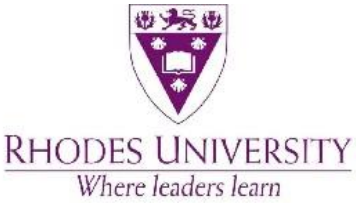

The isolation, genetic characterisation and biological activity of a South African *Phthorimaea operculella* granulovirus (PhopGV-SA) for the control of the Potato Tuber Moth, *Phthorimaea operculella* (Zeller)

A thesis submitted in fulfilment of the

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By

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Abstract

The potato tuber moth, *Phthorimaea operculella* (Zeller), is a major pest of potato crops worldwide causing significant damage to both field and stored tubers. The current control method in South Africa involves chemical insecticides, however, there is growing concern on the health and environmental risks of their use. The development of novel biopesticide based control methods may offer a potential solution for the future of insecticides.

In this study a baculovirus was successfully isolated from a laboratory population of *P. operculella*. Transmission electron micrographs revealed granulovirus-like particles. DNA was extracted from recovered occlusion bodies and used for the PCR amplification of the *lef*-8, *lef*-9, *granulin* and *egt* genes. Sequence data was obtained and submitted to BLAST identifying the virus as a South African isolate of *Phthorimaea operculella* granulovirus (PhopGV-SA). Phylogenetic analysis of the lef-8, lef-9 and granulin amino acid sequences grouped the South African isolate with PhopGV-1346.

Comparison of *egt* sequence data identified PhopGV-SA as a type II *egt* gene. A phylogenetic analysis of egt amino acid sequences grouped all type II genes, including PhopGV-SA, into a separate clade from types I, III, IV and V. These findings suggest that type II may represent the prototype structure for this gene with the evolution of types I, III and IV a result of large internal deletion events and subsequent divergence. PhopGV-SA was also shown to be genetically more similar to South American isolates (i.e. PhopGV-CHI or PhopGV-INDO) than it is to other African isolates, suggesting that the South African isolate originated from South America.

Restriction endonuclease profiles of PhopGV-SA were similar to those of PhopGV-1346 and PhopGV-JLZ9f for the enzymes *Bam*HI, *Hin*dIII, *Nru*I and *Nde*I. A preliminary full genome sequence for PhopGV-SA was determined and compared to PhopGV-136 with some gene variation observed (i.e. *odv-e66* and *vp91/p95*). The biological activity of PhopGV-SA against

P. operculella neonate larvae was evaluated with an estimated LC_{50} of 1.87×10^{8} OBs.ml^{-1} being determined. This study therefore reports the characterisation of a novel South African PhopGV isolate which could potentially be developed into a biopesticide for the control of *P. operculella*.

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List of Abbreviations

General:

$	-	United States dollar
%	-	percentage
×*g*	-	Times gravity
° C	-	Degrees Celsius
µl	-	Microlitre
ApE	-	A plasmid editor
BLAST	-	Basic Local Alignment Search Tool
bp	-	base pair
BV	-	Budded virus
cm	-	Centimetre
CTAB	-	Cetrimonium bromide
D1	-	Dose 1
D2	-	Dose 2
D3	-	Dose 3
D4	-	Dose 4
ddH2O	-	Double distilled water
DNA	-	Deoxyribonucleic acid
dsDNA	-	Double stranded deoxyribonucleic acid
EDDR	-	Egg dip dose response
egt	-	ecdysteroid UDP-glucosyltransferase
EPN	-	Entomopathogenic nematodes
EU	-	European Union
g	-	Grams
GB	-	Gigabase
GV	-	Granulovirus
ha	-	Hectares
LC_{50}	-	Lethal concentration (50 %)
LC_{90}	-	Lethal concentration (90 %)
lef	-	Late expression factor
lef-8	-	Late expression factor-8

lef-9	-	Late expression factor-9
M	-	Molar
mg	-	Milligram
min	-	Minutes
ml	-	Millilitre
mm	-	Millimetre
mM	-	Millimolar
NC	-	Nucleocapsid
NCBI	-	National Center for Biotechnology Information
NGS	-	Next generation sequencing
nm	-	Nanometre
NPV	-	Nucleopolyhedrovirus
NTC	-	No template control
OB	-	Occlusion body
ODV	-	Occlusion derived virus
ORF	-	Open reading frame
PCR	-	Polymerase chain reaction
PIF	-	*per os* infectivity factor
polh	-	polyhedrin
PTM	-	Potato tuber moth
PTW	-	Potato tuber worm
R	-	South African rand
REN	-	Restriction endonuclease
RNA	-	Ribonucleic acid
RT1	-	Rearing technique 1
RT2	-	Rearing technique 2
RT3	-	Rearing technique 3
SDS	-	Sodium dodecyl sulphate
SEM	-	Scanning electron microscopy
SNP	-	Single nucleotide polymorphism
TEM	-	Transmission electron microscopy
v/v	-	volume per volume
w/v	-	weight per volume

Viruses:

AcMNPV - *Autographa californica* multicapsid nucleopolyhedrovirus
AcseNPV - Actias selene nucleopolyhedrovirus
AdorGV - *Adoxophyes orana* granulovirus
AgseGV - *Agrotis segetum* granulovirus
AgseGV - *Agrotis segetum* granulovirus
AgseNPV - Agrotis segetum nucleopolyhedrovirus
BmNPV - *Bombyx mori* nucleopolyhedrovirus
CalGV - *Clostera anastomosis* granulovirus
ChchSNPV - *Chrysodeixis chalcites* single-capsid nucleopolyhedrovirus
ChmuNPV - *Choristoneura murinana* nucleopolyhedrovirus
ChocGV - *Choristoneura occidentalis* granulovirus
ClanGV - *Clostera anachoreta* granulovirus
ClanGV - *Clostera anachoreta* granulovirus
CpGV - Cydia pomonella granulovirus
CrleGV - *Cryptophlebia leucotreta* granulovirus
EpapGV - *Epinotia aporema* granulovirus
ErelGV - *Erinnyis ello* granulovirus
GmMNPV - *Galleria mellonella* multicapsid nucleopolyhedrovirus
HearGV - *Helicoverpa armigera* granulovirus
HearNPV - *Helicoverpa armigera* nucleopolyhedrovirus
MabrNPV - *Mamestra brassicae* nucleopolyhedrovirus
NeseNPV - *Neodiprion sertifer* nucleopolyhedrovirus
PhopGV - *Phthorimaea operculella* granulovirus
PiraGV - *Pieris rapae* granulovirus
PLRV - Potato leafroll virus
PlxyGV - *Plutella xylostell*a granulovirus
PsunGV - *Pseudaletia unipuncta* granulovirus
SeMNPV - *Spodoptera exigua* multicapsid nucleopolyhedrovirus
SpliGV - *Spodoptera litura* granulovirus
XecnGV - *Xestia c-nigrum* granulovirus

Research Outputs

Conferences:

Jukes M.D., Knox C.M., Hill M.P., Moore S.D., (2013). The isolation and genetic characterisation of a novel South African *Phthorimaea operculella* granulovirus. Oral presentation SASM 2013 conference held at Forever Resorts Warm Baths, Bela-Bela, Limpopo province, South Africa. 24-27 November.

Jukes M.D., Knox C.M., Hill M.P., Moore S.D., (2014). The isolation, genetic and biological characterisation of a novel South African *Phthorimaea operculella* granulovirus. Oral presentation SIP 2014 conference held at Johannes Gutenberg-Universität, Mainz, Germany. 3-7 August.

Articles:

Jukes, M.D., Knox, C.M., Hill, M.P., Moore, S.D., (2014). The isolation and genetic characterisation of a South African strain of *Phthorimaea operculella* granulovirus, PhopGV-SA. Virus Res. 183, 85-88.

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Chapter 1

Literature review: The potato tuber moth and the baculoviruses

1.1. Introduction

The agricultural industry in South Africa produced 2.1 million tons of vegetable crops in 2013, totalling approximately R7.96 billion towards the South African economy (DAFF, 2013). This industry is comprised of many products, such as tomatoes, chillies and brinjals, with each contributing millions of rand in produce into the local and international economy. The largest vegetable product which contributed to the South African economy in 2013 was the potato with over 949 thousand tons harvested, resulting in sales of R3.2 billion comprising approximately 40% of the entire vegetable industry output (DAFF, 2013). Potatoes are produced in 16 regions throughout South Africa utilising a total of 52000 ha in 2011 (DAFF, 2012). With the potato representing the largest proportion of vegetable production in South Africa the importance of developing novel methods in order to protect crops from potentially devastating pests is abundantly clear

1.1.1. Pests in the South African potato industry

Potato crops in South Africa are affected by a number of pests, namely, the potato tuber aphid (*Smynthurodes betae* Westwood), the potato tuber moth (*Phthorimaea operculella* Zeller), the Guatemalan potato moth (*Tecia solanivora* Povolny) and the potato ladybird (*Solanophila dregei* Mulsant) (Annecke and Moran, 1982). The damage caused to potato crops by these pests can either be physical due to feeding or through the transmission of diseases to the potato plant, for example, the green peach aphid (*Myzus persicae* Sulzer) is a vector of the potato leafroll virus (PLRV) (Annecke and Moran, 1982; Nikan *et al*., 2013). PLRV severely debilitates the plant resulting in reduced yield and quality of tubers (Jayasinghe, 1988).

Pests which primarily cause physical damage to potato plants include *S. dregei* which defoliates plants by feeding on their leaves and *P. operculella* which causes extensive damage to leaves, stems and the tubers of the plant (**Figure 1.1**) (Annecke and Moran, 1982). *Phthorimaea operculella,* along with *T. solanivora,* often continue to cause damage to tubers once they have been harvested with reported losses of up to 95 % of stored potatoes (MacLeod, 2005; Rondon,

2010; Gómez-Bonilla *et al*., 2012a). As such, control methods for these pests are of great importance for the protection of tubers both in the field and in storage.

Figure 1.1: Damage caused to potatoes by *Phthorimaea operculella* larvae (image taken in this study).

1.2. *Phthorimaea operculella*

The potato tuber moth (PTM), *Phthorimaea operculella (Zeller)* (**Figure 1.2**) is an agricultural pest of solanaceous crops worldwide. *Phthorimaea operculella*, also known as the potato tuber worm (PTW), was first described in 1875 as *Gelechia operculella* by the German entomologist P.C. Zeller (Zeller, 1875). It was later reclassified by Povolný (1964) to its current taxonomic grouping in the genus *Phthorimaea*, with its full taxonomic status shown in **Table 1.1**. *Phthorimaea operculella* belongs to the order lepidoptera. It is further classified into the family Gelechiidae which consists of moths.

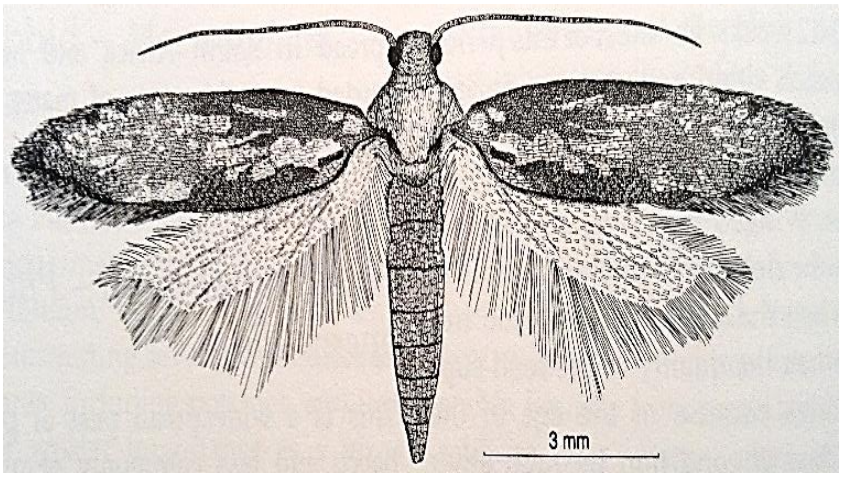


Figure 1.2: The potato tuber moth adult, *Phthorimaea operculella* (Annecke and Moran, 1982)

Phthorimaea operculella has been suggested to have originated in western South America alongside its primary host, the potato, although it is found worldwide today (Rondon, 2010). The first published report of *P. operculella* was by C.H. Berthon (1855) with the pest identified in Tasmania, Australia.

Table 1.1: Taxonomic classification of *Phthorimaea operculella* (Zeller)

Order	Lepidoptera
Family	Gelechiidae
Genus	*Phthorimaea*
Species	*Operculella*
Binomial Name	*Phthorimaea operculella* Zeller

1.2.1. Distribution and host range

Today, *P. operculella* is found worldwide, particularly in tropical and sub-tropical regions. It has been identified in North and South America, Europe, Asia, Australia, New Zealand and across Africa (**Figure 1.3**). As a major pest of potatoes it is often present in areas where this crop is cultivated, however, with more than 60 plants reported as alternative hosts on which *P. operculella* feeds, potatoes are not essential for this pest to establish itself within a region. Of the 60 alternate hosts, several are of economic importance such as tomato (*Solanum lycospersicum* L.), common tobacco (*Nicotiana tabacum* L.), Cape gooseberry (*Physallis peruviana* L.), eggplant (*Solanum melongena* L.) and the chilli (*Capsicum frutescens* L.) among others (Rondon, 2010; Das & Raman, 1994, Annecke & Moran. 1982). Although *P. operculella* is able to feed on a large variety of plants, field studies have shown that it is only able to reproduce when feeding on potato, tomato and eggplant (Rondon, 2010).

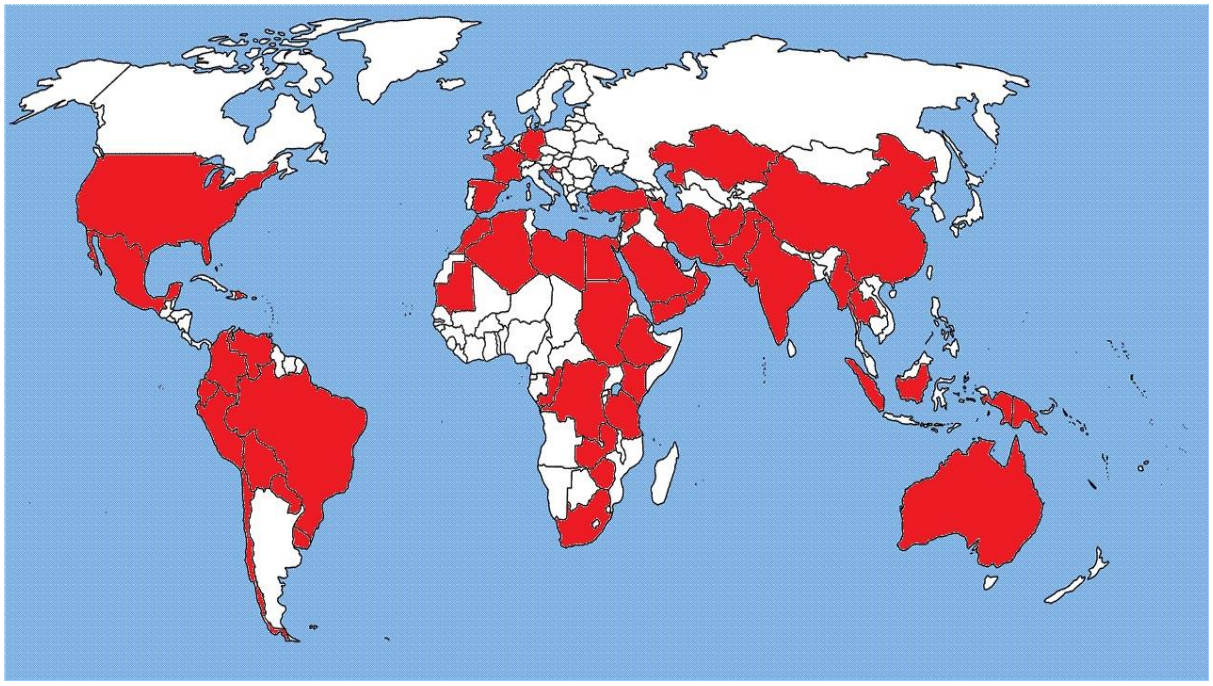


Figure 1.3: *Phthorimaea operculella* global distribution coloured in red (Modified from Rondon, 2010).

1.2.2. Life cycle of *Phthorimaea operculella*

The life cycle of *P. operculella* involves four stages: the adult, egg, larva and pupa and has been reviewed by Rondon (2010). All injury or damage caused to host plants occurs within the larval stage during feeding and, thus, ties in closely with the life cycle. *Phthorimaea operculella* eggs are spherical in shape with a translucent yellow colour and a diameter of less than 0.1 cm (Rondon *et al*., 2007). Adult female moths lay up to 200 eggs, preferably on plant foliage although plant debris, tubers and soil are also used as oviposition substrates (King 2010; Rondon *et* al., 2007; Varela & Bernays 1988). Egg development varies with environmental factors with incubation times of 2.3 to 9.9 days having been recorded (Rondon 2010).

Larvae are light brown in colour changing to pink or greenish (**Figure 1.4a**) as they mature with total larval development occurring between 14 and 33 days depending temperature (Rondon 2010). Larvae mine the leaves and stems of host plants and tunnel into tubers during feeding, causing extensive damage to the host plants during this stage of their life cycle (Rondon *et al*., 2007). Larvae spin a silk cocoon and pupate near the soil surface. Pupae are approximately 0.84 cm long with a smooth brown appearance (**Figure 1.4b**) and a development time which ranges from 6 to 33 days (Rondon, 2010). *Phthorimaea operculella* adults are small moths approximately 0.94 cm in length and a wingspan of approximately 1.27 cm (**Figure 1.4c**) (Rondon *et al*., 2007; Rondon 2010). Males have 2or3 dots whilst the females

exhibit an X on the forewings with moths copulating several hours after eclosion from pupae, followed by the females laying eggs on suitable surfaces.

Figure 1.4: Three of the life stages of *Phthorimaea operculella* (Zeller), A) larva; B) pupa; C) adult moth. Scale – 1 mm. Pictures taken with a Leica (Switzerland) EZ4D microscope in this study.

1.2.3. Agricultural control options

Control methods for pests of the potato plant includes a variety of chemical insecticides, biopesticides, cultural methods and the exploitation of natural predators and parasitoids (Coll *et al*., 2000; Rondon 2010). Cultural practices include planting strategies, crop rotations, alteration to irrigation strategies (i.e. furrow or sprinkler irrigation), hilling and harvesting practices (Hanafi 1999). These practices can improve the yield and quality of potatoes while also limiting the development of pests (Hanafi 1999). Chemical pesticide use has been met with increased concern regarding their safety and environmental effects and has resulted in rigorous restrictions by the European Union (EU). Approximately 67 % of the 1000+ active ingredients used in commercial chemical pesticides have been eliminated from the European market since 1993 placing increased pressure on the agricultural industry to develop new methods to control pests (EU 2009).

A variety of chemical fungicides, herbicides and insecticides are used in the potato industry (Guenthner *et al*., 1999). Chemical pesticides used on potato crops in South Africa are shown in **Table 1.2** with five groups of compounds in use, namely triazines, pyrethroids, pyrazoles, carbamates and organophosphates (Quinn *et al*., 2011). A study by Doğramaci & Tingey (2008) compared the effectiveness of three commercially available chemical insecticides, the organophosphate methamidophos under the trade name Monitor 4® (Bayer Crop Science), the phenylpyrazole fipronil under the trade name Regent 4 SC® (BASF) and the pyrethroid esfenvalerate under the trade name Asana XL® (Dupont Crop Protection) against both field and laboratory reared populations of *P. operculella*. This study found that the laboratory colony was resistant to all three insecticides at the recommended field application rates. Resistance was observed for two of the three insecticides at 10× the recommended field application rates

for the field population. The authors speculated that chemical pesticides within the same class, or those with a similar mode of action, may have resulted in cross-resistance development in the insect population. With increasing limitations and growing resistance to the chemical pesticides currently in use, the need for alternative pest control options is of even greater importance.

Table 1.2: Chemical pesticides used on South African potato crops (Quinn *et al*., 2011).

Chemical	Type	Chemical	Type
Cyromazine	Triazine	Dimethoate	Organophosphate
Cyfluthrin, beta	Pyrethroid	Terbufos	Organophosphate
Cyhalothrin, gamma	Pyrethroid	Phorate	Organophosphate
Cyhalothrin, lamba	Pyrethroid	Fenamiphos	Organophosphate
Cypermethrin, alpha	Pyrethroid	Ethoprop	Organophosphate
Esfenvalerate	Pyrethroid	Disulfoton	Organophosphate
Fenvalerate	Pyrethroid	Chlorpyrifos	Organophosphate
Permethrin	Pyrethroid	Acephate	Organophosphate
Bifenthrin	Pyrethroid	Oxydemetonmethyl	Organophosphate
Chlrofenapyr	Pyrazole	Parathion	Organophosphate
EPTC	Carbamate	Phenthoate	Organophosphate
Bendiocarb	Carbamate	Trichlorfon	Organophosphate
Aldicarb	Carbamate	Demeton-S-methyl	Organophosphate
Carbofuran	Carbamate	Methidathion	Organophosphate

A variety of biological pesticides are currently employed to control *P. operculella* including bacterial, fungal, viral and parasitic approaches. A study by Hassani-Kakhki *et al*. (2013) tested the efficacy of entomopathogenic nematodes (EPNs) against a laboratory population of *P operculella*. Three of the EPN strains were found to be highly pathogenic towards *P. operculella* larvae and prepupae. The use of fungus based biopesticides against *P. operculella* larvae has also been evaluated (Lacey & Neven 2006; Lacey *et al*., 2008). The fungus *Muscodor albus* produces volatile biological compounds which are biocidal towards a number of plant pathogens and when tested as a biofumigant, mean mortality rates of up to 96 % were observed (Lacey & Neven 2006; Lacey *et al*., 2008). The application of *M. albus* as a biofumigant to potatoes in storage may provide an effective means of protecting harvested crops from *P. operculella* infestation. Alternative plant based biopesticides have also been tested against *P. operculella* including neem products such as Nimbecidine® (T. Stanes and Co., India) and Bio-Power® (T. Stanes and Co., India) which showed positive results in reducing the larval mining of treated tubers (Abdel-Razek *et al*., 2014)

Integrated pest management (IPM) incorporate all available methods into a single programme the control of pests. Kogan (1998) defines IPM as "a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analysis that take into account the interests of and impacts on producers, society, and the environment". IPM programmes coordinate pest management and production practices to achieve improved economic protection, minimising human health and environmental risks (Bajwa & Kogan 2002). IPM incorporates all appropriate control methods such as enhancing natural enemies, use of semiochemicals, use of pest resistant crops, cultural methods and the use of pesticides (chemical or biological) (Bajwa & Kogan 2002. IPM programmes have been developed for the management of the potato tuber moth in both the field and in storage (Hanafi 1999). This programme incorporated the use of cultural practices, chemical control, processing practices and biological control (bacterial and viral). Control of *P. operculella* includes the use of biological pesticides such as *Bacillus thuringiensis* (BT) or insect viruses such as baculoviruses, offering an effective means of control while minimising health and environmental risks (Hanafi 1999).

1.3. Insect viruses

Viruses have long been of major interest to researchers, with many affecting one of the most diverse and often economically important groups of organisms, the insects. These viruses are themselves highly diverse, consisting of many distinct families with either RNA or DNA genomes. The virus families *Inflaviridae, Dicistroviridae, Reoviridae* and *Tetraviridae* each consist of RNA genomes and infect a number of insect hosts (Asgari & Johnson 2010; Friesen, 2001). Members of the virus families *Inflaviridae* and *Dicistroviridae* have been reported to infect lepidopteran, hymenopteran and hemipteran insect hosts (van Oers 2010; Bonning & Johnson 2010) while *Reoviridae* and *Tetraviridae* have been reported to infect lepidopteran hosts (Mori & Metcalf 2010; Dorrington & Short 2010). Members of the virus *Tetraviridae* have been considered as biological agents, however, the production of virus particles remains a major problem (Dorrington & Short 2010).

The genomic material of insect viruses can also be DNA, such as with members of the *Ascoviridae, Iridovirudae* and *Baculoviridae* families (Asgari & Johnson 2010). Members of the *Ascoviridae* and *Iridovirudae* infect lepidopteran and dipteran insect hosts respectively with little research into the use of these viruses as biopesticides (Bideshi *et al*., 2010; Williams & Ward 2010). Members of the *Baculoviridae* infect lepidopteran, hymenopteran and dipteran insect hosts, many of which are major pests in the agricultural industry (Possee *et al*., 2010).

Baculoviruses have been extensively researched for their use as biopesticides and have been successfully used in the control of agricultural pests (see **Section 1.4.5**).

1.4. The baculoviruses

The *Baculovirdae* is a family of viruses which has influenced humans in both positive and negative ways. Some of the earliest descriptions of baculoviruses date back to the 16th and 18th centuries and are found in literature describing silkworm rearing. The silkworm, *Bombyx mori*, was an economically important insect in the production of silk and, as such, diseases affecting this insect were of significant human interest. This resulted in the first full description of baculovirus pathology in silkworms by Nysten (1808), with the disease being called jaundice (Harrison & Hoover 2011). Using methods of isolation, examination and experimentation, crystalline polyhedral shaped particles were identified which could induce jaundice in healthy silkworm larvae. These efforts have been continued by modern scientists and have resulted in a greater store of knowledge regarding the *Baculoviridae* and the development of beneficial applications of baculoviruses in a variety of fields. These applications are discussed in greater detail in **section 1.4.5**.

1.4.1. Taxonomy and nomenclature

Since their discovery, baculoviruses have been the focus of many scientists as they attempted to isolate and characterise the many new isolates to provide an improved understanding of their biology and taxonomy. Initially, a single major taxonomic division was recognised within the *Baculoviridae* family resulting in two genera, the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs). This separation was primarily based on their morphological characteristics and has since undergone a further revision to incorporate genetic data. This revision has seen the split of the NPVs into three new genera and the GVs placed into a fourth separate genus (**Table 1.3**). The first of the revised genera is the alphabaculoviruses consisting of lepidopteran specific NPVs. The other two genera consisting of NPVs are the gammabaculoviruses and the deltabaculoviruses specific to hymenopteran and dipteran hosts respectively. The last of the four new genera is the betabaculoviruses consisting of GVs all of which are specific to lepidopteran hosts (Jehle *et al*., 2006a).

Table 1.3: Examples of viruses in the four baculovirus genera (Jehle *et al*., 2006a; Jehle *et al*., 2006b; Herniou *et al*., 2001)

Genus	Species	Abbreviation
Alphabaculoviruses	*Autographa californica* MNPV[*]	AcMNPV
	Helicoverpa armigera NPV[#]	HearNPV
Betabaculoviruses	*Phthorimaea operculella* GV	PhopGV
	Cydia pomonella GV	CpGV
Gammabaculoviruses	*Neodiprion lecontei* NPV	NeleNPV
	Neodiprion sertifer NPV	NeseNPV
Deltabaculoviruses	*Culex nigripalpus* NPV	CuniNPV

[*] Group 1 Alphabaculovirus; [#] Group 2 Alphabaculovirus.

Baculovirus nomenclature has been revised several times with early classifications relying upon the first letters of the host genus and the species from which the virus was first isolated. For example, the GV isolated from *Cydia pomonella* was named *Cydia pomonella* GV (CpGV). This however, resulted in conflicts whereby different baculoviruses were isolated from different hosts with the same first letters for the genus and species, for example, NPVs isolated from *Actias selene* and *Agrotis segetum* would both be AsNPV. This resulted in a change in scientific nomenclature whereby the first two letters of the host genus and species were used whilst the previous two examples became AcseNPV and AgseNPV (Possee *et al*., 2010). Few baculoviruses maintain their original two letter nomenclature with exceptions such as *Autographa californica* MNPV (AcMNPV), *Bombyx mori* NPV (BmNPV), *Galleria mellonella* MNPV (GmMNPV) and *Cydia pomonella* (CpGV) (Jehle *et al*., 2006a). Other baculoviruses such as *Phthorimaea operculella* GV have changed from the two letter naming system, previously PoGV, to the four letter system currently PhopGV (Sporleder *et al*., 2005; Sporleder *et al*., 2007; Carpio *et al*., 2013; Mascarin *et al*., 2010).

