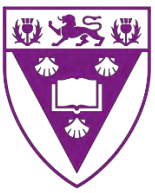

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The isolation, identification, and characterisation of novel insect viruses for the control of emerging and existing agricultural pests

Submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN SCIENCE

at

RHODES UNIVERSITY

By

Tahnee Tashia Bennett

Declaration

I, **Tahnee Tashia Bennett (16B4622)**, confirm that this thesis is my own original work and is submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy. It has not been submitted for the assessment of any degree at any other university, institution, or external organisation.

Author's Signature

01/07/2025

Date

Abstract

The intensification and expansion of agriculture are among the most prominent global changes driven by human activity. As the human population continues to grow, one of the major global challenges is ensuring adequate food production. Insects play an important role in crop production because of the ecosystem functions that they provide. The ecosystem functions that insects provide are often disrupted in agricultural ecosystems. The modification of habitats by humans, such as selecting crops for higher yield, larger size or those that have higher nutritional value, and the cultivation of monocultures for maximum productivity, all contribute to the emergence of insect pests. Additionally, changing climatic conditions or changes in farming practices may result in changes in the status of certain pests in different agricultural crops. In various studies, viruses that are harboured by several agriculturally important pests in South Africa, including *Plutella xylostella*, *Phthorimaea operculella* and *Thaumatotibia leucotreta*, have been isolated and biologically characterised, and some are now commercially available as biopesticides. In addition, the RNA virus family *Dicistroviridae* are viruses that infect arthropods. An example of a virus from this family is the Cricket paralysis virus (CrPV), which was first isolated from Australian field crickets. This virus is known to infect both crickets and fruit flies. As such, the use of this virus may find application in the management and control of fruit flies. To safeguard crop production in South Africa, continued bioprospecting for novel biological control options, particularly viruses, against major, minor, and emerging insect pests is important. The aim of this thesis was to isolate, identify, and characterise novel viruses from various insect pests that already impact, or have the potential to impact one or more important crops.

Serrodes partita (fruit-piercing moth), *Thaumatotibia batrachopa* (macadamia nut borer), *Ectomyelois ceratoniae* (carob moth), and *Acheta domesticus* (house cricket) were the selected species for analysis in this study, because each species contributes meaningfully to the agricultural ecosystem. Species identification of these species was conducted using molecular genetic techniques. Genomic DNA was extracted from the larval samples of *S. partita*, *T. batrachopa*, and *E. ceratoniae*, and the adults of *A. domesticus*. Thereafter, PCR amplification of the *Cytochrome c oxidase subunit I* (*COI*) gene was evaluated. Sequencing and BLAST analysis confirmed the identity of each species.

There have been no reports describing the isolation of viruses from *S. partita*, *E. ceratoniae*, and *T. batrachopa*. Isolating novel baculoviruses from these lepidopteran species holds

potential for their use as microbial control agents and for their development as biopesticides. Symptomatic larval cadavers of *S. partita*, *T. batrachopa*, and *E. ceratoniae* were collected, and baculovirus occlusion bodies (OBs) were purified from these samples. Genomic DNA (gDNA) was extracted from the purified OBs and was used for PCR amplification of the *polyhedrin*/*granulin* (*polh*/*gran*) gene from multiple samples, and the *late expression factor 8* (*lef-8*) and *late expression factor 9* (*lef-9*) genes from select samples. Sanger sequencing of the PCR amplicons was conducted, followed by bioinformatic analysis. Sequencing results suggest that a novel baculovirus has been isolated from *S. partita* larvae. The virus was identified as an *Alphabaculovirus*, and was basal to a monophyletic clade which includes species such as Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) and Lymantria xylina nucleopolyhedrovirus (LyxyNPV), with Kimura two-parameter distance matrices indicating this virus to be a novel species, hereafter referred to as SepaNPV. Whole genome Illumina sequencing was conducted using the SepaNPV genomic DNA, and a *de novo* assembly was conducted to reassemble the genome sequence, using the generated Next-generation sequencing (NGS) data. Results indicate a genome size of 129953 bp, with a GC content of 54.9 %, and a preliminary annotated genome with approximately 128 open reading frames (ORFs). Additionally, infection assays were conducted to test the host range of SepaNPV against four potential host species. Data indicates that *T. leucotreta, Helicoverpa armigera, Spodoptera frugiperda* and *Eldana saccharina* are not susceptible. Multiple *T. batrachopa* larvae were also evaluated and shown to be infected with Cryptophlebia leucotreta granulovirus (CrleGV).

Furthermore, the final component of the study was to develop a screening method to detect the presence of CrPV in *A. domesticus* samples. A plasmid-based two-step reverse transcription polymerase chain reaction (RT-PCR) CrPV positive control sample was effectively developed. Additionally, an RNA isolation protocol was established using Invitrogen™ Phasemaker™ Tubes in conjunction with the Invitrogen™ TRIzol™ reagent for RNA isolation and a RT-PCR amplification. The protocol was used for isolating RNA from symptomatic *A. domesticus* samples; however, CrPV was not detected in the samples.

This research provides new insights into baculovirus diversity and host associations by documenting novel virus-host relationships. It expands the known host range of CrleGV and presents the first identification of a baculovirus from *S. partita*. Through the isolation and characterisation of these viruses, the study enhances our broader understanding of baculovirus biology, host ecology, and insect-virus interactions. Moreover, the development of a screening

method provides a valuable tool for future detection of CrPV infection in symptomatic *A. domesticus* samples.

Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Tables

Chapter 5

Supplementary data

List of Equations

Chapter 4

Conference Presentation Outputs

Bennett, T.T., Hill, M.P., Moore, S.D., Jukes, M.D., & Knox, C. (2023). The isolation, identification, and characterisation of novel insect viruses for the control of emerging agricultural pests. Oral presentation at the Annual Postgraduate Conference, Rhodes University, Makhanda/Grahamstown, Eastern Cape (8-9 September 2023).

Bennett, T., Mushore, T., Hill, M., Moore, S., Jukes, M., Knox, C. (2024). The isolation, identification, and characterisation of a novel *Alphabaculovirus* isolated from *Serrodes partita*. Oral presentation at the International Congress on Invertebrate Pathology and Microbial Control & 56th Annual Meeting of the Society for Invertebrate Pathology. 28 July- 1 August 2024. Technical University of Vienna, Vienna, Austria.

Bennett, T., Mushore, T., Hill, M., Moore, S., Jukes, M., Knox, C. (2024). The isolation, identification, and characterisation of a novel *Alphabaculovirus* isolated from the fruit piercing moth, *Serrodes partita*. Poster presentation at the 12th Citrus Research Symposium. 18-21 August 2024. Champagne Sports Resort, KwaZulu Natal.

List of abbreviations

%	-	Percentage
(-)	-	Negative
(+)	-	Positive
(Pty) Ltd	-	Proprietary Limited
~	-	Approximately/Roughly
±	-	Plus or minus
×	-	Times
×g	-	Times gravity
>	-	Greater than
µg/ml	-	Microgram per millilitre
µl	-	Microlitres
µl/ml	-	Microlitres per millilitre
µM	-	Micromolar
µm	-	Micrometer
AcMNPV	-	Autographa californica multiple nucleopolyhedrovirus
AGE	-	Agarose gel electrophoresis
AgseGV	-	Agrotis segetum granulovirus
AgseNPV-B	-	Agrotis segetum nucleopolyhedrovirus B
AMP	-	Ampicillin
AmpR	-	Ampicillin resistance gene
ATP	-	Adenosine Triphosphate
BCIRL-AG-AM	-	*Anticarsia gemmatalis* cell line
BCIRL-HV-AM1	-	*Heliothis virescens* cell line
BCIRL-Hz-AM1	-	*Helicoverpa zea* cell line
BCIRL-PX2-HNV3	-	*Plutella xylostella* cell line
BLAST	-	Basic Local Alignment Search Tool
BLASTp	-	Protein Basic Local Alignment Search Tool
BmCPV	-	Bombyx mori cytoplasmic polyhedrosis virus
BmNPV	-	Bombyx mori nucleopolyhedrovirus
bp	-	Base pair(s)
BV	-	Budded virus

°C	-	Degrees celsius
C3	-	3-Carbon compound
C4	-	4-Carbon compound
CBC	-	Centre for Biological Control
cDNA	-	Complementary Deoxyribonucleic Acid
CIV	-	Chilo iridescent virus
CO_2	-	Carbon dioxide
COI	-	*Cytochrome c oxidase subunit I*
Col\E1\origin	-	Col E1 origin of replication
CpGV	-	Cydia pomonella granulovirus
CRI	-	Citrus Research International
CrleGV	-	Cryptophlebia leucotreta granulovirus
CrpeNPV	-	Cryptophlebia peltastica nucleopolyhedrovirus
CrPV	-	Cricket paralysis virus
CTAB	-	Cetyltrimethylammonium bromide
CTP	-	Cytidine Triphosphate
CuniNPV	-	Culex nigripalpus nucleopolyhedrovirus
ddH_2O	-	Double-distilled water
DL2	-	*Drosophila* line 2
DNA	-	Deoxyribonucleic acid
DR1	-	*Drosophila* line 1
dsDNA	-	Double stranded deoxyribonucleic acid
dsRNA	-	Double stranded ribonucleic acid
DTT	-	Dithiothreitol
E. coli	-	*Escherichia coli*
e.g.	-	exempli gratia
EDTA	-	Ethylenediaminetetraacetic acid
EFP	-	Envelope fusion protein
egt	-	*Ecdysteroid UDP-glucoslytransferase*
et al.	-	et alia (and others)
g	-	Grams
gDNA	-	Genomic deoxyribonucleic acid
GTP	-	Guanosine triphosphate

GV	-	Granulovirus
GWSS	-	Glassy-winged sharpshooter
H_2O	-	Water
HCl	-	Hydrochloric acid
HearGV	-	Helicoverpa armigera granulovirus
HearNPV	-	Helicoverpa armigera nucleopolyhedrovirus
HMOs	-	Horticulture mineral oils
hr	-	Homologous regions
hr(s)	-	Hour(s)
hrf-1	-	*host range factor 1* gene
HycuGV-Hc1	-	Hyphantria cunea granulovirus
HycuGV-Hc1	-	Hyphantria cunea granulovirus
Hz	-	Hertz
i.e.	-	Id est
ICTV	-	International Committee on Taxonomy of Viruses
IIV-6	-	Invertebrate iridescent virus 6
IIVs	-	Invertebrate iridescent virus
IPM	-	Integrated Pest Management
JM109	-	*Escherichia coli* K-12 strain
K-2-P	-	Kimura-2-parameter
kb/kbp	-	Kilobase pair
km^2	-	Square kilometer
L:D	-	Light:Dark photoperiod
LA	-	Luria Agar
LB	-	Luria Broth
LC_{50}	-	Lethal concentration (50%)
LC_{90}	-	Lethal concentration (90%)
LdMNPV	-	Lymantria dispar multiple nucleopolyhedrovirus
lef	-	*late expression factor*
lef-8	-	*late expression factor 8*
lef-9	-	*late expression factor 9*
LyxyNPV	-	Lymantria xylina nucleopolyhedrovirus
M	-	Molar

MbMNPV	-	Mamestra brassicae multiple nucleopolyhedrovirus
mg/ml	-	Milligrams per microlitre
min	-	Minutes
ml	-	Mililitre
mM	-	Milimolar
mm	-	Millimetre
mm^2	-	Square millimeter
MNPV	-	Multiple nucleopolyhedrovirus
mtDNA	-	Mitochondrial Deoxyribose Nucleic Acid
n	-	number
Na_2EDTA	-	Ethylenediaminetetraacetic acid disodium salt
NaCl	-	Sodium chloride
NCBI	-	National Center for Biotechnology Information
NeleNPV	-	Neodiprion lecontei nucleopolyhedrovirus
ng	-	Nanograms
ng/µl	-	nanograms per microlitre
NGS	-	Next-generation sequencing
nm	-	nanometer
NoarCPV	-	Norape argyrrhorea cypovirus
NPV	-	Nucleopolyhedrovirus
nt	-	Nucleotides
NTC	-	No template control
OBs	-	Occlusion bodies
ODV	-	Occlusion derived virion
OpMNPV	-	Orgyia pseudotsugata multiple nucleopolyhedrovirus
ORF	-	Open reading frame
OrNV	-	Oryctes rhinoceros nudivirus
PAU	-	Punjab Agricultural University
PCR	-	Polymerase chain reaction
pH	-	Potential of hydrogen
PhopGV	-	Phthorimaea operculella granulovirus
pif	-	*Per os* infectivity factor
PlxyGV	-	Plutella xylostella granulovirus

PO_4	-	Phosphate
polh/gran	-	*Polyhedrin/granulin* gene
PR-5	-	*Pieris rapae* cell line
PsunGV	-	Pseudaletia unipuncta granulovirus
PsunNPV	-	Pseudaletia unipuncta nucleopolyhedrovirus
RdRp	-	*RNA-dependent RNA-polymerase*
REN	-	Restriction endonuclease analysis
RNA	-	Ribonucleic Acid
rRNA	-	Ribosomal Ribonucleic Acid
RT-PCR	-	Reverse Transcription Polymerase Chain Reaction
s	-	Seconds
S2	-	Schneider line 2
SASRI	-	South African Sugarcane Research Institute
SDS	-	Sodium Dodecyl Sulfate
SepaNPV	-	Serrodes partita nucleopolyhedrovirus
Sf21	-	*Spodoptera frugiperda* line 21
Sf9	-	*Spodoptera frugiperda* line 9
SIT	-	Sterile insect technique
SNPV	-	Single nucleopolyhedrovirus
SpfrGV	-	Spodoptera frugiperda granulovirus
ssDNA	-	Single-stranded deoxyribonucleic acid
ssRNA	-	Single-stranded ribonucleic acid
T7	-	T7 promoter site
TAE	-	Tris base, acetic acid and EDTA
TEM	-	Transmission electron microscopy
™	-	Trademark
TN-CL1	-	*Trichoplusia ni* cell line
TnGV	-	Trichoplusia ni granulovirus
Tris-HCl	-	Hydroxymethyl aminomethane hydrochloride
U/µl	-	Unit per microlitre
USA	-	United States of America
UTP	-	Uridine triphosphate
V	-	Volts

v/v	-	Volume per volume
VEFs	-	Viral enhancing factors
w/v	-	Weight per volume
XcenGV	-	Xestia c-nigrum granulovirus
XecnGV	-	Xextia c-nigrum granulovirus
YFV	-	Yellow fever virus

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Chapter 1 - Literature Review

1.1. Introduction

Insects represent more than 58 % of all known species on Earth and have successfully adapted to and inhabit nearly all ecological niches (Takov et al., 2021). Only one million out of the estimated population of 2.8 to 10 million insect species have been described. Of these, the number of insect species that have been identified as agricultural pests is approximately 10,000. Under natural conditions, insects typically do not become pests where they are rarely present in large numbers (Jones, 2002). Insects are regarded as pests once there is an increase in their population numbers, and they result in economic damage (Jones, 2002). One of the major contributors to agricultural economic losses is the biological invasion by pest species (Bebber et al., 2014; Diagne et al., 2021; Hulme, 2009; Pimentel et al., 2001). These agricultural pests are responsible for significant crop losses, resulting in about 20-40 % of losses annually during cultivation and post-harvest stages (FAO, 2019). One of the major concerns worldwide is ensuring adequate food production for the expanding global population (Jankielsohn, 2018). There is both a significant social and economic risk as a result of the loss of agricultural biodiversity. This can lead to long-term reductions in agricultural productivity, ultimately threatening global food security (Tscharntke et al., 2005). The losses in crops caused by insect pests have a negative effect on global food security and the increasing demand for food production. Therefore, the effective management of crop losses is essential to ensuring food availability worldwide (Harish et al., 2021).

Evidence on the expansion of arthropods or host plant shifts is sometimes linked to changes in their geographic distribution. This has been documented in various ecological studies (Audusseau et al., 2017; Battisti et al., 2005; Diegisser et al., 2009; Sánchez-Guillén et al., 2016; Singer & Parmesan, 2021). When a phytophagous species expands its geographical range, it will come in contact with novel plant species, potentially leading to changes in its diet breadth (Hill et al., 2011; Jahner et al., 2011; Lancaster, 2020). The ability to shift or expand its host range can enhance the species' chances of establishment by allowing it to utilise previously unavailable resources (Battisti et al., 2005). Global trade and climate change impact the introduction and spread of various pest species. The expansion of international trade, by extensive transportation networks, is moving an increasing number of insect species beyond their native ranges (Hulme, 2021; Pyšek et al., 2020; Seebens et al., 2017). While trade plays a key role in introducing these species to new areas, the local climate conditions influence the

successful establishment and further spread of the insect species (Renault et al., 2018). Moreover, global and regional shifts in climate are further enhancing the likelihood of non-native species establishing and expanding in new regions (Cao & Feng, 2024). Farmers are expected to face severe and new pest problems because of the growing area of host plants shifting, and as a result, insect pests are spreading into new areas. By the year 2055, it is expected that the range of insect pests will move to higher altitudes (Skendžić et al., 2021b). With increasing evidence of insect expansion into new geographic regions, it is crucial to identify species that are extending their ranges. Equally important is the development of new biological control strategies targeting these expanding insect populations to ensure effective protection of vulnerable agricultural crops and to safeguard long-term food security.

1.2. Factors contributing to the movement and emergence of insect pests

When non-native insects are introduced and spread in new regions, it is known as insect invasions, and this can lead to significant economic and environmental impacts (Renault et al., 2018). The rate at which non-native species are introduced and become established continues to rise significantly (Seebens et al., 2017). In Europe, the establishment rate of non-native insects has nearly doubled in recent decades (Roques, 2010). The introductions are referred to as the human-mediated movement of insects across their natural biogeographic boundaries, occurring either accidentally or intentionally (Falk-Petersen et al., 2006; Richardson et al., 2011). Intentional insect introductions usually happen as a result of biocontrol agents being imported into new areas. Whereas accidental insect introductions most likely occur when ornamental plants and wood packaging are being transported (Renault et al., 2018). When arthropods expand their geographical range under natural or climate-driven conditions, they experience a gradual shift in the plant communities that they encounter (Bras et al., 2022). However, when human activities are the drivers towards the invasion of arthropods outside of their native range, these arthropods come in contact with new host plant communities and need to adapt to the plants to survive and establish in the new environment (Hill et al., 2016).

1.2.1. The effect of human-mediated movement on insects

Human activity plays a central role in biological invasions and emergence, introducing species to regions beyond their native ranges and contributing to their further spread (Gippet et al., 2019). Over the past few centuries, the movement of species beyond their historical ranges has been increasingly facilitated by humans (Hulme et al., 2008; Liebhold & Tobin, 2008). The

introduction of new species continues to accelerate with the expansion of global trade and travel (Bertelsmeier et al., 2017; Seebens et al., 2017). Various species introduced into the new ranges succeed in establishing themselves and become invasive, meaning they cause ecological and economic harm in the new environments (Pagad et al., 2015). Insects are typically the most prevalent and destructive group of animal invaders found in terrestrial ecosystems (Bradshaw et al., 2016). There are three human-mediated dispersal phases such as departure, transport, and arrival (Bullock et al., 2018). The departure phase is the start of the dispersal process, which involves the movement of insects either when infested materials are loaded onto a transport vehicle, when insects attach themselves to a carrier, or when insects are collected for shipping. The next phase is transport, which involves the movement of the insects by various modes such as trains, aeroplanes, boats, or cars. The last phase is the arrival phase, which occurs when insects detach from the transport vector either by escaping captivity or through the intentional or accidental release. There are three pathways through which insects are spread, which are grouped into contamination, hitchhiking, and harvest. These pathways influence the three stages of insect dispersal (Pergl et al., 2017). The contamination pathway involves the movement of goods that are unintentionally infested with insects. These are products that are either part of the environment, such as water or soil, or the product is the insect's natural host, such as plants or animals (Meurisse et al., 2019). Additionally, insects attach themselves to objects that are not part of their natural habitat, such as cars, shipping containers, or machinery, and this is how insects are introduced into new regions. This mode of introduction is known as the hitchhiking pathway (Meurisse et al., 2019). The harvesting pathway involves humans deliberately collecting insects, often for commercial purposes such as for biological control or the pet trade (Kumschick et al., 2016). These insects may be introduced into new areas either by escaping from captivity or by being deliberately released into the area (Hulme et al., 2008).

An example of an accidental introduction is *Operophtera brumata* (Linnaeus) (Lepidoptera: Geometridae), which is native to Europe, commonly known as the winter moth, and is a foliage-feeding species. This moth has established itself in eastern Massachusetts in the USA, and over the past several years, it has caused significant forest defoliation (Elkinton et al., 2010, 2014; Renault et al., 2018). An example of an intentional introduction is *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), the harlequin ladybeetle, which was released to control hemipteran pests in various countries (Renault et al., 2018; Roques et al., 2016). The gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Erebidae) is one of the most significantly studied invasive insect pests worldwide. It is an example of an insect that displays both intentional and

accidental introduction. It was initially imported for controlled breeding and silk production; however, it was released into nature accidentally (Liebhold et al., 1989). Human activities within the arthropod's native range can affect the host range of arthropods under various conditions. For instance, the plant defence mechanism can be altered through the selective breeding of crops or the domestication of wild plants, and therefore potentially increasing the susceptibility of these plant varieties to pest attack (Bernal & Medina, 2018; Chen et al., 2015; Turcotte et al., 2017). Human-induced land use changes are another example, such as the expansion of urban areas, bringing rural arthropod species into contact with non-native plants found in gardens and parks (Bras et al., 2022). Furthermore, the modifications of natural habitats caused by humans, particularly through the cultivation of crops selected for traits like larger size, higher yields, and improved nutritional value, contribute to the emergence of insect pests. Furthermore, large monoculture environments are created due to the expansion of orchards and agricultural development. These environments are conducive for insects to thrive, leading to more energy being directed towards reproduction than foraging for food (Jones, 2002). Monocultures function as "biological deserts", supporting few species and offering ideal conditions for certain herbivorous insects to proliferate. These insects can rapidly adapt, evolving into biotypes capable of overcoming challenges such as bypassing natural or engineered plant defences or by resisting pesticides (Jankielsohn, 2018).

1.2.2. The effect of climate change on agricultural pests

Agriculture is also very vulnerable to climate change (Deshar & Koirala, 2019). Over the past few years, there has been an increase in new, emerging and more aggressive pest incidences, which affect the stability and yield levels of crops, which in turn threaten food security (Mafongoya et al., 2019). According to Comoé & Siegrist (2015) and Menapace et al. (2015), the incidence, distribution and intensity of diseases and plant pests were shown to potentially be altered by climate change as well as the emergence of insects on new crop hosts. One of the major risks faced in the southern Africa region and sub-Saharan Africa is climate change (Mafongoya et al., 2019). Agriculture and agricultural insect pests are particularly impacted by global climate changes. Climate-related changes, such as shifts and fluctuations in humidity, temperature and biotic interactions, influence invasive species as their populations respond to these changes. As a result, this may cause the distribution of these invasive species to change (Finch et al., 2021). The probability of an invasive insect establishing in a new geographical region where climate conditions are unfavourable is low. However, this likelihood may be increased because of climate warming (Robinet & Roques, 2010). New ecological niches are

created because of climate warming, and this yields opportunities for the spread and establishment of invasive pests (Skendžić et al., 2021a). The dispersal, reproduction, population dynamics and survival of insect pests are also considerably impacted by climate warming. Additionally, the relationships between pests, their natural enemies and the environment are also impacted by climate warming (Prakash et al., 2014). Climate change may intensify the harmful effects caused by invasive insects, this is due to the decrease in the barriers that aid in their successful dispersal and establishment (Skendžić et al., 2021a). The various drivers of climate change and their impact on insects will be discussed below.

1.2.2.1. Increased carbon dioxide (CO_2) concentration

The impact of the increased levels of CO_2 depends largely on the host plant. The response of plants to elevated CO_2 includes an increase in photosynthetic rate and a change in the chemical composition of leaves. The quality and quantity of vegetation and plants change which directly impacts insects. As a result, the palatability of leaf-eating insects is affected and the nutrient quality of foliage is affected (Lincoln, 1993). In some insect groups, the changes in the chemical composition and nutrient quality in leaves could lead to an increased rate of consumption and plant damage. This is because insects would need to eat more to get equivalent nutrients and level of food (Bezemer et al., 1998; Skendžić et al., 2021b). C3 crops (e.g., cotton, rice, wheat) are impacted at a much greater scale by increased CO_2 levels in comparison to the impact on C4 crops (e.g., sorghum, corn). As a result, the response of insects feeding and herbivory on C3 plants will differ in comparison to that of C4 plants. C4 plants will have a lower response to elevated CO_2 and in turn the likelihood of these plants being impacted by changes in the feeding behaviour of insects is less likely. Whereas elevated CO_2 will likely affect C3 plants positively, but these plants will be negatively impacted by the response of insects to elevated CO_2 (Lincoln et al., 1984).

1.2.2.2. Increased temperature

Due to the ectothermic nature of insects, their physiological processes are primarily affected by temperature. As a result of this, catastrophic effects on agricultural and natural ecosystems can be seen by just minor changes in temperature (Mafongoya et al., 2019). Insect consumption, movement and development has been shown to accelerate due to increases in temperature. The metamorphosis, host availability, mobility and metabolism are furthermore impacted by temperature (Shrestha, 2019). According to Karl & Trenberth (2003), by the end of the current century it is likely that there will be an average of 1.8 to 4 ℃ increase in temperature globally. Due to the physiological limits of individual insect species, the

abundance patterns and distribution of animals and plants are largely affected by temperature. The rates of insect reproduction and development are also accelerated, and this results in more generations per year, which in turn results in an increase in the damage caused to crops (Bale et al., 2002; Yamamura & Kiritani, 1998). Higher temperatures in winter and higher temperatures during the growing season play a significant role in insect species distribution (Mafongoya et al., 2019). Insect population will also colonise crops earlier and develop faster with higher temperatures, and as a result, the damage caused to crops will take place more rapidly. Higher temperatures also affect new emerging species, these species will spread to completely new ecological setting and often their natural enemies are not present in these new settings (Mafongoya et al., 2019).

1.2.2.3. Change in precipitation patterns

Droughts and floods are favoured with changes in rainfall patterns, such as an increase in the intensity of precipitation and a decrease in the frequency of precipitation. Insect species are affected by the occurrence of droughts and floods (Skendžić et al., 2021b). The production of secondary metabolites in plants decreases because of drought. These metabolites play a role in the defence function, and therefore, a decrease in production makes plants more susceptible to attack by insects (Yihdego et al., 2019). Some insect species are also more attracted to plants that are more stressed because of droughts. Some environmental conditions may be more suitable for the growth and development of herbivorous insects, due to dry climates (Skendžić et al., 2021b; Yihdego et al., 2019). Moreover, the diapause of insects is affected, and their survival is threatened because of flooding and prolonged stagnation of water. Flooding and heavy rains may lead to larvae, insect eggs and small-bodied insects being washed away (Pathak et al., 2012; Shrestha, 2019).

1.3. Agriculturally important pests of southern Africa

Various insects already affect the agricultural industry, and with some expanding their geographic range, they present an increasing threat to crop production and overall agricultural sustainability. A diverse range of insect species from various orders are considered pests and pose significant challenges to the South African agricultural industry (Hatting et al., 2019; Prinsloo & Uys, 2015). The most significant pest outbreaks have involved thrips, aphids, red spider mites, and whiteflies, which were recorded in South Africa (Mafongoya et al., 2019). Examples of pests threatening the agricultural industry in southern Africa include the invasive leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), which causes damage to

tomatoes, resulting in losses of 50-100 % (Katsaruware-Chapoto et al., 2017), whiteflies and diamond black moth, *Plutella. xylostella* (Linnaeus) (Lepidoptera: Plutellidae), which cause crop losses on cabbage (Oerke, 2006). Additionally, other insect pests of economically important crops in South Africa include *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) (Grout & Moore, 2015; Newton, 1998), *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Bell & Mcgeoch, 1996; Moran, 1983), *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) (Moore & Jukes, 2023), *Agrotis segetum* (Denis & Schiffermüller) (Lepidoptera: Noctuidae), *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae), and *Spodoptera exempta* (Walker) (Lepidoptera: Noctuidae) (Prinsloo & Uys, 2015). The polyphagous feeding behaviour of insect pests such as bollworms, aphids, and whiteflies affect the increased occurrence of these insect pests, because it enables them to infest a wide range of horticultural and field crops (Sharma et al., 2011). Moreover, this study focused on a selected group of insects, *Serrodes partita* (Fabricius) (Lepidoptera: Erebidae), *Thaumatotibia batrachopa* (Meyrick) (Lepidoptera: Tortricidae), *Ectomyelois ceratoniae* (Zeller, 1839) (Lepidoptera: Pyralidae), and *Acheta domesticus* (Linnaeus) (Orthoptera: Gryllidae), chosen for their importance to the southern African agricultural sector and their availability for research. *Serrodes partita* causes considerable damage on citrus in the Eastern Cape Province during outbreak years. This moth is considered a sporadic pest (Goddard et al., 2019). *Thaumatotibia batrachopa* is a pest of macadamia and lychee in South Africa (Timm et al., 2007). *Ectomyelois ceratoniae* is currently considered a minor pest in South Africa, however it was noted that the status of this insect could escalate to major under favourable climatic conditions that support rapid growth and reproduction (Morland, 2015). *Serrodes partita*, *T. batrachopa* and *E. ceratoniae* inflict damage to crops which causes economic losses because the produce is unsuitable for export or consumption. Isolating viruses from these species could offer potential for use as microbial control agents and biopesticides. Additionally, an insect that is vital in the developing insects-as food market, is *A. domesticus* (van Huis & Tomberlin, 2017). This species is susceptible to entomopathogenic infection. The CrPV has been isolated from field crickets and is known to be pathogenic to insect pests that affect agriculture (Bonning & Miller, 2010). To decrease the impacts caused by new and existing pests in plant production systems, it is important to implement mitigation and adaptation strategies (Mafongoya et al., 2019). Furthermore, before the damage caused to crops leads to economic losses, it is vital to establish effective monitoring and management strategies for controlling these emerging pests.

1.4. Integrated Pest Management (IPM) of agricultural insect pests

With climate change affecting agricultural insect pests, as previously mentioned, it is vital that correct management strategies are put in place to control these pests. The history of the management of pests started with the emergence of agriculture (Dent & Binks, 2020). A method of pest management known as IPM is a holistic approach to control pests by using a combination of different methods to manage a whole spectrum of pests within a particular cropping system (Dent & Binks, 2020). In Africa, international agricultural research centres and national programs have conducted a significant number of research projects in IPM or IPM-related research. Research in pest management has concentrated on chemical control, cultural control, host plant resistance breeding, and biological control (Abate et al., 2000). Entomopathogens such as nematodes, fungi, protozoa, bacteria, and viruses naturally exist within insects or their environment (Harper, 1987; Shermani & Khan, 2015). Of these entomopathogens a select few are also known as microbial control agents. The use of microbial control agents offers an alternative approach to using chemical insecticides. Microbial control agents work similarly to chemical insecticides (Dolinski & Lacey, 2007; Dutta, 2015; Hunter-Fujita et al., 1998; Moore, 2010). It is necessary to isolate and characterise the entomopathogens from cadavers of diseased host insects, to identify these microbial agents (Harper, 1987; Jaques, 1983; Shermani & Khan, 2015). A few entomopathogenic viruses effectively regulate insect populations, therefore playing a significant role in maintaining environmental and agricultural sustainability (Harish et al., 2021). The *Densoviridae*, *Reoviridae*, *Baculoviridae*, and *Entomopoxvirinae* are the major families of viruses that are known to cause epizootic infections among insects. These have been extensively studied and commercially developed for pest management (Harish et al., 2021). Due to the effectiveness of the families *Baculoviridae* and *Reoviridae* as bioinsecticides, these virus families are considered the most significant (Kalawate 2014).

1.5. Entomopathogenic viruses

Viruses belonging to about 39 families have been identified to be associated with arthropods, functioning to varying degrees as insect pathogens (Table 1.1). Several of these insect viruses have been identified as significant insect pathogens and are used as important entomopathogenic bioinsecticides, belonging to the *Baculoviridae* and *Cypoviridae*. The family that has been studied extensively at the molecular, biological, functional, and ecological levels is the *Baculoviridae*. Details on the various insect virus families are discussed below.

Table 1.1: Main virus families infecting invertebrates (ICTV 2025b, c)

Nucleic Acid		Family
DNA	dsDNA	*Asfarviridae, Baculoviridae*, *Polydnaviriformidae*, *Hytrosaviridae*, *Iridoviridae*, *Ascoviriadae*, *Nudiviridae*, *Poxviridae, Nimaviridae, Malacoherpesviridae, Filamentoviridae, Eupolintoviridae**
	ssDNA	*Alphasatellitidae, Bacilladnaviridae, Genomoviridae, Anelloviridae, Geminiviridae, Circoviridae*, *Parvoviridae*, *Bidnaviridae, Adamaviridae*, Anicreviridae*, Draupnirviridae*, Gandrviridae*, Geplanaviridae*, Kanorauviridae*, Mahapunaviridae*, Ouroboviridae**
RNA	dsRNA	*Birnaviridae*, *Artiviridae, Inseviridae, Lebotiviridae, Orthototiviridae, Spinareoviridae, Sedoreoviridae, Spiciviridae, Chrysoviridae*
	ssRNA (+)	*Alphatetraviridae*, *Solinviviridae*, *Dicistroviridae*, *Nodaviridae*, *Carmotetraviridae*, *Permutotetraviridae*, *Iflaviridae, Noraviridae, Kitaviridae, Flaviviridae, Mesoniviridae, Sinhaliviridae, Tymoviridae, Roniviridae, Sarthroviridae, Togaviridae, Hypoviridae, Polycipiviridae, Abyssoviridae*, Euroniviridae*, Mononiviridae*, Medioniviridae**
	ssRNA (- or -/+)	*Rhabdoviridae, Artoviridae, Orthomyxoviridae, Peribunyaviridae, Phasmaviridae, Nairoviridae, Nyamiviridae, Tospoviridae, Phenuiviridae, Cruliviridae, Mymonaviridae, Aliusviridae*, Chuviridae*, Crespuscuviridae*, Lispiviridae*, Mypoviridae*, Myriaviridae*, Natareoviridae*, Qinviridae*, Wupedeviridae*, Xinmoviridae*, Yueviridae**
	ssRNA-RT	*Pseudoviridae, Metaviridae, Belpaoviridae**

*Unknown viruses as indicated by the ICTV

1.5.1. Reoviruses

Reoviruses are double-stranded ribonucleic acid (dsRNA) viruses with icosahedral virions, which are non-enveloped. There are two families within the order *Reovirales*, these are the *Sedoreoviridae* and the *Spinareoviridae*. Within these two families, there are 16 genera, seven within the *Sedoreoviridae* and nine within the *Spinareoviridae* family (ICTV 2025d). Viruses

within this order infect a wide range of hosts, which include algae, arthropods, birds, fish, fungi, mammals, plants, and reptiles. The cypoviruses, a genus within the *Spinareoviridae* family, are known to be pathogenic and infect only arthropods. The genome length varies between the different cypoviruses (ICTV 2025d). There are 16 member species within the genus *Cypovirus*. These viruses are uniquely occluded within a proteinaceous crystalline structure known as polyhedral inclusion bodies, either singly or multiply (Mori & Metcalf, 2010). An example of a cypovirus is the Bombyx mori cytoplasmic polyhedrosis virus (BmCPV), which infects the silkworm (Jiang & Xia, 2014). It is not common to use these viruses as viral insecticides despite their high infectivity and long-term persistence in insect hosts. This is because they induce chronic infections in comparison to rapid and severe symptom expression characteristic of acute infections. The Matsukemin was the only registered *Cypovirus* product used for the control of the pine caterpillar, *Dendrolimus spectabilis*, (Butler) (Lepidoptera: Lasiocampidae) and was used in France, Japan, and China (Kunimi, 2007). However, in 1995, the registration of this product was withdrawn. In Peru, the devastative oil palm defoliator pest was controlled using the Norape argyrrhorea cypovirus (NoarCPV).

1.5.2. Dicistroviruses

Dicistroviruses are single-stranded positive-sense RNA viruses. These viruses have small, isometric, nonenveloped, and roughly spherical virions that have a diameter of 30 nm with a monopartite (Carrillo-Tripp et al., 2015). The length of the genome of these viruses is between 8-10 kb. The Acute bee paralysis virus was the first dicistrovirus discovered in 1963 from honeybees (Bailey et al., 1964). There are 15 species of dicistroviruses, which are divided into the genera *Triatovirus*, *Cripavirus*, and *Aparavirus*. Various natural hosts of dicistroviruses belong to the insect orders Lepidoptera, Hymenoptera, Diptera, Coleoptera, Hemiptera, and Orthoptera. Some viruses are also pathogenic to agricultural pests (Carrillo-Tripp et al., 2015). According to Harish et al. (2021), certain dicistroviruses are found in healthy arthropod hosts as persistent, asymptomatic infections, eventually resulting in the host's death over time. Various advantageous characteristics, such as the host specificity of several members of dicistroviruses, make them suitable candidates for development as biopesticides for insect pest management. The dicistrovirus Homalodisca coagulate virus 1, is an example that was evaluated against the glassy-winged sharpshooter (GWSS). This insect feeds on nearly 100 plant species, serves as a vector for the plant pathogenic bacterium *Xylella fastidiosa*, which causes serious diseases such as citrus variegated chlorosis and Pierce's disease in grapevines (Hunnicutt et al., 2008).

1.5.3. Baculoviruses

Baculoviruses are viruses that are enveloped, rod-shaped, and large. Arthropods are infected by baculoviruses, particularly the insects in the orders Lepidoptera, Diptera, and Hymenoptera (Cory & Myers, 2003; Federici, 1997; Moscardi et al., 2011; O'Reilly et al., 1992). More than 600 viruses belong to the *Baculoviridae*, and these infect 700 insect species globally. The most diverse and largest group of DNA viruses is the baculoviruses. There are four genera that baculoviruses are grouped into, which are based on their genomic organisation and the classification of their insect hosts, and these groups are *Alphabaculovirus*, *Betabaculovirus*, *Deltabaculovirus*, and *Gammabaculovirus*. The formation of OBs is what is used to characterise *Baculoviridae* viruses. With members classified into two morphological groupings, which are the granuloviruses (GVs) and the nucleopolyhedroviruses (NPVs) (Moore & Jukes 2023). Single virions form granules in the occlusions (protein matrix) of GVs, in contrast to NPVs, which have multiple virions in the occlusions. The size of GV OBs is 0.3-0.5 μm, and the NPV OBs are 0.15-15 μm (Cory & Myers, 2003; Possee et al., 2010). More than 100 insect species, mainly Lepidoptera, are infected by GVs (Murphy et al., 1995). Numerous species in the orders Lepidoptera, Diptera, and Hymenoptera are infected by NPVs (Herniou et al., 2012; Murphy et al., 1995; Possee, 1997). The genomes of baculoviruses are large, supercoiled, double-stranded, and circular (Harrison et al., 2018). The size of the genome ranges between 80-180 kbp (Harrison et al., 2018). The first discovery of baculoviruses was in the 19th century from the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) (Linnaeus) (1758), which was known as the 'wilting' disease. Crystalline polyhedral bodies were observed when examining the 'wilting' disease; these polyhedral bodies were, therefore, believed to cause the disease in silkworms. Since the successful reduction of the population of the velvet bean caterpillar, *Anticarsia gemmatalis* (Hübner 1818) (Lepidoptera: Noctuidae), when a baculovirus was tested for the control by treating soybean in Brazil, baculoviruses have become more increasingly recognised as an alternative to chemical insecticides for control (Moscardi, 1999; Szewczyk et al., 2009). The replication of a naturally occurring virus within the host needs to occur to identify and isolate a baculovirus. When an insect host is infected with a baculovirus, the host's physiology and behaviour are manipulated by the virus, in some cases causing the host to climb to the highest point in the vegetation (Knipe et al., 2007; Lacey et al., 2008). Additionally, baculovirus infection causes the appearance of the larvae to change to be milky or glossy, and they swell and eventually lead to liquefaction. These are typical baculovirus infection 'wilting' symptoms (Knipe et al., 2007; Lacey et al., 2008). Therefore,

examining the pathology of the infected insects is the first step to baculovirus identification (Hunter-Fujita et al., 1998).

