survival between virus isolates. A survival analysis with pooled data was also conducted in SPSS. Finally, a stratified analysis accounting for replicate effects was conducted using Cox' proportional hazards model using the R package "survival" (Therneau & Grambsch, 2000; Therneau, 2015; Crawley, 2013).

2.5. Genetic analysis

Additional cleaning of OBs was performed to extract DNA. A purification buffer (TE pH7.5, 0.5%SDS, 0.1% Triton-X100) was added to the OB suspension and placed at 37ºC for 1h in a shaking incubator. The suspension was subsequently centrifuged at room temperature for 10 minutes at 5300 rpm. Pellets were re-suspended in 2 ml milli-Q water. A volume of 4 ml sucrose cushion (30% w/v) was pipetted in 12 ml tubes. Then, the OBs suspension was poured gently over the sucrose cushion and centrifuged at room temperature for 15 min at 5300 rpm. The pellets were washed twice with 5 ml milli-Q water and spun down at room temperature for 8 min at 5300 rpm. The OB-containing pellets were re-suspended in 2 ml milli-Q water in a clean tube.

Extraction of Occlusion Derived Virus (ODV) from OBs

A viral dose of 5×10^8 OBs in 2 ml of milli-Q water was mixed with an equal volume (2 ml) of OB dissolution buffer (0.1M Na_2CO_3; 0.01M Na_2EDTA; 0.17 M NaCl pH 10.8) and incubated for 10 min at room temperature until OB dissolution was completed (monitored by eye or phase contrast microscopy). The suspension was then neutralized by the addition of 1/10th volume (450 µl) of 0.5 M Tris-HCl pH7.5. The tube was placed on ice to reduce enzymatic activity. Insoluble debris was removed by low speed centrifugation (2 min, 800 g). Supernatant was transferred in an SW32 tube. Pellets were again flushed with 5 ml of 0.01M Tris-HCl pH7.5 and centrifuged at 800g for 2 min and the supernatant was added to an SW32 tube.

The supernatant with released ODVs was then supplemented with 0.01 M Tris-HCl pH7.5 to a volume of 25 ml and transferred to a SW32 Beckman tube. A volume of 10 ml of sucrose cushion (20%) was pipetted under the ODVs by stabbing a pipet with sucrose solution

to the bottom of the tube and releasing the sucrose solution gently. The ODVs were pelleted by centrifugation in a SW32 Beckman rotor at 95,000 g for 1 h. The supernatant was removed carefully and pellets were re-suspended in 500 µl of 0.01 M Tris-HCl, pH7.5. The pellets were stored overnight at 4ºC for further resuspension of the ODVs.

Extraction of viral DNA from ODVs

The ODVs (from 5×10^8 OBs) were incubated at 60ºC for 5 minutes to reduce nuclease activity. The ODV sample was then incubated in 1.0% SDS (add 0.1 vol 10% SDS) and 5mg/ml proteinase K (Sigma) at 37ºC for 2 h. Viral DNA was extracted twice with an equal volume of phenol:chloroform:isoamylalcohol (50:48:2) and once with an equal volume of chloroform:isoamlyalcohol, rotated for 30 min and centrifugation for 5 min at 14000 rpm. The phenol/chloroform phases were extracted again with 300 µl TE, rotation for 10 min and centrifugation for 5 min at 14000 rpm. Supernatants were pooled and volume adjusted. After chloroform isoamylalchohol extraction of the phenol, the DNA was precipitated overnight with ice.

PCR amplification of partial 'ORF24' **and polyhedrin genes**

Wild-type virus isolates were checked through PCR and sequencing. Previously isolated DNA from SpltNPV-Pak-BNG, SpltNPV-G1 and AcMNPV was used as templates for PCR. A primer pair that consisted of 5' GGACGGCCCGTTTGTGGAC 3' and 5' GTCTCCGACGCGCTTCGTG 3' was used to amplify a partial portion of the SpltNPV-specific 'ORF24' gene encompassing nucleotides 24586-25785 in the SpltNPV-G2 genomic sequence (Pang et al., 2001). A SpltNPV-specific primer derived from the polyhedrin gene was used to confirm the presence of SpltNPV-Pak-BNG, SpltNPV-G1 and AcMNPV. This primer consisted of 5' GCGAGGCCGACGAGCGTG 3' and 5' CGCGGGACCCGTGTATAG 3', which amplified a portion of the SpltNPV polyhedrin gene including nucleotides 131-744. The AcMNPV polyhedrin was used as control to confirm the identity of the SpltNPV isolates. Amplification reaction was performed using the PCR cycling program; step 1: 94ºC for 3 minutes, step 2: 94ºC for 50 seconds, annealing temperature of 53ºC for 50 seconds, elongation temperature of 72ºC for 1 minute (step 2 cycles of 10 minutes) and a further 35 cycles as above. PCR product was run on an agarose gel (0.7% in TAE buffer) to confirm the expected length of DNA band.

Restriction endonucleases analysis (REN)

Viral DNA (0.5 µg) was cleaved after incubation with *Eco*R1 (10U) and *Bam*H1 (10U) at 37ºC for 4 h. The reaction was stopped by the addition of 1/6 volume of loading buffer (2.5 µg/ml bromophenol blue and 40 mg/ml sucrose). The generated DNA fragments were separated by 0.7% agarose gel electrophoresis carried out at 20 Volts overnight, using 1× TAE buffer. The phage lambda *Pst*1 DNA ladder was used as molecular size marker.

DNA sequencing

'ORF24', *polyhedrin*, *DNApol*. *lef-8* and *pif-2* sequences of both SpltNPV-Pak-BNG and SpltNPV-G1 genomes were obtained by Illumina MiSeq V3 sequencing with 2*300 nucleotide Paired Ends reads (coverage about 4000/base on average) according to the manufacturer's instruction and *de novo* assembly of a consensus genome sequence. The sequences are provided in a supplementary file. The consensus sequences of the above ORFs were compared against the SpltNPV-G2 (Accession number AF325155.1) and SpliNPV-AN1956 (Accession number JX454574.1) in NCBI reference genome database (www.ncbi.nlm.nih.gov/genome/) and the nucleotide sequence identity tabulated.

3. Results

Biological activity of SpltNPV-Pak-BNG and SpltNPV-G1

Mortality increased with dose for both virus isolates (Fig. 1). The slopes were similar suggesting similar susceptibility among the larvae of *S. litura* for the two tested isolates of SpltNPV (Table 1). In the first and second replicate, the LD_{50} values of the tested isolates did not differ significantly ($P \geq 0.05$). However, in the third replicate, the LD_{50} values of the tested isolates were significantly different and the hypothesis of equality was rejected ($P \leq 0.05$). When the data of the three replicates were combined for an analysis of pooled data, the LD_{50} values were not significantly different between the tested isolates ($P \geq 0.05$) (Table 2).

The stratified analysis with logistic regression resulted in three competing models with similar support from the data according to AIC ($\Delta AIC < 2$) (Table 3). The competing models were model 1, which assumed only a dose effect, model 2, which accounted for a dose effect and a difference between the two virus genotypes, and model 5, which accounted for interactive effects of virus strains and replicates (Table 3).

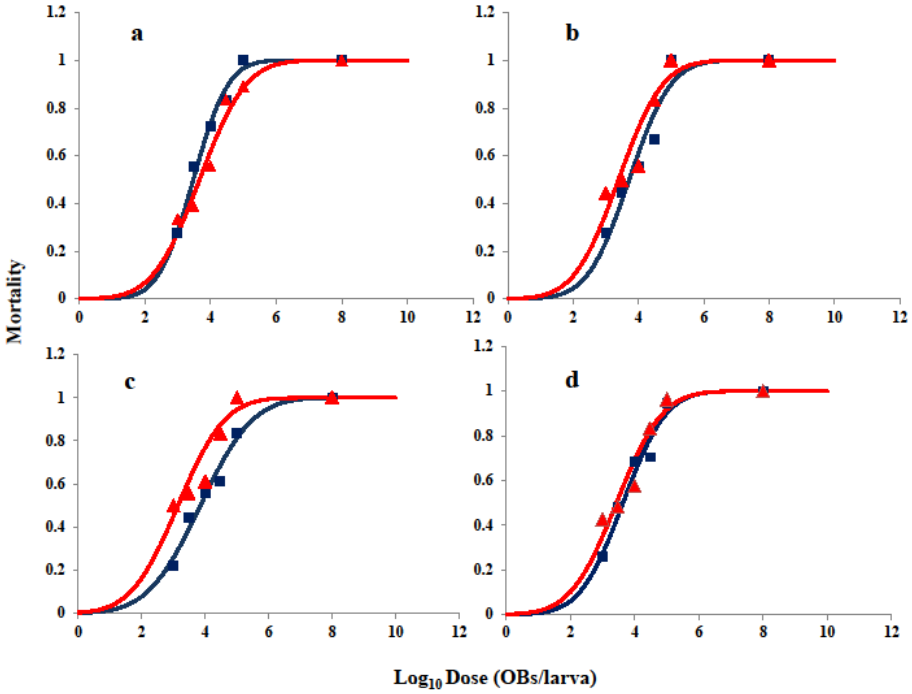

Figure 1. Dose-mortality response for third instar larvae of *S. litura* to SpltNPV-Pak-BNG (■) and SpltNPV-G1 (▲). Three replicate experiments were conducted (panels a-c). Panel d shows the dose response for pooled data.

Table 2. Dose-mortality response (OBs/larva) of 3rd instar larvae of *S. litura* to SpltNPV-Pak-BNG and SpltNPV-G1 isolates.

Replicate	SpltNPV isolate	LD_{50} (OBs/ml/larva)	95% confidence limits		Slope±SE[b]
			Lower	Upper	
Replicate 1	BNG	2.91×10^3 [a*]	1.22×10^3	5.23×10^3	1.20±0.26
	G1	4.43×10^3 [a]	1.58×10^3	9.13×10^3	0.90±0.21
Replicate 2	BNG	5.00×10^3 [a]	2.12×10^3	9.71×10^3	1.01±0.23
	G1	2.61×10^3 [a]	1.70×10^2	7.31×10^3	0.94±0.23
Replicate 3	BNG	7.31×10^3 [a]	2.56×10^3	1.75×10^4	0.77±0.20
	G1	1.80×10^3 [b]	3.05×10^2	4.07×10^3	0.86±0.23
Pooled data	BNG	4.55×10^3 [a]	2.82×10^3	6.8×10^3	0.95±0.13
	G1	2.76×10^3 [a]	6.51×10^2	6.05×10^3	0.89±0.13

[a] LD50's with the same letter within the same replicate (superscript a or b) are not significantly different at $P<0.05$. [b]SE is the standard error of the slope

The Chi squared test was used to test significance of the virus effect in model 2 when compared to the null model 1. The difference in deviance of 2.24 was not significant ($P > 0.05$) (Table 4). Likewise, the reduction in deviance when adding the interaction was not significant (Table 4). Hence, logistic regression supports the conclusion that the two virus genotypes have a similar dose response, which is in agreement with the probit analysis of pooled data done in PoloPlus.

Table 3. Ranking of the models for SpltNPV-Pak-BNG and SpltNPV-G1 in 3rd instar larvae of *S. litura*.

Model	Effects	Df	AIC
1	Dose	2	120.6
2	Dose + Virus	3	120.4
3	Dose + Rep	4	124.4
4	Dose + Virus + Rep	5	124.2
5	Dose + Virus * Rep	7	120.7
6	Dose * Virus * Rep	12	128.8

Table 4. Analysis of deviance of three competing models for dose-response of SpltNPV-Pak-BNG and SpltNPV-G1 in 3rd instar larvae of *S. litura*.

Model	D.f.	Residual Deviance	Pr (>Chi)
1	40	34.16	
2	39	31.92	0.134
5	35	24.25	0.104

A viral dose of 1×10^8 OBs/larva was used to observe the time mortality response (ST_{50}). This dose achieved 100% mortality in 3rd instar larvae of *S. litura*. In each of the three replicates of the experiment, survival was shorter after exposure to SpltNPV-BNG than after exposure to SpltNPV-G1. In the first replicate the median survival time (ST_{50}) (Kaplan-Meier estimator) was 96 hours post infection (hpi) for SpltNPV-Pak-BNG and 108hpi for SpltNPV-G1, significantly different according to the log-rank test ($P = 0.044$) (Fig. 2a). In the second replicate, the ST_{50} was 84hpi for SpltNPV-Pak-BNG and 108hpi for SpltNPV-G1, significantly different in the log-rank test ($P \leq 0.0001$) (Fig. 2b). In the third replicate, the ST_{50} was 84hpi for SpltNPV-Pak-BNG and 108hpi for SpltNPV-G1, again significantly different in the log-rank test ($P = 0.02$) (Fig. 2c). When pooling the data of the three replicates, the ST_{50} was 84 hpi for SpltNPV-Pak-BNG and 108hpi for SpltNPV-G1 infected larvae of *S. litura*, significantly different in the log-rank test at $P \leq 0.0001$) (Fig. 2d).

Cox' Proportional hazards model found no significant difference in survival time in replicate 1 between SpltNPV-Pak-BNG and SpltNPV-G1 ($P>0.05$), but significant differences in survival time between SpltNPV-Pak-BNG and SpltNPV-G1 were found in replicates 2 and 3 ($P<0.05$).

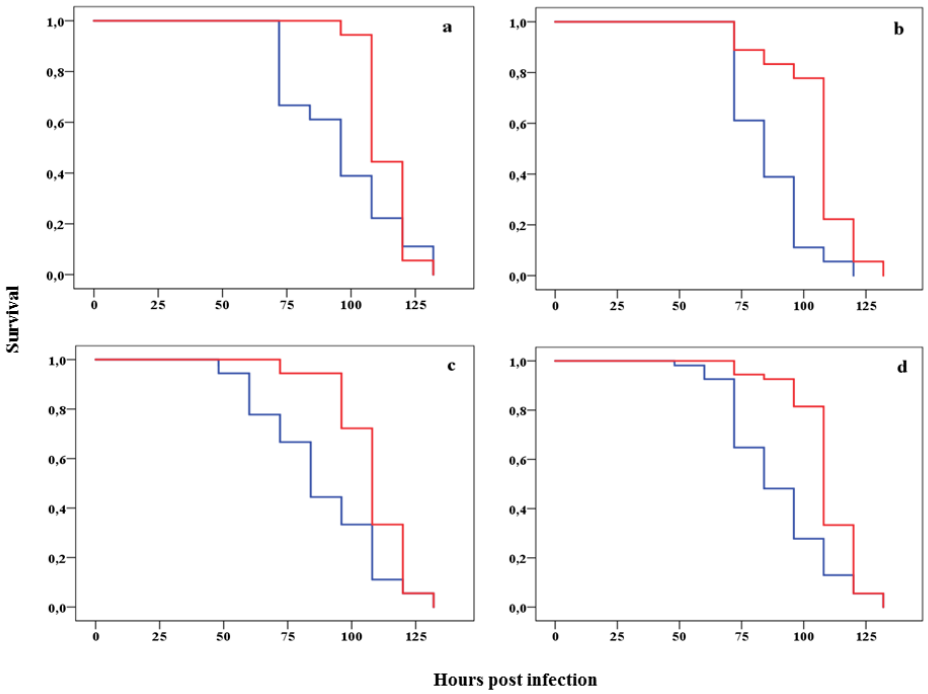

Figure 2. Survival of *Spodoptera litura* L3 infected with SpltNPV-Pak-BNG (blue colour) and SpltNPV-G1 (red colour). Three replicates are shown (panels a-c). Panel d show the survival curves for pooled data of the three replicates.

In an overarching Cox regression of the data of the three replicates, there was a highly significant effect of isolates on the survival time ($P < 0.001$), and a marginally significant effect of replicates ($P = 0.052$) but no interaction between replicates and virus isolates. The overarching Cox regression thus supports the overall highly significant difference in survival time between SpltNPV-Pak-BNG and SpltNPV-G1, identified in the analyses per replicate.

PCR amplification of ORF24

ORF24 and ORF21 appeared to be a unique homolog for SpltNPV (Pang et al., 2001) and SpliNPV (Breitenbach et al., 2013), respectively, and good markers for their identity.

Table 5. Analysis of deviance for Cox regression model comparing survival time following exposure of *S. litura* L3 to SpltNPV-Pak-BNG and SpltNPV-G1 in three replicate experiments. Terms were added sequentially to the model.

Effect	-log(likelihood)	$\chi 2$	df	P
Intercept	400.93			
Isolates	395.34	11.18	1	0.0008
Replicate	392.39	5.89	2	0.052
Isolates × Replicates	391.46	1.86	2	0.39

Two sets of primers, one for the SpltNPV-specific ORF-24 gene and one for the SpltNPV-specific polyhedrin gene (Pang et al., 2001), were used to confirm the identity of SpltNPV-Pak-BNG, SpltNPV-G1. AcMNPV was used as a negative control for the presence of SpltNPV ORF24. The 'ORF-24' set of primers annealed to DNA sequence within the coding region and produced an amplified product of 1120 bp for SpltNPV-Pak-BNG and slightly smaller amplified product for SpltNPV-G1. However, a negative response using AcMNPV DNA as template was observed. The polyhedrin-based primer annealed to DNA sequences within the coding region of the polyhedrin gene and produced an amplified product of 613bp for SpltNPV-Pak-BNG, SpltNPV-G1 and AcMNPV (Fig. 3).

Restriction enzyme and sequence analysis

Restriction endonucleases (REN) were used to further authenticate SpltNPV-Pak-BNG and SpltNPV-G1. The electrophoresis profiles of genomes following digestion with *Eco*R1 and *Bam*H1 are shown in Fig. 4. The *Eco*R1 and *Bam*H1 REN profiles of SpltNPV-Pak-BNG were quite different from those of SpltNPV-G1, with very few fragments in the same position. The fragment patterns for *Bam*H1 for both tested viruses were also very different suggesting all in all that SpltNPV-Pak-BNG and SpltNPV-G1 are different virus species.

To further investigate the nature of the two SpltNPV isolates, the sequences of 'ORF24' and four conserved baculovirus core genes, *polyhedrin*, *DNApol*, *pif-2* and *lef-8*, were analysed from a de novo assembled consensus sequence from Illumina MiSeq analysis of the respective SpltNPV genomes and compared (Table 6; Appendix 1 for sequences).

The SpltNPV-Pak-BNG nucleotide sequences of these genes were >98% identical to the sequence of SpltNPV-G2 (Pang et al., 2001), whereas the SpltNPV-G1 we researched was

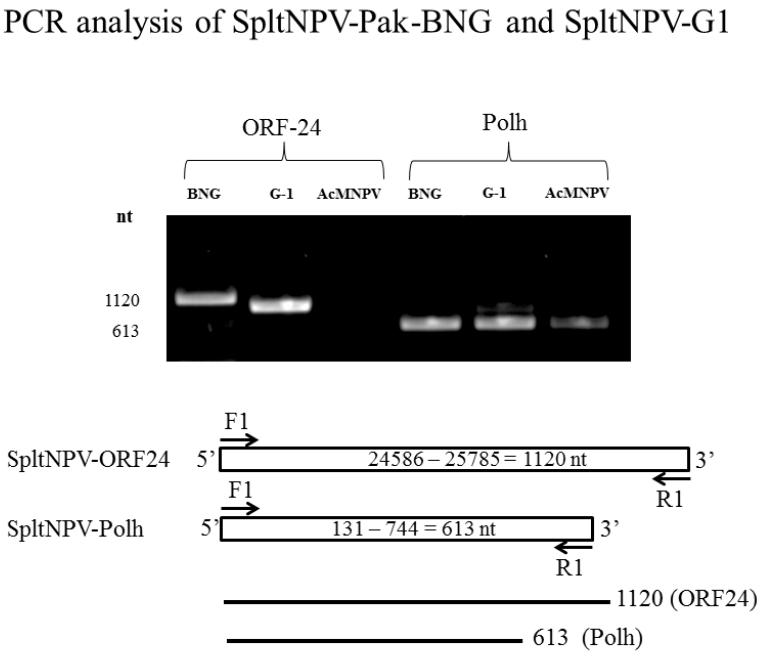

Figure 3. PCR analysis of SpltNPV-Pak-BNG and SpltNPV-G1.

>99% identical to the SpliNPV-AN-1956 isolate (Breitenbach et al., 2013). The nucleotide identity of the *polyhedrin*, *DNApol*, *pif-2* and *lef-8* sequences of SpltNPV-Pak-BNG and SpltNPV-G1 were 94%, 87%, 89% and 88%, respectively (Table 6). The sequences of the 'ORF24' of SpltNPV-Pak-BNG and SpltNPV-G1 had a nucleotide sequence identity of 76%, which is slightly higher than reported (Breitenbach et al., 2013) for ORF24 of SpltNPV-G2 and its homolog ORF21 in SpliNPV-AN1956. Sub-molar bands were observed in the REN profiles of SpltNPV-Pak-BNG and SpltNPV-G1 DNA indicating mixed genotypes within the isolates analyzed. On the basis of the overall REN patterns SpltNPV-Pak-BNG and SpltNPV-G1 are designated as two distinct SpltNPVs.

4. Discussion

Two isolates of Spodoptera litura nucleopolyhedrosis virus (SpltNPV) were analyzed and compared in this study, SpltNPV-Pak-BNG and SpltNPV-G1. SpltNPV-Pak-BNG is an authentic SpltNPV from Pakistan and a potential candidate for control of the cotton leafworm.

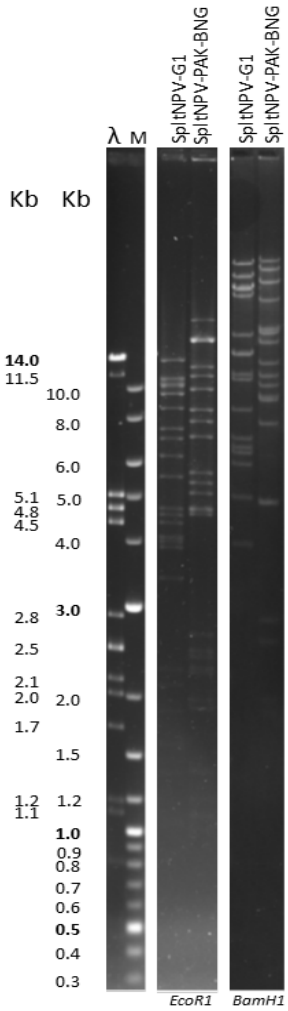

Figure 4. Restriction fragment pattern of SpltNPV-Pak-BNG and SpltNPV-G1 genomic DNA using *Eco*R1 and *Bam*H1. The generated DNA fragments were separated over 0.7% agarose gel electrophoresis carried out at 20 Volts overnight, using 1× TAE buffer. The phage lambda *Pst*1 DNA ladder was used as molecular size marker.

These two viruses showed a similar dose-mortality response (not significantly different) (Table 1; Fig 1). The new isolate SpltNPV-Pak-BNG killed 3rd instar larvae of *S. litura* about 24 h faster (84 h) than the reference isolate SpltNPV-G1 (108 h) (Table 2; Fig. 2). The latter also holds for the dose mortality and time-to-death response of 2nd and 4th instars (see Appendices) to SpltNPV-Pak-BNG.

Table 6. Pairwise nucleotide sequence identity of 'ORF24', *polyhedrin*, *DNApol*, *lef-8* and *pif-2* of *de novo* assembled SpltNPV-Pak-BNG and SpltNPV-G1 genomes from a MiSeq analysis.

	SpltNPV-Pak-BNG	SpltNPV-G2	SpltNPV-G1	SpliNPV-AN1956
	%	%	%	%
ORF24 / **DNApol**				
SpltNPV-Pak-BNG		99	76	76
SpltNPV-G2	**99**		76	76
SpltNPV-G1	**86**	**87**		99
SpliNPV-AN1956	**86**	**87**	**99**	
pif-2 / **lef-8**				
SpltNPV-Pak-BNG		99	89	89
SpltNPV-G2	**99**		89	89
SpltNPV-G1	**88**	**88**		99
SpliNPV-AN1956	**88**	**88**	**99**	
polh				
SpltNPV-Pak-BNG		99	94	93
SpltNPV-G2			94	94
SpltNPV-G1				99
SpliNPV-AN1956				

Note: Bolded numbers refer to bolded ORFs in the left column; unbolded numbers refer to unbolded ORFs in the left column.

Previous studies demonstrated no evidence of a difference in LD_{50} and ST_{50} of geographically distinct SpltNPV isolates from Japan, Vietnam, Malaysia and India collected from diseased *S. litura* larvae and an Egyptian *S. littoralis* NPV isolate (Takatsuka et al., 2003). However, our results show that SpltNPV-Pak-BNG kills *S. litura* larvae considerably faster than SpltNPV-G1. Previous studies demonstrate that the variation in biological activity of nucleopolyhedroviruses may depend on the insect colonies (Barrera et al., 2011; Erlandson, 2009). Our local *S. litura* biotype from the NARC colony expresses greater sensitivity to SpltNPV-Pak-BNG than to the exotic isolate SpltNPV-G1 and may be useful to study the biological properties of SpltNPVs.

The nucleotide identity of the 'ORF24'-like sequences of both viruses suggests that they are highly related and representative for SpltNPV- and SpliNPV-like viruses (Pang et al., 2001; Breitenbach et al., 2013). The smaller size PCR product of the 'ORF24'-like with SpltNPV-G1 is due to a few gaps in the latter as compared to SpltNPV-Pak-BNG. Genetic analysis showed much larger differences between the two virus isolates, e.g. in the REN profile pattern. The REN pattern of SpltNPV-Pak-BNG corresponds to the REN patterns of SpltNPV-Type-B (Kamiya et al., 2004) and Japanese SpltNPVs (Takatsuka et al., 2003; Takatsuka et al., 2016) on the basis of visual inspection of the REN digests. On the other hand, the SpltNPV-G1 REN

profiles are very different from SpltNPV-Pak-BNG, but they appear closely related to SpltNPV-Type-A (Kamiya et al., 2004) and SpliNPV-AN-1956 (Breitenbach et al., 2013). Sequence analysis of the baculovirus core genes *polyhedrin*, *DNApol*, *lef-6* and *pif-2* (Table 6) supports this observation. Therefore the SpltNPV isolate from the Darmstadt baculovirus depository should be renamed SpliNPV-G1 rather than SpltNPV-G1. It cannot be excluded, though, that the SpltNPV-G1 was isolated originally from *S. littoralis*, as Takatsuka et al. (2016) also found a SpliNPV in a field isolate from Japan, which also contained a SpltNPV Sequencing of host DNA present in the Darmstadt SpltNPV-G1 sample should clarify this point (Rohrmann et al., 2014).

Whole genome sequencing of both SpltNPV-Pak-BNG and SpltNPV-G1 should indicate to what extent these two viruses are variants of SpltNPV and a SpliNPV, respectively. It has been shown that large genetic variation exists between SpltNPV and a SpliNPV field isolates (Kamiya et al., 2004; Takatsuka et al., 2003; Takatsuka et al., 2016) and that biological variation between isolates exists (Takatsuka et al., 2016). So, it is worthwhile to obtain further SpltNPV isolates from the field in Pakistan for genetic and biological analysis to select the best biotype for biocontrol. In addition, this information may render further support for the supposition that SpltNPV and SpliNPV are different baculovirus species, but within the same clade.

In a previous study SpltNPV and SpliNPV were isolated from *S. litura* in Japan (Takatsuka et al., 2016). This study found that SpliNPV is more infectious and killed *S. litura* larvae faster than SpltNPV. Here we find that SpltNPV-Pak-BNG is fast acting compared to SpliNPV-G1. SpliNPV-G1 may originally have been found in *S. litura*, as this has also been the case in Japan as mixed infections with SpltNPV (Takatsuka et al., 2016). If our conclusion turns out to be correct, it may be advisable to use SpltNPV isolates to control *S. litura* rather than using SpliNPV isolates, because the former virus is faster-acting. It would also be interesting to compare these two viruses side-by-side in *S. littoralis* to see whether the response is reciprocal. Lastly, SpltNPV-Pak-BNG is a natural Pakistani isolate and probably better adapted to the local ecosystem.

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Chapter 3

Biological activity of a Pakistani isolate of Spodopera litura nucleopolyhedro-virus in second, third and fourth instar larvae of *Spodoptera litura*

Adapted from:
Ghulam Ali, Just M. Vlak and Wopke van der Werf. Biological activity of Pakistani isolate SpltNPV-Pak-BNG against second, third and fourth instar larvae of *Spodoptera litura*. Manuscript submitted.

Abstract

Here, we examined the biological activity of a recently described Pakistani isolate of Spodoptera litura nucleopolyhedrovirus (SpltNPV-Pak-BNG) against second (L2), third (L3) and fourth instar (L4) larvae of the leafworm *S. litura* to measure biological properties that are relevant for use of this virus for pest control under field conditions. The median lethal dose for L2 and L3 instar larvae was similar, but significantly lower than for L4 larvae. Likewise, the survival time was similar for L2 and L3 larvae (84h), but was significantly longer for L4 instar larvae (108h). Thus, in terms of efficacy, *S. litura* L2 and L3 instar larvae are the preferred targets for *S. litura* control with SpltNPV-Pak-BNG in field crops in Pakistan.

Keywords: *Spodoptera litura* nucleopolyhedroviruses; *S. litura*; larval instars; biological activity, potency

1. Introduction

A novel Pakistani isolate of Spodoptera litura nucleopolyhedrovirus (SpltNPV), named SpltNPV-Pak-BNG, has been recently described (Ali, van der Werf & Vlak, 2017). This indigenous isolate is a potential biocontrol agent of the leafworm *S. litura,* an emerging pest in cotton and vegetable crops in Pakistan. In contrast to the cotton bollworm *Helicoverpa armigera*, the leafworm can feed on Bt-cotton, as it is insusceptible to the Bt-toxin. However, further information on the biological properties, in particular the potency against various instars of the leafworm are lacking. Therefore this study was undertaken.

2. Materials and methods

The rearing of *S. litura* and the origin of SpltNPV-Pak-BNG and the production of occlusion bodies (OBs) was described previously (Ali, van der Werf & Vlak, 2017). SpltNPV-Pak-BNG was administered at the appropriate dose to three larval instars of *S. litura* to characterize the median lethal dose and median lethal time to death. For each replicate, thirty newly moulted second and third instar larvae were placed in Petri dishes, while fourth instar larvae were placed individually in 24 well plates to avoid cannibalism. The larvae were starved overnight for 15h prior to infection, after which they were fed with inoculated leaf discs and mortality was observed until death or pupation as described previously (Ali, van der Werf & Vlak, 2017).

Statistical analysis of dose responses was conducted with probit analysis and logistic regression as described (Ali, van der Werf & Vlak, 2017). Survival analysis with data per replicate and pooled data was done in SPSS while a stratified analysis across replicates was made using Cox' proportional hazards model using the R package survival.

3. Results

Dose response of SpltNPV-Pak-BNG for three larval instars of *S. litura*

Second and third instar larvae showed a similar dose-mortality response, but fourth instar larvae were less susceptible than L2 and L3 larvae (Table 1). The slopes were similar for second and third instar larvae, but were slightly lower for fourth instars. This suggests greater variability in susceptibility between individuals in the L4 stage as compared to those in the L2 and L3 stages (Fig 1). In each of the three replicates, the LD_{50} values were similar for L2 and L3 ($P \geq 0.05$) but different for the 4^{th} larval instar ($P \leq 0.05$) (Table 1). In the pooled analysis, there was a

Table 1. Dose-mortality response (OBs/larva) of second, third and fourth instar larvae of *S. litura* to SpltNPV-Pak-BNG

Replicates	Larval stage	LD_{50} (OBs/ml/larva)	95% confidence limits		Slope ±SE
			Lower	Upper	
1	L2	2.34×10^{3} [a]	9.39×10^{2}	4.18×10^{3}	1.25±0.27
	L3	3.46×10^{3} [ab]	1.63×10^{3}	6.04×10^{3}	1.62±0.26
	L4	1.06×10^{4} [b]	3.90×10^{3}	2.93×10^{4}	0.71±0.18
2	L2	3.26×10^{3} [a]	1.40×10^{3}	5.90×10^{3}	1.16±0.25
	L3	5.00×10^{3} [ab]	2.12×10^{3}	9.70×10^{3}	1.01±0.23
	L4	1.51×10^{4} [b]	6.90×10^{3}	3.97×10^{4}	0.85±0.20
3	L2	4.23×10^{3} [a]	2.00×10^{3}	7.53×10^{3}	1.19±0.25
	L3	5.53×10^{3} [ab]	2.45×10^{3}	1.06×10^{4}	1.02±0.22
	L4	3.62×10^{4} [b]	1.82×10^{4}	1.12×10^{5}	0.99±0.22
Pooled data	L2	3.17×10^{3} [a]	1.65×10^{3}	5.17×10^{3}	1.19±0.15
	L3	4.53×10^{3} [ab]	2.25×10^{3}	7.85×10^{3}	1.08±0.13
	L4	1.88×10^{4} [b]	8.42×10^{3}	5.65×10^{4}	0.82±0.11

[a]Values followed by the same letter are not significantly different in pairwise t-test at $P\geq0.05$.

significant difference between L2 and L4 stages only, with L3 intermediate, but not significantly different from L2 or L4 in the median lethal dose.

Several models were fitted in the stratified analysis, and the best of those was a model with dose, instar and replicate as additive effects (model 4; Table 2). That is: all dose-response curves had a common slope, but the intercept varied according to additive influences of replicate and instar. Intercept parameter estimates for L2 and L3 larvae were similar, and not significantly different in t-test ($P > 0.05$) (Table 3). The intercept parameter for L4 larvae was substantially negative and significantly different from those for L2 and L3, confirming the lower susceptibility of the L4 stage to the virus (Table 3). The logistic regression indicated that the susceptibility to virus increased from replicate 1 to replicate 2 to replicate 3, with a significant difference between replicate 1 and 3, but not between replicates 1 and 2 or between replicates 2 and 3.

In summary, both probit analysis and logistic regression indicates that there is no difference in dose-mortality response between L2 and L3 but L4 was less susceptible than the earlier instars tested.

Table 2. AIC of competing models for SpltNPV-Pak-BNG in second, third and fourth instar larvae of *S. litura*.

Model	Effects	D.f.	AIC
1	Dose	1	218.4
2	Dose + Instar	4	175.1
3	Dose + Rep	4	213.61
4	Dose + Instar + Rep	7	169.75
5	Dose + Instar × Rep	10	175.79
6	Dose × Instar × Rep	9	186.32

Table 3. Parameter estimates in the best logistic regression model (model 4; Table 2) of mortality response to dose of SpltNPV-Pak-BNG in three replicate experiments with second, third and fourth instar larvae and of 3 replicates of *S. litura.*

Parameter	Coefficient	Z value
Intercept	-5.6367	-11.125 ***
Dose	1.7185	13.356 ***
L3	-0.3173	-1.539
L4	-1.35	-6.393 ***
Replicate 2	-0.3163	-1.536
Replicate 3	-0.6263	-3.036 **

** $P < 0.01$; *** $P < 0.001$

Time mortality response of SpltNPV-Pak-BNG for three larval instars of *S. litura*

A viral dose of 1×10^8 OBs/larva was used to determine the time mortality response. This dose achieved 100% mortality in each of the three larval stages. In the first replicate, the median survival time (ST_{50}) response of SpltNPV-Pak-BNG was 84 h post infection (hpi) in both L2 and L3, and 108 hpi in L4. For L3 larvae, this is the same ST_{50} as described in Ali et al., 2017 in a separate experiment. The survival times were different between L2 and L3 (log-rank test, $P = 0.027$) and between L2 and L4 (log-rank test, $P \leq 0.0001$). However, there was no significant difference in survival time between L3 and L4 (log-rank test, $P = 0.91$) (Fig. 4a).

In the second replicate, the ST_{50} of SpltNPV-Pak-BNG was 72 hpi in L2, 84 hpi in L3, and 108 hpi in L4. The survival times of the second and third larval instars were not significantly different (log-rank test, $P = 0.088$), but those of the second and fourth larval instars were significantly different (log-rank test, $P \leq 0.0001$). Those of L3 and L4 larval instars were also significantly different (log-rank test, $P = 0.012$) (Fig. 4b).

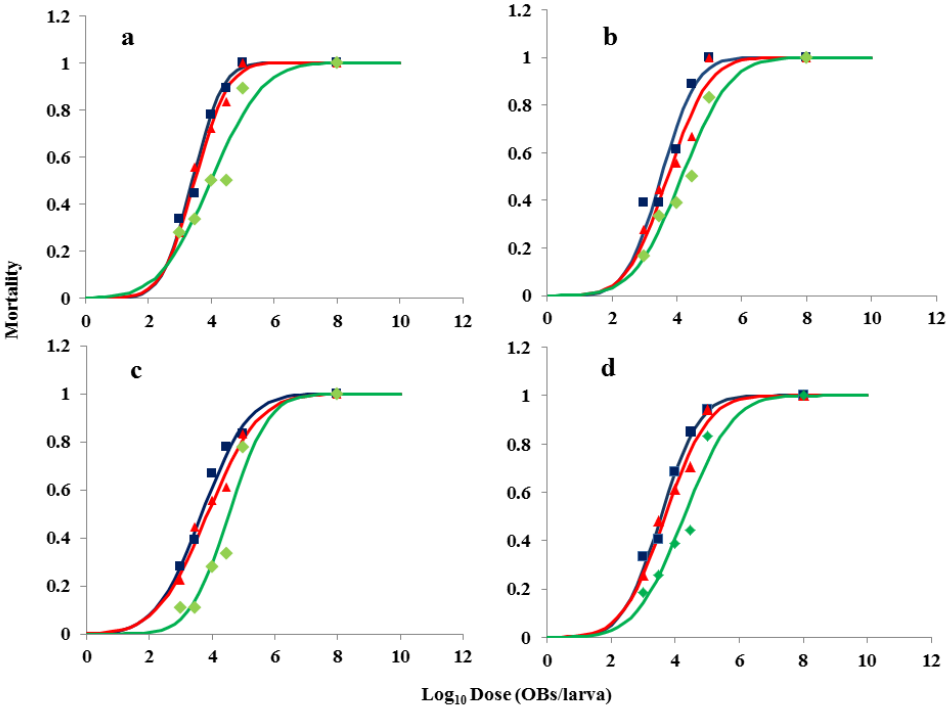

Figure 1. Dose-mortality response for second (■), third (▲) and fourth (◆) instar larvae of *S. litura* to SpltNPV-Pak-BNG. Results of three replicate experiments are shown in panels a-c. Panel d shows the dose-response for the pooled data of the three replicates

In the third replicate, the median survival time values were the same as in replicate 1. The survival times were not significantly different between L2 and L3 (log-rank test, P = 0.077), highly significantly different between second and fourth instar larvae (log-rank test, P = 0.001) and also highly significantly different between the third and fourth instar (log-rank test, P = 0.004) (Fig. 4c). When the data were pooled, The median survival times were the same as in replicates 1 and 3, with no significant difference in ST_{50} between L2 and L3 (log-rank test, P = 0.023), but with a significant difference between second and fourth instar larvae (log-rank test, $P \leq 0.0001$) and between third and fourth instar larvae (log-rank test, P = 0.002) (Fig. 4d).

When the data of the three replicates were combined in a stratified analysis with Cox' regression, survival time differed highly significantly between the larval stages. Significant differences in survival time were also found among the three replicates. There was no significant interaction between larval stages and experimental replicates (Table 4).

Table 4. Analysis of deviance for the Cox regression model comparing survival time following exposure to SplitNPV-Pak-BNG in second, third and fourth instar larvae of *S. litura*.

Effect	Log likelihood	$\chi 2$	df	P
Intercept				
Larval stage	652.51	26.27	2	0.000001***
Replicate	649.04	6.94	2	0.031*
Larval stage × Replicate	646.29	5.49	4	0.24

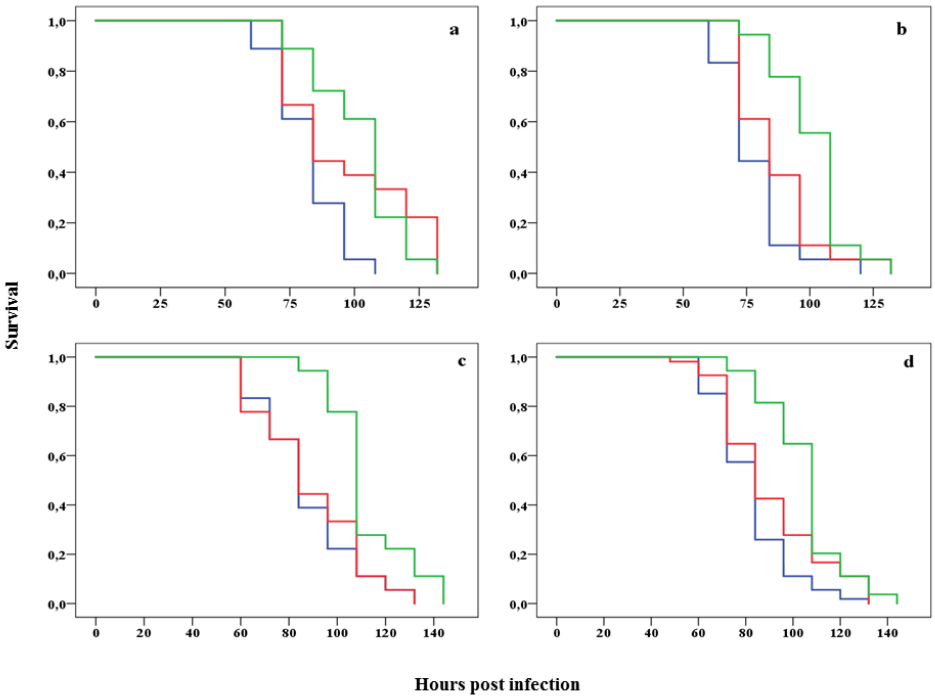

Figure 2. Survival of second (■), third (▲) and fourth (◆) instar larvae of *S. litura* infected with SpltNPV-Pak-BNG. Panels a-c show results in three replicates. Panel d shows survival curves for pooled data

4. Discussion

The susceptibility of different larval instars of *S. litura* to the wild-type SpltNPV-Pak-BNG was studied by quantifying dose-mortality response and speed of kill. The second and third instar larvae showed similar dose-mortality response, but fourth instar larvae were less susceptible. Median survival times followed the same pattern as the median lethal time values and were

similar for L2 and L3 instars, but longer for L4 instar larvae. Our results support previous studies demonstrating that susceptibility to viral infection decreases with larval age both within and between larval instars (Smits and Vlak, 1988; Ali et al., 1991; Martinez et al., 2003; Kouassi et al., 2009; Bernal et al., 2013). Similar susceptibility between the second and third larval stage to SpltNPV-Pak-BNG, might be an advantage of applying viral insecticide to infected crops. This will offers a prolonged period for infection to susceptible larval stages of *S. litura* and helps to control different larval stages following a single viral application of SpltNPV-Pak-BNG. Time and dose mortality responses of the second, third and fourth instar larvae of *S. litura* to the SpltNPV-Pak-BNG isolate suggest that the best time of virus application to infected crops is at the second and or third larval stage of *S. litura*, and even with a low concentration of virus based active ingredient for optimal insect pest management. Applying SpltNPV-Pak-BNG to later stages of *S. litura* is impractical and would not be cost-effective to manage insect pest. These findings were based on laboratory study.