1.4.2. Morphological characteristics

Two main morphological structures are associated with baculovirus virions, the occlusion derived virion (ODV) and the budded virion (BV) (**Figure 1.5a**). Both the ODV and BV consist of cylindrical nucleocapsids surrounded by a lipid envelope. Nucleocapsids (NCs) are comprised of proteins arranged in rings which produce a capsule ranging in size from 40 nm to 60 nm in width and 250 nm to 300 nm in length. This capsule contains the baculovirus genome (see **Section 1.3.3**). The BV envelope surrounds a single nucleocapsid and has several embedded proteins, such as the gp64 or F protein, which assist in cell recognition. The ODV envelope varies in the number of nucleocapsids contained within, ranging from a single

nucleocapsid (e.g. *Plutella xylostella* GV ODV) to many (e.g. *Autographa californica* MNPV ODV).

ODVs are surrounded by a large proteinaceous structure referred to as occlusion bodies (OBs) containing either one ODV as is seen in the GVs or containing multiple ODVs as is seen in NPVs (**Figure 1.5b**). Furthermore, ODVs in NPV OBs can contain either single (SNPVs) or multiple nucleocapsids (MNPVs). The primary protein involved in the OB structure differs between these groups with *granulin* found in the GV OBs and *polyhedrin* (*polh*) found in NPV OBs.

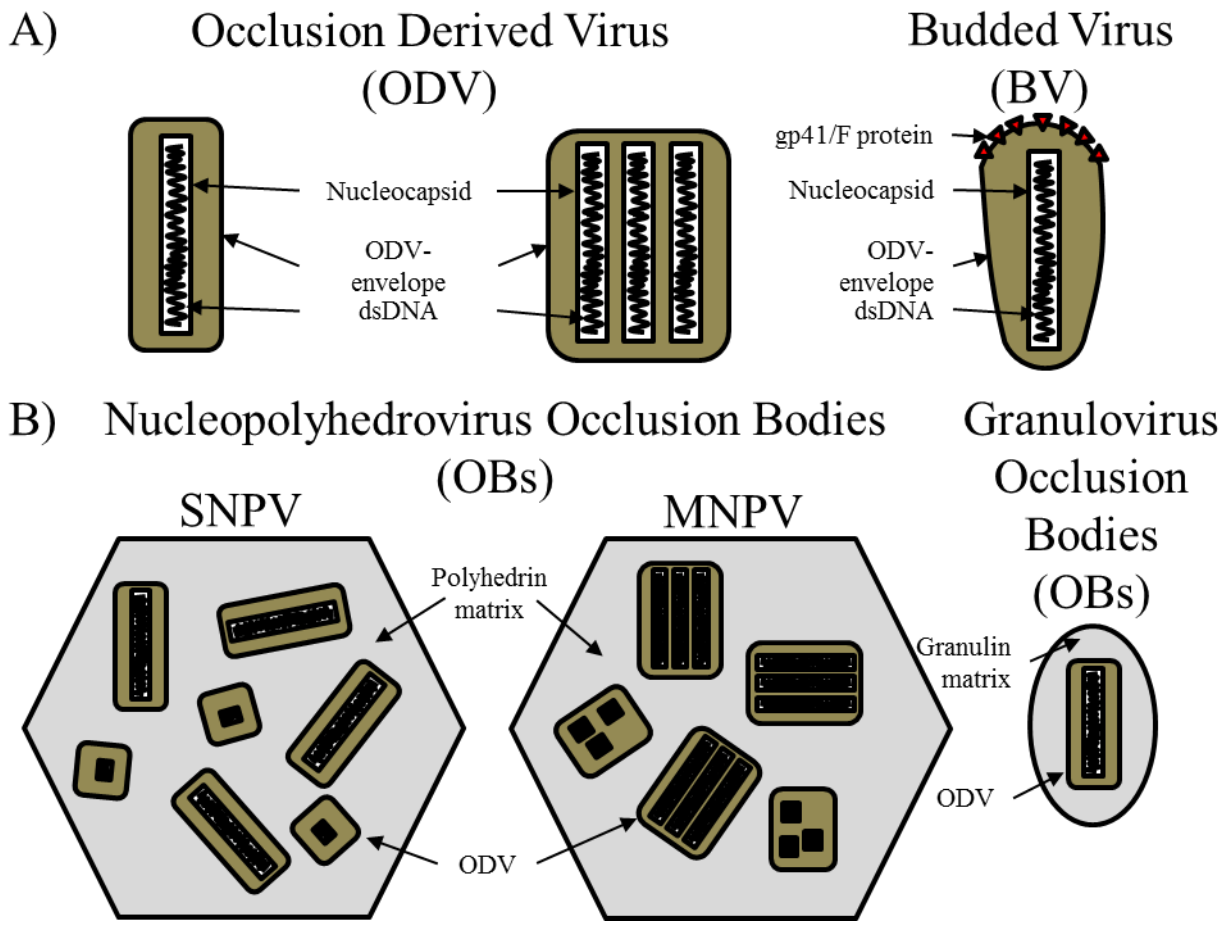


Figure 1.5: Diagram of baculovirus occlusion derived virus and budded virus particles (A) and occlusion bodies for SNPVs, MNPVs and GVs (B). Modified from van Oers *et al*. (2011).

1.4.3. Genetic characteristics

The baculovirus genome consists a large circularised double-stranded deoxyribose nucleotide (dsDNA) molecule. The first baculovirus genome to be fully sequenced was that of *Autographa californica* MNPV (AcMNPV) by Ayres *et al*. (1994) and was found to have a genome size of

133894 bp which contained 156 genes and has since been deposited into the National Center for Biotechnology Information's (NCBI, USA) GenBank (NC_001623.1). As of January 2015, 65 individual baculovirus genomes had been fully sequenced and deposited into GenBank. Genome sizes range from the smallest, that of *Neodiprion lecontei* NPV (NC_005906.1), at 81755 bp to the largest, that of *Xestia c-nigrum* GV (NC_002331.1), at 178733 bp with each containing 89 and 181 genes respectively. Baculovirus genes occur on both strands of the DNA molecule without an apparent organisation regarding temporal expression (Friesen 2001). A comparison of the genes within the various baculovirus genomes has identified several genes which are conserved throughout and are referred to as the core genes (Herniou *et al*., 2003; Miele *et al*., 2011; van Oers and Vlak, 2007). These core genes are regarded as essential for the basic functioning of the virus and influence aspects such as transcription, replication, infection, assembly and the structure of the virus.

1.4.3.1. Transcription genes

The transcription of baculovirus genes is temporally regulated into three classes, early, late and very late (Friesen 2001). Early genes are transcribed by the host protein RNA polymerase II while late and very late genes are transcribed by the viral RNA polymerase (Herniou *et al*., 2003). The viral RNA polymerase is comprised of four sub-units, each of which is a product of a core gene, namely *p47*, *lef*-4, *lef*-8 and *lef*-9 (Herniou *et al*., 2003; Guarino *et al*., 1998). Transcription of late and very late genes is dependent upon viral late expression factors (lef) genes (Titterington *et al*., 2003) of which 22 have been identified including *p47*, *lef*-4, *lef*-8 and *lef*-9 (Li *et al*., 1999; Lu & Miller, 1995; Rapp *et al*., 1998; Todd *et al*., 1995).

1.4.3.2. Replication genes

The replication of the dsDNA genome is an essential aspect of the baculovirus lifecycle relying upon the products of several genes. This includes four of the baculovirus core genes, namely *lef*-1, *lef*-2, *dnapol* and *helicase* (Herniou *et al*., 2003) alongside additional genes such as *lef*-3 and *ie1* (Rohrmann 2014). The product of the *ie1* gene may function by binding to the origin of replication, partially unwinding the dsDNA, allowing helicase to bind and continue to further unwind the molecule (Rohrmann 2014). The products of *lef*-1 and *lef*-2, primase and the primase accessory factor, are recruited to the unwound DNA which enables dnapol to bind to a single stand of the DNA initiating replication of the genome (Rohrmann 2014). *Lef*-3 produces a single stranded binding protein which is believed to prevent the formation of secondary structures during DNA replication (Rohrmann 2014).

1.4.3.3. Structural genes

A number of baculovirus genes are involved in the structural composition of the virus with at least 9 core genes involved (Herniou *et al*., 2003). These include genes such as *gp41*, the product of which is involved in the formation and functioning of BVs and *p6.9*, *vp39*, *vp1054* and *vp91* which are all involved in the formation and functioning of NCs (Rohrmann 2014). While not forming part of the core genes, the products of the *granulin* or *polh* genes are major structural components of OBs in GVs and NPVs respectively.

Several structural genes are *per os* infectivity factors (PIFs) which are essential for infection of the host cells by the baculoviruses and form part of the core genes. PIF genes such as *pif*-1, *pif*-2, *pif*-3 and *pif*-4 form products which associate with the ODV envelope forming complexes on the virion surface (Rohrmann 2014). A study by Pijlman *et al.,* (2003) showed that the deletion of one of these PIF genes, specifically *pif*-2, resulted in the loss of oral infectivity of *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) OBs in *Spodoptera exigua* larvae.

1.4.3.4. Genes affecting the insect host

Baculoviruses have also been observed to affect and alter typical host behaviour. Viral genes have been shown to play a major role in accomplishing this, such as the viral *protein tyrosine phosphatase* (*ptp*) gene which induces hyperactivity in larvae (Kamita *et al*., 2005; van Houte *et al*., 2012). The manipulation of the host by the virus may serve an evolutionary advantage with hyperactivity potentially increasing virus dispersal during late infection, a stage at which larvae would typically be preparing for pupation. Another gene involved in host manipulation is the *ecdysteroid UDP-glucosyl transferase* (*egt*) gene. This gene manipulates host metamorphosis, particularly moulting, and has been shown to decrease the speed of kill in all larval instars (Cory *et al*., 2004; Wilson *et al*., 2000). This change in behaviour may allow for higher OB production during infection as a result of the prolonged life span and increased feeding time of the host.

1.4.4. Life cycle and transmission

The baculovirus life cycle has been the subject of many studies and is reviewed by Friesen (2001); Harrison & Hoover (2011) and Possee *et al*., (2010). Its life cycle begins with the consumption of plant material contaminated with baculovirus OBs by larvae susceptible to the virus, resulting in primary infection (**Figure1.6a**). Once ingested, OBs enter the alkaline environment of the larval midgut where the proteinaceous component (either *granulin* or *polh*)

is dissolved due to the high pH, resulting in the release of ODVs. ODVs target the columnar cells which line the midgut, gaining access to microvilli tips which penetrate the peritrophic matrix (**Figure 1.6b**). Primary infection is dependent upon *per os* infectivity factors (PIFs) which assist in the recognition and binding of ODVs to host cells (Kuzio *et al*., 1989; Haas-Stapleton *et al*., 2004) allowing the entry of the viral NCs. Once entry into the host cells is achieved NCs migrate from the exterior region of the cell to the nucleus (retrograde transport) possibly accomplished through the polymerisation of actin and use of host myosin. Once NCs have migrated to the nucleus, uncoating is initiated followed by gene expression and replication (**Figure 1.6c**).

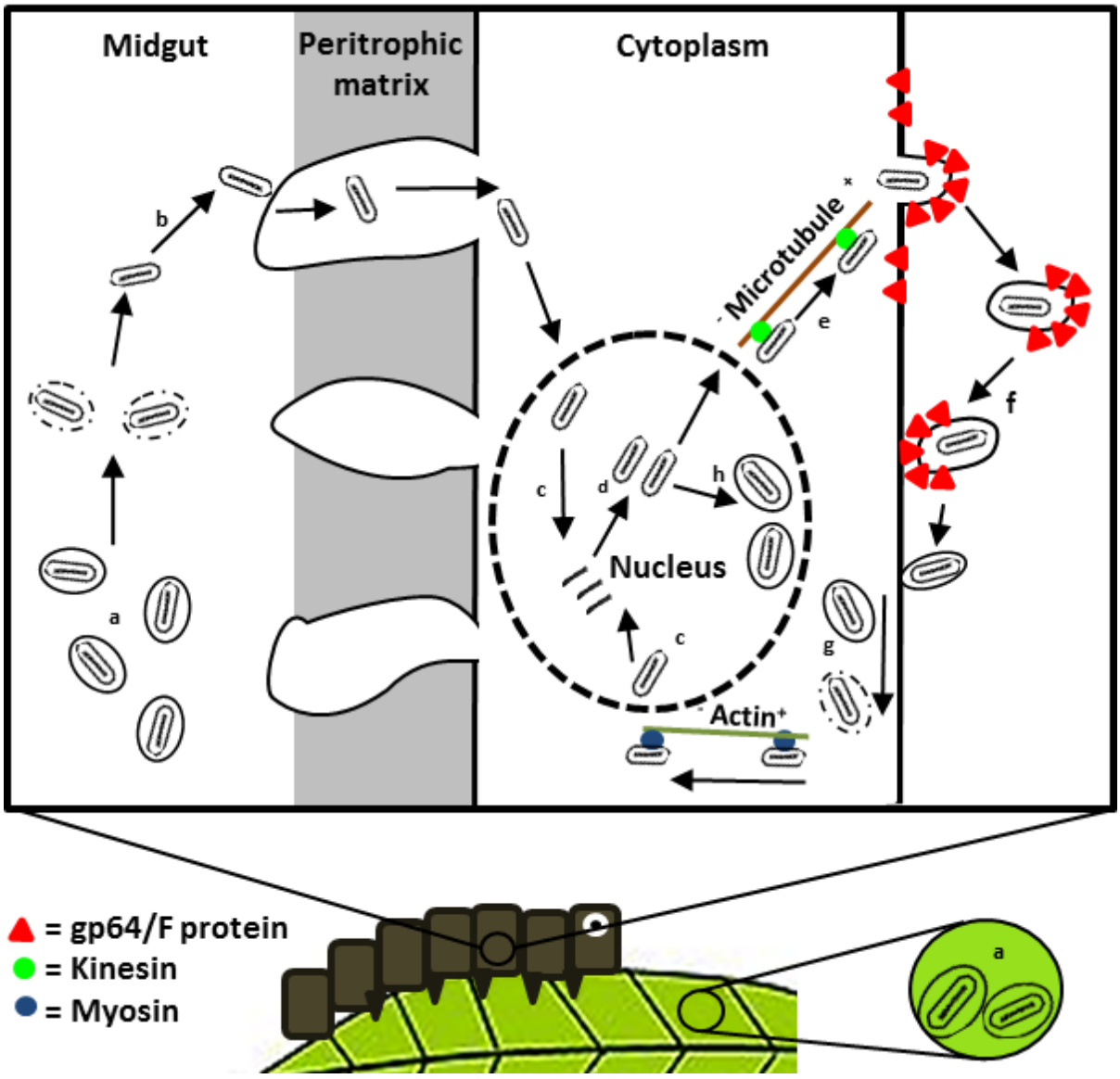


Figure 1.6: Baculovirus life cycle once ingested by a host larva. Adapted from Possee *et al*. (2010).

The virus enters the first part of its biphasic life cycle, the budded virion (BVs), whereby assembled NCs (**Figure 1.6d**) are transported along the microtubule network using host kinesin to the basal membrane (anterograde transport) (**Figure 1.6e**). NCs associate with a baculovirus envelope protein (either gp64 or F protein) which was inserted into the basal membrane during early virus gene expression and transcription. This association initiates budding of NCs which acquire an envelope containing either gp64 or F protein forming BVs (**Figure 1.6f**). BVs released from infected cells initiate a secondary infection resulting in the systemic spread of the virus through the host. The gp64 or F protein are essential for BV recognition, attachment, fusion and entry into target cells through the endocytic pathway during the secondary infection (**Figure 1.6g**).

During late stage infection NCs are retained within the nucleus of infected cells initiating the second part of the biphasic life cycle, the formation of OBs (**Figure 1.6h**). NCs acquire an envelope in the peristromal region of the nucleus which contain several PIFs resulting in the formation of ODVs. ODVS are then further encapsulated by either *granulin* or *polh* (for GVs and NPVs respectively) forming OBs. Nuclear disintegration then occurs and cell lysis begins to occur releasing OBs into the host cavities. Continual cell lysis results in the complete liquefaction of the host releasing OBs into the environment further contaminating plant material. Consumption of contaminated plant material results in the horizontal transfer of the virus to new hosts initiating the life cycle (**Figure 1.6a**).

1.4.5. Applications of Baculoviruses

1.4.5.1. Expression vectors

Baculoviruses have been used extensively as a tool in gene expression due to high levels of late gene expression. These expression systems have been used in protein function studies, diagnostics and in the production of animal and human vaccines (e.g. vaccines against cervical cancer, classical swine fever and prostate cancer) and is reviewed by van Oers (2011). Baculovirus expression technology has resulted in the development of artificial bacterial chromosomes which contain the genome of AcMNPV referred to as Bacmids. The viral genome in the bacteria can be manipulated using site directed transposition and transfer vectors allowing the insertion of target genes into the bacmids. Bacmid DNA is then used to transfect insect cells where transcription begins and the target gene is expressed. Bacmid technology has been further developed to include additional baculovirus genomes as well as improved transfer vectors.

1.4.5.2. Biopesticides

Baculoviruses are used in the biopesticide industry due to their host range which includes many significant agricultural pests. Several NPVs and GVs have been formulated into commercial products shown in **Table 1.4** while many others are used without a brand name. *Helicoverpa armigera* NPV (HearNPV) has been used to protect 100000 ha of vegetable crops in China (Sun & Peng 2007) and on citrus crops in South Africa under the commercial names Helicovir™ (River Bioscience (Pty) Ltd. South Africa) and Bolldex® (Andermatt-Biocontrol AG Switzerland) isolated from and for the control of the pest *Helicoverpa armigera* (the African bollworm) (Moore & Kirkman 2010; Knox *et al*., 2014). According to Moscardi *et al*. (2011) there exists a huge application potential for HearNPV based biopesticides in Africa, Asia and Australasia where considerable crop loss occurs.

A commercial isolate of *Cydia pomonella* GV has also been formulated under the brand name Madex® (Andermatt-Biocontrol AG Switzerland) and used to protect more than 250000 ha of fruit crops in Europe and South Africa from *Cydia pomonella* (Vincent *et al*., 2007, Knox *et al*., 2014). Citrus crops in South Africa are further protected from *Thaumatotibia leucotreta* (false codling moth) by two commercially formulated *Cryptophlebia leucotreta* GV products, Cryptogran® (River Bioscience (Pty) Ltd. South Africa) and Cryptex® (Andermatt-Biocontrol AG Switzerland) (Moore *et al*., 2004; Kessler & Zingg 2008). Lastly, potato crops in South America are protected from *P. operculella* using a GV developed by the International Potato Center (CIP) with a commercial product developed under the name Matapol® (Raman *et al*., 1992; Moscardi *et al*., 1999; Sosa Gómez *et al*., 2008).

Table 1.4: Examples of commercial baculovirus based biopesticides for use against agricultural pests (adapted from Moscardi *et al*., 2011)

Host Insect	Baculovirus	Product Name	Reference
Adoxophyes orana	GV	Capex® 2	Cunningham 1995 Erlandson 2008
Anticarsia gemmatalis	NPV	Coopervirus®	Moscardi 1999, 2007
Cydia pomonella	GV	Madex®, Virosoft®	Vincent *et al*. 2007
Thaumatotibia leucotreta	GV	Cryptex® Cryptogran®	Kessler & Zingg, 2008 Moore *et al*. 2004
Helicoverpa zea	NPV	Elcar™ GemStar®	Moscardi 1999 Erlandson 2008
Helicoverpa armigera	NPV	Helicovir™	Moore & Kirkman 2010
Lymantria dispar	NPV	Gypcheck®	Erlandson 2008
Neodiprion abietis	NPV	Abietiv®	Lucarotti *et al*. 2007
Neodiprion lecontei	NPV	Lecontvirus	Erlandson 2008
Phthorimaea operculella	GV	Matapol®	Moscardi 1999
Spodoptera exigua	NPV	Spod-X®, Ness-A®	Erlandson 2008

Baculovirus based biopesticides offer several advantages, particularly, a narrow host range which enables targeted control of pests, the ability to propagate through pest populations by horizontal transmission after application, minimised health risks and little to no environmental damage (Moscardi 1999; Erlandson *et al*., 2008; Szewczyk *et al*., 2006; Szewczyk *et al*., 2008). With increasing concern over the use of chemical pesticides in the agricultural industry, international organisations such as the European Union have begun to place new restrictions on their use (EU 2009) consequently increasing the vulnerability of crops to pest infestations. Biopesticides offer a potential solution to this problem while maintaining a more environmentally-friendly approach.

There are also disadvantages involved in the use of baculovirus as biopesticides such as the cost of production, field stability, host resistance to virus strains and the speed of kill. For example, the overuse of a specific baculovirus isolate may lead to the development of resistance as was observed with the commercial product Madex®, whereby resistance was reported in codling moth populations in Europe (Fritsch *et al*., 2006; Fritsch *et al*., 2007; Jehle *et al*., 2006; Jehle 2008). This might be prevented through the application of different baculovirus isolates to prevent prolonged exposure and subsequent resistance development. This strategy could be incorporated into management programmes such that isolates are used on a rotational basis and applied with careful consideration to ensure optimal effect. These disadvantages can be

supressed or overcome through the continued development of novel isolates into new biopesticide products.

1.4.6. *Phthorimaea operculella* granulovirus

Phthorimaea operculella granulovirus (PhopGV) has been isolated from various countries worldwide such as South Africa (Broodryk & Pretorius 1974), Tunisia (Taha *et al*., 2000), Costa Rica (Gómez-Bonilla *et al*., 2011; Gómez-Bonilla *et al*., 2012b), Peru (Sporleder *et al*., 2007; Sporleder *et al*., 2005), Brazil (Mascarin *et al*., 2010), Colombia (Espinel-Correal *et al*., 2010) and Indonesia (Zeddam *et al*., 1999). Many of these isolates have been evaluated for use as biopesticides (Moscardi 1999; Arthurs *et al*., 2008; Carpio *et al*., 2013; Espinel-Correal *et al*., 2012; Zeddam *et al*., 2013; Mascarin *et al*., 2010; Gómez-Bonilla *et al*., 2011; Espinel-Correal *et al*., 2010) as they are able to infect pests such as the potato tuber moth species *Phthorimaea operculella* (Zeller) and *Tecia solanivora* (Povolny) although not able to infect a third species, *Symmetrischema tangolias* (Gyen) (Sporleder & Kroschel 2008). During the final stages of PhopGV infection, larvae change colour from their usual green-brown to a milky white (Laarif *et al*., 2006). Changes in larval behaviour also occur during late stage infection with larvae becoming sluggish and weak (Laarif *et al*., 2006).

Three types of pathology are reported for GV diseases with each type affecting different tissues shown in **Table 1.5** (Federici 1997). All three GV types result in a primary infection in the midgut epithelium, however only type 3 produces OBs in this tissue. Granulovirus types 1 and 2 show a transient midgut infection before spreading to other tissues such as the fat body. Type 2 also infects various other tissues including the hemocytes, epidermis and tracheal matrix.

Table 1.5: Types of pathology reported for granulovirus diseases (Modified from Federici 1997) with transient infection marked with an *

Granulovirus type	Midgut epithelium	Hemocytes	Fat body	Epidermis	Tracheal matrix
Type 1	*	-	+	-	-
Type 2	*	+	+	+	+
Type 3	+	-	-	-	-

Larvae which come into contact with PhopGV show varying susceptibility depend on their age. Susceptibility to the virus greatly decreases once larvae pass approximately 30 % of their development, showing a three-fold increase in resistance to the virus at 37 % development compared to neonate larvae (Sporleder *et al*., 2007). Furthermore, larvae which reach 75 % of

their total development were observed to be almost entirely resistant to PhopGV infection (Sporleder *et al*., 2007).

1.5. Justification for study

Phthorimaea operculella is a major pest of solanaceous crops world-wide capable of causing extensive damage to both field and stored produce. A number of chemical insecticides are currently available for the control of this pest, however, increasing concern over the potential health risks and environmental damage caused as a result of their use is leading to increased international restriction and regulation. Biopesticides are becoming increasingly used as an alternative to chemical pesticides and with continued development and incorporation into control programmes these will offer effective methods in controlling pests. No biopesticides are used for the control of *P. operculella* in South Africa with this field requiring further development. This study aimed to isolate a baculovirus from *P. operculella* larvae followed by the morphological, genetic and biological characterisation of purified virus laying down the ground work for the development or import of biopesticides for use in potato crops and storage.

1.6. Chapter outline: aims and objectives

The overall aim of this study was to isolate and genetically characterise a South African PhopGV and test its virulence against a laboratory colony of the insect host.

The specific objectives were:

1) To establish and maintain a laboratory population of *P. operculella.*
2) To collect larval cadavers from the insect population from which virus OBs can be purified and morphologically examined.
3) To genetically characterise the purified virus through the PCR amplification and sequencing of selected genes.
4) To characterise the virus genome by restriction endonuclease analysis of genomic DNA and full genome sequencing.
5) To evaluate the biological activity of the purified virus against *P. operculella*.

Chapter 2 describes the establishment of a *P. operculella* laboratory colony and collection of larval cadavers used in the purification of OBs by glycerol gradient centrifugation. Successfully isolated virus particles were imaged with a transmission election microscope and enumerated for downstream applications

The genetic characterisation of purified virus is discussed in **Chapter 3**. Specific virus genes were PCR amplified and sequenced with the initial identification achieved through BLAST analysis (Identified as PhopGV-SA). Sequence data was examined for the presence of SNPs and used in the construction of phylogenetic trees.

The genome of PhopGV-SA was characterised in **Chapter 4** by restriction endonuclease analysis with the profiles generated compared against the profiles of other PhopGV isolates. The complete genome sequence was also obtained for PhopGV-SA with differences to the reference isolate PhopGV-1346 discussed in this chapter.

Chapter 5 describes the development of a biological assay used to evaluate the virulence of PhopGV-SA against the insect host.

Chapter 6 is a general discussion of the results from the previous chapters focussing on the development of PhopGV-SA into a biopesticide for use in South Africa. Future recommendations are made in terms of improving the results obtained in this study.

Chapter 2

Isolation and morphological identification of a baculovirus isolated from a laboratory colony of *Phthorimaea operculella*

2.1. Introduction

Phthorimaea operculella is a major pest of solanaceous crops and poses a major threat to farming industries and global food stability. Such a threat highlights the need for continued development of control methods for this pest such as the use of baculoviruses as biopesticides. A GV infecting *P. operculella* in South Africa was first discovered in 1974 and observed using electron microscopy (Broodryk & Pretorius, 1974). However, the virus was not genetically characterised and its biological activity was not evaluated. No further research has been conducted on this virus in South Africa.

In order to isolate and identify a baculovirus capable of infecting *P. operculella*, it is essential to establish a stable colony of the host which is collected from a population in the region in question, in this case, South Africa. Many studies regarding baculoviruses in *P. operculella* have utilised laboratory colonies to describe the procedures necessary for rearing the host and virus isolation from diseased or dead larvae (Gómez-Bonilla *et al*., 2011; Zeddam *et al*., 2013; Sporleder *et al*., 2005; Espinel-Correal *et al*., 2012; Gómez-Bonilla *et al*., 2012b).

A study carried out by King (2010) on *P. operculella* described three rearing methods using three geographically distinct South African populations with aspects such as eggs laid per female moth, sex ratio, survival rate and duration from egg to adult monitored in great detail. The first method (RT1) was an adaptation of a method described by Briese (1980) and Visser (2004) and involved the transfer of neonate larvae to fresh potatoes which were left in a controlled environment at 24 ° C with a 12 hour: 12 hour photoperiod until adults developed. Adults were transferred to an egg laying cage whereby eggs were laid on the floor of the cage and collected.

This first rearing method resulted in low survival rates and consequently a second rearing method (RT2) was developed by adapting work done by Visser (2004) and Headrick and Jones (2007). This second method involved the placement of eggs directly onto pierced potatoes allowing neonates to hatch and develop without interference in a controlled environment. Once

pupae developed they were transferred to an emergence container where moths were left to eclose and lay eggs on filter paper placed on the top of the container.

A third rearing method (RT3) was developed through a combination of RT1 and RT2 and involved the placement of eggs directly onto pierced potatoes which were left to develop into moths in a controlled environment. Moths were then transferred to an egg laying container where eggs were laid on filter paper placed on top of the container. Of these three rearing methods, RT3 produced the most stable *P. operculella* colony achieving survival rates of 90 % on average, as well as achieving the highest number of eggs per female moth. The benefits and problems for each rearing method were also investigated with RT3 having significant benefits such as being the least labour intensive with minimal contamination whilst the primary problem was a potential increase in stress during the transfer and handling of adult moths to the emergence container.