1.5.4. Iridoviruses

Iridoviridae have a linear, dsDNA genome which ranges between 140-303 kb in size (Goorha & Murti, 1982; Papp & Marschang, 2019). The viral particles have a diameter ranging between 120-300 nm, and these are nonoccluded, nonenveloped, and icosahedral in shape (Harish et al., 2021). These viruses are classified into two subfamilies and five genera. The *Alphairidovirinae* consist of the genera *Megalocytivirus*, *Lymphocystivirus*, and *Ranavirus*, which infect poikilothermic vertebrates. The *Betairidovirinae* consist of the genera *Iridovirus*, *Decapodiridovirus*, and *Chloriridovirus*, which infect invertebrates (Chinchar et al., 2017a; Chinchar et al., 2017b; İnce et al., 2018). Only a few invertebrate iridescent viruses (IIVs) have been sequenced (İnce et al., 2018). The Invertebrate iridescent virus 6 (IIV-6) has been identified in the *Iridovirus* genus and was the first IIV isolated. The isolation of IIV-6 was from diseased larvae of the rice stem borer *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae), and this virus is also known as Chilo iridescent virus (CIV) (Fukaya & Nasu, 1966). Greater than 100 insect species within the orders Diptera, Hymenoptera, Orthoptera, Hemiptera, Lepidoptera, and Coleoptera can be infected by IIV-6 (Williams, 2008). Furthermore, the cell lines *Spodoptera frugiperda* line 21 (Sf21), *Aedes albopictus*, *Aedes aegypti*, *Anticarsia gemmatalis* (BCIRL-AG-AM), *Trichoplusia ni* (TN-CL1), *Spodoptera frugiperda* line 9 (Sf9), *Drosophila* line 1 (DR1), *P. xylostella* (BCIRL-PX2-HNV3), *Pieris rapae* (PR-5), *Drosophila* line 2 (DL2), *Heliothis virescens* (BCIRL-HV-AM1), and *Helicoverpa zea* (BCIRL-Hz-AM1) are all susceptible to infection by IIV-6 (Constantino et al., 2001). The highest susceptibility was shown in the DL1 and DL2 lines from *Drosophila* (İnce et al., 2018). The use of IIVs in pest control has been proposed by either using these viruses as recombinant vectors engineered to express toxins (Nalcacioglu et al. 2016) or in their wild-type form (Henderson et al., 2001; Jakob et al., 2001; Kleespies et al., 1999).

1.5.5. Nudiviruses

Nudiviruses infect insect and crustacean orders, and the host range of these viruses is broader than other arthropod viruses. The genome of nudiviruses is circular dsDNA, which ranges from 96-232 kbp, and they have a bacilliform nucleocapsid that is enveloped, and rod-shaped or ellipsoidal (Harish et al., 2021). According to Thézé et al. (2011), due to the close phylogenetic relationship, nuclear replication, similar virion morphology, large genome size, the

hypertrophy of infected arthropod cells, and the distinct evolutionary lineage, nudiviruses were initially classified in the *Baculoviridae* family. After phylogenetic analysis was conducted with the nudiviruses, it was shown that it grouped into a distinguishable monophyletic group with baculoviruses (Wang et al. 2007). The Tipula oleracea nudivirus (ToNV) generated occlusion bodies, which are characteristic of baculoviruses (Bézier et al. 2015). There are two genera that the nudiviruses are divided into, which are the *Alpha-* and *Betanudivirus* (Bézier et al., 2015). There are 33 core genes among the nudiviruses, and 21 of these genes are shared with all members of the *Baculoviridae* family (Rohrmann, 2013). Therefore, indicating a close evolutionary relationship between *Baculoviridae* and *Nudiviridae*, which are considered sister clades. Insect hosts of nudiviruses have been identified within the orders Diptera, Orthoptera, Lepidoptera, and Coleoptera. For decades, the Oryctes rhinoceros nudivirus (OrNV) has played a significant role in biological control, managing rhinoceros beetle populations. Mainly due to the high pathogenicity towards the larvae. However, in the Solomon Islands, recent studies have suggested that various rhinoceros beetle populations may have developed resistance to the virus (Etebari et al., 2020).

1.6. Insect virus discovery and production of biopesticides

With evidence on the different viruses associated with insects, various insect viruses have been identified and developed into biopesticides and are used for the control of various agricultural pests. *Baculoviridae* is a group of viruses that have been commercialised as microbial control agents. The most prominent group of viruses used against insect pests are the baculoviruses (Grzywacz, 2017). In South Africa, the first baculovirus to be reported was the Helicoverpa armigera nucleopolyhedrovirus (Whitlock, 1974). It was not until twenty years after this virus was first discovered that researchers evaluated its effectiveness as a biological control agent in the field (Moore et al., 2004b). Following promising results from earlier studies assessing HearNPV's (Helicoverpa armigera nucleopolyhedrovirus) potential as a biocontrol agent, River Bioscience developed a biopesticide formulation named Helicovir™ based on this virus (Moore & Kirkman, 2010). Thereafter, Andermatt (Switzerland) developed two additional HearNPV-based products registered in South Africa, which are Grabol® and Boldex®. Furthermore, in South Africa, CrleGV was first recorded by Whitlock (1980). This virus was also the first African baculovirus that was developed and marketed as a commercial product (Moore & Hattingh, 2012). A novel South African isolate of CrleGV was discovered by Moore (2002), and Singh et al. (2003) characterised this isolate. In South Africa, the three CrleGV-based biopesticides, Cryptogran™ (River Bioscience, South Africa), Gratham® and Cryptex®

(both Andermatt Biocontrol, Switzerland), have been registered for the control of *T. leucotreta* and are extensively used (Hatting et al., 2019). Additional baculoviruses that have been isolated in South Africa include the Plutella xylostella granulovirus (PlxyGV), Phthorimaea operculella granulovirus (PhopGV), Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV), and the Cydia pomonella granulovirus (CpGV) (Hatting et al., 2019).

In comparison to industrialised countries, there is a significantly lower cost associated with the production of biopesticides in Africa (Grzywacz et al., 2023). As a result, the price of the production of baculovirus biopesticides in Africa would be much lower in comparison to those manufactured outside the continent and imported. Therefore, presenting an opportunity for increased local use and potential exporting of these biopesticides. Mafongoya et al. (2019) stated that several of the recorded pests act as vectors for harmful viral pathogens. With evidence on the isolation and development of entomopathogenic viruses, it is therefore essential to identify and characterise viruses with the potential to serve as effective control agents against existing and emerging pests.

1.7. Motivation and Aim

There are numerous insect pests of economically important crops in South Africa, such as *T. leucotreta, H. armigera* and *C. pomonella*. There are different systems put in place to control insect pests in South Africa, and these include a combination of cultural techniques, chemical pesticides, sterile insect techniques, as well as biological control (Bloem et al., 2007). Biopesticides, in the form of viruses in particular, offer an alternative to reduce the utilisation of chemical pesticides. Research on these serious pests in South Africa has resulted in the isolation and characterisation of baculoviruses that are now commercially available as biopesticides. Examples of baculovirus biopesticides in South Africa are Cryptogran® (CrleGV) against *T. leucotreta*, Helicovir® (HearNPV) against *H. armigera* and Madex® (CpGV) against *C. pomonella*. However, there are a number of other insect pests which pose a threat to agriculture for which no virus has been isolated or characterised; examples of these insects include *Lobesia vanillana* (De Joannis) (Lepidoptera: Tortricidae) (grapevine moth), *E. ceratoniae* (carob moth), *Archips occidentalis* (Wals.) (Lepidoptera: Tortricidae) (citrus leafroller), and *Tortrix capensana* (Walker) (Lepidoptera: Tortricidae) (apple leafroller). Emerging agricultural pests may be impacted by climate change. Due to climate change, the geographical range of these agricultural pests may change, as well as how these pests interact with natural enemies and host plants (Skendžić et al., 2021b). It is therefore important to

identify and characterise viruses that have the potential to control these existing and emerging insect pests. The aim of this study is therefore to isolate, identify, and characterise novel viruses from various insect pests that already impact, or have the potential to impact, one or more important agricultural crops.

1.8. Thesis outline

This thesis is divided into six chapters. The general introduction is followed by four data chapters and a general discussion chapter.

Chapter 1: Literature review

Provides an overview and rationale of the study, an overview of relevant literature emphasising the significance of insect pests in agriculture, how insects expand and spread due to human activity and climate change, the management of pests, and the importance of isolating entomopathogenic viruses for the control of agricultural pests. The aim of the study and thesis outline is provided.

Chapter 2: Molecular identification of *Serrodes partita*, *Thaumatotibia batrachopa*, *Ectomyelois ceratoniae*, and *Acheta domesticus* using COI gene sequencing and analysis

This chapter provided an overview of the insect species selected for analysis in the study. The identification of these species was also confirmed using molecular genetic techniques using the *COI* gene as a genetic marker.

Chapter 3: Screening larval cadavers of agriculturally important lepidopteran pest for novel baculoviruses using PCR amplification of selected gene sequences

Within this chapter, *S. partita*, *T. batrachopa*, and *E. ceratoniae* were screened for baculovirus infection. Virus particles were purified from symptomatic larvae using a crude extraction method, followed by DNA extraction. Thereafter PCR amplification using the *polh*/*gran*, *lef-8*, and *lef-9* oligonucleotides was conducted. Positive PCR amplicons were subjected to sequencing and BLAST analysis to identify novel baculoviruses.

Chapter 4: The morphological, genetic, and biological characterisation of a novel alphabaculovirus (SepaNPV) isolated from *Serrodes partita*

Within this chapter, the novel virus isolated from *S. partita* was analysed further and characterised by whole genome sequencing and phylogenetic analysis was conducted to determine the novelty of the virus and its close relatives. The biological activity of the virus

was evaluated by assessing its host range and evaluating its potential as a biological control agent.

Chapter 5: Development and optimisation of a RT-PCR assay for the detection of cricket paralysis virus (CrPV) from *Acheta domesticus* samples

This chapter reports on the development of a screening method to detect CrPV from *A. domesticus* samples. A synthetic plasmid positive control sample was developed by conducting transformation, extraction, restriction digestion, and T7 transcription. Subsequently, RNA was extracted from *A. domesticus* samples, treated with DNase I, and analysed using a two-step RT-PCR assay.

Chapter 6: General discussion

This chapter offers a comprehensive interpretation of the findings, discusses potential avenues for future research, and provides suggestions for improving the outcomes of the study.

Chapter 2 – Molecular identification of *Serrodes partita*, *Thaumatotibia batrachopa*, *Ectomyelois ceratoniae*, and *Acheta domesticus* using *COI* gene sequencing and analysis

2.1. Introduction

As highlighted in the previous chapter, insects play an important role in crop production because of their ecosystem functions (Jankielsohn, 2018). Various insect pests impact a range of economically significant crops in South Africa, such as citrus, pome fruits, and nuts. Many of these pests are polyphagous, and to maintain the high productivity needed to meet global demand, these crops require effective protection against pests. The insects of interest to the current study, and those that require identification, are *S. partita*, *T. batrachopa*, *E. ceratoniae*, and *A. domesticus*. Each of these species plays a significant role in agriculture. The biology of these insects and their role in agriculture and control are discussed below. According to Jinbo et al. (2011) and Armstrong & Ball (2005), it is essential for effective control that pests are identified, as knowing the species identity guides the method of control and ensures that the biosecurity measures are appropriate. Two methods for insect pest identification are morphological identification and DNA barcoding.

2.1.1. Morphological identification of insects

Traditionally, insect pests are identified using various morphological traits (Jinbo et al., 2011), and mainly only the adult stages are identified using morpho-taxonomic keys (Barrett & Hebert, 2011). Studies on phylogenetics and classification primarily rely on observable morphology. However, a specialist's subjective judgement and a strong knowledge of taxonomy are essential to identify subtle differences between closely related species (Kaur & Singh, 2020). There are limitations to using morphometric identifications for insect pests. For example, when using morpho-taxonomic keys to identify economically important pests, it is difficult even for experts, due to cryptic species (Busvine, 1980; Clark et al., 2005; Della Torre et al., 2002). Furthermore, a drawback to using morphology for species identification is when the specimens are degraded or damaged (Smith et al., 2022). The identification of citrus infesting Lepidoptera using identification guides or morphological keys is based on the morphology of the adults. Identification is therefore limited because samples that are usually

collected in the field are either eggs or larvae. Therefore, to identify these samples they first need to be reared to adults, which is often unsuccessful and time-consuming (Marsberg et al., 2015). According to Walter & Winterton (2007), the inaccessibility to relevant information, and the lack of expertise, time and funding make it challenging to use traditional taxonomy where species identification is based on morphology. The combined use of modern identification techniques, for example, DNA barcoding, morphometrics and interactive identification keys, in conjunction with traditional taxonomy is known as integrative taxonomy. Integrative taxonomy can assist the principle of traditional taxonomy (Walter & Winterton, 2007).

2.1.2. Insect identification by *COI* analysis

An alternative method to morphological identification is that of DNA characterisation. This method can quickly and accurately identify organisms at any life stage (Briski et al., 2011; Hebert & Gregory, 2005; Jinbo et al., 2011; von Cräutlein et al., 2011). According to Bellmann (2006) and Ramme (1921), if there is no bioacoustics data available for species, or only museum specimens or subadults are available, then it is not easy to identify species using traditional methods. To overcome the drawbacks of using morphological species identification, the use of molecular tools is key (Hebert et al., 2003; Hebert & Gregory, 2005; Pentinsaari et al., 2016). A simple definition of DNA barcode is the identification of species by using one or more short sequences derived from a standardised region of the genome (Kress & Erickson, 2012; Savolainen et al., 2005), that allows for information about the species to be retrieved. The discovery of thousands of unnamed species can also be potentially accelerated (Kress & Erickson, 2012). Additionally, potential synonymous, cryptic and even extinct species can be identified through DNA barcoding (Baker et al., 2005; Campbell et al., 2008; Lambert et al., 2005; Lawrence et al., 2009), and immature stages can be linked with the adult specimens (Waugh, 2007). Large numbers of specimens, such as those collected from Malaise traps and other mass samples, newly caught specimens in the absence of taxonomic experts, and historical specimens in collections can all be identified using DNA barcoding (Morinière et al., 2016). The use of DNA markers for the identification of species has become more frequently used because these methods produce rapid and accurate results, any life stage can be identified using this method, and the methods are easy to implement and replicate (Hebert et al., 2004; Kress et al., 2005; Rubinoff, 2006). Mitochondrial DNA (mtDNA) is preferred because it is maternally inherited and seldom experiences recombination (Birky, 2001; Saccone et al., 1999). There are also hundreds to thousands of copies of mtDNA per cell, which makes it relatively abundant (Hoy, 2003) and therefore making the isolation from small animal body

parts easy (Stoeckle & Hebert, 2008). Furthermore, during DNA synthesis, the mitochondrial DNA does not have proofreading mechanisms and as a result evolves rapidly (Hoy, 2003).

Characteristics that make an ideal marker for DNA barcoding include using one that has sufficient variation of a sequence to distinguish between species, one that can be amplified easily, and exists in a single form per cell or organism. An example is the mitochondrial *COI* gene (Kress & Erickson, 2012). All eukaryotic life forms have the *COI* gene region, and this is why this gene region is preferred. The occurrence of insertions and/or deletions in this gene region are rare and the sequence variation in this fragment is enough to differentiate closely related species (Hebert et al., 2003). Additional characteristics of the mitochondrial *COI* gene that make it suitable as a molecular marker include, at the biochemical level, that it has been extensively studied, with its structure and size appearing to be conserved across all aerobic organisms studied (Saraste, 1990). At the DNA level, there is high variability in the *COI* gene; however, at the amino acid level, it is conserved (Simon et al., 1994). This is because mitochondrial DNA experiences a high rate of substitution at the third codon position (wobble position) of protein-coding genes, making it a widely used marker for species-level phylogenetic studies (Hwang & Kim, 1999; Navajas et al., 1996). There are three mitochondrial-encoded cytochrome oxidase subunits, the largest being the *COI* gene, and in the metazoan mitochondrial genome, it is one of the biggest protein-coding genes (Kaur & Singh, 2020). Therefore, many more nucleotides can be amplified and sequenced compared to any other mitochondrial gene (Lunt et al., 1996). Furthermore, because the amino acid sequence evolves slowly, it has been used to distinguish species groups at higher taxonomic levels (Hwang & Kim, 1999). Within the class Insecta, this gene has been extensively used in studies on identification and phylogenetics (Kaur & Singh, 2020). The *COI* gene region was selected in this chapter as the target region for species identification.

2.2. Motivation, aim and objectives

The insects *S. partita*, *T. batrachopa*, *E. ceratoniae* and *A. domesticus*, were selected for analysis in this study as each species plays a significant role in agriculture and their availability for research. For effective and responsible control methods to be implemented, pests must be accurately identified. These insects will be screened for entomopathogenic virus infection. In the event that an entomopathogenic virus is isolated from these insects, then knowing the correct identification will be important when developing biopesticides for these insects. The traditional identification of insects is through morphological identification; however, there are

drawbacks to using this method. When phenotypic plasticity is considered, morphological identification becomes challenging (Murugan et al., 2016). Furthermore, for the effective use of taxonomic keys, a high level of expertise is necessary (Ball & Armstrong, 2006). According to Xia et al. (2012), an effective tool for biodiversity assessments, species identification, identifying cryptic species and linking larval and adult morphologies, is DNA barcoding. The gene that is most used is the mitochondrial *COI* gene (Hebert et al., 2003; Hebert & Gregory, 2005; Pentinsaari et al., 2016; Rodrigues et al., 2017; Strüder-Kypke & Lynn, 2010). Additionally, various research on insect population, taxonomy, and evolution have extensively studied the *COI* gene and used it as a genetic marker (Brown et al., 1994; Funk et al., 1995a, b; Juan et al., 1995; Simons et al., 1994; Sperling et al., 1995; Sperling & Hickey, 1994). Therefore, this chapter aimed to conduct species identification using the *COI* gene as a genetic marker. The specific objectives of this chapter were, (i) to extract genomic DNA from each species, (ii) to perform PCR amplification of the *COI* gene, (iii) to sequence the PCR amplicons by Sanger sequencing and (iv) to analyse the sequence data by BLAST (Basic Local Alignment Seatch Tool) analysis.

2.3. *Serrodes partita*

2.3.1. Taxonomy and distribution

Fruit-feeding moths belong to the family Erebidae and the Calpinae subfamily (Bänziger, 1970, 1973, 1980; Pinhey, 1975; Swart, 1969; Swart et al., 1975; Zaspel, 2008). The fruit-piercing moth is one of the two major groups belonging to the fruit-feeding moths, and the other is the fruit-sucking moths (Jack, 1922; Johannsmeier, 1998). The fruit-sucking moths make up a large portion of the fruit-feeding moths and the fruit-piercing moths are only a small minority (Goddard, 2016). In South Africa, only 10 fruit-piercing moth species have been described. These species fall within the different genera namely, *Oraesia* (formerly *Calpe*), *Pericyma*, *Serrodes*, *Eudocima* (=*Othreis*), and *Egybolis* (Johannsmeier, 1998; Moore, 2010, 2012). These genera fall within the Noctuoidea superfamily, and moths in this superfamily are night flyers (Moore 2010). In South Africa, the fruit-piercing moth species that have been found on citrus are, *Egybolis vaillantina* (Stoll), *Eudocima divitiosa* (Walker), *Eudocima fullonia* (Clerck), *Eudocima materna* (L.), *Oraesia emarginata* (F.), *Oraesia provocans Walker*, *Oraesia triobliqua*, (Saalmüller), *Pericyma atrifusa* (Hampson), *Pericyma mendax* (Walker), *Pericyma scandulata* (Felder & Rogenhofer) and *Serrodes partita* (Fabricius) (Johannsmeier, 1998). Various authors have reported different numbers of moth species as fruit-piercing moth species. These numbers range between 6-88 species (Moore, 2012). The major fruit-piercing moth

species in South Africa is *S. partita*, which causes damage in citrus orchards (Robinson et al., 2012). *Serrodes partita* has also been referred to as *Serrodes nigha* Guenée 1852; *Phoberia korana* Felder and Rogenhofer 1874; *Serrodes campona* Guenée, 1852; *Noctua inara* Cramer, 1779 (India) and *Ophiusa basisgnum* Walker, 1858 (Pinhey, 1975).

The moth has been documented in Australia, throughout Asia and most of the Ethiopian region (Pinhey, 1975). *Serrodes partita* pierces the citrus fruit and has been recorded as a pest in various areas in India and Africa (Johannsmeier, 1998; Moore, 2010; Robinson et al., 2012). *Serrodes partita* populations are mainly found in the little Karoo region in South Africa, where its main host for the larvae occurs, but it can also be found throughout most of southern Africa (Goddard, 2016). Out of all the fruit-piercing moth species found in the Eastern Cape, *S. partita* is the most prevalent (Moore, 2010). *Serrodes partita* is regarded as a sporadic pest, affecting the citrus plant, mainly in the Eastern Cape Province every 5-10 years leading to significant yield losses, particularly to soft-skinned citrus (Moore, 2010; Robinson et al., 2012).

2.3.2. Morphological characteristics

The appearance of the fruit-piercing moths varies (Moore, 2012). Fruit-piercing moths are large and have varying wingspans of 20-90 mm in length (Hofmeyr, 2003; Moore, 2012). The adults of *S. partita* have a distinctive characteristic on the base of the forewing, three black spots that form a triangle (Moore, 2012). The larvae vary in colour, they range from black and grey, and from green to brown (Moore, 2012). On the individual body segments of some larvae, there are eye-shaped spots and other larvae are striped (Moore, 2012). The proboscis of the fruit-piercing moths is specially adapted, which enables the moth to get the fruit juice by perforating the fruit rind and pulp (Johannsmeier, 1998; Moore, 2010; Robinson et al., 2012; Zaspel, 2008). According to Goddard et al. (2019), the morphological features which suggest that *S. partita* pierce mainly soft-skinned fruit are the tearing hooks, furcate erectile barbs and erectile barbs present on the female and male proboscides. Ripening fruit can be detected by migratory fruit-piercing moths as far as 10 km away; this is because of their highly developed sense of smell (Johannsmeier, 1998; Moore, 2010; Robinson et al., 2012). Fruit-piercing moths can locate food due to their ability to migrate extensive distances over one night. For these moths to survive they need to feed frequently with intervals less than 3 days (Johannsmeier, 1976). As these moths migrate, they invade the orchards along the way and cause damage to the fruit and then after several days they continue their migration.

2.3.3. Host range

According to Leong & Kueh (2011), fruit crops throughout the tropical and subtropical belt, from Africa through Southeast Asia and Australia to the Pacific Islands, are affected by fruit-piercing moths, and various genera of fruit-piercing moths have been recorded on deciduous fruit and citrus in South Africa (Johannsmeier, 1998; Moore, 2010; Swart et al., 1975). In the Western and Eastern Cape, the main species that feeds on various deciduous and tropical fruits is *S. partita* (Johannsmeier, 1998; Pinhey, 1975). The adult moths prefer to damage mature citrus fruit; however, they can also damage green and ripe fruit (Ngampongsai et al., 2005). The fruit crops that are also damaged by this pest include guava, citrus, banana, kiwifruit, fig, mango, carambola, and papaya (Leong & Kueh, 2011). Fruit-piercing moths cause damage to most citrus cultivars, with the most susceptible to being damaged, being the early maturing cultivars; limes and lemons are however not attacked (Moore, 2012). The larvae also feed on a wide range of species, but in the Karoo the preferred host is *Pappea capensis*, wild plum (Ecklon & Zeyher) (Johannsmeier, 1998; Moore, 2010; Pinhey, 1975). *Pappea capensis* can be found in the Little Karoo and various areas of the Great Karoo, with the range of this plant covering over 80000 km^2 (Moore, 2012). In the South-Western Cape and Little Karoo, rainfall of 50-70 mm produces a flush of young foliage of the host plant on which *S. partita* larvae feed. The flush of young foliage leads to more food available for the larvae and as a result, causes large summer outbreaks of adult *S. partita* and this typically occurs every 5-10 years (Johannsmeier, 1998; Moore, 2010; Robinson et al., 2012). Millions of moths migrate up to 500 km, two months following the outbreak of the larvae and during this time they migrate into the fruit orchards in the Eastern Cape and feed on the fruit, causing damage to the fruit (Rust & Myburgh, 1986).

2.3.4. Life cycle and biology

Under natural conditions, the life cycle of *S. partita* from egg to adult takes 83-91 days (Figure 2.1) (Tayler, 1951). Individual eggs are laid on the host plant foliage and will hatch after 2-3 days. After hatching larvae will take 51-58 days to develop, through 7 larval instar stages. During larval development, the larvae feed at night on the host plant's foliage. Larvae move to the soil and use dead leaves and sand to make a pupal case prior to pupation. After 20-33 days the adult will eclose and the average adult life span is 56 days (Kriegler, 1957; Tayler, 1951).

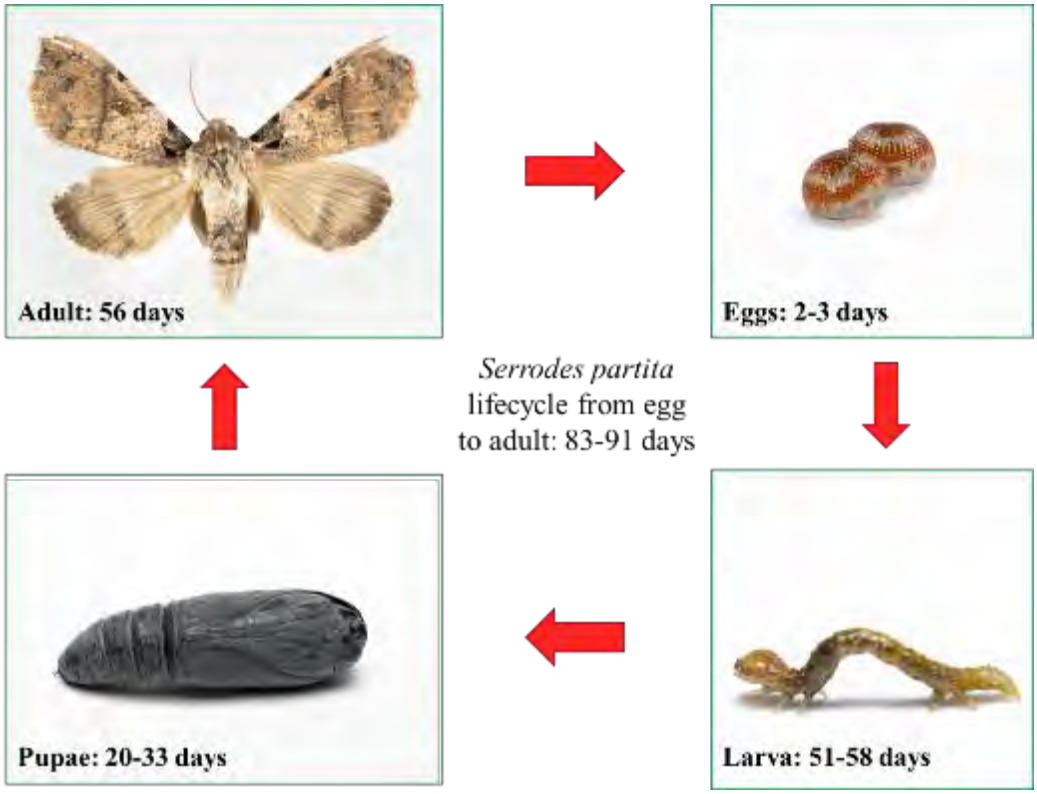

Figure 2.1: Lifecycle of *Serrodes partita* (Pictures taken by David Taylor).

The powerful sclerotised proboscis of the moth is used to pierce the fruit rind to get the fruit sap (Figure 2.2 A) (Büttiker et al., 1996; King & Thompson, 1958; Krenn, 2010; Reddy et al., 2007; Robinson et al., 2012; Zaspel et al., 2011; Zenker et al., 2011). Fruit-piercing moths will pierce through the fruit by breaking the fruit tissue and the juice cells and as a result, the juice is sucked up. The diameter of the feeding holes is 0.7 – 1.2 mm and the shapes are round to oval (Figure 2.2 B). When the fruit has been damaged and the juice sucked up, it will leave the fruit tissue in the damaged area dry and subsequently will lead to this area changing colour to yellow a few days later. The injury sites are entry points for microorganisms. When the fruit-piercing moth damages a fruit, it becomes susceptible to micro-organisms, particularly fungi. These micro-organisms will cause internal fruit decay before the fruit shows signs of decay (Johannsmeier, 1998; Moore, 2010; Robinson et al., 2012). Four to 10 days after being damaged the fruit will often drop off the tree (Moore, 2010). Damaged fruit are undesirable for the export market (Goddard, 2016). Fruit-piercing moths are hosts for various bacteria and fruit rot diseases (Bänziger, 1982; Ngampongsai et al., 2005). Pathogens can be transferred from damaged fruit to healthy fruit, which poses a threat to the export market if a damaged fruit is not detected when packed (Fay & Halfpapp, 1999, 2006). Fruit damage caused by fruit-piercing moths can be up to 70-90 % in orchards during outbreak years (Box, 1941; Gunn, 1929;

Johannsmeier, 1998), however, this is an extreme case. Fruit-piercing moths not only damage fruit during outbreak years, according to Whitehead & Rust (1972), but every year fruit are damaged by the moths in the Little Karoo. Similarly, in the Committees Drift area, it was observed in the study by Mushore (2024) that *S. partita* moths are present each season, occurring at low population densities and causing limited damage. This is because the cultivation of citrus in this area is in close proximity to the larval host plant, *P. capensis* (Mushore, 2024). Fruit-piercing moths often only spend one night in an orchard and do not return to the same orchards in the nights following. In contrast, the study by Mushore (2024) observed a low rate of moth return; *S. partita* likely follows a similar behavioural pattern to the behaviour of the fruit-piercing moths reported by Cochereau (1972) and Johannsmeier (1976), where the moths typically shelter in surrounding vegetation or forests during the day and return to the same orchard at night. The fruit-sucking and fruit-piercing moths are commonly confused by growers, which makes it difficult to monitor the orchards at night to determine which species is attacking the fruit. However, the primary damage to the fruit is caused by the fruit-piercing moth. Prior to implementing any control measures of the moth for citrus fruit, the extent of injury should be evaluated by sampling ripe fruit from the orchard. Ways of determining if a fruit was injured by a piercing moth include visual inspection, placing the fruit in hot water and checking for bubbles coming out, or squeezing the fruit to allow the juice to flow out of the punctures (Johannsmeier, 1998; Robinson et al., 2012; Rust & Myburgh, 1986).

Figure 2.2: *Serrodes partita* piercing citrus with its proboscis (A) and the damage caused to satsuma mandarins by *S. partita* (B). Figure A was taken by David Taylor and Figure B was taken from Moore (2010).

2.3.5. Control of fruit-piercing moths

2.3.5.1. Chemical control

Fruit-feeding moths were controlled in orchards using the classical method of Gunn traps, which were paraffin tins baited with brown sugar syrup or treacle and mixed with sodium arsenite or lead arsenate (Gunn, 1929). *Achaea lienardi*, the fruit-sucking moth was more successfully controlled using Gunn traps than the fruit-piercing moths (Goddard, 2016).

Additionally, Bordeaux mixture used on grapes and DDT and organophosphates on peaches were used as repellents against fruit-feeding moths (Kriegler, 1957).

2.3.5.2. Cultural control

Fruit-piercing moths are not only present in orchards during outbreak years but also during non-outbreak years. However, the numbers are lower, and the damage levels are also considerably low. As a result, moths are not monitored by growers during non-outbreak years (Goddard, 2016). The study by Goddard (2016) observed that damage caused by fruit-piercing moths occurred 6-8 weeks before fruit harvest. This was also observed in Australia by Fay & Halfpapp (2006) where fruit-piercing moths caused damage to the fruit that were starting to ripen (and sometimes unripe fruit), 8 weeks prior to fruit harvest. The scent of decaying and ripe fruit attracts the moths over long distances (Johannsmeier, 1998; Robinson et al., 2012). Therefore, for the moth numbers to remain low in orchards, and for the damage to be decreased, regular orchard sanitation is vital (Johannsmeier, 1998; Robinson et al., 2012). The fungal spore load in orchards, the volume of volatiles released from damaged fruit, the amount of damaged fruit that other pests like false codling moth and fruit flies are attracted to, and the number of moths present in orchards can all be decreased if the damaged fruit are removed from trees in orchards (Goddard, 2016). Starting mid-February, it is recommended that early mandarin orchards should be inspected at night by growers for the presence of fruit-piercing moths (Moore, 2010). Using plant protection products for controlling the larvae of the fruit-piercing moths is impractical because most of the larval host plants are wild host plants (Moore, 2012).

Singh et al. (2023) conducted a study to determine the practicality or achievability of an IPM module designed for fruit-piercing moth control in an eco-friendly manner in citrus. In the study, they tested botanicals, cultural practices, light traps, horticulture mineral oils (HMOs), netting and poison bait traps. The results from the study showed that the treatments tested did not control the fruit-piercing moth but were significant at effectively decreasing the damage caused to the fruit. Trees covered with nets showed significantly low average fruit damage, this was followed by the treatment with HMOs and then the treatments with PAU (Punjab Agricultural University) Homemade Dharek extract and PAU Homemade Neem extract. They concluded that fruit-piercing moths can be effectively and economically managed in citrus by implementing the IPM system that consisted of netting the trees, thereafter, spraying with PAU Homemade Neem extract and PAU Homemade Dharek extract or spraying with HMOs, and then fixing poison bait traps in orchards. They recommended that farmers should adopt these IPM tools as they are economical, effective and easily available (Singh et al., 2023). In the past,

in the tropical regions of West Africa and in South Africa; baits of poisoned fruit have been widely used for controlling fruit-feeding moths (Gunn, 1929).

2.3.5.3. Traps and barriers

Repellent lights are one of the effective methods of controlling fruit-piercing moths in orchards (Johannsmeier, 1998; Whitehead & Rust, 1967, 1971, 1972). Japan was the first to originally propose the use of light barriers for controlling fruit-piercing moths (Johannsmeier, 1998; Kriegler, 1957; Moore, 2010; Robinson et al., 2012). Ultra-violet lights attract noctuid moths. In the 1960s, the fruit-piercing moths were controlled for the first time by using repellent lights (Johannsmeier, 1998; Whitehead & Rust, 1967, 1971, 1972). When the adult fruit-piercing moths are exposed to light barriers, their vision changes physiologically, as they become quiescent when they settle on the trees in the light and will not feed. Additionally, when the moth is exposed to light barriers, they will spiral upwards and away from the light (Moore, 2012). Whitehead & Rust (1967) found that *S. partita* can be effectively controlled using yellow incandescent, paraffin (kerosene) pressure and vapour lamps, and placing the lamps on the edge of the orchard. The activity of *S. partita* moths was shown to be effectively reduced by 50-90 % when using each of the three types of lights (Whitehead & Rust, 1967). To effectively control fruit-piercing moths, lights need to be put on from before dark until midnight, because once the moth starts to feed on the fruit they are not easily disturbed (Johannsmeier, 1998; Moore, 2010; Robinson et al., 2012; Rust & Myburgh, 1986).

In the Little Karoo, moths are mainly attracted to the orchard edges that are alongside rivers and the orchard edge that is downwind from the W to SE breeze that occurs in the area during summer. It was therefore recommended that lights should be placed by these edges when using this form of control (Whitehead & Rust, 1972). The study by Whitehead & Rust (1972), found that a total of 60-90 % reduction in the number of moths present in an orchard was achieved when the most heavily infested side of the orchard was illuminated, and this was when the moth population was low. The study also found that during outbreak conditions, moth numbers were reduced by 43 % when one side of the orchard was illuminated, and 78 % reduction was achieved when two sides of the orchard were illuminated. Studies have also shown that a total of 80-90 % reduction in *S. partita* moth numbers was achieved in apricot and peach orchards when electric lights were used as a control method (Whitehead & Rust, 1967). In addition, fruit-piercing moths are attracted mostly by fresh bananas in orchards (Goddard, 2016). Therefore, this can be used as a bait option in traps.

2.3.5.4. Biological control

There is limited available information on biotic control factors against fruit-piercing moth (Moore, 2012). Viral and bacterial diseases may infect the larvae of the *S. partita* (Moore, 2012). Parasitoids such as flies and wasps are also known to attack the fruit-piercing moth larvae (Moore, 2012; Rust & Myburgh, 1986; Swart et al., 1975). It is however challenging to manipulate these factors because of the widespread nature of the pest (Moore, 2012). Additionally, bats have been used in orchards to catch the adult moths (Johannsmeier, 1976). Parasitism of up to 80 % by a tachinid fly has been documented during outbreak years. However, parasitism of less than 1 % has also been documented when the larval densities were low. Additionally, *S. partita* larvae are frequently killed by viral and bacterial diseases (Swart et al., 1975). Goddard (2016) suggested that studies on using entomopathogenic bacteria, viruses, and fungi should be explored for fruit-piercing moth control. The control of this pest remains a challenge.

2.4. *Thaumatotibia batrachopa*

2.4.1. Taxonomy and distribution

Thaumatotibia batrachopa is commonly known as the macadamia nut borer and belongs to the Tortricidae family (Schoeman, 2009; Timm et al., 2007) and the subfamily Olethreutinae (Brown, 2005; Karisch, 2003). Other known names of this moth include *Cryptophlebia batrachopa*, *Argyroploce colivora* (Meyrick 1932), and *Enarmonia batrachopa* (Meyrick 1908) (Brown, 2005; Karisch, 2003). The native range of *T. batrachopa* is Africa and it is found throughout southern, eastern, and western Africa (La Croix & Thindwa, 1986; Prinsloo & Uys, 2015). The moth has also been recorded in São Tomé and Madagascar (Prinsloo & Uys, 2015). *Thaumatotibia batrachopa* was first described in South Africa in 1999 from the Eastern Cape Province, as a pest of macadamia (Prinsloo & Uys, 2015).

2.4.2. Host range

In South Africa, the host plants of *T. batrachopa* include lychee and macadamia (La Croix & Thindwa, 1986; Rentel, 2013; Timm et al., 2007). In 1972, *T. batrachopa* was reported in citrus, however, no further record has been documented since then (de Villiers, 2001). *Thaumatotibia batrachopa* is a major pest of lychee and macadamia in Malawi and de Villiers (2001) noted that there was 20 % crop loss in macadamia in Malawi. It has also been recorded on Mauritius thorn (*Caesalpinea decapetala*), feijoa (*Feijoa sellwiana*) and snot apple (*Azanza garckeana*) in Malawi (de Villiers, 2001).