Field studies are needed to further consolidate these laboratory results. The results obtained so far indicate that L2 and L3 instar larvae are the preferred stage for control in the field using SpltNPV-Pak-BNG or isolates with similar biological properties. Successful and timely application though requires detailed knowledge of the population build-up and structure of the pertinent host, *S. litura*. A field survey will be set up to isolate further Splt-Pak NPV field isolates with improved insecticidal characteristics.

Acknowledgements

We would like to thank Dr Ehsan-ul-Haq for providing the insect rearing facility in the biological control laboratory of the National Agricultural Research Centre, Islamabad, Pakistan.

Chapter 4

Genotype assembly, biological activity and evolution of spatially separated isolates of Spodoptera litura nucleopolyhedrovirus

Adapted from:
Ghulam Ali, Marleen H.C. Abma-Henkens, Lia Hemerik, Wopke van der Werf and Just M. Vlak. 2017. Genotype assembly, biological activity and evolution of spatially separated isolates of Spodoptera litura nucleopolyhedrovirus. Journal of Invertebrate Pathology, in revision

Abstract

The cotton leafworm *Spodoptera litura* is a polyphagous phytophagous insect. It has recently made a comeback as a primary insect pest of cotton in Pakistan due to reductions in pesticide use on the advent of genetically modified cotton, resistant to *Helicoverpa armigera*. Spodoptera litura nucleopolyhedrovirus (SpltNPV) infects *S. litura* and is recognized as a potential candidate to control this insect. Twenty-two NPV isolates were collected from *S. litura* from different agro-ecological zones (with collection sites up to 600 km apart) and cropping systems in Pakistan to see whether there is spatial dispersal and evolution of the virus and/or adaptation to crops. Therefore, the genetic make-up and biological activity of these isolates was measured. Among the SpltNPV isolates tested for speed of kill in 3rd instar larvae of *S. litura*, TAX1, SFD1, SFD2 and GRW1 were significantly faster killing isolates than most other Pakistani isolates. Restriction fragment length analysis of the DNA showed that the Pakistan SpltNPV isolates are all variants of a single SpltNPV subtype. The isolates could be grouped into three genogroups (A-C). The speed of kill was higher in genogroup A than in group C, according to a Cox' proportional hazards analysis. Sequence analysis showed that the Pakistan SpltNPV isolates are more closely related to each other than to the SpltNPV type species G2 (Pang et al., 2001). This suggests a single introduction of SpltNPV into Pakistan. The SpltNPV-PAK isolates are distinct from Spodoptera littoralis NPV. There was a strong correlation between geographic spread and SpltNPV genogroup, and a marginally significant correlation between the latter and the cropping system. The faster killing isolates may be good candidates for biological control of *S. litura* in Pakistan.

Keywords: Spodoptera litura nucleopolyhedroviruses; regional isolates; genotyping, biological activity; cross tabulation

1. Introduction

The alphabaculoviruses (Baculoviridae) are pathogenic to lepidopteran larvae and have potential as bio-insecticides due to their high virulence and host specificity (Moscardi, 1999). Baculovirus isolates usually contain multiple genotypes and there is diversity among the genotypes found at different geographical locations (Cory, 2005; Figueiredo et al., 2009; Redman et al., 2010; Rowley et al., 2011). Different genotypes of baculoviruses differ in dose response and time to kill (Figueiredo et al., 2009; Alexandre et al., 2010). The collection of isolates from the field is important to obtain isolates that have exploitation potential as a biological control agent because they combine high infectivity with a high speed of kill.

The cotton leafworm, *Spodoptera litura*, is a polyphagous insect pest feeding on more than 300 plant species and is widely distributed in Asia, Africa, North America and Oceania islands (Tuan et al., 2013). It is a very destructive insect causing economic yield losses up to 100% to susceptible field crops like cotton, groundnut, soybean, tobacco and vegetables (Qin et al., 2004). *S. litura* has recently staged a comeback as a primary insect pest of cotton in Pakistan due to the lower pesticide use after wider use of genetically modified cotton resistant to cotton bollworms, such as *Helicoverpa armigera* (Ahmad et al., 2007). A survey in Pakistan showed low incidence of cotton bollworms (*H. armigera, P. gosyppiella, E. vitella, E. insulana*) and a high incidence of armyworms (*S. litura* and *S. exigua*) in Bt cotton (Arshad et al., 2009). Studies on the effect of Bt cotton on *S. litura* during 2002-2005 in the cotton-planting region of the Yangtze River valley of China showed that there was no significant difference in larval population densities in conventional and Bt cotton fields. This indicates that *S. litura* is not affected by Bt Cry1Ac toxin (Wan et al., 2008).

Spodoptera litura nucleopolyhedrovirus (SpltNPV) is most frequently found in *S. litura* but can also infect *Spodoptera exigua* (Feng et al., 2007; Takatsuka et al., 2007). It has been tested and recognized as a potential candidate to control *S. litura* in Japan (Okada, 1977) and has been successfully used in China as biological insecticide against *S. litura* (Chen et al., 1998). Despite the use of SpltNPV as a bio-insecticide in these countries, little information is available on its genetic and biological diversity. Takatsuka et al. (2003) found no evidence of variation in biological activity, based on infectivity and survival time, among 10 geographically distinct SpltNPV and SpliNPV isolates. However, Maeda et al. (1990) found genetic diversity in the wild-type isolates of SpltNPV collected from Japan. These isolates were characterized through restriction endonuclease (REN) analysis and placed in four distinct groups: (I) NPV isolates corresponding to Autographa californica multiple nucleopolyhedroviruses (AcMNPV)

(Miller and Dawes, 1978; Smith and Summers, 1979), (II and IV) two different groups of SpltNPV, characterized previously as group II (SINPV-B) and IV (SINPV-A) (Kislev and Edelman, 1982; Cherry and Summers, 1985; Croizier et al., 1989) and (III) isolates with no homology to any reported virus groups.

Baculoviruses in agro-ecosystems may evolve over time and space and may adapt to the cropping system. The baculoviruses from different ecological regions may result in isolates with different biological traits. Harrison et al. (2016) reported that geographically distinct Lymantria dispar MNPV isolates exhibited different biological activities. Genetic analysis of Mamestra brassicae NPV indicated that the restriction fragment length patterns change progressively over a large geographical range (from UK to Ukraine) suggesting mutation, adaptation and establishment of incoming virus (Van Oers and Vlak, 2007).

It is advantageous to try and identify a potential biological control agent from within Pakistan to avoid the risks associated with importation and release of an alien organism (Muñoz and Caballero, 2000; Louda and O'Brien, 2002). Furthermore, a locally collected virus isolate may possess traits that support its cycling in the agro-ecosystem within it was collected, thus contributing to sustainable inoculative control (Erlandson, 2009; Barrera et al., 2011). To date, SpltNPV has not been used for biological control in Pakistan, but recently a SpltNPV isolate has been reported (Chapter 2; Ali et al., 2017). It is unknown how widespread SpltNPV is in Pakistan and no studies have been done.

We surveyed host crops and natural vegetation in regions of Pakistan to collect virus infected *S. litura* larvae in order to characterize the diversity of insect viruses and to see whether the diversity can be correlated with geographical locations or crop systems. Twenty-two (22) natural SpltNPV isolates have been obtained from 2011-2013 from different ecological regions and cropping systems in Pakistan (Table 1). It was found earlier that the SpltNPV isolate from Pakistan (SpltNPV-Pak-BNG had superior biological characteristics over the reference strain (Ali et al., 2017) giving the impetus to survey for more isolates. Potentially baculovirus isolates can be found that may be developed into a biological control product for *S. litura*. Here, we describe the survey approach, and we characterize the biological activity of the collected virus strains in terms of survival time distribution after exposure to a lethal virus dose. We describe the genetic variation among the virus isolates obtained. Genetic diversity among the collected isolates is characterized by analysis of diversity of banding patterns upon restriction enzyme analysis and sequence analysis.

2. Material and methods

2.1. Field Survey to collect wild-type SpltNPV isolates

Field survey was carried out from 2011 through 2013 from different cropping systems and host plants including cotton and vegetables to collect the dead and symptomatic larvae of *S. litura* with typical signs of polyhedrosis. Symptomatic and dead larvae with typical signs of polyhedrosis were collected individually from 11 different locations. The survey was conducted in Punjab and the Khyber Pakhtunkhwa (KPK) provinces of Pakistan. These provinces have different cropping patterns and climatic conditions. The Punjab consists mostly of irrigated land while KPK constitutes the uplands and is mostly rainfed. These two provinces represent different agro-ecological zones with differences in cropping patterns (spatial and temporal combination of crops in a field for one year) and climatic characteristics (http://pmd.gov.pk).

The Punjab is the main cropland of Pakistan with an altitude ranging from 80m to 508m, an average rainfall of 628.8 mm and temperature ranging from 4ºC in winter to 48ºC in summer. The main crops are wheat, rice, maize, cotton, sugarcane, citrus, mangoes, and vegetables. In Punjab province, three regions were distinguished (Fig. 1). Punjab-1 comprises the northern part of the province, Punjab-2 the central part and Punjab-3 the south. The northern Punjab has a humid climate during summer. The annual daily mean temperature ranges from 22-24°C and mean rainfall is 800-1000 mm. It grows mostly wheat-rice in a double cropping system. Vegetables and minor crops (chickpea, lentil, sesame, and linseed) are also grown in northern region. The central Punjab has a semi-arid climate. The annual daily mean temperature ranges from 24-26°C and the mean rainfall is 400-600 mm. The mixed cropping patterns (wheat, cotton, rice and vegetables) are grown in a double cropping system. The southern Punjab has a semi-arid to arid climate. The annual daily mean temperature ranges from 24-26°C and the mean rainfall is 200-400 mm. Double cropping of wheat and cotton (i.e. wheat followed by cotton within one year) is common in the southern Punjab. Vegetables and minor food crops are grown.

KPK is at higher altitudes with a more temperate and wetter climate than the Punjab region. The main crops are apricot, apples, maize, wheat and vegetables. The altitude ranges from 165m to 1088m, and the average rainfall is 800 mm. Temperature ranges from 3°C in winter to 36°C in summer. The upland of KPK-1 is humid with mild summers and cold winters. The annual daily mean temperature ranges from 16-18°C and the mean rainfall is 1000-1200 mm. Vegetables production shares the land use with maize cultivation and fruit orchards.

We sampled *Brassicae oleracea* and *Raphanus sativus* in wheat-rice, multiple and vegetable cropping systems and *Gossypium hirsutum* in wheat-cotton cropping pattern for *S. litura* with symptoms of baculovirus infection. The areas visited were Gujrnwala and Ghakhar (both in northern Punjab) for wheat-rice, Multan and Bahawalnagar (both in southern Punjab) for wheat-cotton and Islamabad, Rawalpindi, Taxila, Haripur, Mansehra and Bherkund for vegetable cropping systems (Fig 1).

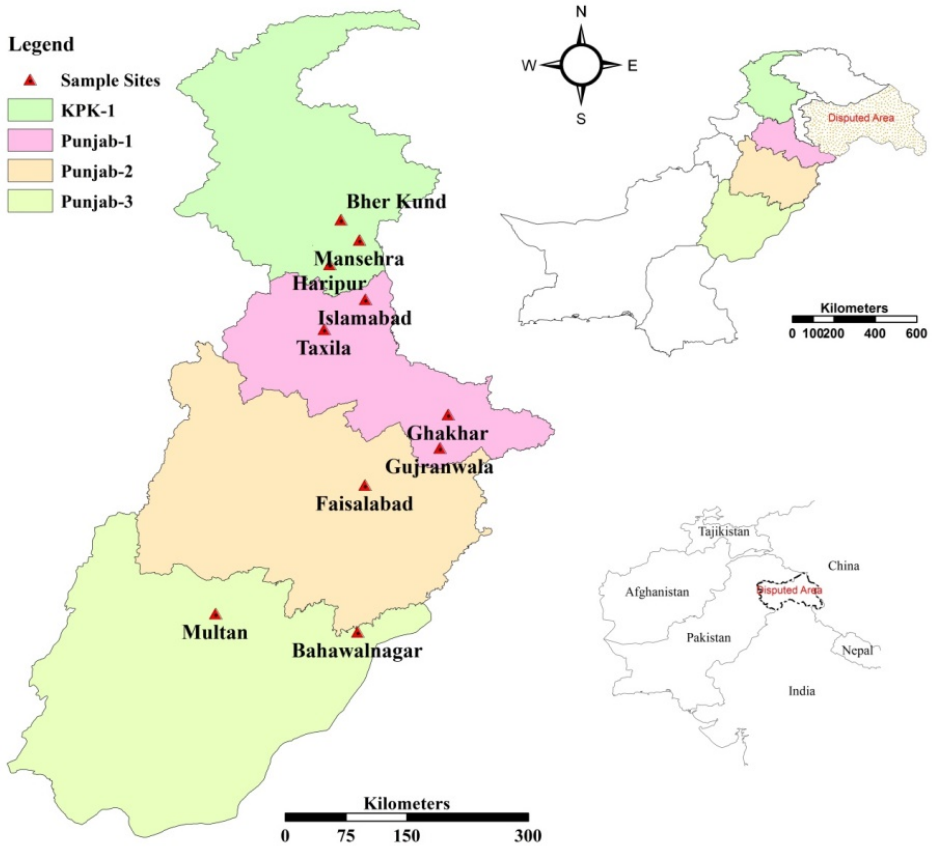

Figure 1. SpltNPV collection sites from Punjab and Khyber Pakhtunkhawa in Pakistan

Table 1. SpltNPV isolates, host plants, cropping system, sites and year of collection.

Isolate	Host plant	Cropping pattern	Collection site	Latitude	Longitude	Ecological zone	Collection date
BKM1	*Raphanus sativus*	Vegetable	Bher Kund	34.370420N	73.205176E	KPK-1	July, 2013
MNS1	*Brassicae oleracae*	Vegetable	Mansehra	34.323319N	73.224272E	KPK-1	August, 2012
MNS3	*Brassicae oleracae*	Vegetable	Mansehra	34.323433N	73.207060E	KPK-1	August, 2012
HRP6	*Brassicae oleracae*	Vegetable	Haripur	33.995371N	72.931084E	KPK-1	August, 2012
HRP7	*Brassicae oleracae*	Vegetable	Haripur	33.995371N	72.931084E	KPK-1	August, 2012
HRP10	*Brassicae oleracae*	Vegetable	Haripur	33.990511N	72.951662E	KPK-1	August, 2012
TAX1	*Brassicae oleracae*	Vegetable	Taxila	33.734947N	72.800213E	Punjab-1	July, 2013
TAX2	*Brassicae oleracae*	Vegetable	Taxila	33.734947N	72.800213E	Punjab-1	July, 2013
ISD	*Brassicae oleracae*	Vegetable	Islamabad	33.659710N	73.167585E	Punjab-1	April, 2012
GRK1	*Brassicae oleracae*	Rice-wheat	Ghakar	32.309925N	74.144323E	Punjab-1	Oct., 2013
GRK2	*Brassicae oleracae*	Rice-wheat	Ghakar	32.309925N	74.144323E	Punjab-1	Oct., 2013
GRK4	*Brassicae oleracae*	Rice-wheat	Ghakar	32.301886N	74.145481E	Punjab-1	Oct., 2013
GRK5	*Brassicae oleracae*	Rice-wheat	Ghakar	32.301886N	74.145481E	Punjab-1	Oct., 2013
GRW1	*Brassicae oleracae*	Rice-wheat	Gujranwala	32.131205N	74.174233E	Punjab-1	April, 2013
GRW2	*Brassicae oleracae*	Rice-wheat	Gujranwala	32.132154N	74.172897E	Punjab-1	April, 2013
GRW3	*Brassicae oleracae*	Rice-wheat	Gujranwala	32.132154N	74.172897E	Punjab-1	April, 2013
SFD1	*Brassicae oleracae*	Multiple crops	Faisalabad	31.728073N	73.578177E	Punjab-2	Apri, 2013
SFD2	*Brassicae oleracae*	Multiple crops	Faisalabad	31.728073N	73.578177E	Punjab-2	April, 2013
BNG	*Gossypium hirsutum*	Cotton-wheat	Bahawalnagar	30.020496N	73.240792E	Punjab-3	Nov, 2011
MTN1	*Gossypium hirsutum*	Cotton-wheat	Multan	30.282947N	71.647677E	Punjab-3	April, 2012
MTN2	*Gossypium hirsutum*	Cotton-wheat	Multan	30.282947N	71.647677E	Punjab-3	April, 2012
MTN4	*Gossypium hirsutum*	Cotton-wheat	Multan	30.079634N	71.494290E	Punjab-3	April, 2012
G1	*Gossypium hirsutum*		China			Guangzhou	2001

Six field visits were conducted from November 2011 through October 2013, to collect symptomatic and dead larvae of *S. litura* for the isolation of SpltNPV (Table 1). *S. litura* hibernates from November to March while field populations are low in Pakistan during the months of May and June due to high temperature and low humidity. The collections were therefore made at times that higher population densities were expected in the field, i.e. in April and in July - October. Symptomatic *S. litura* larvae with typical signs of polyhedrosis were collected and placed in a plastic vial covered with wire-meshed lid, and dead larvae in Eppendorf tubes. The sample information was marked on the plastic vials and Eppendorf tubes as; collection site, date, host plant, cropping system. The symptomatic collected larvae were kept individually at circa 26°C in the insect rearing laboratory at CABI Pakistan till the pupation or death of the larvae. The dead larvae were preserved at 4°C in the dark until further diagnosis.

A total of twenty-two SpltNPV isolates were harvested from the ten different locations of the surveyed area. Each strain isolated from a single cadaver, represents a single isolate. The viral isolate collection sites, host plants and cropping systems are presented in Table 1. Field collected isolates were tagged as; Bahawalnagar (BNG), Multan (MTN), Faisalabad (SDF), Gujranwala (GRW), Ghakhar (GRK), Islamabad (ISD), Taxila (TAX), Haripur (HRP), Mansehra (MNS) and Bherkund (BKM). SpliNPV-G1 was previously labelled as SpltNPV-G1, and obtained from the baculovirus depository at the Julius Kuhn Institute for Biological Control in Darmstadt (Ali et al., 2017).

2.2. Insects rearing and production of SpltNPV isolates

An *S. litura* colony was maintained in the insectary of the National Agricultural Research Centre (NARC), Islamabad at 26±2°C, 50-60% relative humidity and 14h:10h day-night photoperiod on a semi-synthetic diet (annex-1), referred as NARC colony. The insect colony was started using pupae from the Entomology Department, University of Arid Agriculture Rawalpindi. Polyhedral occlusion bodies (OBs) were isolated from the field-derived cadavers according to procedures presented by Cory et al. (2005) with slight modifications as described in Chapter 2 (Ali et al., 2017). OB samples of each isolate were used to infect 500 healthy late-third instar *S. litura* larvae by offering them artificial food contaminated with OBs. OB stocks of each isolate were sequentially produced to avoid cross contamination and stored at -20°C.

Speed of kill of different SpltNPV isolates in *S. litura* larvae

Speed of kill of wild-type SpltNPV isolates, including a Chinese reference isolate SpliNPV-G1 (Ali et al., 2017), were compared in an assay in which 3rd instar larvae of *S. litura* from the NARC colony were exposed to the wild-type isolates using the droplet feeding method (Hughes et al., 1986). Fifty newly molted 3rd instar larvae were placed in petri dishes, and starved for 12hr. The larvae were then fed with a viral suspension containing $1x10^8$ OBs/ml, 10% sucrose and food color (0.05%w/v) for 15 minutes. Pilot assays had shown that a concentration of $1x10^8$ OBs/ml in this test situation provides 100% mortality. Thirty inoculated larvae (with filled stomach) were transferred to 6-well tissue culture plates with the same diet as described above. The wells were covered with parafilm and tissue paper and by the original lid. A mock was established using 30 additional larvae which were fed on 10% sucrose solution and food color without virus. Larvae were incubated at 26±2°C and a 14h:10h day-night photoperiod in a climate chamber, and survival was checked every 24hr during the first two days and subsequently every 12hr until death or pupation. The experiment was completed in three replicates.

Restriction endonucleases (REN) analysis

Extraction of occlusion-derived virions (ODVs) from OBs, DNA isolation and restriction enzyme analysis was carried out as described in detail in Chapter 2 (see also Ali et al., 2017, submitted). The viral DNA was treated with *Eco*RI and the fragments were separated by 0.7% agarose gel electrophoresis. Each lane was computationally sectioned out of a photo and aligned side-by-side to obtain maximum similarity between the profiles. The *Eco*RI profile of SpliNPV-G1 (Ali et al., 2017) was used as a reference.

2.3. Statistical analysis

We explored associations between genogroup and the geographical regions of origin of the isolate, and between genogroup and the cropping system from which the isolate originated by constructing contingency tables of geographic region vs genogroup and cropping system vs genogroup. Significance was tested using the χ^2 test for contingency tables. The median survival time (ST_{50}) was determined using survival analysis, as implemented in R (R Development Core Team, 2008). The specific packages used were MASS (Venables and Ripley, 2002) and survival (Terry and Thomas, 2011). The larvae surviving the full experimental period were not included in the analysis on the assumption that they had not

ingested a sufficient dose. Initially, the ST_{50} of individual replicates was calculated for each of the 22 SpltNPV isolates plus a reference isolate SpliNPV-G1, to determine whether the survival data of the individual replicate for each virus isolate could be pooled before further analysis. As there was a significant difference in survival between the replicates with the log rank test, results from different replicates were not pooled.

We used the Cox proportional-hazards model to analyze survival and test the significance of differences in the survival curve between virus isolates and the genogroups. The model is defined as $h(t, z_1,..., z_p) = h_0(t)\exp(\sum_{i=1}^{p} \beta_i z_i)$, where h is the hazard rate, which depends on the time since exposure (t) and p categorical covariates z_i (0 or 1, for i = 1,...,p) that might affect a baseline hazard $h_0(t)$. The hazard rate represents the conditional probability of death per unit time, given the subject is alive. The effect of the covariables is expressed via an exponential function $\exp(\sum_{i=1}^{p} \beta_i z_i)$, whose argument is a linear combination of the covariables. The effect size per unit change in z_i is $\exp(\beta_i)$. The categorical variable z represents here the isolate of the virus. If $\exp(\beta_i z_i)$ has a value close to 1, this means that the isolate i has a hazard rate function (=mortality rate) close to the base line. Values of $\exp(\beta_i z_i)$ below one indicate lower hazard rate than the baseline, i.e. longer survival than the reference isolate. The effect of replicate was not multiplicative and we used therefore a stratified Cox model defined as $h_g(t, s_2,..., s_p) = h_{0g}(t)\exp(\sum_{i=2}^{p} \beta_i s_i)$ with g (=1,2,3) for the three different replicate, s_i is 1 if the isolate is isolate i and 0 otherwise. The TAX1 isolate was taken as the baseline in the analysis shown in Table 2 (below). To allow pairwise testing between all isolates of the virus in the Cox regression model, the model was furthermore refitted with all the other isolates as a baseline. In the stratified analyses, each replicate has a different baseline hazard rate ($h_{0g}(t)$), and it is assumed that the isolates effects are the same in the three different replicates $h_{0g}(t)$. Subsequently, genogroup was included as a categorical covariable in the model to test whether there are differences in speed of kill between the genogroups.

DNA sequence analysis

'ORF4' (2217 nts), 'ORF92' (369 nts), 'ORF106' (822 nts) and 'ORF110' (1167 nts) (annotation SpltNPV-G2, Pang et al., 2001) homologs of SpltNPV-Pak-BNG, SpltNPV-Pak-

TAX, and SpliNPV-G1 genomes were obtained from the consensus genome sequence of these viruses described in Chapter 2. Homologs of these four ORFs of SpltNPV-Pak-SFD1 were obtained via PacBio sequencing (not described). The nucleotide sequences of the above ORFs were compared against each other and to the SpltNPV-G2 (Accession number AF325155.1) and SpliNPV-AN1956 (Accession number JX454574.1) genomes in the NCBI reference genome database (www.ncbi.nlm.nih.gov/genome/). The homology of these ORFs was determined as nucleotide identity and tabulated.

The phylogenetic relationship of the SpltNPV-PAK isolates with the SpltNPV-G2 isolate was determined using the four ORF homologs of 'ORF4, 'ORF92', 'ORF106' and 'ORF110'. SpliNPV-G1 and SpliNPV-AN1956 were used as out-group. Sequences were translated in frame to proteins and aligned using MAFFT version 7 (Katoh et al., 2017) with default settings, for each of the ORFs. The protein alignments were converted back into the corresponding codon alignment using PAL2NAL (Suyama et al., 2006). Subsequently, the four alignments were concatenated and Gblocks (Castresana, 2000) was used for trimming sequences to select conserved domains. PAUP* version 4.0a (Swofford, 2002) was used to select the optimal evolution model by critically evaluating the selected parameters (Swofford and Sullivan 2009). The General Time Reversible Model with a gamma distribution of among-site rate variation (GTR + G) was selected and used to conduct Bayesian inference using MrBayes 3.1.2. (Ronquist and Huelsenbeck 2003). Analyses were initiated from random starting trees. Two separate Markov chain Monte Carlo (MCMC) runs, each composed of four chains (one cold and three heated), were run for 6,000,000 generations. The cold chain was sampled every 100 generations, and the first 15,000 generations were later discarded (a burn-in of 25%). Posterior probabilities were computed from the remaining trees.

3. Results

Speed of kill (ST_{50}) **of different SpltNPV-Pak isolates**

Virus-induced mortality ranged between 75 and 100% of the inoculated third instar larvae of *S. litura*. Median time to death (ST_{50}) values for wild-type isolates ranged from 108 h for SFD-2 to 132 h for MTN4, HRP7 and GRK4, while most of the isolates showed intermediary ST_{50} values (Fig. 2). TAX1 was the fastest acting isolate (Fig. 2) while isolates SFD1, SFD2 and GRW1 were not significantly different from TAX1 as shown by the z-values ($P \geq 0.05$) in the survival analysis with TAX1 as reference strain (Table 2). TAX1 was significantly faster killing than the Pakistani reference isolate BNG. The fast acting isolates TAX1 and GRW1 were from

Punjab-1 with wheat-rice double cropping system and SFD1 and SFD2 were from Punjab-2 with mixed cropping pattern (wheat, cotton, rice and vegetables). However, the slow acting isolates GRK4 and GRK5 were also from Punjab-1 region suggesting consisting effect of isolates collection region and cropping system on biological activity.

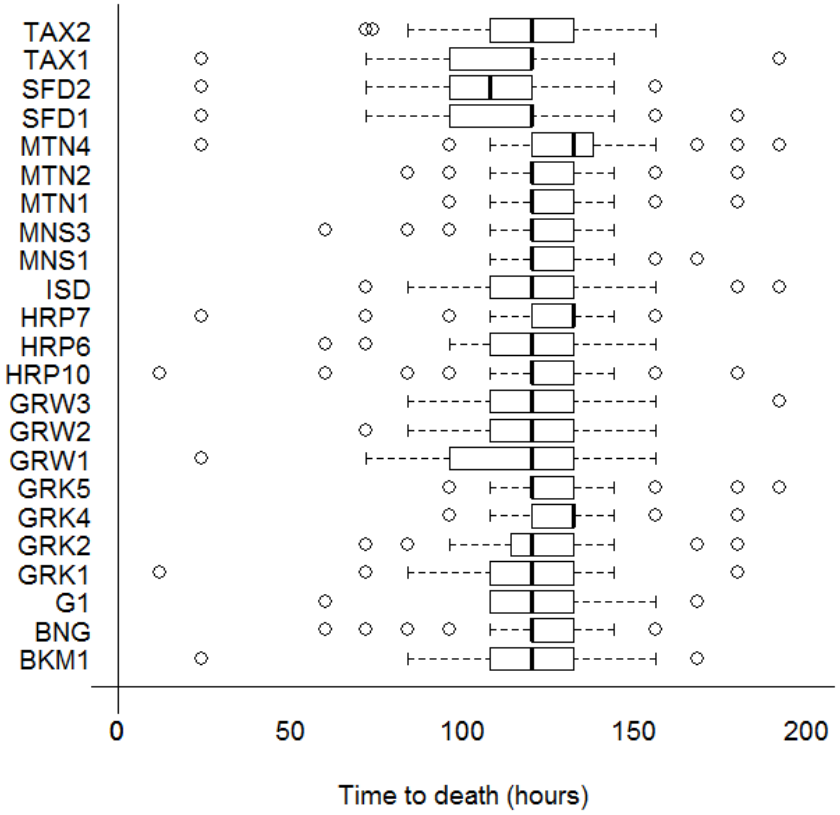

Figure 2. Box and whiskers plot of the time-to-death (ST50) for SpltNPV isolates: the bold vertical lines represent the median times, whereas the box shows the interquartile range (IQR) which is defined as the difference between the first quartile (Q1) or (equivalently) 25% point and the third quartile (Q3) or 75% point. Fifty percent of the observations are in the interquartile range, while 25% of the observations are outside the box at either side. The whiskers extend from the nearest quartile (Q1 or Q3) to the most extreme data value that is less than the IQR away from this nearest quartile (Q1 or Q3). Observations that are more than one IQR away from the nearest quartile are shown as circles.

Table 2. Effect sizes from the stratified survival analysis of wild-type SpltNPV isolates, associated z score test values and their significance for the difference with TAX1 (=baseline strain) at 75-100% mortality.

SpltNPV isolates	Effect size exp(β)	95% confidence limit Lower	Upper	significance	Ecological zones	Z	P-value
MTN4	0.32	0.23	0.44	a	Punjab-3	–7.14	<< 0.0001
GRK4	0.43	0.32	0.58	b	Punjab-1	–5.71	<< 0.0001
HRP7	0.41	0.30	0.56	b	KPK-1	–5.54	<< 0.0001
MNS1	0.45	0.33	0.61	b,c	KPK-1	–5.09	<< 0.0001
HRP10	0.45	0.33	0.62	b,c	KPK-1	–5.04	<< 0.0001
GRK5	0.46	0.34	0.64	b,c	Punjab-1	–4.68	<< 0.0001
MTN1	0.47	0.35	0.64	b,c	Punjab-3	–4.79	<< 0.0001
MNS3	0.48	0.35	0.66	b,c,d	KPK-1	–4.49	<< 0.0001
MTN2	0.48	0.36	0.66	b,c,d	Punjab-3	–4.67	<< 0.0001
GRK2	0.50	0.36	0.68	b,c,d	Punjab-1	–4.37	<< 0.0001
BKM1	0.53	0.39	0.71	b,c,d,e	KPK-1	–4.15	<< 0.0001
ISD	0.54	0.40	0.74	b,c,d,e	Punjab-1	–3.85	<< 0.0001
HRP6	0.54	0.40	0.74	b,c,d,e	KPK-1	–3.91	<< 0.0001
TAX2	0.56	0.41	0.76	b,c,d,e	Punjab-1	–3.73	<< 0.0001
BNG	0.56	0.42	0.78	b,c,d,e	Punjab-3	–3.55	0.0004
GRK1	0.57	0.42	0.78	b,c,d,e	Punjab-1	–3.57	0.0004
G1	0.58	0.43	0.79	c,d,e	Guangzhou	–3.56	0.0004
GRW3	0.59	0.43	0.81	c,d,e,f	Punjab-1	–3.30	0.0010
BKM2	0.65	0.48	0.89	d,e,f,g	Punjab-1	–2.72	0.0064
GRW2	0.69	0.51	0.94	e,f,g	Punjab-1	–2.38	0.0173
GRW1	0.75	0.55	1.02	f,g,h	Punjab-1	–1.82	0.0687
SFD1	0.81	0.60	1.10	g,h,i	Punjab-2	–1.34	0.1798
SFD2	0.92	0.68	1.26	h,i	Punjab-2	–0.51	0.6113

Cox Proportional-Hazards model was used to calculate the mortality rate variation in the larvae compared to TAX1, and is reported with 95% confidence interval. Different letters represent significant differences between isolates, based on different analyses with different strains as baseline. Z-score and P-value refer to the comparison of an isolate to the reference isolate TAX1.

Restriction endonucleases (REN) and sequence analysis

The genomic DNA of each of the twenty two field collected SpltNPV isolates from different agro-ecological regions and one reference wild-type-SpliNPV isolate (SpliNPV-G1) was cleaved with *Eco*R1 to compare the restriction profiles (Fig 3a). From this analysis it can be seen that the SpltNPV isolates from Pakistan are genetically very similar but not identical. This restriction fragment length polymorphism among baculovirus field isolates is characteristic for baculoviruses. Submolar or supramolar bands were observed at low level, suggesting the isolates are relatively genetically homogeneous.

Treatment of the viral DNAs with *Bam*HI and *Pst*I resulted in a small number and very similar patterns (not shown) and thus were not suitable for comparative analysis. The restriction

fragment length pattern of these isolates was completely different from isolate SpliNPV-G1, in line with previous observations for SpltNPV-Pak-BNG (Chapter 2; Ali et al., 2017).

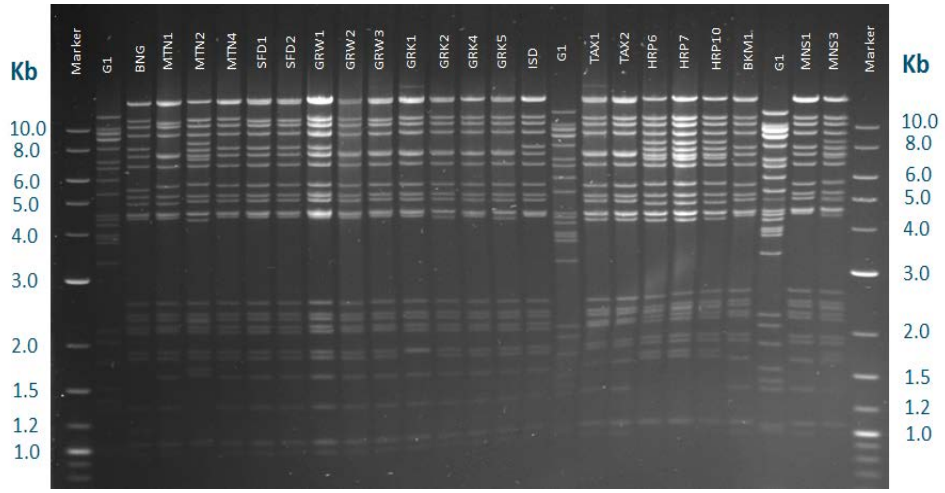

Figure 3a. Restriction endonucleases (REN) analysis of 22 wild-type SpltNPV isolates cleaved with *Eco*R1.

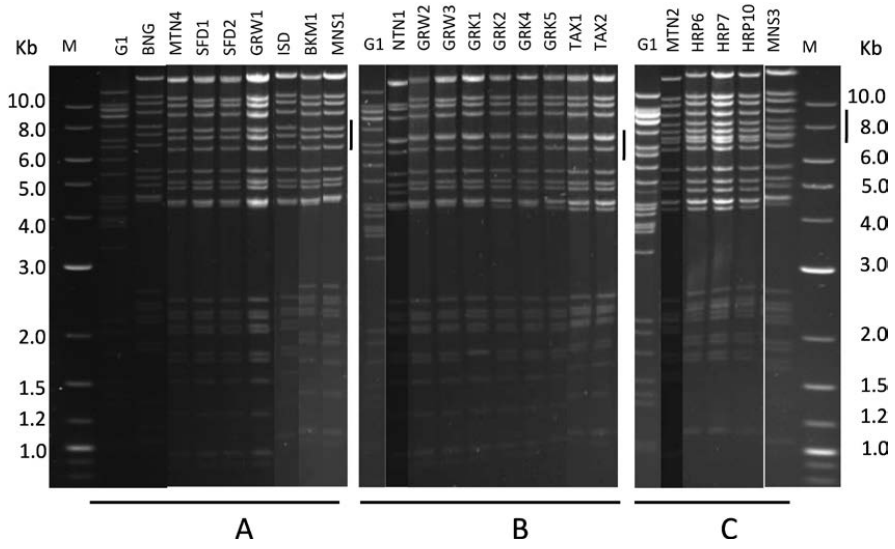

Figure 3b. Restriction endonucleases (REN) analysis of 22 wild-type SpltNPV isolates cleaved with EcoR1 and organized in genogroups (genogroup A, genogroup B and genogroup C). The critical bands have been marked by vertical black bars.

Table 3. Pairwise nucleotide sequence identity (%) of 'ORF4', 'ORF92', 'ORF106', 'ORF110', 'ORF1' and 'ORF24' of *de novo* assembled SpltNPV-Pak-BNG, SpltNPV-Pak-TAX1, SpltNPV-G1 genomes from a MiSeq analysis, SpltNPV-PAK-SFD1 from PacBio analysi,s and SpltNPV-G2 and SpliNPV-AN1856 genomes from the NCBI database.

ORF4 / **ORF92**	SpltNPV-Pak-BNG	SpltNPV-Pak--TAX1	SpltNPV-Pak-SFD1	SpltNPVG2	SpliNPVG1	SpliNPV-AN1956
	%	%	%	%	%	%
SpltNPV-Pak-BNG		99	99	96	80	81
SpltNPV-Pak-TAX1	**99**		98	94	80	81
SpltNPV-Pak-SFD1	**99**	**100**		95	80	81
SpltNPV-G2	**97**	**98**	**98**		79	79
SpliNPV-G1	**86**	**85**	**85**	**84**		99
SpliNPV-AN1956	**86**	**85**	**85**	**83**	**99**	
ORF106 / **ORF110**						
SpltNPV-Pak-BNG		99	99	97	77	76
SpltNPV-Pak-TAX1	**97**		99	94	76	76
SpltNPV-Pak-SFD1	**97**	**99**		96	76	76
SpltNPV-G2	**96**	**94**	**94**		75	75
SpliNPV-G1	**82**	**82**	**82**	**82**		97
SpliNPV-AN1956	**82**	**82**	**82**	**82**	**97**	
ORF1 / **ORF24**						
SpltNPV-Pak-BNG		100	100	99	94	93
SpltNPV-TAX1	**99**		100	99	94	94
SpltNPV-SFD1	**99**	**99**		99	94	94
SpltNPV-G2	**99**	**99**	**99**		94	94
SpliNPV-G1	**76**	**85**	**84**	**76**		99
SpliNPV-AN1956	**76**	**85**	**84**	**76**	**99**	

A further analysis was performed by comparing the restriction fragment length patterns of all twenty-two SpltNPV isolates from Pakistan for maximum similarity (Fig. 3b). On the basis of this similarity three groups of SpltNPVs could be identified, assigned as genogroup A, B and C. Sequence analysis of four SpltNPV homologs with a relatively low degree of sequence homology ('ORF4', 'ORF92', 'ORF106' and 'ORF110' (SpltNPV-G2 annotation (Pang et al., 2001) were compared with the SpltNPV-PAK isolates BNG, TAX1 and SFD and tabulated in Table 3. 'SpltNPV 'ORF4' encodes the HOAR protein (Le et al., 1997), which has some similarity to ubiquitin ligase. 'ORF106' encodes an alkaline nuclease and is a conserved gene in all baculoviruses to date, whereas 'ORF92' encodes a 12 kDa protein of unknown function. The function of 'ORF110' is also not known. The SpltNPV ORF-homologs of the SpltNPV-PAK isolates had a high degree of homology (nucleotide sequence identity) with each other and with SpltNPV-G2, but a significantly lower degree of homology with SpliNPV-G1 and SpliNPV-AN1956. A phylogenetic tree was constructed using the four concatenated ORFs of each virus (Fig. 4). The SpltNPV-PAK isolates were in the same clade as SpltNPV-G2, but were separated from the clade containing SpliNPV-G1 and SpliNPV-AN1956. This confirms that the SpltNPV-PAK isolates are variants of the same baculovirus species, SpltNPV.

Figure 4. Bayesian phylogeny for SpltNPV-Pak-BNG, SpltNPV-Pak -TAX1 and SpltNPV-Pak -SFD1, SpltNPV-G2, SpliNPV-G1 (Darmstadt depository) and SpliNPV-AN1956 using the concatenated nucleotide alignment of SpltNPV-G2 homologs 'ORF4', 'ORF92', 'ORF106' and 'ORF110'. Numbers on the nodes indicate Bayesian posterior probabilities. The scale bar represents 0.1 expected substitutions per site.

To see if there is a spatial dispersal of SpltNPV from a single introduction, a comparison was made between SpltNPV genotypes from different geographic locations. When the geographic origin of the various SpltNPV-PAK isolates was compared to the genogroup based

on REN analysis (SpltNPV-PAK A-C), there was a strong correlation between genogroup and region of origin (χ^2 = 18.27; P = 0.0056) (Table 4).

To investigate the possibility that a genogroup adapted to cropping system a comparison between genogroups and cropping system was made. We found a marginally significant association between genogroup and cropping system of origin of the isolate (χ^2 = 12.58; P = 0.0502) (Table 5).

Table 4. Cross-tabulation of geographic origin of virus isolates and their genogroup according to the results of REN analysis

Region / site	Isolate	Genotype cluster		
		A	B	C
KPK-1	MNS1	X		
	MNS3			X
	HRP6			X
	HRP7			X
	HRP10			X
	BKM1	X		
Punjab-1	ISD	X		
	GRW1	X		
	GRW2		X	
	GRW3		X	
	TAX1		X	
	TAX2		X	
	GRK1		X	
	GRK2		X	
	GRK4		X	
	GRK5		X	
Punjab-2	SFD1	X		
	SFD2	X		
Punjab-3	MTN1		X	
	MTN2			X
	MTN4	X		
	BNG	X		

Survival analysis with genogroup as a covariable demonstrated a significant difference in time to death between genogroups A and C ($\beta = 0.13 \pm 0.060; {\chi_1}^2 = 4.71; P = 0.029$). The hazard rate was 12% lower in group C than in Group A. Group B was more similar in speed of kill to group A than to group C (Figure 5), but there were no significant pairwise differences in time to kill amongst groups A and B ($\beta = 0.035 \pm 0.051; {\chi_1}^2 = 0.46; P = 0.50$) or between groups B and C ($\beta = 0.096 \pm 0.059; {\chi_1}^2 = 2.65; P = 0.103$).