A study by Burden *et al.* (2003) described the presence of long-term persistent *Mamestra brassicae* NPV infection in *Mamestra brassicae*, referred to as covert infections, which were triggered into lethal overt infections when exposed to another baculovirus. Studies by Abdulkadir *et al*. (2013) and Opoku-Debrah *et al*. (2013) showed that stress caused due to the overcrowding of host insect colonies resulted in the outbreak of *Plutella xylostell*a GV (PlxyGV) and *Cryptophlebia leucotreta* GV (CrleGV) infections in *Plutella xylostella* and *Thaumatotibia leucotreta* larvae respectively. The occurrence of CrleGV outbreaks in *T. leucotreta* larvae when reared on a non-sterile *Rhizopus* sp. fungus inoculated diet have also been reported, with these outbreaks disappearing when the larvae were reared on a sterile diet (Moore *et* al., 2011). Each of these studies describe the use of specific stress factors which can be applied to a stable insect colony to attempt to illicit a baculovirus outbreak.

Following a baculovirus outbreak, the isolation and morphological characterisation of potential virus pathogens can proceed. Isolation techniques for GVs from larval cadavers have been extensively refined and are well described in the literature. Many studies utilise gradient centrifugation whereby different concentrations of a solution are added to a centrifugation tube forming a column with increasing density in which particles of different sizes and mass are separated (Zeddam *et al*., 2013; Gómez-Bonilla *et al*., 2011; Léry *et al*., 1997a; Espinel-Correal *et al* 2010; Taha *et al*., 1999). Gradients employed vary in concentration such as 40-80 % (v/v), 15-45 % (v/v) or 30-70 % (v/v) using either sucrose or glycerol in the solution (Carpio *et al*.,

2013; Espinel-Correal *et al*., 2012; Sporleder *et al*., 2005; Moore *et al*., 2002; Abdulkadir *et al*., 2013).

The morphological characterisation of virus particles purified through gradient centrifugation is often attained using either transmission electron microscopy (TEM) or scanning electron microscopy (SEM) (Zeddam *et al*., 1999; Léry *et al*., 1997a; Léry *et al*., 1997b; Laarif *et al*., 2006; Lacey *et al*., 2011; Hunter *et al*., 1975). Morphological characteristics such as shape and size can assist in the early identification of viruses by comparing these characteristics to the characteristics of other known viruses. Furthermore, the concentration of viral particles present in host cadavers can also be evaluated using either spectrophotometry or light microscopy. Granuloviruses isolated from *P. operculella* have been enumerated using both these methods, measuring absorbance at 450 nm (OD_{450}) using spectrophotometry or counting baculovirus particles directly on a counting chamber with a light microscope under dark field illumination (Zeddam *et al.*, 1999; Sporleder *et al*., 2007; Mascarin *et al*., 2010).

The overall aim of this chapter was to establish a colony of *P. operculella* and recover baculovirus particles for morphological characterisation. The first specific objective involved the development and maintenance of a stable insect colony for virus production and downstream biological assays. Secondly, baculovirus particles were recovered from diseased larvae, purified and examined by transmission electron microscopy. Particles from samples were also examined using light microscopy and spectrophotometry to determine the viral concentration which further assisted in downstream applications such as bioassays.

2.2. Material and Methods

2.2.1. Rearing of *Phthorimaea operculella*

Phthorimaea operculella larvae were collected from a commercial plantation in the town of Patensie (33° 45’ 20’’S; 24° 49’ 6’’E) in the Eastern Cape of South Africa by King (2010) (**Figure 2.1)**. A slightly modified version of the rearing method RT3 as described by King (2010) was used to maintain a colony of *P. operculella*.

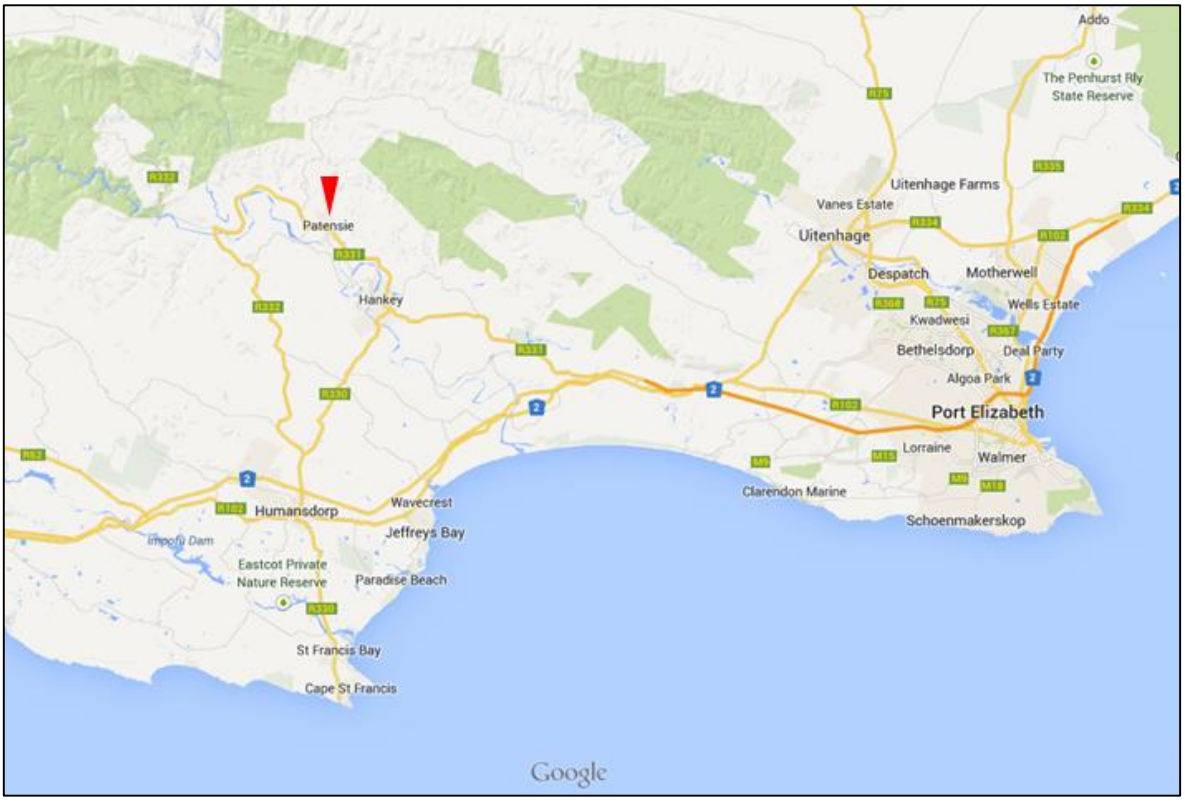


Figure 2.1: Collection site for *Phthorimaea operculella* in Patensie, Eastern Cape, South Africa (33° 45' 20''S; 24° 49' 6''E) (Map data ©2013 AfriGIS (Pty) Ltd, Google).

Insects were maintained in a controlled environment of 24.5 ± 2.5 °C, 50 ± 20 % relative humidity and a 12h:12h light to dark cycle. The rearing of *P. operculella* was carried out in plastic containers alternating between two generations, Generation A or Generation B (**Figure 2.2**). Beginning with **step 1** in Generation A, 200 g of fresh baby potatoes (Sifra, class 1) were pricked approximately 40 times each, with a clean pin providing entry holes for the neonate larvae. A 1 cm layer of perlite was added to the developmental container on top of which the potatoes were placed. Approximately one hundred *P. operculella* larvae were added to the container which was then covered with a piece of fine cloth and fastened with an elastic band. The colony was initially established using larvae collected by King (2010) which were maintained by River Bioscience (Pty) Ltd. who generously provided approximately 200 larvae at the start of this study.

The developmental container was then placed into the controlled environment room and larvae were left to mature and pupate in the perlite layer as shown in **step 2**. Following pupation, moths soon eclose as shown in **step 3**, to then be collected and transferred to a clean egg laying container. This container had a single hole in the side through which additional moths could be transferred by way of a funnel. The hole was sealed using a piece of cotton wool which was

kept moist with ddH_2O to prevent the moths from dehydrating. The container was covered with a piece of coarse cloth and fastened with two elastic bands. Sections of filter paper were placed on top of the cloth and fastened with a second layer of cloth and elastic bands. Moths were left to lay eggs on the filter paper which was collected regularly as shown in **step 4**. Egg sheets were preferably used immediately or stored at 4 °C for up to 60 days in a clean petri dish.

Finally, for **step 5**, 200 g of fresh baby potatoes were pricked approximately 40 times each with a clean pin providing entry holes for the neonate larvae. A 1 cm layer of perlite was added to the container on top of which the potatoes were placed. Sections of filter paper with approximately 100 *P. operculella* eggs were cut and placed atop the potatoes and left to hatch. These larvae form Generation B as shown in **step one**, which continue to follow the same procedure as Generation A.

Once Generation B was established, Generation A was observed for any larval cadavers which were collected non-discriminately and stored at -20 ° C for later use in downstream applications such as OB purification. The containers were cleaned in preparation for the next cycle of Generation A. At the end of Generation B, eggs were collected in the same manner as Generation A and used to start Generation A.

Information regarding the start and end date of each Generation, the egg sheet date and the date the first moth appeared was recorded. In total each generation consisted of 10 developmental containers, each with 100 larvae. Two egg laying containers were used, with container 2 being used if container 1 became overcrowded. Moths were collected until a sufficient number of eggs had been produced to supply the next Generation as well as any required for downstream experiments.

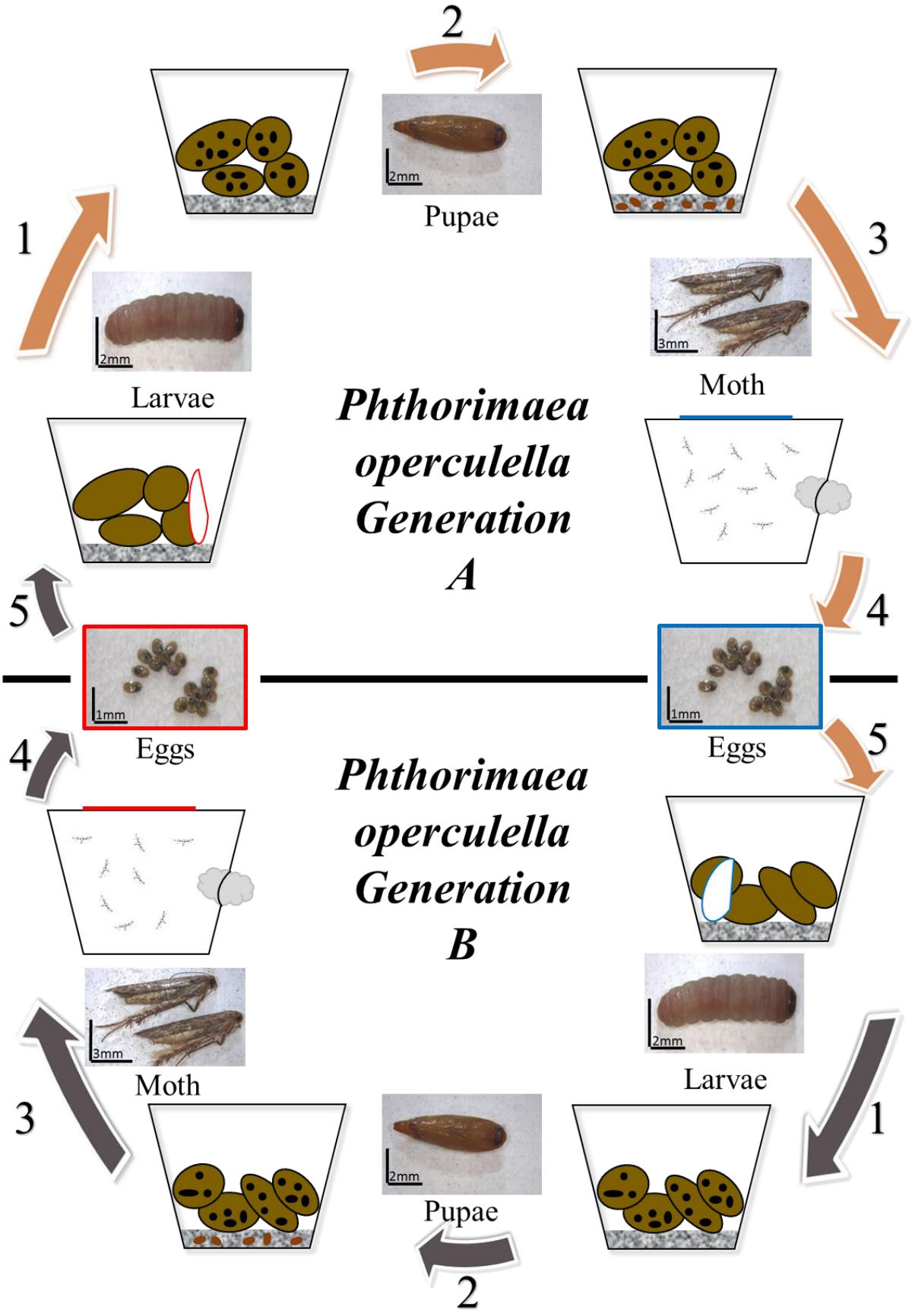


Figure 2.2: The rearing protocol for *Phthorimaea operculella* illustrating the alternating generations and various life stages throughout the cycle.

2.2.2. Occlusion Body Isolation and Purification

Baculovirus OBs were isolated and purified from larval cadavers using a modified 30-80 % (v/v) glycerol gradient protocol described by Hunter-Fujita *et al*., (1998) and Moore (2002). Two gradients were prepared in 14 mm × 89 mm ultracentrifuge tubes by layering 80-70-60-50-40-30 % (v/v) glycerol solutions and stored overnight at 4 °C prior to use. Between 350 mg and 1000 mg of insect cadavers were homogenised in 6 ml of 0.1 % (w/v) sodium dodecyl sulphate (SDS) using a mortar and pestle. Homogenate was filtered through cheese cloth to remove large debris. Filtrate was equally distributed into two JA-20 centrifuge tubes which were filled with ddH_2O and centrifuged at 7840 $\times g$ for 30 min at 4 °C in a Beckman Coulter Avanti® J-E centrifuge. The supernatant was discarded from each and the pellets suspended in ddH_2O and centrifugation repeated. Once again the supernatants were discarded and each pellet was suspended in 1.5 ml ddH_2O with suspensions being carefully added to each of the glycerol gradients. Gradients were centrifuged at 27,783 $\times g$ for 15 min at 4 °C in a Beckman Coulter Optima™ L-90 K ultracentrifuge.

Occlusion bodies forming a whitish brown band across the middle of the tube were collected using an auto-pipette and distributed into two clean JA-20 tubes. These tubes were filled with ddH_2O and centrifuged at 7840 $\times g$ for 30 min at 4 °C. The supernatant was discarded from each and the pellets suspended in ddH_2O for a second round of centrifugation. Pellets were combined into a single JA-20 and suspended in ddH_2O prior to a final third round of centrifugation. The resultant pellet was suspended in 750 µl of ddH_2O in a 1.5 ml tube and stored at -20 °C for later use. Occlusion body extractions were performed a number of times from different batches of collected larval cadavers.

2.2.3. Morphological Identification

Purified OBs were imaged using a Libra 120 (Zeiss) transmission electron microscope. Approximately 5 µl of purified virus sample was placed onto a carbon grid for 30 seconds with excess sample drained off using a section of filter paper. The grids were negatively stained using 5 µl of 1 % uranyl acetate (w/v) applied for 30 seconds with excess stain drained using a section of filter paper. Grids were stored in a grid chamber and viewed the following day. Images were captured using Mega view (G2) Olympus analysis software and the size of OBs was measured using Olympus analysis software (Olympus SZX16). Data was further analysed in Microsoft Excel® 2013.

2.2.4. Occlusion body enumeration

Occlusion body enumeration was achieved using two independent methods, namely spectrophotometry and light microscopy, in order to accurately attain the concentration of virus. Initially a 1:5 dilution of virus was prepared by mixing 20 µl of purified OBs with 80 µl ddH_2O in a new 1.5 ml tube and mixed until homogenous. To the 1:5 dilution, 400 µl 0.07 % SDS (w/v) was added to produce a 1:25 virus dilution which was mixed until homogenous followed by sonication for 60 seconds at 60 Hz with a Vibra Cell (Sonics & Materials, USA). A 1:100 sample was prepared by mixing 200 µl of the 1:25 dilution with 800 µl ddH_2O in a new 1.5 ml tube.

2.2.4.1. OB enumeration by spectrophotometry

The first method using spectrophotometry measured the concentration of the virus samples at an absorbance of 450 nm (OD_{450}) in an UVmini-1240 (Shimadzu). A blank for the spectrophotometer was prepared by adding 50 µl 0.07 % SDS to 1200 µl ddH_2O. 1 ml of the 1:100 dilution was measured three times at 450 nm, with the results being averaged to produce a final OD_{450} reading. Using **Equation 2.1** the OBs per ml ($OBs.ml^{-1}$) was calculated (Carpio *et al*., 2013).

$$OBs.ml^{-1} = x \times \frac{679.9 \times 10^6 OBs}{1\ ml} \times D$$

Equation 2.1: Equation for the determination of OBs per ml using spectrophotometry. Where x = average OD_{450} reading and D = dilution factor.

In this equation, an OD_{450} value of 1 corresponds to 679.9×10^6 ±53.9 OBs (Carpio *et al*., 2013) with 1 ml of dilute virus suspension measured.

2.2.4.2. OB enumeration by microscopy

The second method used to calculate the concentration of the virus sample utilised a Thoma bacterial counting chamber with a depth 0.02 ml and a light microscope with dark field illumination. Initially, 10 µl of the 1:25 virus dilution was added to 890 µl and 790 µl of ddH_2O in clean 1.5 ml tubes and mixed until homogenous to produce a 1:2250 and 1:2000 virus dilution respectively. The counting chamber was cleaned using 70 % ethanol (v/v) and inspected to check cleanliness. A coverslip was partially placed over the chamber and 5 µl of the 1:2250 or 1:2000 dilution was loaded into the chamber via capillary action. The coverslip was slid to fully cover the chamber and the slide was allowed to sit for 5 minutes prior to viewing. Moving particles were counted in the large squares in top left, top right, bottom left,

bottom right and one random square from the centre using a hand held tally counter. The dilution which offered approximately 7 OBs per Small Square was used (the 1:2250 for batch 1 and 1:200 for batch 2 and 3). Data was recorded from three 5 µl samples for each extract and an average of the three counts was used to calculate the concentration of OBs using **Equation 2.2** (River Bioscience [Pty] Ltd., unpublished).

$$OBs.ml^{-1} = \frac{(D \times x)}{(N \times V)}$$

Equation 2.2: Equation for the determination of OBs per ml of extract using a counting chamber. Where D = dilution factor, x = Average No. of OBs counted, N = Number of small squares and V = Volume.

The number of small squares counted was 80 (16 per large square) and the volume was 0.00000005 ml.

2.2.4.3. OB concentration per mg of larval cadaver

The concentration of OBs per mg of larval cadaver tissue was determined using **Equation 2.3**. To do this, the mean of the spectrophotometry OB concentration and microscopy OB concentration was determined.

$$OBs.mg^{-1} = \frac{Avg.OBs.ml^{-1} \times 0.75\ ml}{Weight\ of\ cadavers}$$

Equation 2.3: Equation for the determination of OBs per gram of larval cadavers.

As all OB extracts were suspended in 0.75 ml ddH_2O, the total amount of OBs obtained could be determined for each extract. The weight of cadavers used for each of the extracts was 350 mg, 1000 mg and 635 mg for batch 1, 2 and 3 respectively.

2.3. Results

2.3.1. Insect rearing

A colony of *P. operculella* was successfully maintained for the entire duration of this study by using the rearing method RT3 (King, 2010) providing a stable supply of eggs and larvae on which downstream experiments were performed. The colony produced copious amounts of eggs which were laid on sections of filter paper. Eggs were easily collected from the egg laying container and subsequently used to establish the next generation of the colony. Extra sheets of eggs were stored at 4 °C and were found to be viable (whereby neonates successfully hatch when returned to 24.5 °C) for up to 60 days after their initial storage. The length of development

from egg to moth was approximately 23 days. Moths were transferred to the egg laying container using a funnel in an attempt to reduce stress during rearing. Overcrowding of the colony resulted in the occurrence of many larval cadavers which were collected and stored at -20 °C. These cadavers were typically shrivelled and black in colour and were found amongst the perlite layer.

Insects were observed to cause extensive damage to potatoes as is shown in **Figure 2.3**

Figure 2.3: Potatoes exposed to *Phthorimaea operculella* for 1 day and 21 days.

Fresh potatoes exposed to *P. operculella* for a single day are shown in **Figure 2.3** with no surface damage visible. After 21 days extensive surface and internal damage was observed to tubers in the colony with few resembling that of fresh potatoes.

2.3.2. Occlusion body purification

The isolation of baculovirus like particles was achieved through the gradient centrifugation of macerated larval cadavers. Three batches of purified virus were produced from collected larval cadavers with the process shown in **Figure 2.4**.

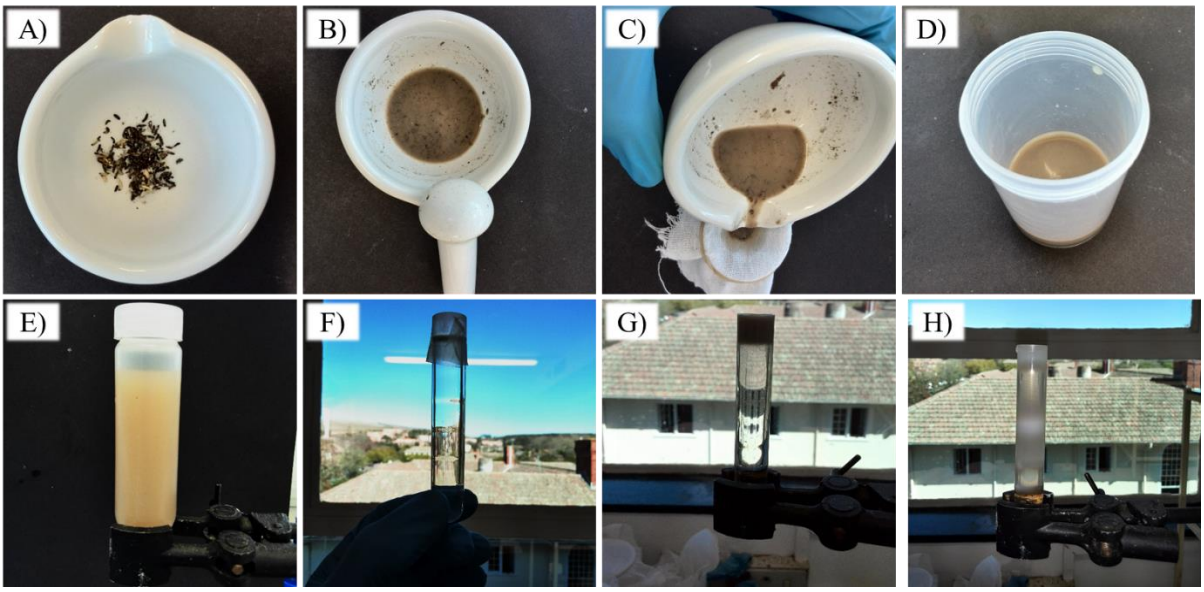


Figure 2.4: Collected *Phthorimaea operculella* cadavers prepared for the isolation of occlusion bodies using a 30 - 80 % glycerol gradient.

Cadavers were typically black in colour (**Figure 2.4 A**) producing a brown fluid once they had been homogenised (**Figure 2.4 D**). Each gradient produced a dense white band in the upper fraction of the gradients, falling within a region identified as the 50 % glycerol layer (**Figure 2.4 G and H**). This band was successfully collected and purified followed by suspension in 750 µl of ddH_2O for further examination.

2.3.3. Morphological identification

Purified virus particles were examined using TEM which revealed a high density of ovocylindrical particles with characteristics similar to that of baculovirus granules (**Figure 2.5**). The average size of these particles, hereinafter referred to as OBs, was 355.9 nm ± 28 nm (n = 50) in length and 188.6 nm ± 22 nm (n = 50) in width.

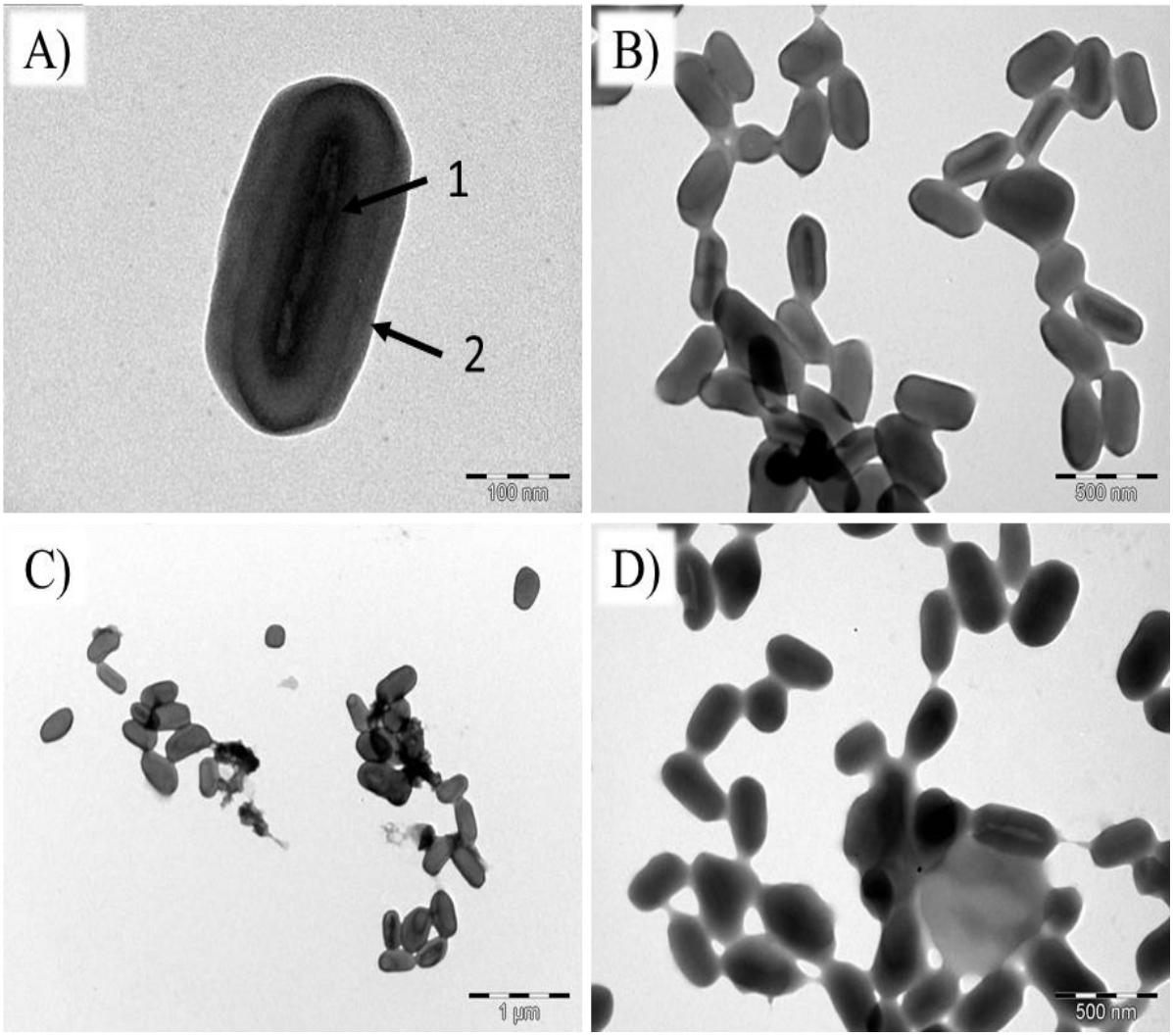


Figure 2.5: Transmission electron micrographs of particles extracted from *P. operculella* larval cadavers. A) Particles with a dense central region (1) and a lighter outer region (2). B-D) Images of particles extracted from separate batches of larval cadavers.