2.4.3. Biology and life cycle

Individual eggs are laid on the fruit's surface, and three eggs can be laid per fruit. The size of *T. batrachopa* eggs is approximately 0.8 × 0.6 mm wide and 0.2 mm high (La Croix & Thindwa, 1986). The eggs are oval, flat, and have a pitted surface (Figure 2.3 C). At 27 °C, the eggs will hatch after 6.8 – 10 days and the number of eggs that the female lays at this temperature is between 20.8 and 35.2 (La Croix & Thindwa, 1986). After eggs hatch, the larvae will move either into the fruit or nut directly under the position of the egg or 4 mm away from the position where the eggs were laid and bore into the fruit or nut husk (La Croix & Thindwa, 1986). The development of the larvae takes between 20.9 to 27.9 days, at 27 °C under controlled laboratory conditions (La Croix & Thindwa, 1986). The head capsule of the larvae is brown, the body of the larvae has dark spots, and the colour of the body is cream to greyish (Figure 2.3 A) (Prinsloo & Uys, 2015). Furthermore, Timm et al. (2007) found that the larvae are elongated with a yellow-brown head. When the larvae pupate on the soil, they use soil particles and silk to create a cocoon, in contrast, a cacoon is not produced when larvae pupate inside the fruit (Prinsloo & Uys, 2015). The colour of the pupae is dark brown and the length ranges between 6-8 mm (Figure 2.3 D) (Prinsloo & Uys, 2015). According to La Croix & Thindwa (1986), the emergence of the moths from pupae occurs gradually over time at 27 °C, under controlled laboratory conditions. Within 9 days 50 % of the moths emerge, at 14 days 80 % of the moths emerge and after 14 days the emergence of the moths will continue over the next 4 months (La Croix & Thindwa, 1986). The size of the moth is usually small with the length of the moth ranging between 16 – 20 mm (Prinsloo & Uys, 2015; Rentel, 2013). The moths are grey to light brown. The wings have noticeable darker and paler markings, and behind the head of the moth there is a saddle-like structure (Prinsloo & Uys, 2015). When the adults are kept in captivity their lifespan is usually between 3-7 days (La Croix & Thindwa, 1986).

When the larvae burrow into the macadamia nut, they cause considerable damage and after burrowing, the fruit often falls off the trees (La Croix & Thindwa, 1986). Larvae eat more than one kernel when the nuts are small. In addition, the larvae cannot penetrate through the nut after the nut shells are hardened. As a result, the development of the larvae will occur within the husk (La Croix & Thindwa, 1986). Once the larvae damage an immature nut and the nut vascular bundle tissue is damaged then the nut cannot continue to develop (Schoeman & de Villiers, 2015).

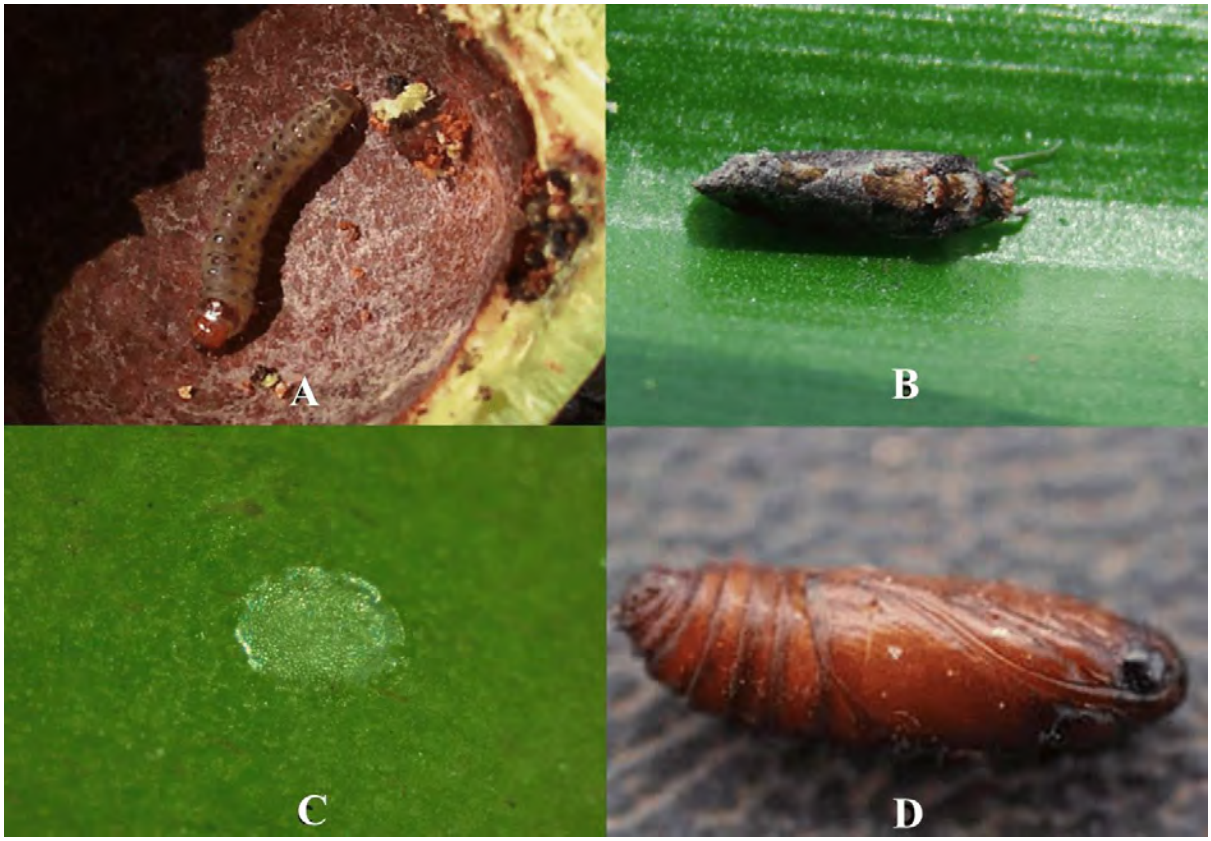

Figure 2.3: The four developmental stages of *T. batrachopa*. A: larva, B: adult, C: egg and D: pupa (Enslin, 2023).

2.4.4. Control

It is challenging to rear a colony of *T. batrachopa* in the laboratory (Timm et al., 2006). As a result, there is a lack of information on the ecological data of *T. batrachopa*, which includes aspects such as population dispersal and gene flow. Such information is vital for control practices to be successfully carried out and maintained (Timm et al., 2006). Pheromone traps can be used to keep track of moth numbers, however, the number of moths caught does not always indicate that the population has reached pest status (Enslin et al., 2024). The three species, *T. batrachopa*, *T. leucotreta,* and *E. ceratoniae* fall within the macadamia nut borer complex. In South Africa, significant economic damage is caused to the macadamia industry by these species (Enslin et al., 2024). The number of active ingredients that are currently registered in South Africa for the control of the macadamia nut borer pest complex is 23 (Schoeman, 2021). According to Schoeman (2021), out of the 23 active ingredients, six are pheromone-based products that are environmentally friendly, five are considered biological products and 12 are considered safe pesticides. The registered product X-Mate™ (Insect Science, South Africa) is used for *T. batrachopa* as a mating disruption product (Enslin, 2023).

2.5. *Ectomyelois ceratoniae*

2.5.1. Taxonomy and distribution

Ectomyelois ceratoniae more generally known as carob moth, is a polyphagous agricultural pest (Vetter et al., 1997). Heinrich (1956) first established the genus *Ectomyelois* (Dueñas-López, 2022). This genus contains six known species: *E. ceratoniae*, *E. austrella*, *E. muriscis*, *E. decolour*, *E. zeteki,* and *E. furvidorsella* (Abedi et al., 2019; Ren & Yang, 2016). The native range of these species is the Neotropics with the exception that the native range of *E. ceratoniae* is Iran and India (Abedi et al., 2019; Ren & Yang, 2016). Zeller (1839) was the first to describe *E. ceratoniae* and the moth was originally referred to as *Myelois ceratoniae* Zeller (Wakil et al., 2015). The following species; *Euzophera zellerella* (Sorhagen, 1881), *Heterographis rivulalis* Warren & Rothschild (1905), *Hypsipyla psarella* Hampson (1903), *Laodamia duradi* D. Lucas (1950), *Myelois oporedestella* Dyar (1911), *Myelois phoenicis* Durrant (1915) (Pintureau & Daumal, 1979), *Myelois tuerckheimiella* Sorhagen (1881), *Phycis ceratoniella* Fischer von Röslerstamm (1839), *Phycita dentilinella* Hampson (1896), *Spectrobates artonoma* Meyrick (1935), and *Trachonitis pryerella* Vaughan (1870), have all been described since *E. ceratoniae* was first described and these species have subsequently been synonymised (Wakil et al., 2015). Other common names for the carob moth include the pomegranate fruit moth (Krasil'Nikova, 1964; Moawad, 1979), the carob bean moth (Gonzalez & Cepeda, 1999), and the pomegranate fruit worm (Al-Jamali, 2006). The native range of *E. ceratoniae* is associated with the original distribution of the pomegranate (*Punica granatum*), its primary host (Abedi et al., 2019; Gothilf, 1970). *Ectomyelois ceratoniae* can be found on all continents but mainly occurs where its host plant is cultivated (Gothilf, 1970). Dry and hot climatic conditions are most suitable for the cultivation of the host plant and subsequently, *E. ceratoniae* will be present in these climatic conditions (Aitken, 1963; Gothilf, 1970). This species occurs in the Oriental and Neotropical regions (Ren & Yang, 2016). *Ectomyelois ceratoniae* can be found in the USA (California), Europe, South Africa, North Africa, Mediterranean countries, South America, Caribbean region, Australia, Hawaii, central and South Asia, China, the Pacific Islands, and Japan (Morland, 2015). The first report of *E. ceratoniae* in South Africa was in 1974. This was when damaged fruit from the Clanwilliam-Citrusdal region in the Western Cape was found to be infested with the moth (Honiball & Catling, 1998). In South Africa, *E. ceratoniae* can now be found in the Eastern Cape Province, the Lowveld region of Mpumalanga Province, the Limpopo Province, the Western Cape Province, and the Vaalharts region of the

Northern Cape Province (Honiball & Catling, 1998; Moore & Hattingh, 2017; Morland, 2015). *Ectomyelois ceratoniae* is regarded as a minor pest in South Africa (Moore, 2012; Moore & Hattingh, 2017). According to Mehrnejad (2001) and Morland (2015), despite the pest status of *E. ceratoniae* being minor in South Africa, if rapid growth and reproduction occurs due to favourable climatic conditions then the status of the moth could shift to being a major pest.

2.5.2. Morphological characterisation

The body length of the adult *E. ceratoniae* is between 6-12 mm, and it has a wingspan that ranges between 24-26 mm. One characteristic of *E. ceratoniae* is that it has extreme polychromy (Abid et al., 2021). The colour of the adults is light grey (Figure 2.4 B) (Dhouibi, 1991; Dhouibi & Jarraya, 1998). The hindwings are homochromatic and lighter in colour than the forewings. The hindwings have long light-coloured hairs and a silky fringe, whereas the forewings have two light lines edged with blackish scales and are pale grey (Dhouibi, 1991; Dhouibi & Jarraya, 1998). The body colour of the larvae is pink-to-white, and they have 12 segments with the addition of the cephalic segment (Figure 2.4 A) (Dhouibi & Jarraya, 1998; Wertheimer, 1958). *Ectomyelois ceratoniae* larvae undergo five larval stages, with each instar increasing in width and length from 0.1-3 mm and 1-18mm respectively. The length of the pupae is between 9-11 mm (Figure 2.4 D) (Abid et al., 2021; Dhouibi, 1991). *Ectomyelois ceratoniae* eggs are roughly 0.56 mm and are oval-shaped (Figure 2.4 C). When the adult first lays the eggs, they are light-coloured but as time passes if the eggs are fertile, they change colour to pink (Dhouibi & Jarraya, 1998; Wertheimer, 1958). The larvae of the carob moth are similar to *T. leucotreta* (Lepidoptera: Tortricidae), they are both pink in colour and their host preferences are similar (Honiball & Catling, 1998). There are however distinct morphological differences, and their mode of feeding is also different (Honiball & Catling, 1998; Morland et al., 2019). *Thaumatotibia leucotreta* larvae penetrate the flesh of the fruit in contrast to *E. ceratoniae* larvae, which usually do not penetrate the flesh. The larvae of *E. ceratoniae* bore into the rind and albedo of the citrus fruit (Morland et al., 2019). The colour of the larvae is pink; they have dark brown bumps on the dorsum and the head of the larvae is brown (Abo-El-Saad & El-Shafie, 2013). Al-Izzi et al. (1987) and Carpenter & Elmer (1978) suggested that *E. ceratoniae* larvae are cannibalistic; this was confirmed by Nay (2006) as he observed date fruit mostly infested with a single larva. The colour of the pupae is light to dark brown (Wakil et al., 2015).

Figure 2.4: *Ectomyelois ceratoniae* life stages. A) larva, B) adult, C) egg and D) pupa (Enslin, 2023).

2.5.3. Host range

Ectomyelois ceratoniae is regarded as an opportunistic feeder because of its broad host range. This allows the moth to get nutrients from various food sources which sustain the moth for reproduction and growth (Wakil et al., 2015). Additionally, the feeding of this moth on non-plant hosts has also been recorded (Wakil et al., 2015). There have been 43 hosts on which *E. ceratoniae* has been identified, belonging to 18 different plant families and 21 plants are economically significant commodities in agriculture (Wakil et al., 2015). This pest affects several commercial nut and fruit crops which include, citrus fruit, stone and pome fruits, walnuts, almonds, figs, pistachios, pomegranates, and dates (Vetter et al., 1997; Warner et al., 1990). In South Africa, *E. ceratoniae* affects crops such as citrus, macadamias, pecans, and pomegranates (Moore, 2012; Moore & Hattingh, 2017). In Europe *E. ceratoniae* affects nuts and stored fruits (Carter, 1984), in the Mediterranean basin it is considered the most economically damaging pest of dates, and in the subtropical regions, it is a major pest of pomegranate crops (Abid et al., 2021). In Iran, *E. ceratoniae* is regarded as a primary pest on

pomegranates with a percentage yield damage of 25-30 % caused by this pest (Morland, 2015). In the Middle East, a 30-80 % yield loss in pomegranates was recorded because of *E. ceratoniae* damage (Hosseini et al., 2017). Additionally, in the USA (California) the percentage of crop damage caused by *E. ceratoniae* in date crops was 10-40 % (Warner, 1988; Warner et al., 1990). In contrast, in the South African context, *E. ceratoniae* is not recognised as a major pest of citrus (Morland et al., 2019). Examples of plants and fruit that *E. ceratoniae* feed on include, *Acacia farnesiana*, *Butea monosperma, Caesalpinia sappan*, *Cassia bicapsularis* [*Senna bicapsularis*], *Ceratonia siliqua, Haematoxylum campechianum*, *Juglans regia*, *Macadamia integrifolia*, *Phoenix dactylifera, Pistacia vera*, *Prosopis juliflora*, *Prunus dulcis*, *Punica granatum*, *Ricinus communis*, and *Samanea saman* (Morland, 2015). In cases where other pests cause primary damage to the fruits, *E. ceratoniae* can accentuate the losses on crops. Examples of crops where this occurs are apples, apricots, peaches, and pears (Millar, 1990).

2.5.4. Life cycle

The duration from egg to adult for the first three generations of *E. ceratoniae* on pomegranates is between 41-59 days. In Autumn, the larvae of the fourth generation enter diapause, which lasts roughly 241 days (Al-Izzi et al., 1985). According to Cox (1979), when temperatures are ≤ 20 ℃ or the duration of daylight is less than 13 h, then the moths enter diapause. Furthermore, in citrus sp., *E. ceratoniae* has two or three generations. (Doumandji, 1981). Maximum, minimum, and average temperatures and their combined effect, greatly impact the seasonal abundance of *E. ceratoniae* (Abid et al., 2021). Temperature in conjunction with relative humidity affects the seasonal abundance of *E. ceratoniae* on dates (Roumani et al., 2018). When rearing conditions are controlled, the average life of the males is 3.5 ± 1.66 days, and of the females is 4.83 ± 1.47 days i.e. longer than the males. On the first- or second-night post-emergence, the adults will start ovipositing (Mehaoua, 2014). In the period between twilight and dark hours, the females will lay eggs (Al-Izzi et al., 1987; Gothilf, 1969; Nay & Perring, 2006). Temperature, diet, and host moisture content also influence the number of eggs that get oviposited by the female moth (Al-Izzi et al., 1987; Gothilf, 1969; Nay & Perring, 2006). Eggs will be deposited by the female *E. ceratoniae* on either the citrus fruit peel or where the fruit splits, and this occurs over 2-8 days and up to three times (Al-Izzi et al., 1987; Mehaoua, 2014). Additionally, eggs are laid on the crown (calyx) of pomegranates or in the cracks found on the fruit peel (Al-Izzi et al., 1985). Individual eggs are also laid on the outer surface of dates and in date openings, wrinkles under the calyx and in the tears of dates (Warner, 1988). Eggs take 4-6 days to hatch, and the larvae will develop from the first to fifth instar between 9-29 days.

The larvae that develop on pomegranates undergo four or five larval stages, and during the winter the majority of the fifth instars and some of the fourth instars hibernate in the fallen pomegranate fruits (Al-Izzi et al., 1985). The development of the larvae on dates is different. The development of the first two instars occurs on the surface of the fruit skin. After that they will penetrate the ripe fruit and develop further inside the dates (Abid et al., 2021). After larval development, they will then either move out of the fruit to the soil where they will pupate or in citrus they will remain in the fruit and pupate. Pupal development takes 4-13 days and there is a pre-pupal stage that takes 1 day. The adults will emerge, and the duration of the adult stage is usually 2-10 days.

The developing larvae of *E. ceratoniae* cause damage to the fruit. It is difficult to detect the first and second instar larvae as they are small. The third to fifth instars are larger, they eat significant amounts of the fruit and large amounts of frass are usually observed after feeding (Figure 2.5) (Wakil et al., 2015). The larvae feed on the fruit pulp, they enter the fruit by boring into developing fruits and pods. Signs of fruit infestation include frass and webbing (Figure 2.5) (Abedi et al., 2019; Morland, 2015). The damage to the fruits caused by the larvae results in early fruit drop, premature fruit yellowing, and contamination with saprophytic fungi (Abedi et al., 2019; Morland, 2015). Saprophytic fungi contaminate the inside of fruit that has been damaged by the larvae or that has been fed on by the larvae. The damage caused both pre- and post-harvest, decreases the commercial value and the quality of the fruit (Abedi et al., 2019). The fruit is, therefore, not suitable for food processing industries and for human consumption (Abedi et al., 2019). *Ectomyelois ceratoniae* is also a pest of stored products; this is because it will infest crops during the ripening stages in the field and will stay inside the fruit through the post-harvest processing (Wakil et al., 2015). *Ectomyelois ceratoniae* can be controlled by implementing various pest management options, which include post-harvest chemical control, release of sterilised male moths and biological control.

Figure 2.5: A: The damage caused by *E. ceratoniae* on citrus (taken from Lloyd, 2017) and B: The larva of *E. ceratoniae* with frass surrounding it (Taken from Wakil et al., 2015).

2.5.5. Control

2.5.5.1. Chemical control

In the USA, *E. ceratoniae* is controlled by frequent application of prophylactic sprays of malathion dust. It was stated however that there is a need to find different control methods that are safer (Mafra-Neto et al., 2013). In South Africa, there are no registered treatments that are targeted at *E. ceratoniae*. Pesticides that have been used for *T. leucotreta* control also affect *E. ceratoniae* and these pesticides include triflumuron, fenpropathrin, teflubenzuron, cypermthrin, and pheromone/permethrin (Dueñas-López, 2022). The infestation of *E. ceratoniae* was reduced in citrus orchards when the chemical sprays, Runner® and Delegate® were used (Thackeray, 2016). These two products are also registered for the use of *T. leucotreta* control (Thackeray, 2016).

2.5.5.2. Physical control

Placing mesh bags around date bunches can be conducted in the field to prevent *E. ceratoniae* from getting to the date branches (Wakil et al., 2015). Less than 0.5 % infestation was recorded when fruit were covered with a mesh of 1.7 mm or smaller in size (Zirari & Laaziza Ichir, 2010). Furthermore, when comparing open paper wraps to closed mesh bags, *E. ceratoniae* moth densities were reduced by 89 % (Perring & Nay, 2015).

2.5.5.3. Cultural control

According to Honiball & Catling (1998), one effective method for controlling *E. ceratoniae* is by orchard sanitation. Before the next growing season, it is important to remove fruit that have been left on trees and those that have fallen (Park & Perring, 2010). In South Africa, orchard sanitation is one of the suggested methods for controlling *E. ceratoniae* on citrus. This method involves the removal of fruit that are infested from trees, fallen fruit, and the remaining unharvested fruit prior to the beginning of the next flowering season (Moore & Hattingh, 2017). Moore (2012) also recommended removing other carob moth host plants near the orchards. These could act as reservoirs or over-wintering sites for these insects (Mozaffarian et al., 2007).

2.5.5.4. Biological control

Several natural enemies have been reported that are affiliated with *E. ceratoniae* (Wakil et al., 2015). Parasitoids within the Hymenoptera order are known to parasitise the larvae, pupae or eggs of *E. ceratoniae* (Gothilf, 1968; Morland, 2015). *Phanerotoma carobivora* van Achterberg and Thackeray (Hymenoptera: Braconidae) and *Phanerotoma ornatulopsis* De Seager (Hymenoptera: Braconidae) are two parasitoid species identified in South Africa. The former species has been discovered on citrus fruits and pecan and the latter species has been reared on acorns in the Citrusdal region (Honiball & Catling, 1998; van Achterberg et al., 2017). A method that seems promising for *E. ceratoniae* control is the augmentative release of mass-reared *Trichogramma* egg parasitoids. This control method will decrease the hatching of *E. ceratoniae* eggs and as a result, the damage caused by larval feeding will also be decreased (Dhouibi et al., 2016). The study by Ben Chaaban & Mahjoubi (2019), found that infestation was reduced by 50 % and 70 % in pomegranates and date palms respectively, when *E. ceratoniae* was controlled through the combination of mass trapping with *Trichogramma cacoeciae*. Additionally, *Bacillus thuringiensis* in the form of a larvicide has the potential to be used as a control option against *E. ceratoniae* (Mnif et al., 2013). When *B. thuringiensis* subspecies *kurstaki* (Delfin) was used in navel oranges, an *E. ceratoniae* control of 53% was achieved (Aytas et al., 1996). Furthermore, Nay & Perring (2005) have documented ant predation in fallen fruit in California.

Additional biological control options include the utilisation of essential oils and plant extracts such as those from *Euclyptus* sp. and essential oils from rosemary (Abada et al., 2019). The essential oils of *Eucalyptus rudis*, *Eucalyptus camaldulensis*, and *Eucalyptus leucoxylon* were shown in two studies to be toxic against *E. ceratoniae* adults (Abid et al., 2021). Other potential

control options include post-harvest fumigation of dates with essential oils of *Eucalyptus* sp., ethyl formate, or carbon dioxide (Abada et al., 2019).

2.5.5.5. Sterile Insect Technique (SIT)

Studies showed that males could be successfully sterilised using gamma radiation. The moth progeny decreased when wild females mated with sterilised males (Al-Izzi et al., 1993). In addition, the moth population densities would continue to decrease with sterile males being continuously released. This is because egg viability is reduced when sterile males mate with wild female moths and this is carried over to subsequent generations (Wakil et al., 2015). A control method for *E. ceratoniae* in pomegranate plantations that has been incorporated into area-wide IPM programmes is that of sterilising moths using gamma radiation before they get released. This has been demonstrated to notably reduce moth density in subsequent generations and reduce adult emergence and progeny (Chakroun et al., 2017).

2.6. *Acheta domesticus*

Entomophagy has been practised around the world for centuries, particularly in areas such as Asia, Latin America and Africa (Qian et al., 2022). In the edible insect market, grasshoppers and crickets comprise 50 % of this market by volume (Pippinato et al., 2020; van Huis, 2020a, 2020b). Crickets (family: Gryllidae) are a common group of insects that are farmed for feed, food and other useful products. This is because they are easy to rear, and their nutritional quality is high (Stull et al., 2018; Wang et al., 2004; Zielińska et al., 2015). Historically, crickets have been consumed as both animal feed and as a human food source (Siddiqui et al., 2024). *Gryllodes sigillatus* Walker (Orthoptera: Gryllidae) (tropical banded cricket), *Gryllus bimaculatus* (Orthoptera: Gryllidae) (African field cricket), *Brachytrupes portentosus* (Orthoptera: Gryllidae) (giant cricket), and *Acheta domesticus* (house cricket) are amongst the most common crickets that are consumed as food and feed (Hall et al., 2017; Malm & Liceaga, 2021; Matandirotya et al., 2022). *Acheta domesticus* has a significant amount of protein and fat as well as a rich array of minerals and vitamins, which gives it an excellent nutritional profile, which has led to the extensive rearing of this species (del Hierro et al., 2020; Lucas et al., 2020; Montowska et al., 2019; Ojha et al., 2021).

2.6.1. Habitat and distribution

Acheta domesticus is native to Eurasia, however, since then it has spread widely due to migration and importation (Magara et al., 2021). The geographical regions where it occurs and

is used for food are Asia, Europe, Oceania, the Americas, and Africa. The countries in Africa include the Democratic Republic of Congo, Kenya, Zambia, Central African Republic, Cameroon, Zimbabwe, and Ghana (Pilco-Romero et al., 2023). *Acheta domesticus* can be found in a variety of habitats, such as in forests, buildings, underground, marshes, bushes, tunnels, beaches and grasslands (Magara et al., 2021).

2.6.2. Biology and ecology

The adults and nymphs of *A. domesticus* are typically grey to brownish and the size can range between 16-21 mm (Figure 2.6). Sexual dimorphism is exhibited by the female and male *A. domesticus* (Hanboonsong & Durst, 2014). The abdomen of the crickets is covered by the wings and the forewings. In males, these are known to wrinkle. Crickets move by both walking and hopping. The males do not use their wings for flying but to attract the females by stridulation (Hanboonsong & Durst, 2014). The duration of *A. domesticus* lifecycle from egg to adult takes 40-45 days. The duration of the egg stage is 10-15 days, followed by the nymphal stage, which takes 6-8 weeks, and the adults typically live for 2 months (Hanboonsong & Durst, 2014). *Acheta domesticus* is frequently used as feed for fish (Lee et al., 2008; Perera et al., 2023), poultry (Kovitvadhi et al., 2019), and pets (Areerat et al., 2021). One of the drawbacks however is that these insects are susceptible to infection by entomopathogens (Duffield et al., 2021). The RNA virus family *Dicistroviridae* are viruses that infect arthropods (Bonning & Miller, 2010). An example of a virus from this family is the Cricket paralysis virus (CrPV), which was first isolated from Australian field crickets. This virus is known to infect both crickets and fruit flies (Bonning & Miller, 2010; Reinganum et al., 1970). The study by Manousis & Moore (1987b) found that adult fruit flies were found to host replication of the cricket paralysis virus. In Sub-Saharan Africa, the horticulture industry is significantly threatened by tephritid fruit flies (Ekesi & Billah, 2006). In addition, citrus in southern Africa is threatened by the three fruit fly species, the oriental fruit fly *Bactrocera* (*Bactrocera*) *dorsalis* (Handel), the Natal fly *Ceratitis* (*Pterandrus*) *rosa* Karsch and the Mediterranean fruit fly (Medfly) *Ceratitis* (*Ceratitis*) *capitata* (Wied.) (Manrakhan, 2022). As such, the use of this virus may find application in the management and control of fruit flies.

Figure 2.6: An adult *A. domesticus* used in the current study (Picture taken by David Taylor).

2.7. Methodology

2.7.1. Insect sample collection

Symptomatic larval cadavers of *S. partita*, *T. batrachopa*, and *E. ceratoniae* and symptomatic adult *A. domesticus* samples were obtained from various locations. Lepidopteran larvae showed symptoms of 'wilting' disease, which included a colour change, being milky or glossy in colour, and black/brown colour. Symptoms of the *A. domesticus* adults included lethargy and paralysis in the legs. *Serrodes partita* larval cadavers that showed symptomology of a baculovirus infection were collected from a colony, as part of a PhD research project, at the Department of Zoology and Entomology, Rhodes University (Makhanda/Grahamstown). *Thaumatotibia batrachopa, E. ceratoniae* and *A. domesticus* samples were obtained from Citrus Research International (CRI) (Port Elizabeth/Gqeberha). The *T. batrachopa* larval samples were collected from infected macadamia nuts that were obtained from various farms around Nelspruit and the Eastern Cape, South Africa. *Ectomyelois ceratoniae* larval samples were collected from the Sundays River Valley, Eastern Cape, and the *A. domesticus* samples were from an insect colony that is being reared at CRI. The *A. domesticus* colony was set up from insects bought at a pet shop (Arthurs Pet Shop) in Gqeberha/Port Elizabeth. These above-mentioned samples were used for subsequent experiments.

2.7.2. DNA extraction

Five random samples for each insect species were selected for the extraction of DNA and species identification. A portion of the larval tissue for each sample and a portion of the adult cricket tissue for each sample were first cut, weighed and recorded. Genomic DNA was extracted following the manufacturer's instructions from the Quick-DNA™ Tissue/Insect

Miniprep kit (D6016) (Zymo Research, USA). DNA was eluted in 100 µl Elution buffer. The gDNA was analysed by 1 % agarose gel electrophoresis and agarose gels were visualised using a ChemiDoc™ XRS+ System with Image Lab™ software (Bio-Rad Laboratories, USA). After analysis, gDNA was stored at – 20 °C.

2.7.3. PCR amplification

A 25 µl PCR reaction mixture was set up, which consisted of 12.5 µl 2 × Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark), 2 µl of each primer (10 µM), 3 µl gDNA template and 5.5 µl ddH_2O. A no-template control (NTC) was also set up using water instead of gDNA template. The primers Cl-J-1751 and Cl-N-2191 were used for the lepidopteran samples for PCR amplification of the target *COI* gene (Table 2.1) and the cycle parameters consisted of an initial denaturation step of 10 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C, and a final elongation step of 10 min at 72 °C. The methodology for these were according to Simons et al. (1994). The COBU and COBL primers were used to amplify the *COI* target gene from the cricket samples, this was according to Hawlitschek et al. (2017) (Table 2.1). The cycle parameters for these primers consisted of an initial denaturation step for 5 min at 95 °C, thereafter, 35 cycles of 45 s at 94 °C, 45 s at 48 °C and 1 min 30 s at 70 °C, followed by a final extension step at 72 °C for 10 min. A MiniAmp thermal cycler (Thermo Fisher Scientific, USA) was used to conduct the amplification.

Table 2.1: Primer pairs used for the amplification of the target *COI* genes from the samples.

Target gene	Primer name	Sequence 5'-3'	Target amplicon size	Reference
COI (Lepidoptera)	Cl-J-1751	GGATCACCTGATATAGCATTCCC	~ 443 bp	Simons et al. (1994)
	Cl-N-2191	CCCGGTAAAATTAAAATATAAACTTC		
COI (Orthoptera)	COBL	TYTCAACAAAYCAYAARGATATTGG	~ 658 bp	Hawlitschek et al. (2017)
	COBU	TAAACTTCWGGRTGWCCAAARAATCA		

2.7.4. Sanger sequencing

Positive PCR amplicons were column purified following the manufacturer's instructions for the DNA Clean & Concentrator®-25 purification kit (Zymo Research, USA). Purified PCR product DNA fragments were eluted in 25 µl DNA elution buffer. The purified DNA was sent for Sanger sequencing at Inqaba Biotechnical Industries (Pty) Ltd (South Africa) in the forward and reverse directions using the primers Cl-J-1751 and Cl-N-2191 for the lepidopteran samples, and COBU and COBL for the cricket samples (Table 2.1). ABI base recall was used to trim

low-quality reads at the beginning and the end of the sequence data (Elyazghi et al., 2017). The trimmed forward and reverse sequences were aligned in Benchling (https://www.benchling.com/) by MAFFT and a consensus sequence was extracted from the alignment. This was followed by conducting a nucleotide BLAST analysis by submitting the consensus sequences to NCBI BLAST.

2.8. Results

2.8.1. DNA extraction

Genomic DNA was successfully extracted from five samples from each insect species. Bands were observed resolving above the DNA ladder indicating the extracted gDNA (Figure 2.7). The bands produced were all greater than 10000 bp for all the samples from each insect species (Figure 2.7).

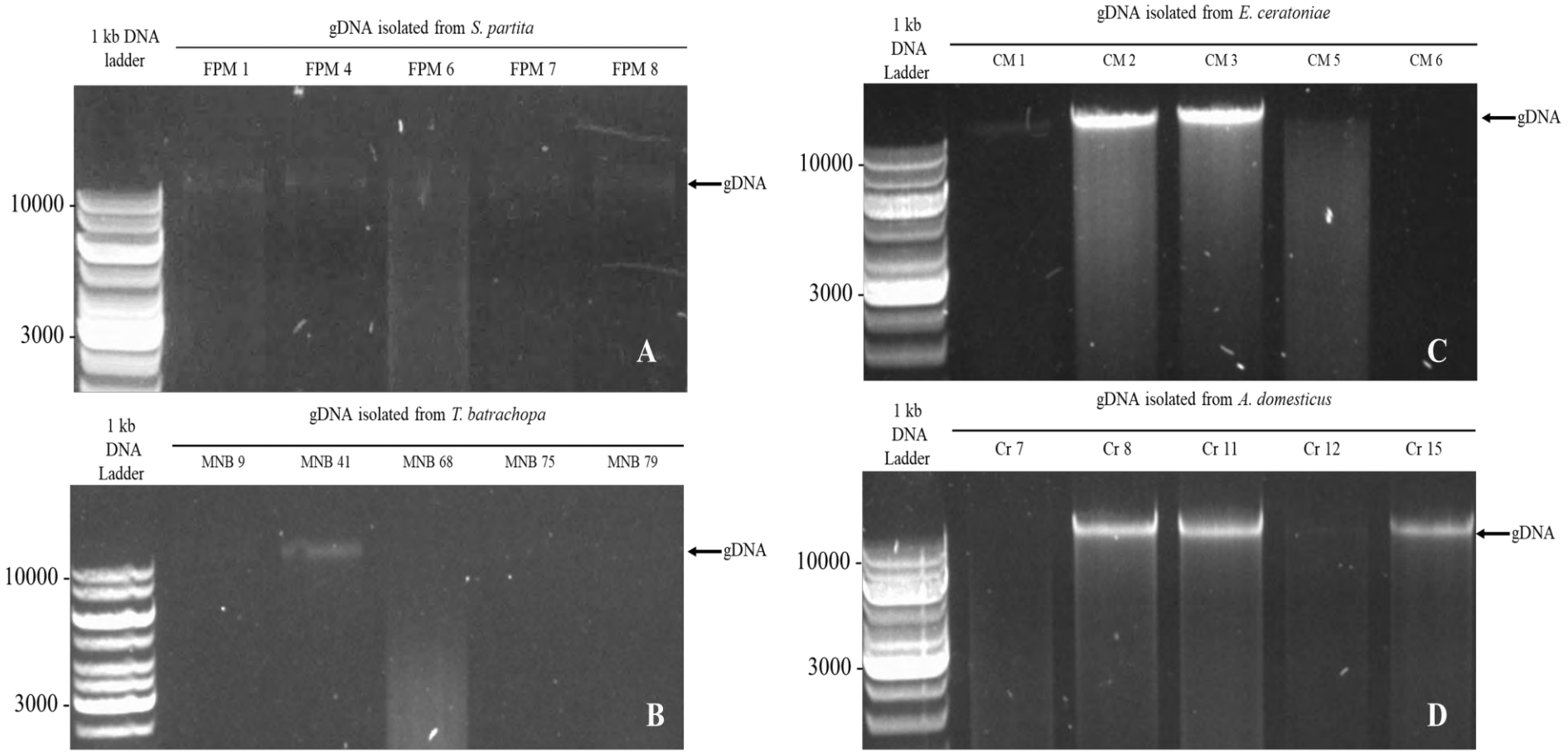

Figure 2.7: Agarose gel electrophoresis (AGE) with ethidium bromide staining, showing the genomic DNA extraction from different insect species. Genomic DNA was extracted from five samples from each species, these are from A: *Serrodes partita* larvae, B: *Thaumatotibia batrachopa* larvae, C: *Ectomyelois ceratoniae* larvae, and D: *Acheta domesticus* adults.

2.8.2. PCR amplification and sequencing

PCR amplification was successful for the *COI* target region, with consistent amplification across all insect samples from each species (Figure 2.8). The amplification products exhibited the expected band size of 440 bp for the lepidopteran samples and 658 bp for the cricket samples on the agarose gel, confirming the presence of the amplifiable DNA (Figure 2.8). The PCR amplicons for the *S. partita* samples are indicated in Figure 2.8 A (FPM 1, FPM 4, FPM 6, FPM 7, and FPM 8), those of *T. batrachopa* are indicated in Figure 2.8 B (MNB 9, MNB 41, MNB 68, MNB 75, and MNB 79), that of *E. ceratoniae* are indicated in Figure 2.8 C (CM 1, CM 2, CM 3, CM 5, & CM 6), and those of *A. domesticus* are indicated in Figure 2.8 D (Cr 7, Cr 8, Cr 11, Cr 12, and Cr 15). The PCR products were subsequently purified for Sanger sequencing. PCR amplicons where non-specific bands were produced, as seen in sample Cr 7 in lane two of Figure 2.8 D were not purified for sequencing.

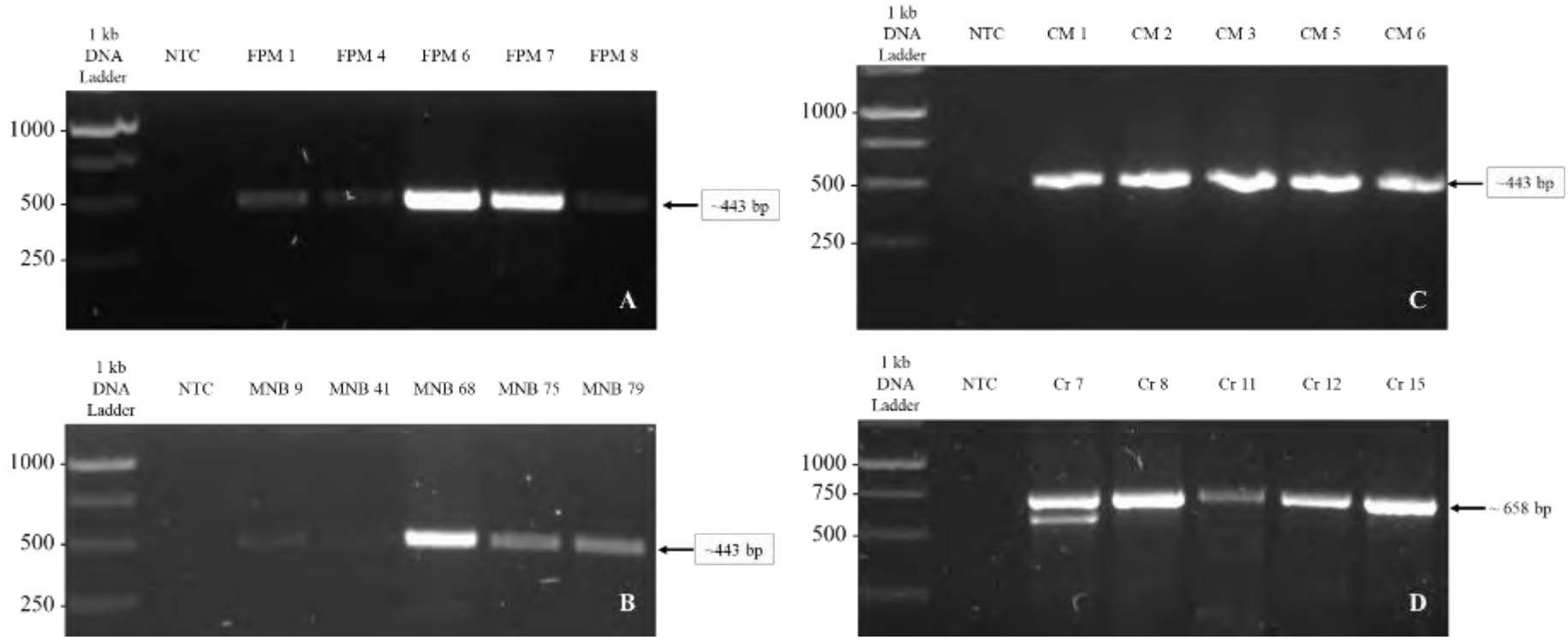

Figure 2.8: PCR amplification of the *COI* gene from various insect samples on a 1 % agarose gel with ethidium bromide staining. The gel shows the successful amplification of the *COI* gene from different insect species, with distinct bands corresponding to the expected amplicon size. The 1 kb DNA ladder (Lane 1, A-D) indicates the size of the amplified products, while the insect sample (Lane 3-7, A-D) show clear PCR products at approximately 443 bp (A-C) and 658 bp (D), confirming the presence of the *COI* gene. Negative controls (Lane 2, A-D) show no amplification. A) *S. partita*, B) *T. batrachopa*, C) *E. ceratoniae,* and D) *A. domesticus*.