Table 5. Cross-tabulation of crop system origin of virus isolates and their genogroup according to the results of REN analysis

Crop system	Isolate	Genotype cluster		
		A	B	C
Cotton-wheat	MTN1		X	
	MTN2			X
	MTN4	X		
	BNG	X		
Vegetable	MNS1	X		
	MNS3			X
	ISD	X		
	HRP6			X
	HRP7			X
	HRP10			X
	TAX1		X	
	TAX2		X	
	BKM1	X		
Rice-wheat	GRW1	X		
	GRW2		X	
	GRW3		X	
	GRK1		X	
	GRK2		X	
	GRK4		X	
	GRK5		X	
Multiple crops	SFD1	X		
	SFD2	X		

4. Discussion

This is the first inventory of SpltNPV baculoviruses from Pakistan, obtained from fields up to 600 km. Via comparative REN analysis all isolates were identifiable as closely related variants of a single SpltNPV biotype. This observation suggests that this is the result of a single introduction of a SpltNPV into Pakistan. Sequence information from a few selected SpltNPV-PAK isolates also indicated that all Pakistani isolates are related to SpltNPV-G2 from China (Pang et al., 2001), but are not the same biotype within the SpltNPV species. This outcome was supported by sequence comparisons (Table 3) using 4 ORFs with a lower percentage of nucleotide similarity and by phylogeny analysis. Sequence comparisons showed 100% nucleotide identity for 'ORF24' (annotation SpltNPV-G2, Pang et al., 2001) for all Pakistani SpltNPV field isolates of *S. litura* as well as SpltNPV-G2 (China) and the reference strain SpliNPV-G1 (Chapter, 2; Ali et al., 2017). Phylogenetic analysis strongly supports the close relationship among the Pakistani SpltNPV isolates.

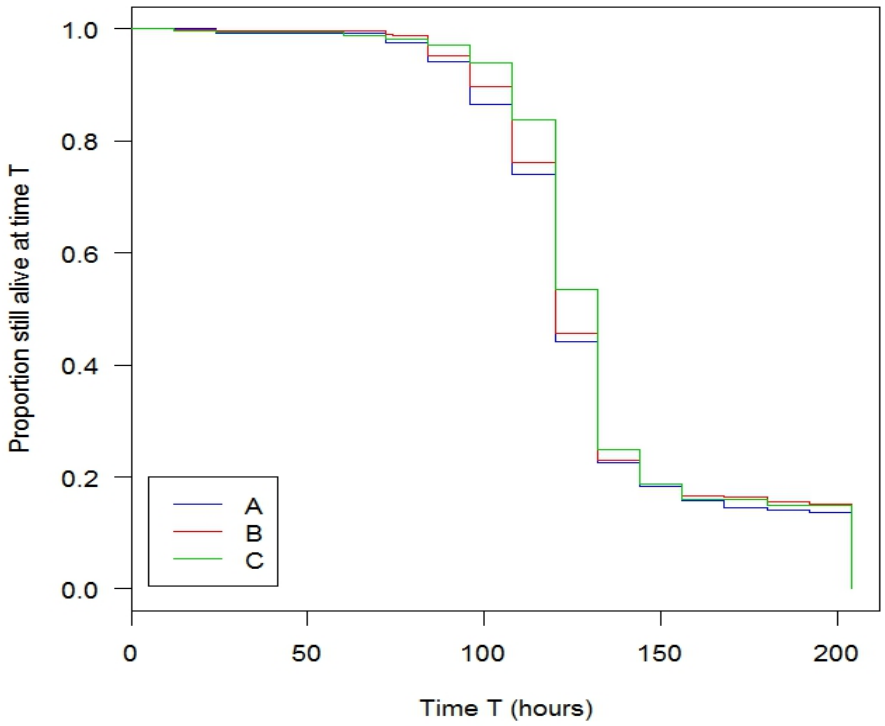

Figure 5: The survivors for the three genogroups, Note that genogroup A is the one with the highest mortality rate, relative to genogroups B and C.

Most of the tested isolates overlapped in their ST_{50} values without expressing significant differences between the median survival times. In general, the isolates can be divided into two groups according to their speed of kill: one group with an ST_{50} of 120 hpi on average and another group with an ST_{50} of 132 hpi on average (Fig. 2). The SpltNPV isolate TAX1 stood out from the other isolates and the survival time distribution analysis revealed that SFD1, SFD2 and GRW1 are similar to the TAX1 isolate in their time mortality response to 3rd instar larvae of *S. litura,* being notably fast killing isolates and were highly significant different ($P \leq 0.001$) from most of the tested isolates (Table 2). In this study, the ST_{50} values for most of the SpltNPV isolates were lower than reported by Kamiya et al. (2004) for their SpltNPV isolates, but this can be explained by a difference in genetic make-up of the virus or the *S. litura* host population.

Previous studies on variability of speed of kill of distinct isolates of SpltNPV showed variable results; from no variation to large variation. Takatsuka, et al. (2003) found no indication of variation in speed of kill for SpltNPV isolates from Japan, Malaysia, Vietnam and India harvested from symptomatic larvae of *S. litura* and an Egyptian isolate of *S. littoralis* NPV. However, Kamiya et al. (2004) reported significant differences in ST_{50} (38.4 h) among different SpltNPV clones. Similarly, Rowley et al. (2011) and Figueiredo et al. (2009) found variation in biological activity among isolates of NPV isolates from heliothine pest insects from different geographical locations. Normally, speed of kill is negatively correlated with occlusion body (OBs) production because larger (longer surviving) hosts can produce a greater number of OBs (Barrera et al., 2011; Hodgson et al., 2004). Thus, fast killing viruses may be at a selection disadvantage (Sun et al., 2005; Georgievska et al., 2010; Zwart et al. 2010) in a natural environment.

By visual inspection of REN profiles the Pakistani S. litura NPV isolates were closely related in restriction enzyme length polymorphism to SpltNPVs isolated by Kamiya et al. (2004) and Lucien et al. (2009), but a genetic comparison is required to substantiate this observation. Our study showed genetic heterogeneity and biological diversity among the geographically distinct SpltNPV isolates within Pakistan, although the sampling was limited. The analysis does show that they are SpltNPV genotypic variants belonging to a single biotype. As this biotype differs from SpltNPV from China (Table 4, Fig. 5), but within a single species (SpltNPV) we propose the term regiotype for such a regional biotype. It would be of interest to analyze the SpltNPVs from elsewhere, e.g. Japan (Takatsuka et al., 2016), to further test the regiotype concept. Regiotype has no taxonomic meaning, but might help to better understand local genetic variation, dispersal and evolution of baculoviruses.

Analysis of MbMNPV isolates over a distance range of about 2500 km showed distinct regiotypes (Van Oers and Vlak, 2006) suggesting these isolates were introduced a longer time ago and had more time to establish locally. Since all SpltNPV-Pak isolates over a wide geographic distance seem to be derived from a single introduction on the basis of REN comparisons, the introduction in Pakistan may also be fairly recent. However, the various genogroups significantly correlate with distance (Table 4) further supporting the view that the virus recently dispersed over large distances, possibly along with its host, *S. litura*, as moths or with infected insects hitchhiking on host plants that are used for planting.

Survival analysis with genogroups exhibited that speed of kill in genogroup A was faster than in genogroup C (Fig. 6). We could not find literature on statistical comparison of genogroups for biological activity. However, the fast acting SpltNPV isolates emerged from genogroups A and B and the genetic variability may have contributed towards fast biological activity as the small variation within genome can result in significant changes in biological activity (Cory et al., 2005). A further study demonstrated that the virulence of LdMNPV is determined only by viral genetics and not by the locality of host population (Martemyanov et al., 2017).

We were expecting more than 22 SpltNPV isolates from our field survey. However, the robustness of the SpltNPV isolates collection results was affected by a range of factors, including zero tolerance for insects, resulting in high use of insecticides on field crops. Farmers apply insecticides at early larval stage of *S. litura* to manage the insect and minimize the crop damage. As a result it is very difficult to find the symptomatic larvae in the field. The climatic conditions (temperature and humidity) play a significant role in building up the *S. litura* field populations. The precipitation was low and temperature was high due to low rainfall in summer of 2012-13. So, there was small window of time during the year (month of April and August – September) to collect the symptomatic larvae from the field.

Most of SpltNPV isolates (79%) were recovered from *S. litura* larvae from *Brassicae oleracea*. *B. oleracea* is a highly preferred host plant for *S. litura* (Ghumare and Mukherjee, 2003). Furthermore, *B. oleracae* has broad leaves and *S. litura* larvae that are hidden under the leaves may escape from mortality due to insecticide applications and survive till polyhedrosis. A minor proportion was recovered from rice or vegetables. The various genogroups do not seem to correlate strongly with cropping system (Table 5), but more intensive sampling would further help to investigate this possibility. Further intensive sampling is also required to find out if there

are certain regions / cropping systems that provide an advantage in the selection of more active isolates.

The SpltNPV-Pak isolates varied both in number and pattern of fragments compared to the SpliNPV-G1 isolate. While this virus was initially obtained as "SpltNPV isolate from China", we conclude that this isolate corresponds to SpliNPV (Chapter 2 and Ali et al., 2017; Breitenbach et al., 2013). When we compared our REN fragments of isolates to other published REN profiles of SpltNPV, the REN patterns of all the collected Pakistani isolates were closely related to known SpltNPV (Lavina et al., 2001; Pang et al., 2001; Takatsuka et al., 2003; Kamiya et al., 2004; Lucien et al., 2009). No larvae infected with SpliNPV were found from *S. litura* in Pakistan, as was the case in Japan (Takatsuka et al., 2016), but more extensive sampling may be required as Pakistan is closer to the natural habitat of *S. littoralis* (Africa).

Interestingly, intraspecific genetic diversity was observed in the tested isolates and a mixture of genotypes was detected, as indicated by the presence of sub-molar fragments in the REN profile. This is common in the wild-type isolates of baculoviruses. Previous studies showed that baculovirus isolates contain a variable number of genotypes from a single larval host (Cory, 2005; Figueiredo et al., 2009; Redman et al., 2010; Rowley et al., 2011). Studies at even geographically smaller scales have shown that baculoviruses vary both within and between populations of the same host species (Parnell et al., 2002; Cooper et al., 2003).

5. Conclusion

In the present study, SpltNPV isolates from different agro-ecological zones of Pakistan were analyzed for survival time and compared for their DNA restriction patterns. There is clear evidence of variations in biological activity; even isolates from the same location differed significantly from each other. We found that isolates TAX1, SFD1, SFD2 and GRW1 showed superior speed-of-kill and may serve as candidates for controlling *S. litura* in Pakistan and elsewhere. In spite of the variation in biological activity among the wild-type isolates, little genetic variation was observed among the Pakistan SpltNPV isolates. The DNA restriction enzyme cleavage patterns demonstrated major differences between the genomes of SpliNPV-G1 (reference strain) and wild-type SpltNPV isolates collected from the field in Pakistan but a close relationship with SpltNPV-G2 from China. However, SpltNPV isolates collected from different agro-ecological zones of Pakistan have small variations in their REN profiles and sequences, allowing the subdivision in genogroups. These genogroups correlate well with geographic regions but less so with cropping system and virulence. This study provides novel

information on the diversity of SpltNPV isolates and is valuable for understanding the evolution of SpltNPVs and for their development as a potential biological insecticide in Pakistan.

Acknowledgements

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Chapter 5

Infection with Spodoptera litura NPV reduces food consumption and weight gain of *Spodoptera litura* larvae

Adapted from:
Ghulam Ali, Wopke van der Werf and Just M. Vlak. 2017. Infection with Spodoptera litura NPV reduces food consumption and weight gain of *Spodoptera litura* larvae. Manuscript in preparation.

Abstract

Insect-pathogenic baculoviruses have established potential as biological insect pest control agents. However, slow speed of kill and continued feeding following application limit their effectiveness in preventing crop losses. Here we studied the food consumption and weight gain of *Spodoptera litura* following application of two viral doses of *Spodoptera litura* nucleopolyhedrovirus (Pakistan isolate SpltNPV-Pak-BNG). Infected larvae with final polyhedrosis exhibited reduced food intake and weight gain. The healthy control and the larvae that survived viral exposure, exhibited the same food consumption and weight gain. This study did thus not elucidate any sub-lethal effects of exposure to the virus on food consumption and weight gain. There was no viral dose dependency observed in food intake or weight gain by infected larvae, suggesting there is no increased crop damage upon virus treatment.

Keywords: *Spodoptera litura* nucleopolyhedroviruses; food consumption; weight gain; *Spodoptera litura*

1. Introduction

The *Baculoviridae* (King et al., 2012) are a large family of invertebrate viruses that are found ubiquitously in insects (notably members of the orders Lepidoptera, Hymenoptera and Diptera) in natural habitats. They are attractive biological control agents for lepidopteran insect pests due to their insecticidal properties, host specificity and safety to non-target animals (Inceoglu et al., 2006; Szewczyk, et al., 2006). However, baculoviruses have limitations, which are restricting their use for insect control in practice. Research is required to mitigate or eliminate these limitations.

A key limitation is a relatively slow speed of kill (Moscardi et al., 2011; Beas-Catena, et al., 2014; Knox et al., 2015). It may take several days to few weeks before the infected larvae die or stop feeding (Vasconcelos et al., 2005). Feeding may continue until the insects eventually die (Glass, 1958; Subrahmanyam and Ramakrishnan, 1981). Crop losses following the application of baculoviruses may therefore be substantial (Bonning and Hammock, 1994; Bianchi et al., 2000[a]). To eliminate this drawback, baculoviruses have been genetically modified to improve the speed of kill and reduce crop losses (Inceoglu et al., 2006; Szewczyk et al., 2006; Sun et al., 2009; Cai et al., 2010; Georgievska et al., 2010). However, these recombinant viruses have reduced within-host fitness compared to their parent wild-type (Cory, 2000; Zwart et al., 2009; Georgievska, 2010). It is difficult to produce them *in vivo* because the yield per cadaver is lower than for wild-type insects (Sun et al., 2005). In addition, such recombinant viruses, being GMO, meets public resistance and is not compatible with organic production methods.

Feeding behaviour of infected larvae determines the extent to which baculoviruses can protect crops against feeding damage (Hoover et al., 1995). Baculoviruses delay the moult of larval hosts (reviewed by Clem and Passarelli, 2013). Normally, insect larvae cease feeding during larval-larval and larval-pupal moults during their life span (O'Reilly and Miller, 1989). However, infection with baculoviruses interferes with this feeding arrest, resulting in a prolonged feeding period after viral infection and increased weight gain by infected larvae (O'Reilly and Miller, 1991). For instance, Subrahmanyam and Ramakrishnan (1981) demonstrated that *Spodoptera litura* larvae infected with Spodoptera litura nucleopolyhedrovirus (SpltNPV) continue feeding until death and consumed more food than the healthy control larvae. On the other hand, Vasconcelos et al. (2005) reported that baculovirus infected *Mamestra brassicae* larvae caused significantly less defoliation in cabbage plants than uninfected larvae.

There are thus contradictory observations in previous studies regarding food consumption and crop damage by baculovirus infected larvae as compared to healthy larvae. A detailed understanding of food consumption following infection by baculoviruses is needed to design effective use strategies for baculoviruses. Therefore, a case-by-case approach is required to study this aspect, in the current case the leafworm *S. litura* and its baculovirus SpltNPV.

This chapter addresses three questions: (i) are food intake and weight gain of larvae reduced if they are infected with SpltNPV? (ii) if there is a reduction in food intake and weight gain, when does it set in following exposure to virus? (iii) is there any difference in food intake and weight gain between healthy control larvae and those that survive exposure to the virus, i.e. sublethal effects on food intake and weight gain in those larvae that do not get lethally infected but have been exposed?

2. Materials and Methods

2.1. Insect rearing and the SpltNPV-Pak-BNG isolate

A *S. litura* colony was maintained in the insectary of the National Agricultural Research Centre (NARC), Islamabad, Pakistan. Insects were reared at 26±2°C, 50-60% relative humidity and 14h:10h day-night photoperiod on a semi-synthetic diet as described in Chapter 2. We used newly moulted 4th instar larvae of *S. litura* to evaluate the feeding and weight gain response by the infected and healthy larvae.

The SpltNPV-Pak-BNG isolate used in this experiment was collected from cotton in the Bahawalnagar district of Punjab, Pakistan, and was amplified *in vivo* in 3rd instar larvae of *S. litura*. Occlusion bodies (OBs) were purified from dead larvae according to Ali et al. (2017) (Chapter 2). The viral concentrations were prepared by counting the OBs in the virus suspension using a haemocytometer (Neubauer) using phase contrast light microscopy. The SpltNPV-Pak-BNG isolate has a median lethal dose (LD_{50}) of 1.88x10^4 OBs / larva and a median survival time (ST_{50}) of 108 hours post infection (hpi) in 4th instar larvae of *S litura* (Chapter 2).

2.2. Bioassay

Two viral stock concentrations, 3×10^6 and 3×10^7 OBs/ml, respectively, were prepared for conducting leaf disc bioassays. In each assay, 45 newly moulted 4th instar larvae were individually placed in 24-well tissue culture plates (bottom diameter of 15.5 mm) to avoid cannibalism, and starved for 15 hr. The larvae were then fed using leaf discs. Fresh tender leaves of young *Ricinus communis* plants were cut into 3 mm^2 pieces and placed on a 1% plant agar-

solidified solution in the 24-well tissue culture plates. The plant agar kept the tender leaves moist and prevented them from drying out. A 3.33 µl volume of viral suspension was placed on each leaf disc and allowed to air-dry. Fifteen leaves were inoculated with the 3×10^6 OBs/ml solution, while fifteen other leaves were inoculated with the 3×10^7 OBs/ml solution. The 15 remaining leaves were mock inoculated with distilled water. The resulting viral doses on the leaf disks were 10^4, 10^5, and 0 OBs, respectively. The overnight-starved larvae were exposed to the leaf discs for 24 hrs and kept at 26±2 °C overnight in a climate chamber. Ten inoculated larvae that had eaten the whole leaf disc (randomly chosen) were weighed and transferred to 6-well tissue culture plates (bottom diameter of 35 mm) having a weighed amount of artificial diet plugs. The 6-wells plates were then covered with parafilm, tissue paper and by the original lid to prevent larval escape. Larvae were incubated at 26±2 °C and a 14h:10h day-night photoperiod in a climate chamber.

Food consumed and weight gain by individual larvae were determined every 24 hrs during the first three days and subsequently every 12 hrs until death or pupation using a PW 214 Analytical Balance with a readability range of 0.1 mg to 210 g (Adam Equipment (Adam Equipment Inc., 26 Commerce Drive, Danbury CT 06810 USA). The remaining diet plugs were weighed to assess the food consumed by the larvae. The larvae were also weighed to determine increase/loss in body weight. The remaining diet plugs and excrements were removed and replaced with fresh, weighed plugs of artificial diet. This process was continued for each larva until death or pupation. The experiment was conducted in three replicates.

2.3. Statistical analysis

Initially, food consumption and weight gain of individual replicates was analyzed for five groups of larvae: infected larvae, exposed but not infected (alive) larvae for each of the two dose treatments and the healthy control. No significant differences were detected in food consumption and weight gain among the replicates. The data from three replicates therefore were combined to determine food consumption and weight gain for all the three treatments, through comparing the mean and by using a univariate generalized linear model in SPSS (IBM Corp., 2012). Pairwise comparisons were conducted using t-tests in R to find differences between the treatments means at $P \leq 0.05$.

3. Results

Mortality increased with viral dose. No mortality was observed in mock-infected larvae (control). The number of larvae dying at the lower dose of 10^4 OBs per larva was 4, 3 and 3 in replicates 1, 2 and 3, respectively. The number of larvae dying at the higher dose of 10^5 OBs per larva was 7, 7 and 10 in replicates 1, 2 and 3 respectively (Table 1).

Table 1. Mortality of 4th instar larvae of *S. litura* challenged with different viral doses (OBs/larva).

Replicates	Control	Low dose (10^4 OBs/larva)		High dose (10^5 OBs/larva)	
	Alive	Dead	Alive	Dead	Alive
Replicate 1	10	4	6	7	3
Replicate 2	10	3	7	7	3
Replicate 3	10	3	7	10	0
Total	30	10	20	24	6

3.1. Food consumption

Exposed larvae that became infected and eventually died ate 59% (dose: $1x10^4$ OBs/larva) or 58% (dose: $1x10^5$ OBs/larva) less for than exposed insects that did not die following exposure. There was thus no viral dose dependency in food intake by infected larvae, but the difference with the control larvae was highly significant ($P \leq 0.05$; Table 2).

Table 2. Food consumption by 4th instar larvae of *S. litura* till pupation or death upon challenge with different viral doses.

Replicates	Mean food consumed (g± SE)				
		Low dose (10^4 OBs/larva)		High dose (10^5 OBs/larva)	
	Control	Dead	Alive	Dead	Alive
Replicate 1	(2.84±0.16)^a (n = 10)	(1.04±0.26)^b (n = 4)	(2.85±0.21)^a (n = 6)	(1.20±0.19)^b (n = 7)	(2.45±0.30)^a (n = 3)
Replicate 2	(2.66±0.27)^a (n = 10)	(1.18±0.49)^b (n = 3)	(2.82±0.32)^a (n = 7)	(1.09±0.32)^b (n = 7)	(3.01±0.49)^a (n = 3)
Replicate 3	(2.66±0.22)^a (n = 10)	(1.32±0.39)^b (n = 3)	(2.80±0.26)^a (n = 7)	(1.14±0.22)^b (n = 10)	- (n = 0)
Combined effect of 3 replicates	(2.83±0.12)^a (n = 30)	(1.17±0.21)^b (n = 10)	(2.83±0.15)^a (n = 20)	(1.15±0.14)^b (n = 24)	(2.73±0.27)^a (n = 6)

S. litura larvae were exposed to different viral doses through leaf disc bioassay (T0=0, T1=10^4 and T2=10^5 OBs/larva). The control was fed with distilled water on the leaf disc. All larvae were given a pre-weighed piece of food. The mean food consumed by infected and healthy larvae is presented. SE represents the standard error of the mean. The food consumed with the same letter is not significantly different at $P \leq 0.05$. Here n is number of larvae.

There was no significant difference between the virus doses. In addition, there was no significant difference in food intake between larvae of the healthy control group and survivors of OB exposure. Differences in cumulative food consumption between infected larvae and those that are either unexposed or that survive following exposure emerge between 72 and 96 hpi (Figure 1). There was no viral dose-food intake dependency among the infected larvae and feeding of infected larvae. The feeding stopped completely at ~120 hpi.

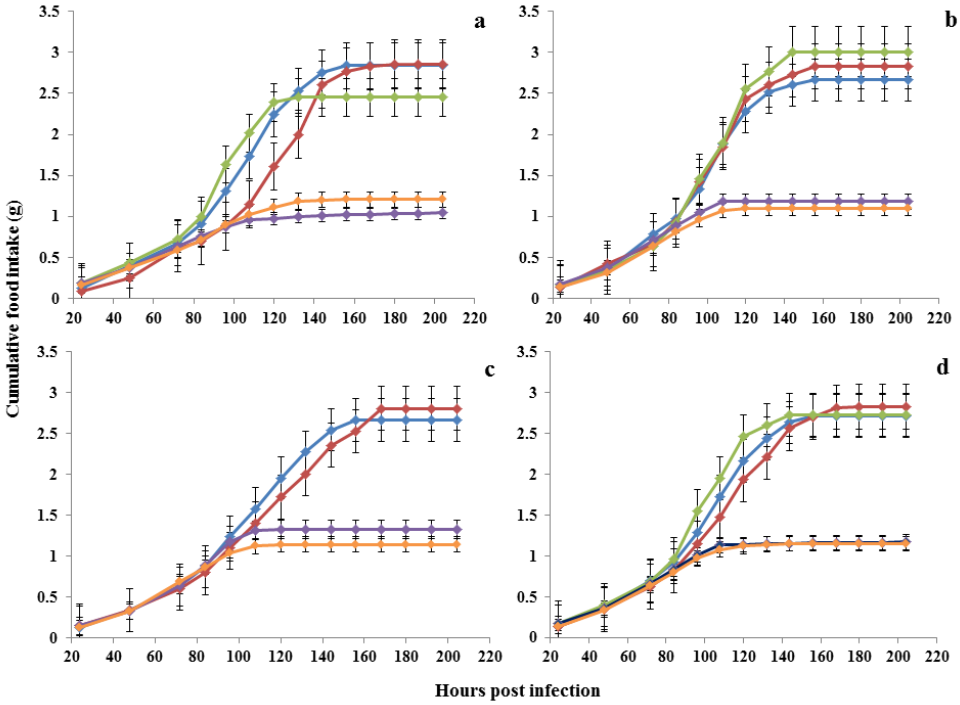

Figure 1. Effect of different viral doses (T0=0, T1=10^4 and T2=10^5 OBs/larva) on cumulative food consumption of healthy and infected larvae of *S. litura* over time. The control (mock infected) is presented in blue colour, T1-alive in red colour, T1-infected in purple colour, T2-alive in green colour and T2-infected in orange colour. The experiment was completed in three replicates. a= replicate 1, b = replicate 2 and c = replicate 3. The data were pooled in panel d to show the combined effect of replicates. Error bars represent standard error of the mean.

3.2. Weight gain

The difference in weight gain between the virus infected larvae that died and those that survived infection was highly significant ($P \leq 0.05$; Table 3). There was no viral dose dependency in weight gain by the infected larvae. There was also no significant difference in weight gain by

larvae of the healthy control and survivors that were exposed but did not get overt infection (polyhedrosis). Differences in cumulative weight gain between infected larvae and those that are either unexposed or that survive following exposure emerged between 72 and 96 hpi (Figure 2).

Table 3. Weight gain by 4th instar larvae of *S. litura* challenged with different viral doses.

Replicates	Mean weight gain (g± SE)				
		Low dose (10^4 OBs/larva)		High dose (10^5 OBs/larva)	
	Control	Dead	Alive	Dead	Alive
Replicate 1	(0.91±0.06)[a] (n = 10)	(0.21±0.09)[b] (n = 4)	(0.85±0.08)[a] (n = 6)	(0.24±0.07)[b] (n = 7)	(0.67±0.11)[a] (n = 3)
Replicate 2	(0.69±0.07)[a] (n = 10)	(0.29±0.13)[b] (n = 3)	(0.85±0.08)[a] (n = 7)	(0.20±0.08)[b] (n = 7)	(0.84±0.13)[a] (n = 3)
Replicate 3	(0.78±0.06)[a] (n = 10	(0.39±0.10)[b] (n = 3)	(0.70±0.07)[a] (n = 7)	(0.25±0.06)[b] (n = 10)	- (n= 0)
Combined effect 3 of replicates	(0.79+0.04)[a] (n = 30)	(0.29+0.06)[b] (n = 10)	(0.80+0.04)[a] (n = 20)	(0.23+0.04)[b] (n = 24)	(0.75+0.08)[a] (n = 6)

S. litura larvae were exposed to different viral doses through a leaf disc bioassay (T0=0, T1=10^4 and T2=10^5 OBs/larva). The control was fed with distilled water on leaf disc. The mean weight gain by infected and healthy larvae is presented. SE represents the standard error of the mean. Weight gains with the same letter are not significantly different at $P \leq 0.05$.

4. Discussion

In the case of SpltNPV-Pak-BNG and *S. litura*, there was a highly significant difference in food intake and weight gain between healthy and infected larvae. Viral infection impaired food intake and reduced weight gain. There was no viral dose dependency in the effects of virus infection on food consumption or weight gain and there was no difference in food consumption and weight gain between healthy and infected larvae during the first 48-72 hrs after OB ingestion. A significant difference between the infected and healthy larvae in food consumption and weight became apparent between 72 and 96 hpi. Infected larvae consumed less food than the exposed larvae that did not become infected (survivors). We found no significant difference in food intake between healthy control larvae and survivors. It is possible that in the latter a latent or persistent infection was established (viral presence), but then this did not affect the feeding behaviour of these survivors. Such infection, if present, can contribute to long-term management of pest populations, as latent or persistent infections can turn into overt infections (polyhedrosis) through biological and environmental stress factors (Milks et al., 1998; Myers et al., 2000; Duan and Otvos, 2001).

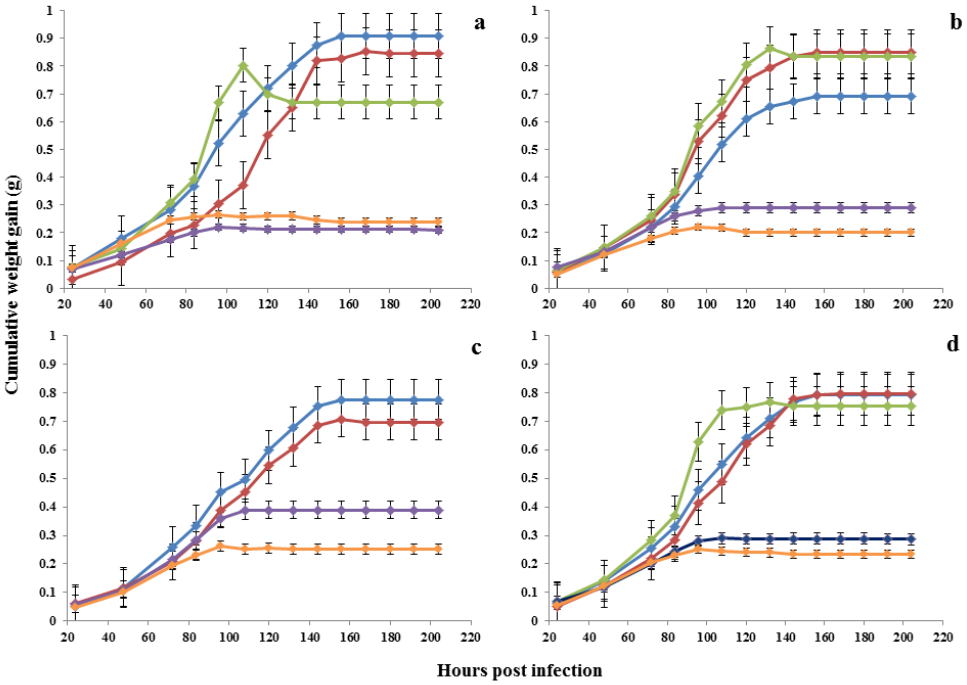

Figure 2. Effect of different viral doses (T0=0, T1=10^4 and T2=10^5 OBs/ml/larva) on cumulative weight gain in healthy and infected larvae of *S. litura* over time. The control (T0) is presented in blue colour, T1-alive in red colour, T1-infected in purple colour, T2-alive in green colour and T2-infected in orange colour. The experiment was completed in three replicates. a= replicate 1, b = replicate 2 and c = replicate 3. The data were pooled to show the combined effect of replicates in panel d. Error bars indicate the standard error of the mean.

The timing of divergence in food consumption and weight gain between survivors and non-survivors in our study agrees with the data of Vasconcelos et al. (2005) for *M. brassicae*. One to two days after exposure is the time when the larval host tries to overcome the viral infections by shedding infected midgut epithelial cells (Keddie et al., 1989). The infected host deploys its resources in this elimination process that would otherwise be used for larval host growth and this may reduce food consumption (Keddie et al., 1989; Cory et al., 1997; Cory and Meyers, 2003).

Previous studies demonstrated that a limitation of naturally occurring wild-type baculovirus is their low speed of kill, which allows the insect pests to significantly damage the crops before they are controlled (Bonning and Hammock, 1994; Bianchi et al., 2000). This is due to expression of the *egt* gene in baculovirus, which blocks moulting and interferes with

normal feeding arrest during larval-larval and larval-pupal moulting (O'Reilly, 1995). This interference results in prolonged feeding and hence greater food consumption in infected larvae within the instar (O'Reilly and Miller, 1991). Subrahmanyam and Ramakrishnan (1981) reported that SpltNPV-infected *S. litura* larvae consumed 66% more food than healthy larvae. It could be that the SpltNPV-Pak-BNG isolate we used resulted in a more reduced survival time (Chapter 2; Ali et al., 2017[a]) and hence reduced food intake. Differences in survival time have been noted among SpltNPV-Pak isolates (Chapter 3; Ali et al., 2017[b]).

Paradoxically, our results reveal that, in spite of low speed of kill, the food intake and weight gain by viral infected larvae is quite low compared to healthy larvae, in agreement with the findings of Vasconcelos et al. (2005). The results demonstrate that the viral applications induce impaired feeding in infected larvae and can significantly lower the food consumption and weight gain in infected compared to healthy larvae, and thereby reduce crop damage. In a previous study (Ali et al., 2017[b]; Chapter 4) I found four isolates of SpltNPV-Pak, that were faster than SpltNPV-Pak-BNG, while having the same median lethal dose. It would be of interest to determine whether these faster acting SpltNPVs further reduce the food uptake and hence further limit crop damage.

Acknowledgements

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Chapter 6

Identification of loci associated with enhanced virulence in *Spodoptera litura* nucleopolyhedrovirus isolates using deep sequencing

Adapted from:
Mark P. Zwart, Ghulam Ali, Elio G.W.M. Schijlen, Wopke van der Werf and Just M. Vlak. Identification of loci associated with enhanced virulence in Spodoptera litura nucleopolyhedrovirus isolates using deep sequencing. Manuscript in preparation.

Abstract

Two genetically distinct Spodoptera litura nucleopolyhedrovirus (SpltNPVs) isolates from Pakistan were investigated by deep sequencing of their full genomes. Previously we reported that isolate SpltNPV-Pak-TAX1 kills *S. litura* (leafworm) larvae significantly faster than SpltNPV-Pak-BNG. Here we find that the consensus sequences of these two virus isolates shared 99% identity, suggesting they are closely related. The major difference between these two isolates is the absence of hr17 (putative enhancer of transcription and origin of replication) in SpltNPV-Pak-TAX1 and the absence of ORF125 with unknown function in SpltNPV-Pak-BNG. Analysis of the rates of nonsynonymous and synonymous single-nucleotide substitutions showed that strong purifying selection predominates. For a small number of genes there was neutral or positive selection. The most striking case is ORF122, which encodes a putative viral fibroblast growth factor-like protein (FGF-like). This protein is known to be involved in the passage of virus from the midgut to the interior of the larva and linked to virus virulence in other baculoviruses. We found very little polymorphism within both virus isolates, a result at odds with observations for other baculoviruses, possibly suggesting recent dispersal of the virus in Pakistan from a single source. We have thus identified two loci possibly and one ORF linked to the enhanced virulence of the SpltNPV-Pak-TAX1. This information could help to understand the enhanced activity of SpltNPV-Pak-TAX1 and to select better SpltNPV isolates for the control of *S. litura* in Pakistan and elsewhere.

Keywords: *Spodoptera litura* nucleopolyhedroviruses, loci, virulence, deep sequencing

1. Introduction

The leafworm *Spodoptera litura* is an emerging pest insect in Pakistan, more specifically in cotton and arable crops. Control of this insect is predominantly by the use of chemical insecticides. However, this strategy is unsustainable in the long run due to resistance developing in the insect as well as the negative impact of chemical agents on human health and the environment. Safe, effective and sustainable alternatives therefore are being sought. Baculoviruses comply with these characteristics and are therefore being investigated for control of *S. litura* in Pakistan.

In a field survey in 2011-2013 twenty-two baculovirus isolates were obtained from *S. litura*, from different cropping systems (Chapter 4). These isolates turned out to be nucleopolyhedroviruses (NPVs) and members of the Genus Alphabaculovirus within the Baculoviridae family (ICTV, 2015). While the high sequence identity (> 98%) suggests that these isolates can be considered to belong to the *Spodoptera litura* nucleopolyhedrovirus or SpltNPV species (Chapter 4), hese isolates also represent a cluster of genotypic variants that are closely related amongst themselves and are distinct from the species type member (SpltNPV-G2, NC_003102.1; Pang et al. 2001). Within these twenty-two Pakistan isolates, three genogroups were identified from restriction fragment length polymorphism (RFLP) analysis (Chapter 4). These genogroups strongly correlate to the regions where they were found and to a much lesser extent to the cropping system. It was also found that four SpltNPVs showed a faster speed-of-kill than the other SpltNPVs and all of these originated from the Punjab (Chapter 4, Table 1). The close relatedness among the Pakistan SpltNPV isolates and their more distant relatedness to the type species from China and SpltNPV isolates from Japan, suggests that SpltNPV may have recently been introduced to Pakistan from a single source. The Pakistan SpltNPVs may be in an early stage of an adaptive radiation, providing a unique opportunity to study this process.

Next-generation sequencing (NGS) is the very high throughput sequencing of the genetic material of an organism or a community of organisms, using massively parallel sequencing and typically employing a shotgun approach to library preparation. Due to the volume of sequence data generated, the introduction of NGS has also lead to the rapid development of bioinformatic tools to quickly analyze large sequencing datasets. The decrease in time and cost of sequencing samples have brought about a revolution in virology (Acevedo et al. 2014, Roossinck et al. 2015, Cuevas et al. 2015, Shi et al. 2016), whilst the technology is consistently and continuously being improved (Acevedo and Andino 2014). An equally

important advantage of NGS is the possibility of identifying rare polymorphisms in mixed-genotype virus populations by increasing the coverage with which they are sequenced. For example, traditional virological approaches, such as restriction fragment length polymorphism (RFLP) analysis on clones, allowed for the identification of considerable genetic variation in baculovirus populations (Knell and Summers, 1981, Smith and Crook 1988, Cory et al. 2005). However, ultra-deep sequencing can be used to identify quantitatively genetic variation present until down to a frequency of 0.00025 (Chateigner et al. 2015).

In this chapter we characterize the full-genomes of the SpltNPV-Pak-BNG and SpltNPV-Pak-TAX1 isolates, with low and high speed-of-kill respectively, using an NGS approach. We also seek to further detail the differences between the SpltNPV genogroups A and B, by studying the above isolates that are representative for each group. When whole genome sequences are considered, the question is whether these isolates in entirety share 99% nucleotide identity as suggested by the sequencing of a limited number of open reading frames (Chapters 2 and 4). In addition, the question is at what loci on the genome do genetic differences occur? Second, we are interested in determining how much genetic diversity there is within each isolate. Do the Pakistani SpltNPV isolates contain considerable genetic variation, as has been found for many other baculoviruses? Finally, we sought to better understand the genetic basis of the difference in speed of kill of the above two isolates. Can we identify loci associated with the higher virulence of the SpltNPV-Pak-TAX1 isolate relative to SpltNPV-Pak-BNG?

2. Material and methods

2.1. SpltNPV isolates, insect rearing, virus amplification and DNA extraction

The SpltNPV isolates SpltNPV-Pak-BNG and SpltNPV-Pak-TAX1 were obtained from Bahawalnagar and Taxila (Punjab, Pakistan), respectively, as described in Chapters 2 and 4, and they fall in genogroups A and B, respectively, as described in chapter 4 (Ali et al., 2017[b]). The viruses were propagated in third instar *S. litura* larvae at the National Agricultural Research Center in Islamabad, Pakistan as described in Chapter 2 (Ali et al., 2017[a]). The DNA was extracted from occlusion-derived virions (ODV), which were in turn were isolated from occlusion bodies (OBs) as described in Chapter 2 (Ali et al., 2017[a]).

2.2. Library preparation and deep sequencing

Libraries for deep sequencing were prepared using the Nextera XT kit (Illumina), following the manufacturer's instructions and using recommended adapters. These libraries were then

sequenced by paired end Illumina MiSeq, in a flow cell together with libraries from other AcMNPV and SeMNPV samples. A high concentration of PhiX control was used because of the expected imbalance in GC content. Library preparation and deep sequencing were performed by the Bioscience unit at Wageningen University and Research.

2.3. Analysis of deep sequencing data

For primary analyses of the MiSeq data, we used CLC Genomics Workbench v10.1.1 (Qiagen Genomics, Aarhus, Denmark). We used two different approaches to analyze the data: (i) resequencing", in which reads are mapped to an existing reference genome, and (ii) a *de novo* assembly approach, in which the reads were assembled without the help of a reference genome.

For analyzing the data with a resequencing approach, the demultiplexed reads were trimmed using the trim sequences tool with default settings, except that the minimum Phred score was raised to 30 (and the expected error rate lowered to $\leq 1/1000$) because we expected to have high coverage. Next, the trimmed reads were mapped to the Spodoptera litura nucleopolyhedrovirus (SpltNPV) reference genome (NC_003102.1, Pang et al. 2001) and low frequency variant detection was performed, both using default settings. For further analysis of the data, we then exported a detailed mapping report (*.tsv) and the low frequency variant detection tables to R v3.4.2 (R Development Core Team, 2008).

For analyzing the data with a *de novo* assembly approach, the reads were trimmed as for the resequencing approach. Subsequently a *de novo* assembly was run using the default CLC Genomics Workbench settings, but ignoring contigs shorter than 1000 bp. The consensus sequence of the contig corresponding to the full-length virus genome (see Results) was then extracted. We set the start codon of the polyhedrin gene (ORF1) as the first reference position in the circular genome. We then used these consensus sequences for two subsequent analysis. First, to identify differences between the two isolates, we performed a reciprocal mapping of reads (i.e. BNG reads on the TAX1 *de novo* assembly consensus sequence, and vice versa), followed by low frequency variant detection. Second, to identify within-isolate polymorphism we mapped the reads of an isolate to its own *de-novo*-assembled consensus sequence.

2.4. Identification of mutations

We classified mutations as being small or large, based on the methods used to detect them. Small mutations are short enough to be identified using individual reads by the low frequency variant detector in CLC Genomics Workbench, and in this case included single nucleotide

polymorphisms (SNPs), multinucleotide polymorphisms (MNPs) and short indels (< 100 bp). The high frequency variant detector does not report indels that span over regions longer than the typical read length, and so we had to resort to other methods. The method used to detect large indels was different for the resequencing and de novo assembly approaches.