A dense central region was observed in many of the particles extracted as shown **Figure 2.5A**. Particles were observed to have a consistent shape and size between various batches of the virus extracts as shown in **Figure 2.5B-D**.

2.3.4. Occlusion body enumeration

The concentration of OBs was measured using two independent techniques, namely, spectrophotometry and light microscopy. The concentration of each batch of purified virus was measured at a wavelength of 450 nm in a spectrophotometer and calculated according to **Equation 2.1,** as well as being measured by light microscopy with a counting chamber and calculated using **Equation 2.2**. The results are shown in **Table 2.1** along with the average number of OBs per mg of larval tissue calculated using **Equation 2.3**.

Table 2.1: Concentrations of OBs extracted from three batches of *P. operculella* cadavers.

Batch	[OB] by spectrophotometry		[OB] by light microscopy		Avg. OBs.mg^{-1}
	Avg. OD_{450}	OBs.ml^{-1}	Avg. OBs	OBs.ml^{-1}	
1	1.1216	7.63×10^{10}	132.80	7.47×10^{10}	1.62×10^{8}
2	0.5808	1.74×10^{10}	110.13	5.51×10^{10}	3.55×10^{7}
3	0.3877	2.64×10^{10}	86.40	4.32×10^{10}	4.11×10^{7}

Avg. OD_{450} = Average of 3 OD_{450} measurements. Avg. OBs = Average of 3 OB counts

The average concentration of OBs for each of the three extracts was determined to be 7.55×10^{10} OBs.ml^{-1}, 4.73×10^{10} OBs.ml^{-1} and 3.48×10^{10} OBs.ml^{-1} with an overall average of 5.25×10^{10} OBs.ml^{-1}. The average amount of OBs per milligram of larval cadaver was also determined to be 7.94×10^{7} OBs.mg^{-1}. Concentrations were all within the same order of magnitude while the first batch used had a slightly higher amount of OBs per mg of larval tissue.

2.4. Discussion

A laboratory colony of approximately 1000 *P. operculella* insects was successfully reared for the entire duration of this study through approximately 24 generations, with each lasting for around a month. The rearing method RT3 as described by King (2010) allowed for the establishment of a stable colony with minor modifications made, primarily to the technique used to transfer adult moths between the developmental container and the egg laying container. This modification incorporated the use of a funnel instead of a net, allowing the moths to freely and easily move into the egg laying container with minimal interference reducing any unwanted stress. The colony was consistently maintained using freshly laid eggs with stored eggs serving as a redundancy in case the colony unexpectedly failed.

The colony alternated between two generations with each split into smaller sub-colonies of 100 individuals. To ensure that the division of the colony into sub-colonies did not affect the genotypic make-up of the colony, adult moths were collected and mixed into one of two egg laying containers allowing individuals from different sub-populations to mate before laying eggs. Furthermore, this design of smaller sub-colonies allowed the colony to be manipulated while ensuring it did not crash.

The colony was not intentionally subjected to stress factors, however, in review of the rearing process it was observed that the colony had been overcrowded as a result of excess eggs placed

in each container. The rearing method RT3 recommends a ratio of 2 g of fresh potato per larva with 100 larvae per container (King 2010). In practice, the number of larvae was later estimated to be much higher than the recommended amount, being closer to 400 larvae per container. As such, overcrowding may have been the primary stress factor placed on the colony, resulting in increased larval mortality. Larval cadavers were collected and were observed as typically black in colour with a shrivelled and dry appearance. This description differs to other PhopGV infections in *P. operculella*, which exhibit as swollen larvae with a clear milky white colour (Laarif *et al*., 2006). A possible explanation for this difference in appearance is the manner in which the larvae were collected from the colony, whereby individuals were only collected after the next generation had been established resulting in cadavers lying for several days in the open. This time difference may have allowed cadavers to dry up and darken thereby losing their characteristic symptomology of a baculovirus infection.

Cadavers were collected from the colony over the entire duration of this study, with a total of three batches undergoing virus purification using gradient centrifugation. While sucrose gradients are predominantly used to isolate PhopGV from *P. operculella* larvae (Zeddam *et al*., 2013; Gómez-Bonilla *et al*., 2011; Léry *et al*., 1997a; Espinel-Correal *et al.*, 2010), glycerol gradients have also been used to isolate baculoviruses (Abdulkadir *et al*., 2013; Opoku-Debrah *et al*., 2013), as was done in this study. Following maceration and centrifugation of cadavers, a dense white virus band was observed in each gradient which was further purified and washed.

Particles from this band were imaged using TEM from each batch of larval cadavers and were observed to have ovocylindrical particles approximately 350 nm by 190 nm in size. NPV OBs are typically cuboidal in shape and range in diameter from 400 nm to 2.5 µm while GV OBs are typically ovocylindrical with a width of 120 nm to 350 nm and a length of 300 nm to 500 nm (Harrison & Hoover, 2011). The virus particles were similar in size and shape to GV OBs, and only slightly smaller in size to PhopGV OBs extracted from *P. operculella by* Laarif *et al*. (2006). Several imaged particles appeared to have a dark, dense central region which may represent the baculovirus nucleocapsid, however, not all particles showed such a region and no technique was used to verify the exact nature of this central region. This information did however indicate the presence of a baculovirus, specifically a GV, in the *P. operculella* larval cadavers although the identity of this GV could not be determined by examining the morphology alone.

The concentration of viral particles present in the purified samples was also determined for use in downstream applications such as DNA extractions and biological assays. This was done using two independent techniques, namely spectrophotometry and light microscopy. These methods have been used to determine virus concentrations in samples purified from *P. operculella* and can be used validate the accuracy of one another (Zeddam *et al.*, 1999; Sporleder *et al.*, 2007; Mascarin *et al.*, 2010). These techniques indicated similar concentrations of OBs from different batches with each measurement falling within the same order of magnitude. An overall average concentration of 5.25×10^{10} OBs.ml^{-1} was achieved through the purification process. This concentration of OBs was sufficient for downstream applications such as in genomic DNA extraction for genetic characterisation and for use in biological assays against *P. operculella*.

Furthermore, OB concentrations were also used to estimate the average concentration of OBs per mg of larval tissue at 7.94×10^{10} OBs.mg^{-1}. This may assist in determining the feasibility of developing the baculovirus into a biopesticide by providing information on the expected yield of virus from insect cadavers. For example, if 1000 litres of biopesticide is to be produced with a concentration of 1.5×10^{6} OBs.ml^{-1}, approximately 13000 mg of larval tissue would be required. Whilst the values given here are arbitrary, additional information from bioassays coupled with the virus concentrations given here, could assist in making informed decisions in the future production of biopesticides.

In this chapter the overall aim to establish and maintain a laboratory colony of *P. operculella* was achieved. The overcrowding of the colony led to the recovery and purification of a baculovirus from larval cadavers which were similar in shape and size to GV particles. The concentration of these particles was also successfully measured using two independent techniques. The following chapter involves the first step in genetic characterisation of the GV using PCR amplification, sequencing and analysis of specific target genes.

Chapter 3

The genetic characterisation of a novel granulovirus recovered from *Phthorimaea operculella* larvae

3.1. Introduction

The genetic characterisation of baculovirus isolates is an essential step for biopesticide development. The molecular characterisation of viruses offers a highly reliable method to characterise unknown isolates. Baculovirus identity can be determined through a variety of methods, such as amplification and sequencing of specific viral genes and the construction of phylogenetic trees using sequence data. These methods rely upon obtaining partial or complete gene sequences or preferably complete genome sequences (Herniou *et al*., 2003; Lange *et al*., 2004; Jehle *et al*., 2006a; Herniou *et al*., 2004; Jehle *et al*., 2006b). Once obtained, sequence data can be used in varying forms of analyses, such as their submission to the NCBI's Basic Local Alignment Search Tool (BLAST) to search for similar sequences in the NCBI's GenBank database providing a list of sequences with a high percentage of identity.

Sequence data from other isolates with a high percentage of identity can be aligned against data from purified viruses in order to identify the presence of single nucleotide polymorphisms (SNPs), which are individual nucleotide changes occurring throughout the genome and can subsequently cause changes in the translated amino acid sequence (Carpio *et al*., 2013). The degree of variation would be expected to be less between isolates of a single species increasing between isolates of different species. It is important to note that complete genome sequence data is available for numerous baculovirus isolates including PhopGV-1346, originating in Tunisia, which has been adopted as the reference isolate for the species PhopGV on GenBank. Available genome data can be used in alignments allowing for the detection of SNPs within any gene successfully sequenced from a purified baculovirus. This assists in identification of the virus as well providing information on the degree of variation a purified virus has with closely related isolates.

A broader view of evolutionary relationships among groups of organisms can be determined through the phylogenetic analysis of genetic sequence data. A comparison of complete baculovirus genomes by Herniou *et al*. (2003) was used to infer baculovirus phylogeny, revealing 30 core genes (see **Section 1.4.3**) as well as providing initial data on the cladistics of

baculoviruses. An alternative to the use of complete genomes is the use of specific genes as it is cheaper, faster and requires less advanced equipment and techniques. A study by Lange *et al*. (2004) identified three genes which can reliably infer baculovirus phylogeny, namely *polyhedrin/granulin,* (*polh*/*granulin*), *late expression factor* 8 (*lef*-8) and *late expression factor* 9 (*lef*-9). These genes were shown to be highly conserved among baculoviruses, providing a unique basis on which to evaluate phylogeny between all baculovirus isolates (Lange *et al*., 2004, Herniou *et al*., 2003). Phylogenies constructed with gene sequences require that each is individually translated and aligned prior to being concatenated into a single amino acid sequence for comparison. Phylogenies produced using concatenated *polh*/*granulin, lef*-8 and *lef*-9 sequences from various isolates showed no discrepancies when compared to those constructed using all thirty core genes.

The phylogeny of 117 baculovirus isolates (71 of which were previously unclassified) have since been inferred using maximum parsimony and distance methods based on concatenated amino acid sequences of *granulin/polh, lef*-8 and *lef*-9 from each isolate (Jehle *et al*., 2006b). The result was a set of criteria according to which isolates are assigned to a specific species of baculovirus or whether they constitute a newly identified baculovirus species. The use of these genes provides an easy, accurate and rapid method of determining the identity of a baculovirus isolate before attempting more expensive and time consuming methods such as complete genome sequencing.

While the *granulin/polh, lef*-8 and *lef*-9 genes have been shown to be important in studying baculoviruses, another gene of great interest is the *ecdysteroid UDP-glucosyl transferase* (*egt*) gene, particularly in the genetic characterisation of PhopGV isolates. The *egt* gene has been shown to vary in size between PhopGV isolates due to a major deletion/insertion in the 3' region of the gene (Carpio *et al*., 2013; Espinel-Correal *et al*., 2010; Gómez-Bonilla *et al*., 2012b). A recent study by Carpio *et al*. (2013) using an alignment of several *egt* sequences identified three types of *egt* gene structures, namely egt I, egt II and egt III with each of these genes having a characteristic length of 1305 bp, 1353 bp and 1086 bp, respectively.

An analysis of *egt* sequences by Espinel-Correal *et al*. (2010) using primers designed to amplify part of the 3' region identified five types of *egt* gene structures named egt type I to egt type V. Each amplicon showed a characteristic length for each egt type with sizes of 937 bp, 723 bp, 1023 bp, 569 bp and 869 bp for types I to V respectively. The only common *egt* sequence between these two studies was that of the reference isolate PhopGV-1346 which was identified

as an egt type I in both, with the size difference due to the use of partial sequences by Espinel-Correal *et al*. (2010). A third study using the same primer set as Espinel-Correal *et al*. (2010) identified all five egt types along with a sixth type with a length of 783 bp. An advantage of using the *egt* gene in the characterisation of the isolated baculovirus is the sheer volume of sequence data available for the gene on GenBank, with complete sequences available for all the isolates used in the studies by Carpio *et al*. (2013) and Espinel-Correal *et al*. (2010).

The overall aim of this chapter was to genetically characterise the novel baculovirus isolate by analysis of specific gene sequences and phylogenetic reconstruction. The objectives were first to purify viral genomic DNA (DNA) from purified OBs and PCR amplify the *granulin*, *lef*-8, *lef*-9 and *egt* gene sequences. The PCR amplicons were sequenced and submitted to BLAST in order to identify the novel baculovirus isolate. Secondly, alignments of these genes were carried out to detect single nucleotide polymorphisms (SNPs) and amino acid changes as well as to infer phylogeny based on concatenated granulin, lef-8, and lef-9 amino acid sequences. Finally, the *egt* gene was also analysed by PCR amplification and sequencing to determine which egt group the isolated virus belonged to, as well as infer inter-species phylogeny based on egt amino acid sequences.

3.2. Material and Methods

3.2.1. DNA extraction

DNA was extracted from OBs using a modified CTAB DNA extraction protocol described by Opoku-Debrah *et al*. (2013). 200 µl of purified OBs were placed in a 1.5 ml tube. To this, 90 µl Na_2CO_3 (1 M) was added prior to incubation at 37 °C in a water bath for 30 min. Following this, 120 µl Tris-HCl (1 M, pH 6.8), 50 µl SDS (10% w/v) and 50 µl Proteinase K (25 mg/ml) were added to the tube and further incubated at 37 °C for 30 min. 10 µl of RNase A (10 mg/ml) was added to the tube and the mixture incubated at 37 °C for 30 min.

The tube was removed from the water bath and centrifuged at 12100 ×*g* in an Eppendorf (Germany) MiniSpin® desktop centrifuge for 3 min with the supernatant being transferred to a 2 ml tube. 400 µl of CTAB buffer (54 mM CTAB, 0.1 M Tris-HCl pH 8.0, 20 mM Na_2EDTA, 1.4 M NaCl, pre-heated to 70 °C) was added to the supernatant and the tube was incubated at 70 °C for 45 min with the tube being inverted regularly. After incubation, 400 µl chloroform (pre-cooled to 4 °C) was added to the tube and inverted several times. The tube was centrifuged at 6700 ×*g* for 10 min and the upper phase of the supernatant was transferred to a new 2 ml tube to which 400 µl of ice-cold isopropanol (-25 °C) was added, mixed and the sample stored

overnight at -25 °C. The following morning the tube was centrifuged at 12100 ×*g* for 20 min and the supernatant discarded. 1 ml of ice-cold ethanol (70% v/v, -25 °C) was added to the tube prior to a final centrifugation at 12100 ×*g* for 5 min. The supernatant was discarded and the pellet was left to dry, ensuring that all ethanol was removed. The pellet was re-suspended in 20 µl Tris-HCl (10 mM, pH 8.0) and stored at -25 °C until use. DNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo-Scientific) and were visualised by 1 % agarose gel electrophoresis at 90 V for 45 min in 1X TAE buffer (40 mM Tris-acetate, 20 mM acetic acid, 1 mM EDTA) stained with ethidium bromide. Gel images were captured with the UVIpro chemi (UVItec) UV trans-illuminator.

3.2.2. PCR amplification of *granulin, egt, lef*-8 and *lef*-9 genes.

PCR amplification of the *granulin*, *egt*, *lef*-8 and *lef*-9 genes was carried out using oligonucleotide primers (**Table 3.1**) based on the PhopGV reference genome (GenBank: NC_004062). Reactions used for the amplification of the *granulin* and *egt* comprised 12.5 µl Taq ReadyMix PCR Kit (Kapa Biosystems, USA), 2 µl forward primer (10 µM), 2 µl reverse primer (10 µM) and approximately 8 ng template genomic DNA. Reactions were made up to 25 µl using ddH_2O and included a no template control. For *granulin* the grnF and grnR primer pair was used while the egtF and egtR primer pair were used for the *egt* reaction. PCR amplification of *granulin* and *egt* sequences was carried out multiple times for each sample of OBs purified from *P. operculella* larval cadavers to ensure consistent identity of the recovered virus.

Reactions used for the amplification of *lef*-8 and *lef*-9 comprised 12.5 µl Maxima™ Hot Start Green PCR Mix (Thermo Scientific, USA), 4 µl and 2 forward primer (10 µM) respectively, 2 µl reverse primer (10 µM) and a range of template DNA (20.8 ng – 5 ng). Reactions were made up to 25 µl using ddH_2O and included a no template control and a positive control. The lef8F and lef8R primer pair was used for amplification of *lef*-8 and the lef9F and lef9R used in the amplification of *lef*-9.

Table 3.1: Oligonucleotide primer pairs used in PCR and sequencing reactions.

Target Gene	Gene position	Direction in genome	Primer name	Position in genome	Primer sequence (5' to 3')	Length (nt)	Amplicon size (nt)
granulin	*1-747*	*5′ - 3′*	grnF	1-23	ATGGGATACAACAAAACTCTGAG	23	747
			grnR	725-747	TTAATAAGCGGGTCCGGTGAAC	22	
egt	*116818-118122*	*3′ - 5′*	egtF	116715-116737	GAGTCGAGCCAATTTTGTTTGCG	23	1471
			egtR	118162-118185	GCAACGATGATCTCATATATGAGC	24	
lef-8	109360-111843	3' - 5'	lef8F	109119-109142	ATTGGAATGAGATCAAGCGCAGTG	24	3014
			lef8R	112109-112132	CGTGCGTTTTACAACTAATCGAAG	24	
lef-9	96371-97864	*5′ - 3′*	lef9F	96313-96334	GCTAGCTTTGGTGGAAAATAGC	22	1620
			lef9R	97909-97932	GTATGGTTGCTATTTAAGGTGTCC	24	

PCR amplification cycle parameters for *granulin*, *egt* and *lef*-9 differed slightly from the *lef*-8 parameters shown below in **Table 3.2**. The parameters used for *granulin*, *egt* and *lef*-9 had a total of 25 cycles with an extension step of 105 seconds while *lef*-8 had a total of 45 cycles and an extension step of 165 seconds. All remaining parameters used were identical with PCR amplification cycles carried out with a MJ Mini™ Gradient Thermal Cycler (BIO-RAD). Amplicons were visualised by 1 % agarose gel electrophoresis at 90 V for 45 min in 1X TAE stained with ethidium bromide and gel images were captured as before.

Table 3.2: PCR cycle parameters for the amplification of the PhopGV *granulin, egt*, *lef*-8 and *lef*-9 genes.

Temperature (°C)	*granulin*/*egt*/*lef-9*		*lef*-8	
	Time (Sec)	Cycles	Time (Sec)	Cycles
95	300	× 1	300	× 1
95	30	× 25/25/30	30	× 45
55	30		30	
72	105		165	
72	300	× 1	300	× 1

3.2.3. Analysis of the *granulin, lef*-8 and *lef*-9 gene sequences.

The *granulin* and *lef*-8 PCR amplicons were sequenced by Inqaba Biotechnical Industries (Pty) Ltd (South Africa). The *granulin* gene was sequenced in the forward direction using the grnF and the *lef*-8 gene was sequenced in the forward and reverse direction using the lef-8F and lef-

8R primers (**Table 3.1**). To acquire the full *lef*-8 gene, three internal primers were designed by Inqaba Biotechnical Industries (Pty) Ltd during the sequencing process.

For each gene, the sequence was assembled by aligning the sequence fragments using Mega 5.2 (Tamura *et al*., 2011). Sequences were examined for any ambiguous nucleotides and these were corrected using the alignments and sequence chromatograms viewed in Chromas Lite. From each alignment, a single consensus sequence was made for the *granulin* and *lef*-8 genes. The PCR amplification of the *lef*-9 gene was unsuccessful. The sequence for this gene was acquired from the complete genome sequence obtained for PhopGV-SA in this study (see **Chapter 4**).

The sequences acquired for *granulin*, *lef*-8 and *lef*-9 were submitted to BLAST to identify similar sequences. These genes were also aligned against the respective genes from the reference isolate genome using Mega 5.2. These alignments were examined for single nucleotide polymorphisms (SNPs) and any amino acid changes resulting from the presence of these SNPs.

3.2.4. Phylogenetic analysis of the granulin, lef-8 and lef-9 genes

Amino acid sequences for granulin, lef-8 and lef-9 were aligned against the respective genes from 15 baculovirus species (**Table 3.3**). These 15 baculovirus species were chosen on the basis of complete genome sequences available for each, with most representing the reference isolate for each of the respective baculovirus species. These alignments were then concatenated into a single amino acid sequence for each isolate and maximum likelihood and maximum parsimony trees were inferred using Mega 5.2. The baculovirus *Neodiprion sertifer* NPV (NeseNPV) was used as an out group for each inference. The best model was identified in Mega 5.2 for the maximum likelihood tree with each phylogenetic tree constructed with 1000 bootstrap replicates.

Table 3.3: List of baculovirus isolates used to infer maximum likelihood and maximum parsimony trees.

Virus Name and Abbreviation		Accession number	Source
Unknown BV isolate			This study
Phthorimaea operculella GV	PhopGV	NC_004062	Taha *et al.,* 1999
Plutella xylostella GV	PlxyGV	NC_002593	Hashimoto *et al.*,2000
Epinotia aporema GV	EpapGV	NC_018875	Ferrelli *et al.*,2012
Clostera anachoreta GV	ClanGV	NC_015398	Liang *et al.*,2011
Pieris rapae GV	PiraGV	NC_013797	Zhang *et al.*,2012
Helicoverpa armigera GV	HearGV	NC_010240	Harrison & Popham 2008
Choristoneura occidentalis GV	ChocGV	NC_008168	Escasa *et al.*,2006
Agrotis segetum GV	AgseGV	NC_005839	Direct Submission - Ai *et al.*,
Cryptophlebia leucotreta GV	CrleGV	NC_005068	Lange & Jehle 2003
Adoxophyes orana GV	AdorGV	NC_005038	Wormleaton *et al.*,2003
Cydia pomonella GV	CpGV	NC_002816	Luque *et al.*,2001
Xestia c-nigrum GV	XecnGV	NC_002331	Hayakawa *et al.*,1999
Spodoptera litura GV	SpliGV	DQ_288858	Wang *et al.,* 2008
Pseudaletia unipuncta GV	PsunGV	EU_678671	Unpublished - Li *et al.*,
Neodiprion sertifer NPV	NeseNPV	NC_005905	Garcia-Maruniak *et al.*,2004

3.2.5. Analysis of the *egt* gene sequences.

The *egt* sequence was acquired several times from different batches of OBs and was sequenced in both the forward and reverse direction using the egtF and egtR primers by Inqaba Biotechnical Industries (Pty) Ltd (South Africa). These were merged into a single consensus sequence using Mega 5.2. The acquired *egt* gene sequence was aligned against all *egt* gene sequence data available on GenBank (**Table 3.4**). This table shows the name for each isolate, the size of the *egt* gene, GenBank accession number and the source of the data. The presence of the various egt types previously described by Carpio *et al*. (2013) and Espinel-Correal *et al*. (2010) were identified among the aligned sequences. Silent and non-silent SNPs were identified between the SA *egt* sequence and the other sequences in the alignment. Gene sequences were translated using Mega 5.2 and isolates with identical amino acid sequences

were placed into one of four groups, namely A, B, C and D. Isolates with unique amino acid sequences were not placed into a group.

Table 3.4: Data available on GenBank for *Phthorimaea operculella* GV *egt* gene indicating the size, accession number and source for each (Jukes *et al*., 2014).

Isolate	Location	Size (nt)	GenBank No.	Source	Group [a]
	South Africa	1353		This study	
VG003	Colombia	1353	HQ166268.1	Espinel-Correal *et al*., (2010)	
INDO	Indonesia	1353	HQ317413.1	Zeddam *et al*., (1999)	
KEN	Kenya	1353	HQ317414.1	CIP	
JLZ9f	Ecuador	1353	HQ317403.1	Carpio *et al*., (2013)	C
VZ	Venezuela	1353	HQ317415.1	INIA	C
CHI	Chile	1353	HQ317406.1	Carpio *et al*., (2013)	C
1346	Tunisia	1305	NC004062.1	CIP	A
1390-2	Peru	1305	HQ317419.1	Vickers *et al*., (1991)	A
TUTA	Peru	1305	HQ317412.1	Carpio *et al*., (2013)	A
VG002	Colombia	1092	HQ166267.1	Espinel-Correal *et al*., (2010)	
YEM	Yemen	1086	HQ317411.1	Kroschel *et al*., (1996)	
TANG	Ecuador	1086	JX082398.1	Carpio *et al*., (2013)	D
GV5	Ecuador	1086	HQ317417.1	Carpio *et al*., (2013)	D
TESO	Ecuador	1086	HQ317408.1	Carpio *et al*., (2013)	D
EUQU	Peru	1086	HQ317409.1	Carpio *et al*., (2013)	D
HUAN	Peru	1086	HQ317405.1	Carpio *et al*., (2013)	D
AUS	Australia	1086	HQ317407.1	Briese (1981)	D
EGY	Egypt	1086	HQ317420.1	Carpio *et al*., (2013)	D
PADE	Bolivia	1086	HQ317416.1	Carpio *et al*., (2013)	D
GUA	Guatemala	1086	HQ317410.1	CIP	D
TUR	Turkey	1086	HQ317404.1	Carpio *et al*., (2013)	D
GV6	Ecuador	1086	HQ317418.1	Carpio *et al*., (2013)	D
VG004	Colombia	861	HQ166269.1	Espinel-Correal *et al*., (2010)	B
VG005	Colombia	861	HQ166270.1	Espinel-Correal *et al*., (2010)	B

[a] Groups with Identical amino acid sequences are denoted as groups A, B, C and D

3.2.6. Phylogenetic analysis of the egt gene sequences

The translated sequence data for egt from the isolates shown in **Table 3.4** were aligned in Mega 5.2. Isolates with identical amino acid sequences were represented as groups A, B, C, and D. A maximum likelihood tree was inferred from an alignment of the egt amino acid sequences. The best model was identified in Mega 5.2 and the phylogenetic tree constructed with 100 bootstrap replicates.

3.3. Results

3.3.1. DNA extractions

DNA was successfully extracted from samples of purified OBs using the CTAB method described in section 3.2.1. **Figure 3.1** below shows the typical appearance of DNA once extracted from OBs forming a dense, bright band in lane 1 above the 10000 bp marker. The actual size of the DNA could not be determined due to its large size and limitations in gel resolution. The concentration of DNA extracted from batches of purified OBs ranged from 384.4 to 856.9 ng/µl.

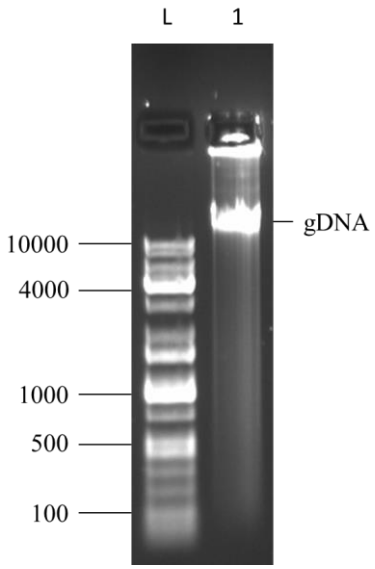


Figure 3.1: PhopGV DNA extractions using the CTAB method run on a 1% agarose gel L – Kapa universal ladder (Kapa Biosystems, USA), 1 – extracted DNA.

3.3.2. PCR amplification of *granulin*, *egt* and *lef*-8

The *granulin* and *egt* genes were successfully amplified using DNA extracted from purified OBs. **Figure 3.2** below shows the amplicons from the amplification of these genes from DNA extracted from two separate batches of purified OBs. Lanes 2 and 3 each have a single band around the 750 bp marker from the amplification of the *granulin* gene and lanes 5 and 6 each have a single band around the 1500 bp marker for the amplification of the *egt* gene. No bands are present lane 8 which represents the no template control. Lanes 1, 4 and 7 did not contain any sample and were left blank.