Two samples for *S. partita* (FPM 6 and FPM 7), and *A. domesticus* (Cr 8 and Cr 15) and five samples for *T. batrachopa* (MNB 9, MNB 41, MNB 68, MNB 75 and MNB 79) and *E. ceratoniae* (CM 1, CM 2, CM 3, CM 5 & CM 6) were sent for Sanger sequencing in both the forward and reverse direction. An alignemnet of the forward and reverse sequences was conducted to generate a consensus sequence. The consensus sequence data were subjected to BLAST analysis, with each sample matching closely to the corresponding correct species (Table 2.2).

Table 2.2: The top BLAST match performed on the sequence data for samples from each species from the sequencing of the partial *COI* gene

	Sample name	Description	Scientific name	Percentage identity	E-value	Accession number
***Serrodes partita* samples**	FPM 6 consensus sequence	*Serrodes partita* voucher L11 cytochrome oxidase subunit I (COI) gene, partial cds: mitochondrial	*Serrodes partita*	98.89 %	0.0	KP083423.1
	FPM 7 consensus sequence	*Serrodes partita* voucher L11 cytochrome oxidase subunit I (COI) gene, partial cds: mitochondrial	*Serrodes partita*	96.82 %	0.0	KP083423.1
***Thaumatotibia batrachopa* samples**	MNB 9 consensus sequence	*Thaumatotibia batrachopa* cytochrome oxidase subunit I gene, partial cds; mitochondrial	*Thaumatotibia batrachopa*	99.55 %	0.0	EF584429.1
	MNB 41 consensus sequence	*Thaumatotibia batrachopa* cytochrome oxidase subunit I gene, partial cds; mitochondrial	*Thaumatotibia batrachopa*	98.64 %	0.0	EF584429.1
	MNB 68 consensus sequence	*Thaumatotibia batrachopa* cytochrome oxidase subunit I gene, partial cds; mitochondrial	*Thaumatotibia batrachopa*	99.55 %	0.0	EF584429.1
	MNB 75 consensus sequence	*Thaumatotibia batrachopa* cytochrome oxidase subunit I gene, partial cds; mitochondrial	*Thaumatotibia batrachopa*	99.09 %	0.0	EF584429.1
	MNB 79 consensus sequence	*Thaumatotibia batrachopa* cytochrome oxidase subunit I gene, partial cds; mitochondrial	*Thaumatotibia batrachopa*	99.77 %	0.0	EF584429.1

***Ectomyelois ceratoniae* samples**	CM 1 consensus sequence	*Ectomyelois ceratoniae* voucher MNB8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Ectomyelois ceratoniae*	99.78 %	0.0	OQ836367.1
	CM 2 consensus sequence	*Ectomyelois ceratoniae* voucher MNB10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Ectomyelois ceratoniae*	99.77 %	0.0	OQ836368.1
	CM 3 consensus sequence	*Ectomyelois ceratoniae* voucher MNB10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Ectomyelois ceratoniae*	99.77 %	0.0	OQ836368.1
	CM 5 consensus sequence	*Ectomyelois ceratoniae* voucher MNB10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Ectomyelois ceratoniae*	99.77 %	0.0	OQ836368.1
	CM 6 consensus sequence	*Ectomyelois ceratoniae* voucher MNB10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Ectomyelois ceratoniae*	99.77 %	0.0	OQ836368.1
***Acheta domesticus* samples**	Cr 8 consensus sequence	*Acheta domesticus* mitochondrial partial COI gene for cytochrome oxidase subunit 1, strain RI_001	*Acheta domesticus*	100 %	0.0	LR585071.1
	Cr 15 consensus sequence	*Acheta domesticus* voucher FDA:CFSAN OR 35-001 cytochrome oxidase subunit 1 (COI) gene, partial cds;	*Acheta domesticus*	100 %	0.0	MG458975.1

2.9. Discussion

The aim of this chapter was to conduct species identification using molecular genetic techniques by extracting gDNA from the larval samples of *S. partita*, *T. batrachopa,* and *E. ceratoniae* (lepidopteran samples) and the adults of *A. domesticus*, followed by PCR amplification of the *COI* gene and then sequencing and BLAST analysis. This ensured that we had a 100% accurate identification of the species for the research that follows.

The AGE results for the gDNA extraction showed high molecular weight from all the *S. partita*, *T. batrachopa, E. ceratoniae,* and *A. domesticus* samples, which were larger than 10000 bp in size. Therefore, the extraction of gDNA was successful, as the genomes of lepidopterans are small, ranging between ~246-809 Mb (Triant et al., 2018) and according to Szrajer et al. (2024) the genome of crickets ranges between 1.6-2.3 Gb. The AGE of the PCR amplicons showed that the targeted *COI* PCR amplicons for the lepidopteran samples were consistent with the 443 bp reported by Timm et al. (2007) and Simons et al. (1994) and the cricket samples were consistent with the 658 bp reported by Pan et al. (2006) and Huang et al. (2013). Therefore, the correct target region has been successfully amplified for all samples. For applied taxonomy users, barcoding is a useful tool for confirming the purity and identity of biological products and for the identification of regulated species, for example, endangered and invasive species (Kress & Erickson, 2012). The two basic steps of the DNA barcoding process involve firstly building a barcode library of species that are known and then secondly using the barcode sequence of the sample that is unknown and then matching or assigning it against the barcode library to identify the unknown sample (Kress & Erickson, 2012). BLAST is a programme used to search for similarities between sequences (Altschul et al., 1990, 1997). To confirm the species identity, the *COI* sequence obtained from the respective *S. partita, T. batrachopa*, *E. ceratoniae,* and *A. domesticus* specimens, were compared to sequences in GenBank using the BLAST search tool. GenBank provides a matching tool known as BLAST which can be used to search for similarities between a sequence library and a query sequence (Kress & Erickson, 2012). In this process, nucleotide queries are compared with a nucleotide database. BLAST will identify short matches between two sequences and use them to initiate alignments (Ye et al., 2006). The BLAST results revealed that the *S. partita* query sequences shared identity between 96.82 % and 98.89 % respectively with the top BLAST hit of the partial sequence of the *S. partita COI* gene. Similarly, the *T. batrachopa* query sequences shared identity between 98.64 % - 99.77 % respectively, with the top BLAST hit of the partial sequence of the *T. batrachopa COI* gene. The *E. ceratoniae* query sequences shared identity of 99.77 % and 99.78

% respectively with the top BLAST hit of the partial sequence of the *E. ceratoniae COI* gene and the *A. domesticus* query sequences shared a 100 % identity with the top BLAST hit of the partial sequence of the *A. domesticus COI* gene. Each sample species matched closely to previous sequences in the GenBank database. Therefore, confirming the correct species identification of each sample species.

In conclusion, the BLAST analysis confirmed the identity of the sample species as *S. partita*, *T. batrachopa*, *E. ceratoniae,* and *A. domesticus,* respectively, based on the high identity match to the reference sequence. This result is consistent with the species identification provided by DNA barcoding using the mitochondrial *COI* gene. The next chapter aims to screen each of the *S. partita*, *T. batrachopa,* and *E. ceratoniae* lepidopteran samples for novel baculovirus infection. If a novel baculovirus is isolated, then knowing the correct identification will be important when developing biopesticides for these insects.

Chapter 3 – Screening larval cadavers of agriculturally important lepidopteran pest for novel baculoviruses using PCR amplification of selected gene sequences

3.1. Introduction

As highlighted in Chapter 2, *S. partita*, *T. batrachopa*, and *E. ceratoniae* play a significant role in agriculture. These insects cause damage to crops rendering them undesirable for consumption or export, leading to economic losses. To date, there have been no reports in the literature describing viruses infecting these insects, but isolating novel baculoviruses from these lepidopteran species could potentially be used as microbial control agents and characterised as biopesticides.

Of the control products available on the market, baculoviruses are the most preferred and successfully commercialised viral control products (Lacey & Shapiro-Ilan, 2008). For this reason, the focus of this chapter is on the isolation and identification of novel baculoviruses from these three lepidopteran species. Federici (1997) suggested that in nature, there could be thousands of unique baculovirus species because of the diversity of Lepidoptera. Although there are numerous baculoviruses in nature, only a small fraction of these have been studied (Jehle et al. 2016). There is a total of 107 baculovirus species that infect the insect orders Diptera, Lepidoptera, and Hymenoptera (ICTV 2025a). The majority of baculoviruses that infect insects have been recorded from Lepidoptera, accounting for 90 %, from 34 distinct Lepidopteran families (Martignoni & Iwai 1981). Various characteristics make baculoviruses ideal candidates for microbial control. These include being host-specific; safe to beneficial insects, animals and humans; having a protein coat that enables their survival outside of their hosts; and they are capable of replicating and persisting in the environment, therefore the need for frequent spraying is reduced (Moscardi, 1999; Moscardi et al., 2011). Over the last 20 years, in regions such as Europe, South and North America, and Asia, there have been several baculovirus species that have been tested, developed into formulations, and sold as commercially (Haase et al., 2015; Lacey et al., 2001; Sun & Peng, 2007; Szewczyk et al., 2006). Additionally, since the 1960s in Africa, baculoviruses have been explored as potential

biopesticides for managing agricultural pests. The first identification of CrleGV was isolated from larvae of the *T. leucotreta* in the Ivory Coast (Angélini et al., 1965a).

Today, a handful of lepidopteran pests are controlled by using a few baculoviruses, with advances in DNA technology allowing for rapid isolation, identification, and characterisation of viruses (Cory & Myers, 2003; Knox et al., 2015). In African countries, there are eleven species of baculovirus that have been, or are currently being studied to be used as biopesticides (Moore & Jukes, 2023). In South Africa, examples of successful baculovirus biopesticides that are commercially available are Cryptogran® (River Bioscience, South Africa) formulated with CrleGV which is used against *T. leucotreta*, Carpovirusine® and Madex® (Andermatt-Biocontrol, AG Switzerland) formulated with CpGV which is used against *C. pomonella*, Helicovir® and HeliMax™ (River Bioscience, South Africa) formulated with HearNPV which is used for the control of *H. armigera* (Knox et al., 2015; Moore et al., 2014.), MultiMax™ and CodlMax™ (River Bioscience, South Africa) formulated with CrpeNPV which is used against *T. leucotreta* and other agriculturally important pests, Codlgran™ (River Bioscience, South Africa), formulated with CpGV and used against *C. pomonella*, and CryptoMax™ (River Bioscience, South Africa) used to control *T. leucotreta* and formulated with CrleGV.

Gene cloning and sequencing, molecular phylogeny, and DNA restriction endonuclease analysis are different molecular methods that are useful for characterising genes and genomes for virus identification (Lange et al., 2004). Additionally, virus identification can be achieved through PCR, which was demonstrated in Autographa californica multiple nucleopolyhedrovirus (AcMNPV) and a few other nucleopolyhedroviruses (NPVs) (de Moraes & Maruniak, 1997). The method of using degenerate PCR in conjunction with molecular phylogeny allows for the rapid and definitive identification of baculoviruses, which requires only minimal sample amounts, even in cases where the virus cannot be isolated (Lange et al., 2004). Polymerase chain reaction is used to identify a baculovirus, where a characteristic baculovirus gene is targeted and amplified using specific primers. Examples of target genes include the *lef-8*, *ecdysteroid UDP-glucoslytransferase* (*egt*) or *polh/gran* genes. The presence of the baculovirus is confirmed when one or more of the target gene regions is successfully amplified (Hunter-Fujita et al., 1998). The DNA from species without prior sequence information can be sequenced and identified using universal PCR primers (Simons et al., 1994). Universal or degenerate oligonucleotide primers are utilised when sequence data is unavailable for a target sequence of a PCR-amplified gene of a novel virus. Various conserved and related genomic sequences can be amplified using degenerate primers.

The goal of the study by Lange et al. (2004), was to develop a rapid, high-throughput screening method, which would be used for lepidopteran baculovirus identification and classification. Lange et al. (2004) developed oligonucleotides through comparative analysis of sequenced lepidopteran-specific baculovirus genomes, and these oligonucleotides were designed to amplify specific regions of the baculovirus genome through the methods of PCR amplification and DNA sequencing. The targeted genes for the amplification were the highly conserved genes namely, *lef-8*, *polh/gran*, and the *lef-9* genes, which are encoded by genomes of GVs and NPVs specific to Lepidoptera. The *polh/gran* is also found in baculoviruses of sawflies (Estrada-Emigdio et al. 2023). The viral DNAs extracted from historical field samples were used to test the designed oligonucleotides, and the results successfully amplified PCR products from 8 GV and 12 NPV DNAs. The oligonucleotides developed remain highly specific to baculovirus DNA despite it being highly degenerate (Lange et al., 2004). The study by Jehle et al. (2006b), isolated baculovirus DNA from historic virus samples from a wide range of hosts and geographic locations and used the degenerate oligonucleotides described by Lange et al. (2004) to PCR amplify partial gene sequences. This study validated the use of the degenerate primers for the PCR method for baculovirus identification. Additionally, the gene-specific binding regions were maintained as previously described for the degenerate primers. Therefore, these three degenerate oligonucleotides designed by Lange et al. (2004) were selected for PCR amplification for the current chapter to identify novel baculoviruses. Whole genome sequences of baculoviruses have become more readily accessible because of the advancements in DNA sequencing techniques (Ayres et al., 1994). Many fully sequenced baculovirus genomes have been deposited on Genbank. GenBank is an extensive database that hosts nucleotide sequences for more than 340000 formally described species, which are publicly available (Benson et al., 2007; Clark et al., 2016). The function of GenBank is both as a reference database for the research community and as an archive for sequence data published in the scientific literature (Federhen et al., 2016). GenBank offers a tool called BLAST, which allows users to search for similarities between a query sequence and a sequence library (Kress & Erickson, 2012). As such, BLAST analysis will be used to identify novel baculoviruses from sequenced PCR amplicons.

The chapter aims to isolate novel baculoviruses from symptomatic larvae of *S. partita*, *T. batrachopa*, and *E. ceratoniae*. The objectives of the chapter are, i) crude purification of virus particles, ii) CTAB DNA extraction from purified OBs, iii) PCR amplification of the *polh/gran*, *lef-8* and *lef-9* gene regions and iv) Sanger sequencing and BLAST analysis.

3.2. Methodology

3.2.1. Insect sample collection

Symptomatic larval cadavers of *S. partita*, *T. batrachopa*, and *E. ceratoniae* were obtained from various locations (Chapter 2, section 2.7.1). The larval symptomology was that of the 'wilting' disease which included being milky or glossy, or black/brown in colour. Seven individual *S. partita* larvae and eighty-two individual *T. batrachopa* larvae were screened for baculovirus infection. Nine tubes each containing multiple *E. ceratoniae* larvae were screened for baculovirus infection.

3.2.2. Crude purification

The virus OBs were isolated from larval cadavers following a crude extraction method taken from Bennett (2022), which was originally adapted from Wennmann & Jehle (2014). An individual larval cadaver was placed into a 2 ml tube and homogenised in 1 ml 0.1 % SDS (sodium dodecyl sulphate) using a sterile microtube pestle. The homogenate was vortexed for 1-2 min, followed by centrifugation at 100 ×g for 60 s. The supernatant was collected into a new 2 ml tube and the pellet was resuspended in 1 ml 0.1 % SDS and centrifuged for 60 s at 100 ×g. The supernatant was pooled together with the previously collected supernatant and thereafter centrifuged for 5 min at 2500 ×g. The supernatant was then discarded, and the pellet was resuspended in 1 ml ddH_2O and centrifuged at 12000 ×g for 5-10 min. The supernatant was discarded, and the final pellet was resuspended in 500 µl ddH_2O.

3.2.3. Cetyltrimethylammonium bromide (CTAB) DNA extraction

The purified OBs were used for DNA extraction following the CTAB method (Opoku-Debrah et al., 2013). In a 1.5 ml tube, 90 µl of 1M Na_2CO_3 was mixed with 200 µl purified OBs and then incubated at 37 ℃ for 30 min. After incubation, the suspension was neutralised with 120 µl Tris-HCl (1M, pH 6.8) followed by the addition of 20 µl Proteinase K (25 mg/ml) and 90 µl 10 % SDS (w/v), followed by incubating at 37 ℃ for 30 min. An additional 10 µl RNase A (10 mg/ml) was added and thereafter the sample was incubated again at 37 ℃ for 30 min. The sample was then centrifuged at 12100 ×g for 3 min and the resulting supernatant was placed into a new tube. 400 µl pre-warmed (70 ℃) CTAB buffer (54 mM CTAB, 0.1 mM Tris-HCl, 20 mM Na_2EDTA, 1.4 M NaCl) was added and the sample was then incubated for a final time at 70 ℃ for 45 min. An addition of 400 µl pre-cooled (4 ℃) chloroform was added, and the tube was inverted a few times followed by centrifugation at 6700 ×g for 10 min. The upper aqueous phase was carefully pipetted into a new 2 ml tube and 400 µl of isopropanol (ice-cold,

-20 °C) was added. The sample was incubated overnight at -20 °C to allow the DNA to precipitate. After the overnight incubation step, samples were centrifuged at 12100 ×g for 20 min and the supernatant was discarded. The resulting pellet was resuspended in 250 µl 70 % ethanol (ice-cold, -20 °C) and centrifuged at 12100 ×g for 5 min. The supernatant was carefully discarded, and the pellet was air dried, before resuspending in 20 µl ddH_2O. The extracted DNA was stored at – 20 °C. A Nanodrop™ Lite Plus Spectrophotometer (Thermo Scientific, USA) was used to measure the concentration of gDNA extracted from the samples, and this was conducted in triplicate.

3.2.4. Occlusion body purification and DNA extraction of a positive control sample

Purified CrleGV OBs were provided by Rhodes University, South Africa. *Thaumatotibia leucotreta* larval cadavers infected with CrleGV were obtained from a colony at Rhodes University and purified by glycerol gradient centrifugation (Hunter-Fujita et al. 1998; Jukes 2015). Larval cadavers were homogenised in 6 ml 0.1 % SDS using a sterile pestle and mortar. A cheese cloth was used to filter the homogenate into a beaker, divided into two JA-20 centrifuge tubes equally and topped with ddH_2O. Tubes were centrifuged in a Beckman Coulter Avanti® J-E centrifuge for 30 min at 7840 ×g and 4 °C. The supernatant was removed, ddH_2O was used to resuspend the pellet and tubes were centrifuged again for 30 min at 7840 ×g and 4 °C. The supernatant was removed and 1.5 ml ddH_2O was used to resuspend the pellet. In two ultracentrifuge tubes a continuous 30-80 % (v/v) glycerol gradient using 0.1 % SDS was prepared, and the substrate was pipetted on top of the gradient. Gradients were centrifuged using a Beckman Coulter Optima™ L-90K Ultracentrifuge for 15 min at 27783 ×g. The whitish-brown OB-forming bands were collected and transferred into two clean JA-20 tubes. Each tube was filled to the top with ddH_2O and then centrifuged at 7840 ×g for 30 min at 4 °C. The supernatant was discarded, the pellets were resuspended in ddH_2O, and the centrifugation step was repeated. Afterwards, the supernatant was removed again, and the pellets were combined into a single JA-20 tube, resuspended in ddH_2O, and centrifuged one final time for 30 min at 7840 ×g at 4°C. The resulting pellet was resuspended in 750 µl of ddH_2O and transferred to a 1.5 ml microcentrifuge tube. The purified OBs were stored at -20 °C prior to gDNA extraction.

The purified OBs were used for gDNA extraction. A sodium carbonate pre-treatment step was conducted by adding 50 µl purified OBs, 50 µl ddH_2O and 45 µl 1M Na_2CO_3 in a 1.5 ml tube and incubated at 37 °C for 15 min. 60 µl 1M Tris-HCl (pH 6.8) was added to the sample to

neutralise the pH. Thereafter, the manufacturer's instructions for the Quick-DNA™ Miniprep Plus kit (Zymo Research, USA) was followed to extract gDNA. The centrifugations were all performed at 12000 ×g and 50 µl (pre-heated to 70 °C) DNA elution buffer was used to elute the DNA. Extracted DNA was stored at -20 °C prior to subsequent experiments.

3.2.5. PCR amplification

Each 25 µl PCR reaction consisted of 12.5 µl Taq DNA polymerase 2× Master Mix RED (Ampliqon, Denmark), 2 µl 10 µM forward oligonucleotide (Table 3.1), 2 µl 10 µM reverse oligonucleotide (Table 3.1), 3 µl template gDNA (-0.67 to 71.45 ng/µl) and 5.5 µl ddH_2O. Each oligonucleotide contained the M13 universal forward and reverse adapter sequences which are target-specific regions for sequencing using the M13 forward and reverse sequencing oligonucleotides. For each PCR reaction, no template controls (NTC) were used and for these reactions in which template gDNA was replaced with ddH_2O. Positive controls were used for each PCR reaction using gDNA of a known CrleGV sample (Section 3.2.4). The oligonucleotide set targeting the *polh/gran* gene was used for the amplification from multiple samples (Table 3.1). The following cycle parameters were used as indicated by Lange et al. (2004): an initial denaturation step at 95 °C for 3 min, thereafter 36 cycles of 95 °C for 30 s, 70 °C for 1 min, 50 °C for 1 min and a final elongation step for 10 min at 72 °C. The oligonucleotide sets for the PCR amplification of the *lef-8* (Table 3.1) and the *lef-9* (Table 3.1) genes were used for selected samples. The cycle parameters for the *lef-8* (prL8) and *lef-9* (prL9) oligonucleotide sets were followed according to Lange et al. (2004). The cycle parameters for the prL8 oligonucleotide set consisted of an initial denaturation step of 95 °C for 4 min, 35 cycles of 2 min at 95 °C, 1 min at 72 °C, and 1 min at 38 °C, and a final elongation step of 2 min at 72 °C. The prL9 cycle parameters consisted of an initial denaturation step for 4 min at 95 °C, 35 cycles of 2 min at 95 °C, 1 min at 72 °C, and 1 min at 45 °C, and a final elongation step of 2 min at 72 °C. The resultant PCR products were analysed by 1 % AGE. The agarose gel was visualised using a ChemiDoc™ XRS+ System with Image Lab™ software (Bio-Rad Laboratories, USA).

Table 3.1: The degenerate oligonucleotide sets used for the amplification of the target gene regions

Target gene	Oligonucleotide name	Sequence 5'-3'	Target amplicon size	Reference
polh/gran	prPH-1	TGTAAAACGACGGCCAGTNRCNGARGAYCCNTT	507-510 bp	Lange et al. 2004
	prPH-2	CAGGAAACAGCTATGACCDGGNGCRAAYTCYTT		
lef-8	prL8-1	TGTAAAACGACGGCCAGTCAYGGHGARATGAC	681-771 bp	
	prL8-2	CAGGAAACAGCTATGACCAYRTAS$_1$GGRTCYTCS$_2$GC		
lef-9	prL9-1	CAGGAAACAGCTATGACCAARAAYGGITAYGCB$_3$G	258-273 bp	
	prL9-2	TGTAAAACGACGGCCAGTTTG$_4$TCDCCRTCRCARTC		

The standard sequencing primers (-21) M13 forward and (-29) M13 reverse are indicated by the underlined nucleotides.

(I = Inosin; B = C, G or T; D = A, G, or T; H = A, C, or T; N = A, C, G or T; R = A or G, S = C or G; Y = C or T)

(B_3 = A in AdhoNPV; G_4 = A in PhopGV and HzSNPV; S_1 = T in CrleGV; S_2 = T in AdhoNPV)

3.2.6. Sanger sequencing

Positive PCR amplicons were purified before sequencing. Gel purification was done following the manufacturers' instructions of the GeneJET Gel Extraction kit (Thermo Fischer Scientific, USA), and purified DNA was eluted in 50 µl Elution buffer. This method was used for multiple samples. The manufacturer's instructions for the DNA Clean & Concentrator®-25 kit (Zymo Research, USA) were followed for column purification and purified DNA was eluted in 25 µl DNA Elution buffer. This method of purification was used for selected samples. Purified samples were sent for Sanger sequencing at Inqaba Biotechnical Industries (Pty) Ltd (South Africa) in the forward direction using the M13 sequencing oligonucleotides which bind to the M13 sequences on the degenerate oligonucleotides. Low-quality reads at the beginning and the end of the sequence data were trimmed using the ABI base recall software (Elyazghi et al., 2017), followed by conducting a nucleotide BLAST analysis by submitting the sequences to NCBI BLAST. The *S. partita polh/gran*, *lef-8*, and *lef-9* sequences were aligned with the sequences produced by Mushore (2024) on Benchling (https://www.benchling.com/) by conducting a pairwise alignment using the MAFFT method.

3.3. Results

3.3.1. CTAB DNA extraction

Genomic DNA was extracted from seven crude purified *S. partita* larval samples, eighty-two *T. batrachopa* and nine *E. ceratoniae* samples (Figure 3.1). The gDNA resolved above 10000 bp for all samples. The concentration of the template gDNA ranged between 3.5 ng/µl to 10.1

ng/µl for *S. partita* gDNA samples, 0 ng/µl to 71.45 ng/µl for *T. batrachopa* gDNA, and 0 ng/µl to 56.5 ng/µl for *E. ceratoniae* gDNA samples.

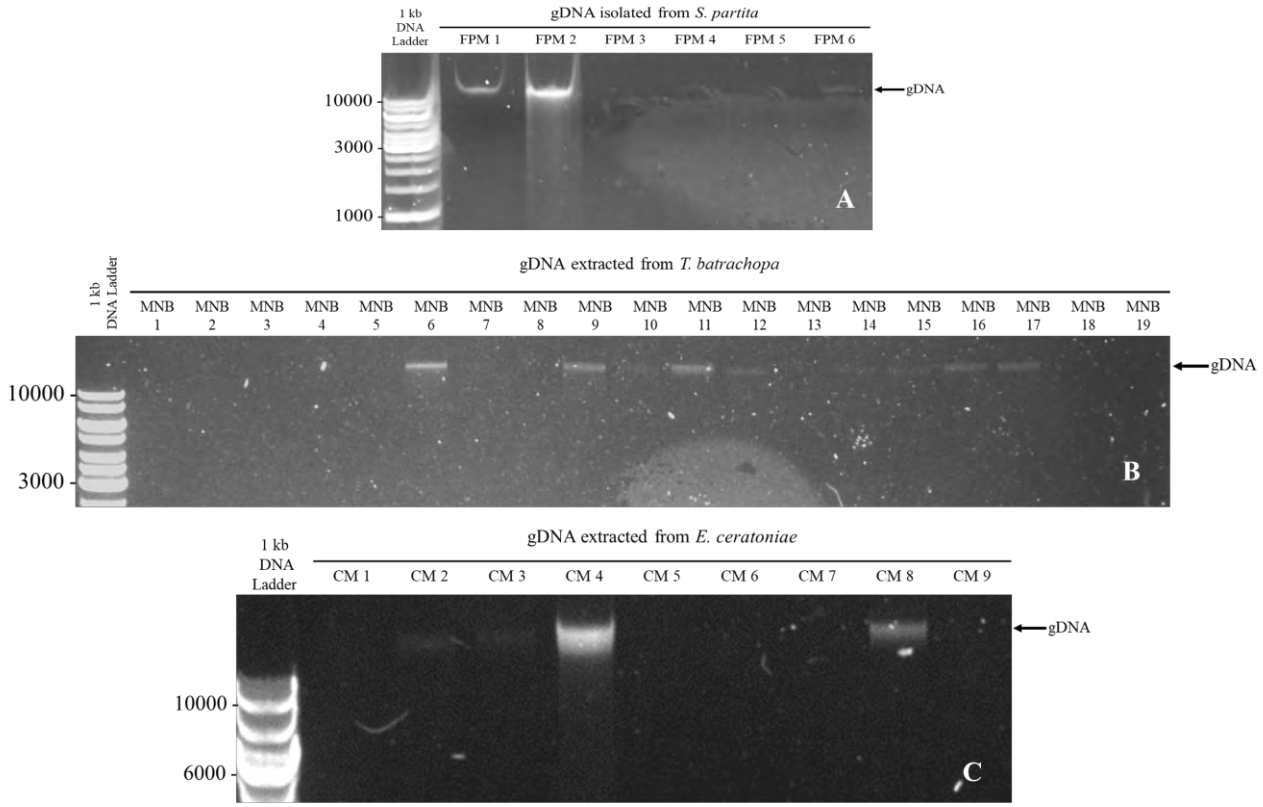

Figure 3.1: AGE with ethidium bromide staining of the extracted viral DNA from OBs purified from A: *S. partita* OB samples, B: *T. batrachopa* OB samples and C: *E. ceratoniae* OB samples. Images from gel A, B, and C are representative of the viral DNA extracted from all the OB samples.

3.3.2. PCR amplification

Polymerase chain reaction amplification was performed to amplify baculovirus DNA using degenerate oligonucleotides targeting conserved genome regions. PCR amplification of the *polh/gran* target gene was successful for five of the *S. partita* samples and sixty-six of the *T. batrachopa* samples. The amplicon sizes were at the expected band size of ~510 bp (Figure 3.2 A and B), shown in lanes 2-3 and lanes 2-4 in Figure 3.2 A1 and A2 respectively for the *S. partita* samples, and in lanes 3-19 respectively in Figure 3.2 B for the *T. batrachopa* samples. Non-specific and faint bands were observed for the *E. ceratoniae* samples (Figure 3.2 3.2 C). No amplicons were observed for the NTCs in lane 1 for all the insect species (Figure 3.2 A, B, and C). Amplicons of approximately 510 bp were produced for the positive controls for both

the *T. batrachopa* and *E. ceratoniae* PCR reactions observed in lane 2 in Figure 3.2 B and C, and these were of the expected band sizes.

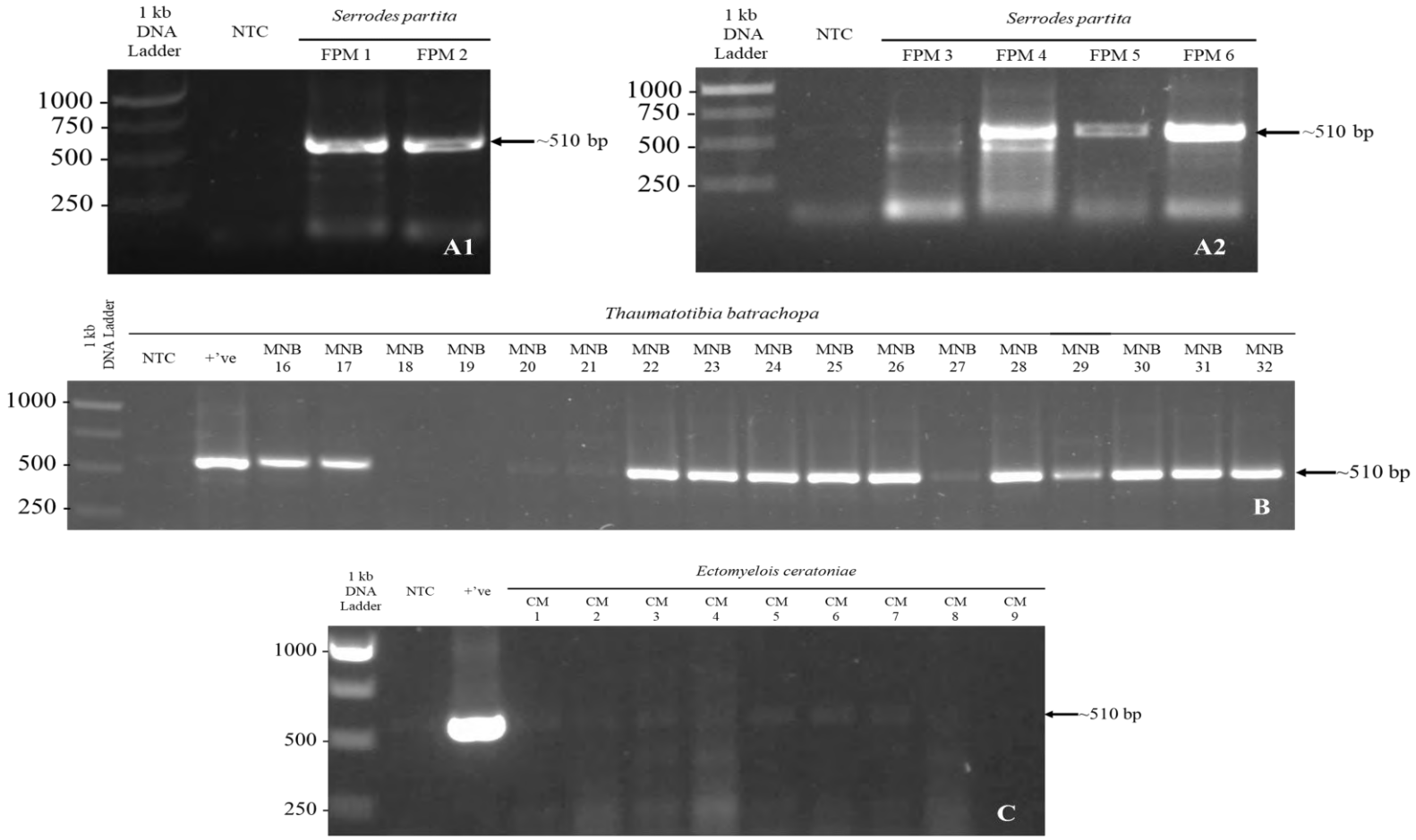

Figure 3.2: AGE with ethidium bromide staining of PCR amplification using the *polh/gran* oligonucleotide set. A1 and A2: PCR amplification of the gDNA extracted from OBs purified from *S. partita* larval samples, B: A representative gel for the PCR amplification of the gDNA extracted from OBs purified from *T. batrachopa* larval samples and C: PCR amplification of the gDNA extracted from OBs purified from *E. ceratoniae* larval samples. The expected amplicon size is ~510 bp.

The *lef-8* and *lef-9* oligonucleotide sets were used for the *S. partita* samples and the *E. ceratoniae* samples. Amplicons of the approximate expected sizes for the *lef-8* oligonucleotides (~760 bp) were observed for sample FPM 1 and FPM 6 for the *S. partita* samples (Figure 3.3 A), indicating the successful amplification of this target gene region. Amplification of the target *lef-9* region was successful for *S. partita* samples FPM 1, FPM 2, and FPM 6 (Figure 3.3 B), with an amplicon size of ~270 bp obtained. No bands were observed for the NTC reactions (Figure 3.3). No amplicons were obtained for the *E. ceratoniae* PCR amplification using the *lef-8* oligonucleotide set, and for the *E. ceratoniae* PCR amplification targeting the *lef-9* gene non-specific bands were observed.

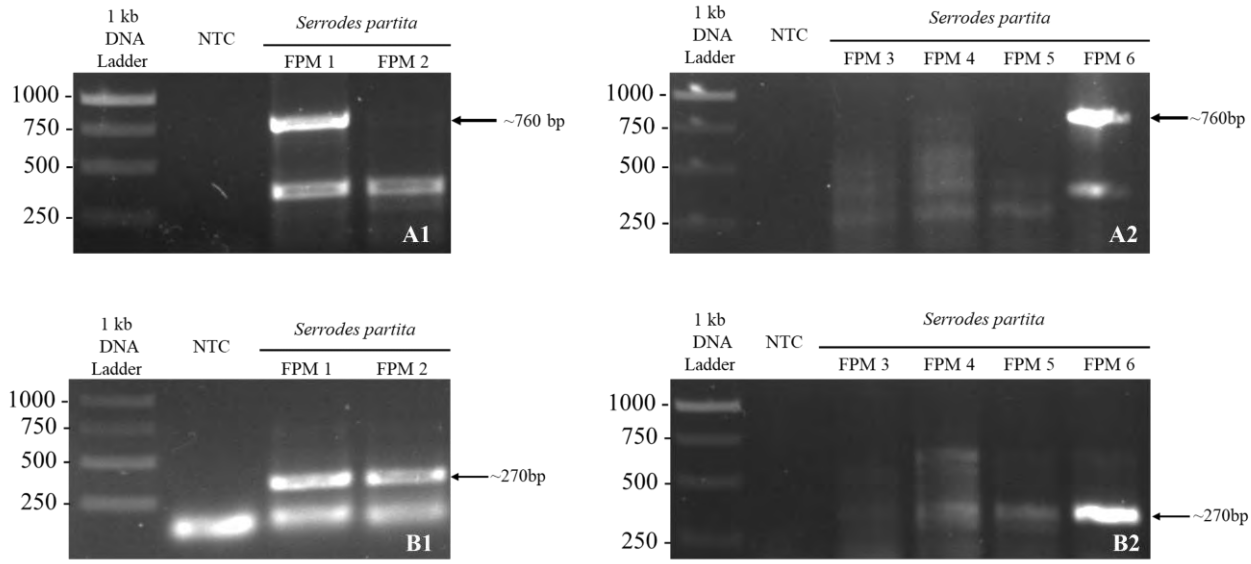

Figure 3.3: AGE with ethidium bromide staining of PCR amplification from *S. partita* larval samples using the *lef-8* and *lef-9* oligonucleotide sets. A1 and A2) *lef-8* PCR amplicons with an expected amplicon size of ~760 bp. B1 and B2) *lef-9* PCR amplicons with an expected amplicon size of ~270 bp.

3.3.3. Sanger sequencing

Fourteen of the positive *T. batrachopa* PCR amplicons were sent for sequencing, and sequence results were subjected to nucleotide BLAST analysis (Table 3.2). All samples matched closely with the Cryptophlebia leucotreta granulovirus, with the percentage identity ranging between 96.22 % to 99.38 %.

Table 3.2: *Thaumatotibia batrachopa* BLAST results showing the closest match for the sequenced PCR amplicon samples

Sample Name	Description	Scientific name	Percentage Identity	E-value	Accession number
MNB 6	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	98.97 %	0.0	AY293731.1
MNB 7	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	98.10 %	0.0	AY293731.1
MNB 8	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	96.22 %	0.0	AY293731.1
MNB 9	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	99.38 %	0.0	AY293731.1
MNB 10	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	99.17 %	0.0	AY293731.1
MNB 72	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	99.38 %	0.0	AY293731.1
MNB 74	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	98.93 %	0.0	AY293731.1
MNB 75	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	99.17 %	0.0	AY293731.1
MNB 76	Cryptophlebia leucotreta granulovirus, complete genome	Cryptophlebia leucotreta granulovirus	99.17 %	0.0	NC_005068.1

MNB 77	Cryptophlebia leucotreta granulovirus, complete genome	Cryptophlebia leucotreta granulovirus	99.38 %	0.0	NC_005068.1
MNB 78	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	98.99 %	0.0	AY293731.1
MNB 79	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	99.38 %	0.0	AY293731.1
MNB 81	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	99.17 %	0.0	AY293731.1
MNB 82	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA granulin gene, complete cds	Cryptophlebia leucotreta granulovirus	99.15 %	0.0	AY293731.1

The *polh/gran*, *lef-8* and *lef-9* PCR amplicons for FPM 1 and FPM 6 of the *S. partita* samples were sent for sequencing, and sequence results were subjected to nucleotide BLAST analysis (Table 3.3). The results from the BLAST analysis of the *S. partita* larval samples show that the *polh/gran* PCR amplicon sequences for both FPM 1 and FPM 6 samples matched closely with Peridroma alphabaculovirus, the sequenced *lef-8* PCR amplicons for both FPM 1 and FPM 6 samples matched closely with Olene mendosa nucleopolyhedrovirus and the sequenced *lef-9* PCR amplicons for both FPM 1 and FPM 6 samples matched closely with Phalera bucephala nucleopolyhedrovirus. The percentage identity for all the sequenced *S. partita* PCR amplicons was less than 85 % (Table 3.3). The *polh*/*gran* sequences had a 91.04 % pairwise identity, the *lef-8* sequences had a 90.13 % pairwise identity match, and the *lef-9* sequences had a 91.95 % pairwise identity match when aligned with the sequences by Mushore (2024) (Figure S1, Figure S2, Figure S3). Sequencing was not conducted on *E. ceratoniae* samples due to non-specific bands obtained for the *polh/gran* PCR amplification.