To map large indels in the resequencing analysis, we scanned for low coverage regions (< 10 reads) in the mapping of the reads to the reference genome. The coverage in these low coverage regions was essentially zero (see Results section), and therefore the overall occurrence of deletions was unambiguous. Upon discovering a low coverage region, we found that its edges reads only mapped until a defined position. With the analysis settings used, reads can partially map to the reference sequence, and the non-mapping portion of the read is ignored in the analysis. We then searched for the consensus sequence of the non-mapping portion of reads next to the low coverage area in the genome. In most cases found this corresponded to the sequence adjacent to the opposite end of the low coverage region. Similarly, we checked where the split-mapped reads were mapping, and this provided further support for the occurrence of deletions. (i.e., pair-end reads can map on both sides of the deletion, and because of the distance covered on the reference genome can be considered split-mapped reads.). To map large indels for the *de novo* assembly approach, we aligned the consensus *de novo* assembly sequence to the reference genome, and then checked for gaps.

2.5. dN/dS and dI/dS analyses

The ratio of the rate of nonsynonymous to the rate of nonsynonymous substitution (dN/dS) is a widespread tool to identify the kinds of selection acting on genomes or genes. The main assumption behind this analysis is that nonsynonymous substitutions can be advantageous or disadvantageous because they lead to changes in the amino acid sequence, whereas synonymous changes are neutral because they do not alter the amino acid sequence. When dN/dS < 1 there is purifying selection (more synonymous mutations then expected by chance), whereas when dN/dS > 1 there is directional selection (more nonsynonymous mutations then expected by chance). When dN/dS = 1, there is neutral evolution, as mutations that are assumed to be neutral or not occur at the same rate. Following Tenaillon et al. (2012), we also performed a dI/dS analysis to investigate the rate of evolution in intergenic sequences (dI), using the rate of synonymous mutations in coding regions to establish a baseline.

Prior to the dN/dS and dI/dS analysis, we had to identify which observed mutations would be included in the analysis. For the data from the *de novo* assembly, all homozygous

SNPs were included. However, for the resequencing analysis, mutations could be the result of errors in the reference sequence or true differences between the reference virus and the Pakistani virus isolates. The SpltNPV reference sequence (NC_003102.1, Pang et al. 2001) was obtained by Sanger sequencing and we could not verify its quality independently. To exclude errors, we therefore only considered mutations, with respect to the reference sequence (NC_003102.1) that were found only in BNG or TAX1, and not in both as the latter might represent sequencing errors in the reference genome. Using this criterion will not only eliminate mutations that are due to errors, but it will also exclude any mutations as compared to the reference that are present in both Pakistani virus isolates. The analysis thus focuses on the mutations that occurred within the Pakistani clade of the virus.

To perform a dN/dS analysis on the whole genome and individual genes, we followed an approach similar to that used by Tenaillon et al. (2012). First we generated an expectation for the number of possible synonymous (*S*) mutations within open reading frames (ORFs) that do not lead to a change in the amino acid sequence and nonsynonymous (*N*) mutations within ORFs that do lead to changes in the amino acid sequence, using the reference genome (NC_003102.1). We tallied the number of times each codon was used in the whole genome or in a gene, and multiplied this number by the synonymous or nonsynonymous mutations possible for that particular codon. The sum of these products is then the numerator for each rate. The synonymous or nonsynonymous mutations for each codon were weighted by the probability of the underlying nucleotide substitution. We are not aware of any mutational spectrum data that could be used to infer the mutational biases for an alphabaculovirus and we therefore simply assumed a transition to transversion ratio (TTR) of either 1, 3 or 5, and generated predictions for each value. Custom R scripts were used to generate these predictions. Finally, the numerator for each rate is obtained by counting the number of synonymous or nonsynonymous substitutions observed in each gene, or over the whole genome. Some genes contained non-synonymous mutations but no synonymous mutations. To be able to approximate dS in these cases, we calculated the expected number of synonymous mutations in the gene, given the genome-wide occurrence of synonymous mutations and the length of the gene.

In the dI/dS analysis, the denominator (dS) is exactly the same as in the dN/dS analysis. The rate of intergenic mutations (dI) was determined by first generating a prediction for the number of possible intergenic substitutions, by considering the frequency of all nucleotides in intergenic regions (i.e., all positions outside of ORFs). All possible mutations were also weighted for mutational bias, assuming different TTR values. Finally the numerator, dI, was

obtained by counting all intergenic mutations. We only performed this analysis for the whole genome, since assigning regulatory regions to specific genes is a non-trivial task.

3. Results

3.1. Sequencing results overview

We deep sequenced two Pakistani SpltNPV isolates: SpltNPV-Pak-BNG and SpltNPV-Pak-TAX1, and analyzed the data through two different approaches: a 'resequencing' approach that employs a reference sequence (NC_003102.1, Pang et al., 2001), and a de novo assembly approach. As we deep sequenced purified virus DNA, despite using only high quality reads (Phred ≥ 30), we still had high coverage of the viral genome (resequencing mean coverage ±SD: SpltNPV-Pak-BNG: 4645 ±1079; SpltNPV-Pak-TAX1: 5746 ±1154). For the resequencing analysis, for both isolates there are approximately 2000 bases in the reference genome without any coverage (Fig. 1A-B). Likewise, for the *de novo* assembled genomes, the total genome length for the two Pakistani isolates (SpltNPV-Pak-BNG: 137,155 bp, SpltNPV-Pak-TAX1: 137,655 bp) is shorter than the reference genome SpltNPV-G2 (139,342 bp) (Pang et al., 2001). Therefore, both approaches suggest there are indels present in both of the Pakistani genotypes as compared to the reference genome. To gauge whether further genomic variation identified by both methods was similar, we compared the number of homozygous SNPs unique to SpltNPV-Pak-BNG and SpltNPV-Pak- SpltNPV-Pak-TAX1 identified by each method. The total number of intergenic (I), synonymous (S) and nonsynonymous (N) mutations identified was similar (resequencing: I = 76, S = 257, N = 107; de novo assembly: I = 87, S = 247, N = 111). The total number of mutations found in each ORF was high similar for the two approaches (Spearman rank correlation: $\rho = 0.990$, n = 242, $p < 0.001$; Fig 1C). There does appear to be a conflict for ORF125 (Fig 1C), but this discrepancy between the two approaches can be explained by the occurrence of a large deletion at this locus (See Section 3.2). As a final validation of our deep sequencing analyses, we compared the *Eco*RI restriction fragment length profiles (RFLP) predicted by the consensus sequences for the *de novo* assemblies of the two viruses. The results were similar (Table S1, in the appendix to this chapter), providing further confirmation of the accuracy of the deep sequencing analysis and the two assembly protocols.

The SpltNPV-Pak-BNG and SpltNPV-Pak-TAX1 genomes have been annotated (see Table S2, in the appendix to this chapter) and compared to the reference strain SpltNPV-G2 (Pang et al., 2001) and to the genome of a closely related species, S. littoralis NPV (SpliNPV) (Breitenbach et al., 2013).

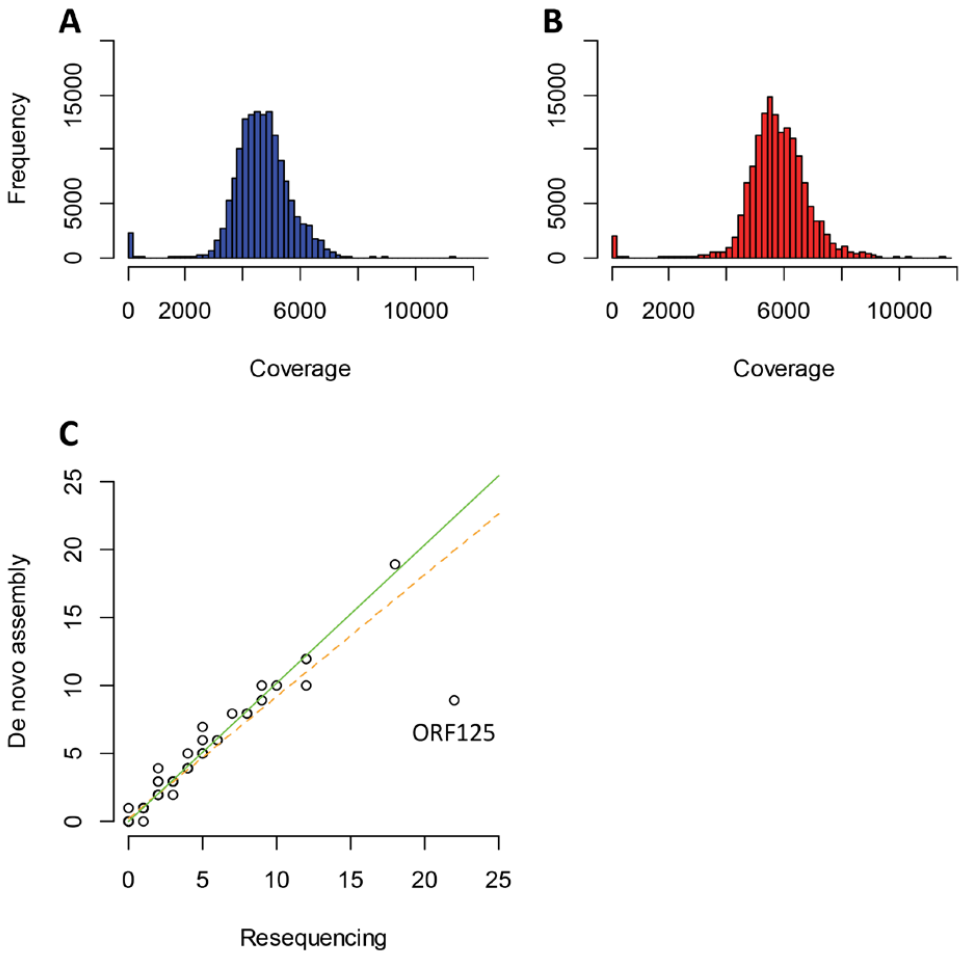


Figure 1. Histograms of the coverage per nucleotide position in the reference genome for the resequencing analysis is given for isolates SpltNPV-Pak-BNG (Panel A) and SpltNPV-Pak-TAX1 (Panel B). Note the similarity of the distributions for SpltNPV-Pak-BNG and SpltNPV-Pak-TAX1, and that approximately 2000 positions have little or no coverage. In Panel C, the total number of unique synonymous and nonsynonymous mutations per ORF (i.e., the sum for SpltNPV-Pak-BNG and SpltNPV-Pak-TAX1) is plotted for the resequencing (x-axis) and de novo assembly (y-axis) approaches. The dotted orange line is the model 2 regression line using all data, whilst the green line is the model 2 regression line excluding ORF125 (data point labelled), which appears to be an outlier. For all data, the slope of the regression line is 0.897, whereas without ORF125 the slope is 1.011. With the exception of ORF125, there seems to be very good agreement between the two deep sequencing analysis methods.

In addition to the loss of a limited number of ORFs due to the large deletions (Section 3.2), SpltNPV-Pak-TAX1 is missing ORF9. The latter is a very small ORF and may be not functional. However, overall the organization of the SpltNPV-Pak-BNG and SpltNPV-Pak-TAX1 genomes is highly similar to SpltNPV-G2.

3.2. Occurrence of large indels in both virus isolates and the deletion of hr17 in SpltNPV-Pak-TAX1

Both sequencing methods suggest that there are indels in both SpltNPV-Pak-BNG and SpltNPV-Pak-TAX1, both with respect to each other and to the reference sequence SpltNPV-G2. For the resequencing data, plotting the coverage per position shows that low coverage positions cluster, and that there are two large deletions with respect to the reference sequence in each Pakistani isolate (Fig. 2, see also Fig. S1, in the appendix of this chapter).

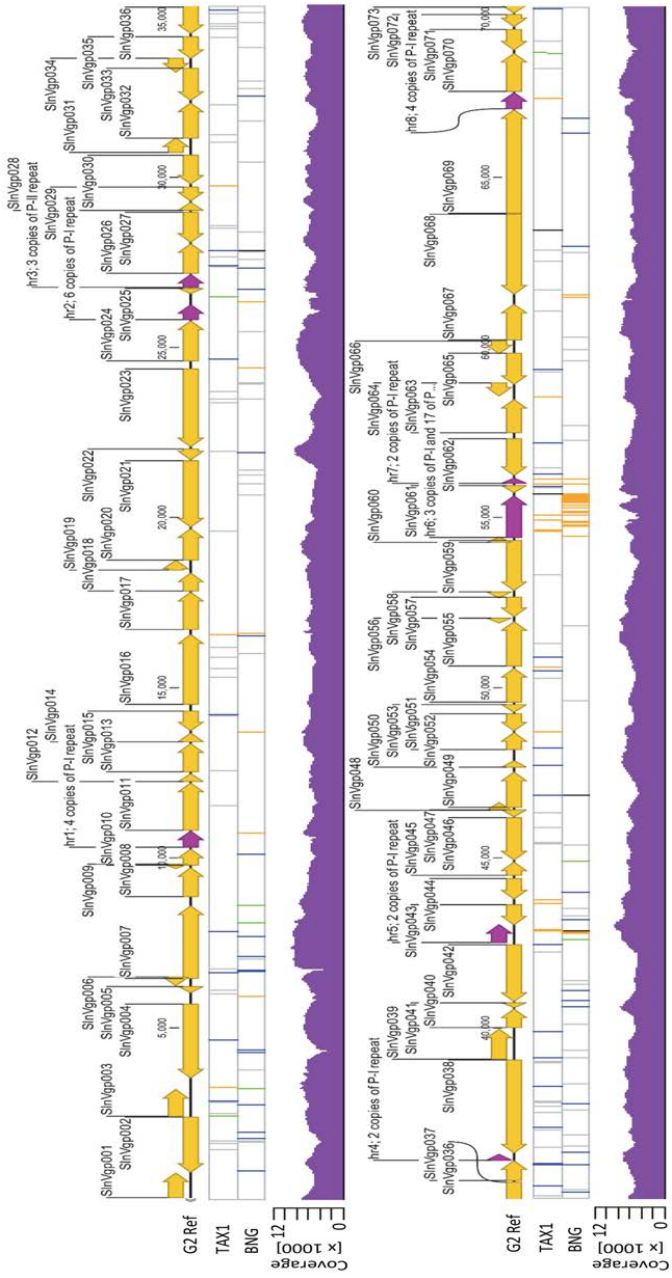
The exact positions of these indels were determined using both sequence analysis methods (see Materials and Methods section), and found to be identical (Table 1). For both isolates, the largest indel results in the loss of the entire ORF126. In SpltNPV-Pak-BNG, another indel partially removes ORF125, whereas in SpltNPV-Pak-TAX1 a second indel entirely removes hr17, one of 17 homologous regions found in the SpltNPV reference sequence and isolate BNG. The (putative) functions of ORFs 125 and 126 are unknown.

Table 1: Deletions in the BNG and Tax1 isolates.

Isolate	Start Deletion	End Deletion	Size	Locus and notes
BNG	122,674	123,167	493	ORF125 (broB), partial deletion
	123,500	125,232	1732	ORF126 deleted
TAX1	123,500	125,232	1732	ORF126 deleted
	137,732	138,050	318	hr17 deleted, 33 bp insert

3.3. High similarity between the two Pakistani virus isolates

The majority of mutations detected by the resequencing approach are not unique to either SpltNPV isolate. For example, a total of 2116 SNPs are detected in the two isolates, whilst there are 219 unique SNPs in SpltNPV-Pak-BNG and 221 unique SNPs in SpltNPV-Pak-TAX1 but 1674 SNPs that are shared between the two isolates. However, the SpltMNPV reference sequence (NC_003102.1, Pang et al., 2001) was determined using different sequencing methods at the time, and it is therefore difficult to know whether mutations detected here using the resequencing analysis are due to mistakes in the reference genome sequence or *bona fide* genetic differences.


SlnVgp001
SlnVgp002
SlnVgp003
SlnVgp004
SlnVgp005
SlnVgp006
SlnVgp007
SlnVgp008
SlnVgp009
hr1; 4 copies of P-I repeat
SlnVgp010
SlnVgp011
SlnVgp012
SlnVgp013
SlnVgp014
SlnVgp015
SlnVgp016
SlnVgp017
SlnVgp018
SlnVgp019
SlnVgp020
SlnVgp021
SlnVgp022
SlnVgp023
SlnVgp024
SlnVgp025
hr2; 6 copies of P-I repeat
SlnVgp026
SlnVgp027
hr3; 3 copies of P-II repeat
SlnVgp028
SlnVgp029
SlnVgp030
SlnVgp031
SlnVgp032
SlnVgp033
SlnVgp034
SlnVgp035
SlnVgp036
SlnVgp037
SlnVgp038
hr4; 2 copies of P-I repeat
SlnVgp039
SlnVgp040
SlnVgp041
SlnVgp042
hr5; 2 copies of P-I repeat
SlnVgp043
SlnVgp044
SlnVgp045
SlnVgp046
SlnVgp047
SlnVgp048
SlnVgp049
SlnVgp050
SlnVgp051
SlnVgp052
SlnVgp053
SlnVgp054
SlnVgp055
SlnVgp056
SlnVgp057
SlnVgp058
SlnVgp059
SlnVgp060
SlnVgp061
hr6; 3 copies of P-I and 17 of P...
SlnVgp062
SlnVgp063
SlnVgp064
hr7; 2 copies of P-I repeat
SlnVgp065
SlnVgp066
SlnVgp067
SlnVgp068
SlnVgp069
SlnVgp070
SlnVgp071
hr8; 4 copies of P-I repeat
SlnVgp072
SlnVgp073
5,000
10,000
15,000
20,000
25,000
30,000
35,000
40,000
45,000
50,000
55,000
60,000
65,000
70,000
G2 Ref
TAX1
BNG
Coverage
[x 1000]
12
0

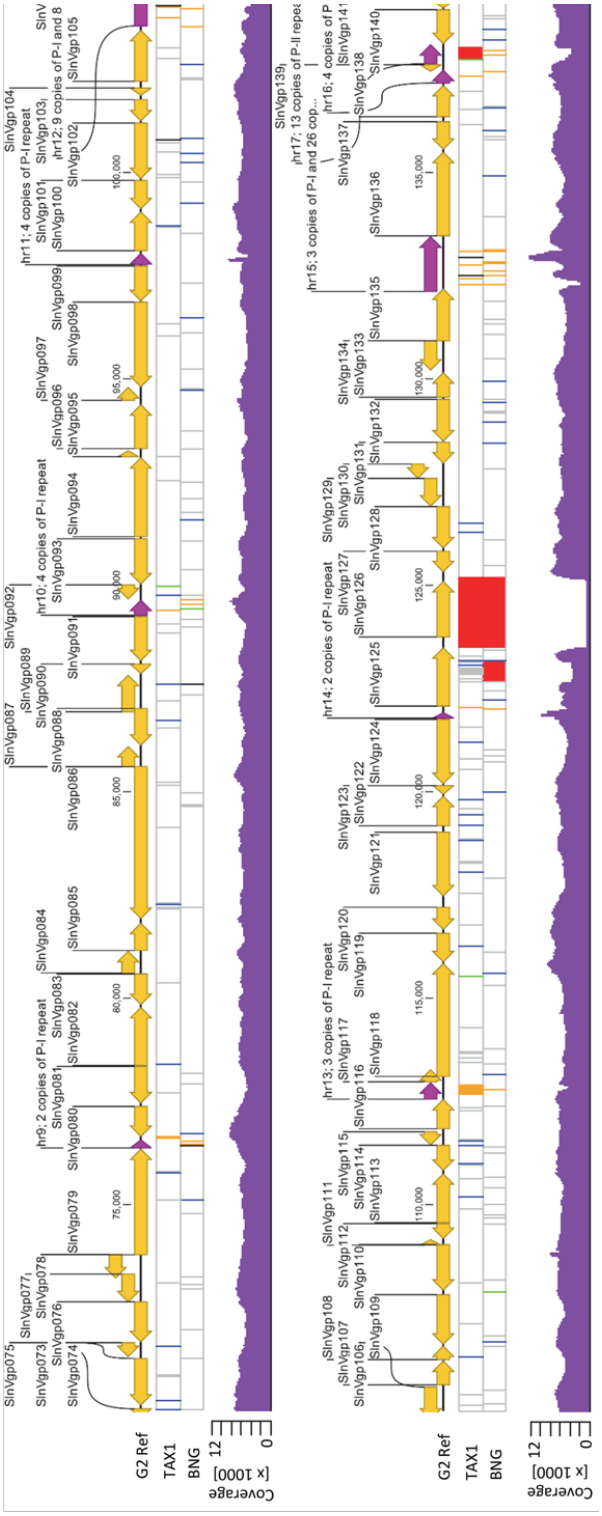

SlnVgp075
SlnVgp077
SlnVgp073
SlnVgp078
SlnVgp076
SlnVgp074
SlnVgp079
75,000
hr9; 2 copies of P-I repeat
SlnVgp080
SlnVgp081
SlnVgp082
SlnVgp083
SlnVgp084
SlnVgp085
SlnVgp086
80,000
85,000
SlnVgp087
SlnVgp089
SlnVgp090
SlnVgp088
SlnVgp091
SlnVgp092
hr10; 4 copies of P-I repeat
SlnVgp093
SlnVgp094
90,000
SlnVgp097
SlnVgp096
SlnVgp095
SlnVgp098
95,000
SlnVgp099
hr11; 4 copies of P-I repeat
SlnVgp101
SlnVgp100
SlnVgp104
SlnVgp103
hr12; 9 copies of P-I and 8
SlnVgp102
100,000
SlnV
SlnVgp105
G2 Ref
TAX1
BNG
Coverage
[x 1000]
12
0
SlnVgp108
SlnVgp107
SlnVgp106
SlnVgp112
SlnVgp110
SlnVgp109
SlnVgp111
SlnVgp115
SlnVgp114
SlnVgp113
110,000
hr13; 3 copies of P-I repeat
SlnVgp117
SlnVgp116
SlnVgp118
SlnVgp120
SlnVgp119
115,000
SlnVgp123
SlnVgp122
SlnVgp121
SlnVgp124
120,000
hr14; 2 copies of P-I repeat
SlnVgp125
SlnVgp129
SlnVgp127
SlnVgp130
SlnVgp126
SlnVgp131
SlnVgp128
125,000
SlnVgp134
SlnVgp133
SlnVgp132
130,000
SlnVgp139
hr17; 13 copies of P-II repea
hr15; 3 copies of P-I and 26 cop...
SlnVgp135
SlnVgp137
hr16; 4 copies of P
SlnVgp141
SlnVgp138
SlnVgp140
SlnVgp136
135,000
G2 Ref
TAX1
BNG
Coverage
[x 1000]
12
0

Figure 2 (at page 89 and 90). Overview of the mutations in isolates SpltNPV-Pak-BNG and Splt-Pak-TAX1. The genome is depicted linearly, in four different sections. In each of these sections, the upper panels show the key features of the SpltNPV-G2 reference genome (Pang et al. 2001). ORFs are yellow and homologous regions (HRs) are magenta, and they are labelled according to the NCBI annotation. The numbers directly above the line are nucleotide coordinates in the reference genome. The middle panels show homozygous mutations detected in the Pakistani isolates. The mutations shown were detected using the resequencing approach, but only mutations that occur in only one of these isolates is shown. These mutations include intergenic SNPs (orange), synonymous SNPs (grey), non-synonymous SNPs (blue), multi-nucleotide polymorphisms (MNPs, black), insertions (green) and deletions (red). In the bottom panels, the mean coverage for the two isolates is plotted in purple.

We therefore cannot draw conclusions on the differences between the Pakistani and Chinese SpltNPV isolates, although the data clearly suggest that the two Pakistani isolates are much more similar to each other than to the Chinese isolate SpltNPV-G2. Although the latter isolate has been sequenced more than 17 years ago, based on the ORF annotation and high similarity with the Pakistani isolates the SpltNPV-G2 sequence appears to be quite accurate. If we focus only on the two Pakistani isolates, we find very few differences besides the unique indel in each deletion (Fig. 2), for example only 440 homozygous SNPs detected by the resequencing approach. Over the entire genome, SpltNPV-Pak-BNG and SpltNPV-Pak-TAX1 share 98.6% identity, or 99.2% identity excluding the two large indels (Table 1). The two Pakistani isolates are therefore highly similar, an observation congruent with previous results of RFLPs and Sanger sequencing of a limited number of ORFs (Chapter 4) (Ali et al., 2017c).

3.4. Limited within-isolate polymorphism

We considered whether there was polymorphism within each Pakistani isolate by mapping reads against their respective *de novo* assembled genome. In our experience, mutations identified as being homozygous by the low frequency variant detector (see Materials and Methods) are rarely artefacts of poor mapping. However, mutations identified as being heterozygous are sometimes mapping artefacts, and for all heterozygous variants called at a frequency > 0.02 we therefore manually curated the read mappings. This led to discarding most variants, typically because they appeared to be artefacts from the mapping of reads to regions with repeats (see Table S3, in the Appendix to this chapter). After this curation, we found surprisingly little polymorphism, and only SNPs (Table 2).

For SpltNPV-Pak-BNG, there were four SNPs at frequencies ranging between 0.021 and 0.470; for SpltNPV-Pak-TAX1 there was only a single SNP at a frequency of 0.139. The patterns in coverage for the mapping of reads against their respective *de novo* assembled genome (Fig. S2) do not suggest that there is any copy number variation in the population. For

Table 2: Within-isolate polymorphism

Isolate	Mutation	Count/Coverage	Frequency	Forward/Reverse	ORF: AA change[a]
BNG	c35,000a	155/3881	0.0399	44.19%	ORF36
	c35,202t	1623/3455	0.4698	49.97%	ORF36
	g41180a	60/2910	0.0206	47.76%	ORF42
	c111904t	407/3822	0.1065	49.34%	ORF116: S45F
TAX1	c98204a	416/2995	0.1389	43.56%	

[a] The ORF in which the mutation occurs is given for mutations in coding regions, and amino acid changes are given when mutations are non-synonymous.

both Pakistani isolates, there is therefore very limited genetic variation within each virus population, and the genotypes present must be very closely related, as they differ only by 1 to 4 nucleotides.

3.5. Identification of loci associated with virulence of the SpltNPV-Pak- isolates

We performed dN/dS and dI/dS analyses to determine what types of selection have been acting on the whole genome and specific loci in the two Pakistani isolates. For the resequencing analysis results, we could consider each isolate separately by looking only at mutations with respect to the reference genome SpltMNPV-G2 (Pang et al., 2001) that were unique for that isolate. For the *de novo* assembly, we could only consider the differences between the two Pakistani isolates. When considering the whole genome, the excess of synonymous mutations suggests there is strong purifying selection acting on coding regions (Table 3). For noncoding regions, purifying selection is weaker and not significant when a higher transition to transversion ratio (TTR = 5) is assumed.

Table 3: Whole genome dN/dS and dI/dS analyses

			Obs. mutations[b]		dN/dS or dI/dS		
Analysis	Approach[a]	Isolate	NS/I	S	TTR[c]=1	TTR[c]=3	TTR[c]=5
dN/dS	Resequencing	BNG	50	129	0.103^{***}	0.136^{***}	0.152^{***}
		TAX1	57	128	0.119^{***}	0.157^{***}	0.175^{***}
		Both	107	257	0.111^{***}	0.146^{***}	0.163^{***}
	de novo Assembly	Both	111	247	0.120^{***}	0.158^{***}	0.176^{***}
dI/dS	Resequencing	BNG	40	129	0.520^{***}	0.642^{*}	0.695^{*}
		TAX1	36	128	0.471^{***}	0.583^{**}	0.630^{*}
		Both	76	257	0.495^{***}	0.612^{***}	0.663^{**}
	de novo Assembly	Both	87	247	0.590^{***}	0.730^{*}	0.789

[a] Approach refers to the primary data analysis approach. [b]The observed mutations in each class: NS = nonsynonymous, I = intergenic, and S = synonymous. Note NS or I is given depending on the analysis. [c]The transition to transversion ratio assumed in the dN/dS or dI/dS analysis. *** Indicates a corresponding binomial p value < 0.001 comparing the observed and expected values, ** indicates a p value < 0.01 and * indicates a p value < 0.05. Note that for the de novo assembly, only an analysis comparing the two Pakistani isolates can be done.

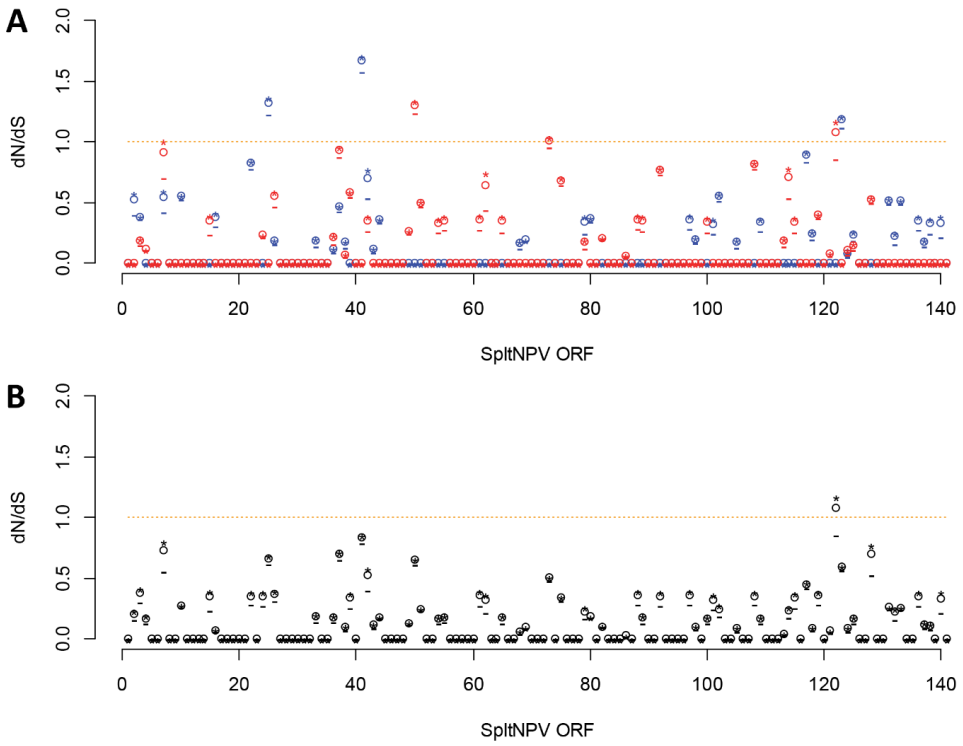


Figure 3: dN/dS analyses for homozygous SNPs done separately for each isolate (Panel A) and for the two isolates together (Panel B). On the x-axis is the ORF number corresponding to the SpltMNPV-G2 reference, and on the y-axis is the dN/dS value. In panel A, the blue data points correspond to SpltNPV-Pak-BNG, and the red points to SpltNPV-Pak-TAX1. In both panels, dN/dS values assuming different mutational biases are present: transition to transversion ratio (TTR) values of 1 (lines), 3 (circles) and 5 (asterisks). The orange line indicates dN/dS = 1, which indicates neutral evolution. When dN/dS < 1, there is purifying selection, as the amino acid sequence tends to be conserved. When dN/dS > 1 there is positive selection, as more coding mutations occur then under the neutral expectation.

When we performed the dN/dS analysis on individual genes using the resequencing approach for primary analysis of the data, we found that in most cases dN/dS < 1 (Fig. 3), a similar result to the whole genome. However, for a small number of genes, when the two isolates are analyzed separately dN/dS ≥ 1 (Table 4), it seems that neutral evolution or positive selection might be occurring at these loci. When the data from the two isolates were combined, only ORF122 gave a dN/dS ≥ 1. However, there are no unique mutations in this gene in SpltNPV-Pak-BNG (as compared to the reference sequence SpltNPV-G2), and this result therefore is driven entirely by Splt-Pak-Tax1. In no cases were these differences significantly greater than the null expectation (neutral evolution, see Materials and Methods for a description of the binomial test procedure), a result that is not surprising given the relatively small number

Table 4: Summary of genes under neutral or purifying selection.

Isolate	ORF[a]	Function	Mutations[c]		dN/dS		
			NS	S	TTR[d]=1	TTR[d]=3	TTR[d]=5
BNG	25	Unknown, unique to SpltNPV	1	0	1.229	1.328	1.375
	41	Unknown, found only in SpltNPV and SpliNPV	1	0	1.576	1.670	1.715
	123	Unknown	1	0	1.125	1.187	1.216
TAX1	7	Unknown, found only in SpltNPV and SpliNPV	5	2	0.698	0.912	1.01
	37	Late-expression factor 12	2	0	0.876	0.939	0.968
	50	Unknown, Ac55-like protein, non-essential in BmNPV (Ono et al. 2012, Rohrmann 2013)	1	0	1.234	1.306	1.340
	73	Unknown, DUF843 superfamily	1	0	0.955	1.014	1.042
	122	Fibroblast growth factor, determinant of speed of kill (Yin et al. 2016).	3	1	0.862	1.079	1.179

[a] Only ORFs a dN/dS value that exceeds 0.95 for any TTR value are included here. [c]The observed mutations in each class: NS = nonsynonymous and S = synonymous. [d]The transition to transversion ratio (TTR) assumed in the dN/dS analysis. Note that no dN/dS results were significantly higher than the null expectation.

of mutations observed. We therefore cannot ascertain with statistical certainty whether directional selection occurred, but only report a trend that is in stark contrast to the rest of the genome. Finally, note that only the dN/dS results for the resequencing approach are presented here, but the *de novo* assembled assembly gave highly similar results to the combined analysis. Of the genes with higher dN/dS values, the results for ORF7 and ORF122 in SpltNPV-Pak-TAX1 are probably the most robust, because multiple nonsynonymous substitutions were found in each gene. Interestingly, we also found three nonsynonymous mutations and a somewhat elevated dN/dS result in the ORF7 of SpltNPV-Pak-BNG (dN/dS: 0.418-0.606). We only found a SpltMNPV ORF7 homolog in SpliNPV, hence this ORF is specific for the Splt/SpliNPV species (Pang et al., 2001). We detected mutations in ORF122 only for SpltNPV-Pak-TAX1, the faster killing isolate of the two sequenced Pakistani isolates, as compared to the reference strain SpltNPV-G2, and the fastest killing SpltNPV isolate characterized so far (Chapters 2 and 4). ORF122 codes for a viral fibroblast growth factor-like protein (FGFlike). Knockouts of HearNPV (group II alphabaculoviruses) *fgf* resulted in a slower speed of kill (Yin et al. 2016), as do knockouts of its homolog in Group I alphabaculoviruses (Detvisitsakun et al. 2007, Means and Passarelli 2010).

4. Discussion

We characterized and compared the SpltNPV-Pak-BNG and the fast-acting isolate SpltNPV-Pak-TAX1 isolates using a deep sequencing approach, and used two divergent approaches for data analysis: resequencing by mapping of reads to the SpltNPV reference sequence and *de novo* assembly. The results of these two approaches were similar, in terms of the presence or absence of large indels and the homozygous SNPs identified. This similarity highlights the power of a *de novo* assembly based approach – despite the presence of repeat regions –, provided there is sufficient sequence coverage. The two Pakistani isolates shared approximately 99% nucleotide identity, as had been anticipated from sequencing of a small number of loci (Chapters 2 and 4).

The variation between the two isolates included two indels, a partial deletion of ORF125 in SpltNPV-Pak-BNG and a complete deletion of homologous region hr17 in SpltNPV-Pak-TAX1, as well as approximately 250 SNPs, the majority of which were synonymous. Baculovirus homologous regions (hrs) are thought to be origins of DNA replication and transcriptional enhancers, though no single homologous region appears to be essential (Carstens and Wu, 2007, Rohrmann 2013). As hr17 might control transcription levels, it may also effect the virulence of the virus as a whole. When it serves as replication origin, it may affect the replication speed and final outcome of the infection. dN/dS analysis suggests that ORF122 – coding for a vFGF – of SpltNPV-Pak-TAX1 is not subject to strong purifying selection, and possibly even subject to directional selection. In other studies, both vFGFs and homologous regions have been linked to baculovirus virulence and speed of kill (Wang et al. 2016), making it plausible that these two loci contribute somehow to the increased speed of kill of the SpltNPV-Pak-TAX1 isolate relative to SpltNPV-Pak-BNG. Mutational analysis of ORF122, ORF7 or hr17 using SpltNPV bacmids to be constructed or via CRISPR-CAS9 mutagenesis would be required to substantiate this claim. It is interesting to note that differences in biological activity were found between spatially separated isolates of *Lymantria dispar* MNPV in conjunction with differences in whole genome sequence information (Harrison et al., 2016; Martemyanov et al., 2017). By applying dN/dS analysis it is possible that other virulence genes come to light, an approach that has worked well when comparing different baculovirus species (Harrison and Bonning 2003).

We found surprisingly little genetic variation within each isolate, with only a single polymorphic SNP in BNG and four polymorphic SNPs within SpltNPV-Pak-TAX1, as compared to the reference strain SpltNPV-G2 (Pang et al., 2001). Although we had reasonably

high sequencing coverage, in another study much higher coverage (> 100,000) has been used to identify very rare variants (Chateigner et al. 2015). Nevertheless, our results contrast with those generally found for other baculoviruses (Knell and Summers 1981, Smith and Crook 1988, Cory et al. 2005, Chateigner et al. 2015), where considerable genotypic variation is typically found within an isolate. However, in the case of these two Pakistani SpltNPV isolates, there is very limited polymorphism. Although we cannot gauge the generality of this result given our sample size, it is still interesting to speculate on specific or general causes. The limited polymorphism observed could very well be due to a small number of viral founders of infection in the larvae sampled (Zwart et al. 2009). The circulating virus population could also stem from a single, recent introduction, because after a narrow genetic bottleneck one would expect to find limited genetic variation in a virus population with a low mutation rate. Hence, a recent outbreak and subsequent dispersal of a SpltNPV-Pak ancestor could explain the limited variation found in Pakistani isolates. Alternatively, in another study the frequency of mixed-genotype infections was found to be lower during disease outbreaks (Hoa et al. 2011), possibly due to the fixation of high virulence strains in epidemic conditions.

Regardless of how it all has come about and notwithstanding differences between the two Pakistani isolates, the lack of polymorphism observed means that there is probably very little variation in speed-of-kill within these two isolates. Hence, using a laboratory evolution approach to select for variants with a faster speed-of-kill will be entirely dependent on the occurrence of *de novo* mutations and may therefore take considerable evolutionary time given the low mutation rates for DNA viruses. To use such an approach, it may be advisable to search for isolates with greater standing genetic variation, or alternatively, to mutagenize the virus population.

One important constraint in our study was the lack of data on mutational biases in insect DNA viruses in general. Assuming different transition to transversion ratios clearly affects the results of the dN/dS analyses, but in the absence of any data, we could only perform the analysis for a range of values. For the dN/dS analysis on the whole genome, we found good evidence for strong purifying selection, regardless of assumptions on mutational bias. On the other hand, for the dI/dS analysis, the statistical significance of purifying selection decreases as higher transition to transversion ratios are assumed (Table 4), calling into question whether this trend has biological meaning. These complications highlight the need for the investigation of the mutational spectrum (e.g., Tromas and Elena, 2010) for insect DNA viruses. We used a simple

counting method for the dN/dS analysis, but given the small number of mutations observed, and the fact that they affect different codons, this approach appears to be justified.

In conclusion, deep NGS sequencing of two Pakistani SpltNPV isolates with differences in speed of action revealed limited genetic variation within isolates. There was neutral to positive selection on two loci in one of the isolates, SpltNPV-Pak-TAX1: ORF7 (unknown function) and ORF122 (*fgf-like*). The fact that SpltNPV-Pak-TAX1 is more virulent than SpltNPV-Pak-BNG suggests that one or more of these loci might be determinants of virulence. However, it could be that hr17 is also involved in virulence, alone or in concert with the products of ORF122 and/or ORF7. It should be worthwhile to deep-sequence other SpltNPV-Pak isolates with different speeds of action, such as SpltNPV-Pak SFD1 and GRW1, to see whether the above observations have general validity. In addition it would be of interest to (re)sequence SpltNPV isolates outside of Pakistan, e.g. from China (SpltNPV-G2) or Japan, to see the extent of variation within these isolates and which ORFs are subjected to positive selection.

Chapter 7

General Discussion

1. General discussion

This thesis describes the discovery, genetic identification and biological characterization of nucleopolyhedroviruses of *Spodoptera litura* in Pakistan. Due to cultivation of Bt-cotton, cotton bollworm *Heliothis armigera* populations are effectively controlled, but *S. litura* has filled the niche left by *H. armigera* and has resurged as a major insect pest in cotton and other arable crops in the Indian continent (Arshad et al., 2009). The application of synthetic insecticides is considered the most commonly used method for controlling insect pests and *S. litura* has developed resistance against these synthetic insecticides (Saleem et al., 2008; Abbas et al., 2012; Shad et al., 2012). Novel insecticides are either not available, not allowed or too expensive for low-income farmers in developing countries, including Pakistan. In addition, the use of chemical insecticides will not only generate resistance, but will also have a negative impact on non-target organisms and the environment. So, there is an urgent need to explore and implement more eco-friendly methods to manage resurging *S. litura* populations. Biologically-based insect control methods, involving the use of parasites, predators and microbials, are more sustainable and ecologically safe.

The baculovirus Spodoptera litura nucleopolyhedrovirus (SpltNPV) is a potential candidate to control *S. litura* (Pang, 1994). The virus has been used successfully in China and Japan (Yang et al., 2012; M. Nakai, personal communication). The respective strains were natural endemic SpltNPV isolates, which were most likely better adapted to the local environment and agro-ecological circumstances. However, there is no information available on the natural presence of SpltNPV in Pakistan neither on its (commercial) use. Therefore, a research program was initiated to obtain such SpltNPV isolates from Pakistan and to identify and select proper isolates for development as biocontrol agent for *S. litura*. Furthermore, when such isolates are found, attempts should be made to obtain information on the genetics underpinning the biology and performance of such SpltNPV-Pak isolates.

Baculovirus field isolates contain a mixture of genotypes (Erlandson, 2009), which as an assembly determine the biological performance of the virus. The potential of field isolates of SpltNPV in the control of *S. litura* has not yet been explored, as there is no knowledge on the presence, biology and genetic diversity of SpltNPV isolates from Pakistan. So, it is not only important to study the genetic diversity among different SpltNPV isolates, but also the genetic variation within each isolate. There has been no commercial introduction of SpltNPV in Pakistan to date. Therefore, it was postulated that SpltNPV isolates, when found, would have been fairly recently introduced and are not yet fully adapted to the local environment.