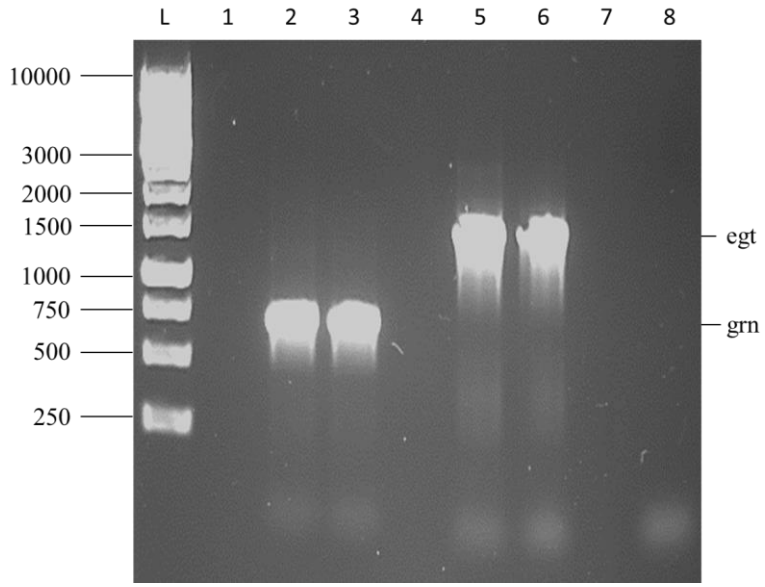


Figure 3.2: PCR amplification of PhopGV *granulin* and *egt* from DNA extracted from two batches of OBs. L – GeneRuler 1Kb DNA ladder, 1 - blank, 2 - *granulin* using (batch 06/03), 3 - *granulin* using (batch 16/07), 4 - blank, 5 - *egt* using (batch 06/03), 6 - *egt* using (batch 16/07), 7 - blank, 8 - no template control.

The 750 bp amplicons in lanes 2 and 3 match the expected size of the *granulin* amplicon based on information from the reference isolate shown in **Table 3.1**. Similarly the 1500 bp amplicons in lanes 5 and 6 match the expected size of the *egt* amplicon. The PCR amplicons for both the *granulin* and *egt* genes were sent off for sequencing by Inqaba Biotechnical Industries (Pty) Ltd (South Africa). Additional *egt* amplicons were amplified from successive OB extracts and were also sent off for sequencing (Data not shown). The length of the *granulin* sequence acquired was 699 bp with BLAST results showing an identity of 100 % (E value of 0.0) when aligned against the reference isolate PhopGV-1346. The *egt* sequence acquired was 1482 bp with BLAST indicating an identity of 99 % (E value of 0.0) when compared against the reference isolate. This high degree of identity reveals the recovered virus to be a novel member of the species PhopGV and was thereafter named PhopGV-SA. Consensus sequences for the *granulin* and *lef*-8 genes were uploaded to the NCBI GenBank with the accession numbers KF724710.1 and KF724712.1, respectively.

Amplification of *lef*-8 was problematic and achieved by varying the DNA concentration, adjusting primer concentrations and the number of cycles used. A range of dilutions, 1:0, 1:10, 1:20, and 1:50 of extracted DNA from purified OBs was prepared for use in the amplification of *lef-8*. The number of cycles was increased to 45 (**Table 3.2)** and additional forward primer (lef8F) was added as the concentration was observed to be lower than that of the reverse (lef8R) (Data not shown). A positive control and no template control were included.

Figure 3.3 shows the PCR amplification of *lef*-8 using the four dilute DNA samples as template. Lane 1 and 2 show the positive and no template controls respectively with the positive control producing a single band and no band occurring within the no template control as expected. Lanes 3 to 6 show the amplicons for the PCR amplification of *lef*-8 from the dilute DNA samples with lane 3 using a 1:0 dilution, lane 4 a 1:10 dilution, lane 5 a 1:20 dilution and lane 6 a 1:50 dilution of template DNA. Lanes 3 to 5 did not produce any bands while lane 6 produced 2 bands, a faint band around the 5000 bp marker and an intense band around the 3000 bp marker.

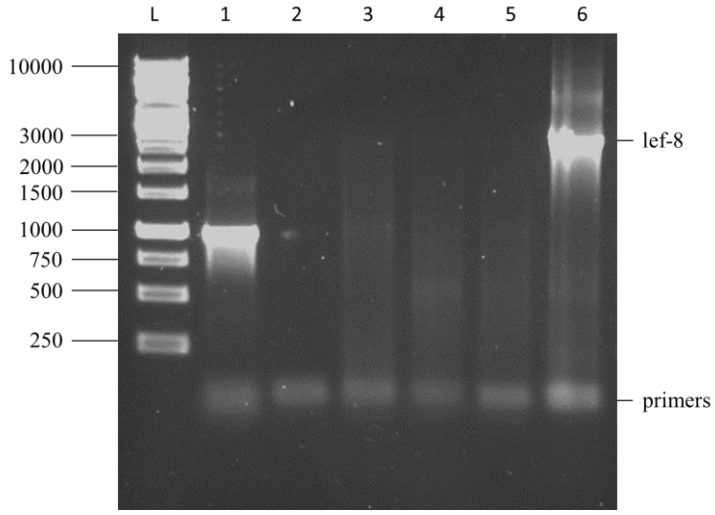


Figure 3.3: Agarose gel of the *lef*-8 PCR amplicons using varying concentrations of template DNA. L – GeneRuler 1Kb DNA ladder, 1 – positive control, 2 – no template control, 3 – 1:0 DNA template, 4 – 1:10 DNA template, 5 – 1:20 DNA template, 6 – 1:50 DNA template

The 3000 bp amplicon in lane 6 of **Figure 3.3** matches the expected size of the *lef*-8 amplicon based on the reference isolate 1346 shown in **Table 3.1.** The length of the sequence acquired for *lef*-8 was 2928 bp with BLAST indicating an identity of 99 % (E value of 0.0) against the reference isolate 1346.

PCR amplification of the *lef*-9 gene was unsuccessful (data not shown). The sequence for this gene was acquired from the complete genome sequence of PhopGV-SA (see **Chapter 4**) and was 1494 bp in length. BLAST indicated an identity of 100 % (E value of 0.0) against the reference isolate.

3.3.3. Analysis of the *granulin*, *lef*-8 and *lef*-9 gene sequences.

An alignment of the partial *granulin* and complete *lef*-9 genes from the isolated baculovirus against the complete *granulin* and *lef*-9 genes from the reference isolate, PhopGV-1346, showed a 100 % identical sequence with no SNPs detected.

Analysis of the alignment between the complete PhopGV-SA *lef*-8 gene and the complete reference isolate *lef*-8 gene revealed a total of six SNPs detected at nucleotides 100G→A, 813T→G, 981C→A, 1071T→A, 1332A→G and 2118T→C with two of these resulting in amino acid changes at positions 34A→T and 271H→Q. Analysis of the alignment between the complete PhopGV-SA *lef*-8 gene and the partial PhopGV-C4 isolate *lef*-8 gene sequenced by Herniou *et al.* (2004) revealed a total of four SNPs detected at nucleotides 1644G→T, 1647C→T, 2064A→T and 2067A→G with none resulting in amino acid changes.

3.3.4. Phylogenetic analysis of the granulin, lef-8 and lef-9 sequences

Phylogenetic analysis of concatenated amino acid sequence alignments for granulin, lef-9 and lef-8 were performed and are shown below in **Figure 3.4**. For each data set a maximum likelihood consensus tree and a maximum parsimony consensus tree were created each with 1000 bootstrap replications. The Jones-Taylor-Thornton (JTT) model was identified as the best model used with frequencies and discrete Gamma distribution for the maximum likelihood tree determined with the model select feature in Mega 5.2.

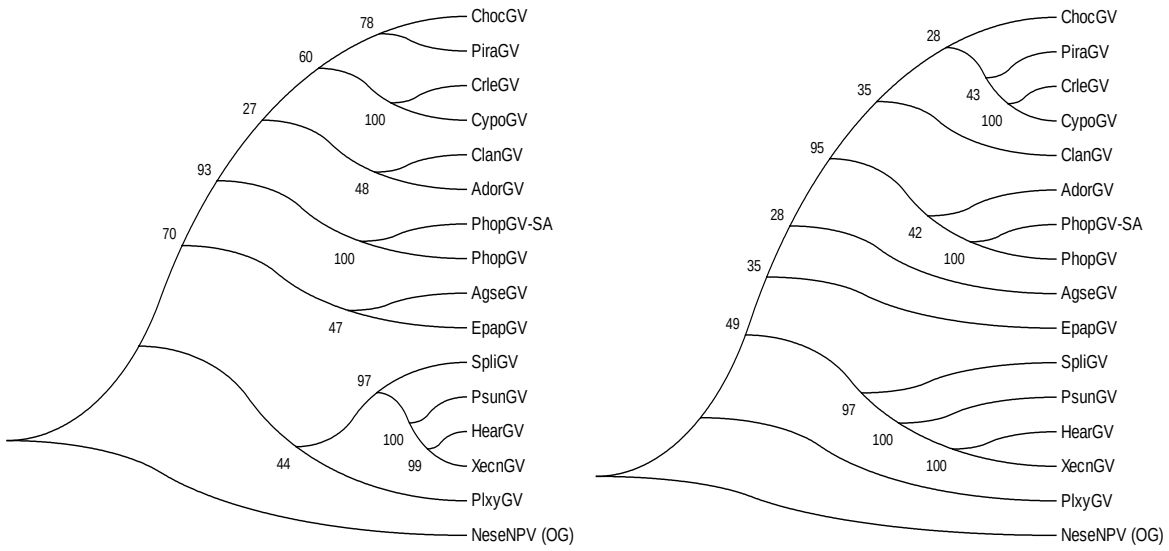


Figure 3.4: Phylogenetic reconstruction inferred using A) Maximum Likelihood and B) Maximum Parsimony of concatenated granulin, lef-8 and lef-9 amino acid sequences. Bootstrap values based on 1000 replicates, NeseNPV set as the out group (OG)

In both the maximum likelihood tree and maximum parsimony tree PhopGV-SA grouped with PhopGV-1346 with a strong bootstrap support value of 100. Both of the trees created had similar overall clustering with NeseNPV set as the out group.

3.3.5. Analysis of the *egt* gene sequence.

Alignment of the SA isolate *egt* gene sequence against *egt* sequences from other isolates available on GenBank showed the SA isolate to be similar in structure to several isolates identified as type II egt genes by Carpio *et al*. (2013). These PhopGV isolates are CHI, JLZ9F, VZ, INDO, and KEN along with a single sequence published by Espinel-Correal *et al*. (2013) from the isolate VG003. The reference isolate 1346 was identified as an egt type I by Carpio *et al*. (2013) and can only be aligned against egt II sequences up to nucleotide 1262 due to the modifications in the 3' region. Within this region, a single SNP was identified between isolates SA and 1346 at position 1059A→G which did not result in an amino acid change.

Among the egt type II isolates additional SNPs were identified. Isolates SA and VG003 had SNPs at position 1282C→G, SA and CHI at position 969C→A; 1053T→C, SA and VZ at position 969C→A, SA and KEN at position 327G→A; 647C→T; 736G→A; 909C→T, SA and INDO at position 615G→C; 646G→A and between SA and all egt type IIs at position 1059A→G. Amino acid changes were identified between isolates SA and KEN at position 109M→I; 216A→V; 246D→N, SA and INDO at position 205W→C; 216A→T and SA and VG003 at position 428P→A. The abundance of SNPs both silent and non-silent are summarised in **Figure 3.5**.

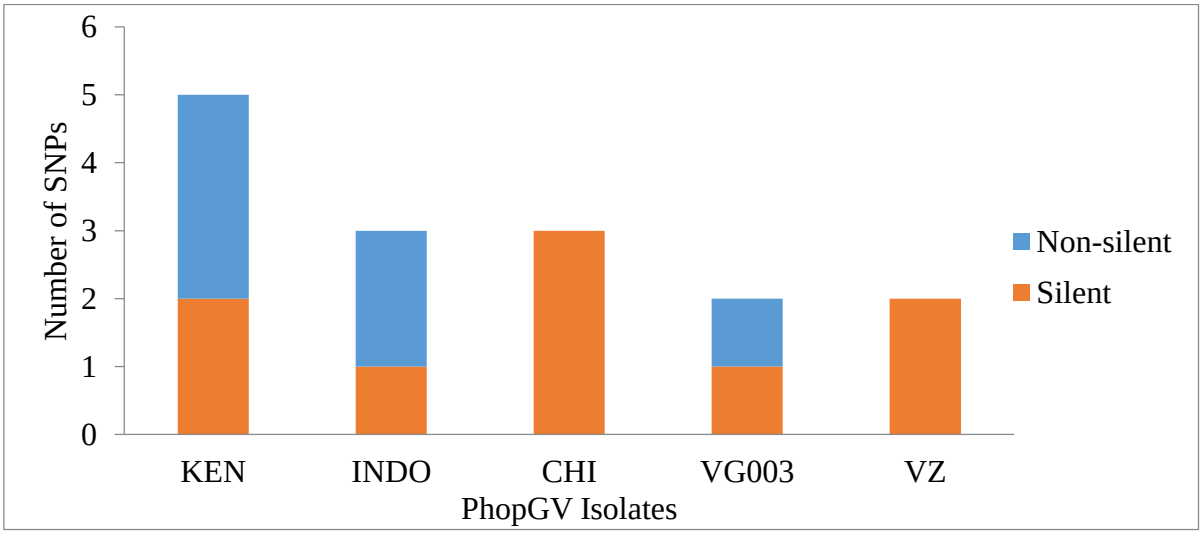


Figure 3.5: The number of non-silent and silent SNPs in the PhopGV-SA *egt* gene sequence compared against *egt* gene sequences from other egt II isolates.

A total of five egt types were identified in the *egt* alignment including the three identified by Carpio *et al*. (2013) with sizes of 1305 bp, 1353 bp, 1086 bp, 1092 bp and 861 bp for types I to V respectively. Each was investigated using the sequence obtained for the SA isolate as the primer design resulted in a larger amplicon than the length of the *egt* gene itself and consequently provided information about the region flanking the 5, and 3' regions of the gene. These flanking regions are shown in **Figure 3.6** as the region left of the start codon (marked with a green bar) and the region right of the stop codon (marked with a ref bar) in PhopGV-SA. The alignment of a representative *egt* gene sequence from each egt type to the SA *egt* gene resulted in the 3' region of type I aligning alongside the stop codon of the SA isolate and types III and IV aligning beyond the stop codon of the SA isolate. The 5' region for types I, III and IV aligned up to nucleotides 1262, 1053 and 1053 of the *egt* sequence from PhopGV-SA respectively. The 3' region of type V aligned to the middle of the sequences for types I to IV with the stop codon at position 1015 (relative to the SA isolate) while the 5' region aligned up to nucleotide 840 of the SA isolate gene sequence. The type II sequence aligned perfectly with the SA isolate with both having a length of 1353 bp. **Figure 3.6** also shows the nucleotide sequences for the regions at the start of and end of the deletions/insertions with the translated amino acid sequence given below. In comparison to the SA isolate/type II sequence, the deletion/insertion event in types I, III, IV and V all result in a frame shifts producing unique 3' motifs in each type.

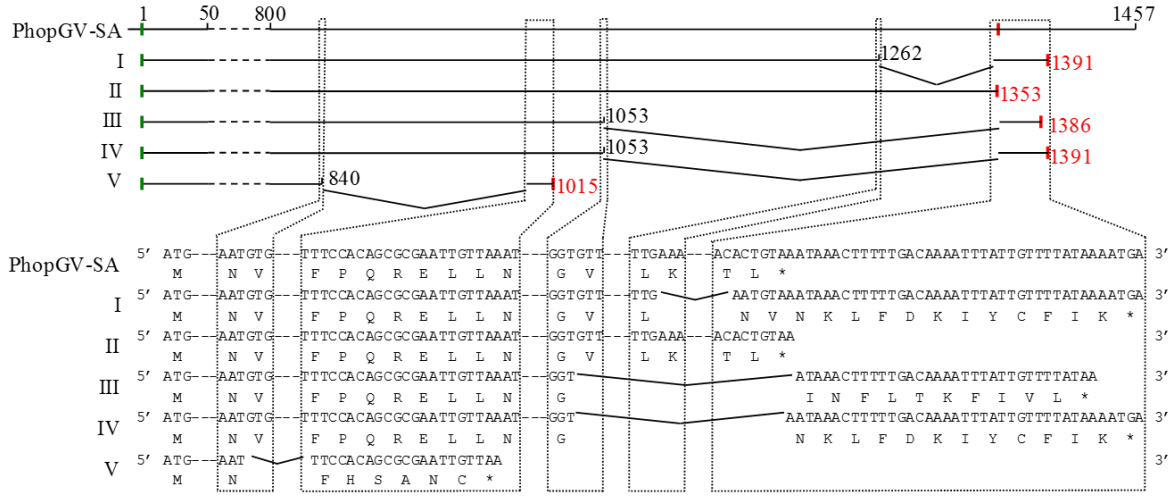


Figure 3.6: The PhopGV-SA *egt* gene aligned against the five egt types with the green bars indicating the start codon, red bars indicating the stop codon (and position), v indicating a deletion. Nucleotide sequence alignments are shown below with the translated amino acids for PhopGV-SA and the five egt types (Jukes *et al*., 2014).

The nature of the nucleotide changes in the various egt types and the unique manner in which each egt type aligned with the region upstream of the SA isolate stop codon indicated that the modifications in types I, III and IV were a result of simple deletion events from the type II gene rather than several insertion and deletion events. The mechanism by which the egt type V arose remains unknown.

3.3.6. Phylogenetic analysis of the egt sequences.

The Whelan and Goldman model was found to best represent the egt data from which a maximum likelihood tree was constructed. Analysis of egt amino acid sequences using the gene data shown in **Table 3.4** resulted in the formation of two primary clades, Clade 1 which includes all egt II isolates (including SA) and Clade 2 which consists of all egt I, III, IV and V isolates (**Figure 3.7)**. The formation of these clades had a strong bootstrap support value of 100 while support values for branches within each clade were typically lower.

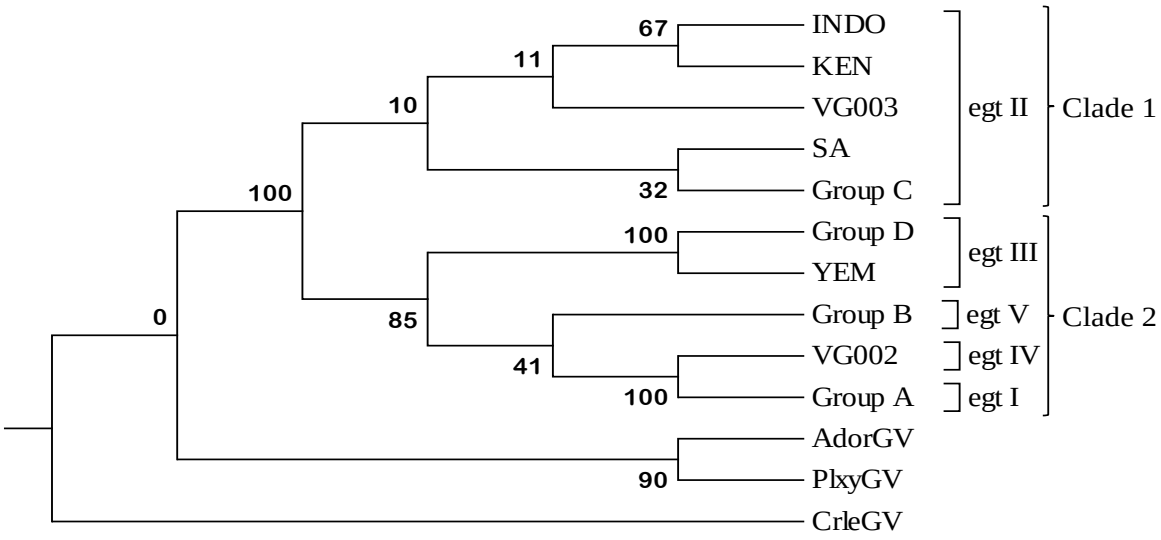


Figure 3.7: Phylogenetic reconstruction inferred by using maximum likelihood method based on the Whelan and Goldman model (Whelan and Goldman, 2001) of aligned PhopGV egt amino acid sequences (see **Table 3.4** for isolate data) (Jukes *et al*., 2014).

Within Clade 1 the SA isolate was found to group closely to isolates in group C, these being isolates JLZ9F, VZ and CHI however the bootstrap support value for this was low at 32. The consensus sequence for the *egt* gene was uploaded to the NCBI GenBank, accession number KF724711.1.

3.4. Discussion

The overall aim of this chapter was to genetically characterise the novel baculovirus recovered from *P. operculella* larval cadavers and was achieved through the comparison of gene sequences to that of other isolates and, furthermore, through the construction of phylogenetic trees. As discussed in the introduction to this chapter, specific genes, *granulin lef*-8 and *lef*-9 have been described to accurately infer baculovirus phylogeny and consequently represent important targets in the study of these viruses. The *granulin* and *lef*-8 genes were successfully PCR amplified and sequenced while the amplification of the *lef*-9 gene was not successful. The gene sequence was however acquired through the sequencing of the full genome of the isolated virus (see **Chapter 4**) and used in the phylogenetic analysis of the virus. Early identification of the isolated baculovirus was achieved through the submission of the *granulin* and *lef*-8 gene sequences to BLAST, whereby the top search results for each of these genes were sequences from PhopGV. The *granulin* and *lef*-8 genes from the reference isolate appeared in the BLAST results for each of the respective genes, with identity scores of >99 % and E values of 0.0. This high degree of identity suggested that the novel isolate was a member of the PhopGV species and was named *Phthorimaea operculella* granulovirus-South Africa (PhopGV-SA).

A comparison of the *granulin*, *lef*-8 and *lef*-9 nucleotide sequences for the SA isolate and those of the reference isolate revealed a few single nucleotide polymorphisms (SNPs) with only two SNPs resulting in amino acid changes, both within the *lef*-8 gene. The high degree of similarity between the SA isolate and reference isolate further supported the identification of the isolated virus. The phylogeny of this virus was determined by constructing phylogenetic tress using the aligned and concatenated amino acid sequences for these three genes. **Figure 3.4** showed the construction of a maximum likelihood tree and a maximum parsimony tree for 16 baculovirus species (including PhopGV-SA) which resulted in the grouping of PhopGV-SA with the PhopGV reference isolate showing a high degree of association with the species PhopGV. This clustering received a bootstrap support value of 100 indicating that this clustering was present in all replicate trees.

The use of the highly conserved *granulin*, *lef*-8 and *lef*-9, did not offer insight into inter-species relatedness and similarity due to their high degree of conservation coupled with the sparse amount of sequence data available for these genes on GenBank. To investigate this aspect of PhopGV-SA, the *egt* gene was successfully amplified and sequenced. As mentioned in this chapter's introduction, several *egt* gene structures have recently been identified due to modifications in the 3' region of the gene, however, it remains unclear whether these

modifications may affect the functioning of egt (Carpio *et al*., 2013; Espinel-Correal *et al*., 2010). An alignment of all available *egt* gene sequences (25 at the time of writing) identified the PhopGV-SA as a member of egt II with the characteristic size of 1353 bp along with the CHI, KEN, INDO, JLZ9f, VZ and VG003 isolates. The naming system adopted in this study is based upon the system started by Carpio *et al*. (2013) over the system proposed earlier by Espinel-Correal *et al*. (2010) as two sequences described as being different egt types by Espinel-Correal *et al*. (2010) had identical nucleotide sequences on GenBank and consequently the five egt types described could not be replicated. Furthermore, two additional *egt* gene structures were identified in the alignment, the egt IV consisting of the isolate VG002 and the egt V consisting of the isolates VG004 and VG005.

Analysis of the alignment further indicated that the egt II group, of which the SA isolate is a member, represents the prototype gene structure from which egt I, III and IV originate. Egt types I, III and IV arose as a result of a large internal deletions in the 3' region of the prototype gene after which each managed to proliferate and form a stable genotype of the virus. Furthermore, the egt group II gene appears to most closely represent the closest ancestral form with a similar size to the egt gene present in the closely related AdorGV and CrleGV. These results suggest that the groups I, III, IV and V may be a result of recent genetic changes in PhopGV.

Due to the assignment of the SA isolate to the egt type II group, a comparison of the nucleotide sequence of the *egt* gene with that of the reference isolate was only possible up to nucleotide 1262. Within this region only one SNP was detected with no effect on the amino acid sequence. A greater degree of variation was observed within the egt II group (**Figure 3.6**), with the KEN isolate being least similar to the SA isolate and the CHI and VZ isolates were the most similar.

As discussed in the **Chapter 1**, *P. operculella* is native to South America having spread across the globe in the last few centuries. PhopGV most likely spread alongside *P. operculella* resulting in the introduction of the virus into Africa, the Middle East, Australasia and Australia. Considering the amount of variation in the *egt* gene structure between PhopGV isolates, the ancestry of PhopGV-SA can be discussed by postulating how the virus was introduced into the African continent (**Figure 3.8**).

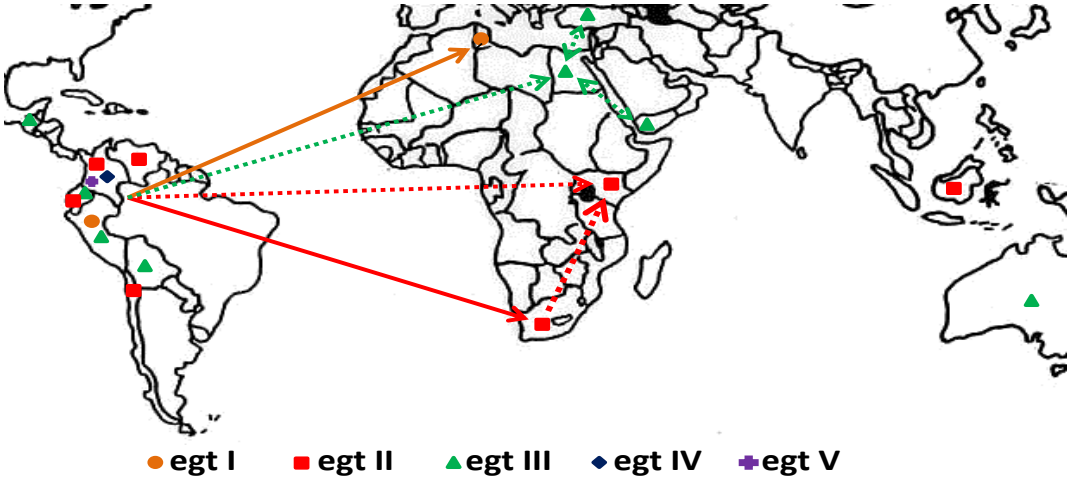


Figure 3.8: World map showing the distribution of *Phthorimaea operculella* granulovirus (PhopGV) isolates by the type of *egt* gene present, represented as egt I, II, III, IV and V. Solid lines indicate predicted introduction events and dashed lines indicate alternate introduction events.

Analysis of the *egt* gene showed PhopGV-SA to have a greater similarity to South American isolates (i.e. VZ or CHI) than to other African isolates (i.e. KEN) indicating a separate introduction event into Africa. The KEN isolate, also a member of the egt II group, had the greatest degree of variation to the SA isolate which may have resulted from an early separation from a South African *P. operculella* population which spread north into a new region. The increased amount of variation may have occurred as the host adapted to its changing environment with the virus adapting alongside its host. Alternatively, the KEN isolate may itself be a second introduction of PhopGV into Africa from South America, again with changes in environment influencing the host biology and consequently causing the increased variation observed. The Tunisian (PhopGV-1346) isolate, an egt I member, marks a third introduction of PhopGV into Africa while the Egyptian isolate (PhopGV-EGY), an egt III member, may have been introduced from South America, Turkey or Yemen. The exact origin of the Egyptian isolate is difficult to interpret as minimal variation was seen among these isolates.

In summary, the aims of this chapter were achieved whereby the previously unknown GV isolated from *P. operculella* larvae was successfully identified as a member of the species PhopGV. The isolate was named PhopGV-SA and was further characterised as a member of the egt II group showing high similarity to the CHI and VZ isolates. From this it was inferred that the South African isolate originated in South America, having a more distant relation to the Kenyan isolate. The next chapter aims to further expand on the genetic characterisation of PhopGV-SA by restriction endonuclease analysis of genomic DNA as well as through the sequencing of the full genome of this novel isolate.