Table 3.3: *Serrodes partita* sequence results showing the closest BLAST match for the sequenced PCR samples

Sample Name	Target gene	Description	Scientific name	Percentage Identity	E-value	Accession number
FPM 1	*polh/gran*	Peridroma alphabaculovirus isolate GR_167, complete genome	Peridroma alphabaculovirus	83.48 %	6×10^{-131}	NC_024625.1
	lef-8	Olene mendosa nucleopolyhedrovirus isolate 435, complete genome	Olene mendosa nucleopolyhedrovirus	73.35 %	6×10^{-69}	NC_077147.1
	lef-9	Phalera bucephala nucleopolyhedrovirus isolate 204 lef-9 gene, partial cds	Phalera bucephala nucleopolyhedrovirus	83.24 %	9×10^{-43}	MH454177.1
FPM 6	*polh/gran*	Peridroma alphabaculovirus isolate GR_167, complete genome	Peridroma sp. nucleopolyhedrovirus	82.36 %	2×10^{-137}	NC_024625.1
	lef-8	Olene mendosa nucleopolyhedrovirus isolate 435, complete genome	Olene mendosa nucleopolyhedrovirus	73.45 %	5×10^{-70}	NC_077147.1
	lef-9	Phalera bucephala nucleopolyhedrovirus isolate 204 lef-9 gene, partial cds	Phalera bucephala nucleopolyhedrovirus	82.68 %	4×10^{-40}	MH454177.1

3.4. Discussion

The chapter aimed to isolate novel baculoviruses from the lepidopteran species *S. partita*, *T. batrachopa*, and *E. ceratoniae*. To achieve this, virus OBs were obtained from symptomatic larval cadavers, followed by gDNA extraction and PCR amplification of selected genes using degenerate *polh/gran*, *lef-8*, and *lef-9* oligonucleotides. Thereafter, sequencing of PCR amplicons and BLAST analysis were performed to identify novel baculoviruses. The extraction of gDNA from purified *S. partita*, *T. batrachopa*, and *E. ceratoniae* larval cadavers was accomplished, yielding extracted gDNA fragments greater than 10000 bp in length. The size of the gDNA fragments falls within the size range of baculovirus genomes, which is between 80 to 180 kbp in length (Harrison et al., 2018). It was later identified upon PCR analysis that the isolation of baculovirus gDNA present on the agarose gels from the *E. ceratoniae* samples could have been a result of the extraction of host DNA. According to Triant et al. (2018), the genome sizes of Lepidoptera are relatively small, ranging between ~ 246-809 MB. The size of the gDNA fragments isolated from *E. ceratoniae* was greater than 10000 bp which falls within the size range of Lepidoptera genomes.

Polymerase chain reaction amplification and sequencing of the target genes, using the oligonucleotides described by Lange et al. (2004) was performed to genetically identify any virus isolated from the three insect species. A key advantage of PCR amplification is that it requires only minimal amounts of the starting material and enables degraded or small individuals to be sequenced (Pääbo, 1990). Therefore, even though gDNA was only observed for a few samples, PCR amplification was still performed on all the gDNA extracted samples. According to Lange et al. (2004), amplifying the targeted *polh* gene from 20 baculoviruses resulted in a PCR amplicon size that ranged between 507 - 510 nucleotides. For the amplification of the targeted *lef-8* gene, the PCR amplicon size ranged between 681 – 771 nucleotides and for the targeted *lef-9* gene, the PCR amplicons ranged between 258 – 273 nucleotides. The size of PCR amplicons of the targeted genes from the *S. partita* and *T. batrachopa* samples in the current study were synonymous with the size range of the target genes in the study by Lange et al. (2004). This indicates the successful amplification of the targeted *polh*/*gran* gene for the *T. batrachopa* and *S. partita* samples and the targeted *lef-8* and *lef-9* genes for the *S. partita* samples. For the rapid identification of uncharacterised baculovirus isolates, using a single target gene, for example, the *polh/gran*, may often be sufficient (Lange et al., 2004). For this reason, only the targeted *polh/gran* gene was initially used for all three insect species samples. Similarly, the study by Marsberg (2016) screened

symptomatic larvae of *C. peltastica* by using the degenerate primers by Lange et al. (2004) for PCR amplification and sequencing of the *gran/polh* gene and identified and characterised a novel SNPV from *C. peltastica.*

PCR amplification using the *lef-8* and *lef-9* oligonucleotides was only used for the *S. partita* and *E. ceratoniae* samples. This was because, after sequencing the *polh/gran* PCR amplicons of the *S. partita* samples, the percentage identity of the closest match was less than 85 %, indicating the possible isolation of a novel baculovirus species and therefore, further analysis using the *lef-8* and *lef-9* oligonucleotides was conducted. PCR amplification of these genes was effective for the *S. partita* samples. Additionally, for the *E. ceratoniae* samples, non-specific bands for the *polh*/*gran* PCR amplification was obtained and because of this PCR amplification of the *lef-8* and *lef-9* genes was further attempted. However, no positive amplicons were observed for the PCR amplification targeting the *lef-8* gene and non-specific bands were observed for the PCR amplification targeting the *lef-9* gene. Successfully amplified PCR amplicons were sent for sequencing. The sequenced PCR amplicon data were subjected to BLAST analysis to determine the baculovirus species it closely matches. All the sequenced *T. batrachopa* samples were found to be infected with CrleGV, and this study is the first to report the isolation of CrleGV from *T. batrachopa*.

Various crops across sub-Saharan Africa are significantly damaged by *T. leucotreta* (Moore, 2011). Additionally, both *T. leucotreta* and *T. batrachopa* are pests on macadamia nuts. Greater than 90 % of the damage to macadamia nut husk and kernels is caused by *T. batrachopa* and *T. leucotreta* (Schoeman et al., 2003; Schoeman, 2009; Timm et al., 2006). Furthermore, a yield loss exceeding 30 % was recorded in infested macadamia farms in South Africa due to *T. leucotreta* (La Croix & Thindwa, 1986). There are various control options for the management of *T. leucotreta*. The use of the Cryptophlebia leucotreta granulovirus is an example of one of the control options used for the management of *T. leucotreta* (Moore et al., 2004a). The South African biopesticide Cryptogran™, formulated with CrleGV, has been used for the control of *T. leucotreta* on grapes, avocadoes, citrus and other crops since 2003 (Grové et al., 2010; Moore et al., 2004a). It is possible that Cryptogran was applied in or near macadamia orchards to control *T. leucotreta*. As such, the larval cadavers of *T. batrachopa* examined in this chapter may have ingested the virus while feeding on macadamia nuts sprayed with CrleGV for *T. leucotreta* management.

The sequenced *polh/gran*, *lef-8*, and *lef-9* PCR amplicons from the *S. partita* samples matched closely with Peridroma sp. nucleopolyhedrovirus, Olene mendosa nucleopolyhedrovirus, and

Phalera bucephala nucleopolyhedrovirus respectively. The Peridroma sp. nucleopolyhedrovirus (PespNPV) was isolated from the cutworm larvae, *Peridroma* species (Lepidoptera: Noctuidae) (Rohrmann, 2013). This virus groups with the group II lineage of alphabaculoviruses and this grouping is based on the partial *lef-8* gene sequence of the virus (Rohrmann, 2013). Additionally, it is most closely related to Spodoptera exigua NPV and Agrotis segetum NPV, both of which are part of the group II lineage of alphabaculoviruses (Rohrmann et al., 2015). The Olene mendosa nucleopolyhedrovirus (OlmeNPV) is an alphabaculovirus isolated from larval cadavers of *Olene mendosa*, the brown tussock moth (Rabindra & Subramaniam, 1974). The insect family that *O. mendosa* belongs to is the Erebidae family and Lymantriinae subfamily (Wang et al., 2015; Zahiri et al., 2012). This virus also groups within the group II NPVs and is most closely related to Lymantria dispar multiple nucleopolyhedrovirus and Lymantria xylina multiple nucleopolyhedrovirus (Harrison & Rowley, 2022a).

In the study by Mushore (2024) *S. partita* larval cadavers were screened for baculovirus infection. They were the first to report the isolation of a novel alphabaculovirus using the degenerate oligonucleotides described by Lange et al. (2004). After sequencing of the amplified *polh/gran*, *lef-8*, and *lef-9* PCR amplicons, Mushore (2024) found that the *polh/gran* gene sequences matched closely with *Alphabaculovirus penudae*, *Alphabaculovirus pendibubdae*, *Alphabaculovirus orpseudotsugatae*, and *Alphabaculovirus spliturae*. The *lef-8* sequences matched closely with *Alphabaculovirus trini* and *Alphabaculovirus lydisparis*, and the *lef-9* sequences matched closely with *Alphabaculovirus leseparatae*. The sequenced samples in both the study by Mushore (2024) and the current study, matched with other known alphabaculovirus species and had a percentage identity score of less than 85 %. When aligning the *polh/gran*, *lef-8*, and *lef-9* sequences with those obtained by Mushore (2024), there was a pairwise identity match of 91.04 %, 90.13 %, and 91.95 %, respectively. However, the matched baculovirus species found in the study by Mushore (2024) was different to the matched species identified in the current study.

In conclusion, occlusion bodies were isolated from the larval cadavers of *S. partita*, and *T. batrachopa*, and genomic DNA was effectively extracted from the purified virus particles. No occlusion bodies were isolated from larval cadavers of *E. ceratoniae.* PCR amplification of the targeted *polh/gran* gene was achieved for *S. partita* and *T. batrachopa* samples, while non-specific amplification was observed for the *E. ceratoniae* samples. Additionally, the targeted *lef-8* and *lef-9* genes were successfully amplified in the *S. partita* samples. Sequence analysis

of the *T. batrachopa* samples confirmed infection with CrleGV and this study is the first to report the isolation of CrleGV from *T. batrachopa*. Sequence analysis of the *S. partita* samples suggests the potential isolation of a novel baculovirus. The next chapter will aim to analyse further and characterise the baculovirus isolated from the *S. partita* samples by whole genome sequencing and phylogenetic analysis.

Chapter 4 – The morphological, genetic, and biological characterisation of a novel alphabaculovirus (SepaNPV) isolated from *Serrodes partita*

4.1. Introduction

The identification of a potential novel baculovirus (SepaNPV) isolated from *S. partita* larval cadavers using *polh*, *lef-8*, and *lef-9* gene sequencing and analysis was described in Chapter 3. With the initial identification of the virus, this current chapter focused on further characterising the virus genetically and analysing its biological activity.

The morphology and size of baculoviruses that have been isolated can be characterised through transmission electron microscopy (TEM) (Ackermann & Smirnoff, 1983; Bayramoğlu et al., 2021; Grasela et al., 2008). The appearance of baculoviruses is distinct and identifiable, and therefore TEM is used to confirm the detection of a baculovirus in a host (Griffith, 1982). There are two groups of baculoviruses characterised based on their morphology: granuloviruses and nucleopolyhedroviruses (Bayramoğlu et al., 2021). The lepidopteran NPVs (*Alphabaculovirus*) are classified based on two morphotypes. The occluded virion of these viruses either has single (SNPV) or multiple (MNPV) nucleocapsids within the virion envelope. Meanwhile, each occlusion body of GVs (*Betabaculovirus*) only has a single virion (Rohrmann, 2014b). The number of virions enclosed within the OB of baculoviruses can be identified by TEM and as a result multiple and single NPVs can be differentiated (Ackermann & Smirnoff, 1983; Hughes & Addison, 1970). The classifications can be identified by sectioning the OBs and examining them by TEM.

An approach for the classification of *Baculoviridae* adopted by the International Committee on Taxonomy of Viruses (ICTV) is based on DNA sequence data. The OB morphology, genome characteristics (especially gene content), phylogeny, and host range are the criteria that the demarcation of the genera of *Baculoviridae* is based on (Jehle et al. 2006a). After the initial identification of a baculovirus, and before it is considered for development as a biopesticide, further genetic characterisation needs to be conducted. Whole genome sequencing is used for the characterisation of baculoviruses and is the more accurate and comprehensive approach (Liu et al., 2011; Mardis, 2008). The sequencing of the whole genome of a virus and the

analysis thereof, using NGS, is the most precise and extensive approach to characterise a virus genetically. The sequencing method that is commonly used is Illumina sequencing. Genome assembly is the procedure where the genome sequence technologies' reads are used by merging and aligning the sequence reads to generate a whole genome sequence (Basantani et al., 2017). *De novo* assembly is an example of a genome assembly, where there is no reference sequence used to reconstruct the genome (Liao et al., 2019). Therefore, there is no reference-bias when generating a genome sequence using a *de novo* assembly (Liao et al., 2019). The standard procedure following *de novo* assembly is to annotate the genome to distinguish the virus further. The genomes of NPVs are analysed by first identifying the ORFs and thereafter locating homologous genes. An annotated genome map is then created using the identified ORFs and therefore the genome structure of the specific baculovirus is illustrated (Ardisson-Araújo et al., 2014; Cuartas et al., 2015). A group of genes known as the baculovirus core genes are considered the genetic backbone of all baculoviruses because they are common to all baculovirus species and these genes have been used in phylogenetic analysis for the classification of the genera within the *Baculoviridae* (Garavaglia et al., 2012; Javed et al., 2017). There are 38 core genes found within all known baculoviruses (Garavaglia et al., 2012; Wennmann et al., 2018).

Phylogenetics is used to determine the novelty of a newly isolated virus and the closely related species of the virus. When phylogenetic analyses are conducted, either multiple genes, single genes, or full genomes are used (Herniou et al., 2011; Jehle et al., 2006a, 2006b; Lange et al., 2004). According to Herniou et al. (2001, 2003), it was previously shown that when studying baculovirus phylogeny, the *lef-8* and *lef-9* genes are highly useful. A pairwise distance calculation and phylogenetic comparison of shared genes, including conserved or core genes, can be performed to deduce the evolutionary history of baculoviruses (Herniou et al., 2001; Lange et al., 2004).

According to Jehle et al. (2006b), the more recent criteria proposed to distinguish alpha- and betabaculovirus species, are based on pairwise nucleotide distances calculated using the Kimura-2-parameter (K-2-P) substitution model. The partial sequences of the three conserved baculovirus genes, *polh/gran*, *lef-9*, and *lef-8,* are used to estimate these phylogenetic distances. Jehle et al. (2006b) conducted alignment of sequences from 117 distinct baculovirus isolates and the phylogeny derived from this alignment is what the proposed criteria are based upon. The model that Jehle et al. (2006b) described for the species distinction states that two baculovirus species are regarded as the same species when the value of the nucleotide distances

between two viruses at the loci are below 0.015 substitutions/site. Furthermore, two viruses are considered as different species when the value of the nucleotide distances between the two viruses exceeds 0.05 substitutions per site. Further characteristics of the viruses must be taken into account, such as host range when the distance value falls between 0.015 and 0.050 substitutions per site to determine the baculoviruses' taxonomic status (Jehle et al., 2006b). The current species classification based on the pairwise distances for the *polh*, *lef-8,* and *lef-9* loci was validated by Wennmann et al. (2018) when the pairwise nucleotide distances across 38 core baculovirus genes from 172 fully sequenced baculovirus genomes were analysed.

Once a virus has been genetically characterised, it is important to further describe the virus biologically by evaluating the host range and the potential of the virus as a biological control agent. To make informed recommendations for the deployment of microbial biocontrol agents, it is important to understand how baculoviruses impact various hosts (Bayramoğlu et al., 2021). When determining how a baculovirus isolate will persist in an ecosystem, it is essential to know the host range, as the presence of the primary and alternative hosts affects the persistence of the virus (Bayramoğlu et al., 2021). NPVs typically have a host range restricted to the host species that the virus was isolated from, or to one or, rarely, more related host species (Moscardi, 1999). When developing an effective commercial biocontrol agent, it is advantageous if it has a wide host range (Brodeur, 2012). In the current study, infection assays were conducted to test the host range of the virus. *Serrodes partita* belongs to the Erebidae insect family. Therefore, host species were selected based on availability and significance to agriculture, starting with species belonging to the Erebidae family, followed by those taxa in closely related families.

The chapter aims to further characterise the virus by whole genome sequencing and to determine the host range of the virus. The aim was achieved through, i) TEM analysis of the virus OBs, ii) whole genome sequencing and genome annotations, iii) phylogenetic analysis and distance matrix analysis, and iv) host range testing through infection assays.

4.2. Methodology

4.2.1. Transmission electron microscopy of virus sample

Transmission electron microscopy (TEM) examined the virus OBs isolated from *S. partita* larval cadavers (Chapter 3, Section 3.2.2), sample FPM 1. The method described by Jukes (2015) was used to prepare transmission electron microscope grids. 5 µl of OB suspension was pipetted onto the grid for 60 s. The excess was drained off using filter paper. 5 µl of 1 % uranyl

acetate (w/v) was pipetted onto the grid and left for 30 s. The excess uranyl acetate was removed using filter paper. Grids were left to dry overnight at room temperature. Transmitted electron images were collected with a ZEISS Libra 120 standard (Zeiss, Germany), and Olympus Megaview G2 camera. Fifteen micrograph images showing various OBs were analysed.

4.2.2. Resin embedding, sectioning and transmission electron microscopy

The morphological characterisation of the virus was determined by using a sectioning protocol. ~200 µl virus OB suspension (sample FPM 1) (approximately half of the isolated virus) were added into a 1.5 ml tube and pelleted at 12000 ×g. The pellet was thereafter immersed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer. The sample was incubated overnight at room temperature. After overnight incubation, the primary fixative was removed. The pellet was then washed using 0.1 M sodium phosphate buffer for 10 min. This step was repeated. 1 % osmium tetroxide in PO_4 was used to immerse the pellet for 90 min, this was a secondary fix solution. The secondary fix was removed before washing the pellet twice with 0.1 M sodium phosphate buffer for 10 min. Five ethanol concentrations were used to dry the pellet by immersing the pellet consecutively in each concentration for 5 min. The pellet was first immersed in 30 % ethanol, followed by 50, 70, 80, and 90 %, with the ethanol being removed before introducing the next concentration. Then, absolute ethanol was added, allowed to sit for 10 min, and removed. This step was repeated. Propylene oxide (pro) was added, left for 15 min, removed and then repeated. A 75:25 pro:resin solution was added, allowed to sit for 90 min, and then removed. This was followed by a 50:50 pro:resin solution and then a 25:75 pro:resin solution, with each left for 90 min before removing. The pellet was then submerged in pure resin and left overnight. The pellet was then placed in fresh pure resin in a 1.5 ml tube and incubated at 60 ℃ for 36 hours.

The resin mould was removed from the 1.5 ml tube and cut to fit into the RMC microtome, which was used to cut sections for TEM. The section thickness was 100 nm. The cut sections were placed on 100 mesh copper grids. Grids were placed face down on a 5 % uranyl acetate drop to stain for 25 min. The grids were removed from the uranyl acetate, washed twice with distilled H_2O, and the excess H_2O was removed using filter paper. The grids were then placed on a drop of lead citrate and stained for 5 min. Grids were removed from the lead citrate, washed three times with distilled H_2O, and placed on filter paper to dry. Grids were visualised using a JEOL JEM-2100 electron microscope at the CHRTEM facility, NMU, South Africa. Five micrograph images showing the sectioned OBs were analysed.

4.2.3. Whole genome sequencing and *de novo* assembly

The DNA extracted from *S. partita* (FPM 1) was sent for sequencing by Inqaba Biotechnical Industries (Pty) Ltd (South Africa) using Illumina (MiSeq) sequencing. The Error Correct & Normalise Reads function in Geneious R11 (v11.1.5) (Biomatters, New Zealand) was used on the sequence reads. The resultant reads from the Error correct & Normalise step were used to conduct a *de novo* assembly in Geneious R11 to generate a single consensus sequence using the *de novo* assembly function with the assembler set to Geneious and the sensitivity set to medium sensitivity/fast.

4.2.4. Genome annotations

The consensus sequence was annotated to create a full genome sequence. The NCBI Open Reading Frame Finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to identify ORFs. A nucleotide sequence FASTA format was uploaded onto the NCBI ORF finder, with the default setting selected. The nucleotide sequence was also submitted and predicted by FgenesV (http://www.softberry.com/berry.phtml?topic=virus&group=programs&subgroup=gfindv).

The predicted ORFs were analysed using BLASTp. ORFs where BLASTp did not detect any sequence similarity and it was at least 50 codons, then it was annotated following the guidelines stated by Harrison & Rowley (2022b). These were that if, (1) an ORF did not overlap a larger ORF by >75 bp or an *hr*, (2) the ORF did not fall within an *hr*, and (3) FgenesV predicted the ORF. An ORF number was allocated to each gene and the *polyhedrin* gene was allocated ORF 1. Geneious R11 (v11.1.5) was used to edit the consensus sequence by annotating the position of the identified ORFs on the genome sequence.

4.2.5. Phylogenetic and distance matrix analysis of the *polyhedrin*, *lef-8* and *lef-9* genes

The *polh*, *lef-8*, and *lef-9* nucleotide and amino acid sequences were extracted from the complete genome sequences of 60 *Alphabaculovirus* species and one species from *Betabaculovirus, Gammabaculovirus* and *Deltabaculovirus* groups on NCBI GenBank database (Benson et al. (2012) - https://www.ncbi.nlm.nih.gov/genbank/). *Betabaculovirus cypomonellae* (Cydia pomonella GV – CpGV), G*ammabaculovirus nelecontei* (Neodiprion lecontei NPV – NeleNPV) and *Deltabaculovirus cunigripalpi* (Culex nigripalpus NPV – CuniNPV) were used as an outgroup. The nucleotide sequences for the three gene regions were concatenated into a single nucleotide sequence for each isolate and this was also done for the

amino acid sequences. Concatenated sequences were aligned using MUSCLE in MEGA 11 (v 11.013).

The aligned amino acid sequences were used for the phylogenetic analysis using IQ-TREE (Wong et al., 2025). The ModelFinder (Kalyaanamoorthy et al., 2017) and ultrafast bootstrap (Hoang et al., 2018) were computed during the phylogenetic analysis on IQ-TREE. The best-fit model according to BIC scores that was used was the Q.INSECT+F+R5 model and the bootstrap method was chosen with 1000 bootstraps. The concatenated and aligned nucleotide sequences were used to conduct a distance matrix. The computed pairwise distances function was selected and the K-2-P model in MEGA 11 was used.

4.2.6. Enumeration of virus sample

The OBs purified from *S. partita* samples (FPM 1 and FPM 6) in Chapter 3, Section (3.2.2.) were used for virus enumeration. In a 1.5 ml tube, 40 µl ddH_2O was mixed with 10 µl of the purified OBs to make a 1:5 dilution of the virus suspension. An additional 1:5 dilution (total 25 × dilution) was made by adding 200 µl of 0.07 % (w/v) SDS to the 50 µl virus suspension and mixing until homogenous. The sample underwent sonication at 60 Hz for four pulses, each lasting 15 seconds. A further four dilutions ranging from 1:80 to 1:50 (total 1:2000 to 1:1250) were prepared in 2 ml tubes by mixing 25.0, 28.6, 33.3, and 20.0 µl sonicated virus suspension with 1975.0, 1971.4, 1966.7, and 980.0 µl ddH_2O respectively. Tissue paper and 70 % ethanol solution were used to clean a Helber counting chamber with a Thoma ruling and a 0.02 mm depth (Hawksley, United Kingdom) and a coverslip. Each virus suspension was tested, beginning with the most diluted and moving to the most concentrated. The virus suspension with approximately 7 OBs per small square (0.0025 mm^2) on the grid was selected for counting. The counting chamber was first partially covered with the coverslip and 5 µl of the virus suspension was pipetted onto the counting chamber. Thereafter, the counting chamber was covered completely with the coverslip and left for 5 min to stand to allow non-virus particles to settle due to Brownian motion before counting. A light microscope at 400 × magnification under dark field microscopy was used to count the OBs. The OBs found in the top left, top right, bottom left, bottom right corners, and one random central large square of the main 4 × 4 grid (each square containing 16 smaller squares of 0.0025 mm^2), were counted (Figure 4.1). Counts were performed three times with the average number of OBs counted calculated. The concentration of the OBs was calculated using Equation 4.1.

Equation 4.1: Calculation for the concentration of the isolated virus

$$Concentration\ (OBs\ per\ ml) = (D\ \times\ \chi) \div (N\ \times V)$$

χ: Average number of OBs counted, D: Dilution factor, N: Number of small squares, and V: Volume of the grid in ml (5×10^{-8} ml)

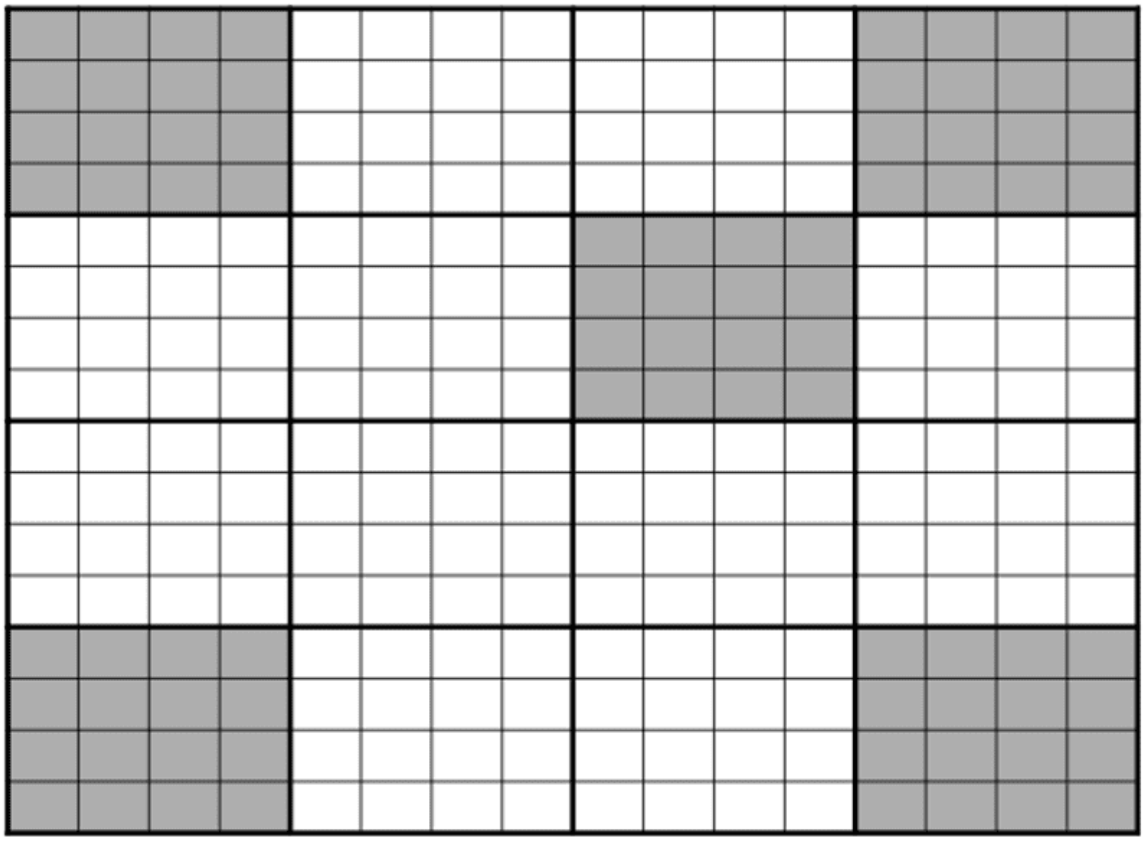

Figure 4.1: Visualisation of the counting chamber squares observed under the microscope. The OBs located in the top left, top right, bottom left, bottom right corners, and the one random central large square, that are shaded, were used for counting.

4.2.7. Host range testing infection assays

The insect species selected for testing the SepaNPV host range were *H. armigera*, *S. frugiperda*, *T. leucotreta*, and *E. saccharina*.

4.2.7.1. Diet preparation and rearing of *Helicoverpa armigera* for infection assays

The diet of *H. armigera* was prepared by first individually autoclaving 16.05 g brewer's yeast, 16.05 g agar, and 27.6 g soya flour. These ingredients were added into a blender along with 209 ml boiling ddH_2O and mixed thoroughly. The mixture was cooled down to 60 °C before

adding 19.25 g wheat germ to the mixture and mixing thoroughly. Lastly, 0.405 g phosphoric acid, 0.425 g propionic acid, and 1.13 g ascorbic acid was added to the mixture and then mixed thoroughly. The diet was either poured into 24-well bioassay plates for infection assays or into glass vials for rearing.

Pupae of *H. armigera* were received from River Bioscience (Gqeberha, South Africa). A petri dish containing *H. armigera* pupae was placed in a petri dish and put inside an insect cage. Wax paper was placed around the cage to act as a substrate for oviposition. The insect cage was observed daily for eggs. *Helicoverpa armigera* diet was prepared and placed into each well of a 24-well bioassay plate, filling roughly half of the well with the diet. The diet was pressed down using the plunger of a 5 ml syringe. Some neonates that hatched from the eggs were used for the infection assays, and the remaining neonates were used to keep the insect colony going. Three neonates were carefully placed onto the diet in each well using a paintbrush. After 3-4 days, the larvae were separated and placed individually into a glass vial containing *H. armigera* diet and plugged with cotton wool at the top. Vials were monitored daily, and pupae were collected. After collecting the pupae, they were placed in a container lined with paper towel, cleaned by gently spraying with distilled water, and then transferred onto a paper towel to drain the excess water. Pupae were then transferred into a petri dish and placed in the insect cage.

4.2.7.2. Diet preparation and rearing of *Spodoptera frugiperda* for infection assays

Ten male and ten female *S. frugiperda* pupae were received from North-West University, South Africa. The rearing of *S. frugiperda* was conducted in a CE room with temperature set at 25 °C; relative humidity of 39.0 % and 12:12 (L:D) photoperiod. The petri dish containing the pupae was placed inside an insect cage. Wax paper was placed around the cage to act as a substrate for oviposition. A 50 % honey solution soaked in cotton wool was placed in a petri dish inside the cage. This was a food source for the moths. The insect cage was checked daily for eggs and the honey solution soaked in cotton wool was replaced every second day. Stonefly Heliothis Diet (Ward's Science, USA catalog number: 470146-944) was prepared in a dish by adding 50 g diet and mixing it with 100 ml ddH_2O, creating a dough-like consistency, and thereafter placed into each well of a 24-well bioassay plate, filling roughly half of the well with diet. The diet was pressed down using the plunger of a 5 ml syringe. Some neonates that hatched from the eggs were used for the infection assays. The remaining neonates were used to keep the insect colony going, and this was done by carefully placing three neonates onto the diet in each well using a paint brush. After 3-4 days, the larvae were separated and placed individually into a glass vial containing the stonefly Heliothis diet and plugged with cotton

wool at the top. Larvae need to be separated from the larval instar stage 3 as they are cannibalistic. Pupae were collected ~14-20 days after separating the larvae into individual glass vials. After collecting the pupae, they were cleaned by spraying them gently with distilled water and the excess water was drained off by transferring the pupae onto a paper towel. Pupae were then transferred into a petri dish and placed in the insect cage.

4.2.7.3. Diet preparation for *T. leucotreta*

Eggs of *T. leucotreta* were obtained from Rhodes University, Department of Zoology and Entomology. Eggs were incubated at 25 ℃ until they hatched, and neonates were used for the infection assays. *Thaumatotibia leucotreta* artificial diet was prepared by mixing 250 ml ddH_2O with 250 g *T. leucotreta* diet (210 g maize meal, 200 g wheat germ, 100 g brewer's yeast, 36.5 g milk powder, 15 g nipagin, and 6.5 g sorbic acid) in a baking tray forming a paste. The baking dish was covered with foil and baked at 200 ℃ for 20 min.

4.2.7.4. Diet preparation and rearing of *Eldana saccharina*

Eggs of *E. saccharina* and diet were received from the South African Sugarcane Research Institute (SASRI). Eggs were incubated at 25 ℃ until they hatched, and neonates were used for the infection assays.

4.2.7.5. Host range infection assay

Infection assays for each host species tested were carried out in two 24-well bioassay plates, one plate served as the treatment and the other as a control plate. The diet for each respective insect species was placed into the bioassay plates, filling roughly half of each well. 50 µl SepaNPV OBs (9.375 $\times 10^6$ OBs/ml) was evenly pipetted onto the diet in each well of the treatment plate and 50 µl ddH_2O was pipetted onto the diet in each well of the control plate. A single neonate larva was placed on the surface of the diet in each well of both the treatment and control plates. Bioassay plates were securely closed and incubated at 25 ℃ for 7 days. The larval mortality was recorded by carefully dissecting the diet. Infection assays were performed three times for each host insect tested. Statistical analysis was performed using TIBCO STATISTICA© (version 14.1.08). Independent t-tests were used to test the differences between the data of the control and treatment plates for each host species.

4.3. Results

4.3.1. Transmission electron microscopy of virus sample

TEM of the isolated FPM 1 sample confirmed the presence of a baculovirus sample. The average size of the OBs, measuring the diameter at the widest points, was found to be 1.99 ± 0.35 µm (n = 10) (mean ± SD) (Figure 4.2 A). The OBs were irregular in shape, typical of *Alphabaculovirus* morphology. The sectioned OBs were viewed by TEM, and single nucleocapsids were observed within the OB, and the average diameter of the sectioned OBs was 1.63 ± 0.13 µm (n = 5) (mean ± SD) (Figure 4.2 B).

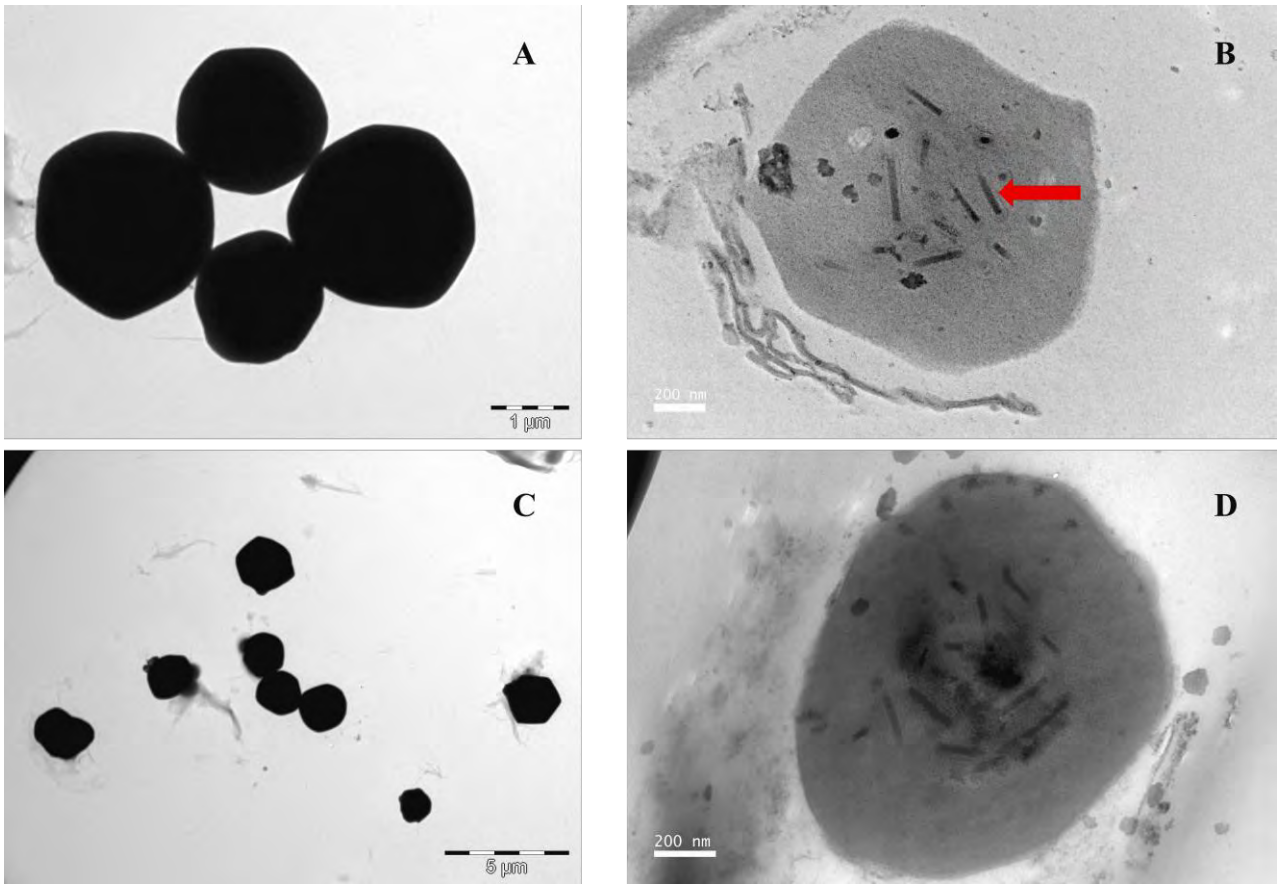

Figure 4.2: Transmission electron micrographs of SepaNPV OBs isolated by crude extraction. A and C: Unsectioned OBs. B and D: sectioned OB after resin-embedding. The virions are indicated by the red arrow.

4.3.2. Whole genome sequencing & genome annotations

Paired reads were created from sequence data, with a total of 7294628 reads with a minimum length of 35 nt. The error correction and normalise function reduced the number of sequences

to 105462. The *de novo* assembly produced 1103 contigs. The longest contig had a length of 132178, 89540 sequences, identical sites of 25440 and pairwise percentage identifty of 93.3 %. The mean percentage coverage of the 132178 bases was 97.0 % with standed deviation of 28.0. The consensus sequence was 129953 bp in length, and has a GC % of 54.9 %. The confidence mean is 2908.3 and the Q20, Q30, and Q40 is 99.7 %, 99.7 % and 99.6% respectively. A circular genome was obtained. One hundred and twenty-eight ORFs were preliminary annotated (Figure 4.3), which included 30 identified core baculovirus genes (Table 4.1).