Regardless of frequent variation in natural populations of baculoviruses, there have been inadequate systematic studies of SpltNPV diversity in general and its importance to insect-pathogen interactions, except for a recent study in Japan (Takatsuka et al., 2016). This justifies the attention to SpltNPVs from Pakistan, virgin territory to the virus until today. My hypothesis is that when there has been a recent natural introduction of SpltNPV in Pakistan, then the molecular and biological characteristics of these isolates may reflect this course of events and allow monitoring the geographic dispersal, and possibly evolution, of SpltNPV over space and time.

The research described in this thesis aims to provide answers to a number of key questions on the occurrence and biological and genetic diversity of SpltNPV isolates within Pakistan. Finally, twenty-two SpltNPV isolates were obtained from various regions and cropping systems within Pakistan, and a few potential candidates were identified to control *S. litura* (Chapters 2 and 4). Relevant genetic diversity among these SpltNPVs was investigated using restriction enzyme and sequence analysis, and some biological characteristics, such as dose response, time-to-kill and feeding behaviour of infected *S. litura* larvae (Chapters 2-5). Also, deep genomic sequence analysis of some of the SpltNPV isolates from Pakistan was performed and comparisons with the SpltNPV reference isolate SpltNPV-G2 (Pang et al., 2001) and other baculoviruses were made.

In this final chapter I will first discuss the main findings of this thesis (section 7.1), secondly the genetic diversity associated with virulence of SpltNPV isolates (section 7.2), thirdly the prospects of using SpltNPV, and baculoviruses in general in Pakistan (section 7.3). Finally I will discuss the current state and way forward to future research of SpltNPV as potential biocontrol agent (section 7.4).

2. Main questions addressed by this thesis

2.1. Are *S. litura* NPVs present in Pakistan?

Baculoviruses occur widely among Lepidoptera, Hymenoptera and Diptera, and cause epizootics in outbreak insect populations (Cory and Meyers, 2003). These insect-specific viruses have proven potential for development as biological control agents of insect pests in the context of sustainable integrated pest management systems (Moscardi, 1999; Erlandson, 2008; Szewczyk et al., 2009; Moscardi et al., 2011). The selection of a SpltNPV isolate with appropriate insecticidal properties entails comparative evaluation of naturally occurring isolates with a reference isolate from elsewhere (Pang et al., 2001). Isolate SpltNPV-Pak-BNG was the

first and obtained from a dead cadaver of *S. litura* in a cotton field near Bahawalnagar, Pakistan (Chapter 2). This isolate was compared to a reference isolate SpltNPV-G1, obtained from the Darmstadt baculovirus depository. These two viruses showed a similar dose-mortality response, but SpltNPV-Pak-BNG killed 3rd instar larvae of *S. litura* about 24 h faster than the reference isolate SpltNPV-G1. The enhanced speed of kill of SpltNPV-Pak-BNG might have been due to adaption to local insect biotype. Other studies show that the variation in biological activity of NPVs depends on the insect colonies (Erlandson et al., 2009; Barrera et al., 2011). In experiments described in Chapter 2, the local insect biotype expressed greater susceptibility to SpltNPV-Pak-BNG than to the exotic isolate (SpltNPV-G1). The rapid speed of kill of SpltNPV-Pak-BNG might be due to enhanced peroral and intera-hemocoelic infection, resulting in a quicker systemic infection within *S. litura* larvae. It could be that more budded viruses (BVs), which are responsible for horizontal infection (within the insect body), are produced to achieve a lethal infection (Volkman, 2007). Therefore, faster passaging of the midgut barrier or enhanced levels of BVs may provide an explanation for the faster speed of kill of SpltNPV-Pak-BNG versus SpltNPV-G1.

The Pakistani isolate SpltNPV-Pak-BNG was considered the SpltNPV biotype from Pakistan and a potential candidate for control of the *S. litura.* An additional 21 isolates were obtained from different regions and cropping systems in Pakistan and characterized in Chapter 4. This successful collection effort showed that SpltNPV is naturally present in large parts of Pakistan and in a variety of crops. Since there was extensive chemical control of *S. litura*, it was difficult to find cadavers with disease symptoms. A future alternative to obtain more samples is a more extensive survey or involvement of extension workers or farmers, or to collect apparently healthy larvae and 'stress out' the virus, e.g. by crowding of starvation in insect rearing facilities. Baculoviruses often occur in a latent state or covert infections in insects (Cory and Hails, 1997).

Genetic and sequence analysis showed large differences between the two virus isolates SpltNPV-Pak-BNG and the reference isolate SpltNPV-G1, strongly suggesting that they are related, but also that they belong to two different recognized baculovirus species, SpltNPV and SpliNPV, respectively (ICTV, 2015). Previous studies demonstrated considerable genetic variation between SpltNPV and a SpliNPV field isolates, respectively (Kamiya et al., 2004; Takatsuka et al., 2003; Takatsuka et al., 2016) and also biological variation between SpltNPV isolates (Takatsuka et al., 2016). Unfortunately, the ORF originally chosen in 2012 to identify SpltNPVs (ORF24) was based on the uniqueness of this ORF for SpltNPV (Pang et al., 2001;

Chapter 2). Later on (Breitenbach et al., 2013) showed that SpliNPV had an ORF24 homolog with 99% nucleotide sequence identity, whereas the majority of other ORFs showed much more nucleotide sequence diversity. Therefore, ORF24 was an unfortunate choice in hindsight.

In the end the reference isolate SpltNPV-G1, obtained from the Darmstadt baculovirus depository, was classified a SpliNPV (Chapter 2). It may originally have been found in *S. litura*, as this has also been the case in Japan as mixed infections with SpltNPV (Takatsuka et al., 2016). However, none of the available Pakistani SpltNPV isolates had a SpliNPV genotype. Therefore, it is advisable in general to have more ORFs sequenced prior to biological experiments, to prevent erroneous selection of test strains.

2.2. Is SpltNPV-Pak-BNG a potential candidate to control *S. litura*?

Susceptibility of the insect host to viral infections is a key aspect in managing pest insect populations through microbial control. In the baculovirus case, it is important to evaluate the efficacy of the viral pathogen as an active ingredient against different larval stages of an insect biotype. A specific larval stage can affect the mortality response and time to death of virus-infected hosts (Milks et al., 1997; Erlandson et al., 2009). In this thesis, the susceptibility of different larval instars of *S. litura* to SpltNPV-Pak-BNG was evaluated by quantifying dose-mortality response and speed of kill. Second and 3rd instar larvae showed a similar dose-mortality response, but 4th instar larvae were less susceptible. Median survival time (ST_{50}) followed the same pattern as LD_{50} and was similar for L2 and L3, but longer for L4. A similar pattern of age related susceptibility has been observed in *Helicoverpa armigera* larvae challenged with HearMNPV (Rovesti et al., 2000). Our results support previous studies, which demonstrated that susceptibility to viral infection decreases with larval age both within and between larval instars (Smits and Vlak, 1988; Ali et al., 1991; Martinez et al., 2003; Kouassi et al., 2009; Bernal et al., 2013). Here we can conclude that virus-induced mortality increased with viral dose within larval stage, but decreased with host age.

This developmental resistance is due to the infection process in the larval midgut (Haas-Stapleton et al., 2003). The infection progress may result from an increased rate of sloughing of infected midgut epithelial cells in the later stages of the larval host (Washburn et al., 1998; Rohrmann, 2008) and the variation in the porosity of the peritrophic matrix (Lehane, 1997; Hegedus et al., 2009). Peritrophic matrix act as a physical barrier to baculovirus infection (Levy et al., 2007; Plymale et al., 2008) and larvae in later stages resist to viral infection with increasing thickness and remoulding response of peritrophic matrix to midgut infections (Levy

et al., 2007; Zhu et al., 2007). An alternative explanation is that apoptosis of infected tracheal epidermal contribute to age-dependent developmental resistance (Haas-Stapleton et al., 2003; McNeil et al., 2010).

Normally, speed of kill is negatively correlated with occlusion body (OB) production, as there is less time to replicate for fast killing viruses in host insects and hosts could not reach optimum body size for maximum OB production (Barrera et al., 2011; Hodgson et al., 2004). Hence, lower speed of kill might be advantageous to the virus as the insect larvae can produce higher amounts of OBs for secondary transmission in the following generations of insect pest in the field. The trade-off between OB production and speed of kill is an important aspect to secure its survival in nature. This aspect thus needs to be studied further in the case of SpltNPV-Pak-BNG when further development of this virus as viral insecticide in agro-ecosystems is considered.

A mixture of larval stages may be present at the same time and place in the field due to overlapping generations. Similar susceptibility between 2nd and 3rd larval stage to SpltNPV-Pak-BNG (Chapter 3) might be an advantage upon application of a viral insecticide to crops infested with insects. This will offer a prolonged period for infection to susceptible larval stages of *S. litura* and helps to control different larval stages following a single virus application. The time (ST_{50}) and dose (LD_{50}) mortality responses of SpltNPV-Pak-BNG isolate to 2nd stage larvae of *S. litura* suggests that the best time of virus application to affected crops is at the 2nd larval stage of *S. litura*, and even with a low concentration of virus based active ingredient for optimal insect pest management. SpltNPV-Pak-BNG application to later stages of *S. litura* is impractical and would not be cost-effective to manage *S. litura* infestations, because the cost of producing higher doses of SpltNPV-Pak-BNG required to infect the later stages of *S. litura* would be high. Furthermore, the increased interval between infection and death in later larval stage would allow the later stage larvae to continue feeding on the crop causing damage. However, these findings were based on studies (Chapter 3). However, field studies should be conducted to support these laboratory results. Further SpltNPV isolates were collected from the field in Pakistan for genetic and biological analysis to select the best biotype for biocontrol.

2.3. Do *S. litura* NPV isolates from Pakistan exhibit relevant biological and genetic diversity?

In Chapter 4 twenty-two isolates of SpltNPV from different agro-ecological zones of Pakistan were investigated. This is the first inventory on the presence of SpltNPV baculoviruses in

Pakistan, with sampled fields up to 600 km apart from each other. In the present study, SpltNPV isolates were analysed for survival time and compared for their DNA restriction patterns. Most of the tested isolates overlapped in their ST_{50s} without expressing significant differences between the median survival times. However, some SpltNPV isolates (TAX1, SFD1, SFD2 and GRW1) emerged as comparatively fast acting isolates. Previous studies on variability of speed of kill of distinct isolates of SpltNPV showed variable results, from no variation to large variation. Takatsuka et al. (2003) found no indication of variation in speed of kill for SpltNPV isolates from Japan, Malaysia, Vietnam and India and an Egyptian isolate of *S. littoralis* NPV. However, Kamiya et al. (2004) reported significant differences in ST_{50} (38.4 h) among different SpltNPV isolates. Similarly, Rowley et al. (2011) and Figueiredo et al. (2009) found variation in biological activity among NPV isolates from heliothine pest insects from different geographical locations.

Sequence information from a few selected SpltNPV-Pak isolates indicated that all Pakistani isolates were related to SpltNPV-G2 from China (Chapter 4; Pang et al., 2001), but were not the same biotype because of a lower percentage of nucleotide similarity by sequence comparisons and by phylogenetic analysis. The SpltNPV-Pak isolates varied considerably both in number and pattern of REN fragments compared to the SpliNPV-G1 isolate (Chapter 2), REN profiles being a distinctive and robust characteristic of representatives of these two species, SpltNPV and SpliNPV. Phylogenetic analysis supported the close relationship among the Pakistani SpltNPV isolates (Chapter 4). There was limited intraspecific genetic diversity in the tested SpltNPV isolates. Sometimes a mixture of genotypes was detected, as indicated by the presence of sub-molar fragments in the REN profile of a pertinent virus. Due to this different genetic population structure among SpltNPV isolates, each isolate can have different biological potential. This has often been the basis for selection of isolates for application against insect pest populations (Arrizubieta et al., 2013).

Previous studies showed that baculovirus isolates contain a variable number of genotypes and there is diversity among genotypes found at different geographical locations (Cory, 2005; Figueiredo et al., 2009; Redman et al., 2010; Rowley et al., 2011). Studies at even geographically smaller scales have shown that baculoviruses vary both within and between populations of the same host species (Parnell et al., 2002; Cooper et al., 2003). There is a possibility that infection by multiple genotypes might actually be beneficial to the performance of the baculovirus. The current study demonstrated that there is clear evidence of variations in biological activity and even isolates from the same location differed significantly from each

other in this aspect (Chapter 4). A correlation between biological performance and genetic structure of SpltNPV still needs to be determined. It would, among others, require next generation sequencing and bioinformatic analysis to support this contention.

2.4. Does genetic diversity of SpltNPV exist in reference to geographical location and cropping system?

In this thesis I also describe genetic heterogeneity and biological diversity among the geographically distinct SpltNPV isolates within Pakistan. The sampling was limited due to the low incidence of affected cadavers and the inaccessibility of certain regions of contention within Pakistan. The analysis does show, however, that the SpltNPV genotypic variants belong to a single biotype within the species SpltNPV. The biotype differs from SpltNPV from China (Pang et al., 2001) and was regionally confined. Here the term regiotype is proposed for such a regional biotype. It would be of interest to analyse the SpltNPVs from elsewhere, e.g. Japan (Takatsuka et al., 2016) and China, to name a few, to further test the regiotype concept. Regiotype has no taxonomic meaning, but this term might help to better understand local genetic variation and dispersal of baculoviruses. Analysis of MbMNPV isolates over a distance range of about 2500 km also showed distinct regiotypes (Van Oers and Vlak, 2007) suggesting that (the ancestors of) these isolates were introduced a longer time ago and had ample time to establish locally. Similarly, HearNPV in three different locations from Iberian Peninsula (Figueiredo et al., 2009) and SfMNPV from three geographical regions of Colombia exhibited distinct regiotypes (Barrera et al., 2011).

All SpltNPV-Pak isolates over a wide geographic distance seem to be derived from a single ancestor on the basis of REN comparisons, because the dominant genotypes are closely related and distinct from SpltNPVs from other countries. The introduction of SpltNPV into Pakistan may also be fairly recent considering the low genetic divergence among SpltNPV isolates. It is not known exactly where and when the initial introduction in Pakistan occurred, but genetic ancestry analysis may shed light on this aspect, as has been done for other viruses, including HIV, Chikungunya, etc. Although the introduction may have been recent, the virus has already spread over quite some distance allowing speciation to occur, e.g. in genogroups. The various genogroups significantly correlate with distance, further supporting the view that the virus recently dispersed over large distances. This may be along with its host, *S. litura*, or with infected insects hitchhiking on host plants that are used for planting or may be dispersed

with insectivorous birds (Entwisted et al., 1993) and these incoming SpltNPVs may have evolved locally upon arrival.

The various genogroups have marginal correlation with cropping system, but more intensive sampling would further help to investigate this possibility. This information can contribute to understand how SpltNPV varies spatially and can contribute to design effective pest management programs. Further intensive sampling is also required to find out if there are certain regions / cropping systems that provide an advantage in the selection of more active SpltNPV isolates.

2.5. Does SpltNPV infection alter feeding behaviour of *S. litura* larvae?

There was a highly significant difference in food intake and weight gain between healthy and infected larvae (Chapter 5). Viral infection impaired food intake and reduced weight gain in the infected larvae of *S. litura*. There was no difference in food consumption and weight gain between healthy and infected larvae during the first 48-72 hours. A significant difference between the infected and healthy larvae to food consumption and weight gain became apparent between 72 and 96 hpi. This is the period when the larval host tries to overcome the viral infection by eliminating the invading virus by shedding infected midgut cells. The infected host deploys its resources in this elimination process that would otherwise be used for larval host growth and may reduce food consumption (Keddie et al., 1989; Cory et al., 1997; Cory and Meyers, 2003).

Infected larvae consumed less food than the exposed larvae that did not become infected (survivors). No significant differences in food intake between healthy control larvae and survivors were noted. This suggests that there might not be any latent or persistent infections. The timing of divergence in food consumption and weight gain between survivors and non-survivors in our study agrees with the data of Vasconcelos et al. (2005). Previous studies demonstrated that a limitation of naturally occurring baculoviruses is their low speed of kill, which allows the insect pest insects to significantly damage the crops before they are killed by the virus (Bonning and Hammock, 1994; Bianchi et al., 2000). This study revealed that, in spite of a low speed of kill, the food intake and weight gain by viral infected larvae is quite low compared to healthy larvae, in agreement with the findings of Vasconcelos et al. (2005). The results demonstrate that the viral applications induce impaired feeding in infected larvae and can significantly lower the food consumption and weight gain in infected larvae as compared to healthy larvae, and thereby reduce crop damage. Previously reported crop damage

experiments showed that the crop damage was significantly less by viral infected compared to non-infected larvae (Inceoglu et al., 2001; Vasconcelos et al., 2005). This information can contribute to understand the insecticidal properties of a Spodoptera litura nucleopolyhedrovirus in terms of food consumption (reduced) and in the design of baculovirus-based pest control programs. However, the current study was conducted in controlled laboratory conditions and field trials are required to justify the laboratory results. We found SpltNPV isolates TAX1, SFD1, SFD2 and GRW1 as fast acting and we need similar further studies to investigate the feeding behaviour of *S. litura* infected with some of these fast acting SpltNPV isolates.

3. Genetic diversity associated with virulence of SpltNPV isolates

In this thesis interspecific genetic diversity among the SpltNPV isolates was found. Some of these isolates differed significantly in speed of kill and SpltNPV-Pak-TAX1 and SpltNPV-Pak-BNG have emerged as fast and slow acting isolates, respectively (Chapter 4). REN analysis showed that these two isolates fell into different genogroups corresponding to different ecological regions in Pakistan. Further genomic sequence analysis of SpltNPV-Pak-TAX1 and SpltNPV-Pak-BNG isolates showed limited genetic heterogeneity within the isolate (Chapter 6), but more than 99% nucleotide sequence homology between these two isolates. One of the main questions is what genetic changes then contribute to the speed of kill in SpltNPV-Pak-TAX1 isolate. Previous study showed that small variations within the genome could result in significant changes in biological activity (Cory et al., 2005). In our case there is evidence of a partial deletion of ORF 125 in SpltNPV-Pak-BNG and a complete deletion of homologous repeat region 17 (hr17) in SpltNPV-TAX1. Furthermore, a strong positive selection was found for the *fgf* gene (viral fibroblast growth factor) (ORF122) in SpltNPV-Pak-TAX1 as compared to SpltNPV-Pak-BNG. These two loci may have been involved towards more speed of kill in SpltNPV-Pak-TAX1 as compared to SpltNPV-Pak-BNG. Hr17 might control transcription levels and may affect the virulence of the virus as a whole. When it serves as replication origin, it may affect the replication speed and final outcome of the infection. However, previous studies on the function of vFGF demonstrated that this protein enhances baculovirus infection and propagation in insect larvae (Means and Passarelli, 2010; Yin et al., 2016). The faster speed of kill of SpltNPV-Pak-TAX1 infected 3rd instar larvae of *S. litura* compared to SpltNPV-Pak-BNG could thus be due to deletion of homologous region (hr17) and/or to an altered vFGF of SpltNPV-Pak-TAX1. Further studies are required to experimentally link the genetic changes in

SpltNPV-Pak-TAX1 genome with the differences in speed of kill, e.g. mutational analysis of SpltNPV bacmids to be constructed or via CRISPR-CAS technology.

Recent studies on Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) isolates have demonstrated that the geographic distribution of isolates is not an important driver of LdMNPV virulence, and that the virulence of LdMNPV is determined by viral genetics (Harrison et al., 2016; Martemyanove et al., 2017). There is surprisingly little variation within each SpltNPV isolate, with only a single polymorphic SNP in SpltNPV-Pak-BNG and four polymorphic SNPs within SpltNPV-Pak-TAX1. The little variation might be due to the recent introduction of SpltNPV into Pakistan and it will take time for the virus to establish in the agro-ecosystem. The SpltNPV genogroups in Pakistan exhibited diversity in speed of kill. There was a significant difference in time to death between genogroup A and C. Group B was more similar in speed of kill to group A than to group C. However, there were no significant differences in time to kill amongst group A and B or between group B and C. Are SpltNPV isolates undergoing selection and evolved to a particular geographical region? We need further studies to explore the mechanism that maintains the spatial diversity among the SpltNPV isolates. This fascinating perspective invites for more testing and selection of SpltNPV isolates for *S. litura* population control.

4. Prospects of baculoviruses use in Pakistan

Pakistan produces tropical and sub-tropical agricultural crops and faces a number of challenges including frequent incidence and resurgence of insect pests. Among farmers the use of pesticides is considered the quickest method to control insect pest. This heavy reliance on pesticides has led to serious environmental, ecological and health concerns. Public perception of food safety and development of resistance in insect pests are the two major prevailing concerns. The development of eco-friendly insect management technologies is needed for sustainable crop management, but it remains a major concern due to technical and economic difficulties for commercialization in the country.

Baculoviruses occur naturally and have been used for insect pest control in many countries, but not (yet) in Pakistan. According to my knowledge, SpltNPV has not been introduced commercially in the country. I explored and studied the *S. litura* NPV from Pakistan and found a number of potential candidate isolates to control this insect. Despite its effectiveness, several factors still need to be considered for its commercial use in Pakistan. The limiting factors are: over-reliance of farmers on pesticides, technical and economic difficulties

for commercial production of baculoviruses, and the registration and marketing of baculovirus as a biological insecticide. The most important aspect is to present SpltNPV as a good alternative option to insecticides for policy makers. Policy makers have to choose an option with increasing and speedy returns, but introduction of biological insecticides, such as SpltNPV, may take time to be a success story in Pakistan, as both farmers and policy makers have to be convinced about the biocontrol potential of baculoviruses.

5. Concluding remarks and the way forward

In this thesis I studied the occurrence, genetic diversity and biological activity of a number of SpltNPV isolates from Pakistan. Furthermore, the feeding behaviour of virus infected *S. litura* larvae was assessed to determine optimal OB dose and target instar. Previous studies demonstrated the genetic and biological diversity among the wild-type baculoviruses (Cory et al., 2005; Barrera et al., 2011), but there have been limited systematic studies of SpltNPV genetic and biological diversity and its importance to insect-pathogen interactions. The fundamental questions 1) "Can indigenous *S. litura* NPV isolates be found in Pakistan?" and 2) "Does genetic diversity of SpltNPV exists in relation to geographical distribution and cropping system?" have been answered in section 7.1. I discovered S. litura NPV in Pakistan and characterized the SpltNPV biotype from Pakistan. In Chapter 4 I discovered three genogroups of SpltNPV forming regiotypes within the SpltNPV species and found strong correlation between the genogroups and the geographical regions suggesting regional adaption. However, the various genogroups have a marginal correlation with the cropping system, but more intensive sampling would further help to investigate the possibility that SpltNPV adapts to the crops. SpltNPV-Pak-TAX1 emerged as a fast acting isolate (Chapter 4). Its genomic sequence was compared with the geographically distinct isolate SpltNPV-Pak-BNG and genomic analysis identified the gene that may be responsible for the higher virulence of SpltNPV-Pak-Tax1 (Chapter 6). Surprisingly, I found little genetic variation within each isolate suggesting a recent introduction of SpltNPV in Pakistan.

Further genetic analysis of additional spatially separated SpltNPV isolates would be required to translate the genetic diversity into meaningful practical application. However, there are a number of questions that need to be answered, such as why and how this SpltNPV diversity is maintained and what mechanism is involved to relate spatial diversity and variation to virulence. Furthermore, the influence of host plants/crop on baculovirus biological activity and genetic diversity within and among SpltNPV isolates should be investigated. Pakistan may be

a case where a baculovirus recently emerged and started to evolve and adapt to a new agro-ecological setting. Further and more intensive sampling should be conducted around the country to find out if there are certain regions/cropping systems that can provide an advantage in the selection of more active SpltNPV isolates. The biological activity of SpltNPV-Pak isolates was tested in controlled conditions but now field trials are required to confirm the laboratory results described in this thesis. Finally, the efforts should lead to sustainable application of SpltNPV as a bio-insecticide to control the leafworm *S. litura* in Pakistan and elsewhere.

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Appendices

Appendix for Chapter 2

SpltNPV-Pak-BNG		
ORF	**Name**	**Sequences**
"1"	***Polyhedrin***	ATGTATAGTCGTTATAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTA TTACAAAAATCTAGGTCACGTGATCAAAAATGCTAAACGCAAACACGATGCTCTCGAACGCGAGGCCGAC GAGCGTGAGCTCGATCACCTCGACAAGTATCTGGTCGCAGAGGATCCGTTCATGGGTCCTGGTAAAAATC AAAAGTTGACTCTGTTCAAAGAGATTCGTAACGTGAAGCCCGACACGATGAAGCTGATCGTCAATTGGAA CGGTAAAGAGTTTCTGCGCGAAACTTGGACTCGCTTTATGGAAGACAGCTTCCCCATTGTGAACGATCAA GAAGTGATGGACGTGTTTTTAGTGGTAAACATGCGTCCCACTAGACCAAACCGTTGCTTTAGATTTTTAGC GCAGCACGCGCTCCGATGCGATCCCGAGTACGTTCCTCACGACGTGATCCGTATCGTAGAACCGTCGTAC GTCGGCACCAATAACGAATACCGCATCAGTCTGGCTAAGAAGGGCGGCGGTTGTCCCGTGATGAACCTGC ACGCCGAATACACCACGTCGTTCGAGAGCTTCATCGACAAGGTGATATGGTACAACTTTTACAAGCCCAT CGTGTACGTGGGCACCGACTCGGCTGAAGAGGAGGAGATCCTTCTCGAAGTGTCGCTCGTGTTCAAGATT AAAGAGTTTGCTCCCGACGCGCCTCTATACACCGGTCCCGCGTATTAA
"24"		ATGGACGGCCCGTTTGTGGACTACGACGTAAAGTTTAATTATTCTATTGAATCCATCGGCGGATCTACGAA TTTTATCATCAACCACAGTTTGCCGACGGTGAACTTGTTGAAAAATAACATGGGAATGTTGCGTCGAAAG ATTGAAAAATATTTCGATTGCGAAGCCGATTTGAGAAGACATTTGATCAAGCTAAAAATGCTTCTATTGTC CAAATACAAGGAATACATTTTGATAATAATATTGAAAAAACTCTTTAATCGAGACTTGATCATGGTGGCG CCGATGTGCAACCATTTCTTCAATCTACACGATCAATACATGTTTACGTTTGGAAAAATATTTTACACACG TTTACATATAAAATTAGAATGTTTGATATTTTTCGATTTGAACAATAATACTCTGTTCCTTGTTCACAAGAC TTTGGATGATTTTCAATTGGCAGAATTCATATCTTCGTATCTGACTAAATACGGATTGAAACAGATCGAAT TGAGAGTGAACGGTTCCACCGAAACGCAGTACATGGAACACGACATAAATGATTTGGACGATTATGTGTG CTTCAAGGAAGATCCAATAGTGATTGATGCTAATAATAAATTTAAAGATTTAAAATTATACTATTCAAAAT TTATCCATTGTATTGACTCGCGAAAAGAATTGGATGTTAAATTGAAAGAGATTAGAAATCTTTTGAAACG AGTCAAGTGTGCGCACACTTTGAATGCAAATAAAGAAAACGAAAACGGTTTGCAAGTGGACGTTTGTAAA ATTATGCAAGACGCCGTCGAACGAATAACCATAATTAATAACAAGTACATGATCGATAAAGTTAGAAAA ATGTCAAAGCATCTTGACGACGAGACCGATGTTTCCCCAGCTAAGAGGAAAAAAATAAACGTTGCACAGC AGCAAAGTGTTGAAGAATCCCGACCGTTTGTGCACGAAAACCCTGACGATTTCAATTACACACATCCTCC GATATCGTGCATGTTCAATTTACAATGCGACAGATCATCTTTGAATGTGACCACATTGATGATGTTTTCAA AAAATTCATCGTGGAGCAAAGATTACGCCGACGACAACGACGACAAGAACACGAAGCGCGTCGAAGACA TCAAGAAACTGATCAATTCGAAAAATTTCAAAATGTTTAAACCTTTTCTTAAAAGACAATACGAATATGA TGAATAA
"38"	***lef8***	TCAATTTACAATGACATTGTTACAATGCAAAAGACTGGTGAGCACTTTGTATTCGCCAGATGGATCGGGC AAATTGGTACCGCCGCGGACGCACTGCAGACTCTCGGCCCATCTGTTGCCGGCGAAAGATTGATTGATCG ACCATTGATCGAGTCGAGTTCCTTCGATCTTTTCGTGTCCCGTGTAATTATGTCTGTTGAACTCTTTGTAAA TGTTGTCGGGCGTATTGTTGAACAGCATGTACGGTATGTTGAACATTGGATACCGGACGGCCGACGGATC GGTGTGATCGCCGCCGCGCACCACGTACTTTCGTTCGATATAATCAAAGTTTGACTGCCTCGAGAGAAAC GAAACGGGCGACAGGCAGATTTGGGCGTGTGTGCCGTCCTCGGCCATCCATTCGGTCAGATCCTCCGAAC TGTTCATAACTCCCTTCTGGCCGTGTATGCCGCAAATCTTTATGCCGTCCAGATTGTTGGTCGATGTGACG ATCGAAAACTTTACGTACACGAAATCATTGAGCGTGCTCAGTTTTGCGTCTATAAATTCTATTCGCTGCGA TTTGATTCTTCTGAGAAACGTGTAAATTTTAAAAATGTAAAACGACTTGTTCTTGCACTGTTCGATCTTGTA TCGCTTTCCGTCGTGCGTCCAATTTATTTTCACGTTCGACACGAGCACTCCCGCCATGGCTAGGACGTGGC CCTCGTTCGTGTCGACAAAATTGTTGCTTTCGCTCTTGAAAAATTTTACAATGGGCGTTTTAGATTTTGACG CGTACACCATCTTTCCTTTGAGACGGTTCACCTTGTTATTGAACAGTTTCACGGGCAGAGTTACGTTCGGT ATGTACGGATCTTCGGCGGTCATGAGTCGGCTGTCTCGCACCAGAGTCCACAAATTTATCATTTTATTGTT GTGAACCGAGCTACGGCTCGCCACCACGGAATTGCCGACAGGCAGGTTTTTAATGAACCTCTCGCGGTTC TCGTTCCATTCGACGTCGTCGTACTCGATTACGGGCATTGCGTTTTTCAAATTTGTCACGGACACGATCAA TTTTGGCACGGGTATCGTAGCGAAAATGTGCAGATGCGATCGGTAATAGTATTGAAGCGTCTTGGACATT

AGCGCAGAAACATGATCAGCGTCGACGATCCGAGCTTTGATTTGAGCAACAATAGATTCGCCGTTGTGGT
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ATACCTTCGTGATGATTGACAAATAGAATATTTTTAAACAGTTTGATCTCGACGGGAAACCGTTTGCGTTT
AAACTCGTACACGATGCGCACTAGATGCTTTCGTTTGCACGAAAACGTCGTAGGTCTATTATTGAAAGCG
ATCATCAACCATTCGTCATCATCACCGCCGCCGCCGCCGCCCAAAGACTTGGTCTCGTTGCCGCTTTC
GTCGTCCTCGTCGCGCCGCACAATCAGATTTGATTCGATTAAATCTTTAAATTTTTTCGCTATAGATTCGTA
ATCGACGTTTGGAAGCCGCACGTCTCGACACAAGAAAAACTTTTTGCCCGCCACCGTCATCTCGCCGTGA
AAGAAACTGTCTACAAATTTTACAAAGTCGCGTTCCTGCTTCAACATGTCCTGTCTCATGTTGTCGTTTATG
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ATTGTCCTGATGATTTAAAAAGTTTTTCTTTTGCTTGCTAAACGTTTTCGATATAACGTGTATCAACTTTCC
GTTGACTATTGTGTCGACAATCTTTTTACAATCCTTGGGATACATCACGCCTCCACTTTGGTTTCCTCCAAA
CTTTTTAAGAGTCACGGAACCGTTCGTCGCGGAACCGTTTGAATCGAACTTGCTAGTCTGATACATTTTTA
AAATCGGCTTGTACAGTAGATACGCCAAATAGGCGTGTTTGTATATGATTTTGTTTGATAGACTATCGATG
GAATAGTTGATTTCGGCGCGCATGATCATTTTGATCCGGTCGAACATTTTGTCCGTTTGATTTTTACCAAA
GTCAAACAAAAAGTTGAGCGGTTCCCATTTTCCGCTGCTTCTCAAATACGTTTCCAACGTTTCGTTGAGAT
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AGTTGGATGGCTCGATTTATGCGTTTCTCCTCGTCCATGTTGCCGTACAGGAACATTCTTTTGCAATTTTTC
GAATAGAGCTTGTCGTAAAAATTGTGCATCAATATATTGTTGTTCATCATCACGTTGGGAAAACTTAAAAA
TCTACCGTCTATCATAAACGTTCCTCTGATGTTGGATCTTTCCTCGATCGCGTCATCGTCATCGTCGTCGCC
GCCGCCGCTTTTGACCACATCACAGCCCGATTGTCTCTTTCTAAATTTTTTGTCCAACCAAGTGCCAAAGA
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TCTCTCTTGCAAATATTTGATGGTGCAAGTATTGTTCGATTTGTCTACGCAGTTTAAATAAAAATTTAAATT
GTAATTATTACATATAATATTATAGACGTGATCGAAATCAGACACAACGTCCGTCAT

"69" ***DNApol*** ACATTGATCTGTATCAATTTCTCAGCTCGGACTCGGAACAGAACGACGTGGAAAATTTTCGTTTGAATACG
GTCGCTCAACACTATTTGAACGATACCAAAGTGGATTTGAAAATTTCCGACATGATCTCGATGTATCGCGA
GAACAGGATGCTAAAGATCATCGAGTACAACGTGCACGATGCCGTCCTACCCATAGAGATTTTTTCAAAA
TTGGAAATTATTGAATTCATGTACACTCAATGTACCGTTCTATTTTTATGCACCGACGACGCGCTAAAAAA
CATCTCACACAAAGTGAGCGTAGTGCTGTTTCAAAAGTCTCTAACGAACGTCAGACACGATGGTACGCCC
GATCCGTACTTTTTTAACAAGTCCGATTTAAACGTGACATCGGGCCGAAGCGGAGGAGGCGGCGATAGAC
AGATTGTAGATTTGACGCAGTTGAATAGAAAATGCATCGTTCCCACAAACATGATACCCCAAACGGCGAT
CAAGTTGTGTGCGCGCCGAGAAAGATGTGTGTACAAGGGAGGCAAAGTGATTGCGCCCATACCCGGCAT
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AAAAAGATTGAATCGATGAGCAACGACGCGACGATACTCGAGGATTTTGGTCTGTCGAGAATCAAATTCC
GAGTCGTGTACGGAGATACGGACTCTTCGTTTATTCAAGTGGATTACGAGTCGAACGAGATCGACGAAAC
GCTTAGACACGAAACCGTAGAACGCATCGTGAATGGATACGTGTTGAAAAAGTTAAACGCTTCGTGGGAC
GGATACAAAATGGCGTTAGAAAACGTTATGCAATCGCTCATTTTATTGAAGAAGAAAAAGTATTGCTATT
TGAACACAGAGAATCGGATCAAGTACAAGGGCTGGCTGGTAAAGAAGGACATGCCGATTTTTATGCGTA
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GAACACCAGCACGGACTTTTTGCCGGGCAATGGTGACAGAATTCCGTACCTGTTGAGAGACGAAGCCGGC
AGCGTTACGAAAAAAGCGTATCCGACACACGCGTTTGATCCCCGAATCATGGCGGTCAGTTGGACCAAAC
ACGTCGGCATCATGTGTTCGTTTATTAACGAACTGATTCAAGTGTTGGGAGATCGCGGCGAGTTTCAATTG
TACTTTAACGAAATTTGCAATGTGTACAATTCAAAGATGATGTACGACGTCCGAGTTCCGGTCCTAAAGG
AAATCACCGAATTGGTCGCGAGAAAAATTAGGGGCGGAGGCAAGAAGCAAAAGGGAGACATCGAGACC

GACGATGACGACTGCGGCGGTGACGACGACGACAATTGTGATTCTGATTGTGATTCAGACAGTGTTGGTT
TCAAATGTCAATTTTCAATGTTAAAACGAGAGCCTAAGACGGCTATAAAGATAAAATTTAACAGACCCGA
TGTGATTGTTTGTGATGTTTGTTCGAATAAATGA

"135" ***pif2*** ATGAACGGTTTTGGTCAACGCCGACATGTTGCCTATGCGATCGGTAAACTAAGTATGGTGGACGGCGCCA
TGTACGTGGTGATTTTTATATTGTTGATAATTTTTGTGTTGGCGTTGGCGCGTCCTATGCAAGTCGCGTACG
AGACAATACGCAAACGACAAATTGATTACGAGAATCGAATCGATGAACGGATCGGCTACATGCAAAACG
TGCTCAGCCGACGGCGCTACGTGCCGCTGAGCTCGTTGCCTAACATTGATTTTGGTGCCACGCTCGAGACG
ATCTCCGAAGGAGAGATTCGATGTCTGTCCGTGCCGGCGTTTGTGAGTCGATTCAACACGCCCAACTTTGA
TTGTACCGAAATGTGCGACGATCCTTCGGCGTCGTACTTTTACGTAGGACCGTTCGATCGGTTTGTAGTGA
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CAAAACATGATCCAAACCGCGGGCAGGCAACATATCGACTTTATACGTAGCGACGAGATCGATAGGATTG
TACTGTTCGATCGTCAATTGGGCGTACCCGTGGACGTGTCTCGTAACACATTTCGGAGCCATTGGGACGAG
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GATCCGAACGATCCCAGTTCGATGTGCGCTTCGATTGTGCACAATTTGAATCAAAGCGAGCTAACGTACG
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CGATCATTTGATGGAATCGACAGGCGACGCGGCATACACTTTTGTGATGCCCGGCGCGTTTCCAATGTCAT
CGAACGGTATAGAGGAGCCAAACTACAGACTATGGTTGGACGTGAGGAACAGAGTCAACTTTTTCCAACG
AAGTCATTATGAATCTGTTAGCGCCGAAGCGACTCAAGCAATGGCCAAATTCAAATACCCAACGAAAAGT
CAGACAAGTTAA

SpltNPV-Pak-G1

"1" ***Polyhedrin*** ATGTATAGTCGCTACAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGT
ATTACAAAAATTTAGGTCACGTGATCAAAAATGCTAAGCGCAAACACGATGCTCTCGAACGCGAGGCCGA
CGAGCGCGAGCTCGATCACCTAGACAAGTATCTAGTCGCCGAAGATCCGTTCATGGGTCCCGGTAAAAAT
CAAAAGTTGACTCTGTTCAAGGAGATCCGTAACGTGAAACCCGACACGATGAAGCTGATCGTCAACTGGA
ACGGCAAAGAGTTTCTCCGTGAGACTTGGACCCGTTTCATGGAAGACAGCTTCCCCATCGTGAACGATCA
AGAAGTGATGGACGTGTTTCTAGTGGTGAACATGCGTCCCACTAGACCGAACCGTTGCTTTAGATTTTTGG
CGCAACACGCGCTCCGATGCGACCCCGAGTACGTTCCCCACGACGTGATCCGCATCGTCGAACCGTCGTA
CGTCGGCACCAACAATGAATACCGCATCAGTCTCGCCAAGAAAGGTGGCGGCTGTCCCGTGATGAACCTG
CACGCCGAATACACCACTTCGTTTGAGAGTTTCATCGACAAGGTGATATGGTACAACTTTTACAAGCCCAT
CGTGTACGTGGGCACCGATTCGGCCGAAGAGGAGGAGATCCTTCTCGAAGTGTCGCTCGTGTTCAAGATC
AAAGAGTTTGCTCCCGACGCGCCACTCTACACCGGTCCCGCGTACTAA

"24" ATGGACGCCGGTGTAAAATTTAATTATTCCATTGAATCTACCGCAAATTTTGTCATAAATCACAGTTGGCC
TATGGTGAAGATATATGATTTGGAAAAGTTAAAAGAATCATCATCATCATCGTATTTAAAGATTAAAAAA
TATTTCGAGTGTGAATCGGATCTAAAGAGACATTTGAAAAGATTAAAGATTCTTTTCGTGTTCAATTACAA
GGAATCCGTTTTGAATGAAACAATCAAAAGACTATTGAATCCCGACTTGATTATGGTGGCGCCGATATGC
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GGCGACGCAACGACCGAGACTCGGTACAGGGAATGTAGAATTGATGATATGGACGATTACGTGCACAGC
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GTAAAATTGTACAAGACGTCTATGAAAAAATTATATTAATCAATAACGAGTACATGATGGAAAAAGTTAA
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AAAATGAACGCGCCGCAGCCTCGTAATGTTGAGGAGTCGCAGCCTCGGAATGTTTACGAAAATTCCGACG
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GATTTGACCACGTTGATGATATTTTCCAAAAATTCATTATTCAGCCCAGATTACGCAGATGACGATGACAA
GAACACGCAGCGTATCGAAGACATCAAGAAACTGATAAAATCGAAAAATTTTAAAATGTTTAAACCTTTT
CTTAAACGCCAATACCAATAA

"38" ***lef8*** TTAATTTACAATGACATTGTTACAATGCAAAAGACTGGTGAGCACTTTGTATTCGCCAGACGAATCGGGC
AGATTGGTGCCGCCGCGAACGCACTGTAGACCCTCGGCCCATCTGTTGCCGGCGAAAGATTGATTGATAG
ACCATTGATCGAGACGGGTTCCTTCGATTTTTTCGTGACCCGTGTAATTGTGTCTGTTGAATTCTTTGTAAA
TATTTTCGGGCGTGTTGTTGAACAGCATGTACGGTATGCGGAACATTGGATAGCGAACGGCCGACGGATC
GGTGTGGTCGCCGCCGCGCACCACGTATTTTCGTTCGATATAATCAAAGTTTGACTGCCTCGACAGAAACG
AAACGGGCGATAGACAAATTTGCGCGTGCGTGCCGTCTTCGGCCATCCATTCGGTCAGATCTTCCGAACT
GTTCATGACACCCTTCTGGCCGTGTATTCCGCAAATCTTTATACCGTCCAGATTGTTGGTGGACGTGACGA
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CTAATCTTTCTGAGAAACGTGTAAATTTTAAAAATGTAAAACGACTTGTTCTTGCACTGTTCAATCTTGTA
CCGCTTTCCGTCGTGTGTCCAATTTATTTTCACGTTCGACACGATCACGCCCGCCATGGCGAGAACGTGAC
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CGTGCTTGTATATGATCTTGTTGGACAGGCTGTCGATGGAATAGTTGATTTCGGCGCGCATGATCATTTTG
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CTTTATGGTGCAAACGTTGTTCGATCTGTCTGCGCAATTTAAATAATAGTTTAAATTGTAATTATTACGTAT
AATATTATAGACGTGATCGAAATCAGACACAACGTCCGTCAT