Chapter 4

Genomic analysis of *Phthorimaea operculella* granulovirus-South Africa

4.1 Introduction

Chapter three described the identification and genetic characterisation of a novel South African *P. operculella* granulovirus (PhopGV-SA) by comparison of selected gene sequences with PhopGV-1346 and phylogenetic analysis. Another commonly used method to genetically characterise novel baculovirus isolates is to enzymatically fragment genomic DNA and separate the fragments by agarose gel electrophoresis to generate distinct profiles. Restriction endonuclease (REN) analysis has been used in many studies examining the genomes of a variety of PhopGV isolates, producing profiles for many commonly available restriction enzymes including *Bam*HI, *Hin*dIII, *Nde*I and *Nru*I (Gómez-Bonilla *et* al., 2012b; Espinel-Correal *et al*., 2010; Carpio *et al*., 2012; Gómez-Bonilla *et al*., 2011; Zeddam *et al*., 2013). The production of these profiles has resulted in the identification of several viral genotypes to which different PhopGV isolates belong. Comparison of these profiles assists in determining the novelty of newly recovered viruses as differences in the patterns observed are a result of changes to the genomic sequence such as insertions, deletions and point mutations (Jehle 1996; Munoz *et al*., 1997; Espinel-Correal *et al*., 2010; Carpio *et al*., 2013). Such changes to the genomic structure of baculoviruses may affect the pathogenicity of the virus and emphasises the need to identify novel genotypes especially when prospecting for viruses intended to be used as biopesticides.

While the comparison of profiles generated has long been used to identify novel virus, single restriction enzyme digestion of genomic DNA only provides an estimate of band sizes. The production of *in silico* REN profiles can easily be performed by computationally analysing the complete genome sequences available for viruses including baculoviruses. For example, Espinel-Correal *et al*. (2010) and Zeddam *et al*. (2013) produced accurate reference profiles to which five new PhopGV isolates were compared using the complete genome sequence of PhopGV-1346 (Croizier & colleagues, unpublished). This method of analysis provides highly accurate profiles for any number of restriction enzymes to which REN profiles generated *in vitro* for isolates which are yet to be fully sequenced can be compared.

While the production of *in vitro* profiles has become common practice in baculovirus research, the use of *in silico* profiles has yet to become mainstream because of the lack of full genome sequences. This however may begin to change with the rapidly expanding field of next generation sequencing (NGS) technologies which is changing the way researchers approach genomic and genetic research. In recent years several new NGS platforms have been introduced and utilised in research and commercial communities such as the Roche (Switzerland) 454 genome sequences, Life Technologies (Thermo Scientific, USA) Ion Torrent and the Illumina (USA) sequences (Shokralla *et al.*, 2012; Quail *et al*., 2012). These platforms are based on the polymerase chain reaction (PCR) and produce several thousand short reads between 100 bp to 800 bp resulting in up to 600 gigabases (GB) worth of sequence data within a single sequencing run. This technology is becoming increasingly prominent in baculovirus research with platforms by both Roche and Illumina having been used to determine a number of genome sequences. These baculoviruses include both NPVs such as *Chrysodeixis chalcites* SNPV (ChchSNPV), *Choristoneura murinana* NPV (ChmuNPV), and *Mamestra brassicae* NPV (MabrNPV) and GVs such as *Clostera anastomosis* GV (CalGV), *Clostera anachoreta* GV (ClanGV), *Erinnyis ello* GV (ErelGV) and *Agrotis segetum* GV (AgseGV) (Liang *et al*., 2013; Bernal *et al*., 2013; Rohrmann *et al*., 2014; Choi *et al*., 2013; Ardisson-Araújo *et al*., 2014; Liang *et al*., 2011; Zhang *et al*., 2014). With the continual advancement in NGS technologies, sequencing costs are rapidly decreasing from several $1000 per GB to less than $100 per GB (Quail *et al.*, 2012) allowing smaller laboratories the ability to produce genome sequences for novel virus isolates further expanding the field of baculovirus research.

The overall aim of this chapter was to further characterise the genome of PhopGV-SA by restriction endonuclease analysis of genomic DNA and complete genome sequencing. Specific objectives were first to generate DNA profiles for PhopGV-SA using the restriction enzymes *Bam*HI, *Hin*dIII, *Nru*I and *Nde*I for comparison with *in silico* profiles of PhopGV-1346. The second objective was to obtain a complete PhopGV-SA genome sequence and generate *in silico* profiles for comparison with PhopGV-1346. *In silico* profiles of PhopGV-SA will be used to validate the PhopGV-SA *in vitro* profiles produced by single restriction enzyme digestion. Lastly, the degree of similarity between the PhopGV-SA genes and those of the reference strain were also determined.

4.2. Materials and Methods

4.2.1. Restriction endonuclease analysis.

Genomic DNA was digested using the restriction enzymes *Bam*HI (Thermo Scientific, USA), *Hin*dIII (Thermo Scientific, USA), *Nru*I (Thermo Scientific, USA) and *Nde*I (Thermo Scientific, USA). Restriction reactions comprised template DNA (208 ng for *Bam*HI; 4.6 µg for *Hin*dIII and 2.3 µg for *Nru*I/ *Nde*I), 3 µl buffer and 3 µl of the respective restriction enzyme with each reaction having a no template control. Reactions were made up to 30 µl and were visualised by 0.6 % agarose gel electrophoresis at 30 V for 16 hours in 1X TAE stained with ethidium bromide and gel images were captured with a UVIpro chemi (UVItec) UV trans-illuminator.

Profiles were separated alongside two DNA ladders, a high range ladder and a low range ladder. For the *Bam*HI, *Hin*dIII and *Nru*I profiles the high range ladder used was the Lambda Mix Marker, 19 (Fermentas, USA) while the high range ladder used for the *Nde*I profile was the GeneRuler High Range DNA Ladder (Thermo Scientific, USA). The GeneRuler 1Kb DNA Ladder (Thermo Scientific, USA) was used as the low range ladder for all profiles. Estimated band sizes were determined using the UviBand software (UVItec). All profiles were performed on genomic DNA extracts from a single batch of purified OBs. *In silico* digests using the complete genome sequence of the reference isolate PhopGV-1346 (GenBank: NC_004062) were carried out using the A Plasmid Editor (ApE) v2.0.47 software for each of the four restriction enzymes used.

4.2.2. Complete genome analysis of PhopGV-SA

The full genome of PhopGV-SA was sequenced by Inqaba Biotechnical Industries (Pty) Ltd (South Africa) using a MiSeq Desktop Sequencer (Illumina). Genomic DNA, approximately 100 ng, extracted from PhopGV-SA OBs (see **Section 3.2.1)** was sequenced producing a total of 1220000 paired reads. These reads were paired together and 60 % of the data was used to produce contigs through the de novo assembly of the raw reads. A total of 10000 contigs were assembled and mapped to the complete genome sequence of the reference isolate with medium sensitivity and a total of 5 iterations producing a single consensus sequence. A pairwise alignment using the progressive mauve algorithm was performed between the consensus sequence and the genome sequence of PhopGV-1346. The consensus sequence was annotated using annotation data from the reference isolate. The percentage similarity transferred from the reference isolate to PhopGV-SA for each gene was determined based on similarities in gene

amino acid sequences. All genome analysis was carried out in Geneious (New Zealand) version R7 (Kearse *et al*., 2012). The percentage of transferred similarity for each gene was plotted in Microsoft Excel (USA) with outlying genes being analysed for SNPs and amino acid changes. *In silico* digests using the complete genome sequence of the reference isolate PhopGV-1346 and the complete genome sequence of PhopGV-SA were carried out using ApE software (USA) for each of the four restriction enzymes used and *in vitro* profiles compared to the *in silico* profiles.

4.3. Results

4.3.1. Restriction endonuclease profiles.

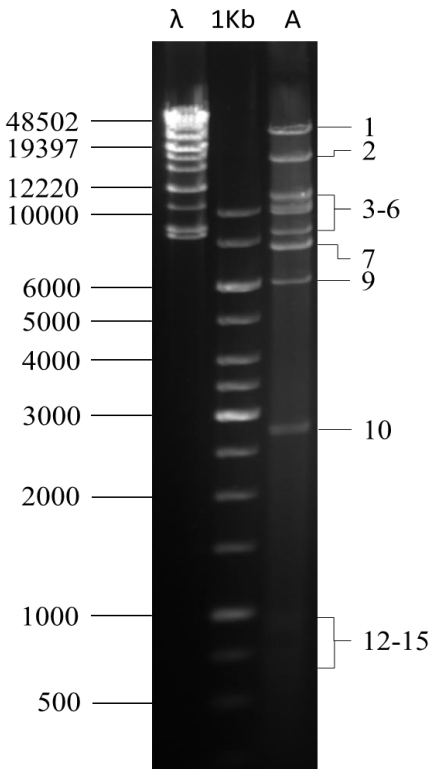
4.3.1.1. *Bam*HI restriction endonuclease profile

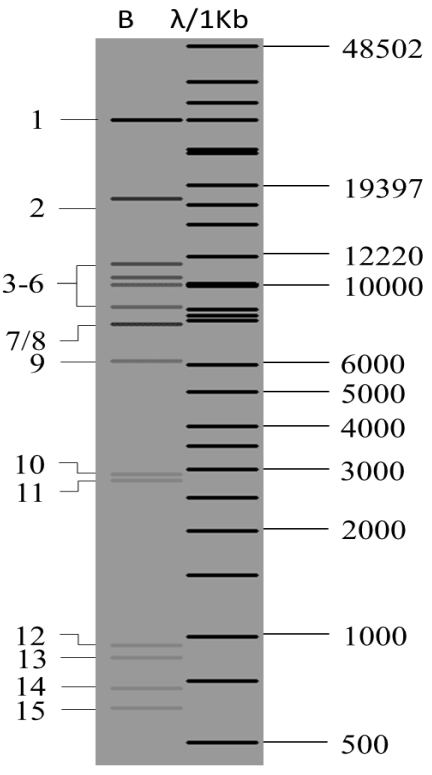
The restriction profile generated for PhopGV-SA using the enzyme *Bam*HI is shown below in **Table 4.1** alongside the *in silico* digest of PhopGV-1346. A total of 13 bands were detected in the profile of the SA isolate shown in lane A and a total of 15 bands were present in reference isolate in lane B.

Table 4.1: Comparison of *in vitro* PhopGV-SA and *in silico* PhopGV-1346 genomic REN profiles using Bam*HI.*

Restriction endonuclease analysis of PhopGV using B*am* HI (sizes in bp)

PhopGV-SA *In vitro*	A	Band	B	PhopGV-1346 *In silico*
	27067	**1**	29906	
	16879	**2**	17766	
	10924	**3**	11561	
	10086	**4**	10577	
	10000	**5**	10129	
	8529	**6**	8725	
	7751	**7**	7792	
		8	7789	
	6230	**9**	6114	
	2831	**10**	2901	
		11	2806	
	970	**12**	943	
	900	**13**	870	
	750	**14**	712	
	650	**15**	626	
	103567	**Size**	119217	
	13	**Bands**	15	





λ - Lambda Mix Marker 19, 1Kb - GeneRuler 1Kb DNA Ladder

Each of the 13 bands detected in the SA isolate could be matched to similar sized bands present in the reference isolate when digested with *Bam*HI. Bands 8 and 11 of the reference isolate were not detected in the *in vitro* analysis of PhopGV-SA. Band 7 and 10 in lane A were of greater intensity when compared to the bands immediately above, indicating the possibility that they represent doublets. The approximate size of the genome is 112000 bp if putative doublet bands are taken into account whereas the exact size of PhopGV-1346 is 119217 bp.

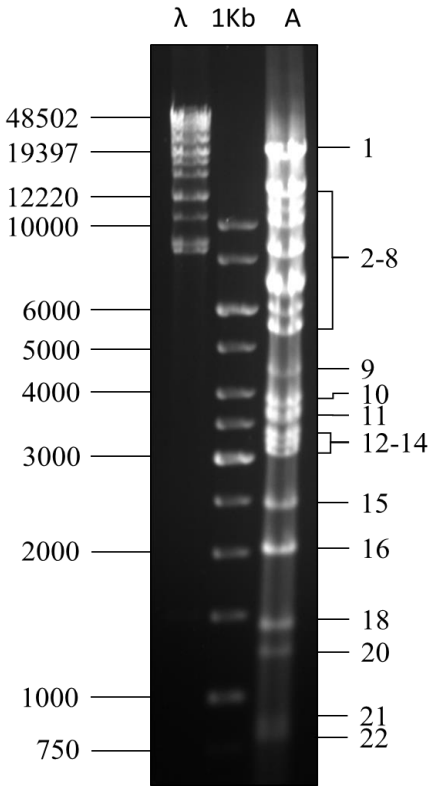
4.3.1.2. *Hin*dIII restriction endonuclease profile

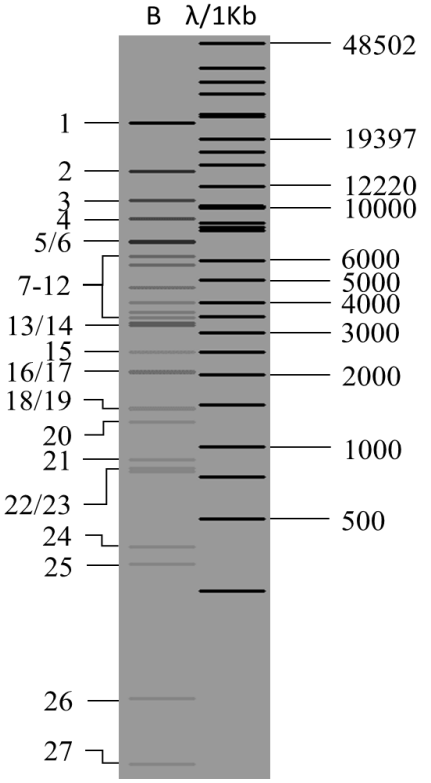
The *Hin*dIII profile of PhopGV-SA had a total of 20 detectable bands compared to a total of 27 bands present in the *in silico* analysis of PhopGV-1346 (**Table 4.2**).

Table 4.2: Comparison of *in vitro* PhopGV-SA and *in silico* PhopGV-1346 genomic REN profiles using *Hin*dIII.

Restriction endonuclease analysis of PhopGV using *Hin* dIII (sizes in bp)

PhopGV-SA *In vitro*	A	Band	B	PhopGV-1346 *In silico*
	17990	**1**	22634	
	12778	**2**	14177	
	10704	**3**	10705	
	10000	**4**	8969	
	8168	**5**	7248	
	7044	**6**	7126	
	6043	**7**	6275	
	5494	**8**	5736	
	4467	**9**	4627	
	3871	**10**	4020	
	3659	**11**	3644	
	3381	**12**	3474	
	3249	**13**	3298	
	3138	**14**	3216	
	2487	**15**	2476	
	2052	**16**	2072	
		17	2036	
	1458	**18**	1449	
		19	1441	
	1264	**20**	1266	
	846	**21**	881	
	800	**22**	812	
		23	791	
		24	384	
		25	324	
		26	89	
		27	47	
	108893	**Size**	119217	
	20	**Bands**	27	





λ - Lambda Mix Marker 19, 1Kb - GeneRuler 1Kb DNA Ladder

Similar to the *Bam*HI profiles, each band present in the PhopGV-SA *Hin*dIII profile could be matched to a similarly sized band present in the reference profile with the exception of bands 4 and 5 which were larger in size in the SA isolate. Bands 17, 19 and 23 to 27 of the reference isolate were not detected in the SA isolate. Band 16 in lane A may represent a doublet band due to increased band intensity when compared to upper bands. The size of PhopGV-SA was estimated to be approximately 108000 bp based on visible bands and 112000 bp including possible doublet bands.

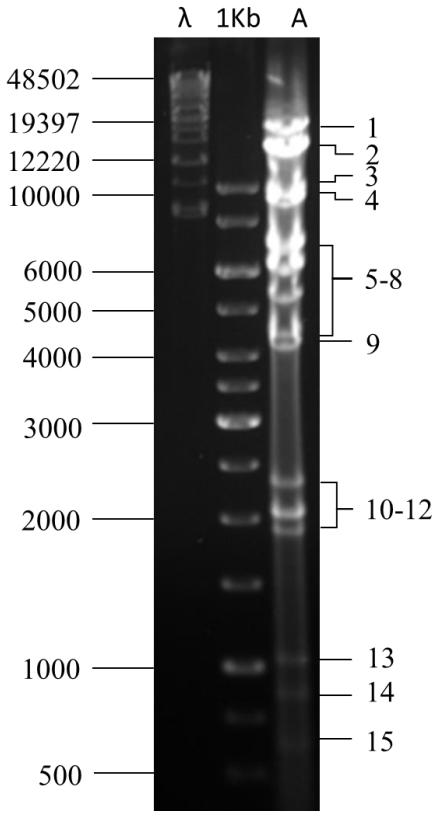
4.3.1.3. *Nru*I restriction endonuclease profile

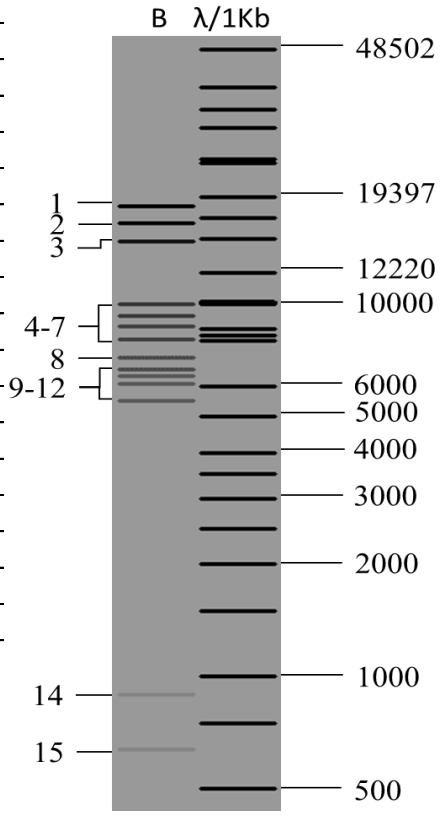
Analysis of the *Nru*I profile generated for PhopGV-SA showed the presence of 15 bands in lane A compared to 14 bands in lane B of the reference isolate (**Table 4.3**). The additional band detected in the SA isolate was approximately 1009 bp in size marked as band 13 in lane A.

Table 4.3: Comparison of *in vitro* PhopGV-SA and *in silico* PhopGV-1346 genomic REN profiles using *Nru*I.

Restriction endonuclease analysis of PhopGV using *Nru* I (sizes in bp)

PhopGV-SA *In vitro*	A	Band	B	PhopGV-1346 *In silico*
	17816	**1**	18349	
	13132	**2**	16487	
	9565	**3**	14772	
	8614	**4**	10034	
	6967	**5**	9333	
	6054	**6**	8749	
	5218	**7**	8063	
	4398	**8**	7210	
	4208	**9**	6670	
	2313	**10**	6403	
	2000	**11**	6104	
	1862	**12**	5511	
	1009	**13**		
	818	**14**	895	
	622	**15**	637	
	84596	**Size**	119217	
	15	**Bands**	14	





λ - Lambda Mix Marker 19, 1Kb - GeneRuler 1Kb DNA Ladder

Unlike the *Bam*HI and *Hin*dIII profiles, the bands produced in the *Nru*I profile for the SA isolate could not be easily matched to the bands of the reference isolate with many bands differing in size by several thousand base pairs. The total genome size for PhopGV-SA was significantly smaller than that of the reference isolate with all the bands totalling approximately 84000 bp compared to the 119217 bp for the reference isolate.

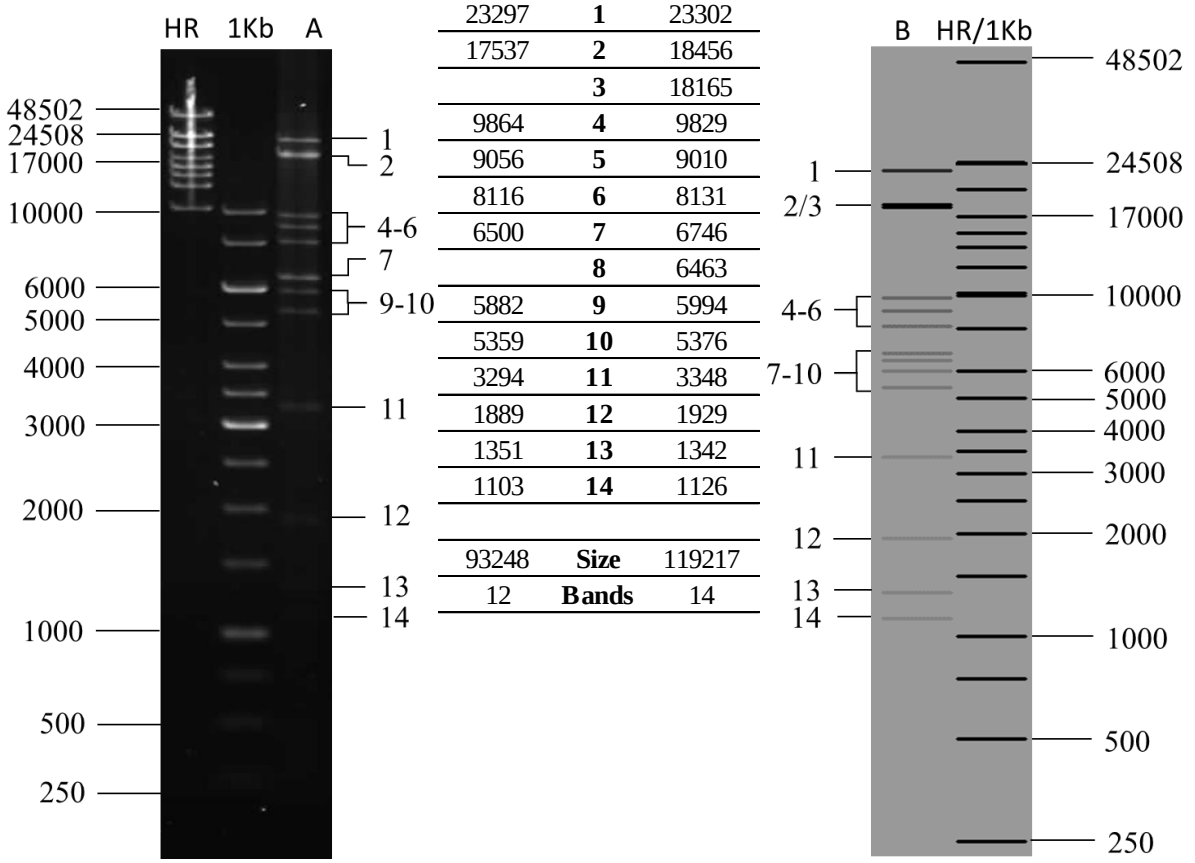
4.3.1.4. *Nde*I restriction endonuclease profile

The PhopGV-SA *Nde*I profile had a total of 12 bands shown in lane A of **Table 4.4**. In comparison the *in silico* analysis of the reference isolate produced a total of 14 bands shown in lane B.

Table 4.4: Comparison of *in vitro* PhopGV-SA and *in silico* PhopGV-1346 genomic REN profiles using *Nde*I.

Restriction endonuclease analysis of PhopGV using *Nde* I (sizes in bp)

PhopGV-SA *In vitro*		A	Band	B	PhopGV-1346 *In silico*
HR 1Kb A		23297	**1**	23302	B HR/1Kb
		17537	**2**	18456	
			3	18165	
		9864	**4**	9829	
		9056	**5**	9010	
		8116	**6**	8131	
		6500	**7**	6746	
			8	6463	
		5882	**9**	5994	
		5359	**10**	5376	
		3294	**11**	3348	
		1889	**12**	1929	
		1351	**13**	1342	
		1103	**14**	1126	
		93248	**Size**	119217	
		12	**Bands**	14	



HR - GenRuler High Range DNA Ladder, 1Kb - GeneRuler 1Kb DNA Ladder

The 12 bands present in the SA isolate match 12 of the bands in the reference isolate, each having similar sizes. Bands 3 and 8 of the reference isolate were not detected in the SA isolate. However, bands 2 and 7 in lane A appear to have greater intensity than the upper bands and

may represent doublets. The total size of the PhopGV-SA genome was calculated to be approximately 116500 bp taking into account putative doublet bands.

4.3.2. Full genome sequencing of PhopGV-SA

A preliminary genome sequence for PhopGV-SA was produced using the Illumina MiSeq desktop sequencer. A total of 1.22 million paired reads were sequenced of which 709785 were used in the *de novo* assembly producing a total of 29025 contigs of which 10001, those rated with the highest quality in Geneious R7, were stored. These stored contigs were mapped to the reference isolate genome sequence producing a single consensus sequence of 118138 bp. Following de novo assembly and mapping, a total of 370637 reads were used to produce the consensus sequence with a coverage of 887 ± 337.4 and a GC percentage of 35.7 %. Alignment of the consensus sequence for PhopGV-SA against the full genome sequence of PhopGV-1346 showed a pairwise identity of 98.4%.

4.3.3. In silico restriction analysis of PhopGV-SA

In silico analysis of the PhopGV-SA genome sequence produced profiles based on the *Bam*HI, *Hin*dIII, *Nru*I and *Nde*I restriction sites (**Table 4.5**). Profiles for the enzymes *Bam*HI, *Nru*I and *Nde*I produced an equal number of bands for both PhopGV-SA and PhopGV-1346. Furthermore these bands typically differed by less than 50 bp (marked with a red icon) with only a few bands equal in size (marked with a green icon). The few remaining bands differed in size by more than 50 bp, such as band number 11 in the *Nru*I profile which differed by 771 bp and band number three in the *Nde*I profile which differed by 471 bp.

Comparison of the *Hin*dIII profiles revealed the addition of a band (number 24) in PhopGV-SA with a size of 787 bp. Of the remaining bands 11 of the fragments in the *Hin*dIII profiles were identical in size and 10 differed by less than 50 bp and 7 differed by more than 50 bp such as band 9 with difference of 607 bp observed.

Table 4.5: In silico digest of the PhopGV-SA and PhopGV-1346 genome sequences based on the *Bam*HI, *Hin*dIII, *Nru*I and *Nde*I restriction sites.

Band	*Bam* HI		*Hin* dIII		*Nru* I		*Nde* I	
	SA	1346	SA	1346	SA	1346	SA	1346
1	29700	29906	22557	22634	18223	18349	23339	23302
2	17770	17766	14269	14177	16432	16487	18064	18456
3	11593	11561	9935	10705	14681	14772	17694	18165
4	10549	10577	8971	8969	10034	10034	9793	9829
5	10129	10129	7236	7248	9365	9333	9002	9010
6	8653	8725	7108	7126	8758	8749	8112	8131
7	7783	7792	6250	6275	8123	8063	6568	6746
8	7777	7789	5663	5736	7223	7210	6457	6463
9	5344	6114	4020	4627	6671	6670	5994	5994
10	2883	2901	3803	4020	6430	6403	5376	5376
11	2806	2806	3658	3644	5333	6104	3348	3348
12	943	943	3456	3474	5333	5511	1929	1929
13	870	870	3298	3298	895	895	1342	1342
14	712	712	3197	3216	637	637	1120	1126
15	626	626	2522	2476				
16			2072	2072				
17			2036	2036				
18			1449	1449				
19			1440	1441				
20			1266	1266				
21			881	881				
22			812	812				
23			791	791				
24			787					
25			384	384				
26			324	324				
27			89	89				
28			44	47				
Size	118138	119217	118318	119217	118138	119217	118138	119217
Bands	15	15	28	27	14	14	14	14

= Fragments with identical bp = Fragments with similar bp

4.3.4. Preliminary analysis of the PhopGV-SA genome

Genes within the preliminary genome of PhopGV-SA were annotated using the gene data from the reference sequence PhopGV-1346 (**Figure 4.1)**. A total of 128 genes were identified out of the 130 genes present in the complete sequence of PhopGV-SA with genes 54 and 128 missing. Identified genes were confirmed to have complete coding regions from start codon to stop

codon. Importantly the *egt* sequence in the PhopGV-SA genome was identical to the *egt* sequences produced through independent PCR amplification (as discussed in **Section 3.3.5)** maintaining the SA isolate in the type II egt group.