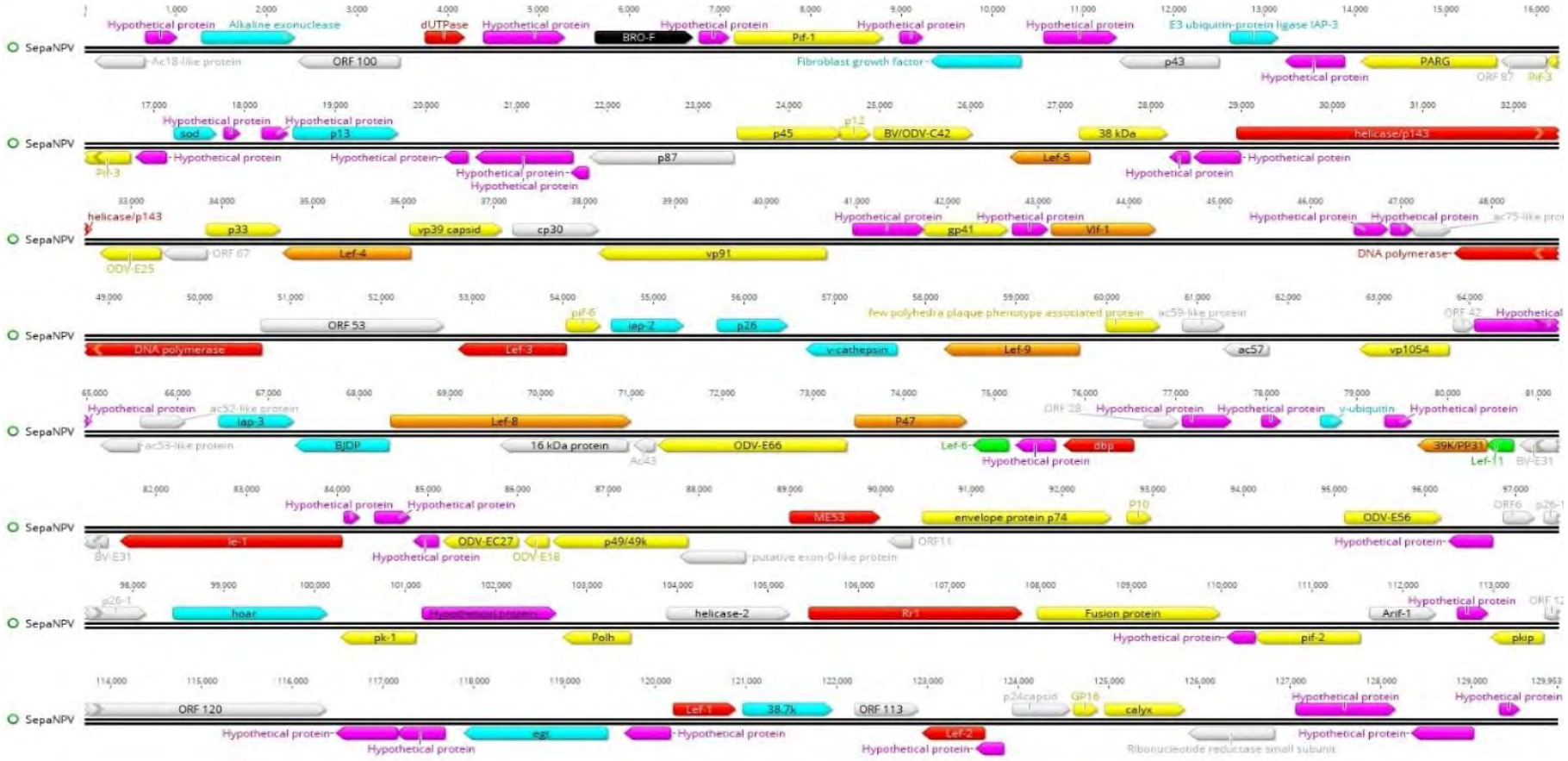

Figure 4.3: Linearised genome map of SepaNPV, with the genes represented by different coloured arrows. Structural genes in yellow, replication genes in red, auxiliary genes in light blue, transcription genes in orange, gene expression in green, *bro* genes in black, hypothetical proteins in purple, and genes with unknown function in grey.

From the 128 annotated ORFs, 28 were identified as structural genes, 10 are involved in replication, 14 are auxiliary genes, 8 are transcription genes, 2 are involved in gene expression, 1 is a *bro* gene, 28 have an unknown function, and 37 are hypothetical proteins, which includes 13 unique hypothetical genes to SepaNPV (Table 4.1).

Table 4.1: Genes identified in SepaNPV genome arranged by function and respective ORF numbers indicated. Groups by which ORFs are arranged include, Structural genes (yellow), replication genes (red), auxiliary genes (light blue), transcription genes (orange), gene expression (green), *bro* genes (black), unknown function (grey), and hypothetical proteins (purple) and the unique hypothetical regions identified are in red text.

Function	Gene	ORF		Gene	ORF
Structural	*polh*	ORF 1	Unknown Function	*p26-1*	ORF 5
				ORF 6	ORF 6
	*odv-e56**	ORF 8		*ORF 11*	ORF 11
	p10	ORF 9		*putative exon-0-like protein*	ORF 13
	*envelope protein p74**	ORF 10		*bv-e31*	ORF 21
	*p49/49k**	ORF 14		*ORF 28*	ORF 28
	*odv-e18**	ORF 15		*ac43*	ORF 34
	*odv-ec27**	ORF 16		*16kda protein*	ORF 35
	odv-e66	ORF 33		*ac52-like protein*	ORF 39
	*vp1054**	ORF 43		*ac53-like protein**	ORF 40
	*pif-6**	ORF 51		*ORF 42*	ORF 42
	*gp41**	ORF 60		*ac57*	ORF 44
	vp91	ORF 62		*ac59-like protein*	ORF 45
	few polyhedral plaque phenotype associated protein	ORF 46		*ORF 53*	ORF 53
	*vp39capsid**	ORF 64		*ac75-like protein*	ORF 55
	*p33**	ORF 66		*cp30*	ORF 63
	*odv-e25**	ORF 68		*ORF 67*	ORF 67
	*38 kda**	ORF 72		*p87*	ORF 77
	*bd/odv-c42**	ORF 74		*ORF 87*	ORF 87
	p12	ORF 75		*p43*	ORF 91
	*p45**	ORF 76		*ORF 100*	ORF 100
	*pif-3**	ORF 86		*ac18-like protein*	ORF 103
	parg	ORF 88		*ribonucleotide reductase small subunit*	ORF 107
	*9**	ORF 95		*p24capsid*	ORF 110
	calyx	ORF 108		*ORF 113*	ORF 113
	gp16	ORF109		*ORF 120*	ORF 120
	pkip	ORF 121		*arif-1*	ORF 123
	*pif-2**	ORF 124		*helicase-2*	ORF 128
	fusion protein	ORF 126	Hypothetical Regions	*Hypothetical protein*	ORF 2, 7, 17, 18, 19, 24, 26, 30, 27, 41, 56, 57, 59, 61, 70, 71, 78, 79, 80, 82, 83, 85, 89, 92, 94, 96, 98, 102, 104, 105, 106, 111, 116, 118, 119, 122, 125
Replication	*me53*	ORF 12			
	ie-1	ORF 20			
	dbp	ORF 29			
	lef-3	ORF 52			
	*dna polymerase**	ORF 54			
	*helicase/p143**	ORF 69			
	dutpase	ORF 99			
	*lef-2**	ORF 112			
	*lef-1**	ORF 115			
	rr1	ORF 127			
Auxiliary	*hoar*	ORF 4			
	bjdp	ORF 37			
	iap-3	ORF 38			
	sod	ORF 84			
	v-ubiquitin	ORF 25			
	v-cathepsin	ORF 48			
	p26	ORF 49			
	iap-2	ORF 50			
	p13	ORF 81			
	e3 ubiquitin-protein ligase iap-3	ORF 90			
	fibroblast growth factor	ORF 93			
	*alkaline exonuclease**	ORF 101			
	38.7k	ORF 114			
	egt	ORF 117			
Transcription	*pk-1*	ORF 3			
	39k/pp31	ORF 23			
	*p47**	ORF 32			
	*lef-8**	ORF 36			
	*lef-9**	ORF 47			
	*vlf-1**	ORF 58			
	*lef-4**	ORF 65			
	*lef-5**	ORF 73			
Gene Expression	*lef-11**	ORF 22			
	lef-6	ORF 31			
bro genes	*bro-f*	ORF 97			

*Core baculovirus genes

4.3.3. Phylogenetic analysis

Phylogenetic analysis of SepaNPV using the concatenated *polh*, *lef-8*, and *lef-9* gene amino acid sequences from 60 alphabaculovirus species alongside SepaNPV, and using CpGV, CuliNPV, and NeleNPV as outgroups was performed (Figure 4.4). SepaNPV was determined to be basal to two monophyletic clades. The first, with a bootstrap support of 75%, included group II alphabaculoviruses isolated from species in the families Geometridae (i.e. *Ectropis obliqua* and *Sucra jujuba*), Bombycoidea (i.e. *Clanis bilineata* and *Perigonia lusca*), and Erebidae (i.e. *Lymantria sp., Olene mendosa*, and *Serrodes partita*). The second clade, having a bootstrap support of 81%, included a few group II alphabaculoviruses isolated from species in the families Lasiocampidae (i.e. Malacosoma Neustria) and Tortricidae (i.e. *Adoxophyes sp.* and *Cryptophlebia peltastica*) along with all group I alphabaculoviruses.

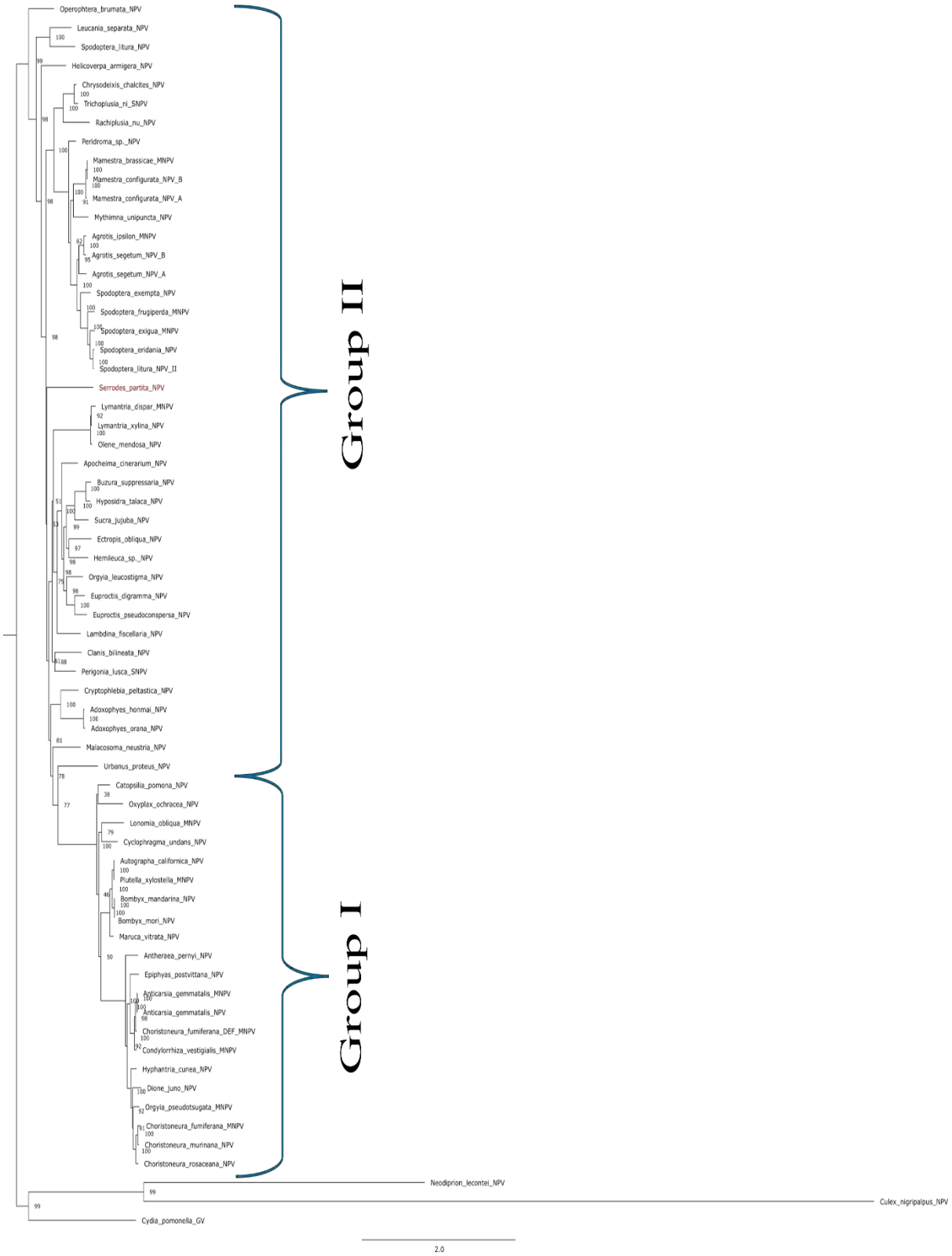

Figure 4.4: Phylogenetic analysis of SepaNPV. Constructed using a maximum likelihood inference analysis of the concatenated *polh*, *lef-8*, and *lef-9* amino acid sequences from 60 alphabaculoviruses and SepaNPV, with CpGV, CuliNPV, and NeleNPV as outgroups. SepaNPV indicated in red.

Aligned concatenated *polh*, *lef-8*, and *lef-9* nucleotide sequences from SepaNPV and 60 alphabaculoviruses were used to conduct the K-2-P distance matrix in Mega 11. The estimated distances between SepaNPV and alphabaculoviruses were greater than 0.05 substitutions per site. The biggest K-2-P estimated distance was between SepaNPV and Epiphyas postvittana NPV which was 2.52240 ± 0.17880. The smallest K-2-P estimated distance was between SepaNPV and Peridroma sp. NPV which was 0.41113 ± 0.01701 (Supplementary data - Table S1). The K-2-P distances indicate that SepaNPV is a new *Alphabaculovirus* species.

4.3.4. Host range testing

The biological activity of SepaNPV was tested against *T. leucotreta*, *S. frugiperda*, *H. armigera* and *E. saccharina*. The average mortality from the control plates for *T. leucotreta*, *S. frugiperda*, *H. armigera*, and *E. saccharina* was 12.50 % (± 4.17), 9.72 % (± 2.41), 12.50 % (± 4.17), and 8.33 % (± 7.22) respectively. The recorded average mortality for the treatment plates for *T. leucotreta*, *S. frugiperda*, *H. armigera* and *E. saccharina* was 6.94 % (± 2.41), 9.72 % (± 6.36), 15.28 % (± 10.49), and 11.11 % (± 12.03) respectively (Figure 4.5 and Table 4.2). There was no significant difference ($p > 0.05$) between the data from the control and treatment for all the host species tested. A p-value of 0.12, 1.0, 0.69, and 0.74 was obtained for *T. leucotreta*, *S. frugiperda*, *H. armigera*, and *E. saccharina,* respectively (Table 4.2).

Table 4.2: Statistical analysis of the average mortality obtained for the control and treatment for each host species.

Host species	Mean control (%)	Mean treatment (%)	p-value	Std. Dev. Control (%)	Std. Dev. Treatment (%)
Thaumatotibia leucotreta	12.50	6.94	0.12	4.17	2.41
Helicoverpa armigera	12.50	15.28	0.69	4.17	10.49
Spodoptera frugiperda	9.72	9.72	1.00	2.41	6.36
Eldana saccharina	8.33	11.11	0.74	7.22	12.03

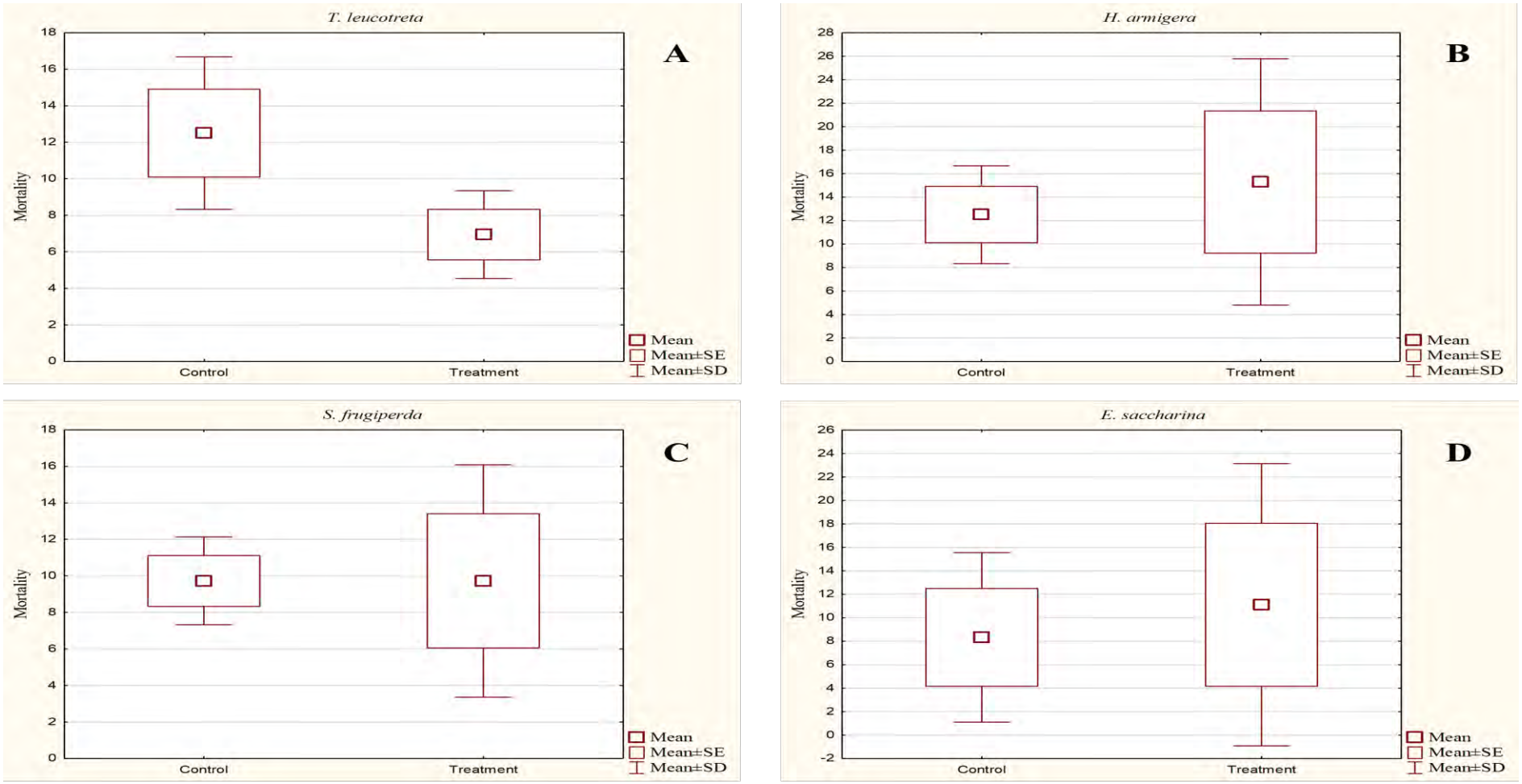

Figure 4.5: Infection assays analysis of SepaNPV conducted on four insect species, with the average percentage mortality (standard deviation) indicated. Infection assays conducted against A: *T. leucotreta*, B: *H. armigera,* C: *S. frugiperda*, and D: *E. saccharina* larvae. No significant difference was found between the control and treatment for SepaNPV tested on each host species.

4.4. Discussion

The aim of this chapter was to further characterise a potentially novel baculovirus, SepaNPV, isolated from *S. partita*. This involved TEM of the isolated virus OBs, whole genome sequencing and annotation, phylogenetic analysis, distance matrix analysis, and lastly host range testing through infection assays. A crucial aspect of baculovirology is sequencing baculovirus genomes, which is considered essential for the identification and phylogenetic analysis of baculoviruses. It also enables the study of the gene composition and genome organisation of baculoviruses (Roelvink et al., 1995; Rohrmann, 2013).

The first objective was to identify SepaNPV morphologically by TEM. Transmission electron microscopy is used to obtain an initial identification of baculovirus infection and is used by examining the macerated symptomatic larvae (Griffith, 1982). Additionally, the morphological characteristics of the virus OBs used to identify the type of baculovirus (NPV or GV) and the presence of a baculovirus in a sample can be confirmed through TEM (Erlandson & Theilmann, 2009; Kool et al., 1995; Possee & Rohrmann, 1997; Rohrmann, 1986; van Oers et al., 2004). Lepidopteran-specific baculoviruses are classified into two of the four genera, which are either NPVs or GVs (Knox et al., 2015; Landwehr, 2021). The size range of GV OBs is between 0.13 to 0.5 µm (Haase et al., 2015). The shape of these OBs is ovocylindrical, and the OB occludes a single virion and a single nucleocapsid (Rohrmann, 2019). According to Harrison & Rowley (2022b), the shape of NPV OBs is polyhedral. The size range of NPVs is 0.15-15 µm in diameter (Cory & Myers, 2003; Possee et al., 2010). Herniou et al. (2011) stated that these viruses contain multiple virions in the OB. Either single or multiple nucleocapsids are found within each enveloped virion (ODV) inside the OBs of alphabaculoviruses (Rohrmann, 2014). The size range of the SepaNPV OBs falls within the size range typical of the genus *Alphabaculovirus*, and the irregular shape observed of the OBs is also typical of that of an alphabaculovirus. TEM images revealed single nucleocapsids within the OB confirming that the isolated virus is an SNPV.

The second objective was to conduct whole genome sequencing and annotation of SepaNPV. Next generation sequencing makes use of a high throughput approach, where short reads are generated from a fragment ‘library’, therefore enabling millions of these parallel short sequence reads to be processed simultaneously. A whole genome sequence is generated by *de novo* assembly using the short reads (Liu et al., 2011; Mardis, 2008). The genomes of viruses in the *Baculoviridae* family are double-stranded and range between 80-180 kbp (Rohrmann, 2014,

2019). Between 90 and 180 encoded genes are found in fully sequenced baculoviruses (Rohrmann, 2019). The genomes of baculoviruses consist of 38 core genes and 79 known genes, with the lepidopteran baculoviruses having an additional 23 conserved genes (Javed et al., 2017; Li et al., 2019), and only among the group I alphabaculoviruses, are an additional 11 genes present (Garavaglia et al., 2012; Herniou et al., 2003). The core genes are commonly involved in the transmission cycle and the infection cycle of viruses (Garavaglia et al., 2012) The preliminary full genome size of SepaNPV was found to be 129953 bp and 128 ORFs were annotated, which falls within the size range and number range of encoded genes of baculoviruses. The recorded ORFs were grouped by function. According to Vlak & Smith (1982), the nucleotide considered as the first base in any genome of baculoviruses is the adenine of the start codon, ATG, of the *granulin* or *polyhedrin* gene. Therefore, ORF number 1 is the *granulin* or *polyhedrin*. Similarly, ORF 1 in SepaNPV is the *polyhedrin* gene.

Of the 128 ORFs preliminary annotated in the genome of SepaNPV, 30 core genes were identified using Rohrmann (2019) and Garavaglia et al. (2012) as reference for the identification of the core genes. The essential functioning of baculovirus core genes is involved in the process of the host gut infection, virion structure, and expression of late genes (Ferrelli et al., 2012; Rohrmann, 2014a). Examples of core genes not identified in the genome of SepaNPV that have been previously identified in other baculovirus species are *lef-12*, *desmoplakin*, *p6.9*, *p40*, *p95*, *per os* infectivity factor 4 (*pif-4*), *pif-7*, *ac81*, *p18*, and *ac78* (Garavaglia et al., 2012; Rohrmann, 2019). Further genome analysis needs to be conducted to determine if these genes are present within the SepaNPV genome as these are core genes. The unidentified genes could be linked to the hypothetical regions. The protein P6.9 is a basic DNA-binding protein which is essential for virus assembly and packaging. This protein is small, and in DNA condensation it has a predicted role (Tweeten et al., 1980; van Oers & Vlak, 2007; Wilson et al., 1987; Wilson & Price, 1991; Witteveldt et al., 2005). The *pif-4* gene is a component of a core complex, alongside *pif-1* and *pif-3*, and during the process of the dissolution of polyhedral in the midgut of insects, it may aid in offering proteolytic resistance to the core structure (Boogaard et al., 2018; Rohrmann, 2019). The *pif-7* gene forms part of the *pif* genes essential for baculovirus infection when insects are infected with the virus (Rohrmann, 2019). As previously mentioned, the group II NPVs have the fusion protein and do not have the *gp64* gene (Rohrmann, 2014b). SepaNPV lacks the *gp64* gene but has the fusion protein, therefore validating the grouping of this virus into the group II NPVs.

Additional genes also present in lepidopteran NPVs that were not identified in the SepaNPV genome are the *lef-10*, *fp25k*, *pp34/pep, odvp-6e*, and *orf1629* genes. The genes *p95*, *odvp-6e*, and *p6.9* are structural genes. The *lef-10* gene plays a role in transcription and is essential for the late gene expression (Lin & Blissard, 2002; Passarelli & Miller, 1994). The *fp25k* encodes for structural proteins. It plays a role in the viral structural protein intracellular transport (van Oers & Vlak, 2007). A phosphoprotein is encoded by the *pp34/pep* gene and this phosphoprotein is a major component of the polyhedron envelope (van Oers & Vlak, 2007; Zuidema et al., 1989). Furthermore, the *orf1629* gene encodes for a phosphoprotein that is linked with the nucleocapsid and plays a role with *bv/odv-c42* (Braunagel et al., 2001; van Oers & Vlak, 2007). Furthermore, examples of non-essential genes that are involved in the stimulation of DNA replication (de Jong et al., 2005) that were not identified in SepaNPV are *lef-7*, and *pe38*. The *lef-7* is essential in the baculovirus host range (Nguyen et al., 2013). The host's multi-protein complexes of G-actin get stored in the nucleus, and this process is the essential function of the *pe38* gene (Ferrelli et al., 2012; Nguyen et al., 2013). Various baculovirus genes are not present in the genome of AcMNPV, these genes have either been previously studied in other baculovirus species, or they are homologous to genes that are well-known in other organisms (Rohrmann, 2019). Examples of these genes that were also identified in SepaNPV genome are: *hoar*, *helicase-2*, and *ribonucleotide reductase small subunit*. Furthermore, the *polh* gene identified in SepaNPV also corroborates the classification of this virus as an alphabaculovirus and not a betabaculovirus.

Following the whole genome sequencing and annotation, the next objective was to evaluate the phylogeny of SepaNPV. Phylogenetic analysis conducted on the concatenated *polh*, *lef-8*, and *lef-9* amino acid sequences revealed that SepaNPV clustered with the group II alphabaculoviruses. There are two groups that the lepidopteran NPVs are divided into. There are 12 distinct genes that the group I alphabaculoviruses contain, with the envelope fusion protein GP64 being the most notable. In contrast, the group II alphabaculoviruses have a fusion protein known as F protein and do not have the GP64 protein, and this group of viruses are more diverse (Rohrmann, 2014b; Thézé et al., 2018). SepaNPV was identified to have the F protein and therefore corroborating the grouping within group II alphabaculovirus, as it lacks the *gp64* gene found in group I alphabaculoviruses. Additionally, phylogenetic results also showed that SepaNPV was basal to a monophyletic clade which includes species such as Lymantria dispar MNPV and Lymantria xylina NPV. Lymantria dispar MNPV was isolated from the moth *L. dispar*, commonly known as the spongy moth. This moth is a pest on

deciduous trees (Elkinton & Liebhold, 1990) and several industrial, food and forestal crops. Additionally, the Lymantria xylina MNPV (LyxyMNPV) was isolated from infected *L. xylina* larvae (Wu & Wang, 2005). The host plants of *L. xylina* include acacia (*Acacia confusa*) forests, guava (*Psidium guajava* L.), Lychee (*Litchi chinensis* Sonn.), casuarina (*Casuarina equisetifolia*), and longan (*Euphoria longana* Lam.) (Shen et al., 2003, 2006). Based on phylogenetic analysis it was found that *L. xylina* and *L. dispar* are closely related (Wu & Wang, 2005) and LyxyMNPV and LdMNPV were also found to be closely related (Nai et al., 2010). According to Zahiri et al. (2012), when molecular phylogenetics was evaluated based on mitochondrial and nuclear genes, it resulted in the *Lymantria* genus being classified into the Erebidae. Therefore, SepaNPV matched closely to other Erebidae infecting viruses. The K-2-P nucleotide distance comparisons confirmed the novelty of the virus. With the pairwise distance threshold found to be greater than 0.050 when comparing SepaNPV with 60 different alphabaculovirus species which falls within the distance criteria indicated by Wennmann et al. (2018) and Jehle et al. (2006b) for a different virus species. The study conducted by Mushore (2024) evaluated an initial phylogenetic analysis of SepaNPV using the concatenated partial *polh*/*gran*, *lef-8*, and *lef-9* gene sequences of SepaNPV and seven other alphabaculovirus species obtained from GenBank. The results from the study found that SepaNPV formed a monophyletic clade with OpMNPV and LdMNPV.

The four families, *Baculoviridae*, *Hytrosaviridae*, *Nudiviridae*, and *Filamentoviridae,* are grouped within the order *Lefavirales* (van Oers et al., 2023; Varsani et al. 2025). Virus species that belong to the order *Lefavirales* have a standardised binomial naming system. The name of the virus species are comprised of two distinct words written in italics, with the first letter of the first word being capitalised. The two worded virus species name consists of: 1) The genus of the virus species forms the first word, and 2) the species name of the host from which the virus was isolated from is structured to form the second word. The first 2-4 letters of the second word correspond to the first 2-4 letters of the genus of the host species, directly followed by the species name of the host (van Oers et al., 2023). Therefore, following this naming system, the proposed species name of SepaNPV would be *Alphabaculovirus separtitae*.

The last objective was to conduct infection assays with the virus against *T. leucotreta*, *S. frugiperda* (JE Smith) (Lepidoptera: Noctuidae), *H. armigera* and *E. saccharina* Walker (Lepidoptera: Pyralidae) to determine its host range. It is crucial to know the information on the host range of a virus when understanding the characteristics of a microbial biocontrol agent

(Bayramoğlu et al., 2021). The widespread use and adoption of baculoviruses as viral insecticides has been limited, due to factors such as their narrow host range, where one formulation targets only specific insect species, and due to their slow speed of kill (Rohrmann, 2019). Baculoviruses typically exhibit a narrow host range. Especially when the host range is compared to other microbial control agents or synthetic chemical insecticides. The host range within *Baculoviridae* is, however, variable (Cory et al., 2000). Granuloviruses have a restricted host range compared to NPVs, with the host of GVs being limited mainly to a single species (Rao et al., 2015). Nucleopolyhedroviruses often have a host range that limits them to infecting one or more species that belong to the same genus or family as the homologous host (Doyle et al., 1990; Groner, 1986; Hostetter & Puttler, 1991; Tanuja et al., 2019). Euproctis chrysorrhoea NPV, Lymantria dispar NPV (Gani et al., 2017), and Spodoptera exigua NPV are examples that have a very narrow host range (Gelernter & Federici, 1986). There are a few NPV species that have a much larger host range in comparison to what is typical of NPVs. Examples include, Anagrapha falcifera NPV, Autographa californica MNPV, and Mamestra brassicae MNPV. More than 31 species of Lepidoptera belonging to ten insect families are infected by A. falcifera NPV. Additionally, 30 lepidopteran species belonging to 10 insect families are infected by A. californica MNPV, and the host range of M. brassicae MNPV includes 32 lepidopteran species belonging to four different families (Doyle et al., 1990; Groner, 1986; Hostetter & Puttler, 1991).

In weed biological control, test plant selections are based on a centrifugal phylogenetic method. Which involves testing plants, starting with those that are most closely related to the target weed and then spiralling outwards towards the taxa that are more distantly related to the target weed (Wapshere, 1974). The sequence of selection involves starting with 1) additional forms of the same species, 2) species belonging to the same genus, 3) species belonging to the same tribe, 4) thereafter, those belonging within the same subfamily, 5) moving to those in the same family, and finally 6) species that are within the same insect order (Wapshere, 1974). This method of approach was used to select hosts for testing the host range of SepaNPV in the current study. No insect species within the Erebidae with sufficient numbers required to conduct the infection assays were available during the study period. Therefore, hosts within this insect family could not be evaluated. Additionally, due to the sporadic nature of the homologous host *S. partita*, the difficulty acquiring *S. partita* and there not being an artificial diet to establish a laboratory colony, further infection assays, lethal concentration or lethal time bioassays with SepaNPV on this host could not be evaluated. There was no significant

difference obtained between the control and treatment results for each host species, with the p-value obtained all being greater than 0.05. Results from the current study indicated that *T. leucotreta*, *S. frugiperda*, *H. armigera*, and *E. saccharina* are not susceptible. Erebidae, Noctuidae, Nolidae, and Euteliidae, are among the insect moth families grouped into the Noctuoidea superfamily (Zahiri et al., 2011). *Serrodes partita*, *S. frugiperda*, and *H. armigera* are grouped within the same insect superfamily, hence the selection of *S. frugiperda* and *H. armigera* as test species for host range testing. Additionally, despite *T. leucotreta* and *E. saccharina* not grouping within the same insect superfamily, these insects were also subjected to host range testing because they are important agricultural pests and because laboratory cultures of these insect species have been established and are available in South Africa.

In conclusion, an extensive characterisation of SepaNPV was carried out. Morphological identification by TEM showed that the virus is an SNPV. The genetic characterisation of SepaNPV was evaluated by whole genome sequencing and *de novo* assembly to establish the genome sequence. The phylogenetic results indicate the classification of SepaNPV as a potential novel baculovirus species. Infection assays were conducted to test the host range of SepaNPV against four potential host species, however, none were found to be susceptible to infection.

Chapter 5 – Development and optimisation of a RT-PCR assay for the detection of cricket paralysis virus (CrPV) from *Acheta domesticus* samples

5.1. Introduction

The cricket paralysis virus is a single-stranded positive-sense RNA virus characterised as a member of the virus family *Dicistroviridae* (Kerr et al., 2015; Nayak et al., 2010). According to Tate et al. (1999), one of the most well-defined and studied members of a group of small, RNA-containing insect viruses, commonly known as "picorna-like viruses", is CrPV. The initial isolation of the CrPV was from the field crickets *Teleogryllus commodus* and *Teleogryllus oceanicus*, (Reinganum et al., 1970). The host range of CrPV is broad, with various insect species being infected, and these belong to the orders Orthoptera, Hemiptera, Lepidoptera, Diptera and Hymenoptera. Additionally, various cultured insect cell lines are also infected by CrPV (King et al., 2011).

The cricket paralysis virus is known to be pathogenic to some insect pests that affect agriculture (Bonning & Miller, 2010), and studies have shown that within both fruit flies and field crickets, CrPV produces lethal infections (Manousis & Moore, 1987b; Reinganum, 1975). In the study by Manousis & Moore (1987b), the olive fruit fly (*Dacus oleae* Gmel) was used to test various families of insect viruses for pathogenicity and growth. The cricket paralysis virus, a naturally occurring virus of the olive fruit fly, was also used in the analysis. The replication of CrPV within adult flies was demonstrated in the study. The study found that within five days, a mortality rate of 50 % was observed in adult flies. The study also found that in laboratory-maintained colonies of olive fruit fly, CrPV causes significant mortality. Additionally, CrPV could play an important role as part of an integrated pest management system (Manousis & Moore, 1987a). In Africa, most of the fruit fly pest species belong to four genera, *Dacus*, *Bactrocera*, *Trirhithrum* and *Ceratitis* (Badii et al., 2015). As previously stated, the three fruit fly species, *B. dorsalis*, *C. rosa*, and *C. capitata,* threaten citrus in southern Africa (Manrakhan, 2022). In South Africa, poisoned protein baits, either applied as sprays or in bait stations, have been used for the control of fruit flies (Barnes, 2000), but with evidence of the pathogenicity of CrPV, the question arises as to whether this virus holds potential for use in fruit fly management and control.

Evaluating the potential of CrPV as a biocontrol agent requires the ability to isolate and detect the virus from symptomatic insects. A suitable method for the isolation of viral RNA should comply with the following criteria which include (i) that high-quality RNA containing no contaminants is produced, (ii) that the chance of RNA degradation is restricted (Tattersall et al., 2005), (iii) that a reasonable quantity of RNA is produced, and (iv) that the method is robust and consistent (Ridgeway & Timm, 2014). The use of TRIzol for extracting RNA from various biological samples has been demonstrated to be a reliable method in numerous studies (Heidary & Kakhki, 2014). After isolating total RNA from an insect using TRIzol, the isolated RNA needs to be identified using molecular techniques. A reverse transcriptase polymerase chain reaction (RT-PCR) is conducted when RNA viruses such as CrPV are being targeted for detection (Ozoemena et al., 2004). One of the approaches is a two-step RT-PCR which involves the synthesis of complementary DNA (cDNA) from an RNA template as the first step. Thereafter, the second step involves a PCR amplification reaction where the synthesised cDNA is used as template DNA. These two steps are conducted in two separate reaction tubes (Avashia & Garibyan, 2013; Wacker & Godard, 2005).

For the detection of pathogens using PCR-based methods, it is essential to include a positive control ensuring the reliability of the diagnostic test (Moldovan & Moldovan, 2020). A positive control is necessary in situations where the presence of the target is uncertain. Not having access to genomic material of a sample, or native microorganisms pose challenges to molecular methods (Zeinoddini et al., 2020). Artificial or synthetic positive control constructs can be used to overcome these challenges (Zeinoddini et al., 2020). Kim et al. (2024) stated that when detecting DNA- and RNA-type pathogen genes, a useful tool is to use plasmid DNA as a PCR positive control. The synthetic vectors can be designed to contain various genes from one organism, which will allow for a more accurate detection of the organism. Alternatively, the synthetic vectors could be designed to incorporate the gene sequences from different organisms, and this will allow for multiple organisms to be detected from a single control vector (Caasi et al., 2013; Charrel et al., 2004; Nordstrom et al., 2007; Skottman et al., 2007). RNA viruses have a unique hallmark gene known as the *RNA-dependent RNA polymerase* (*RdRp*). The RdRp is a protein found among all RNA viruses that is structurally conserved (Jácome et al., 2022; Mönttinen et al., 2021). This gene is also widely used as a marker for identifying RNA viruses (Charon et al., 2024; Wolf et al., 2018). Therefore, this gene was chosen for CrPV detection due to its conserved nature.

The aim of this chapter was to develop a screening method to detect the presence of CrPV from *A. domesticus* samples. The objectives of the chapter were i) to create a positive control for downstream experiments using molecular techniques, ii) to extract total RNA from symptomatic insects, and iii) to develop a two-step RT-PCR assay for the detection of CrPV in *A. domesticus* samples.

5.2. Methodology

5.2.1. Sample collection

Acheta domesticus samples were provided by Citrus Research International (Port Elizabeth/Gqeberha) (Chapter 2, Section 2.7.1), and individual symptomatic *A. domesticus* samples were screened for CrPV infection. Symptoms included lethargy and paralysis in the legs.

To preserve *A. domesticus* samples, *T. leucotreta* larvae infected with CrleGV and healthy larvae were used for the testing of the RNA isolation protocols. *Thaumatotibia leucotreta* eggs were provided by the Centre for Biological Control (CBC), Department of Zoology and Entomology, Rhodes University and were incubated at 25 ℃ until hatching. Once the eggs hatched, two 24-well bioassay plates were prepared for infecting the neonates with CrleGV, one plate for the control and the other for the infection treatment. In a baking tray, 100 ml ddH_2O was mixed well with 100 g of dry *T. leucotreta* diet (210 g maize meal, 100 g brewer's yeast, 6.5 g sorbic acid, 36.5 g milk powder, 15 g nipagin and 200 g wheat germ) (Moore et al., 2014) and baked at 200 ℃ for 20 min. The diet was cut using a modified 10 ml syringe and pressed down into each well of the bioassay plates. For the control plate, 50 µl of ddH_2O was pipetted onto the surface of the diet in each well and for the treatment plate, 50 µl of CrleGV OB suspension (2.5×10^6 OBs/ml) was pipetted onto the surface of the diet in each well. A single neonate was placed on the surface of the diet in each well of both the treatment and control plate. Bioassay plates were securely closed and incubated at 25 ℃ for 4 days. Infected and healthy larvae were collected, placed individually in a 1.5 ml tube, and stored at – 20 ℃.