"69" ***DNApol*** ATGAAGGTGAAGATTCAAAATTTTTATCAATTGCAACGAGAGATGGCAGCCGAAAACGGTTGCCGCAAA
GGCGATGTGGTTATATCGACACGCGACGTGTTTCGTATTACGAGAATGTGCTACCGAAACAAATGTCTTTA
CGTGTTCATGACTGGCTATCTCGAGTCCGATCCGGACAGAGTGTTTCAATTTTACATGCGCAGTTCGTGCG
TTCTGTACTCGTACAGGTCTTGCTATAACGTACACATGAATTCGTCGTGCCAATGTCGCAGCTACAAGACT
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CCATACCCGCCGTGGTCTCGTGTTACGACATTGAGACTTATACGAACGGCATGCGAATGTCGGCGCCCGA
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ATACGGCTCAGGGACCGGCTCGAGATTTGTTCGACGGCGACGGCGATGGTGAAACCGTTCACGTCGTATA
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ACATGGCCGACTATTGTTTCAGTATGACTTACAACGAGAATCCGGGAGGGAAGAAGCGAAAGTCGGCGTT
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CGACGACGACGAAGACGACGGCGACGATCAGGATTCTGATTGTGATTCGGACGGCGGTACGCGCGACAT
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AATAGACCCGATGTGGTCGTTTGTAATGTTTGTTTAAATAAATGA

"135" ***pif2*** ATGGTGGACGGCGTCATGTACGTGGTGATTTTTATATTACTGATAATTTTCGTGTTGGCGTTGGCGCGTCC
GATGCAAATCGCGTACGAGACCATACGTGGGCGACAAATTGATTACGAGAACCGAATCGATGAACGGAT
AAACTATATGCAAAACGTGCTAGGCCGACGGCGCTACGTGCCTCTGAGCTCGCTGCCCAACATTGACTTT
GGGGCCACGCTCGAGACGATATCCGAGGGCGAGATTCGATGTCTGTCCGTGCCGGCGTTTGTGAGCCGAT
TCAACACGCCCAATTTCGATTGTACCGAAATGTGCGACGATCCGTCGGCGACGTATTTTTACGTCGGCCCA
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GCCAATGCAACCGCGAGACTTCGGTGATCGTGCACGGTCTCAATCAGTGGACGTGCATAGCCGAAGACCC
TAGATACTTTGCCGGCGAACAGAACATGGTCCAAACGGCGGGCAGGCAGCATGTCGACTCGATCCGTCCC
GACCAGATCGATAGAATCGTACTGTTCGATCGCCAACTGGGCATGCCCGTGGACGTGTCTCGTAACACGT
TCCGCAGCCACTGGGACGAGACACTGTCGGACGGATCGAGACGGTTCGAAGTAAGATGCGACGCTCGGG
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CGTGCAATACGTCCATCCGGCGGTCAGGCCGGATTTCGAACGGGGCGTGTGCGACTGCGGCGATCCGGAC
GTGACCAGAGTCGTTCACGTCGATCCGAACGATCCGAGTTCCATGTGCGCTTCGATCGTGCACAATTTGAA
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ACAAACATGCTGATGTGTCCCGAGCATTTGATGGAATCGACCGGCGACGCGGCATACACATTCGTGATGC
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TAGAGTCAACTTTTTCCAACGAAGACACGACACCATTACATCCGAAGCGGCTCTAGCAATGGCCAAATTC
AAAATTCCCAACAAAAAGTCAGACAAGTTTGAATCGAACGCGGCCGACTTTTCGTACAGACAGAATAACA
TTTGA

Appendix for Chapter 4

ORF 1 *(Polyhedrin)*	**Sequences**
SpltNPV-Pak-BNG	ATGTATAGTCGTTATAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATT ACAAAAATCTAGGTCACGTGATCAAAAATGCTAAACGCAAACACGATGCTCTCGAACGCGAGGCCGACGAGC GTGAGCTCGATCACCTCGACAAGTATCTGGTCGCAGAGGATCCGTTCATGGGTCCTGGTAAAAATCAAAAGTT GACTCTGTTCAAAGAGATTCGTAACGTGAAGCCCGACACGATGAAGCTGATCGTCAATTGGAACGGTAAAGA GTTTCTGCGCGAAACTTGGACTCGCTTTATGGAAGACAGCTTCCCCATTGTGAACGATCAAGAAGTGATGGAC GTGTTTTTAGTGGTAAACATGCGTCCCACTAGACCAAACCGTTGCTTTAGATTTTTAGCGCAGCACGCGCTCCG ATGCGATCCCGAGTACGTTCCTCACGACGTGATCCGTATCGTAGAACCGTCGTACGTCGGCACCAATAACGAA TACCGCATCAGTCTGGCTAAGAAGGGCGGCGGTTGTCCCGTGATGAACCTGCACGCCGAATACACCACGTCGT TCGAGAGCTTCATCGACAAGGTGATATGGTACAACTTTTACAAGCCCATCGTGTACGTGGGCACCGACTCGGC TGAAGAGGAGGAGATCCTTCTCGAAGTGTCGCTCGTGTTCAAGATTAAAGAGTTTGCTCCCGACGCGCCTCTA TACACCGGTCCCGCGTATTAA
SpltNPV-Pak-TAX1	ATGTATAGTCGTTATAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATT ACAAAAATCTAGGTCACGTGATCAAAAATGCTAAACGCAAACACGATGCTCTCGAACGCGAGGCCGACGAGC GTGAGCTCGATCACCTCGACAAGTATCTGGTCGCAGAGGATCCGTTCATGGGTCCTGGTAAAAATCAAAAGTT GACTCTGTTCAAAGAGATTCGTAACGTGAAGCCCGACACGATGAAGCTGATCGTCAATTGGAACGGTAAAGA GTTTCTGCGCGAAACTTGGACTCGCTTTATGGAAGACAGCTTCCCCATTGTGAACGATCAAGAAGTGATGGAC GTGTTTTTAGTGGTAAACATGCGTCCCACTAGACCAAACCGTTGCTTTAGATTTTTAGCGCAGCACGCGCTCCG ATGCGATCCCGAGTACGTTCCTCACGACGTGATCCGTATCGTAGAACCGTCGTACGTCGGCACCAATAACGAA TACCGCATCAGTCTGGCTAAGAAGGGCGGCGGTTGTCCCGTGATGAACCTGCACGCCGAATACACCACGTCGT TCGAGAGCTTCATCGACAAGGTGATATGGTACAACTTTTACAAGCCCATCGTGTACGTGGGCACCGACTCGGC TGAAGAGGAGGAGATCCTTCTCGAAGTGTCGCTCGTGTTCAAGATTAAAGAGTTTGCTCCCGACGCGCCTCTA TACACCGGTCCCGCGTATTAA
SpltNPV-Pak-SFD1	ATGTATAGTCGTTATAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATT ACAAAAATCTAGGTCACGTGATCAAAAATGCTAAACGCAAACACGATGCTCTCGAACGCGAGGCCGACGAGC GTGAGCTCGATCACCTCGACAAGTATCTGGTCGCAGAGGATCCGTTCATGGGTCCTGGTAAAAATCAAAAGTT GACTCTGTTCAAAGAGATTCGTAACGTGAAGCCCGACACGATGAAGCTGATCGTCAATTGGAACGGTAAAGA GTTTCTGCGCGAAACTTGGACTCGCTTTATGGAAGACAGCTTCCCCATTGTGAACGATCAAGAAGTGATGGAC GTGTTTTTAGTGGTAAACATGCGTCCCACTAGACCAAACCGTTGCTTTAGATTTTTAGCGCAGCACGCGCTCCG ATGCGATCCCGAGTACGTTCCTCACGACGTGATCCGTATCGTAGAACCGTCGTACGTCGGCACCAATAACGAA TACCGCATCAGTCTGGCTAAGAAGGGCGGCGGTTGTCCCGTGATGAACCTGCACGCCGAATACACCACGTCGT TCGAGAGCTTCATCGACAAGGTGATATGGTACAACTTTTACAAGCCCATCGTGTACGTGGGCACCGACTCGGC TGAAGAGGAGGAGATCCTTCTCGAAGTGTCGCTCGTGTTCAAGATTAAAGAGTTTGCTCCCGACGCGCCTCTA TACACCGGTCCCGCGTATTAA
SpltNPV-G2	ATGTATAGTCGTTATAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATT ACAAAAATCTAGGTCACGTGATTAAAAATGCTAAACGCAAACACGATGCTCTCGAACGCGAGGCCGACGAGC GTGAGCTCGATCACCTCGACAAGTATCTGGTCGCAGAGGATCCGTTCATGGGTCCTGGTAAAAATCAAAAGTT GACTCTGTTCAAAGAGATTCGTAACGTGAAGCCCGACACGATGAAGCTGATCGTCAATTGGAACGGTAAAGA GTTTCTGCGCGAGACTTGGACTCGCTTTATGGAAGACAGCTTCCCCATCGTGAACGATCAAGAAGTGATGGAC GTGTTTTTAGTGGTAAACATGCGTCCCACTAGACCGAACCGTTGCTTTAGATTTTTAGCGCAGCACGCGCTCCG ATGCGATCCCGAGTACGTTCCTCACGACGTGATCCGTATCGTAGAACCGTCGTACGTCGGCACCAATAACGAA TACCGCATCAGTCTGGCTAAGAAGGGCGGCGGTTGTCCCGTGATGAACCTGCACGCCGAATACACCACGTCGT TCGAGAGCTTCATCGACAAGGTGATATGGTACAACTTTTACAAGCCCATCGTGTACGTGGGCACCGACTCGGC TGAAGAGGAGGAGATCCTTCTCGAAGTGTCGCTCGTGTTCAAGATCAAAGAGTTTGCTCCCGACGCGCCTCTA TACACGGGTCCCGCGTATTAA
SpliNPV-G1	ATGTATAGTCGCTACAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATT ACAAAAATTTAGGTCACGTGATCAAAAATGCTAAGCGCAAACACGATGCTCTCGAACGCGAGGCCGACGAGC

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	GTTTCTCCGTGAGACTTGGACCCGTTTCATGGAAGACAGCTTCCCCATCGTGAACGATCAAGAAGTGATGGAC
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	ATGCGACCCCGAGTACGTTCCCCACGACGTGATCCGCATCGTCGAACCGTCGTACGTCGGCACCAACAATGAA
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	TACACCGGTCCCGCGTACTAA
SpliNPV-AN1956	ATGTATAGTCGCAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATTACA
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	TCTCCGTGAGACTTGGACCCGTTTCATGGAAGACAGCTTCCCCATCGTGAACGATCAAGAAGTGATGGACGTG
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	CGACCCCGAGTACGTTCCCCACGACGTGATCCGCATCGTCGAACCGTCGTACGTCGGCACCAACAATGAATAC
	CGCATCAGTCTCGCCAAGAAAGGTGGCGGCTGTCCCGTGATGAACCTGCACGCCGAATACACCACTTCGTTTG
	AGAGTTTCATCGACAAGGTGATATGGTACAACTTTTACAAGCCCATCGTGTACGTGGGCACCGATTCGGCCGA
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	ACCGGTCCCGCGTACTAA

ORF 4 (*hoar*)	**Sequences**
SpltNPV-Pak-BNG	ATGCTGTCCAATTATTATATCGCGTTTGAAGAGTCAAAGTCGATGCAGTGCATTAACGTATTTTTAGTGCCGGC
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	GTGACAAAGACTCAGTACAATGTGTATAGTGATACTCTAGTTCAATTTAAACAAAACTTGTGCCGTGTTCCGT
	TCGAGGCAAAGTTAAACAAAAAACTAAACGACCTTTTAACCGATCTGACCCGTTTTAAGATTGTCGAGATACT
	CTTGGTAAAAATGGACGAATTGCAAAAGGAACATGAGAATACCACGTACGACGACAGCGAGTATTACTGTTT
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TTCGATGTCAAGTTGAACAAAAAACTCAATGACCTCTTGACCGACATGACCCGATTTAAACTTGTCGAAATAC
TATTGGTGAAGATGGACGAGCTGCTGAGGAGCCACGAGAGGACCACTTACGATGACAGTGAATATTATCGTT
TGGTACGTAAATTTTTACAAATTGTCGAGACTGCTCAAAGCCACAAGGAGATAACCATTGCCGCAACCGAATT
GGGCATCACTCTGATGGAGAAATGTAAATATCAAAATTTAAATTTTTACACTAATTTTCTATGTTCCGAATCGA
TTCACAACGAGGTCTATATTATGAAAGATAAACTTAGACCGGCCATACTGAATGCCCGTCAAGTATTAGAGGC
TAAGATCAATCTGTCTATCAACGAGCTCAAGTCTGTGATAATATTGTACCAATTGCGCGTAGACGCGATCTTG
TGCGAATCTTGCAATGAAAAATCAGCCTCTCAACTTATGACGTGTGGACATAGATTCTGCGTCGATTGTATAT
ACGAAAAGATTGTAGAGGAGATTAAAGAACATGTGAATTATTCATGTATCATTTGCGAAAAAATGAATTATTT
CGTATCACTAAATGTTGGTCCCATGAACGAGTTTATGAGCGATTTTTATAACCAGCTGCGCGGCGCTACTAAC
AATGCTGCCAAAAAAATTGGAATTCTCGCCGACGACGTAGATGATTCGGGCGTGGCGACGGCCGCATCTGAC
TCTTCTTCGGCAGTGAGCACCAAGGCCAAGAGCAGGAAACGATCCGCATCTACCGCTACCGCCAAATCATTAA
AGTCACGTAAGTTGTCACGGTCAATACCGATTACACCTGCAATTGTTTATTCTTCTGACGATGATGATGATGAT
GATGATAAAGTTCGGCCGGACACCATATCGGTAGTAAATATAGACAAATTAAAATCTAATACTAACTGTTCTG
TACAACTTAATAGCGACGAGTCTACAGATTATGATGCCGACGACGACGATAACATTCCGCCGAACCAGGAGG
CTTTCAATAATACCGAAAAGTCCCCGCCCGAATCTGCGGCCGAATCTGAGCCACCGCAGATTGCGCCCGTTTT
CGAACCATTTGAGTATCTAGACTATTTGCCCGATTACGAGCCGCCGCCAACGCTACAGCCCGAGCCCGAGACT
TCCGAGACGCATGAAGCAGCAGAGTCCGAGTCTACGACCGAGCCAGAGTCTGCGACCGAGCCCGAGACGCTG
CATGAAGCAGAGTCCGAGTCTACTACGACCGAGCCGCCGCCACAGGCAGAGTCCGAGTCTGCGACCGAGCCG
CCGCCGCCGCCGCAGTCAGAGTCATCATCTCAGTCCGATGAACCTCTCAGGTACGATGATTCTTGTGTTGTTCC

	GTACAAAGAGCCCGTTGTTGTAGACGATAATGATGTGGTCATTAAAAAGGAAATCATATCCGATCCGGACGT
	AATTGATCAATATTATAAAACTAAATTTGATCAGACAGTAACGATTAATAATGATGATGATGATGACGATGAC
	GACGATGATATTGAATTTGTTAGCTGCAAGGATAAAGATGGCAAAGACATGGACGTGCGTGACATAAAATTG
	CACAAGGGCTTTGAATGTTTGTACAGATTACCGGGCACAAACGAGTTTCAGTCAATTATTATGGAGAAGGTGA
	TGATTAAAGATGAGAATAATGGGACCAATGGTGTGCCGGCTCTGTTCGATTCCATAGATCGGTACATGTGTCA
	AGCGCGCAACATACATTACACTCGGCGTATCGAACGCCGTAGAGGTATCGACGAAAACATTATTATGAATGA
	GTTACAGCGCAAACAGCAGCCGGCGTCGCCGCAGCCACCGTCTACATCGGCCGCGTCTCCGCAGCCTAAGCC
	ACCATCTACATCGACGCCGCCGCAACCACCGTCGCCGCAGCCACCGTCTACATCGGCCGCGTCTCCGCAGCCT
	AAGCCTAAGCAGCCTAAGCCTAGGCAGCCTAAGCCCGCCGGTGCCGCGTCTTCCATTTCTAAATCCAAGTCGA
	CAATTGCCGCGTCTTCCATTTCTAAATCCAAGTCCAAGTCTAAATATAGGCAGGCGACAATATCCGAGTGTAA
	CACGACGCGTTTGTATCCCGATGGAAAAGTGAAGTTTTTAACTCAGTAA

ORF 24	**Sequences**
SpltNPV-Pak-BNG	ATGGACGGCCCGTTTGTGGACTACGACGTAAAGTTTAATTATTCTATTGAATCCATCGGCGGATCTACGAATTT
	TATCATCAACCACAGTTTGCCGACGGTGAACTTGTTGAAAAATAACATGGGAATGTTGCGTCGAAAGATTGAA
	AAATATTTCGATTGCGAAGCCGATTTGAGAAGACATTTGATCAAGCTAAAAATGCTTCTATTGTCCAAATACA
	AGGAATACATTTTGATAATAATATTGAAAAAACTCTTTAATCGAGACTTGATCATGGTGGCGCCGATGTGCAA
	CCATTTCTTCAATCTACACGATCAATACATGTTTACGTTTGGAAAAATATTTTACACACGTTTACATATAAAAT
	TAGAATGTTTGATATTTTTCGATTTGAACAATAATACTCTGTTCCTTGTTCACAAGACTTTGGATGATTTTCAAT
	TGGCAGAATTCATATCTTCGTATCTGACTAAATACGGATTGAAACAGATCGAATTGAGAGTGAACGGTTCCAC
	CGAAACGCAGTACATGGAACACGACATAAATGATTTGGACGATTATGTGTGCTTCAAGGAAGATCCAATAGT
	GATTGATGCTAATAATAAATTTAAAGATTTAAAATTATACTATTCAAAATTTATCCATTGTATTGACTCGCGAA
	AAGAATTGGATGTTAAATTGAAAGAGATTAGAAATCTTTTGAAACGAGTCAAGTGTGCGCACACTTTGAATGC
	AAATAAAGAAAACGAAAACGGTTTGCAAGTGGACGTTTGTAAAATTATGCAAGACGCCGTCGAACGAATAAC
	CATAATTAATAACAAGTACATGATCGATAAAGTTAGAAAAATGTCAAAGCATCTTGACGACGAGACCGATGT
	TTCCCCAGCTAAGAGGAAAAAAATAAACGTTGCACAGCAGCAAAGTGTTGAAGAATCCCGACCGTTTGTGCA
	CGAAAACCCTGACGATTTCAATTACACACATCCTCCGATATCGTGCATGTTCAATTTACAATGCGACAGATCA
	TCTTTGAATGTGACCACATTGATGATGTTTTCAAAAAATTCATCGTGGAGCAAAGATTACGCCGACGACAACG
	ACGACAAGAACACGAAGCGCGTCGAAGACATCAAGAAACTGATCAATTCGAAAAATTTCAAAATGTTTAAAC
	CTTTTCTTAAAAGACAATACGAATATGATGAATAA
SpltNPV-Pak-TAX1	ATGGACGGCCCGTTTGTGGACTACGACGTAAAGTTTAATTATTCTATTGAATCCATCGACGGATCTACGAATTT
	TATCATCAACCACAGTTTGCCGACGGTGAACTTGTTGAAAAATAACATGGGAATGTTGCGTCGAAAGATTGAA
	AAATATTTCGATTGCGAAGCCGATTTGAGAAGACATTTGATCAAGCTAAAAATGCTTCTATTGTCCAAATACA
	AGGAATACATTTTGATAATAATATTGAAAAAACTCTTTAATCGAGACTTGATCATGGTGGCGCCGATGTGCAA
	CCATTTCTTCAATCTACACGATCAATACATGTTTACGTTTGGAAAAATATTTTACACACGTTTACATATAAAAT
	TAGAATGTTTGATATTTTTCGATTTGAACAATAATACTCTGTTCCTTGTTCACAAGACTTTGGATGATTTTCAAT
	TGGCAGAATTCATATCTTCGTATCTGACTAAATACGGATTGAAACAGATCGAATTGAGAGTGAACGGTTCCAC
	CGAAACGCAGTACATGGAACACGACATAAATGATTTGGACGATTATGTGTGCTTCAAGGAAGATCCAATAGT
	GATTGATGCTAATAATAAATTTAAAGATTTAAAATTATACTATTCAAAATTTATCCATTGTATTGACTCGCGAA
	AAGAATTGGATGTTAAATTGAAAGAGATTAGAAATCTTTTGAAACGAGTCAAGTGTGCGCACACTTTGAATGC
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	CATAATTAATAACAAGTACATGATCGATAAAGTTAGAAAAATGTCGAAGCATCTTGACGACGAGACCGATGT
	TTCCCCAGCTAAGAGGAAAAAAATAAACGTTGCACAGCAGCAAAGTGTTGAAGAATCCCGACCGTTTGTGCA
	CGAAAACCCTGACGATTTCAATTACACACATCCTCCGATATCGTGCATGTTCAATTTACAATGCGACAGATCA
	TCTTTGAATGTGACCACATTGATGATGTTTTCAAAAAATTCATCGTGGAGCAAAGATTACGCCGACGACGACG
	ACGACAAGAACACGAAGCGCGTCGAAGACATCAAGAAACTGATCAATTCGAAAAATTTCAAAATGTTTAAAC
	CTTTTCTTAAAAGACAATACGAATATGATGAATAA
SpltNPV-Pak-SFD1	ATGGACGGCCCGTTTGTGGACTACGACGTAAAGTTTAATTATTCTATTGAATCCATCGGCGGATCTACGAATTT
	TATCATCAACCACAGTTTGCCGACTGTGAACTTGTTGAAAAATAACATGGGAATGTTGCGTCGAAAGATTGAA
	AAATATTTCGATTGCGAAGCCGATTTGAGAAGACATTTGATCAAGCTAAAAATGCTTCTATTGTCCAAATACA
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	CCATTTCTTCAATCTACACGATCAATACATGTTTACGTTTGGAAAAATATTTTACACACGTTTACATATAAAAT
	TAGAATGTTTGATATTTTTCGATTTGAACAATAATACTCTGTTCCTTGTTCACAAGACTTTGGATGATTTTCAAT
	TGGCAGAATTCATATCTTCGTATCTGACTAAATACGGATTGAAACAGATCGAATTGAGAGTGAACGGTTCCAC
	CGAAACGCAGTACATGGAACACGACATAAATGATTTGGACGATTATGTGTGCTTCAAGGAAGATCCAATAGT
	GATTGATGCTAATAATAAATTTAAAGATTTAAAATTATACTATTCAAAATTTATCCATTGTATTGACTCGCGAA
	AAGAATTGGATGTTAAATTGAAAGAGATTAGAAATCTTTTGAAACGAGTCAAGTGTGCGCACACTTTGAATGC
	AAATAAAGAAAACGAAAACGGTTTGCAAGTGGACGTTTGTAAAATTATGCAAGACGCCGTCGAACGAATAAC
	CATAATTAATAACAAGTACATGATCGATAAAGTTAGAAAAATGTCAAAGCATCTTGACGACGAGACCGATGT
	TTCCCCAGCTAAGAGGAAAAAAATAAACGTTGCACAGCAGCAAAGTGTTGAAGAACCCCGACCGTTTGTGCA
	CGAAAACCCTGACGATTTCAATTACACACATCCTCCGATATCGTGCATGTTCAATTTACAATGCGACAGATCA
	TCTTTGAACGTGACCACATTGATGATGTTTTCAAAAAATTCATCGTGGAGCAAAGATTACGCCGACGACGACG
	ACGACAAGAACACGAAGCGCGTCGAAGACATCAAGAAACTGATCAATTCGAAAAATTTCAAAATGTTTAAAC
	CTTTTCTTAAAAGACAATACGAATATGATGAATAA
SpltNPV-G2	ATGGACGGCCCGTTTGTGGACTACGACGTAAAGTTTAATTATTCTATTGAATCCATCGGCGGATCTACGAATTT
	TATCATCAACCACAGTTTGCCGACTGTGAACTTGTTGAAAAATAACATGGGAATGTTGCGTCGAAAGATTGAA
	AAATATTTAGATTGCGAAGCCGATTTGAGAAGACATTTGATCAAGTTAAAAATGCTTCTTTTGTCCAAATACA
	AGGAATACATTTTGATAATAATATTGAAAAAACTCTTTAATCGAGACTTGATCATGGTGGCGCCGATGTGCAA
	CCATTTCTTCAATCTACACGATCAATACATGTTTACGTTTGGAAAAATATTTTACACACGTTTACATATAAAAT
	TAGAATGTTTGATATTTTTCGATTTGAACAATAATACTCTGTTCCTTGTTCACAAGACTTTGGATGATTATCAA
	TTGGCAGAATTCATATCTTCGTATCTGACTAAATACGGATTGAAACAGATCGAATTGAGAGTGAACGGTTCCA
	CCGAAACGCAGTACATGGAACACGACATAAATGATTTGGACGATTATGTGTGCTTCAAGGAAGATCCAATAG
	TGATTGATGCTAATAATAAATTTAAAGATTTAAAATTATACTATTCAAAATTTATCCATTGTATTGACTCGCGA
	AAAGAATTGGATGTTAAATTGAAAGAGATTAGAAATCTTTTGAAACGAGTCAAGTGTGCGCACACTTTGAATG
	CAAATAAAGAAAACGAAAACGGTTTGCAAGTGGACGTTTGTAAAATTATGCAAGACGCCGTCGAACGAATAA
	CCATAATTAATAACAAGTACATGATCGATAAAGTTAGAAAAATGTCGAAGCATCTTGACGACGAGACCGATG
	TTTCCCCAGCTAAGAGGAAAAAAATAAACGTTGCACAGCAGCAAAGTGTTGAAGAACCCCGACCGTTTGTGC
	ACGAAAACCCTGAAGATTTCAATTACACACATCCTCCGATATCGTGCATGTTCAATTTACAATGCGACAGATC
	ATCTTTGAATGTGACCACATTGATGATATTTTCAAAAAATTCATCGTGGAGCAAAGATTACGCCGACGACGAC
	GACAAGAACACGAAGCGCGTCGGAGACATCAAGAAACTGATCAATTCGAAAAATTTCAAAATGTTTAAACCT
	TTTCTTAAAAGACAATACGAATATGATGAATAA
SpliNPV-G1	ATGGACGCCGGTGTAAAATTTAATTATTCCATTGAATCTACCGCAAATTTTGTCATAAATCACAGTTGGCCTAT
	GGTGAAGATATATGATTTGGAAAAGTTAAAAGAATCATCATCATCATCGTATTTAAAGATTAAAAAATATTTC
	GAGTGTGAATCGGATCTAAAGAGACATTTGAAAAGATTAAAGATTCTTTTCGTGTTCAATTACAAGGAATCCG
	TTTTGAATGAAACAATCAAAAGACTATTGAATCCCGACTTGATTATGGTGGCGCCGATATGCGACCGCTTCTT
	TAATCTGCGCGATCGATACATGTTCACGCTTGGAAAAATATTTTACACGCCTATGCGTATAAAACTTGAATGTT
	TGATATTTTTTGATCTAAAGAATGACACTTTGTTCTTTGTTCACAAGACTTTGGACGATTCTCGATTGGCCGAA
	TTCATATCTTTGTATTTGGCCAAATATGGATTGAAACAGATTGAATTCAAAGTGGGCGACGCAACGACCGAGA
	CTCGGTACAGGGAATGTAGAATTGATGATATGGACGATTACGTGCACAGCGACGGTGATCCTCTGGTGATTGA
	TACTAATAATAAATTTAAAAATTTACATTTTCATTTTACAGAGTTGTTGACGCGCAAAAGAAAAGATGATTAT
	TTGCGCACGATCAATGTTAAATTGAATAAAATTAAAAAATTATTGAAACAGGTCAAGTACGCGTACGCTTTGA
	ATGTAAACAAAGAAAACGAATACGGTCTTGAAGTGGACATTTGTAAAATTGTACAAGACGTCTATGAAAAAA
	TTATATTAATCAATAACGAGTACATGATGGAAAAAGTTAAAGAAATGTCAGAATTCCTTTGCAAATATTACGG
	CGACGCGGAGGACGGCGTCTCCCCTGCTAAAAAGAGAAAAATGAACGCGCCGCAGCCTCGTAATGTTGAGGA
	GTCGCAGCCTCGGAATGTTTACGAAAATTCCGACGACTTTGATCCGTGCCATTATACATATCCTCCGATATCGT
	GCATGTTCAATTTCAAATGCGACAGATCATTAGATTTGACCACGTTGATGATATTTTCCAAAAATTCATTATTC
	AGCCCAGATTACGCAGATGACGATGACAAGAACACGCAGCGTATCGAAGACATCAAGAAACTGATAAAATCG
	AAAAATTTTAAAATGTTTAAACCTTTTCTTAAACGCCAATACCAATAA
SpliNPV-AN1956	ATGGACGCCGGTGTAAAATTTAATTATTCCATTGAATCTACCGCAAATTTTGTCATAAATCACAGTTGGCCTAT
	GGTGAAGATATATGATTTGGAAAAGTTAAAAGAATCATCATCATCATCGTATTTAAAGATTAAAAAATAT
	TTCGAGTGTGAATCGGATCTAAAGAGACATTTGAAAAGATTAAAGATTCTTTTCGTGTTCAATTACAAGGAAT

	CCGTTTTGAATGAAACAATCAAAAGACTATTGAATCCCGACTTGATTATGGTGGCGCCGATATGCGACCGCTT CTTTAATCTGCGCGATCGATACATGTTCACGCTTGGAAAAATATTTTACACGCCTATGCGTATAAAACTTGAAT GTTTGATATTTTTTGATCTAAAGAATGACACTTTGTTCTTTGTTCACAAGACTTTGGACGATTCTCGATTGGCC GAATTCATATCTTTGTATTTGGCCAAATATGGATTGAAACAGATTGAATTCAAAGTAGGCGACGCAACGACCG AGACTCGGTACAGGGAATGTAGAATTGATGATATGGACGATTACGTGCACAGCGACGGTGATCCTCTGGTGA TTGATACTAATAATAAATTTAAAAATTTACATTTTCATTTTACAGAGTTGTTGACGCGCAAAAGAAAAGATGA TTATTTGCGCACGATCAATGTTAAATTGAATAAAATTAAAAAATTATTGAAACAGGTCAAGTACGCGTACGCT TTGAATGTAAACAAAGAAAACGAATACGGTCTTGAAGTGGACATTTGTAAAATTGTACAAGACGTCTATGAA AAAATTATATTAATCAATAACGAGTACATGATGGAAAAAGTTAAAGAAATGTCAGAATTCCTTTGCAAATATT ACGGCGACGCGGAGGACGGCGTCTCCCCTGCTAAAAAGAGAAAAATGAACGCGCCGCAGCCTCGTAATGTTG AGGAGTCGCAGCCTCGGAATGTTTACGAAAATTCCGACGACTTTGATCCGTGCCATTATACATATCCTCCGAT ATCGTGCATGTTCAATTTCAAATGCGACAGATCATTAGATTTGACCACGTTGATGATATTTTCCAAAAATTCAT TATTCAGCCCAGATTACGCAGATGACGATGACAAGAACACGCAGCGTATCGAAGACATCAAGAAACTGATAA AATCGAAAAATTTTAAAATGTTTAAACCTTTTCTTAAACGCCAATACCAATAA
ORF 92 (*p12*)	**Sequences**
SpltNPV-Pak-BNG	ATGAGTATAATAGTGACACCCGATGACATCCTGTCTGGCGGCGGCGGCGGCGGCGGAAGAAACCGTTCGCGT CGTCGCAGGGGAGATGCCGGCGGAAACGATGACTTTGATGTCGCGAACAACATTGATCCATCTCAAATTATA AACGAACTCAACGACGTGAACACGAGCGCGCACGCAGTTCTCGCCGATCGATCCGACAACCGATTGGAAACG CTGCAAGTTTTGTCAAATCAATCAAACATTGCGAAAAAAAATTGTAACCGCCGCTAACAATCGCGATGATCGTG TAAAATTCAACGTCGTCGAAACAATGGAACTGTTGCGTCTAATGACGGATCTTTATGATAATAAATTTCTAAT TGTCGAATAA
SpltNPV-Pak-TAX1	ATGAGTATAATAGTGACACCCGATGACATCCTGTCTGGCGGCGGCGGCGGCGGAAGAAACCGTTCGCGTCGT CGCAGGGGAGATGCCGGCGGAAACGATGACTTTGATGTCGCGAACAACATTGATCCATCTCAAATTATAAAC GAACTCAACGACGTGAACACGAGCGCGCACGCAGTTCTCGCCGATCGATCCGACAACCGATTGGAAACGCTG CAAGTTTTGTCAAATCAATCAAACATTGCGAAAAAAAATTGTAACCGCCGCCAACAATAGCGATGATCGTGTAA AATTCAACGTCGTCGAAACAATGGAACTGTTGCGTCTAATGACGGATCTTTATGATAATAAATTTCTAATTGTC GAATAA
SpltNPV-Pak-SFD1	ATGAGTATAATAGTGACACCCGATGACATCCTGTCTGGCGGCGGCGGCGGCGGAAGAAACCGTTCGCGTCGT CGCAGGGGAGATGCCGGCGGAAACGATGACTTTGATGTCGCGAACAACATTGATCCATCTCAAATTATAAAC GAACTCAACGACGTGAACACGAGCGCGCACGCAGTTCTCGCCGATCGATCCGACAACCGATTGGAAACGCTG CAAGTTTTGTCAAATCAATCAAACATTGCGAAAAAAAATTGTAACCGCCGCCAACAATAGCGATGATCGTGTAA AATTCAACGTCGTCGAAACAATGGAACTGTTGCGTCTAATGACGGATCTTTATGATAATAAATTTCTAATTGTC GAATAA
SpltNPV-G2	ATGAGTATAATAGTGACGCCCGATGACATCCTGTCTGGCGGCGGCGGCGGAAGAAACCGTTCGCGTCGTCGC AGGGGAGATGCCGGCGGAAACGATGACTTTGATGTCGCGAACAACATTGATCCATCTCAAATTATAAACGAA CTCAACGACGTGAACACGAGCGCGCACGCAGTTCTCACCGATCGATCCGACAACCGATTGGAAACGCTGCAA GTTTTGTCAAATCAATCAAACATTGCGAAAAAAATTGTAACCGCCGCCAACAATCGCGATGATCGTGTAAAAT TCAACGTCGTCGAAACAATGGAACTGCTGCGTCTAATGACGGATCTTTATGATAATAAATTTCTAATTGTCGA ATAA
SpliNPV-G1	ATGAGTATAATAGTGACACCCGATGACATCTTGTCCGGCGGCGGTGGCGGTCGCGGTGGAAGAAACCGTTCG CGTCGTCGGCGGGAAGAGGACGGCGCCGACGACGATGCCGCGAACAATATCGATCCGTCTCAAATCATTAAC GAGCTCAACGACGTGAACACAAGCGCACATGCCATCCTCGCCGATCGGTCCGACAACCGATTGGAGACGCTG CAACTTTTGGCCAACCAGTCAAACATTGCGAAAAAAATTGTAACGTCTGTGAACAACCGTGATGATCGTGTAA AATTCAATGTCGTCGAAACCATGGAACTGTTGCGTCTAATGACGGATCTTTATGATAATAAATTTTTAGTCACC GAATAA
SpliNPV-AN1956	ATGAGTATAATAGTGACACCCGATGACATCTTGTCCGGCGGCGGTGGCGGTCGCGGTGGAAGAAACCGTTCG CGTCGTCGGCGGGAAGAGGACGGCGCCGACGACGATGCCGCGAACAATATCGATCCGTCTCAAATCATTAAC GAGCTCAACGACGTGAACACAAGCGCACATGCCATCCTCGCCGATCGGTCCGACAACCGATTGGAGACACTG CAACTTTTGGCCAACCAGTCAAACATTGCGAAAAAAATTGTAACGTCTGTGAACAACCGTGATGATCGTGTAA

	AATTCAATGTCGTCGAAACCATGGAACTGTTGCGTCTAATGACGGATCTTTATGATAATAAATTTTTAGTCACC GAATAA
ORF 106	**Sequences**
SpltNPV-Pak-BNG	ATGGGAAATTTATTGAATAAATTATTCCGCCGTGAAAATTATGTTTTCGAGAAGACCGATTCCGAAACAACCA ACAAGAGCGATCTGGACGATTTTGAAAAAAATCATATTTTCGAAAATACCGATTCTGATTCGAGCGGTGCCGA TGACAGCGGCGACGACGGTGACGACGAGAAAGATGCAACCATCTGTACGGACGAGAGTTACGAGATAGACG AATCGAACATGTTCGATGAATCTATTACGACTTTGGAGCAAAAAATTGAAAAGGCACGAGAAATCTTCACCC AACGGTTTGAGCGCTTCGGGTACGATTTAAAGTTTGACGGATACGATGATACAGATTATGGTGAAGATTATGA TAATTATTGCTGCGAACATCATCCAAATTGGTATTTGACGAGCAAGAGCCATTGTTTCTATAGTGTATCGACCG TTGTTAAAACCGTCGAAAAATGGTACAAGGAACTGCATCGTCGGGGCGGATTTGAAAACTTGGAAGATTTTGT AAAAGTGTTTCACGCGACCCTGCCGGTAAACGTGTACGTGGCCATGTTTGATGTCGATTTAAAGTATTGGAAA GATTGCTATGTATGCGACGCAAAATATCAATATAGAGACAACCACGTTTACCCATTAAAGATGTGTTTTTGCT CAGAATGCGCAACTCAATTATTTTACGAGCGTTACGACATTACGGAAAAGATTGTTAAAAGTTTTTGCACTTG TTCCAAGACTGAATTTGTCTATTTGAATGTACTTAGAGAAATATATTGTATAACATGTAAACGTGTTAAAATTT TTAAATATATTTATAATTAA
SpltNPV-Pak-TAX1	ATGGGAAATTTATTGAATAAATTATTCCGCCGTGAAAATTATGTTTTCGAGAAGACCGATTCCGAAACAACCA ACAAGAGCGATCTGGACGATTTCGAAAAAAATCATATTTTCGAAAATACCGATTCTGATTCGAGCGGTGCCGA TGACAGCGGCGACGACGGTGACGACGAGAAAGATGCAACCATCTGTACGGACGAGAGTTACGAGATAGACG AATCAAACATGTTCGATGAATCTATTACGACTTTGGAGCAAAAAATTGAAAAGGCACGAGAAATCTTCACCC AACGGTTTGAGCGCTTCGGGTACGATTTAAAGTTTGACGGATACGATGATACAGATTATGGTGAAGATTATGA TAATTATTGCTGCGAACATCATCCAAATTGGTATTTGACGAGCAAGAGCCATTGTTTCTATAGTGTATCGACCG TCGTTAAAACCGTCGAAAAATGGTACAAGGAACTGCATCGTAGGGGCGGATTTGAAAACTTGGAAGATTTTG TAAAAGTGTTTCACGCGACCCTGCCGGTAAACGTGTACGTGGCCATGTTTGATGTCGATTTAAAGTATTGGAA AGATTGCTATGTATGCGACGCAAAATATCAATATAGAGACAACCACGTTTACCCATTAAAGATGTGTTTTTGC TCAGAATGCGCAACTCAATTATTTTACGAGCGTTACGACATTACGGAAAAGATTGTTAAAAGCTTTTGCACTT GTTCCAAGACTGAATTTGTCTATTTGAATGTACTTAGAGAAATATATTGTATAACATGTAAACGTGTTAAAATT TTTAAATATATTTATAATTAA
SpltNPV-Pak-SFD1	ATGGGAAATTTATTGAATAAATTATTCCGCCGTGAAAATTATGTTTTCGAGAAGACCGATTCCGAAACAACCA ACAAGAGCGATCTGGACGATTTTGAAAAAAATCATATTTTCGAAAATACCGATTCTGATTCGAGCGGTGCCGA TGACAGCGGCGACGACGGTGACGACGAGAAAGATGCAACCATCTGTACGGACGAGAGTTACGAGATAGACG AATCAAACATGTTCGATGAATCTATTACGACTTTGGAGCAAAAAATTGAAAAGGCGCGAGAAATCTTCACCC AACGGTTTGAGCGCTTCGGGTACGATTTAAAGTTTGACGGATACGATGATACAGATTATGGTGAAGATTATGA TAATTATTGCTGCGAACATCATCCAAATTGGTATTTGACGAGCAAGAGCCATTGTTTCTATAGTGTATCGACCG TTGTTAAAACCGTCGAAAAATGGTACAAGGAACTGCATCGTAGGGGCGGATTTGAAAACTTGGAAGATTTTGT AAAAGTGTTTCACGCGACCCTGCCGGTAAACGTGTACGTGGCCATGTTTGATGTCGATTTAAAGTATTGGAAA GATTGCTATGTATGCGACGCAAAATATCAATATAGAGACAACCACGTTTACCCATTAAAGATGTGTTTTTGCT CAGAATGCGCAACTCAATTATTTTACGAGCGTTACGACATTACGGAAAAGATTGTTAAAAGCTTTTGCACTTG TTCCAAGACTGAATTTGTCTATTTGAATGTACTTAGAGAAATATATTGTATAACATGTAAACGTGTTAAAATTT TTAAATATATTTATAATTAA
SpltNPV-G2	ATGGGAAATTTATTGAATAAATTATTCCGCCGTGAAAATTATGTTTTCGATAAAACCGATTCCGAAACAACCA ACAAGAGCGATCTGGACGATTTCGAAAAGATCGATTCTGATTCGAGCGGTGCCGATAACAGCGGCGACGACG GTGACGACGAAAAAGATGCAACAATCTGTACGGACGAGAGTTACGAGATAGACGAATCGAACATGTTCGATG AATCTATTACGACTTTGGAGCAAAAAATTGAAAAGGCACGAGAAATCTTCACCCAACGGTTTGAGCGCTTCGG GTACGATTTAAAGTTTGACGGATACGATGATACAGATTATGGTGAAGATTATGATAATTATTGCTGCGAACAT CATCCAAATTGGTATTTGACGAGCAAGAGCCATTGTTTCTATAGTGTATCGACCGTCGTTAAAACCGTCGAAA AATGGTACAAGGAACTGCATCGTCGGGGCGGATTTGAAAACTTGGAAGATTTTGTAAAAGTGTTTCACGCGAC CCTGCCGGTAAACGTGTACGTGGCCATGTTTGATGTCGATTTAAAGTATTGGAAAGATTGCTATGTATGCGAC GCAAAATATCAATATAGAGACAACCACGTTTACCCATTAAAGATGTGTTTTTGCTCAGAATGCGCAACTCAAT TATTTTACGAGCGTTACGACATCACGGAAAAGATTGTTAAAAGCTTTTGCACTTGCTCCAAGACTGAATTTGTC