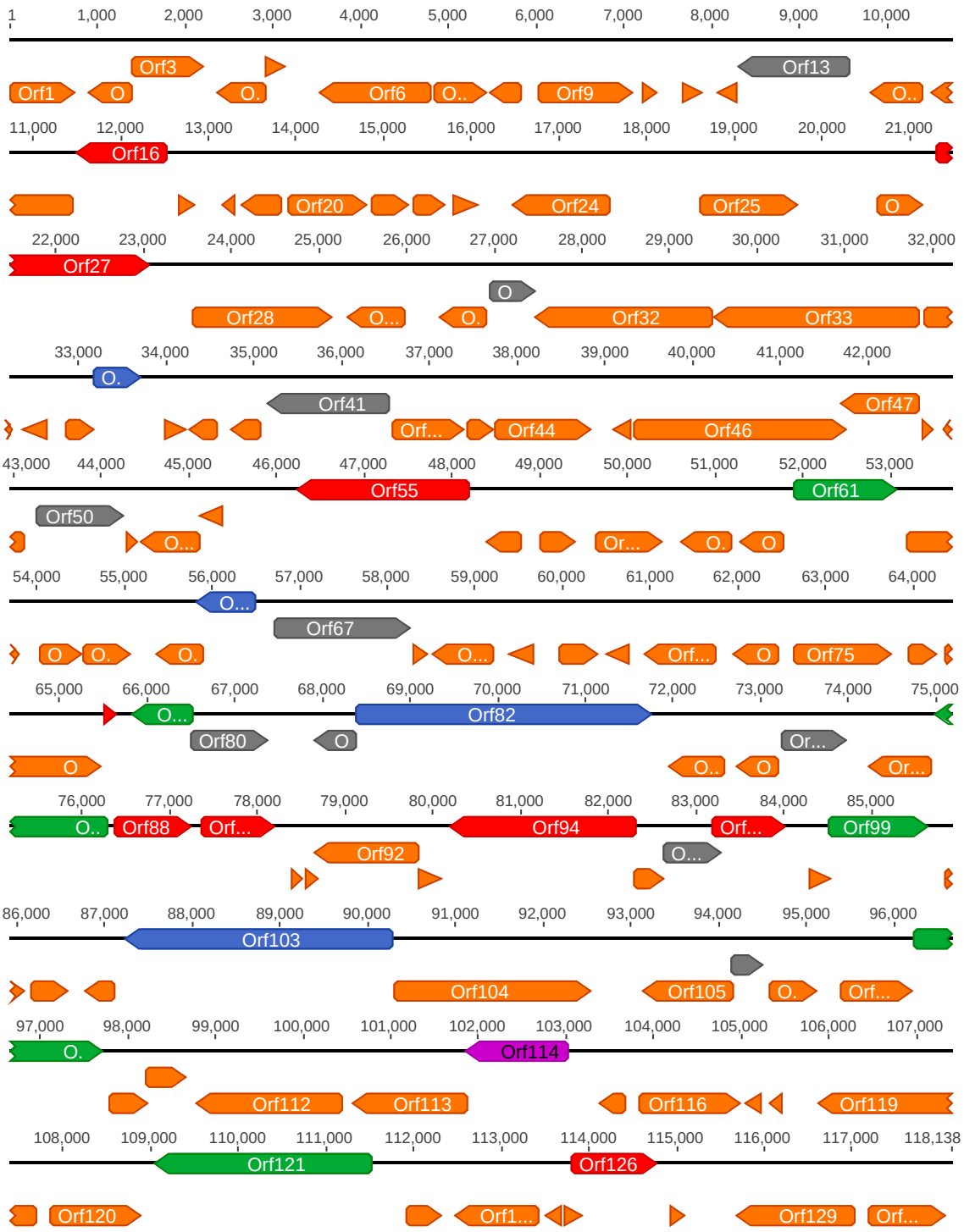


Figure 4.1: Preliminary genome organisation in PhopGV-SA with Orf1 representing the *granulin* gene. Core genes involved in replication (blue) transcription (green), structural (red), auxiliary (pink) and those with unknown functions (grey) are highlighted (see **Table 4.6)**

Genes which were previously identified as core genes by Herniou *et al*. (2003) were identified within PhopGV-SA (shown in **Figure 4.1**), with replication genes highlighted in blue, transcription genes highlighted in green, structural genes highlighted in yellow, auxiliary genes highlighted in pink and core genes with unknown functions highlighted in grey. Details for each of these core genes is described in **Table 4.6** and are presented in order of appearance. Genes described as having an unknown function by Herniou *et al*. (2003) were assessed by looking at the respective products in AcMNPV (Rohrmann 2014). However, they were not assigned to the replication, transcription, structural and auxiliary groups as most of these functions have not been confirmed in PhopGV.

Table 4.6: Core genes identified in PhopGV-SA with the products and open reading frames (ORFs) for replication genes (blue) transcription genes (green), structural genes (red), auxiliary genes (pink) and genes with unknown functions (grey) shown (Herniou *et al*., 2003; Rohrmann 2014).

	Gene product	ORF		Gene product	ORF		Gene[#]	ORF	Gene product[*]
Replication	lef-2	37	Structural	odv-e56	16	Unknown	ac142	13	p49
	lef-1	66		fusion	27		ac115	31	pif-3
	helicase	82		p74	55		ac22	41	pif-2
	dnapol	103		p6.9	78		ac109	50	odv-ec43
Transcription	p47	61		vp39	88		ac119	67	pif-1
	lef-5	79		odv-ec27	89		ac98	80	38k
	lef-4	87		vp91/p95	94		ac96	81	pif-4
	vlf-1	99		gp41	97		ac92	85	p33
	lef-9	109		vp1054	126		ac81	96	-
	lef-8	121	aux	alk exo	114		ac68	106	pif-6

[#]Genes with 'ac' refer to respective ORFs in AcMNPV

[*]Products described for the respective genes in AcMNPV by Rohrmann, (2014)

The percentage similarity between the amino acid sequences for each gene of the reference sequence and that of PhopGV-SA was determined and is shown in **Figure 4.2.** Sixty seven genes were 100 % similar to the reference genes with fifty four genes greater than >98.2 % similar. Five genes in PhopGV-SA showed a higher degree of variation (<98.2 %) to the respective reference genes.

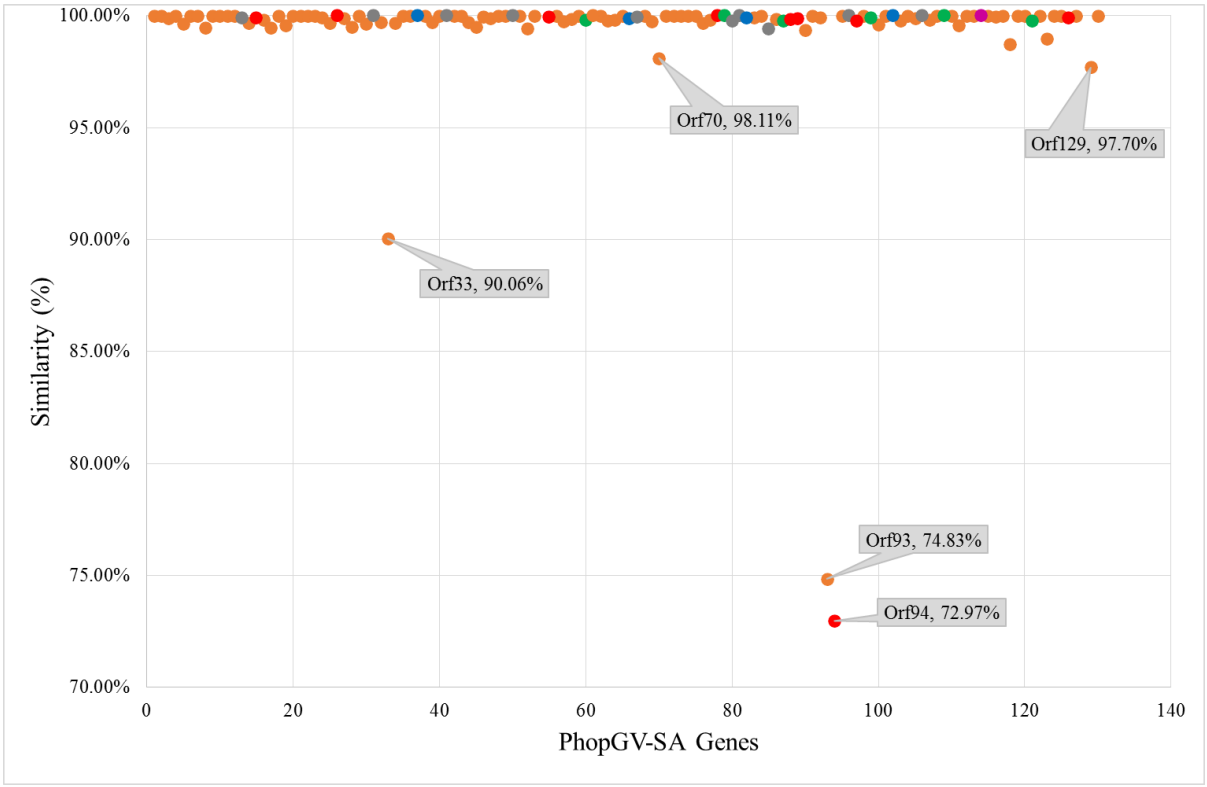


Figure 4.2: Scatter graph of the percentage similarity for the genes in PhopGV-SA to genes in PhopGV-1346. Five outlying open reading frames are shown with the respective percentage and core genes are highlighted (see **Table 4.6**)

The first of these five genes, ORF33 which codes for the *occlusion derived virus envelope protein 66* (odv-e66, ac46), was observed to have two variable regions from nucleotides 29 – 327 and 472 - 483 which were not present in the SA gene sequence while a third region from nucleotides 376 - 381 was not present in the reference gene sequence. These changes in the nucleotide sequence did not disrupt the open reading frame for this gene. The next gene was ORF70 which is predicted to code for a hypothetical protein. Changes in the nucleotide sequence were observed which resulted in amino acids 23 and 24 of the reference isolate to be missing in PhopGV-SA while still maintaining a correct reading frame. The same was found for ORF93 and ORF94 which code for a hypothetical protein and *capsid protein vp91/p95 (ac83)* respectively. Amino acids 64 to 69 were observed to be missing in ORF93 and amino acids 276 - 281 to be missing in ORF94 for PhopGV-SA with both changes maintaining the reading frame. The last gene to show a high degree of variation is ORF129 which codes for *ecdysteroid UDP-glucosyltransferase* and is discussed in greater detail in **Chapter 3**.

4.4. Discussion

To investigate the genome of PhopGV-SA, genomic DNA was extracted from purified OBs and used to produce restriction profiles for the enzymes *Bam*HI, *Hin*dIII, *Nde*I and *Nru*I which were compared against *in silico* profiles of the reference isolate. These reference profiles were produced by computationally digesting the complete genome sequence for PhopGV-1346 for each of the enzymes used producing accurate and reliable reference profiles on which to compare the novel isolate PhopGV-SA. Three of the *in vitro* profiles of PhopGV-SA, the *Bam*HI, *Hin*dIII and *Nde*I profiles, were observed to have similar banding patterns to the *in silico* profiles generated for the reference isolate PhopGV-1346, indicating similarities between the genomes of these isolates.

There are, however, several problems associated with *in vitro* restriction endonuclease profiles which are commonly used as a means of comparing virus isolates. These include difficulties in detecting fragments with low molecular weights, fragments of similar sizes being indistinguishable from one another (referred to as doublet bands, triplet bands, etc.), difficulties in accurately sizing fragments, poor gel resolution, differences in experimental technique and low or high enzyme specificity. Bands identified to be absent in the PhopGV-SA *in vitro* profiles when compared against the PhopGV-1346 *in silico* profiles for the enzymes *Bam*HI, *Hin*dIII and *Nde*I were missing either as a result of low molecular weights such as bands 23 to 25 in the *Hin*dIII profile or to have likely formed doublet bands. Bands which were speculated to form doublets were often brighter than the upper bands, such as band 16 of the *Hin*dIII profile and band 2 of the *Nde*I profile. Conversely, the PhopGV-SA profile generated for the enzyme *Nru*I differed significantly from the profile generated for the PhopGV-1346 with a size discrepancy of 35000 bp observed which could not be accounted for. The likely reason for this is an incomplete digestion of the DNA by the *Nru*I restriction enzyme.

Such a discrepancy can be resolved by determining the full genome sequence of the virus in question using NGS technologies. The genetic novelty of PhopGV-SA was further investigated by producing a preliminary full genome sequence which was assembled with a size of 118000 bp and was used to produce *in silico* profiles for the same set of enzymes used in the *in vitro* analysis. The *in silico* profiles for the SA isolate produced banding patterns with similar sizes and number of bands for the enzymes *Nde*I, *Nru*I and *Bam*HI when compared to the respective *in silico* profiles of the reference isolate. The *Hin*dIII profile also produced similar banding patterns with the SA isolate observed to have an additional sub-molar band. Additionally, bands predicted to be doublet bands in the *in vitro* analysis were all identified in the *in silico* profiles

addressing the differences in genome sizes observed between PhopGV-SA and PhopGV-1346. The differences observed between the *Nru*I *in vitro* profile of PhopGV-SA and the *in silico* profile of PhopGV-1346 were not observed when comparing the *in silico* profiles for both isolates. The differences observed in the *Nru*I *in vitro* profile was likely a result of an experimental flaw such as the overloading of DNA into the reaction. The *in silico* analysis of PhopGV-SA and PhopGV-1346 showed these two isolates to have similar genome organisation which is somewhat unexpected considering the earlier identification of these isolates belonging to egt groups II and I respectively in **Chapter 3**.

The *in silico* profiles produced for PhopGV-SA were further compared to profiles of various isolates available in the literature. A study by Zeddam *et al*., (2013) produced profiles using the enzymes *Bam*HI and *Hin*dIII for the isolate JLZ9f which were identified as having similar banding patterns to the respective profiles for the SA isolate. The *Nru*I and *Nde*I profiles could not be compared due to discrepancies in the *in silico* reference profile for this study. Another study by Espinel-Correal *et al*. (2010) of five Colombian isolates (VG001-VG005) were also analysed with the same set of enzymes used on the SA isolate. The profiles for each of these isolates differed from the respective SA profiles, particularly the *Nde*I profile which had additional bands not present in the *Nde*I profile for the SA isolate. A discrepancy was observed in the *Nru*I profile of PhopGV-1346 in this study with the total size equalling 119207 bp compared to the expected size of 119217 bp and as such was not used in the comparison. The PhopGV-SA profiles were further compared against those in a study by Carpio *et al*. (2013), in which profiles were generated for 7 isolates using the enzyme *Nde*I. Two isolates, PhopGV-1346 and PhopGV-GV6 produced similar profiles to PhopGV-SA. The *Nde*I profile for JLZ9f, which was described as having similar *Bam*HI and *Hin*dIII profiles in the study by Zeddam *et al*., (2013), had an additional band at approximately 4300 bp which was not present in the SA isolate. Lastly, a study by Gómez-Bonilla *et al*. (2012b) using the enzyme *Nde*I for five Costa Rican isolates (CR1-CR5) was compared against the *Nde*I profile of PhopGV-SA. Isolates CR2 and CR5 were observed as having similar profiles to that of the SA isolate. For each of the profiles using *Nde*I mentioned above, the second band likely represents a doublet band as was observed when comparing the *in silico* profiles of PhopGV-1346 with PhopGV-SA. The profiles for PhopGV-JLZ9f, PhopGV-GV6, PhopGV-CR2, PhopGV-CR5 and the reference PhopGV-1346, all of which are South American isolates, were typically more similar to the SA isolate than the profiles of other isolates. This result further supports a South American

origin of PhopGV-SA with the isolate PhopGV-JLZ9f (also an egt II member) possibly representing the closest relative to PhopGV-SA.

The full genome also offered insight into the degree of gene variation between the reference isolate PhopGV-1346 and the SA isolate. Of the 128 genes identified, five were observed to vary compared to the respective genes from the reference isolate. Of these genes ORF94 which codes for the capsid protein vp91/p95 and has also been identified as a core baculovirus gene by Herniou *et al*. (2003), was the most variable out of all the genes when compared to their respective counterparts in PhopGV-1346. This gene codes for a structural protein involved in the nucleocapsid of the baculovirus ODV and forms part of the *per os* infectivity factor (PIF) complex (Rohrmann 2014). Another gene identified which showed variation to that of the respective reference gene was that of ORF33 which codes for the *occlusion derived virus envelope protein 66* (odv-e66). This protein, while not forming part of the baculovirus core genes, was identified by Herniou *et al*. (2003) to be present in all lepidopteran baculoviruses. Also forming part of the PIF complex, odv-e66 is a component of the ODV envelope and has been shown to be involved in recognition or entry into the mid-gut along with regulating cytokines and growth factors (Rohrmann 2014). The PIF complex is a set of factors which have been identified as essential components for infection of host insects (Rohrmann 2014), with the variations observed in vp91/p95 and odv-e66 likely evolutionary responses to PhopGV-SA infecting a South African population of *P. operculella* compared to PhopGV-1346 which infects a Tunisian population of the pest. The remaining two genes, ORF70 and ORF93 are both hypothetical proteins with unknown function with the increased variation observed promoting the idea that these genes are important factors in baculovirus biology.

In conclusion, the novel PhopGV-SA isolate was further characterised by REN analysis of genomic DNA and assembly of a preliminary full genome sequence. A comparison of the genes present in PhopGV-SA and PhopGV-1346 was completed resulting in the identification of five genes with variation. The following chapter describes the development of bioassays to evaluate the biological activity of PhopGV-SA virus against neonate *P. operculella* larvae in a laboratory setting.

Chapter 5

Development of a biological assay to evaluate the virulence of PhopGV-SA against neonate larvae

5.1. Introduction

PhopGV-SA was shown to be genetically similar to other PhopGV isolates in chapters 3 and 4, however, it can be further characterised through evaluation of its biological activity in order to determine its virulence against the host organism. This is an essential step in developing the virus into a biopesticide, providing valuable information for future formulation and application. Whilst many PhopGV isolates have been evaluated, with one strain formulated into the commercial biopesticide Matapol® (Moscardi 1999), the continued evaluation of novel isolates and their development into biopesticides may prove necessary should resistance occur in target insect populations towards biopesticide already in use.

The virulence of different PhopGV isolates towards *P. operculella* neonate larvae has been widely investigated in bioassays using two inoculation methods, namely, surface dose and egg dipping. Surface dose bioassays either involve the dipping of tubers or leaf material into either liquid or powder formulations (Broodryk and Pretorius, 1974; Sporleder *et al*., 2007; Arthurs *et al*., 2008; Zeddam *et al*., 1999; Mascarin *et al*., 2010) or through application of the virus by way of a nebulizer apparatus which sprays a controlled concentration of OBs onto the plant/tuber surface (Gómez-Bonilla *et al*., 2012b; Carpio *et al*., 2013; Espinel-Correal *et al*., 2012; Espinel-Correal *et al*., 2010; Gómez-Bonilla *et al*., 2011; Zeddam *et al*., 2013). Egg dipping involves the immersion of insect egg sheets in concentrations of virus suspensions, and thus, exposing larvae to the virus during hatching (Sporleder *et al*., 2005).

Both the surface dose and egg dipping methods are employed to evaluate the virulence of baculovirus isolates and is measured by the lethal concentration of virus required to achieve 50 % mortality (LC_{50}) of the target pest. Previous surface dose bioassay studies of PhopGV isolates on *P. operculella* through the application of OBs using a nebulizer have reported LC_{50} values ranging between 7 and 30 OBs per mm^2 (Gómez-Bonilla *et al*., 2012b; Carpio *et al*., 2013; Espinel-Correal *et al*., 2012; Espinel-Correal *et al*., 2010; Gómez-Bonilla *et al*., 2011; Zeddam *et al*., 2013). Surface dose bioassays involving the dipping of tubers into PhopGV solutions have reported LC_{50} values of 4.11×10^4 OBs per ml (Mascarin *et al*., 2010) and

between 15.6 and 18.5 larval equivalents per 100 ml (Zeddam *et al*., 1999). A study by Sporleder *et al*. (2005) using the egg dipping method reported an LC_{50} of 5×10^6 OBs per ml.

The aim of this chapter was to develop an egg dipping protocol to determine the LC_{50} value of PhopGV-SA on *P. operculella* neonate larvae. The first specific objective was to test a range of virus doses and analyse the data by probit analysis in order to determine the LC_{50} value. The second objective was to recover OBs from larvae which survived the bioassay and attempt to amplify the *egt* sequence to determine either the presence or absence of virus.

5.2. Materials and Methods

5.2.1. Dose preparation for a neonate egg dip bioassay

A virus stock solution was prepared by adding 100 µl of purified OBs (batch three described in **Section 2.3.4**) with a concentration of 3.48×10^{10} OBs.ml^{-1} to 400 µl ddH_2O creating a 1:5 dilution. The 1:5 dilution was further diluted by added 400 µl to 1600 µl 1 % SDS (w/v) to produce a 1:25 dilution. A final dilution was made by adding all 2000 µl of the 1:25 into 6000 µl ddH_2O to produce a 1:100 virus stock suspension, referred to as dose 1 (D1) with a concentration of 3.48×10^8 OBs.ml^{-1}. Three ten-fold serial dilutions were made from D1 producing four additional doses, namely, dose 2 (D2) at 3.48×10^7 OBs.ml^{-1}, dose 3 (D3) at 3.48×10^6 OBs.ml^{-1} and dose 4 (D4) at 3.48×10^5 OBs.ml^{-1} (**Figure 5.1)**. These dilutions were prepared by adding 100 µl of D1 into 900 µl ddH_2O, producing D2. D2 was mixed before adding 100 µl to 900 µl ddH_2O, producing D3 with this procedure being repeated to produce D4.

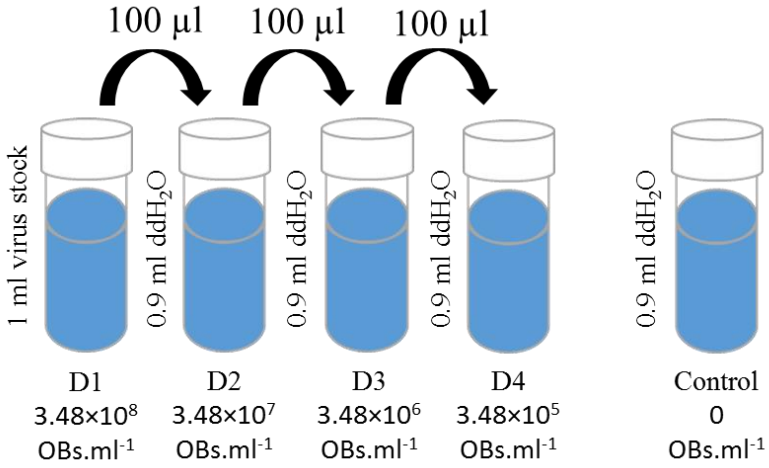


Figure 5.1: Diagram of PhopGV-SA dose preparation with three ten-fold dilutions prepared from a 3.48×10^8 OBs.ml^{-1} virus stock sample. D1 – dose 1, D2 - dose 2, D3 - dose 3, D4 - dose 4. A control dose was prepared with 0.9 ml ddH_2O.

5.2.2. Egg dip bioassay with neonate larvae

Freshly laid egg sheets were collected from the *P. operculella* laboratory colony and 18 sections of filter paper, with 30 eggs each, were carefully selected and cut from the rest of the egg sheet using a Leica (Switzerland) EZ4D microscope. Each section of filter paper was large enough to be handled without disrupting the eggs as well as ensuring that the eggs were not clustered together. The sections were divided among five sterile petri dishes, three per dish, with each petri dish labelled D1, D2, D3, D4 and control respectively.

Three samples of virus suspensions were prepared for each dose, with each sample being applied to one of the three sections of eggs in the corresponding petri dish. Egg sheets in the different petri dishes represent the different doses with each egg sheet in a single petri dish representing one of three replicates for that specific dose. Egg sheets in the fifth petri dish were inoculated with ddH_2O and represent the control replicates. Egg sheets were inoculated for 15 min with excess liquid removed before being left to dry. One random egg sheet from each replicate and the three control egg sheets, were re-examined under a microscope to ensure that all 30 eggs were still present.

Five containers were prepared by adding a 1 cm layer of pearlite on which 60 g of washed baby potatoes (pierced with a clean pin) were placed. An egg sheet which had been dipped in D1 was carefully pinned to the surface of a potato in one of the five containers and the container was correspondingly labelled to the dose for that egg sheet. This was repeated for each of the remaining three doses and the control with all five containers forming replicate one. This process was repeated for both replicates two and three. Mesh cloth was used to cover each container and the experiment was placed in a controlled environment at ±25 °C with a relative humidity of approximately 45 %. The bioassay was evaluated by determining the survival of larvae by counting those which emerged from the potatoes in each container. These larvae were collected and stored at -20 °C in 1.5 ml tubes for further evaluation by way of PCR amplification. All three replicates were run and monitored regularly for 30 days with temperature and humidity readings taken weekly basis.

5.2.3. Data analysis and calculations

A probit analysis of the data obtained from the egg dip dose response bioassays was performed using Proban statistical software (Van Ark, 1995). For each replicate, dose D4 was excluded due to mortality rates observed to be lower than those of the control's with the remaining doses transformed to $\log_{10}$ and percentage mortality to empirical probits. Abbott's formula was used

to calculate the corrected mortality by using the percentage mortality observed in the control and each treatment for all three replicates (Abbott, 1925).

$$Corrected\ mortality\ (\%) = \frac{Treatment\ mortality\ (\%) - Control\ mortality\ (\%)}{100 - Contol\ mortality\ (\%)} \times 100$$

Equation 5.1: Abbott's formula for the calculation of corrected mortality

Regression lines obtained were analysed by Bartlett's test to determine whether they were comparable with the Chi squared value, degrees of freedom and approximate probability recorded. For lines which were comparable the mean LC_{50} and LC_{90} values and the respective upper and lower fiducial values were calculated.

5.2.4. PCR amplification of the *egt* gene

To test for the presence or absence of virus, OBs were extracted from 15 individual larvae collected from each treatment in all three replicates. Each larva was macerated in 500 µl 0.1 % SDS (w/v) in a 2 ml tube before centrifugation at 400 ×g for 30 sec. The supernatant was collected in a new 2 ml tube and the pellet suspended in 500 µl 0.1 % SDS (w/v) and centrifuged at 400 ×g for 30 sec. The supernatant was collected and combined with the previously collected supernatant. The pellet was suspended and centrifuged a further two times with the supernatant collected and combined each time. The combined supernatant was centrifuged at 10000 ×g for 30 min. The resultant supernatant was discarded and the pellet suspended in 100 µl ddH_2O, producing a final OB extract for each larvae treated.

Genomic DNA was extracted using the CTAB method described in **Chapter 3**, **section 3.2.1**, and stored at -25 °C. PCR amplification of the *egt* gene was performed as described in **Chapter 3**, **section 3.2.2** and **Table 3.2**. Amplicons were visualised by way of 1 % agarose gel electrophoresis at 90 V for 120 min in 1X TAE and stained with ethidium bromide. Gel images were captured on a ChemiDoc™ XRS+ system (Bio-Rad Laboratories, Inc. USA) with the Image Lab (v5.1) software (Bio-Rad Laboratories, Inc. USA).

5.3. Results

5.3.1. Egg dip dose-response bioassay

An egg dip dose-response bioassay was performed on 30 larvae for four virus dosages ranging from 3.48×10^8 OBs.ml^{-1} to 3.48×10^5 OBs.ml^{-1} (D1-D4) and a control. Three replicates were performed and the experiment was monitored for 30 days and evaluated by the emergence of larvae from the potatoes. The percentage mortality for D4 was observed to be lower than that

of the control for all three replicates and, consequently, excluded from the Proban analysis (**Figure 5.2)**. Mortality was observed to increase with increasing concentration of virus with each replicate in the highest dose showing considerable variation.