5.2.2. Development of a positive control for application in RT-PCR assays

5.2.2.1. Synthetic plasmid and oligonucleotide design

A synthetic plasmid (pCrPV) was constructed with the CrPV *RdRp* gene synthesised within the pMA-RQ vector and then ordered from Invitrogen GeneArt (Thermo Fisher Scientific, USA). The *RdRp* sequence for the CrPV (NC_003924.1) was extracted from NCBI GenBank. Oligonucleotides were designed to bind to target regions of the extracted *RdRp* gene sequence.

Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al., 2012) was used for the design of the oligonucleotide sets by uploading the extracted sequence onto the Primer-BLAST interface. Inqaba Biotechnical Industries (Pty) Ltd (South Africa) synthesised the selected oligonucleotide set (Table 5.1).

Table 5.1: Oligonucleotide set targeting a region of the *RdRp* gene of the Cricket paralysis virus

Target gene	Oligonucleotide name	Sequence 5'-3'	Target amplicon size	Tm (℃)
RdRp	RdRp-F	AGAGTTTTCTCAGCGGGACC	407 bp	53.83
	RdRp-R	GGGTTTCCTGAAGGTTGGGA		53.83

5.2.2.2. Transformation into JM109 competent cells

The synthetic plasmid was transformed into JM109 competent cells. A 1.5 ml tube containing 50 µl competent cells was placed on ice to thaw, to which 2 µl (50 ng/µl) of the pCrPV DNA was added to the competent cells. A control reaction was also set up by adding 2 µl of ddH_2O to 50 µl competent cells. The tubes were then incubated on ice for 15 min. This was followed by heat shock at 42 ℃ for 45 s. The tubes were placed back on ice for 2 min. 450 µl Luria Broth (LB) (1.25 g NaCl, 1.25 g yeast extract, 2.5 g tryptone and 250 ml ddH_2O, autoclaved) was added, followed by incubation at 37 ℃ with shaking for 30 min. 100 µl of the transformation reaction mixture was spread-plated on Luria Agar (LA) (1.25 g NaCl, 1.25 g yeast extract, 2.5 g tryptone, 5 g agar and 250 ml ddH_2O, autoclaved) plates containing 100 µg/ml ampicillin. The remaining transformation reaction mixture was centrifuged at 1000 ×g for 3 min. The supernatant was carefully poured out, leaving ~100 µl. The pellet was carefully resuspended and spread-plated on LA plates containing 100 µg/ml ampicillin. Plates were incubated overnight (~16 hrs) at 37 ℃.

5.2.2.3. Colony PCR

A 20 µl colony PCR reaction was set up in PCR tubes by adding 10 µl *Taq* DNA polymerase 2 × Master Mix RED (Ampliqon, Denmark), 0.4 µl RdRp-F (10 µM) (Table 5.1), 0.4 µl RdRp-R (10 µM) (Table 5.1) and 9.2 µl ddH_2O. Four distinct colonies were selected from the transformation and transferred into the PCR mixture using a pipette tip. The PCR amplification was done using a MiniAmp Thermal Cycler (Thermo Fisher Scientific, USA). The cycle

parameters consisted of an initial denaturation step of 95 ℃ for 3 min followed by 25 cycles of 95 ℃ for 1 min, 48 ℃ for 30 s and 72 ℃ for 1 min and a final elongation step at 72 ℃ for 2 min. Colony PCR amplicons were evaluated by 1 % agarose gel electrophoresis with ethidium bromide staining in 1 × TAE buffer run for 35 min at 90 V and visualised using a ChemiDoc™ XRS+ System with Image Lab™ software (Bio-Rad Laboratories, USA).

5.2.2.4. Plasmid extraction and restriction digestion

Colonies identified as positive by colony PCR were used to inoculate 5 ml LB containing 100 µg/ml ampicillin. These were incubated at 37 ℃ overnight (~16 hrs) with shaking. 3 ml of the cultured cells were harvested in a microcentrifuge tube by centrifugation for 2 min at 6800 ×g. The supernatant was discarded, and plasmids were extracted from the cells following the manufacturer's protocol of the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA). All centrifugation steps were carried out at 12000 ×g, 50 µl elution buffer was used to elute the plasmid DNA, and all samples were stored at -20 ℃.

A 20 µl restriction digestion reaction was set up in a PCR tube, which consisted of 2 µl 10 × FastDigest Buffer (Thermo Fisher Scientific, USA), 1 µl FastDigest EcoRI (10 U/µl) (Thermo Fisher Scientific, USA), 1 µg plasmid DNA and up to 20 µl ddH_2O. Reaction mixtures were gently mixed, centrifuged for ~5 s and incubated at 37 ℃ for 15 min. The restriction enzymes were inactivated at 80 ℃ for 5 min. Extracted plasmids and restriction digests were analysed by 1 % agarose gel electrophoresis with ethidium bromide staining in 1× TAE buffer run for 35 min at 90 V and visualised using a ChemiDoc™ XRS+ System with Image Lab™ software.

5.2.2.5. T7 Transcription and purification of synthesised RNA

Linearised plasmids were used as template DNA for the T7 transcription reactions. The manufacturers' instructions for the HiScribe® T7 High Yield RNA synthesis kit (New England Biolabs, USA) were followed by setting up 20 µl reactions. In a PCR tube, 2 µl 10 × reaction buffer, 2 µl ATP (100 mM), 2 µl GTP (100 mM), 2 µl UTP (100 mM), 2 µl CTP (100 mM), 1 µg template DNA, 1 µl DTT (0.1 M), 2 µl T7 RNA polymerase mix and up to 20 µl nuclease-free water was added. A control reaction was also set up using the FLuc control template DNA provided in the kit. The reactions were mixed thoroughly, followed by a quick spin down. Reactions were incubated at 37 ℃ for 2 hrs in a MiniAmp Thermal Cycler PCR instrument. The RNA transcripts were prepared for analysis by gel electrophoresis by mixing 1 volume of the RNA transcript with 1 volume of 2 × RNA loading dye and then heated for 10 min at 70 ℃. This was followed by chilling on ice for 3 min. After that, a quick spin down was performed

before loading the samples on a 1 % agarose gel. The RiboRuler™ RNA ladder (Thermo Fisher Scientific, USA) was prepared for electrophoresis as per the manufacturer's instructions. The prepared RNA was evaluated by 1 % agarose gel electrophoresis stained with ethidium bromide in 1 × TAE buffer for 45 min at 64 V. The RNA ladder was used to estimate the transcript size. The agarose gel was visualised using a ChemiDoc™ XRS+ System with Image Lab™ software (Bio-Rad Laboratories, USA).

RNA transcripts were purified by phenol: chloroform extraction and ethanol precipitation. The RNA transcription reaction volume was adjusted to 180 µl by adding nuclease-free water, followed by adding 20 µl of 3 M sodium acetate, pH 5.2 and mixing thoroughly. An equal volume of 1:1 phenol/chloroform mixture was added, and the sample was mixed followed by collecting the upper aqueous phase and placing it into a new 1.5 ml tube. An equal volume of chloroform was added, and tubes were inverted a few times followed by collecting the upper aqueous phase and placing it into a new 1.5 ml tube. The extraction with chloroform was repeated. Two volumes of ethanol were added to the aqueous phase to precipitate the RNA, by incubating at – 20 ℃ for 30 min. The samples were then centrifuged at 12000 ×g for 20 min and the supernatant was removed. The pellet was then rinsed with 500 µl cold 70 % ethanol, followed by centrifugation for 5 min at 12000 ×g. The supernatant was removed, and the pellet was air dried, followed by resuspending the RNA pellet in 50 µl of 0.1 mM EDTA. RNA was stored at – 20 ℃. A Nanodrop™ Lite Plus Spectrophotometer (Thermo Scientific, USA) was used to measure the concentration of the T7 transcript RNA sample, and this was conducted in triplicate.

5.2.3. Development of RNA isolation technique

Two RNA isolation methods were first tested, the first method was RNA isolation using TRIzol™ reagent in conjunction with Phasemaker™ tubes, and the second method was using TRIzol™ reagent. Healthy and CrleGV infected *T. leucotreta* larvae were used to test the two methods. RNA was isolated following the manufacturer's protocol of either the Phasemaker™ tubes (ThermoFisher Scientific, USA) or the TRIzol™ reagent (ThermoFisher Scientific, USA). Thereafter, RNA was isolated from the *A. domesticus* samples using the protocol for the TRIzol™ reagent in conjunction with Phasemaker™ tubes.

5.2.3.1. RNA isolation using TRIzol™ reagent in conjunction with Phasemaker™ tubes

RNA was isolated from insect samples using TRIzol™ reagent (Thermo Fisher Scientific, USA) in conjunction with Phasemaker™ tubes (Thermo Fisher Scientific, USA). RNA was isolated

following the manufacturer's protocol of the Phasemaker™ tubes. All centrifugation steps were carried out at room temperature. Phasemaker™ tubes were centrifuged at 12000 ×g for 30 s prior to use. A whole insect sample was placed into a 2 ml tube, 1 ml of TRIzol™ reagent per 50-100 mg of cricket sample was added, and the sample was homogenised using a microtube pestle. The tube containing the sample was centrifuged at 12000 ×g for 5 min, thereafter up to 1.4 ml of the clear supernatant was transferred into the Phasemaker™ tube. The sample was incubated at room temperature for 5 min. 0.2 ml of chloroform per 1 ml TRIzol™ reagent used for lysis, was added to the sample, followed by vigorously shaking for 15 s by hand, and then incubating at room temperature for 15 min. The sample tube was centrifuged at 12000 ×g for 5 min, and the upper aqueous phase was transferred into a new 1.5 ml tube. Isopropanol (0.5 ml per 1 ml of TRIzol™ reagent used for lysis) was added to the aqueous phase and incubated at room temperature for 10 min. The tube was centrifuged at 12000 ×g for 10 min, a white gel-like pellet is formed at the bottom of the tube, which is the total RNA, thereafter the supernatant was removed using a micropipette. 1 ml of 75 % ethanol per 1 ml TRIzol™ used for lysis was added to resuspend the pellet, the sample was mixed briefly followed by centrifugation at 7500 ×g for 5 min. A micropipette was used to carefully remove the supernatant, and the pellet was left to air dry for 10 min at room temperature. The final pellet was resuspended in 40 µl RNase-free water, followed by incubating for 15 min in a heating block set at 60 ℃. A Nanodrop™ Lite Plus Spectrophotometer (Thermo Scientific, USA) was used to measure the concentration of RNA isolated from the samples, and this was conducted in triplicate.

5.2.3.2. RNA isolation using TRIzol™ reagent

RNA was isolated from samples using TRIzol™ reagent (Thermo Fisher Scientific, USA). RNA was isolated following the manufacturer's protocol for TRIzol™ reagent. All centrifugation steps were carried out at room temperature. A whole insect sample was placed into a 2 ml tube, 1 ml of TRIzol™ reagent per 50-100 mg of sample was added, and the sample was homogenised using a microtube pestle. The tube containing the sample was centrifuged at 12000 ×g for 5 min, thereafter the clear supernatant was transferred into a new tube. The sample was incubated at room temperature for 5 min. 0.2 ml of chloroform per 1 ml TRIzol™ reagent used for lysis, was added to the sample, followed by vigorously shaking for 15 s by hand, and then incubating at room temperature for 2-3 min. The sample tube was centrifuged at 12000 ×g for 15 min, and the upper aqueous phase was transferred into a new 1.5 ml tube. Isopropanol (0.5 ml per 1 ml of TRIzol™ reagent used for lysis) was added to the aqueous phase and incubated at room temperature for 10 min. The tube was centrifuged at 12000 ×g for 10 min, a white gel-like

pellet is formed at the bottom of the tube, which is the total RNA, thereafter the supernatant was removed using a micropipette. 1 ml of 75 % ethanol per 1 ml TRIzol™ used for lysis was added to resuspend the pellet, the sample was mixed briefly followed by centrifugation at 7500 ×g for 5 min. A micropipette was used to remove the supernatant carefully, and the pellet was left to air dry for 10 min at room temperature. The final pellet was resuspended in 40 µl RNase-free water, followed by incubating for 15 min in a heating block set at 60 ℃. A Nanodrop™ Lite Plus Spectrophotometer (Thermo Scientific, USA) was used to measure the concentration of RNA isolated from the samples, and this was conducted in triplicate.

5.2.4. DNase I treatment and evaluation by agarose gel electrophoresis

In an RNase-free tube, a 10 µl DNase I reaction was set up. Each reaction consisted of up to 1 µg template RNA, 1 µl 10 × reaction buffer, 1 µl DNase I and up to 10 µl DEPC-treated water. The reaction was incubated for 30 min at 37 ℃. 1 µl of 50 mM EDTA was added, and the reaction was incubated for a further 10 min at 65 ℃. A spike control sample was also set up by replacing the template RNA with a 1:1 ratio of RNA isolated from the cricket samples and mixing it with the positive T7 transcript sample. The RNA samples were prepared for electrophoresis by mixing 1 volume of the sample with 1 volume of the 2 × RNA loading dye and then heating for 10 min at 70 ℃. This was followed by chilling on ice for 3 min. Thereafter, a quick spin down before loading the samples on an agarose gel. The RiboRuler™ RNA ladder (Thermo Fisher Scientific, USA) was prepared for electrophoresis in the same way as the RNA samples, replacing the RNA sample with the RNA ladder. The prepared RNA was evaluated by 1 % agarose gel electrophoresis stained with ethidium bromide in 1 × TAE buffer (1 mM EDTA, 20 mM acetic acid, 40 mM Tris-acetate) for 45 min at 64 V. The RNA ladder was used to estimate the amplicon size. The agarose gel was visualised using a ChemiDoc™ XRS+ System with Image Lab™ software (Bio-Rad Laboratories, USA).

5.2.5. Two-step RT-PCR assay

The DNase I treated isolated RNA was used as template RNA for the two-step RT-PCR amplification reaction. For the first step, the manufacturers' instructions for the ProtoScript® II First Strand cDNA Synthesis kit (New England BioLabs, USA) was followed for the cDNA synthesis reaction. A 20 µl cDNA synthesis reaction was set up in a PCR tube by adding 1 µg Template RNA, 5 µl RdRp-R (1 µM) oligonucleotide (Table 5.1), 10 µl ProtoScript II reaction mix (2 ×), 2 µl ProtoScript II enzyme mix (10 ×) and up to 20 µl of Nuclease-free water. A No-RT negative control reaction was also set up, adding all the components except the ProtoScript

II Enzyme mix. Additionally, DNase treated positive control reaction was set up by using 1 µg of the T7 transcript RNA sample (Section 5.2.2.5) as template RNA. Additionally, a spike control reaction was set up by using the spike control sample as template RNA, and this spike control sample consisted of a 1:1 ratio of DNase I treated RNA isolated from cricket samples and mixed with the DNase I treated positive T7 transcript sample. The reaction mixtures were mixed, followed by a quick spin down. The reaction mixtures were incubated for 1 hr at 42 ℃ followed by 5 min at 80 ℃ to inactivate the enzyme. The resultant cDNA was used as template DNA for PCR amplification in the second RT-PCR step.

A 25 µl PCR reaction was set up in a PCR tube by adding 12.5 µl Taq DNA polymerase 2 × Master Mix RED (Ampliqon, Denmark), 2 µl RdRp-F oligonucleotide (10 µM) (Table 5.1), 2 µl RdRp-R oligonucleotide (10 µM) (Table 5.1), 3 µl (cDNA) template DNA and 5.5 µl ddH_2O. No template control was also set up by replacing the template DNA with ddH_2O. A positive control reaction was also set up using the cDNA generated from the T7 transcript RNA sample as template DNA. Furthermore, a spike reaction was also set up by using the cDNA generated from the spike control sample, which consisted of a 1:1 ratio of DNase I treated RNA isolated from cricket samples and mixed with the DNase I treated positive T7 transcript sample. Reactions were mixed carefully, followed by quick centrifugation of ~5 s. A MiniAmp thermal cycler was used for PCR amplification. The cycle parameters consisted of an initial denaturation step at 95 ℃ for 3 min, followed by 25 cycles of 95 ℃ for 1 min, 48 ℃ for 30 s, and 72 ℃ for 1 min and a final elongation step at 72 ℃ for 2 min. The PCR amplicons were analysed by 1 % agarose gel electrophoresis with ethidium bromide staining in 1 × TAE buffer run for 35 min at 90 V and visualised using a ChemiDoc™ XRS+ System with Image Lab™ software.

5.3. Results

5.3.1. Development of positive control

5.3.1.1. Oligonucleotide design

A set of oligonucleotides was designed targeting a region in the *RdRp* gene of the CrPV genome sequence, based on criteria for optimal binding. The criteria included a melting temperature between 57-63 ℃, a PCR product size between 70-1000 bp, and a GC percentage between 50-60 %. The selected oligonucleotide set amplified a 407 bp amplicon (Table 5.1). The schematic diagram below illustrates the binding of the oligonucleotide set (Figure 5.1**)**.

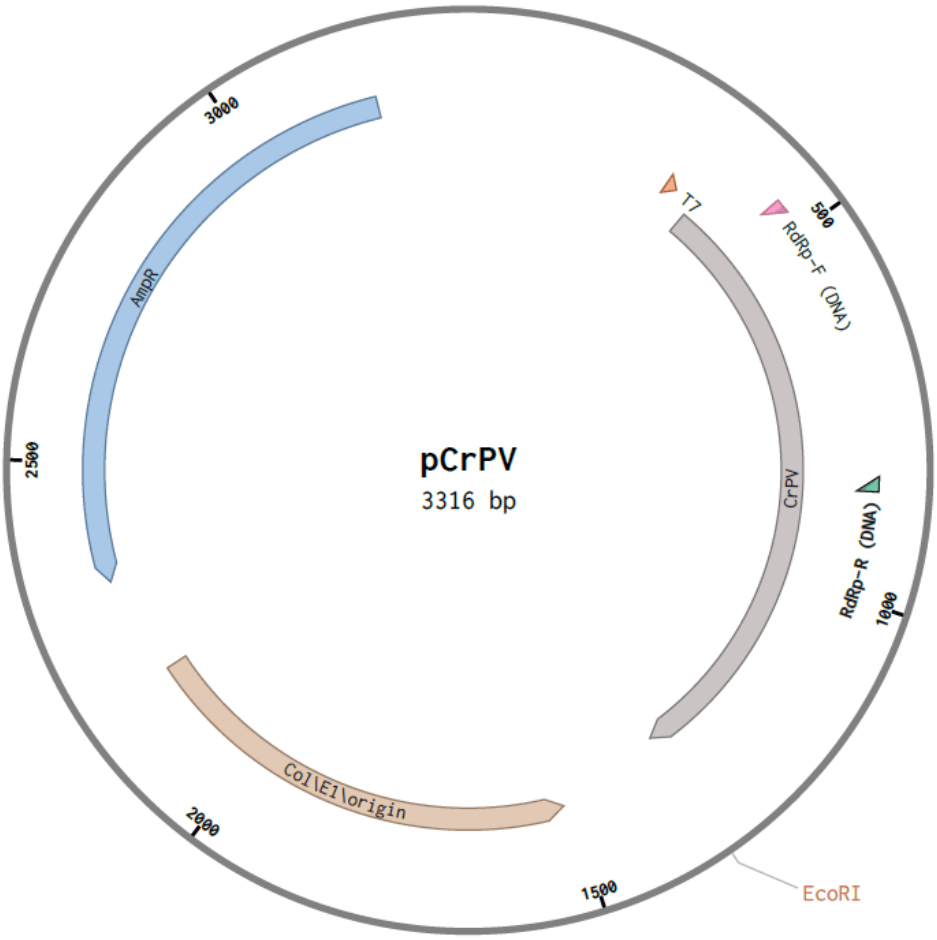

Figure 5.1: Synthetic plasmid constructed with the cricket paralysis virus *RdRp* gene indicated by CrPV region. The binding sites of the oligonucleotide set are indicated by the RdRp-F and RdRp-R. The annotations of the plasmid include the Col E1 origin of replication (Col\E1\origin), ampicillin resistance gene (ampR), cricket paralysis virus *RdRp* gene (CrPV), T7 promoter site (T7), the oligonucleotide binding sites, and the EcoRI restriction enzyme binding site. Image created using Benchling (https://www.benchling.com/).

5.3.1.2. Colony PCR

After transforming pCrPV into JM109 competent cells, colony PCR was conducted using the RdRp oligonucleotide set. All four selected colonies produced amplicons of the expected size, approximately 407 bp (Figure 5.2).

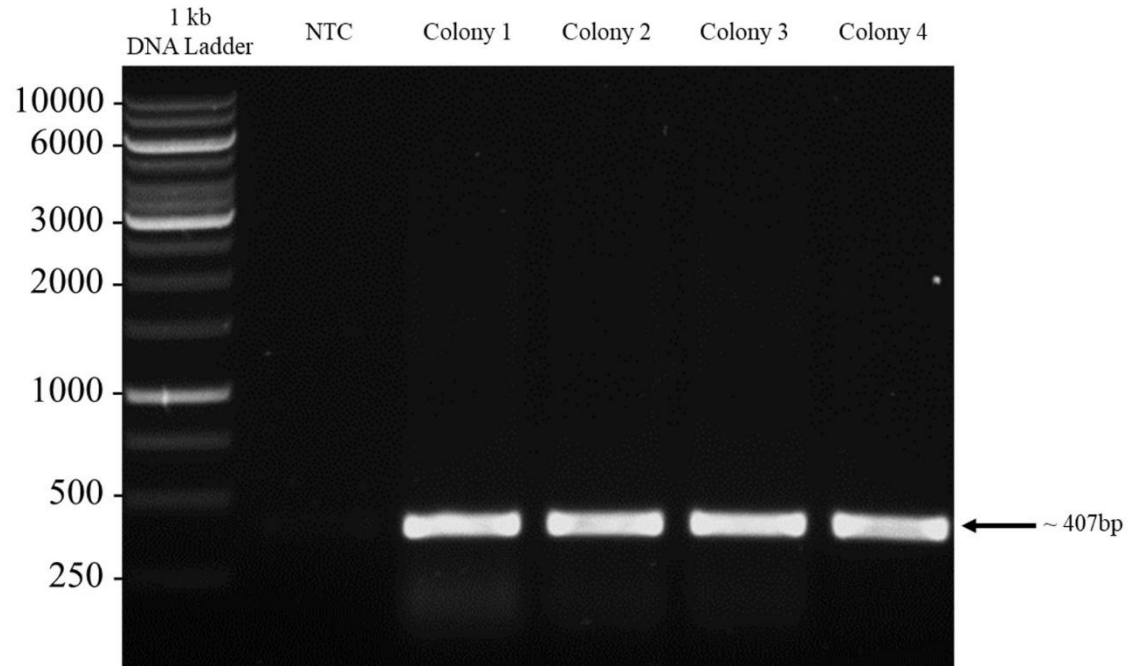

Figure 5.2: Agarose gel electrophoresis (1 %) with ethidium bromide staining, showing the results of the colony PCR amplification of four positive colonies selected.

5.3.1.3. Plasmid extraction and restriction digestion

Following colony PCR, the same four colonies were individually cultured in liquid broth overnight and subjected to plasmid extraction. Subsequent restriction digestion of extracted plasmid DNA with the FastDigest EcoRI restriction enzyme was performed, with the undigested plasmids, along with the digested plasmids, resolved by 1 % agarose gel electrophoresis (Figure 5.3). The different plasmid conformations, open circular, linear, and supercoiled, were observed for the undigested plasmids in lanes 1, 3, and 5. These conformations were not visible for the plasmid extracted for colony 4, lane 7. Restriction digestion of the plasmids produced a linear band of approximately 3316 bp in length for all digests (Figure 5.3).

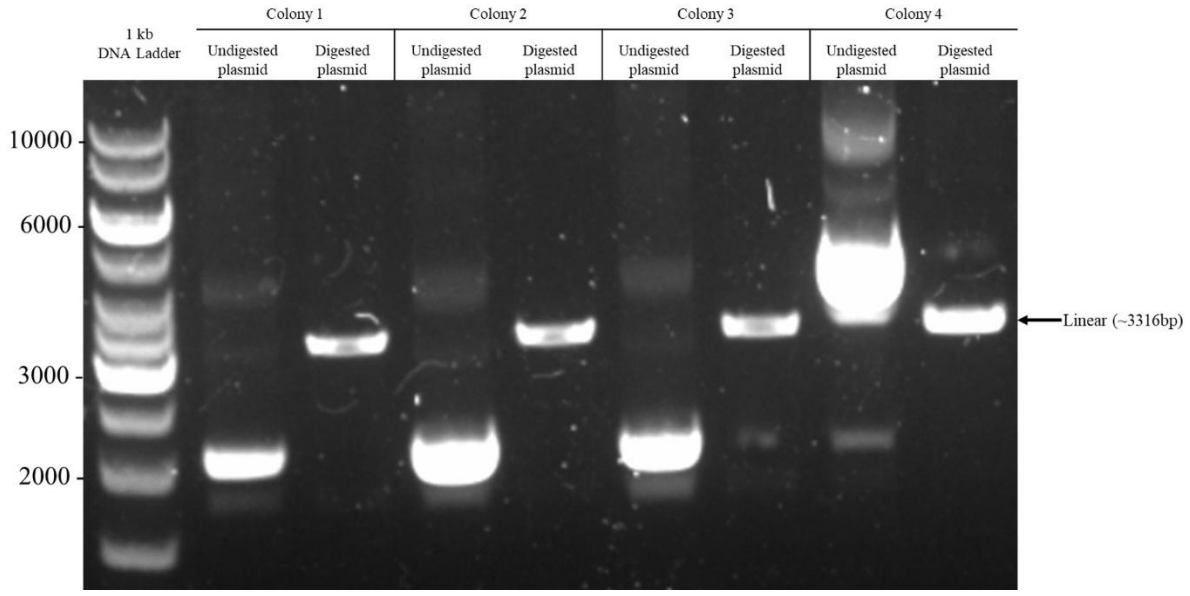

Figure 5.3: Agarose gel electrophoresis (1 %) with ethidium bromide staining showing undigested and digested plasmids extracted from four different bacterial colonies.

5.3.1.4. T7 Transcription of the positive control sample

The digested plasmid for colony 2 (pCrPV_colony2) was selected for T7 transcription. The integrity and size of the RNA transcripts were confirmed by agarose gel electrophoresis (Figure 5.4). The expected amplicon size for the pCrPV_colony2 T7 transcript was approximately 1004 bp in length, and the control transcript's expected size was 1800 bp. The presence of a single band for pCrPV_colony2 T7 transcript indicates that an RNA transcript was successfully generated (Figure 5.4). RNA yield was quantified, and the average concentration was measured at 3.739 µg/µl. RNA was treated with DNase I in preparation for subsequent RT-PCR experiments.

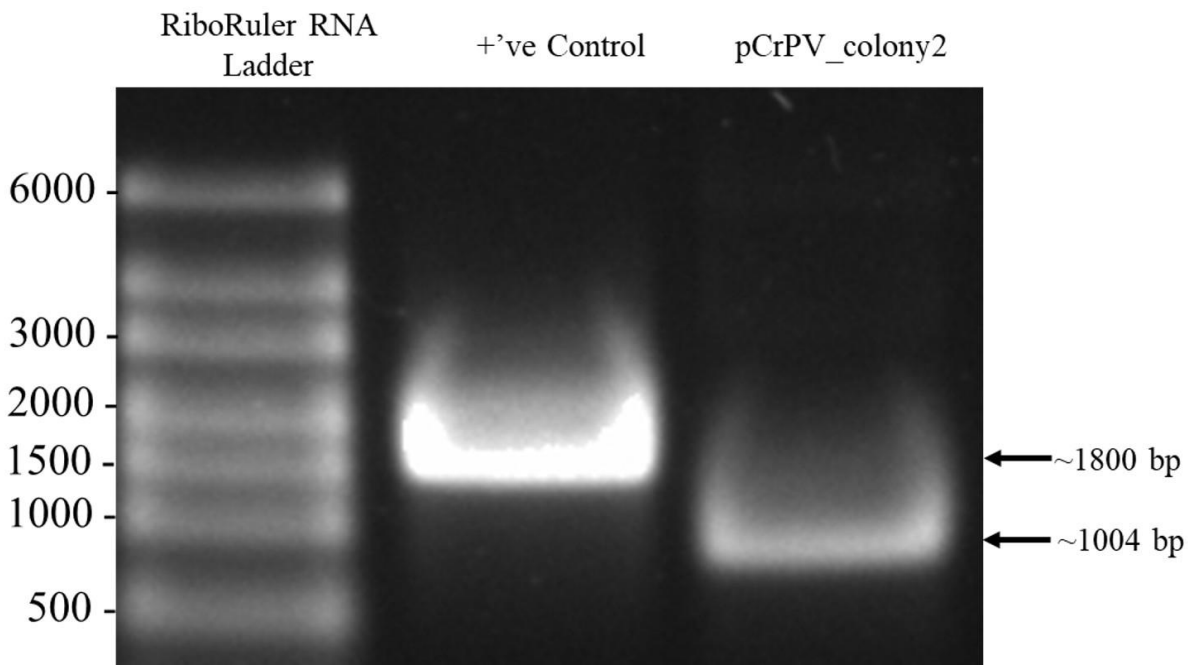

Figure 5.4: Agarose gel electrophoresis (1 %) with ethidium bromide staining showing the result of T7 transcription. The amplicon size for the control transcript is approximately 1800 bp and the pCrPV_colony2 T7 transcript is approximately 1004 bp.

5.3.1.5. RT-PCR for testing the positive control sample

The pCrPV_colony2 T7 transcript was used to generate cDNA, and this was used as template DNA for PCR amplification using the *RdRp* oligonucleotide set. The no template control reaction worked as expected with no amplicon observed (Figure 5.5). A PCR amplicon of approximately 407 bp was obtained for the pRdRp_colony2 cDNA sample, indicating the successful amplification of the target region of the *RdRp* gene from the cDNA sample (Figure 5.5). Therefore, indicating the successful development of a positive control sample for use in downstream experiments.

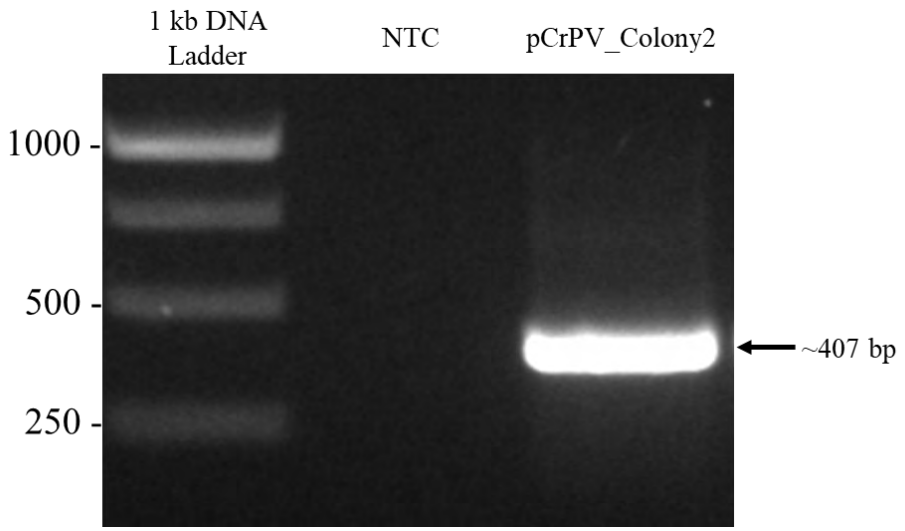

Figure 5.5: Agarose gel electrophoresis (1 %) with ethidium bromide staining. PCR amplification of the *RdRp* gene from template cDNA pCrPV_Colony2 sample.

5.3.2. RNA Isolation method testing from *Thaumatotibia leucotreta* samples

To preserve *A. domesticus* samples, the RNA isolation method was first tested on *T. leucotreta* samples. The two methods tested for the isolation of RNA showed effectiveness in the extraction. The isolated RNA, along with the DNase I treated RNA, were resolved by 1 % agarose gel electrophoresis (Figure 5.6). The first method tested using TRIzol reagent in conjunction with Phasemaker™ tubes is represented by Figure 5.6 A. For this method, bands of approximately 2000 bp were visible in lanes 2, 3, 4, and 6, and faintly in lanes 5 and 7 (Figure 5.6 A). The second method tested using TRIzol reagent is represented in Figure 5.6 B. Similarly, for this method, bands of approximately 2000 bp were visible in lanes 2, 3, and 4, and faintly in lane 5 (Figure 5.6 B).

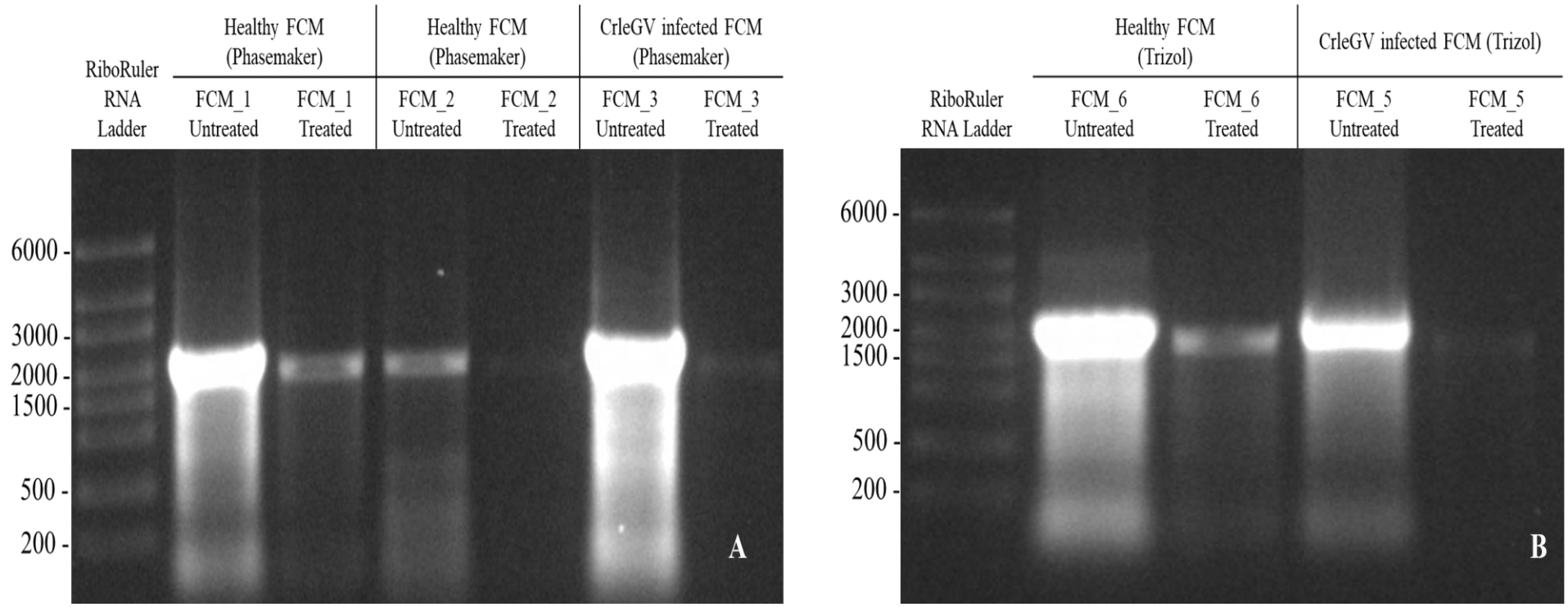

Figure 5.6: Agarose gel electrophoresis comparing the isolation of RNA from *Thaumatotibia leucotreta* samples using two different extraction methods. Figure A represents RNA extracted from samples using the method with TRIzol reagent in conjunction with Phasemaker™ tubes. Lanes 2, 4, and 6 show isolated RNA before DNase I treatment and lanes 3, 5, and 7 show DNase I treated RNA. Figure B represents RNA extracted from samples using the method with TRIzol reagent. Lanes 2 and 4 show isolated RNA before DNase I treatment, and lanes 3 and 5 show DNase I treated RNA.

5.3.3. RNA isolation from *Acheta domesticus* samples

RNA was extracted from *A. domesticus* samples. The isolated RNA, along with the DNase I treated RNA, were resolved by 1 % agarose gel electrophoresis (Figure 5.7). RNA bands were visible in lanes 2, 3, 6, 7, 8, and 9 for cricket samples Cr18, Cr20, and Cr21, respectively.

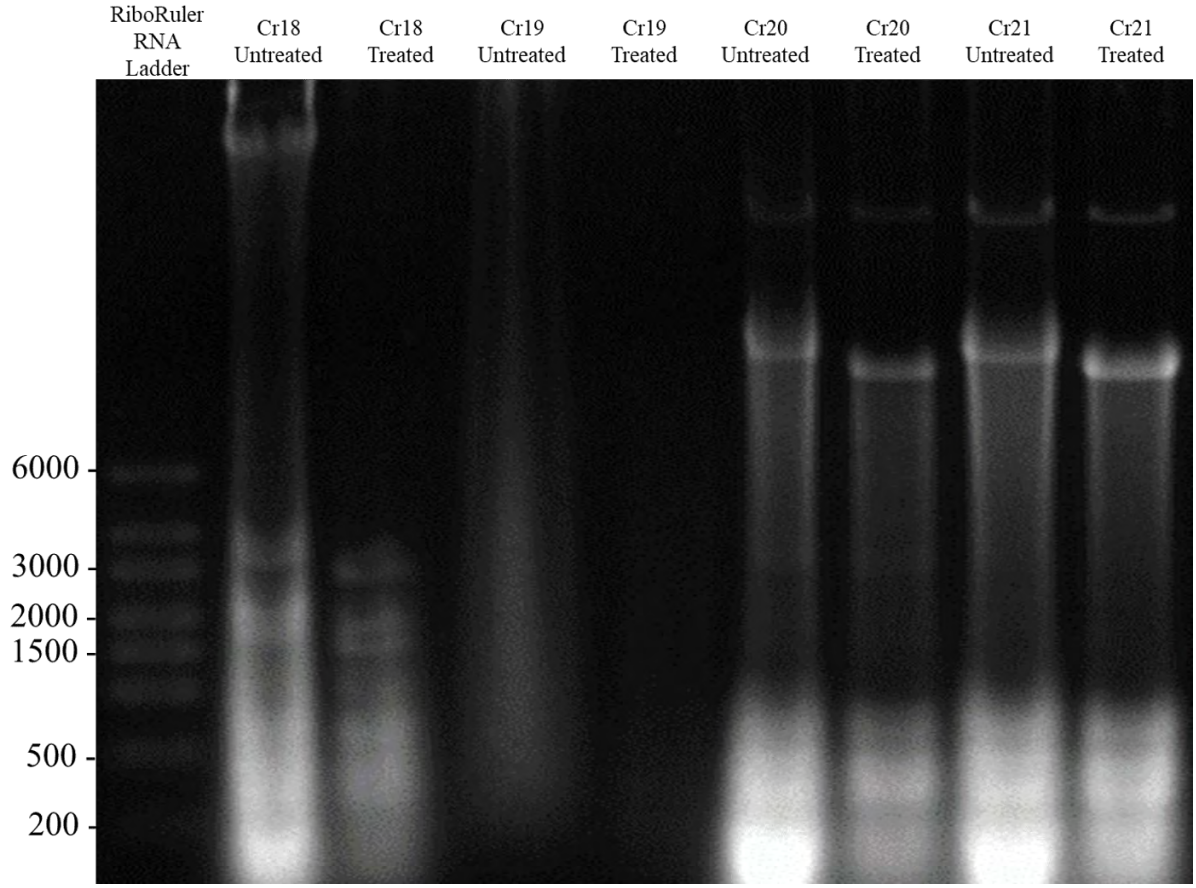

Figure 5.7: RNA isolated from *Acheta domesticus* samples using TRIzol reagent in conjunction with Phasemaker™ tubes. The isolated RNA, along with the DNase I treated RNA for each sample, were resolved by 1 % agarose gel electrophoresis.