	TATTTGAATGTACTTCGAGAAATATATTGTATAACATGTAAACGTGTTAAAATTTTTAAATATATTTATAATTA A
SpliNPV-G1	ATGGGAAATTTACTGAATAAATTATTTGGTAATAATCAAACGGACAAGATCATCGATCTGGACACTCTTGAAT CGAATAATACCGAAACGGACACGATCGATACCGAAACGGACAAGACGGATCTGGACAATACCGATGACAAG ACGGATCTGGACAATACCGATGACAAGACGGATCTGGACAATACCGATCTGGACAAGACCGATCTGGACAAT ACCGATGACAAGACCGATCTGGACACTACCGATGACAAGACGGATTTGAAAGATTCCGAGACAGACAAAAGC TCGGAGTACGACGGCGACGAGAGCTACGAAATAGACGAATCGAACATGTTCGATGAATCGATCGTGACTTTG GATCAAAAAATTGAAAAGGCGCGAGAAATTTTTACTGAGCGGTTCGAGCACTTTGGGTACAATCTAAAGTTTG ACGACTATGACGACACGAGCTACGAGTACGATCCCGAATATTATTGTTGCGAACACCATCCATACTATTATGC GGAAAGCAACAAAAATTGTTTCTATAACATGTACGCCGTCGTGGGGGCCGTCGAAAATTGGTACAAGGAACT GAACCGGCGCGGCGGATTCGAAAACTTTAAAGATTTCGCAAAAGTGTTTCACGCGACCCTACCGAACAACGT GTACGTGTCCATGTTCGACATCAGTTCGATGTATTCGCAGATTTGCTACGTGTGTGACACCGAATATGAAAAG GACGAACATGACGTTTACCCGTTAAAAATGTGTTTTTGTTCAGAATGCGCAGTAGAATTATTTTTCGATCCGGA CTTACGCAAGAAAAAGATTGTTAAAAACTTTTGCACTTGTTCTCCATCTGAATCAGAATTTGAATGCCTGCATG TACTAGAAGAAGAATTTTGCAACACATGTAAACGTGTAAAAATTTTCAAATACATTTATTAA
SpliNPV-AN1956	ATGGGAAATTTACTGAATAAATTATTTGGTAATAATCAAACGGACAAGATCATCGATCTGGACACTCTTGAAT CGGACACGATCAATTTGAATAATACCGAAACGGACACGATCGATACCGAAACGGACAAGACGGATCTGGACA ATACCGATCTGGACAATACCGATGACAAGACGGATCTGGACAATACCGATCTGGACAATACCGATGACAAGA CGGATCTGGACAATACCGATCTGGACAATACCGATGACAATACCGATCTGGACAATACCGATCTGGACAATA CCGATGACAATACCGATCTGGACAATACCGATCTGGACAAGACCGATCTGGACAATACCGATCTGGACACTA CCGATGACAAGACGGATTTGAAAGATTCCGAGACAGACAAAAGCTCGGAGTACGACGGCGACGAGAGCTAC GAAATAGACGAATCGAACATGTTCGATGAATCGATCGTGACTTTGGATCAAAAAATTGAAAAGGCGCGAGAA ATTTTTACTGAGCGGTTCGAGCACTTTGGGTACAATCTAAAGTTTGACGACTATGACGACACGAGCTACGAGT ACGATCCCGAATATTATTGTTGCGAACACCATCCATACTATTATGCGGAAAGCAACAAAAATTGTTTCTATAA CATGTACGCCGTCGTGGGGGCCGTCGAAAATTGGTACAAGGAACTGAACCGACGCGGTGGATTCGAAAACTT TAAAGATTTCGCAAAAGTGTTTCACGCGACCCTACCGAACAACGTGTACGTGTCCATGTTCGACATCAGTTCG ATGTATTCGCAGATTTGCTACGTGTGCGACACCGAATATGAAAAGGACGAACATGACGTTTACCCGTTAAAAA TGTGTTTTTGTTCAGAATGCGCAGTAGAATTATTTTTCGATCCGGACTTACGCAAGAAAAAGATTGTTAAAAA CTTTTGCACTTGTTCTCCATCTGAATCAGAATTTGAATGCCTGCATGTACTAGAAGAAGAATTTTGCAACACAT GTAAACGTGTAAAAATTTTCAAATACATTTATTAA
ORF 110	**Sequences**
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SpltNPV-Pak-TAX1	ATGTCGCAAATGGTCAACATGTTTCAAGTCGATTCCCATGACGGCGAGTCGATCACGTTTGAGCCGCCTCAAG TGAAGACTACAATAGTAAAAAATCAATTTCAAATGGAGATTATGTTTCCATTTAACATTAAAAATCTTATATT GGAAAAAATTTACAAAAACAAAAAAAGCGTAATTTCATTGAGCGACGAAGAAAGAGAACAAATTGCCAACG

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AATTGTAA

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SpliNPV-AN1956 ATGGCCAATGAAAACGACGACGGCGACGGCGACAAAGTGGCAAATGAAAACGACGGCGACGACAACAACGA
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Appendix for Chapter 6 (Supplementary Material)

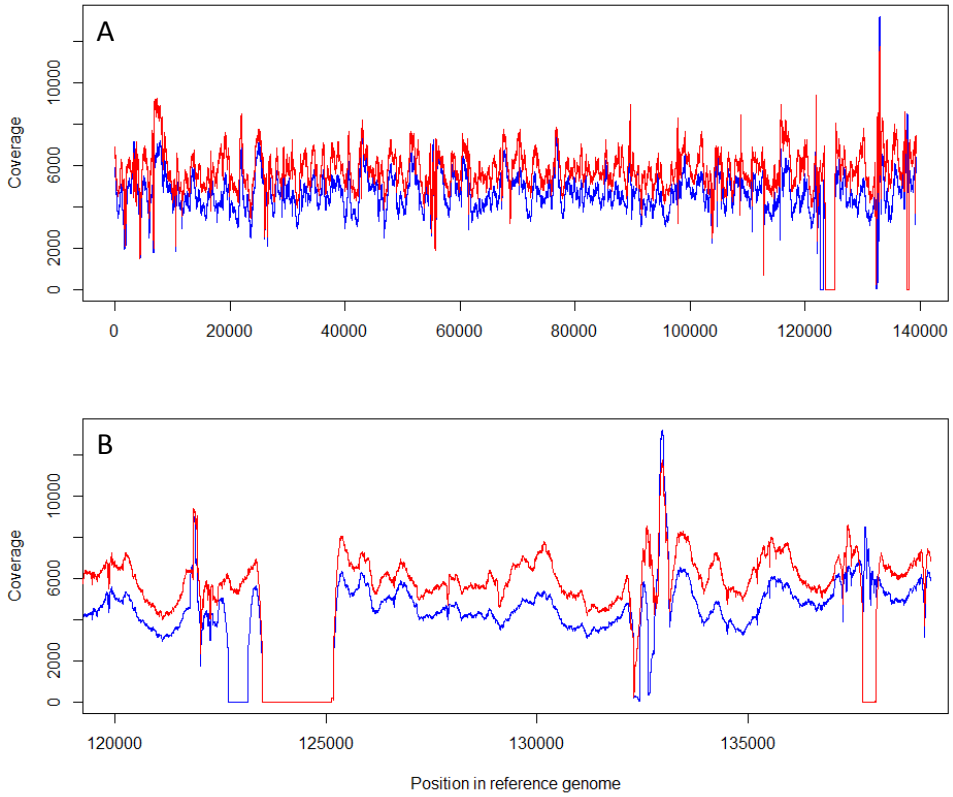

Figure S1: Plotting of the resequencing analysis coverage (y-axis) per nucleotide site (x-axis) in the whole genome (panel A), and in a part of the genome in which large deletions appear to occur in both virus isolates (panel B). The blue lines indicate coverage for the SpltNPV-Pak-BNG isolates, whereas the red lines indicate coverage for the SpltNPV-Pak-TAX1 isolate. Note the similarity in coverage between the two virus isolates, and that the largest indel is present in both.

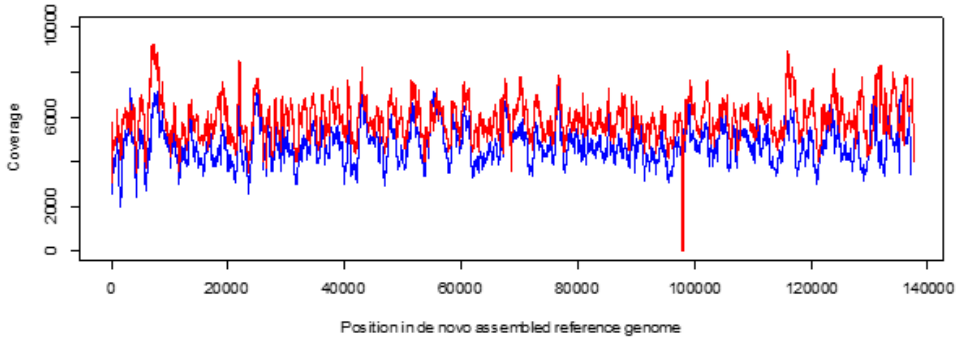

Figure S2: Plotting of the coverage (y-axis) per nucleotide site (x-axis) for the mapping of reads to their corresponding de novo assembled reference. The blue lines indicate coverage for the SpltNPV-Pak-BNG isolates, whereas the red lines indicate coverage for the SpltNPV-Pak-TAX1 isolate.

Table S1: Comparison of EcoRI RFLP predictions based on experimental results and de novo assembled sequences. All lengths are in nucleotide bases. "ND?" indicates we suspect the predicted band corresponds to multiple observed bands that are not distinguishable on the gel.

BNG		TAX1	
Predicted	Observed	Predicted	Observed
16028	15000	16021	15000
11420	10500	11386	10500
10496	ND?	10480	10000
10220	10000	10217	ND?
9386	9000	9393	8250
7918	8000	7765	8000
7458	7500	7458	7500
6779	6500	6795	6500
5533	5250	5675	5500
5389	ND?	5250	5250
5250	ND?	5078	5000
5010	4900	5058	ND?
4621	4750	4617	4500
4613	4500	4614	ND?
4493	4250	4421	4250
2612	2500	2612	2500
2423	2300	2423	2300
2370	2250	2370	ND?
2266	2100	2260	2250
2186	2000	2198	2100
1941	1750	1938	2000
1886	1600	1886	1750
1357	1300	1663	1600
1171	1200	1357	1300
1062	1100	1062	1200
1061	ND?	1061	1100
976	No longer visible	976	No longer visible
351		391	
243		351	
222		243	
218		222	
196		218	
		196	

Table S2: ORF comparison SpltNPV-PAK-BNG with SpltNPV-G2 (China) and SpliNPV-AN1956

ORF or feature	Namea	Positionb	# of aa (size, Da)	Promoter motifsc	Comparison with related NPVs					
					SpltMNPV-G2			SpliMNPV-AN1956		
					ORF (aa) or *hr*	% aa ID (range)	% nt ID (range)	ORF (aa) or hr	% aa ID (range)	% nt ID (range)
1	*polh*	1 → 750	249 (29,233)	L	*splt1* (249)	100 (244–249)	99 (774-750)	*Spli1 (248)*	99 (248-249)	93 (700-750)
2	*orf1629*	747 ← 2,402	551 (61,212)	e	*splt2* (548)	97 (537-551)	98 (1624-1656)	*Spli2 (544)*	81 (342-424)	83 (677-814)
3	*pk-1*	2,401 → 3,216	271 (31,683)		*splt3* (270)	99 (267–270)	99 (808-816)	*Spli3 (278)*	86 (234-272)	87 (708-811)
4	*hoar*	3,524 ← 5,758	744 (84,458)	EC	*splt4* (731)	94 (628–670)	96 (2146-2245)	*Spli4 (792)*	83 (379-458)	81 (846-1040)
5		6,110 ← 6,312	67 (7,368)		*splt5* (67)	88 (55-66)	99 (203-204)	*Spli5 (64)*	63 (34-54)	89 (91-102)
6		6,309 ← 6,593	94 (11,059)		*splt6* (94)	99 (93–94)	99 (283-285)			
7		6,529 → 8,685	718 (81,269)	L	*splt7* (717)	97 (594–611)	97 (2112-2169)	*Spli6 (754)*	60 (394-661)	83 (1133-1367)
8	*ie-0*	8,940 → 9,812	290 (33,449)		*splt8* (289)	99 (287–290)	99 (862-873)	*Spli7 (291)*	90 (261-291)	86 (755-873)
					Splt9(51)					
9	*dutpase*	9,864 → 10,361	165 (18,034)	e, L	*splt10* (164)	99 (151–152)	99 (492-498)	*Spli8 (171)*	82 (141-171)	87 (368-424)
hr1	*(5 P-I repeats)*	10,794…11,070			*hr1* (4 P-I repeats)			hr1		
10		10,866 → 12,275	469 (55,223)	EC, L	*splt11* (469)	99 (468–469)	99 (1404-1410)	*Spli9 (473)*	97 (460-473)	91 (1265-1391)

11	*odv-e18*	12,299 → 12,550	83 (9,222)	L	*splt12* (83)	100 (83–83)	100 (252-252)	*Spli10 (83)*	100 (83-83)	98 (244-250)
12	*odv-ec27*	12,589 → 13,440	283 (28,994)	–	*splt13* (283)	100 (283–283)	99 (851-852)	*Spli11 (284)*	95 (271-284)	91 (778-85)
13		13,472 → 13,753	93 (10,802)	EC	*splt14* (93)	100 (93–93)	99 (281-282)	*Spli12 (93)*	94 (87-93)	89 (248-280)
14		13,797 ← 14,396	199 (22,074)	L	*splt15* (200)	99 (199–200)	99 (599-603)	*Spli13 (204)*	80 (164-206)	84 (520-622)
15	*i.e.-1*	14,570 → 16,639	689 (78,048)	e, L	*splt16* (688)	99 (654–656)	99 (2059-2070)	*Spli14 (749)*	71 (516-727)	89 (1187-1339)
16	*odv-e56*	16,764 → 17,873	369 (40,010)	e, L	*splt17* (371)	99 (348–351)	98 (1099-1116)	*Spli15 (370)*	93 (327-351)	89 (988-1113)
17		17,888 → 18,439	183 (21,093)	–	*splt18* (183)	99 (182–183)	99 (549-552)	*Spli16 (183)*	95 (173-183)	91 (504-552)
18	*p10*	18,504 →18,821	105 (11,266)	EC, L	*splt19* (105)	100 (74–74)	100 (318-318)	*Spli17 (104)*	99 (79-80)	91 (286-316)
19		18,799 → 19,740	313 (36,217)		*splt20* (314)	98 (308–315)	98 (926-948)	*Spli18 (323)*	79 (256-323)	83 (817-980)
20	*p74*	19,768 ← 21,741	657 (75,873)		*splt21* (657)	99 (655–657)	99 (1955-1974)	*Spli19 (658)*	93 (614-661)	90 (1782-1971)
21		21,744 ← 22,090	115 (13,114)		*splt22* (114)	94 (104–111)	97 (337-348)			
22	*rr1*	22,130 ← 24,442	770 (86,806)	L	*splt23* (770)	99 (765–770)	99 (2291-2313)	*Spli20 (828)*	85 (690-812)	88 (2016-2290)
23		24,650 → 25,852	400 (47,503)		*splt24* (399)	98 (393–400)	99 (1190-1203)	*Spli21 (406)*	58 (239-415)	76 (834-1104)
hr2	*(10 P-I and 6 P-II-like repeats)*	26,117…27,290			*hr2 and hr3* (6 P-I and 4 P-II repeats)					
24		26,629 ← 26,805	58 (6,674)	L	*splt25* (59)	95 (52–55)	97 (170-175)			
25		27,218 → 28,111	297 (34,854)	e	*splt26* (297)	99 (295–297)	99 (887-894)	*Spli22 (334)*	88 (280-319)	87 (845-973)

26	*me53*	28,132 ← 29,037	301 (36,295)		*splt27* (301)	100 (301–301)	99 (903-906)	*Spli23 (304)*	94 (283-301)	92 (838-914)
27		29,069 → 29,329	86 (10,530)		*splt28* (86)	100 (86–86)	99 (259-261)	*Spli24 (68)*	76 (52-68)	87 (155-179)
28	*lef-6*	29,343 ← 29,780	145 (16,992)	e	*splt29* (145)	99 (143–145)	99 (434-438)	*Spli25 (153)*	71 (113-159)	86 (285-332)
29	*dbp*	29,843 ← 30,715	290 (33,270)		*splt30* (290)	99 (289–290)	99 (868-873)	*Spli26 (286)*	80 (233-290)	85 (712-841)
30		30,817 → 31,234	139 (16,183)	EC, e, L	*splt31* (149)	97 (148–153)	99 (427-430)	*Spli27 (133)*	90 (120-133)	88 (357-404)
31	*ubi/gp37*	31,203 → 32,258	351 (39,896)		*splt32* (351)	99 (350–351)	99 (1047-1056)	*Spli28 (337)*	92 (311-337)	88 (892-1014)
32	*39k*	32,313 ← 33,287	324 (36,785)		*splt33* (322)	94 (306–325)	98 (961-978)	*Spli29 (330)*	75 (256-341)	82 (818-1001)
33	*lef-11*	33,154 ← 33,582	142 (17,493)		*splt34* (144)	94 (135–144)	99 (428-432)	*Spli30 (143)*	86 (123-143)	90 (390-432)
34		33,552 ← 34,214	220 (26,559)		*splt35* (220)	100 (220–220)	99 (660-663)	*Spli31 (226)*	86 (195-226)	86 (589-681)
35	*p47*	34,284 ← 35,552	422 (49,315)		*splt36* (422)	99 (419–422)	99 (1257-1269)	*Spli32 (843)*	88 (370-422)	85 (1082-1266)
36	*lef-12*	35,579 → 36,184	198(23,597)	L	*splt37* (201)	99 (200–201)	99 (604-606)	*Spli33 (198)*	85 (168-198)	85 (492-582)
hr3	*(1 P-1 repeat)*	36,506…36,547								
37	*lef-8*	36,389 ← 39,142	917 (106,084)	L	*splt38* (918)	99 (913–918)	99 (2738-2757)	*Spli34 (911)*	90 (828-920)	88 (2443-2767)
38	*bjdp*	39,141 → 40,049	302 (34,633)	EC	*splt39* (302)	100 (302–302)	99 (905-909)	*Spli35 (303)*	91 (277-303)	87 (794-914)
39		40,072 → 40,641	189 (21,637)		*splt40* (189)	100 (189–189)	99 (567-570)	*Spli36 (189)*	88 (167-189)	89 (511-571)
40		40,661 ← 40,825	54 (6,257)	L	*splt41* (54)	96 (52–54)	98 (161-165)	*Spli37 (64)*	63 (40-64)	87 (86-99)

41	*chitA*	40,838← 42,532	564 (62,906)	L	*splt42* (564)	99 (562–564)	99 (1683-1695)	*Spli38 (599)*	92 (538-584)	90 (1532-1711)
hr4	*(5P-I repeats)*	42,837…43,091			*hr4* (2 P-1 repeats)					
42		43,092 ← 43,706	204 (24,517)	L	*splt43* (204)	99 (203–204)	99 (608-615)	*Spli39 (200)*	64 (130-203)	74 (466-627)
43	-	43,850 ← 44,476	208 (25,166)	EC, L	*splt44* (208)	99 (205–208)	99(623-627)	*Spli40 (212)*	80 (170-212)	87 (551-633)
44		44,546 → 44,959	137 (16,284)	e	*splt45* (137)	100 (137–137)	99 (413-414)	*Spli41 (148)*	83 (123-148)	89 (334-374)
45		44,998 ← 46,248	416 (46,671)		*splt46* (422)	91 (384–422)	98 (1243-1269)	*Spli42 (422)*	90 (316-352)	84 (1072-1280)
46		46,253 ← 46,480	75 (9,089)	EC, L	*splt47* (75)	100 (75–75)	100 (228-228)	*Spli43 (75)*	95 (71-75)	94 (214-228)
47	*lef-10*	46,440 → 46,694	84 (9,169)	L	*splt48* (84)	100 (84–84)	100 (255-255)	*Spli44 (82)*	85 (71-84)	91 (232-255)
48	*vp1054*	46,528 → 47,592	354 (40,947)	L	*splt49* (352)	99 (334–337)	99 (1055-1065)	*Spli45 (345)*	90 (306-339)	87 (910-1042)
49		47,713 → 47,928	71 (8,207)	e, L	*splt50* (71)	100 (71–71)	100 (216-216)	*Spli46 (71)*	80 (57-71)	85 (187-219)
50		48,232 → 48,751	173 (20,393)		*splt51* (173)	100 (173–173)	99 (518-520)	*Spli47 (166)*	93 (154-166)	89 (446-500)
51		48,782 ← 49,303	173 (19,341)	e, L	*splt52* (174)	99 (155–158)	97 (514-528)	*Spli48 (189)*	67 (103-153)	93 (208-224)
52		49,328 ← 49,579	83 (9,701)		*splt53* (82)	98 (65-66)	97 (245-252)	*Spli49 (82)*	88 (58-66)	86 (181-211)
53	*cathepsin*	49,626 → 50,639	337 (38,087)		*splt54* (337)	99 (334-337)	99 (1007-1014)	*Spli50 (336)*	94 (316-337)	89 (898-1004)
54	*p49*	50,688 → 52,007	439 (51,074)		*splt55* (439)	99 (438–439)	99 (1312-1320)	*Spli51 (446)*	84 (375-446)	86 (1149-1339)
55		51,946 ← 52,113	55 (6,707)		*splt56* (55)	100 (55–55)	100 (168-168)			

56	*fp25k*	52,135 ← 52,728	197 (23,009)		*splt57* (197)	100 (197–197)	99 (592-594)	*Spli52 (197)*	100 (197-197)	92 (548-593)
57		52,682 ← 52,885	67 (7,733)	L	*splt5* (67)	100 (67–67)	100 (204-204)			
58	*lef-9*	52,911 ← 54,407	498 (57,389)		*splt59* (498)	99 (497–498)	99 (1489-1497)	*Spli53 (498)*	96 (479-498)	90 (1352-1498)
hr5	*(8 P-I repeats)*	54,677…55,304								
59		54,325 → 54,501	58 (6,542)		*splt60* (58)	100 (58–58)	100 (177-177)	*Spli55 (87)*	no similarity	
hr6	*(3 P-I and 10 P-II-like repeats)*	56,009…57,005			*hr6* (3 P-I and 20 P-II repeats)			*hr6*		
60		55,719 ← 55,951	77 (8,445)	e	*splt61* (77)	100 (49-49)	98 (230-234)			
61		56,234 ← 57,337	367 (42,770)		*splt62* (367)	99 (363–367)	99 (1091-1104)	*Spli56 (346)*	57 (213-372)	70 (784-1114)
62	*rr2b*	57,490 → 58,491	333 (38,448)	EC	*splt63* (333)	100 (333–333)	99 (998-1002)	*Spli57 (334)*	90 (302-335)	86 (824-954)
63	*iap3/2*	58,569 ← 58,979	133 (15,619)		*splt64* (136)	99 (135-136)	99 (409-411)	*Spli58 (135)*	72 (99-137)	82 (344-421)
64		58,912 ← 59,853	313 (35,536)		*splt65* (313)	98 (308–313)	99 (934-942)	*Spli59 (313)*	83 (261-314)	86 (794-925)
65		59,813 ← 60,211	132 (15,174)	L	*splt66* (132)	99 (131-132)	99 (395-399)	*Spli60 (138)*	91 (126-138)	94 (344-365)
66	*lef-3*	60,216 → 61,280	354 (41,335)	L	*splt67* (357)	99 (352-357)	98 (1057-1074)	*Spli61 (349)*	89 (315-353)	89 (885-999)
67		61,536 ← 63,908	790 (91,660)	L	*splt68* (790)	99 (784–790)	99 (2354-2373)	*Spli62 (800)*	87 (697-805)	86 (2080-2423)
68	*dnapol*	63,910 → 66,975	1021 (117,867)	L	*splt69* (1022)	99 (1014-1022)	99 (3043-3069)	*Spli63 (1020)*	90 (921-1026)	87 (2685-3089)
hr7	*(1 P-I and 3 P-II-like repeats)*	67,916…68,117			*hr7* (2 P-I repeats)			*hr7*		
69		67,490 → 68,632	380 (44,767)	e, L	*splt70* (380)	99 (378-380)	99 (1136-1143)	*Spli65 (380)*	81 (309-380)	85 (972-1145)
70		68,714 ← 69,343	209 (23,565)	e, L	*splt71* (208)	96 (200-209)	99 (626-630)	*Spli66 (207)*	76 (162-213)	82 (526-644)

71		69,402 ← 69,785	127 (14,647)		*splt72* (127)	100 (127-127)	100 (384-384)	*Split67 (127)*	97 (123-127)	93 (357-384)
72		69,804 ← 70,058	84 (9,715)	L	*splt73* (84)	100 (68-68)	100 (255-255)	*Spli68 (84)*	99 (67-68)	96 (246-255)
73	*vlf-1*	70,123 ← 71,271	382 (44,974)	L	*splt74* (384)	99 (341-342)	99 (1141-1155)	*Spli69 (383)*	97 (331-340)	92 (962-1049)
74		71,292 ← 71,653	120 (13,065)		*splt75* (120)	98 (117-120)	99 (359-362)	*Spli70 (120)*	67 (83-124)	81 (303-375)
75	*gp41*	71,650 ← 72,639	329 (36,839)	EC, L	*splt76* (330)	100 (305-305)	99 (988-993)	*Spli71 (326)*	98 (307-313)	92 (906-990)
76		72,614 ← 73,312	232 (27,218)		*splt77* (232)	99 (225-227)	99 (693-699)	*Spli72 (237)*	86 (200-233)	92 (598-652)
77	*tlp-20*	73,191 ← 73,781	196 (21,630)	e	*splt78* (197)	98 (194-197)	99 (590-594)	*Spli73 (193)*	79 (160-202)	86 (523-610)
hr8	*(6 P-I repeats)*	77,112…77,422			*hr8* (4 P-I repeats)			*hr8*		
78	*vp91capsid*	73,750 → 76,332	860 (97,827)	EC	*splt79* (861)	99 (850-851)	99 (2561-2586)	*Spli74 (856)*	85 (734-861)	86 (2225-2589)
79	*cg30*	76,620 ← 77,372	250 (29,009)	E, L	*splt80* (250)	99 (248-250)	99 (751-753)	*Spli75 (259)*	72 (192-266)	88 (544-619)
80	*vp39*	77,431 ← 78,339	302 (33,875)	L	*splt81* (302)	100 (302-302)	99 (903-909)	*Spli76 (302)*	98 (296-302)	92 (837-909)
81	*lef-4*	78,341 → 79,768	475 (54,707)	L	*splt82* (475)	99 (473-475)	99 (1420-1428)	*Spli77 (489)*	85 (414-489)	85 (1257-1471)
82	*p33*	79,814 ← 80,581	255 (30,662)	EC	*splt83* (255)	99 (254-255)	99 (764-768)	*Spli78 (255)*	97 (247-255)	92 (706-766)
83		80,580 → 81,128	182 (21,402)		*splt84* (182)	99 (181-182)	99 (547-549)	*Spli79 (177)*	93 (169-182)	92 (504-549)
84	*odv-e25*	81,125 → 81,808	227 (24,897)	L	*splt85* (227)	100 (227-227)	99 (681-684)	*Spli80 (226)*	95 (215-227)	92 (628-684)
85	*DNA helicase*	81,897 ← 85,604	1235 (144,651)	EC, L	*splt86* (1235)	99 (1233-1235)	99 (3690-3708)	*Spli81 (1251)*	92 (1155-1251)	91 (3059-3373)

86		85,573 → 86,085	170 (19,203)	L	*splt87* (170)	100 (170-170)	99 (510-513)	*Spli82 (171)*	96 (165-171)	92 (474-516)
87	*38k*	86,092 ← 87,006	304 (35,989)	L	*splt88* (304)	99 (302-304)	99 (913-915)	*Spli83 (305)*	94 (287-305)	91 (839-918)
88	*lef-5*	86,902 → 87,810	302 (34937)		*splt89* (302)	99(301-302)	99 (904-909)	*Spli84 (297)*	84 (256-305)	87 (804-919)
89	*p6.9*	87,828 ← 88,082	84 (9,986)		*splt90* (84)	100 (10-10)	100 (255-255)	*Spli85 (87)*	100(10-10)	88 (235-267)
hr9	*(8 P-l repeats)*	90,305…90,732			*hr9* (2 P-l repeats)					
90	*p40*	88,140 ← 89,234	364 (41,319)	e, L	*splt91* (363)	99 (363-364)	99 (1088-1095)	*Spli86 (367)*	90 (331-367)	90 (998-1105)
91	*p12*	89,648 ← 90,019	123 (13,514)	e	*splt92* (121)	89 (109-123)	97 (362-372)	*Spli87 (122)*	84 (103-123)	86 (310-360)
92	*p45*	90,016 ← 91,137	373 (44,041)	L	*splt93* (373)	100 (373-373)	99 (1118-1122)	*Spli88 (375)*	95 (358-375)	90 (1017-1125)
93	*vp80*	91,163 → 93,100	645 (73,950)	L	*splt94* (644)	99 (620-627)	99 (1919-1938)	*Spli89 (648)*	87 (550-631)	85 (1637-1920)
94		93,100 → 93,267	55 (6,622)	EC	*splt95* (55)	100 (55-55)	99 (167-168)	*Spli90 (55)*	98 (54-55)	96 (161-168)
95	*odv-ec43*	93,299 → 94,384	361 (41,601)	EC	*splt96* (361)	100 (361-361)	99 (1072-1086)	*Spli91 (361)*	99 (357-361)	92 (995-1086)
96		94,465 → 94,803	112 (13,155)	e, L	*splt97* (112)	99 (111-112)	99 (334-339)	*Spli92 (112)*	91 (102-112)	88 (299-339)
97	*odv-e66*	94,793 ← 96,871	692 (78,135)	L	*splt98* (692)	99 (686-692)	99 (2063-2079)	*Spli93 (715)*	90 (652-723)	90 (1920-2126)
hr10	*(8 P-l repeats)*	98,878…99,348			*hr10* (4 P-l repeats)					
98	*p13*	96,874 ← 97,743	289 (33,771)		*splt99* (289)	100 (289-289)	99 (867-870)	*Spli94 (285)*	96 (280-289)	88 (731-833)
99		98,203 → 99,168	321 (37,202)	e	*splt100* (321)	99 (319-321)	99 (961-966)	*Spli96 (322)*	75 (242-322)	82 (792-969)
100		99,217 ← 99,927	236 (27,177)		*splt101* (236)	99 (235-236)	99 (705-711)	*Spli97 (232)*	85 (205-240)	90 (471-524)

101		99,947 ← 101,320	457 (52,159)		*splt102* (457)	98(449-457)	99 (1361-1374)	*Spli98 (469)*	79 (370-470)	81 (1148-1418)
102		101,347 ← 101,886	179 (20,892)	L	*splt103* (179)	100 (179-179)	100 (540-540)	*Spli99 (177)*	88 (158-179)	86 (464-540)
103		101,966 ← 102,163	65 (6,941)		*splt104* (66)	95 (61-64)	98 (197-201)	*Spli100 (65)*	70 (46-66)	80 (156-195)
104		102,301 → 103,557	418 (46,671)	L	*splt105* (418)	99 (417-418)	99 (1250-1257)	*Spli101 (431)*	89 (373-421)	87 (1084-1249)
hr11	*(7 P-I and 24 P-II-like repeats)*	104,511…107,449			*hr11* (4 P-I repeats)					
105		104,797 ← 105,618	273 (32,602)	e	*splt106* (267)	97 (264-273)	96 (793-822)	*Spli102 (349)*	68 (149-218)	77 (493-640)
106	*pif-3*	105,647 → 106,249	200 (22,627)		*splt107* (200)	99 (198-200)	99 (598--603)	*spli103 (200)*	93 (185-199)	88 (531-604)
107		106,259 → 106,611	117 (13,120)	EC, L	*splt108* (117)	100 (114-114)	99 (350-354)	*Spli104 (78)*	85 (66-78)	85 (198-234)
108	*alk-exo*	106,629 ← 107,855	408 (47,370)	EC	*splt109* (408)	99 (403-408)	99 (1214-1227)	*Spli105 (415)*	82 (335-411)	84 (1034-1232)
109		107,940 ← 109,088	382 (43,923)	e	*splt110* (376)	94 (360-384)	96 (1113-1155)	*Spli106 (319)*	74 (171-230)	82 (571-695)
110		109,050 → 109,208	52 (6,012)	e, L	*splt111* (52)	100 (52-52)	99 (157-159)	*Spli107 (63)*	55 (34-62)	87 (97-112)
111		109,211 ← 109,594	127 (14,844)	L	*splt112* (127)	99 (126-127)	99 (382-384)	*Spli108 (131)*	80 (105-131)	86 (272-318)
112		109,596 → 110,801	401 (47,016)		*splt113* (401)	99 (382-386)	99 (1199-1206)	*Spli109 (410)*	84 (331-395)	86 (1056-1233)
113	*lef-2*	110,850 ← 111,482	210 (24,445)		*splt114* (254)	100 (210-210)	99 (630-633)	*Spli110 (258)*	79 (207-261)	88 (580-660)
114		111466 ← 111,813	115 (12,458)	e	*splt115* (114)	98 (113-115)	99 (344-348)	*Spli111 (140)*	83 (95-114)	87 (310-355)
115	*p24capsid*	111861 → 112,595	244 (27,346)	e	*splt116* (244)	99 (243-244)	99 (731-735)	*Spli112 (239)*	91 (219-241)	89 (628-703)

hr12	*(7 P-I repeats)*	115,638…116,055								
116		112,974 → 113,292	106 (12,610)	e	*splt117* (105)	95 (98-103)	98 (313-319)			
117		113,102 → 115,864	920 (105,521)	L	*splt118* (919)	97 (900-927)	98 (2716-2776)	*Spli113 (928)*	84 (763-910)	85 (2346-2747)
118		115,881 ← 116,609	242 (27,715)	L	*splt119* (239)	98 (238-242)	98 (717-729)	*Spli114 (243)*	89 (217-243)	86 (631-732)
119	*bro-a*	116,671 ← 117,230	186 (21,909)		*splt120* (186)	99 (185-186)	99 (559-561)	*Spli115 (184)*	87 (162-186)	88 (497-563)
120	*egt*	117,486 ← 119,057	523 (60,242)		*splt121* (522)	99 (518-523)	99 (1558-1572)	*spli116 (532)*	86 (453-529)	86 (1332-1540)
121	*fgf*	119,185 → 119,919	244 (27,301)	L	*splt122* (246)	97 (246-247)	98 (727-744)	*Spli117 (243)*	81 (199-247)	82 (618-750)
122		119,944 ← 120,177	77 (9,270)	e	*splt123* (77)	99 (76-77)	99 (233-234)	*Spli118 (77)*	90 (69-77)	87 (204-235)
123	*pif1*	120,198 ← 121,778	526 (59,815)	e, L	*splt124* (525)	99 (504-505)	99 (1571-1581)	*Spli119 (525)*	83 (417-505)	84 (1338-1590)
hr13	*(2 P-I repeats)*	125,032…125,152			*hr13* (3 P-I repeats)			*hr13*		
124	*bro-b*	122,303 → 122,919	205 (23,631)		*splt125* (478)	78 (190-244)	91 (560-615)			
125	*38.7k*	123,903 ← 124,928	341 (40,632)	L	*splt128* (342)	99 (341-342)	99 (1023-1029)	*Spli120 (345)*	83 (284-343)	84 (865-1029)
126	*lef-1*	124,915 ← 125,610	231 (27,425)	L	*splt129* (231)	100 (231-231)	99 (695-696)	*Spli121 (231)*	93 (214-231)	91 (631-696)
127		125,591 ← 125,953	120 (14,038)	EC	*splt130* (122)	97 (110-113)	98 (360-369)	*Spli122 (128)*	86 (103-120)	91 (266-291)
128		125,950 ← 126,480	176 (20,971)		*splt131* (176)	99 (174-176)	99 (528-531)	*Spli123 (176)*	90 (170-176)	88 (466-531)
129	*calyx/pep*	126,484 ← 127,521	345 (38,506)	EC	*splt132* (344)	94 (323-345)	99 (1023-1038)	*Spli124 (353)*	94 (262-278)	85 (907-1071)
130	*pkip*	127,550 → 128,167	205 (23,797)		*splt133* (205)	99 (202-205)	99 (611-618)	*Spli125 (179)*	87 (157-180)	87 (474-543)

131	*arif-1*	128,205 ← 128,942	245 (27,656)		*splt134* (245)	99 (244-245)	99 (730-738)	*Spli126 (242)*	88 (219-248)	84 (623-745)
132	*pif-2*	128,916 → 130,193	425 (48,526)		*splt135* (425)	99 (424-425)	99 (1270-1278)	*Spli127 (421)*	92 (371-404)	89 (1106-1247)
hr14	*(2 P-I and 6 P-II-like repeats)*	131,598…132,033			*hr14* (2 P-I repeats)			*hr14*		
133		131,264 → 133,312	682 (76,837)		*splt136* (682)	98 (670-684)	99 (2027-2055)	*Spli128 (678)*	89 (606-683)	87 (1765-2039)
134		133,361 ← 134,056	231 (27,429)		*splt137* (231)	99 (189-191)	99 (688-696)	*Spli129 (242)*	70 (170-243)	77 (561-734)
135		134,154 → 134,933	259 (31,224)		*splt138* (259)	98 (255-259)	99 (772-780)	*Spli130 (258)*	86 (223-259)	86 (670-779)
hr15	*(6 P-II-like repeats)*	136,511…136,711			*hr15* (3 P-I and 26 P-II repeats)			*hr15*		
136		135,314 ← 135,497	61 (7,570)		*splt139* (60)	96 (51-53)	96 (177-184)	*Spli131 (136)*	no similarity	
137		136,027 ← 136,875	282 (34,042)		*splt140* (282)	99 (279-282)	99 (838-849)	*Spli132 (294)*	no similarity	
138		136,898 ← 137,083	61 (7,384)		*splt141* (61)	100 (61-61)	100 (186-186)			

P-I repeat: 5′-GAAAAGTCGGCCAGGTTCGATTCGAACTTGTCTGACTTTTC-3′ (underlined nucleotides differ in the majority sequence of SpltMNPV P-I repeats); P-II-like repeat: 5′-AACATGTT $(N)_{11–34}$.
P-II-like repeat: 5′-AACATGTT (N)11–34.
b
Arrows indicate 5′ → 3′ orientation of the ORFs.
c
EC, early promoter motif; e, CGTGC motif within 210 bp of the initiation codon; L, late promoter

ORF comparison SpltNPV-Pak-TAX1 with SpltNPV-G2 and SpliNPV-AN1956

ORF or feature	Namea	Positionb	# of aa (size, Da)	Promoter motifsc	Comparison with related NPVs					
					SpltMNPV-G2			SpliMNPV-AN1956		
					ORF (aa) or *hr*	% aa ID (range)	% nt ID (range)	ORF (aa) or *hr*	% aa ID (range)	% nt ID (range)
1	*polh*	1 → 750	249 (29,233)	L	*splt1* (249)	100 (249–249)	99 (744-750)	*Spli1*(248)	99 (248-249)	93 (700-750)
2	*orf1629*	747 ← 2,393	548 (60,848)	e	*splt2* (548)	95 (521-595)	98 (1618-1656)	*Spli2* (544)	81 (344-424)	84 (1064-1258)
3	*pk-1*	2,392 → 3,207	271 (31,725)		*splt3* (270)	99 (268–271)	99 (806-816)	*Spli3* (278)	87 (236-272)	88 (712-811)
4	*hoar*	3,523 ← 5,754	743 (84,144)	EC	*splt4* (731)	93 (629 -679)	94 (2119-2249)	*Spli4* (792)	83 (379-458)	82 (1279-1561)
5		6,108 ← 6,309	67 (7,611)		*splt5* (67)	100 (67-67)	99 (201-202)	*Spli5 (64)*	63 (34-54)	89 (91-102)
6		6,306 ← 6,590	94 (11,059)		*splt6* (94)	99 (93–94)	99 (283-285)			
7		6,526 → 8,682	718 (81,136)	L	*splt7* (717)	98(643 - 657)	97 (2109-2166)	*Spli6 (754)*	64 (383-597)	83 (1133-1367)
8	*ie-0*	8,937 → 9,809	290 (33,499)		*splt8* (289)	99 (287–290)	99 (862-873)	*Spli7 (291)*	90 (261-291)	86 (755-873)
					Splt9 (51)					
9	*dutpase*	9,861 → 10,358	165 (18,061)	e, L	*splt10* (164)	100 (152-152)	99 (493-498)	*Spli8 (171)*	83 (142-171)	87 (369-424)
hr1	*(5 P-I repeats)*	10,794…11,070			*hr1* (4 P-I repeats)					