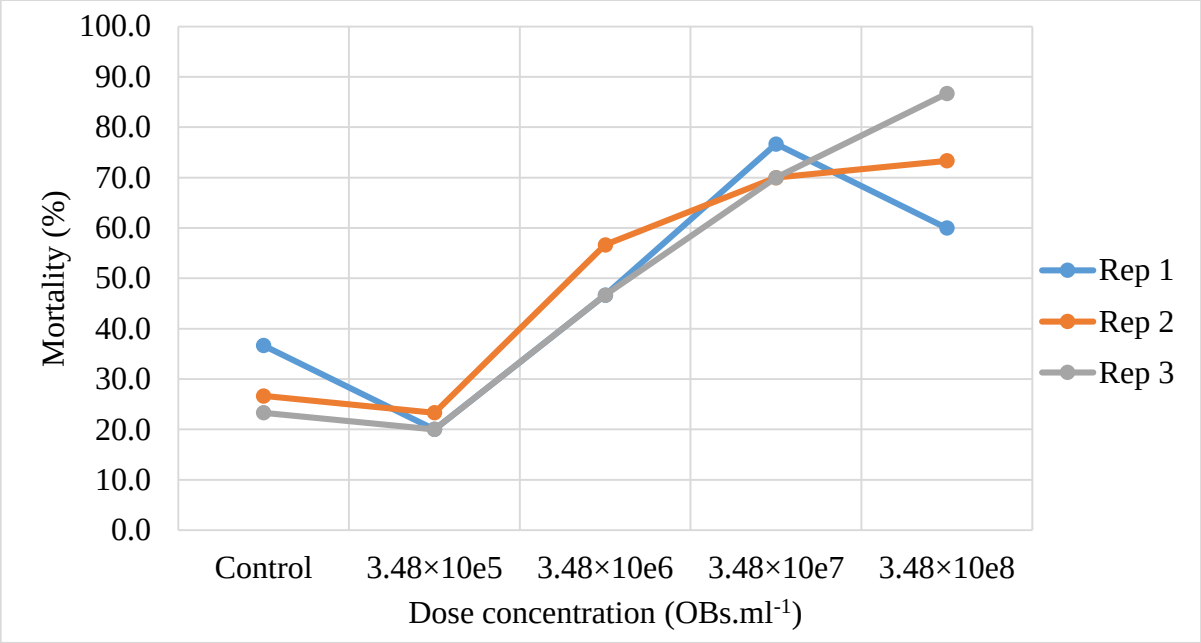


Figure 5.2: *Phthorimaea operculella* neonate mortality once exposed to suspensions of PhopGV-SA for three replicates in an egg dip dose response bioassay.

The first replicate caused a percentage mortality of between 20 and 80 % (**Table 5.1)**. For the lowest dose, D4, a percentage mortality of 20 % was recorded compared to the control at 36.7 %. The corrected mortality for D4 was -26.3 % once transformed using Abbott's formula. Consequently, D4 was excluded from the Proban analysis. Deviations for the regression line were determined to be heterogeneous with G for the fiducial limit calculated to be ≥ 1 with no fiducial limits calculated for replicate 1.

Table 5.1: *Phthorimaea operculella* neonate mortality for replicate 1 of the egg dip dose response bioassay exposed to suspensions of PhopGV-SA

	Dose and concentration (OBs.ml^{-1})	**Number exposed**	**Larval mortality**	**Mortality (%)**	**Corrected mortality (%)**
C	0	30	11	36.7	0.0
D4	3.48×10^{5}	30	6	20.0	-26.3
D3	3.48×10^{6}	30	14	46.7	15.8
D2	3.48×10^{7}	30	23	76.7	63.2
D1	3.48×10^{8}	30	18	60.0	36.8

The second replicate was determined to have a percentage mortality of between 23.3 % and 73.3 % (**Table 5.2)**. As was observed in the first replicate, the lowest dose D4 resulted in a percentage mortality of 23.3 % when compared to the control at 26.7 %. The corrected mortality for D4 was -4.5 % and was consequently excluded from the Proban analysis. Deviations for the regression line were determined to be heterogeneous with G for fiducial limit calculated to be ≥1 with no fiducial limits calculated for replicate 2.

Table 5.2: *Phthorimaea operculella* neonate mortality for replicate 2 of the egg dip dose response bioassay exposed to suspensions of PhopGV-SA

Dose and concentration (OBs.ml^{-1})		Number exposed	Larval mortality	Mortality (%)	Corrected mortality (%)
C	0	30	8	26.7	0.0
D4	3.48×10^{5}	30	7	23.3	-4.5
D3	3.48×10^{6}	30	17	56.7	40.9
D2	3.48×10^{7}	30	21	70.0	59.1
D1	3.48×10^{8}	30	22	73.3	63.6

The third replicate was determined to have a percentage mortality ranging from 20 % to 86.7 % (**Table 5.3)**. As was observed in the first and second replicates for the lowest dose, D4 resulted in a percentage mortality of 20 %, lower than that of the control at 23.3 %. A corrected mortality of -4.3 % was calculated for D4 which was consequently excluded from the Proban analysis. Deviations for the regression line were determined to be homogeneous with G calculated to be 0.3903. Although G was determined to be high by the Proban statistical software the fiducial limits were calculated for replicate 3 (see **Table 5.5** below). The equation for the regression line was $y = 0.7204x + (-0.1905)$ with the standard error of the slope equalling 0.2296.

Table 5.3: *Phthorimaea operculella* neonate mortality for replicate 3 of the egg dip dose response bioassay exposed to suspensions of PhopGV-SA

Dose and concentration (OBs.ml^{-1})		Number exposed	Larval mortality	Mortality (%)	Corrected mortality (%)
C	0	30	7	23.3	0.0
D4	3.48×10^{5}	30	6	20.0	-4.3
D3	3.48×10^{6}	30	14	46.7	30.4
D2	3.48×10^{7}	30	21	70.0	60.9
D1	3.48×10^{8}	30	26	86.7	82.6

The regression lines for all three replicates were compared with the residual variance and determined to be homogenous, with the chi square test for this comparison calculated to be 2.651 with two degrees of freedom and an approximate probability of 0.2650. These lines were determined to be parallel, allowing for comparison of the elevations with no significant difference determined. The mean fiducial limits were not calculated as no limits were obtained for replicate 1 and 2 as a result of these having G ≥1. The results for the three replicates were used to calculate the mean LC_{50} and a predicted LC_{90} for PhopGV-SA shown in **Table 5.4**.

Table 5.4: Mean LC_{50} and LC_{90} of PhopGV-SA on *Phthorimaea operculella* neonate larvae

Lethal concentration	Concentration (OBs.ml$^{-1)}$
LC_{50}	1.87×10^{8}
LC_{90}	6.4×10^{13}

The average LC_{50} and LC_{90} for the three replicates was calculated to be 1.87×10^{8} and 6.4×10^{13} respectively.

The LC_{50} and a predicted LC_{90} values for replicate 3 are shown in **Table 5.5** with the upper and lower fiducial limits determined due to G < 1.

Table 5.5: The LC_{50} and LC_{90} for replicate three of PhopGV-SA on *Phthorimaea operculella* neonate larvae

Lethal concentration	Concentration (OBs.ml$^{-1)}$	Fiducial limits	
		Lower	Upper
LC_{50}	1.6×10^{8}	1.75×107	4.97×10^{8}
LC_{90}	9.6×10^{10}	2.05×10^{9}	2.9×10^{12}

5.3.2. Evaluation of controlled environment

The controlled environment in which the egg dip dose response bioassay was performed was monitored for the entire duration of the experiment. The temperature at the time of measurement (weekly on Monday) along with the maximum and minimum value for each week are shown in **Figure 5.3.**

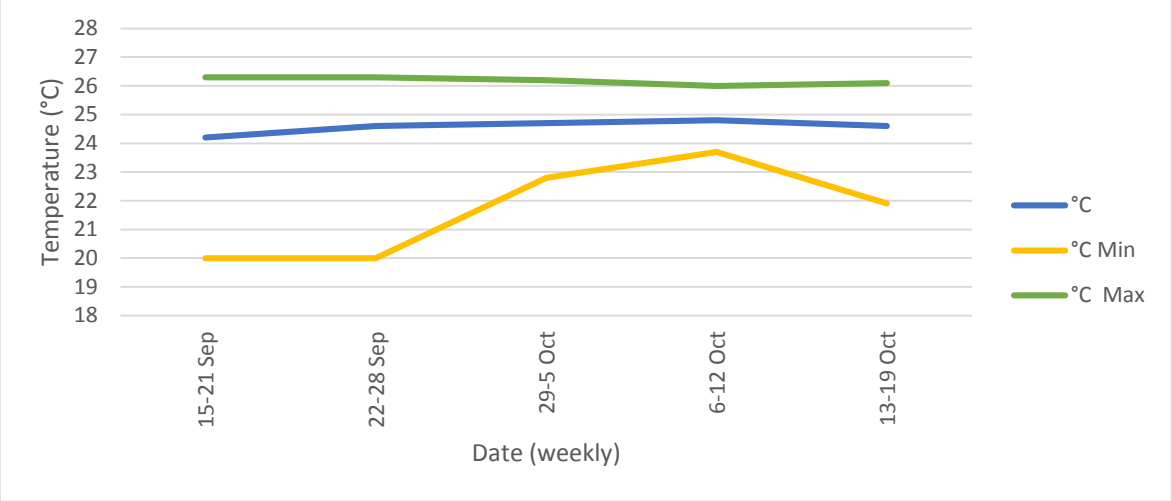


Figure 5.3: Graph showing the temperature of the controlled environment room with the °C indicating the values at the time of measuring and the min and max values indicating weekly variation for the duration of the egg dip bioassay.

The temperature at the time of measurement for the controlled environment remained relatively constant for the duration of the study. The maximum temperature did not exceed 27 °C while the minimum temperature showed slightly more variation within the first two weeks with temperatures as low as 20 °C.

The relative weekly humidity along with the maximum and minimum value for each week are shown in **Figure 5.4.**

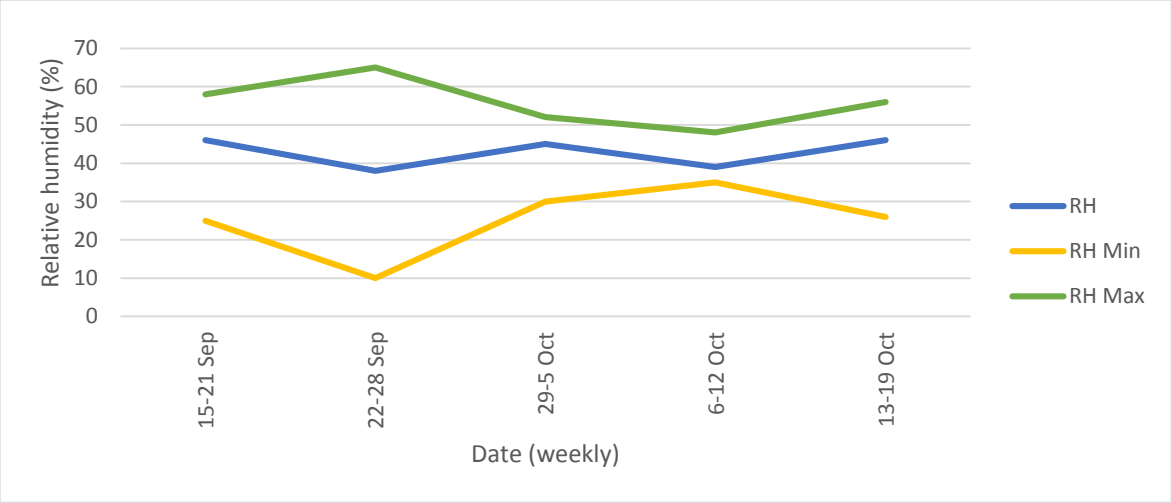


Figure 5.4: Graph showing the relative humidity of the controlled environment room with RH indicating the values at the time of measuring and the min and max values indicating weekly variation for the duration of the egg dip bioassay.

The relative humidity at the time of measurement for the controlled environment remained relatively constant, ranging between 40 % and 50 % for the duration of the study. The highest

and lowest relative humidity values were both recorded in the second week having reached a maximum of 65 % and a minimum of 10 %. For the remainder of the experiment the relative humidity remained between 25 and 60 %.

5.3.3. PCR amplification of *egt*

As mentioned in **section 5.2.2**, bioassays were evaluated by determining the number of larvae which survived exposure to the various virus treatments by collecting and counting those which emerged from the potatoes. A difference in external appearance and response to external stimuli was observed between *P. operculella* larvae which had been exposed to PhopGV-SA compared to those which had not been exposed (**Figure 5.5**).

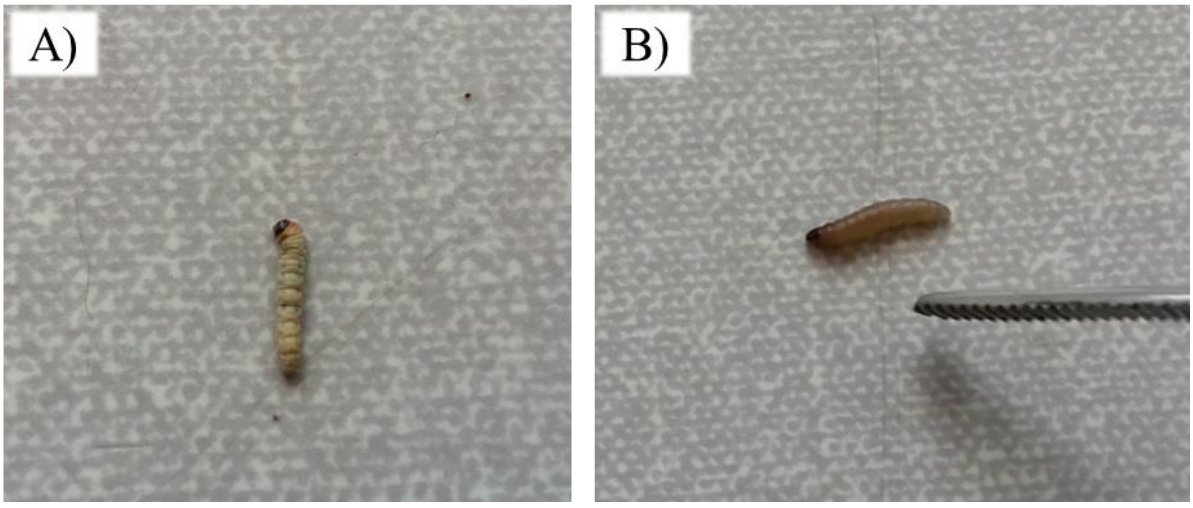


Figure 5.5: Comparison of *Phthorimaea operculella* larvae A) exposed to PhopGV-SA and B) with no exposure to PhopGV-SA

Several larvae collected from the various bioassay treatments were unresponsive to external stimuli, having a flaccid milky white appearance (**Figure 5.5A)**. This differed to the larvae maintained in the colony which were brownish green in appearance and vigorously responded to external stimuli (**Figure 5.5B**). OBs were recovered from single larvae collected for each of the virus treatments and the control across all three bioassay replicates (total of 15). DNA was extracted from OBs and was used as template to amplify the PhopGV *egt* gene (**Figure 5.6**). This was done to determine whether PhopGV was present in the larvae collected from the bioassay.

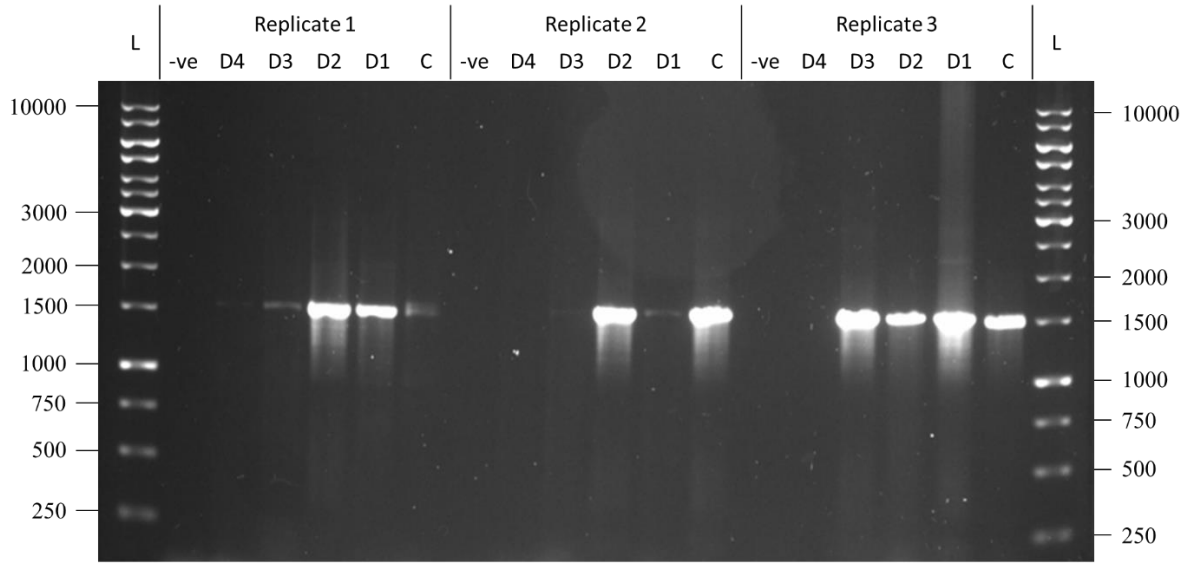


Figure 5.6: PCR amplification of PhopGV *egt* from larvae collected from the egg dip dose response bioassay replicates 1, 2 and 3. L = GeneRuler 1Kb DNA ladder; -ve = no template control; D4 = dose 4; D3 = dose 3; D2 = dose 2; D1 = dose 1; C = control.

Amplicons were present in samples recovered from 13 of the 15 larvae collected from the egg dip dose response bioassay. All five samples recovered from larvae in replicate 1 produced an *egt* amplicon, including the sample recovered from the control larvae indicating the presence of PhopGV. No amplicon was observed in the no template control lane for replicate 1. Samples recovered from larvae in replicate 2 produced 4 egt amplicons including the sample recovered from the control larvae indicating the presence of PhopGV in these larvae. No amplicon was observed in the no template control or the sample recovered from the larvae exposed to dose 4. Lastly, four *egt* amplicons were observed in samples recovered from larvae in replicate three, including the sample recovered from the control larvae indicating the presence of PhopGV. No *egt* amplicon was observed in the no template control or sample recovered from the larvae exposed to dose 4.

5.4. Discussion

In this chapter, a biological assay to evaluate the virulence of PhopGV-SA against *P. operculella* was developed by using an egg dipping dose response method similar to that of Sporleder *et al*. (2005). Several attempts at evaluating the virulence of PhopGV-SA were made with a variety of difficulties experienced. Initially, two bioassays were prepared using surface dose and egg dipping methods with these evaluated by counting the number of moths which emerged. A rapid emergence of a second generation was observed within the bioassays with the number of moths counted exceeding the initial amount of larvae exposed. This was caused

by moths laying a second generation of eggs shortly after emerging, with these eggs progressing through to adulthood within the period of evaluation. Due to an inability to accurately evaluate these bioassays another attempt was made with evaluation once again based on the number of moths recovered. To avoid an emergence of a second generation, funnels were used to allow moths to exit the assay before eggs were laid. This attempt was hindered by the sudden occurrence of a fungal outbreak. Finally, a bioassay was developed using the egg dipping method and evaluated by counting larvae which emerged from the potatoes in an attempt to avoid the occurrence of a second generation. The results of this attempt, hereinafter referred to as the egg dip dose response (EDDR), were reported in this chapter albeit with a variety of associated problems.

Of the four doses evaluated in EDDR, D4 consistently produced a lower mortality than the control experiments within each replicate. This data was therefore excluded from the statistical analysis. A mean LC_{50} of 1.87×10^{8} OBs.ml^{-1} was calculated from the data of the remaining three doses, however, the upper and lower fiducial limits were not successfully determined. This may be due to variation observed within the data obtained for dose 1. The fiducial limits were only obtained from analysis of replicate 3 with an LC_{50} of 1.6×10^{8} OBs.ml^{-1} and limits of 1.75×10^{8} to 4.97×10^{8}. The LC_{50} values obtained for PhopGV-SA are higher than those obtained in other studies such as Sporleder *et al*. (2005) where the LC_{50} of a Peruvian isolate was 5×10^{6} OBs.ml^{-1} and Mascarin *et al*. (2010) where the LC_{50} of a Brazilian isolate was 4.11×10^{4} OBs.ml^{-1}. These results suggest that PhopGV-SA is less virulent than South American isolates of PhopGV prompting further analysis of the bioassay to ensure that the results obtained in EDDR are reliable.

Larvae collected from the bioassay were observed to have a flaccid milky white appearance and were often unresponsive to external stimuli. These observations match the symptomology of PhopGV in *P. operculella* described by Laarif *et al*. (2006) and indicated the presence of virus in the larvae collected. PCR analysis of genomic DNA from OBs recovered from the larvae collected in EDDR produced *egt* amplicons for 13 of the 15 samples. This indicates the presence of PhopGV in these larvae and consequently shows that the LC_{50} values determined are an underestimation for the following reasons. Firstly, the bioassay was evaluated by the number of larvae which survived exposure to PhopGV-SA and the presence of PhopGV in these larvae indicates that many of these larvae were pre-emptively collected and may have succumb to the virus given more time. Had these larvae succumb to the virus, percentage mortality would have been higher for doses 1 to 4 and a lower LC_{50} value would have been

obtained. Secondly, virus was also detected in control larvae in all three replicates even though they had not been exposed. This indicates that the high levels of mortality observed in the control larvae may have been the result of virus infection consequently skewing the results as all data is corrected based upon control mortality. The control is used to determine the percentage of mortality which occurs due to factors other than exposure to the virus in question. The absence of virus in control larvae would tend to lower percentage mortality in the control and therefore result in a lower, but more accurate LC_{50} value estimate.

In conclusion, an egg dipping bioassay was developed to evaluate the biological activity of PhopGV-SA against neonate larvae. Preliminary LC_{50} values were obtained and may be underestimated due to the presence of virus in the control larvae collected. The next chapter discusses the significant results of this study and their relevance to the management of *P. operculella* in South Africa.

Chapter 6

General discussion

6.1. Conclusions

The aim of this study was to recover a baculovirus from field collected *P. operculella* which could further be developed into a biopesticide for the control of this pest on both field and stored potato tubers. The objectives included the establishment of a *P. operculella* colony, collection of larval cadavers and purification of virus particles, the morphological and genetic characterisation of the virus and, lastly, an evaluation of the virulence of the virus against neonate *P. operculella* larvae.

The establishment of a laboratory colony of the host insect was a major objective and was successfully achieved with larvae reared on whole baby potatoes in a controlled environment. The use of whole potatoes as diet was adequate for the establishment of a laboratory colony which was maintained for the full duration of this study. Challenges were encountered when using whole potatoes which primarily affected the outcome of the bioassays. Firstly, without adequate ventilation, moisture quickly built up within the rearing containers which often resulted in fungal outbreaks. This was observed in the second bioassay where containers were designed to trap moths by using a funnel system which inadvertently reduced ventilation. Secondly, the use of whole potatoes presented difficulties in evaluating bioassays as larvae may succumb to the virus before exiting the tuber. Larval cadavers, in particular early instars, are extremely difficult to recover from within the tubers and therefore, bioassays were evaluated by survival rather than mortality. Lastly, potatoes may introduce uncontrollable variables into studies such as contaminants (bacterial or fungal), unwanted insecticide residues or differences in nutritional values which may affect the outcome of experiments. One solution to this problem in future experiments is the use of a sterile artificial diet which can be standardised to ensure consistent nutritional value and reduce potential contamination. An artificial diet for *P. operculella* has been described although differences in life cycle duration and percentage survival were observed when compared to a leaf diet (Kashyap *et al*., 2008). For mass production of a virus as a biopesticide on a commercial scale, the use of an artificial diet is essential as natural diets, such as whole potatoes, may introduce risks such as high cost,

ease of contamination, lack of reproducible results and difficulty in recovering larval cadavers (Moore *et al*., 2014).

Another major objective was the isolation of a baculovirus from larvae collected from the laboratory colony. In retrospect, it was determined that the colony was unintentionally overcrowded when it was first established due to an underestimation of the number of eggs added to the rearing containers. A potential covert baculovirus in the population may have become overt due to this stress factor resulting in larval mortality and the subsequent isolation of the virus (Burden *et al*., 2003; Opoku-Debrah *et al*., 2013). Transmission electron micrographs of the virus revealed GV like occlusion bodies in purified samples.

Genetic characterisation of the GV, PhopGV-SA, was achieved using the *lef*-8, *lef*-9 and -*granulin* gene sequences as described by Lange *et al*., (2004). This virus is the first confirmed PhopGV isolate to be identified in southern Africa following an earlier isolation of a GV from *P. operculella* by Broodryk & Pretorius, (1974), which was not genetically characterised. The virus was further genetically characterised using the *egt* gene, as this gene has been shown to have considerable variation between PhopGV isolates. Previous studies by Espinel-Correal *et al*., (2010), Carpio *et al*., (2013) and Gómez-Bonilla *et al*., (2012b) identified several types of *egt* genes with each differing as a result of an internal deletion/insertion event in the 3' region of the gene. In total, five egt types were identified in this study using gene data from PhopGV-SA and GenBank, with PhopGV-SA belonging to the egt type II group previously described by Carpio *et al*., (2013). Furthermore, based upon sequence data for the flanking regions of the *egt* gene from PhopGV-SA, it was determined that *egt* types I, III and IV diverged from type II as a result of large deletion events suggesting that the type II gene represents the prototype structure. Interestingly, the *egt* genes from the closely related AdorGV and CrleGV have similar lengths to the type II gene in PhopGV and each may represent the earlier ancestral form of the gene.

While another PhopGV isolate with an *egt* type II gene is present on the African continent in Kenya, a greater amount of SNPs were observed between it and the South African isolate than between the South African and South American isolates. Coupled with observations from the REN analysis which showed that the DNA profiles for PhopGV-SA were similar to those of South American isolates, particularly PhopGV-1346 and PhopGV-JLZ9f, these results indicate a South American origin of PhopGV-SA rather than an African origin. Regarding biopesticide application in South Africa, closely related South American PhopGV isolates or those already

developed into commercial biopesticides such as Matapol® could potentially be imported now that the virus has been isolated and identified in South Africa.

While imported PhopGV isolates may offer protection of crops from *P. operculella*, a complete genome analysis of PhopGV-SA showed variation in PIF genes including *vp91/p95* and *odv-e66* when compared against PhopGV-1346. These genes encode proteins which control oral infectivity and the variation observed may represent adaptations towards a South African host population. With this in mind, it is possible that imported isolates may be less virulent to a South African population of *P. operculella* than against their native population due to these genetic differences. The LC_{50} values obtained for PhopGV-SA against neonate larvae in this study were observed to be higher than that of South American isolates reported by Sporleder *et al.*, (2005) using the egg dip method. However, due to problems encountered whilst performing bioassays, it is likely that the LC_{50} values for PhopGV-SA were underestimated and further analysis and improved bioassay design may result in LC_{50} values closer to those of the South American isolates.

In summary, this study has contributed towards the field of baculovirus biology and evolution by identifying and genetically and biologically characterising a novel South African PhopGV isolate. Moreover, given that South Africa is at the forefront of biopesticide development and application (Moore *et al*., 2004; Kessler & Zingg 2008), this study lays the foundation for further and improved control of *P. operculella*, an important pest of one of the country's most economically important crops.

6.2. Future work

The incorporation of an artificial diet is highly recommended for future work on *P. operculella*. The artificial diet described by Kashyap *et al*., (2008) could be used for both the establishment of future *P. operculella* laboratory colonies and in bioassays which could be adapted if necessary. An improved bioassay method is required to obtain a more precise LC_{50} value and determine the LC_{90} and lethal time at 50 % and 90 % (LT_{50} and LT_{90}) values. The incorporation of a virus free insect colony, a modified method for evaluating the assay, artificial diet and further use of the egg dipping method is recommended. The virulence of PhopGV-SA should also be evaluated against later instars (i.e. fourth instar larvae) in future bioassays to determine whether larval development alters insect susceptibility to the virus as was observed by Sporleder *et al*., (2007). The results from these bioassays will assist in continued research and development of PhopGV-SA into a commercial biopesticide. Future studies should include

field trials and trial on stored potatoes as well as investigations into formulation and application. For example, a study by Gómez-Bonilla *et al*. (2012a) tested the use of a PhopGV isolate to protect both stored and field potatoes from *P. operculella* and *T. solanivora* with the application of powdered formulations to tubers resulting in a significant reduction in injury when compared with non-treated tubers.

Regarding the genetic analysis of PhopGV-SA, PCR amplification and sequencing of ORF54 and ORF128 is required to complete the genome sequence. PCR amplification and sequencing of regions in PhopGV-SA which were observed to vary to the reference isolate PhopGV-1346 genome sequence and regions with low coverage should also be performed to ensure the reliability and accuracy of results.

Lastly, it is essential to continue bioprospecting for novel isolates of PhopGV in South Africa from geographically distinct populations of *P. operculella*, with each requiring genetic and biological characterisation. Biological characterisation of PhopGV isolates should involve the evaluation of virulence to each geographic population of *P. operculella* in determining whether certain populations are more or less susceptible. Furthermore, the use of different isolates should be carefully evaluated and considered in order to manage resistance arising towards PhopGV based biopesticides.

Chapter 7

Reference Materials

7.1. References

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