10		10,863 → 12,272	469 (55,623)	EC, L	*splt11* (469)	99 (468–469)	99 (1403-1410)	*Spli9 (473)*	97 (460-473)	91 (1264-1391)
11	*odv-e18*	12,296 → 12,547	83 (9,222)	L	*splt12* (83)	100 (83–83)	100 (252-252)	*Spli10 (83)*	100 (83-83)	98 (244-250)
12	*odv-ec27*	12,586 → 13,437	283 (32,817)	–	*splt13* (283)	100 (283-283)	99 (850-852)	*Spli11 (284)*	95 (271-284)	91 (777-853)
13		13,469 → 13,750	93 (10,802)	EC	*splt14* (93)	100(93-93)	99 (281-282)	*Spli12 (93)*	94 (87-93)	89 (248-280)
14		13,794 ← 14,393	199 (22,101)	L	*splt15* (200)	99 (198-200)	99 (597-603)	*Spli13 (204)*	80 (164-206)	84 (521-622)
15	*i.e.-1*	14,566 → 16,635	689 (78,047)	e, L	*splt16* (688)	99 (654-656)	99 (2057-2070)	*Spli14 (749)*	71 (516-727)	88 (1184-1339)
16	*odv-e56*	16,777 → 17,886	369 (40,010)	e, L	*splt17* (371)	99 (348-351)	99 (1099-1116)	*Spli15 (370)*	93 (327-351)	89 (988-1113)
17		17,901 → 18,452	183 (21,093)	–	*splt18* (183)	99 (182-183)	99 (549-552)	*Spli16 (183)*	95 (173-183)	91 (504-552)
18	*p10*	18,517 → 18,834	105 (11,266)	EC, L	*splt19* (105)	100 (74–74)	100 (318-318)	*Spli17 (104)*	99 (79-80)	91 (286-316)
19		18,812 → 19,753	313 (36,217)		*splt20* (314)	98 (308–315)	99 (925-948)	*Spli18 (323)*	79 (256-323)	83 (816-980)
20	*p74*	19,782 ← 21,755	657 (75,873)		*splt21* (657)	99 (655-657)	99 (1959-1974)	*Spli19 (658)*	93 (614-661)	90 (1782-1971)
21		21,758 ← 22,104	115 (13,126)		*splt22* (114)	95 (105-111)	97 (337-348)			
22	*rr1*	22,144 ← 24,456	770 (86,806)	L	*splt23* (770)	99 (765–770)	99 (2293-2313)	*Spli20 (828)*	85 (690-812)	88 (2019-2290)
23		24,664 → 25,866	400 (46,544)		*splt24* (399)	98 (392–400)	99 (1190-1203)	*Spli21 (406)*	58 (239-415)	76 (835-1104)
hr2	*(10 P-I and 6 P-II-like repeats)*	26,117…27,290			*hr2 and hr3* (6 P-I and 4 P-II repeats)					
24		26,643 ← 26,817	58 (6,735)	L	*splt25* (59)	98 (55–56)	98 (172-175)			
25		27,243 → 28,136	297 (34,813)	e	*splt26* (297)	99 (294–297)	99 (887-894)	*Spli22 (334)*	87 (278-319)	87 (846-973)

26	*me53*	28,157 ← 29,062	301 (36,295)		*splt27* (301)	100 (301–301)	99 (903-906)	*Spli23 (304)*	94 (283-301)	91 (836-914)
27		29,094 → 29,357	87 (10,530)		*splt28* (86)	100 (86-86)	99 (259-261)	*Spli24 (68)*	76 (52-68)	87 (155-179)
28	*lef-6*	29,368 ← 29,805	145 (18,243)	e	*splt29* (145)	99 (143–145)	99 (434-438)	*Spli25 (153)*	71 (113-159)	86 (285-332)
29	*dbp*	29,869 ← 30,741	290 (33,663)		*splt30* (290)	99 (289–290)	99 (869-873)	*Spli26 (286)*	80 (233-290)	85 (711-841)
30		30,831 → 31,260	143 (16,576)	EC, e, L	*splt31* (149)	97 (148–153)	99 (426-430)	*Spli27 (133)*	90 (120-133)	88 (357-404)
31	*ubi/gp37*	31,229 → 32,284	351 (39,896)		*splt32* (351)	99 (350-351)	99 (1046-1056)	*Spli28 (337)*	92 (311-337)	88 (892-1014)
32	*39k*	32,340 ← 33,308	322 (36,551)		*splt33* (322)	99 (321-323)	99 (964-972)	*Spli29 (330)*	76 (258-339)	82 (818-1000)
33	*lef-11*	33,175 ← 33,603	142 (17,122)		*splt34* (144)	94 (135-144)	99 (428-432)	*Spli30 (143)*	86 (123-143)	90 (390-432)
34		33,573 ← 34,235	220 (26,559)		*splt35* (220)	100 (220-220)	99 (661-663)	*Spli31 (226)*	86 (195-226)	86 (589-681)
35	*p47*	34,305 ← 35,573	422 (49,379)		*splt36* (422)	99 (417-422)	99 (1253-1269)	*Spli32 (843)*	88 (370-422)	85 (1082-1267)
36	*lef-12*	35,600 → 36,205	201 (23,605)	L	*splt37* (201)	99 (199–201)	99 (603-606)	*Spli33 (198)*	85 (169-198)	85 (493-582)
hr3	*(1 P-1 repeat)*	36,506…36,547								
37	*lef-8*	36,410 ← 39,160	916 (106,101)	L	*splt38* (918)	99 (911-918)	99 (2729-2757)	*Spli34 (911)*	90 (825-919)	88 (2438-2764)
38	*bjdp*	39,159 → 40,067	302 (34,679)	EC	*splt39* (302)	99 (300–302)	99 (905-909)	*Spli35 (303)*	91 (275-303)	87 (793-914)
39		40,087 → 40,656	189 (21,637)		*splt40* (189)	100 (189-189)	99 (568-570)	*Spli36 (189)*	88 (167-189)	89 (510-571)
40		40,676 ← 40,840	54 (6,209)	L	*splt41* (54)	98 (53-54)	98 (162-165)	*Spli37 (64)*	64 (41-64)	88 (87-99)
41	*chitA*	40,853← 42,547	564 (63,101)	L	*splt42* (564)	99 (563–564)	99 (1684-1695)	*Spli38 (599)*	92 (539-584)	90 (1532-1710)
hr4	*(5 P-l repeats)*	42,837…43,091			*hr4* (2 P-1 repeats)					

42		43,098 ← 43,712	204 (24,534)	L	*splt43* (204)	100 (204–204)	99 (612-615)	*Spli39 (200)*	64 (130-203)	75 (468-627)
43		43,856 ← 44,482	208 (25,151)	EC, L	*splt44* (208)	99 (206–208)	99 (624-627)	*Spli40 (212)*	88 (169-212)	87 (550-633)
44		44,552 → 44,965	137 (16,284)	e	*splt45* (137)	100 (137-137)	99 (413-414)	*Spli41 (148)*	83 (123-148)	89 (334-374)
45		45,003 ← 46,265	420 (47,017)		*splt46* (422)	92 (390–423)	98 (1251-1272)	*Spli42 (422)*	81 (345-427)	83 (1072-1286)
46		46,270 ← 46,497	75 (9,089)	EC, L	*splt47* (75)	100 (75–75)	99 (227-228)	*Spli43 (75)*	95 (71-75)	93 (213-228)
47	*lef-10*	46,457 → 46,711	84 (9,378)	L	*splt48* (84)	100 (84-84)	100 (255-255)	*Spli44 (82)*	86 (72-84)	91 (232-255)
48	*vp1054*	46,545 → 47,600	351 (40,790)	L	*splt49* (352)	99 (333-335)	99 (1053-1059)	*Spli45 (345)*	91 (306-336)	88 (908-1032)
49		47,721 → 47,936	71 (8,233)	e, L	*splt50* (71)	99 (70–71)	99 (215-216)	*Spli46 (71)*	80 (57-71)	85 (187-219)
50		48,238 → 48,757	173 (20,407)		*splt51* (173)	99 (172–173)	99 (517-520)	*Spli47 (166)*	92 (153-166)	89 (445-500)
51		48,788 ← 49,306	172 (19,284)	e, L	*splt52* (174)	98 (155-158)	98 (514-525)	*Spli48 (189)*	67 (103-153)	93 (208-224)
52		49,331 ← 49,582	83 (9,701)		*splt53* (82)	98 (65 - 66)	97 (245-252)	*Spli49 (82)*	88 (58-66)	86 (181-211)
53	*cathepsin*	49,629 → 50,642	337 (37,958)		*splt54* (337)	99 (333-337)	99 (1006-1014)	*Spli50 (336)*	93 (315-337)	90 (899-1004)
54	*p49*	50,691 → 52,010	439 (15,107)		*splt55* (439)	99 (437–439)	99 (1311-1320)	*Spli51 (446)*	84 (374-446)	86 (1148-1339)
55		51,949 ← 52,116	55 (6,707)		*splt56* (55)	100 (55–55)	100 (168-168)			
56	*fp25k*	52,138 ← 52,731	197 (23,009)		*splt57* (197)	100 (197-197)	99 (592-594)	*Spli52 (197)*	100 (197-197)	92 548-593)
57		52,685 ← 52,888	67 (7,733)	L	*splt5* (67)	100 (67–67)	100 (204-204)			
58	*lef-9*	52,902 ← 54,398	498 (57,389)		*splt59* (498)	99 (497–498)	99 (1488-1497)	*Spli53 (498)*	96 (479-498)	90 (1351-1498)

hr5	*(8 P-I repeats)*	54,677…55,304			*hr5* (8 P-1 repeats)					
59		54,316 → 54,492	58 (6,542)		*splt60* (58)	100 (58–58)	100 (177-177)	*Spli55 (87)*		
hr6	*(3 P-I and 10 P-II-like repeats)*	56,009…57,005			*hr6* (3 P-I and 20 P-II repeats)			*hr6*		
60		56,205 ← 57,308	367 (42,772)		*splt62* (367)	98 (361-367)	99 (1089-1104)	*Spli56 (346)*	57 (213-372)	70 (783-1111)
61	*rr2b*	57,461 → 58,462	333 (38,448)	EC	*splt63* (333)	100 (333-333)	99 (999-1002)	*Spli57 (334)*	90 (302-335)	86 (825-954)
62	*iap3/2*	58,540 ← 58,944	134 (15,446)		*splt64* (136)	95 (129-136)	98 (403-411)	*Spli58 (135)*	74 (102-137)	82 (342-419)
63		58,877 ← 59,818	313 (35,605)		*splt65* (313)	98 (307–313)	99 (933-942)	*Spli59 (313)*	83 (261-314)	86 (793-925)
64		59,778 ← 60,176	132 (15,174)	L	*splt66* (132)	99 (131-132)	99 (396-399)	*Spli60 (138)*	91 (126-138)	95 (345-365)
65	*lef-3*	60,181 → 61,245	354 (41,335)	L	*splt67* (357)	99 (352-357)	98 (1057-1074)	*Spli61 (349)*	89 (315-353)	89 (885-999)
66		61,501 ← 63,873	790 (91,678)	L	*splt68* (790)	99 (784–790)	99 (2352-2373)	*Spli62 (800)*	87 (697-805)	86 (2080-2423)
67	*dnapol*	63,875 → 66,940	1021 (117,815)	L	*splt69* (1022)	99 (1016-1022)	99 (3045-3069)	*Spli63 (1020)*	90 (923-1026)	87 (2687-3089)
68		67,455 → 68,597	380 (44,767)	e, L	*splt70* (380)	99 (378 - 380)	99 (1135-1143)	*Spli65 (380)*	81 (307-378)	85 (973-1145)
hr7	*(1 P-I and 3 P-II-like repeats)*	67,916…68,117			*hr7* (2 P-I repeats)			*hr7*		
69		68,684 ← 69,313	209 (23,565)	e, L	*splt71* (208)	96 (200-209)	99 (625-630)	*Spli66 (207)*	76 (162-213)	82 (525-644)
70		69,372 ← 69,755	127 (14,647)		*splt72* (127)	100 (127-127)	100 (384-384)	*Split67 (127)*	97 (123-127)	93 (357-384)

71		69,774 ← 70,028	84 (9,701)	L	*splt73* (84)	99 (67 - 68)	99 (254-255)	*Spli68 (84)*	97 (66-68)	96 (245-255)
72	*vlf-1*	70,093 ← 71,232	379 (44,643)	L	*splt74* (384)	99 (341-342)	98 (1133-1155)	*Spli69 (383)*	97 (331-340)	91 (966-1060)
73		71,253 ← 71,614	120 (13,037)		*splt75* (120)	97 (116-120)	99 (358-362)	*Spli70 (120)*	67 (83-124)	81 (302-375)
74	*gp41*	71,611 ← 72,600	329 (36,839)	EC, L	*splt76* (330)	100 (305-305)	99 (989-993)	*Spli71 (326)*	98 (307-313)	92 (907-990)
75		72,575 ← 73,273	232 (27,218)		*splt77* (232)	99 (225-227)	99 (695-699)	*Spli72 (237)*	86 (200-233)	92 (598-652)
76	*tlp-20*	73,152 ← 73,742	196 (21,630)	e	*splt78* (197)	98 (194 - 197)	99 (590-594)	*Spli73 (193)*	79 (160-202)	86 (523-610)
77	*vp91capsid*	73,711 → 76,293	860 (97,817)	EC	*splt79* (861)	99 (849-861)	99 (2562-2586)	*Spli74 (856)*	85 (733-861)	86 (2223-2587)
hr8	*(6 P-I repeats)*	77,112…77,422			*hr8* (4 P-I repeats)			*hr8*		
78	*cg30*	76,581 ← 77,333	250 (28,961)	E, L	*splt80* (250)	99 (249-250)	99 (752-753)	*Spli75 (259)*	73 (191-262)	88 (544-619)
79	*vp39*	77,392 ← 78,300	302 (33,875)	L	*splt81* (302)	100 (302-302)	99 (905 - 909)	*Spli76 (302)*	98 (296-302)	92 (839-909)
80	*lef-4*	78,305 → 79,729	474 (54,575)	L	*splt82* (475)	99 (472-475)	99 (1417-1425)	*Spli77 (489)*	84 (413-489)	85 (1254-1468)
81	*p33*	79,755 ← 80,542	262 (31,233)	EC	*splt83* (255)	99 (254-255)	99 (763-768)	*Spli78 (255)*	97 (247-255)	92 (705-766)
82		80,541 → 81,089	182 (21,402)		*splt84* (182)	99 (181-182)	99 (547-549)	*Spli79 (177)*	93 (169-182)	92 (504-549)
83	*odv-e25*	81,086 → 81,769	227 (24,897)	L	*splt85* (227)	100 (227-227)	99 (681-684)	*Spli80 (226)*	95 (215-227)	92 (628-684)
84	*DNA helicase*	81,858 ← 85,565	1235 (144,637)	EC, L	*splt86* (1235)	99 (1232-1235)	99 (3688-3708)	*Spli81 (1251)*	92 (1154-1251)	91 (3324-3669)
85		85,534 → 86,046	170 (19,203)	L	*splt87* (170)	100 (170-170)	99 (510-513)	*Spli82 (171)*	96 (165-171)	92 (474-516)

86	*38k*	86,053 ← 86,967	304 (36,058)	L	*splt88* (304)	99 (301-304)	99 (911-915)	*Spli83 (305)*	94 (286-305)	91 (837-918)
87	*lef-5*	86,863 → 87,771	302 (34,937)		*splt89* (302)	99 (301-302)	99 (905-909)	*Spli84 (297)*	84 (270-305)	87 (803-919)
88	*p6.9*	87,789 ← 88,043	84 (9,986)		*splt90* (84)	100 (10-10)	100 (255-255)	*Spli85 (87)*	100 (10-10)	88 (235-267)
89	*p40*	88,101 ← 89,192	363 (41,232)	e, L	*splt91* (363)	100 (363-363)	99 (1087-1092)	*Spli86 (367)*	90 (330-367)	90 (992-1105)
hr9	*(8 P-I repeats)*	90,305…90,732			*hr9* (2 P-I repeats)					
90	*p12*	89,680 ← 90,048	122 (13,387)	e	*splt92* (121)	89 (108 - 122)	98 (362-369)	*Spli87 (122)*	82 (100-122)	85 (307-360)
91	*p45*	90,045 ← 91,166	373 (44,041)	L	*splt93* (373)	100 (373-373)	99 (1120-1122)	*Spli88 (375)*	95 (358-375)	91 (1019-1125)
92	*vp80*	91,192 → 93,141	649 (74,424)	L	*splt94* (644)	98 (621-631)	99 (1922-1950)	*Spli89 (648)*	87 (525-631)	85 (1641-1923)
93		93,141 → 93,308	55 (6,622)	EC	*splt95* (55)	100 (55-55)	99 (166-168)	*Spli90 (55)*	98 (54-55)	96 (162-168)
94	*odv-ec43*	93,340 → 94,425	361 (41,601)	EC	*splt96* (361)	100 (361-361)	99 (1074-1086)	*Spli91 (361)*	99 (357-361)	92 (997-1086)
95		94,507 → 94,845	112 (13,185)	e, L	*splt97* (112)	100 (112-112)	99 (336-339)	*Spli92 (112)*	92 (103-112)	88 (299-339)
96	*odv-e66*	94,835 ← 96,913	692 (78,136)	L	*splt98* (692)	99 (687-692)	99 (2064 - 2079)	*Spli93 (715)*	90 (653-725)	90 (1919-2126)
97	*p13*	96,916 ← 97,785	289 (33,771)		*splt99* (289)	100 (289-289)	99 (868-870)	*Spli94 (285)*	91 (262-289)	88 (732-833)
hr10	*(8 P-I repeats)*	98,878…99,348			*hr10* (4 P-I repeats)					
98		98,188 → 99,153	321 (37,218)	e	*splt100* (321)	99 (318-321)	99 (960-966)	*Spli96 (322)*	75 (241-322)	82 (791-969)
99		99,202 ← 99,912	236 (27,163)		*splt101* (236)	100 (236-236)	99 (707-711)	*Spli97 (232)*	86 (206-240)	90 (472-524)
100		99,932 ← 101,305	457 (52,029)		*splt102* (457)	99 (451-457)	99 (1358-1374)	*Spli98 (469)*	79 (369-470)	81 (1144-1417)

101		101,332 ← 101,871	179 (20,892)	L	*splt103* (179)	100 (179-179)	100 (540-540)	*Spli99 (177)*	88 (158-179)	86 (464-540)
102		101,951 ← 102,148	65 (6,971)		*splt104* (66)	94 (60 - 64)	97 (195-201)	*Spli100 (65)*	73 (46-63)	87 (1085-1249)
103		102,286 → 103,542	418 (46,657)	L	*splt105* (418)	100 (418-418)	99 (1251-1257)	*Spli101 (431)*	89 (373-421)	87 (1085-1249)
hr11	*(7 P-I and 24 P-II-like repeats)*	104,511…107,449			*hr11* (4 P-I repeats)					
104		104,754← 105,575	273 (32,602)	e	*splt106* (267)	97 (264-273)	97 (794-822)	*Spli102 (349)*	67 (140-209)	75 (476-633)
105	*pif-3*	105,604 → 106,206	200 (22,627)		*splt107* (200)	99 (198 - 220)	99 (598 - 603)	*spli103 (200)*	93 (185-199)	88 (531-604)
106		106,216 → 106,568	114 (12,850)	EC, L	*splt108* (117)	97 (113-114)	99 (350-354)	*Spli104 (78)*	85 (66-78)	84 (197-234)
107	*alk-exo*	106,586 ← 107,812	408 (47,338)	EC	*splt109* (408)	99 (404 - 408)	99 (1215 - 1227)	*Spli105 (415)*	82 (336-411)	84 (1029-1231)
108		107,896 ← 109,062	388 (44,509)	e	*splt110* (376)	91 (358 - 392)	94 (1108 - 1179)	*Spli106 (319)*	74 (171-230)	82 (571-695)
109		109,024 → 109,182	52 (6,012)	e, L	*splt111* (52)	100 (52-52)	99 (157-159)	*Spli107 (63)*	52 (32-62)	87 (97-112)
110		109,185 ← 109,568	127 (14,844)	L	*splt112* (127)	99 (126 - 127)	99 (383-384)	*Spli108 (131)*	80 (105-131)	86 (272-318)
111		109,570 → 110,775	401 (46,998)		*splt113* (401)	99 (381-386)	99 (1197-1206)	*Spli109 (410)*	84 (330-395)	86 (1060-1233)
112	*lef-2*	110,823 ← 111,455	210 (24,537)		*splt114* (254)	99 (208 - 210)	99 (630-633)	*Spli110 (258)*	79 (205-261)	88 (580-660)
113		111, 439 ← 111, 786	115 (12,458)	e	*splt115* (114)	98 (113 - 115)	98 (342-348)	*Spli111 (140)*	83 (95-114)	87 (310-355)
114	*p24capsid*	111, 834 → 112, 568	244 (27,346)	e	*splt116* (244)	99 (243 - 244)	99 (730-735)	*Spli112 (239)*	91 (219-241)	89 (627-703)
hr12	*(7 P-I repeats)*	115,638…116,055						*hr12*		
115		112, 945 → 113,262	105 (12,039)	e	*splt117* (105)	96 (101 - 105)	99 (314-318)			

116		113, 072 → 115,846	924 (105,989)	L	*splt118* (919)	96 (895 - 929)	98 (2728-2790)	*Spli113 (928)*	84 (770-913)	85 (2349-2748)
117		115,863 ← 116,588	241 (27,656)	L	*splt119* (239)	98 (237 - 241)	99 (717-726)	*Spli114 (243)*	89 (217-243)	86 (632-732)
118	*bro-a*	116,650 ← 117,210	186 (21,909)		*splt120* (186)	99 (185 - 186)	99 (558-561)	*Spli115 (184)*	87 (162-186)	88 (496-563)
119	*egt*	117,464 ← 119, 032	522 (60,104)		*splt121* (522)	99 (518-522)	99 (1553 -1569)	*spli116 (532)*	86 (454-529)	86 (1331-1540)
120	*fgf*	119, 168 → 119,902	244 (27,329)	L	*splt122* (246)	96 (237 - 247)	97 (723-744)	*Spli117 (243)*	81 (119-247)	82 (618-750)
121		119,927 ← 120, 160	77 (9,222)	e	*splt123* (77)	100 (77-77)	100 (234-234)	*Spli118 (77)*	91 (70-77)	87 (204-234)
122	*pif-1*	120,181 ← 121,761	526 (59,833)	e, L	*splt124* (525)	99 (504-505)	99 (1572-1581)	*Spli119 (525)*	83 (418-505)	84 (1337-1590)
hr13	*(2 P-I repeats)*	125,032…125,152			*hr13* (3 P-I repeats)			*hr13*		
123	*bro-b*	122,209 → 123,639	476 (55,779)		*splt125* (478)	94 (450-478)	95 (1358 - 1432)			
					Splt126 (456)					
124		123,726 ← 124,244	172 (19,868)		*splt127* (172)	99 (171-172)	99 (513-519)			
125	*38.7k*	124,301 ← 125,326	341 (40,632)	L	*splt128* (342)	99 (339-342)	99 (1022-1029)	*Spli120 (345)*	82 (282-343)	84 (862-1029)
126	*lef-1*	125,313 ← 126, 008	231 (27,425)	L	*splt129* (231)	100 (231-231)	99 (695-696)	*Spli121 (231)*	93 (214-231)	91 (631-696)
127		125,989 ← 126,348	119 (13,951)	EC	*splt130* (122)	96 (109 - 113)	97 (359-369)	*Spli122 (128)*	85 (102-120)	92 (268-291)
128		126,345 ← 126,875	176 (20,999)		*splt131* (176)	99 (175-176)	99 (529-531)	*Spli123 (176)*	91 (160-176)	88 (467-531)
129	*calyx/pep*	126,879 ← 127,910	343 (38,188)	EC	*splt132* (344)	97 (335-344)	99 (1025-1035)	*Spli124 (353)*	92 (256-278)	85 (907-1065)
130	*pkip*	127,939 → 128,556	205(20,676)		*splt133* (205)	99 (203-205)	99 (612-618)	*Spli125 (179)*	87 (156-180)	87 (473-543)

131	*arif-1*	128,594 ← 129,331	245 (27656)		*splt134* (245)	99 (244-245)	99 (731-738)	*Spli126 (242)*	88 (219-248)	84 (624-745)
132	*pif-2*	129,305 → 130,582	425 (48,526)		*splt135* (425)	99 (424-425)	99 (1274-1278)	*Spli127 (421)*	92 (371-404)	89 (1104-1247)
hr14	*(2 P-I and 6 P-II-like repeats)*	131,598…132,033			*hr14* (2 P-I repeats)			*hr14*		
133		131,801 → 133,849	682 (76,823)		*splt136* (682)	98 (671-684)	99 (2029-2055)	*Spli128 (678)*	89 (607-683)	87 (1766-2039)
134		133,898 ← 134,593	231 (27,489)		*splt137* (231)	99 (189-195)	99 (689-696)	*Spli129 (242)*	70 (170-243)	77 (562-734)
135		134,691 → 135,470	259 (31,210)		*splt138* (259)	99 (256 - 259)	99 (772-780)	*Spli130 (258)*	86 (223-259)	86 (668-779)
136		135,847 ← 136, 030	61 (7,570)		*splt139* (60)	96 (51 - 53)	96 (177-184)	*Spli131 (136)*	no similarrity	
hr15	*(6 P-II-like repeats)*	136,511…136,711			*hr15* (3 P-I and 26 P-II repeats)			*hr15*		
137		136,233 ← 137, 081	282 (34,010)		*splt140* (282)	99 (281-282)	99 (842-849)	*Spli132 (294)*	no similarrity	
138		137, 104 ← 137, 289	61 (7,384)		*splt141* (61)	100 (61-61)	100 (186-186)			

a
P-I repeat: 5′-GAAAAGTCGGCCAGGTTCGATTCGAACTTGTCTGACTTTTC-3′ (underlined nucleotides differ in the majority sequence of SpltMNPV P-I repeats); P-II-like repeat: 5′-AACATGTT $(N)_{11–34}$.
P-II-like repeat: 5′-AACATGTT (N)11–34.
b
Arrows indicate 5′ → 3′ orientation of the ORFs.
c
EC, early promoter motif; e, CGTGC motif within 210 bp of the initiation codon; L, late promoter

Table S3: Manual curation of within-isolate polymorphisms. Position is the nucleotide position in the corresponding de novo consensus sequence, length is size of the mutation, freq. is the frequency of the mutation (count/coverage), F./R. is the ratio of reads mapping in the forward to reverse orientations, avg. score is the average Phred score, and manual curation gives the results of inspections of the read mappings.

Virus	Position	Class	Length	Reference	New	Count	Coverage	Freq.	F./R.	Avg. Score	Manual curation
BNG	10396	Deletion	1	T	-	221	3915	5.645	0.457	36.742	Artefact repeated T stretch
	10396	Insertion	1	-	T	135	3867	3.491	0.431	37.733	Artefact repeated T stretch
	14399	Insertion	1	-	T	1277	3392	37.647	0.487	37.288	Artefact repeated T stretch
	14399	Deletion	1	T	-	84	3503	2.398	0.447	36.702	Artefact repeated T stretch
	29118	Deletion	1	A	-	106	3447	3.075	0.433	36.434	Artefact repeated A stretch
	35000	SNV	1	C	A	155	3881	3.994	0.442	37.465	Authentic
	35202	SNV	1	C	T	1623	3455	46.975	0.500	37.212	Authentic
	40062	Deletion	1	A	-	173	2940	5.884	0.383	36.457	Artefact repeated A stretch
	41180	SNV	1	G	A	60	2910	2.062	0.478	36.967	Authentic
	45843	Deletion	3	TAG	-	92	3669	2.507	0.472	36.935	Artefact repeated T stretch
	54562	Insertion	1	-	A	792	3571	22.179	0.492	37.556	Artefact repeated A stretch
	54562	Deletion	1	A	-	123	3818	3.222	0.485	36.333	Artefact repeated A stretch
	68688	Deletion	2	AC	-	168	3577	4.697	0.451	37.015	Artefact repeat region
	94442	Deletion	1	T	-	245	4343	5.641	0.436	36.878	Artefact repeated T stretch
	97803	SNV	1	C	T	217	3219	6.741	0.271	37.230	Artefact repeat region
	97957	Deletion	3	TCG	-	364	1382	26.339	0.403	37.357	Artefact repeat region
	97961	Deletion	14	CCACGTTCGATCAT	-	382	1354	28.213	0.413	37.370	Artefact repeat region
	97977	SNV	1	A	C	562	2375	23.663	0.379	37.527	Artefact repeat region
	97977	Deletion	2	AT	-	341	2427	14.050	0.482	37.388	Artefact repeat region
	97978	Deletion	1	T	-	1505	2427	62.011	0.387	36.747	Artefact repeat region
	97979	SNV	1	A	T	558	2467	22.619	0.376	37.427	Artefact repeat region
	104686	Insertion	1	-	A	133	4810	2.765	0.477	37.534	Artefact repeated A stretch
	110733	Deletion	1	T	-	313	4491	6.969	0.427	36.572	Artefact repeated T stretch
	110733	Insertion	1	-	T	98	4461	2.197	0.415	37.153	Artefact repeated T stretch
	111904	SNV	1	C	T	407	3822	10.649	0.493	37.314	Authentic
	112846	Insertion	1	-	T	230	3989	5.766	0.434	37.626	Artefact repeated T stretch
	112846	Deletion	1	T	-	105	4049	2.593	0.458	36.724	Artefact repeat region
	116029	Deletion	3	GAT	-	89	4360	2.041	0.439	36.647	Artefact repeated GAT sequence
TAX1	3227	Deletion	2	GT	-	386	4894	7.887	0.471	35.933	Artefact repeated region
	10393	Insertion	1	-	T	110	4637	2.372	0.459	37.436	Artefact repeated T stretch
	16680	Deletion	2	TA	-	451	4992	9.034	0.453	36.230	Artefact repeated region
	16680	Insertion	2	-	TA	263	4463	5.893	0.389	37.736	Artefact repeated region
	16680	Deletion	4	TATA	-	109	5245	2.078	0.393	36.770	Artefact repeated region
	26850	Deletion	1	T	-	238	5251	4.532	0.476	36.882	Artefact repeated T stretch
	26850	Insertion	1	-	T	106	5185	2.044	0.482	37.585	Artefact repeated T stretch
	29143	Deletion	1	A	-	151	4594	3.287	0.381	36.728	Artefact repeated A stretch
	45848	Deletion	3	TAG	-	153	5202	2.941	0.434	37.013	Artefact related to TAG repeats
	55296	Deletion	1	A	-	161	4978	3.234	0.495	37.366	Artefact repeated region
	68638	Deletion	2	AC	-	200	3684	5.429	0.424	36.700	Artefact repeated region
	91564	Deletion	3	GAC	-	89	4095	2.173	0.418	36.921	Artefact repeated region
	94483	Deletion	1	T	-	298	5559	5.361	0.422	36.990	Artefact repeated T stretch
	94483	Insertion	1	-	T	117	5491	2.131	0.452	37.179	Artefact repeated T stretch
	97943	Insertion	1	-	A	513	544	94.301	0.499	36.690	Artefact repeated region.
	97943	Insertion	1	-	C	27	544	4.963	0.444	37.333	Artefact repeated region
	97944	SNV	1	T	G	540	544	99.265	0.494	36.157	Artefact repeated region.
	98204	SNV	1	C	A	416	2995	13.890	0.436	35.007	Authentic
	113154	Insertion	1	-	T	1961	5016	39.095	0.436	37.303	Artefact repeated T stretch
	115768	Deletion	3	TAC	-	166	6201	2.677	0.476	37.404	Artefact related to TAC repeats
	119268	Deletion	2	CA	-	177	5444	3.251	0.492	36.836	Artefact repeated region
	120114	Deletion	1	T	-	177	6079	2.912	0.477	37.249	Artefact repeated T stretch

Summary

Spodoptera litura is an emerging insect pest in a wide range of crops worldwide. The insect is difficult to control because of resistance development to synthetic insecticides. There is therefore a need to develop biological control agents, preferably from an indigenous source to avoid risks associated with importation of exotic natural antagonists. This thesis describes the search for naturally occurring nucleopolyhedroviruses in *S. litura* collected in different ecological regions and crop systems in Pakistan. The thesis focuses on (i) genetic and biological diversity of SpltNPV to get insight in the genotypic structure among and within SpltNPV isolates that occur in Pakistan, (ii) the relationship between genetic make-up on the one hand and geographical distribution and cropping system on the other, and (iii) the functional significance and biocontrol potential of genetic and biodiversity of SpltNPVs.

In Chapter 2, a first Pakistani isolate, SpltNPV-Pak-BNG, was obtained from the field, characterized biologically and genetically, and compared to a SpltNPV reference isolate, SpltNPV-G1. The dose mortality response (LD_{50}) of SpltNPV-Pak-BNG was not significantly different from that of the reference isolate SpltNPV-G1, but the time-to-death (LT_{50}) was significantly shorter for SpltNPV-Pak-BNG than for SpltNPV-G1. DNA restriction enzyme profiling indicated that SpltNPV-Pak-BNG and SpltNPV-G1 are different viruses. Sequence analysis of 'ORF24', specific for SpltNPV and its orthologue in S. littoralis NPV,ORF21), and the conserved baculovirus core genes *polyhedrin*, *DNApol*, *pif-2* and *lef-8* confirmed that this was indeed the case and that SpltNPV-Pak-BNG is a genuine SpltNPV variant whereas the SpltNPV-G1 isolate used is in fact a SpliNPV variant, hence renamed to SpliNPV-G1. The newly isolated SpltNPV-Pak-BNG has potential for development as a biocontrol agent of *S. litura* in Pakistan.

To obtain biological information on SpltNPV-Pak-BNG, the median lethal dose and median survival time were determined for second (L2), third (L3) and fourth instar (L4) larvae of *S. litura* (Chapter 3). The median lethal dose (LD_{50}) was higher in L4 than in L2 and L3 larvae, while the LD_{50} values for L2 and L3 larvae were not significantly different. Likewise, the survival time was similar in L2 and L3 larvae (84h), but was significantly longer for L4 larvae (108h). Thus, in terms of efficacy, *S. litura* second or third instar larvae are the preferred target for baculovirus control with SpltNPV-Pak-BNG in field crops. This led to further collection of SpltNPV isolates from different geographical regions in Pakistan for genetic and biological analysis to select the best biotype for biocontrol.

In Chapter 4 a batch of twenty-two NPV isolates were collected from *S. litura* from different agro-ecological zones (with collection sites up to 600 km apart) and cropping systems

in Pakistan to see to what extent there is spatial dispersal of the virus and/or adaptation to crops. Among the SpltNPV isolates tested for speed of kill in 3rd instar larvae of *S. litura*, four isolates (SpltNPV-TAX1, -SFD1, -SFD2 and -GRW1 were significantly faster killing isolates than other Pakistani isolates. Restriction fragment length analysis of the DNA showed that the Pakistan SpltNPV isolates are all variants of a single SpltNPV biotype, for which the term 'regiotype' is proposed. There was limited interspecific genetic diversity among the SpltNPV isolates, but enough to be grouped into three so-called genogroups (A-C) corresponding to different ecological regions in Pakistan. Sequence analysis showed that the Pakistan SpltNPV isolates are more closely related to each other than to the SpltNPV type species G2 (Pang et al., 2001). This suggests a single introduction or ancestor of SpltNPV into Pakistan. There was a strong correlation between geographic location and genogroups of SpltNPV, and a marginally significant correlation between the latter and the cropping system. The faster killing SpliNPV isolates are therefore good candidates for biological control of *S. litura* in Pakistan.

Baculoviruses have established potential for biological pest control. However, slow speed of kill and continued feeding after the application limit their effectiveness in preventing crop losses. Chapter 5 presents the food consumption and weight gain of *Spodoptera litura* following application of two viral doses of the SpltNPV-Pak-BNG. Infected larvae with final polyhedrosis exhibited reduced food intake and weight gain. The healthy control and the larvae that survived viral exposure, exhibited the same food consumption and weight gain. There was no viral dose dependency observed in food intake or weight gain by infected larvae.

To identify the genetic basis of the difference in speed of kill, the genomes of SpltNPV-Pak-TAX1 and SpltNPV-Pak-BNG were deep-sequenced and compared, (Chapter 6). These two viruses were genetically closely related, with limited sequence variation within each isolate. The major difference between these two isolates is the absence of homologous repeat region 17 (putative enhancer of transcription and origin of replication) in SpltNPV-Pak-TAX1 and deletion of ORF125 with unknown function in SpltNPV-Pak-BNG. A further difference between the consensus genome of these two viruses is the strong positive selection on open reading frame (ORF) 122. This ORF encodes a putative viral fibroblast growth factor (*fgf*), known to be involved in virus transport with larvae and possibly virulence. SpltNPV-Pak-TAX1 is 24 h faster in 3rd instar larvae of *S. litura* than to SpltNPV-Pak-BNG. This could be due to deletion of hr17 or a mutation in the *fgf* gene of SpltNPV-Pak-TAX1. However, a key question is what drives the selection for increased virulence in one case (region) and not in the other.

The results presented in this thesis show that SpltNPV isolates occur naturally in Pakistan and provides evidence of spatial diversity. The genomic analysis of spatially distinct SpltNPV isolates confirmed heterogeneity in the genomic sequence, which may have contributed towards the evolution towards more speed of kill in certain SpltNPV-Pak isolates. In conclusion, the results presented in this thesis enhanced our knowledge of genetic diversity and biological activity of SpltNPV in general and in Pakistan in particular. This may lead to the development of SpltNPV-based products and strategies to control the leafworm *S. litura* in Pakistan.

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Ghulam Ali Wageningen, March 2018

List of publications

1. **Ali, G**., Poswal, M. A. and Rehman, A. (2002). Issues and challenges of farmer's empowerment and zero tillage technology adoption in rice-wheat systems in Punjab, Pakistan. Proceeding of the national workshop on rice-wheat systems in Pakistan, 11-12 December 2002 Islamabad. Rice-Wheat consortium paper series 15. p.100-102.
2. Jafry, T., Poswal, A., Ahmad, B. R. and **Ali, G**. (2013). Adoption of resource conservation technologies by farmers in the rice wheat system of Pakistan. Natural Resource Conservation, 1: 15 - 20. doi: 10.13189/nrc.2013.010103.
3. **Ali, G**. and Zia, Q. (2014). Working with Water in Cotton; developing water scouting strategies at farm level. Universal Journal of Agricultural Research, 2: 127 - 130. doi: 10.13189/ujar.2014.020401
4. **Ali, G**., M. Henkens, E. Schijlen, W. van der Werf and J.M. Vlak. (2015). Population structure of *Spodoptera litura* multicapsid nucleopolyhedroviruses from Pakistan. Abstract Book of the 48th Annual Meeting of the Society for Invertebrate Pathology, Vancouver, Canada, p.67.
5. **Ali, G**., W. van der Werf and J.M. Vlak. (2017). Biological and genetic characterization of a Pakistani isolate of Spodoptera litura nucleopolyhedrovirus. Biocontrol Science and Technology, in press (doi.org/10.1080/09583157.2017.1409339)
6. **Ali, G**., J.M. Vlak and W. van der Werf. (2017). Biological activity of Pakistani isolate SpltNPV-Pak-BNG against second, third and fourth instar larvae of *Spodoptera litura*, Manuscript submitted.
7. **Ali, G**., Marleen H.C. Abma-Henkens, Lia Hemerik, Wopke van der Werf and Just M. Vlak. (2017.) Genotype assembly, biological activity and evolution of spatially separated isolates of Spodoptera litura nucleopolyhedrovirus. Journal of Invertebrate Pathology, in revision.
8. **Ali, G**., W. van der Werf and J.M. Vlak. (2017). Infection with Spodoptera litura NPV reduces food consumption and weight gain of *Spodoptera litura* larvae. Manuscript in preparation.
9. Zwart, M. P., **G. Ali** , E. Schijlen, W. van der Werf and J.M. Vlak. (2017). Identification of loci associated with enhanced virulence in Spodoptera litura nucleopolyhedrovirus isolates using deep sequencing approaches and analyses. Manuscript in preparation.

PE&RC Training and Education Statement

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

Review of literature (6 ECTS)

- Baculoviruses: experimental evolution, population structure and biofitness

Writing of project proposal (4.5 ECTS)

- Functional diversity and experimental evolution of *Spodoptera litura* nucleopolydrovirus

Post-graduate courses (3.3 ECTS)

- Generalized linear model: PE&RC (2011)
- Mixed linear model; PE&RC (2011)
- Bioinformatics; a user's approach: EPS, WUR (2014)
- Introduction to R for statistical analysis; PE&RC and SENSE, WUR (2014)

Laboratory training and working visits (4.5 ECTS)

- Insect rearing techniques; Biological Control Laboratory; NARC Islamabad, Pakistan (2011)

Deficiency, refresh, brush-up courses (3 ECTS)

- Gene technology (2011)
- Basic statistics (2014)
-

Competence strengthening / skills courses (4.5 ECTS)

- Management course for development practioners; Management development Foundation, Ede (2007)
- Intensive communication skills; Nat-IPM Program NARC, Pakistan (2013)

- Techniques for writing and presenting scientific paper; WUR (2014)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.4 ECTS)

- PE&RC Day: optimization of science: pressure and pleasure (2014)
- PERC Weekend, last year (2014)
- Dutch Entomologendag Day (2014)

Discussion groups / local seminars / other scientific meetings (5 ECTS)

- Experimental evolution discussion group (EEDG) seminars (2011-2014)
- WEES seminars (2011-2015)

International symposia, workshops and conferences (4.5 ECTS)

- International Cotton Advisory Committee meeting (2009)
- Asian Cotton Research & Development Network (2011)
- Society for Invertebrate Pathology Annual Meeting (2014)

Lecturing / supervision of practical's / tutorials (0.6 ECTS)

- Lecture on biological control of insects (2012)
- Lecture on organic agriculture: current and future perspective (2012)

About the author

Ghulam Ali was born on 28th of December 1968 in the Narowal district in the Punjab province of Pakistan. In 1989 he left his hometown to study agriculture at the University of Agriculture Faisalabad. In 1994 he started his Master studies at the Department of Entomology, University of Agriculture Faisalabad, and completed his thesis "Study on the yield losses in winter planted rapeseed and mustard by mustard aphid (*Bravicoryne brassicae*)". In 1998 he obtained his Master degree and joined CAB International on 22nd of June 1999. He worked in Pakistan, Afghanistan, Bangladesh and Vietnam on projects in plant health systems, sanitary and phytosanitary measures and capacity building programs for farmers and agricultural professionals in good agricultural practices at the farm level for more than 15 years. He received a scholarship from the Netherlands Fellowship Program in 2006 and moved to Wageningen International where he obtained a postgraduate diploma in Food and Nutrition Security. In 2007 he obtained a scholarship from the Netherlands Fellowship Program and moved to the Management and Development Foundation (MDF) in Ede, where he completed a certificate course in Project & Program Development and Administration. In 2011 he obtained a sandwich PhD scholarship from the Netherlands Fellowship Program and moved to the Laboratory of Virology, Wageningen University and Research. During his PhD research he studied the diversity and biological activity of nucleopolyhedroviruses of the leafworm *Spodoptera litura* in Pakistan.

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