5.3.4. RT-PCR *Acheta domesticus* RNA samples

Following the isolation of RNA, a target region within the *RdRp* gene was amplified by RT-PCR analysis. The results are presented in Figure 5.8. No PCR amplicons were observed for the cricket samples in lanes 4, 6, and 8 (Figure 5.8). Bands were observed in the spike samples in lanes 5, 7, and 9, indicating that no inhibitors were present during the amplification. An amplicon was also observed for the positive control sample. No bands were present for the no-template control. Amplicons observed were of the expected band size of 407 bp.

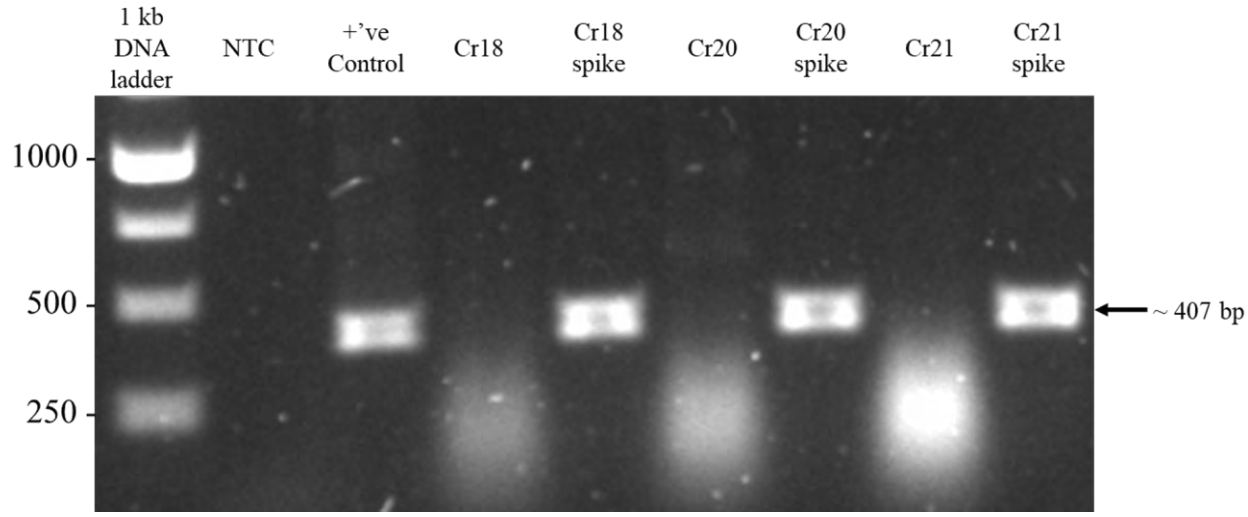

Figure 5.8: Agarose gel electrophoresis for the RT-PCR assay of RNA isolated from *Acheta domesticus* samples.

A further series of RT-PCR amplifications without the spike controls were performed on seventeen of *A. domesticus* samples. No amplicon was detected in the NTC or in any of the *A. domesticus* samples. A positive CrPV *RdRp* amplicon was detected in the positive control (data not shown).

5.4. Discussion

The chapter aimed to develop a screening technique for isolating CrPV from adult *A. domesticus* samples. This was achieved through the development of a positive control by transformation, extraction, restriction digestion, and T7 transcription of a synthetic plasmid containing the CrPV *RdRp* gene. Thereafter, isolation of RNA and DNase I treatment, followed by a two-step RT-PCR assay was applied to several *A. domesticus* samples.

The first objective was the development of a positive control. It is essential to have control DNA, as it corroborates that an assay is working correctly and that specimens do not contain inhibitors. Additionally, it is difficult to validate how well PCR reagents used in laboratories perform when there is no standardised reference material available (Cleveland et al., 2024). Positive controls are used to confirm that PCR inhibitors are not present in the experiment, to verify that the PCR reagents are functioning, that the extension times are adequate, and that suitable annealing temperatures for the primers are used. Samples that have been previously identified as positive through sequencing or plasmids that have been designed to contain the

required sequence can each be used as positive control samples (Moldovan & Moldovan, 2020). When viral and bacterial pathogens are being diagnosed, one of the major difficulties is not having a positive control sample. In this context, a different approach for manufacturing molecular detection kits is the design of artificial sequences and genomes. Specific genomic sequences from various pathogens are found within these constructs. In amplification reactions, these synthetic vectors can be used as positive controls when specific primers are designed and the desired sequences for the synthetic vectors are amplified (Zeinoddini et al., 2020). Several studies have used plasmids as positive controls (Álvarez-Díaz et al., 2019; Inoue et al., 2004; Laiton-Donato et al., 2023; Lee et al., 2011). The standardisation of in-house protocols for routine surveillance and studies on viral activity is hindered by the limited access to positive samples. Therefore, the study by Laiton-Donato et al. (2023) aimed to create and evaluate a synthetic control RNA to detect yellow fever virus (YFV) using real-time RT-PCR. The design of the YFV control plasmid, starting from the 5' end to the 3' end, consisted of a T7 promoter, the target sequence for the primers and probes, followed by the SP6 promoter. The study concluded that research laboratories conducting the molecular detection of YFV for basic research and public health purposes can use the available positive YFV plasmid DNA control described by Laiton-Donato et al. (2023). Additionally, to ensure assay reliability by mitigating the risk of false negatives due to inhibitors or inefficient reaction conditions, Lee et al. (2011) designed standardised positive controls, which were included in RT-PCR assays for the detection of norovirus. The use of the designed standardised positive control plasmids allows for accurate and reliable detection of norovirus by RT-PCR when conducted in various laboratories across different regions. There was no access to either CrPV RNA or the virus, as a result, the current study aimed to develop a positive control by using a recombinant plasmid containing the CrPV *RdRp* gene. The cloning of a DNA template is preferred when the generation of large quantities of RNA is required, as the methods of bacterial culture and plasmid extraction produce ample template DNA (Beckert & Masquida, 2010). Therefore, the development of the positive control sample involved transformation, colony PCR, and plasmid extraction, ensuring the production of an adequate amount of positive control DNA.

The first step was the transformation of the synthetic plasmid, followed by colony PCR. After performing a transformation, colonies of bacteria or yeast that have been grown up on selective media can be rapidly screened through colony PCR to either amplify a portion of a construct or to confirm the presence of a desired genetic construct (Bergkessel & Guthrie, 2013). Colony PCR results from the current study showed the successful amplification of the target regions,

with the amplicons produced being of the expected size. Therefore, indicating a positive transformation of pCrPV. Following colony PCR, the next step was plasmid extraction and restriction digestion of the synthetic plasmid. The bright band of the expected size of 3316 bp on the agarose gel suggests the effective isolation of intact plasmid DNA. Plasmids were successfully digested with EcoRI during restriction enzyme analysis, with distinct bands observed corresponding to the expected size of 3316 bp. The results demonstrated that the plasmids were suitable for further applications, such as T7 transcription.

The linearised plasmids were used to conduct T7 transcription for the development a positive control RNA transcript sample. The T7 promoter site on the constructed synthetic plasmid allows for the synthesis of an RNA transcript. The promoter is the site where the transcription of a gene starts (Epstein & Beckwith, 1968; Pribnow, 1975). During *in vitro* transcription, the T7 RNA polymerase is the most extensively used enzyme (Borkotoky & Murali, 2018; Wang et al., 2018). An RNA molecule is synthesised once the RNA polymerase binds to the promoter DNA (Pribnow, 1975). The T7 RNA polymerase has a strong affinity towards the T7 promoter, and therefore, the initiation of transcription is precise and accurate (Beckert & Masquida, 2010; Borkotoky & Murali, 2018; Wang et al., 2018). Single-stranded transcripts are produced during the *in vitro* transcription of RNA molecules, and these transcripts can range in length from a few nucleotides to thousands of nucleotides (Beckert & Masquida, 2010). The results from using the HiScribe T7 High Yield RNA Synthesis Kit produced a high yield of RNA transcripts with the expected size. The successful synthesis of these transcripts corroborated the effectiveness of the method and supported the development of a reliable positive control sample.

Following the development of a positive control, the next objective was to determine the most effective technique for the isolation of RNA. Two RNA isolation methods were initially evaluated using *T. leucotreta* samples. Both methods produced visible bands at approximately 2000 bp, likely to correspond to the 18S ribosomal RNA (rRNA) subunit. Indicating the isolation of total RNA. Similar to the study by Ridgeway & Timm (2014), RNA isolated from *T. leucotreta* samples using TRIzol produced an 18S RNA band of approximately 2000 nt in length. Since both methods tested exhibited similar results, either method was considered suitable for subsequent RNA isolation from *A. domesticus* samples. RNA was extracted from *A. domesticus* samples using TRIzol Reagent in conjunction with Phasemaker™ tubes. During the isolation process, the homogenate was allowed to separate into three layers, with the DNA and proteins being present in the lower organic layer, an interphase layer, and the RNA being

present in the clear upper aqueous layer. A physical barrier between the lower and upper phases is created when using the Phasemaker™ tubes, making it easier to remove the upper aqueous layer containing the RNA. Hence, this method was used for subsequent extractions from *A. domesticus* samples. Extracted RNA from samples were observed to run above 6000 bp, which is consistent with the size range of dicistroviruses (Bonning & Miller, 2010; Valles et al., 2017). The size of the genomes of dicistroviruses ranges between 8-10 kb (Bonning & Miller, 2010). Additionally, DNA was successfully removed from the isolated RNA samples using DNase I treatment. DNase treatment can be used to easily eliminate small traces of DNA quickly (Chomczynski & Sacchi, 2006). Therefore, eliminating DNA present in the sample, and only RNA remains in the sample.

The next objective was to conduct a two-step RT-PCR assay. The RT-PCR assay was first evaluated using the positive control T7 transcripts and the designed RdRp oligonucleotides. A PCR amplicon was produced with an expected size of approximately 407 bp, confirming the RT-PCR assay's success and the development of a positive control sample. No amplicons were observed for *A. domesticus* samples, therefore, indicating that the samples were not infected with CrPV. To eliminate the possibility of contamination or the presence of inhibitors within the RT-PCR reactions, no template controls and spiked samples were included in the analysis. Negative controls are used in PCR experiments to determine if the reaction mixture contains any contaminants and to ensure that primer-dimers are not forming. In PCR amplification, a negative control reaction consists of molecular-grade water and the PCR reaction mixture (Czurda et al., 2016). The molecular-grade water is equivalent to the volume of the genetic material added to the tubes or wells of the test samples. In all the RT-PCR reactions, no amplicons were present for the negative controls, therefore confirming that no contaminants were present in the reactions.

Furthermore, one of the significant challenges is having inhibitors present in an amplification reaction. Using internal amplification controls can partially address this challenge (Hoorfar et al., 2003, 2004; Wadowsky et al., 1994). Inhibitors can be detected in various ways, such as decreasing the effects of inhibitors by diluting the specimen (Thoreson et al., 1999), or by using target DNA to spike negative samples (Jantos et al., 1998), or through the addition of a control template (Gibb & Wong, 1998; Martineau et al., 1998). Several specimen matrices can contain inhibitors which could prevent PCR amplification by interacting with the DNA and stopping the polymerase activity or the activity of other components in the PCR mixture. Inhibition linked to the processing method, or the specimen matrix, can be recognised when adding

internal (or inhibition) controls directly to the specimen (Hartman et al., 2005; Rosenstraus et al., 1998). A study by Herwegh et al. (2005) aimed to identify inhibitors in clinical samples by developing a detection method. An internal control was designed so that the same primer pair used to amplify the wild-type target DNA would also be used to amplify the internal control. Recombinant PCR was used for the construction of the internal control, by an exogenous DNA fragment being inserted into a target region of a reference strain (Donnenberg et al., 1993; Ho et al., 1989). The PCR reaction mixture contained the exogenous DNA and was co-amplified with the target sequence. The study by Herwegh et al. (2005) managed to effectively implement the detection method during the amplification process of a fragment of *B. pertussis* IS*481* for the diagnosis of whooping cough. Similarly, the current study developed a positive control that was used to spike negative samples by adding the positive control sample RNA with the extracted RNA from *A. domesticus* samples. Bright PCR amplicons were visible for the spiked samples, with an expected size of approximately 407 bp. Therefore, confirming that no inhibitors were present within the reaction mixtures for the two-step RT-PCR amplification reaction.

In conclusion, in this study, a positive control sample was effectively developed by transforming a constructed plasmid into competent cells and confirming the successful transformation by colony PCR. Thereafter, the plasmid was extracted and linearised, and T7 transcription was conducted to obtain a T7 transcript. Additionally, a robust protocol for the isolation of RNA and a two-step RT-PCR assay to detect CrPV from *A. domesticus* samples was successfully implemented. The protocol was used for isolating RNA from symptomatic *A. domesticus* samples, however, CrPV was not detected. A successful detection method has been established, which can be applied in the future for the detection of CrPV from *A. domesticus*.

Chapter 6 – General Discussion

6.1. Thesis overview

Shifts in climate or alterations in farming methods may change the impact and prevalence of specific pests on various agricultural crops. Therefore, to continuously assure that these agricultural pests are controlled, the process of detecting, characterising, and developing entomopathogenic viruses into biopesticides is vital. In various studies such as Moore et al., 2004a), Moore & Kirkman (2010), Abdulkadir et al. (2013), and Marsberg et al. (2018), entomopathogens have been isolated and biologically characterised, and some are now commercially available as biopesticides. This study therefore aimed to isolate, identify and characterise novel and existing viruses from various insects that already impact or have the potential to affect one or more important crops. There are several pests of interest, some of which are emerging pests. This study focused on a selection of these insects: *S. partita, T. batrachopa, E. ceratoniae* and *A. domesticus*. These species were chosen due to their relevance to the southern African agricultural industry and their accessibility for research. Chapter 2 gave an overview of these insect species, and the identification of these species was confirmed using molecular genetic techniques. Symptomatic *S. partita, T. batrachopa* and *E. ceratoniae* larval cadavers were screened for baculovirus infection. Virus particles were isolated from the cadavers, and gDNA was extracted from the purified virus particles. This was followed by PCR amplification of the *polh*/*gran*, *lef-8*, and *lef-9* genes, and lastly sequencing and BLAST analysis of the positive PCR amplicons (Chapter 3). A novel virus isolated from *S. partita* was identified and thereafter further characterised, and the biological activity was analysed (Chapter 4). Lastly, a screening technique for the detection of CrPV infection from *A. domesticus* samples was developed (Chapter 5). This chapter provides an integrated interpretation of the results and reflects on directions for future research.

6.2. Isolation, identification, and characterisation of novel viruses from lepidopteran insects

Several insect pests threaten agriculture, yet no insect viruses have been isolated or characterised for many of them. Among these are the species examined in Chapter 3: *S. partita*, *T. batrachopa* and *E. ceratoniae*. Symptomatic *S. partita*, *T. batrachopa* and *E. ceratoniae* larvae were screened for baculovirus infection. Polymerase chain reaction amplification of the target genes was successfully achieved for the *S. partita* and *T. batrachopa* samples. Non-

specific bands were observed for the PCR amplification from the *E. ceratoniae* samples. Further analysis, such as TEM to confirm if virus OBs are present and PCR optimisation, can be conducted to determine if *E. ceratoniae* samples are infected. If viral particles are confirmed by TEM, PCR optimisation should be conducted to eliminate the chances of obtaining non-specific bands. Additional *E. ceratoniae* samples should be examined to identify potential baculovirus infections in this species, which would have the potential to be developed as a possible biological control agent. The identification of a possible biological insecticide for this species would offer an effective control option for this pest, and subsequently reduce crop damage and increase the availability of crops for both export and human consumption.

The results of Sanger sequencing revealed the isolation of a novel baculovirus isolated from *S. partita* samples, which is described below. Cryptophlebia leucotreta GV was detected in the *T. batrachopa* samples, representing the first documented case of CrleGV isolated from *T. batrachopa*. Cryptophlebia leucotreta GV was isolated first from infected *T. leucotreta* larvae in the Ivory Coast (Angélini et al., 1965b). Subsequently, the South African isolate, CrleGV-SA, was isolated from infected larvae collected from a laboratory colony (Lange & Jehle, 2003; Singh et al., 2003). In the 1980s, in Cape Verde, *T. leucotreta* was controlled for the first time with CrleGV in a small-scale field trial (Fritsch, 1988). This field trial achieved a 65 % and 77 % reduction in the damage caused by *T. leucotreta* in Spanish pepper and citrus, respectively (Fritsch, 1988). Cryptogran, which is formulated with CrleGV, has been used for more than 20 years for the control of *T. leucotreta* (Grové et al., 2010; Moore et al., 2004a). *Thaumatotibia leucotreta* is effectively controlled by CrleGV as this virus is highly specific and effective against *T. leucotreta* (Jehle et al., 1992). The isolation of CrleGV from *T. batrachopa* expands the host range of this virus and presents a new opportunity to explore its potential in controlling *T. batrachopa*. Currently, various chemical and pheromone-based products are available for *T. batrachopa* control (Enslin, 2023; Schoeman, 2021), and CrpeNPV has been registered for the control of *T. batrachopa* on macadamias (Thackeray, 2023).

6.3. Serrodes partita nucleopolyhedrovirus

Serrodes partita is considered a sporadic pest (Goddard et al., 2019). During 1987, the activity of *S. partita* on satsumas in the Sundays River Valley was reported as sporadic, and during this period, the feeding activity of the moths led to greater than 100 fruit drops per tree, every week (Johannsmeier, 1998). An increase in the larval host plant, *Pappea capensis* (wild plum) (Ecklon & Zeyher), after significant rainfall in the Little Karoo region, causes a surge of *S.*

partita, making the moth problematic, and this occurs every five to 10 years (Moore, 2010; Robinson et al., 2012). In the Eastern Cape, significant crop damage is caused by *S. partita* (Robinson et al., 2012). The use of repellent lights in orchards is an effective method for controlling the fruit-feeding moths (Whitehead & Rust, 1967, 1971, 1972). Little is known about the biotic factors that control *S. partita* (Moore, 2012). In a previous study during the investigation of alternative control methods for *S. partita*, a baculovirus was isolated from diseased larvae in laboratory-reared insects (Mushore, 2024), which warranted further investigation. In the current study, this novel baculovirus, named SepaNPV, was genetically characterised, and its host range investigated to assess its potential as a biological control agent.

The genome size of SepaNPV is 129953 bp in length, which is within the typical size range for baculoviruses. Baculovirus genomes generally contain between 90 and 180 genes (Rohrmann, 2019), and the SepaNPV genome was identified to contain 128 annotated ORFs, aligning well with this expected range. The analysis of baculovirus genomes identified 38 conserved genes in all baculoviruses, known as the core genes (Garavaglia et al., 2012). The ORFs predicted in the genome of SepaNPV using NCBI ORF finder and FgenesV, did not identify all the baculovirus core genes. Thirty out of the 38 baculovirus core genes were identified within the SepaNPV genome. No sequence similarity or matches were detected for the unidentified core genes when the predicted ORFs were analysed using BLASTp. The unidentified core genes may be associated with the regions annotated as hypothetical genome regions or along the regions of the genome where no genes have been annotated. Further analysis of the genome is required to identify the additional core genes. Similar to Harrison & Rowley (2022b), HMM-HMM queries with HHpred could be used to evaluate ORFs where no match was obtained in a BLASTp query. Furthermore, Glimmer3 in Geneious or the online tool HMMER could be used to further characterise the genome and to identify additional ORFs that were not identified using NCBI ORF finder or using FgenesV.

All sequenced lepidopteran baculoviruses also share 24 genes (Garavaglia et al., 2012; van Oers & Vlak, 2007). Of these 24 genes, 15 were identified in the genome of SepaNPV. Four *pif* genes were identified in the genome of SepaNPV, *pif-1*, *pif-2*, *pif-3*, and *pif-6*. PIF proteins play a crucial role in initiating infection in insect larvae through oral ingestion, and ten *pif* genes have been identified in baculoviruses (Peng et al., 2010). The peritrophic membrane of the insect is broken down by the enhancin, which is an enzyme classified as a metalloproteinase (Wang & Granado, 1997). The initiation of infection is facilitated when the enhancin degrades the proteins of the host's peritrophic matrix (Toprak et al., 2012; Wang & Granados, 1998).

Similar to Hyphantria cunea granulovirus (HycuGV-Hc1) (Gencer et al., 2020), with the initial annotation of SepaNPV genome, the *enhancin* gene was not detected. Furthermore, the host cell polymerases are responsible for transcribing the early genes *pe38*, *ie-0*, *ie-1*, and *ie-2* (Friesen, 1997). Of these, the *ie-1* gene was the only one detected in the SepaNPV genome. Apoptosis is a vital biological process in insects, which takes place during tissue maintenance and normal development, where unnecessary cells are removed via a programmed form of cell death regulated by genetic mechanisms. Apoptosis also serves as a cellular defence against viral infections. The production of progeny virions is decreased during the early infection of the virus because of apoptosis, and this helps eliminate infected cells from the organism. Viruses have developed genes that encode proteins that can suppress the apoptotic pathway, therefore overcoming the antiviral cellular responses linked to apoptosis, and subsequently the viruses can optimise their replication in infected cells (Everett & McFadden, 2001; Roulston et al., 1999; Teodoro & Branton, 1997). The *p49*, *p35*, and *iap* genes were acquired through the evolution of baculoviruses, inhibiting the apoptosis process and enabling them to adapt. These genes are responsible for the suppression of apoptosis, allowing viral replication to proceed efficiently (Vaux & Strasser, 1996). Baculovirus genomes have been identified to contain five *iap* genes (Ikeda et al., 2004), with only *iap-2* and *iap-3* identified in the SepaNPV genome. During the later stages of infection, the genes that play a vital role in the breakdown of insect tissues and the liquefaction of the host are the *chitinase* and *cathepsin* genes (Hawtin et al., 1997; Slack et al., 1995). The time it takes for the larvae to die is significantly prolonged when either only the *cathepsin* gene is absent or when both genes are absent (Ishimwe et al., 2015). The *cathepsin* gene was identified in SepaNPV, and the *chitinase* gene is absent. The evidence highlighting the roles of various baculovirus genes in viral replication suggests that the presence or absence of specific genes identified in SepaNPV may influence the efficiency of viral infection in the host. Additionally, other genome annotation software should be identified and used for further analysis, with the current sequence forming a preliminary characterisation of the SepaNPV genome. The gene organisation between two viral genomes can be compared by generating gene parity plots (Hu et al., 1998). In addition, gene parity plot analysis can be conducted in future research for SepaNPV to determine the organisation of the genome in comparison to other baculovirus genomes.

Phylogenetic and distance matrix analyses confirmed the novelty of SepaNPV, and showed that SepaNPV was basal to a monophyletic clade which includes species like Lymantria xylina NPV and Lymantria dispar MNPV. There are two subdivisions for the alphabaculoviruses, these are

group I and group II viruses, which are based on single gene phylogenies (Bulach et al., 1999; Zanotto et al., 1993). Furthermore, the grouping is also linked to the distribution of two distinct fusion proteins used by the BV for receptor-mediated endocytosis (Pearson & Rohrmann, 2002). A distinct difference between the two groups is that the envelope fusion protein (EFP) of group I viruses is the GP64 protein, and the EFP of the group II viruses is the F protein (Thézé et al., 2018). SepaNPV contains an F protein gene, indicating that it is a member of the group II alphabaculoviruses. The fusion between the viral envelope and the host cell membrane is facilitated by the F proteins, which are active envelope fusion proteins (Ijkel et al., 2000; Pearson et al., 2000).

When considering the use of a particular NPV against a specific pest or a range of different pest species, it is important to determine the host range of the virus (Abid et al., 2020). Moreover, knowledge of the host range is important when registering and commercialising a baculovirus as a microbial pesticide. Microbial control agents, such as baculoviruses, have a very specific host range, which often makes them considered unfavourable by the market. The potential use is limited to those dealing with the specific target pest. In comparison to chemical pesticides that can be used against multiple pests, attracting a wider market. Consequently, the market for this product is limited, and the development and production may be cost-effective unless the insect that it is targeting is a major pest (Ravensberg, 2011). The host range of NPVs is typically broader in comparison to GVs, and often multiple species within the same genus or family can be infected (Ishii et al., 2003; Rao et al., 2015; Szewczyk et al., 2006). An example of an NPV with a broad host range is the CrpeNPV, isolated from the lychee moth, *Cryptophlebia peltastica* (Marsberg et al., 2018). In South Africa, this virus was recently registered for application against *T. leucotreta* and *T. batrachopa* on macadamias, *C. pomonella* on pome fruit, and *C. peltastica* and *T. leucotreta* on lychees. This virus has been developed into two commercialised CrpeNPV-based biopesticides, Codlmax™ and Multimax™ (River Bioscience, South Africa), which are used for the control of various important agricultural pests (River Bioscience, 2024a, b; Thackeray, 2023). The identification of SepaNPV presented the potential for its development as a biopesticide to manage populations of *S. partita*. The potential market could be expanded if SepaNPV can infect more than one species; as a result, its development would be more commercially viable. The biological activity of SepaNPV was evaluated, however, one major limitation was not being able to evaluate the biological activity of this virus against the homologous host. The development of an artificial diet as well as establishing an insect colony for *S. partita* is required to evaluate the biological activity (i.e.,

lethal time and concentration) of SepaNPV against this pest. Therefore, four other insect host species, *T. leucotreta*, *S. frugiperda*, *H. armigera*, and *E. saccharina*, were selected to test the biological activity of SepaNPV. However, no mortality was observed, indicating that SepaNPV may not be pathogenic to these hosts. Future research should prioritise the development of an *S. partita* colony to enable the evaluation of SepaNPV OBs. It is possible that the isolated OBs are not viable, and application against a homologous host could assist in establishing fundamental biological activity. The biological activity of SepaNPV against other potential host species could then be further evaluated.

Various strategies focusing on broadening the host range of baculoviruses have been explored (Chen et al., 1998; Croizier et al., 1994; Graillot et al., 2017), and this could be applied to SepaNPV in future studies. One strategy focuses on successive passages of viruses. Successive passages of the baculovirus CpGV were conducted in the study by Graillot et al. (2017) to adapt the virus to a semi-permissive host and therefore improve the insecticidal efficiency. An isolate mixture of CpGV was passaged repeatedly 14 times through *Grapholita molesta*. The study found that there was a substantial increase in the mortality rate of *G. molesta*, with LC_{50} and LC_{90} values improving by 54-fold and 450-fold, respectively. Another strategy involves the genetic modification of the virus genome. In the study by Chen et al. (1998), the *host range factor 1* (*hrf-1*) gene of LdMNPV was inserted into the genome of AcMNPV to expand the host range of AcMNPV. *Lymantria dispar* larvae were previously identified as a non-permissive host of AcMNPV, and the genetic modification of AcMNPV enabled the virus to replicate within this host. There were comparable results obtained for the mortality observed in both the permissive host and the adapted host of AcMNPV. Furthermore, a new variant of Bombyx mori nucleopolyhedrovirus (BmNPV) was identified during the analysis of mixed infections between Autographa californica multiple nucleopolyhedrovirus (AcMNPV) and BmNPV. Previously BmNPV was not able to replicate in Sf-21 cells. However, it was found that the new variant was able to replicate in both Sf-21 and BmNPV cells. The altered host range of BmNPV was linked to a 572 bp fragment of the DNA *helicase* gene (Maeda et al., 1993). Further investigation found that the change in host range was because of a single amino acid substitution in the helicase ORF (Kamita & Maeda, 1997). Using a similar strategy, a study was conducted to modify the *helicase* gene of AcMNPV by substituting three specific amino acids with those found at the same position in BmNPV. The change in the *helicase* gene of AcMNPV enabled the replication of AcMNPV in the cells of *Bombyx mori* (Croizier et al., 1994).

Laboratory bioassays have shown that the insecticidal activity of baculoviruses can be enhanced when using baculovirus mixtures in contrast to when one virus is applied for the bioassay (Biedma et al., 2015; Lara-Reyna et al., 2003). Research conducted by Arrizubieta et al. (2016), Carballo et al. (2017), Jukes et al. (2017), and Redman et al. (2016) has shown that the viral activity can be improved when viruses are applied in mixtures of virus isolates, genotypes, or species. It was shown that the larval mortality was improved when mixtures of a GV and NPV were investigated (Jukes et al., 2017; Lara-Reyna et al., 2003; Wennmann et al., 2015). The interaction between the two viruses, Agrotis segetum granulovirus (AgseGV) and Agrotis segetum nucleopolyhedrovirus B (AgseNPV-B), was investigated against the neonate larvae of *Agrotis segetum* in the study by Wennmann et al. (2015). When the two viruses were mixed, a neutral or additive effect was achieved. Furthermore, the combination of CrpeNPV and CrleGV was evaluated in the studies by both Jukes (2018) and Taylor (2021) against the *T. leucotreta* larvae. Two different virus ratios were evaluated in Jukes (2018), and one virus ratio was evaluated by Taylor (2021). Jukes (2018) observed a synergistic effect at LC_{50} and LC_{90} for the 1:3 GV/NPV virus mixture, and an additive and synergistic effect at LC_{50} and LC_{90}, respectively, was observed when using a 3:1 GV/NPV mixture. A 1:1 GV/NPV virus mixture was evaluated by Taylor (2021), and a synergistic effect was observed; however, the lethal time was increased. Based on these findings, future studies could investigate the potential of SepaNPV in combination with other baculoviruses to assess whether these mixtures could enhance the insecticidal activity of SepaNPV.

Furthermore, the OBs of GVs contain proteins that increase the infectivity of other baculoviruses, and these were originally referred to as “Synergistic Factors” (Hara et al., 1976; Tanada et al., 1973). A protein in the occlusion body of Pseudaletia unipuncta granulovirus (PsunGV), was discovered to be a synergistic factor of the virus (Tanada & Hukuhara, 1971). These proteins were identified when mixed infections of PsunGV and Pseudaletia unipuncta nucleopolyhedrovirus (PsunNPV) were applied in bioassays. Similar proteins to the “Synergistic Factors” were discovered in later studies, within the OBs of Xestia c-nigrum GV (XecnGV) and Trichoplusia ni GV (TnGV), and these proteins are known as “enhancins” and “viral enhancing factors (VEFs)” (Derksen & Granados, 1988; Gallo et al., 1991; Goto, 1990). Combining NPVs with extracts from GV occlusion bodies has been employed in numerous studies (Goto et al., 2015; Mukawa & Goto, 2007, 2008, 2010, 2011). One example was when granulovirus proteins from Xestia c-nigrum granulovirus (XcenGV) was effectively applied as additives to Mamestra brassicae multiple nucleopolyhedrovirus (MbMNPV) treatments against

larvae of *Autographa nigrisigna*, *H. armigera* and *Mamestra brassicae* (Goto et al., 2015; Mukawa & Goto, 2007, 2008, 2010, 2011). Studies have evaluated the effect of virus mixtures using various GVs in combination with more than one NPV (Ferrelli & Salvador, 2023). The GVs tested were Helicoverpa armigera GV (HearGV), AgseGV, XcenGV, Spodoptera frugiperda GV (SpfrGV), and PsunGV. When these GVs were applied to non-permissive hosts, they were found to function as synergists, enhancing the infectivity of various NPVs. However, no enhancing effect was observed for AgseGV and PsunGV in *H. armigera* and *B. mori*, respectively. When these GVs were applied to permissive hosts, HearGV, AgseGV, and TnGV had an additive or antagonistic effect. Whereas, in tests involving PsunGV and XcenGV across multiple hosts, synergistic effects are typically limited only to specific larval instars, or synergistic effects are absent (Ferrelli & Salvador, 2023). In light of these findings, future research could evaluate the potential of combining SepaNPV with GV OBs extracts to determine whether the infectivity of SepaNPV can be enhanced through such synergistic interactions.

Despite SepaNPV not showing potential as a biocontrol agent against *S. partita*, this study is the first to describe a novel baculovirus isolated from this insect species. It provides insight into a greater understanding of this species' biology and ecology. Further research is required to test the host range of SepaNPV. Moreover, as presented above, ongoing research on expanding viral host ranges and exploring diverse application methods opens new opportunities to conduct similar studies using SepaNPV.

6.4. RT-PCR assay development and optimisation for the detection of CrPV in *A. domesticus* samples

A method for the detection of CrPV from *A. domesticus* samples was developed, and this protocol was used to screen symptomatic *A. domesticus* samples (Chapter 5). Although CrPV was not detected in the samples screened, an effective plasmid-based RT-PCR CrPV positive control sample was successfully developed. No previously extracted CrPV sample that could be used as a control sample was available before the start of the analysis. Therefore, it was important to develop a positive control sample to use in the detection protocol. Zeinoddini et al. (2020) recommended that for the detection of high-risk or inaccessible pathogens, synthetic constructs should be used as an effective method. Therefore, these synthetic vectors can be included as positive control samples when developing the amplification assays. The development of the positive control ensures the accurate detection of the target virus and

confirms that a negative result is not due to inhibitors present in the reaction. One aspect that could be further improved in this study, is the addition of a dilution factor for the positive control. Studies conducted by Álvarez-Díaz et al. (2019, 2021), Laiton-Donato et al. (2023), and Onosi (2018) have included a dilution series for the positive control sample to determine the lowest concentration of RNA required in the assay for a PCR amplicon to be detected. Similarly, future research should include the dilution series of the positive control RNA when implementing the detection method for CrPV from *A. domesticus* samples, to determine the minimum detection levels.

Following the isolation of RNA, a two-step RT-PCR assay was used to evaluate the presence of CrPV in the isolated RNA samples. Oligonucleotides were designed to amplify a region in the *RdRp* gene using NCBI's Primer-BLAST tool. The design parameters were set to generate amplicons of 70-1000 bp in size with melting temperatures ranging between 57-63 °C. Multiple oligonucleotide pairs were generated based on these criteria. A conserved region of the *RdRp* gene was not identified prior to entering the sequence into Primer-BLAST for analysis. Therefore, primer-BLAST analysed the full *RdRp* gene sequence to find suitable regions where oligonucleotides can bind. The resultant oligonucleotides may not specifically target a conserved region of the gene but rather bind to suitable sites across the full gene sequence as determined by the tool. The lack of identification of CrPV from samples could be because the oligonucleotides were designed targeting a region of the *RdRp* gene synthesised in the positive synthetic plasmid, which may be a variable region in when compared with wild-type viruses. The reliability and precision of the design of an oligonucleotide primer is one of the vital factors influencing a PCR amplification. It is important to design primers targeting a conserved region of a related nucleotide sequence, because it enables the amplification of PCR products from a wide range of organisms, such as poorly studied and/or multiple organisms, using a single primer set (Pessoa et al., 2010). The oligonucleotides used in the current study should therefore be designed to target a conserved region of the *RdRp* gene. An alignment of various *RdRp* genes from different CrPV sequences should be conducted to determine conserved regions, and thereafter, oligonucleotides should be designed to bind to those regions.

Kerr et al. (2015) established two CrPV infectious clones (CrPV-2 and CrPV-3), which were transfected into *Drosophila* Schneider line 2 (S2) cells. Results demonstrated that the S2 cells produced virions of CrPV-2 and CrPV-3, which were found to be infectious, and when these virions were injected into adult *Drosophila,* mortality was observed. The first dicistrovirus infectious clones were created by Kerr et al. (2015). Both the *Drosophila* adult flies and S2

cells are capable of being infected with the CrPV infectious clones. Therefore, these clones provide an essential tool for studying molecular mechanisms of the pathogenesis, life cycle, and interactions with the host of *Dicistroviridae*. In nature, the CrPV is found in various regions globally (Reinganum et al., 1981). The host range of this virus is broad, and out of all invertebrate small RNA viruses, CrPV has the widest host range (Christian & Scotti, 1998). Various cell lines can be infected with this virus (Christian & Scotti, 1998). The insect orders of the natural hosts of CrPV are Hymenoptera, Hemiptera, Orthoptera, Diptera, and Lepidoptera (Bonning, 2009). With evidence showing that CrPV infects the olive fruit fly and causes infection in fruit fly species (Manousis & Moore, 1987b; Reinganum, 1975), and given the wide host range of CrPV, a major motivation for isolating CrPV, whether from *A. domesticus* or another source, is to explore its potential use in fruit fly biocontrol. Therefore, future research could explore the infection of the cell lines from other fruit fly species, such as the Natal fly and Mediterranean fruit fly, using the two CrPV infectious clones. This would help assess CrPV's ability to infect these fruit fly species and evaluate its potential in fruit fly control programmes. Moreover, future studies could investigate the susceptibility of various fruit fly species to CrPV and assess its suitability as a biocontrol agent, particularly if the virus is successfully identified and isolated from *A. domesticus* or acquired through alternative approaches.

Cricket paralysis virus is only spread by horizontal transmission (Bonning, 2009). Infected insects shed the virus particles in their frass. The frass containing the virus particles becomes a source of infection; therefore, if other insects encounter or ingest the frass, they could potentially get infected with the virus (Bonning, 2009). Therefore, an alternative to isolating CrPV from *A. domesticus* adults is that the frass of infected *A. domesticus* samples could be collected and used to isolate CrPV. Additionally, a comparison could be conducted between the isolation of CrPV from adult *A. domesticus* samples and infected frass. Furthermore, to assess the potential of CrPV as a biocontrol agent for fruit fly management and control, more *A. domesticus* samples should be screened for CrPV infection using the RT-PCR assay established in this study. Spiked control samples should be included in these assays to confirm that no inhibitors are present and that the reactions are functioning properly.

The detection method developed in this study is useful not only for detecting CrPV in *A. domesticus* but also for identifying other RNA viruses in insects. This approach, which involves RNA isolation, followed by a two-step RT-PCR assay with the inclusion of a positive control and spiked control samples in the analysis, offers a robust method for detecting viral infections

in diseased insects. This method can be extended to screen for other novel RNA viruses, and it has potential for discovering RNA viruses that could be used as biocontrol agents.

6.5. Conclusion

In conclusion, the overall aim of the study was to isolate, identify, and characterise novel and existing viruses from various insects that already impact or have the potential to affect one or more important crops. The first objective was to confirm the identification of the insect species selected for research in the current study. DNA barcoding using the mitochondrial *COI* gene and BLAST analysis confirmed the species identification of *S. partita*, *T. batrachopa*, *E. ceratoniae* and *A. domesticus*. *Serrodes partita*, *T. batrachopa* and *E. ceratoniae* larval cadavers were screened for baculovirus infection. No baculoviruses were identified from the *E. ceratoniae* samples, CrleGV was identified in the *T. batrachopa* samples and a novel virus, SepaNPV was identified from the *S. partita* samples. This study is the first to report the isolation of CrleGV from *T. batrachopa*, therefore expanding the host range of CrleGV. This study also lays the foundation to further investigate the interaction between CrleGV and *T. batrachopa*. The next objective was to further characterise SepaNPV genetically and to evaluate its biological activity. This objective was successfully achieved, and a preliminary genome has been annotated. The host range of SepaNPV was evaluated, and an interesting observation was that there was no mortality in *T. leucotreta*, *S. frugiperda*, *H. armigera* and *E. saccharina* larvae. This study enhances our understanding of baculoviruses by detailing the characterisation of SepaNPV and expands our knowledge on the ecology of *S. partita* and on host insect interactions. The identification of SepaNPV is the first baculovirus isolated from *S. partita*. The last objective was to develop and optimise an RT-PCR assay for the detection of CrPV from *A. domesticus* samples. A positive control sample was successfully developed. In addition, a protocol for RNA extraction and a two-step RT-PCR assay for detecting CrPV in *A. domesticus* samples was successfully established. CrPV was, however, not detected when screening the *A. domesticus* samples. The development of a screening method offers the opportunity to be implemented for future screening of symptomatic *A. domesticus* samples for CrPV infection.

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Supplementary data

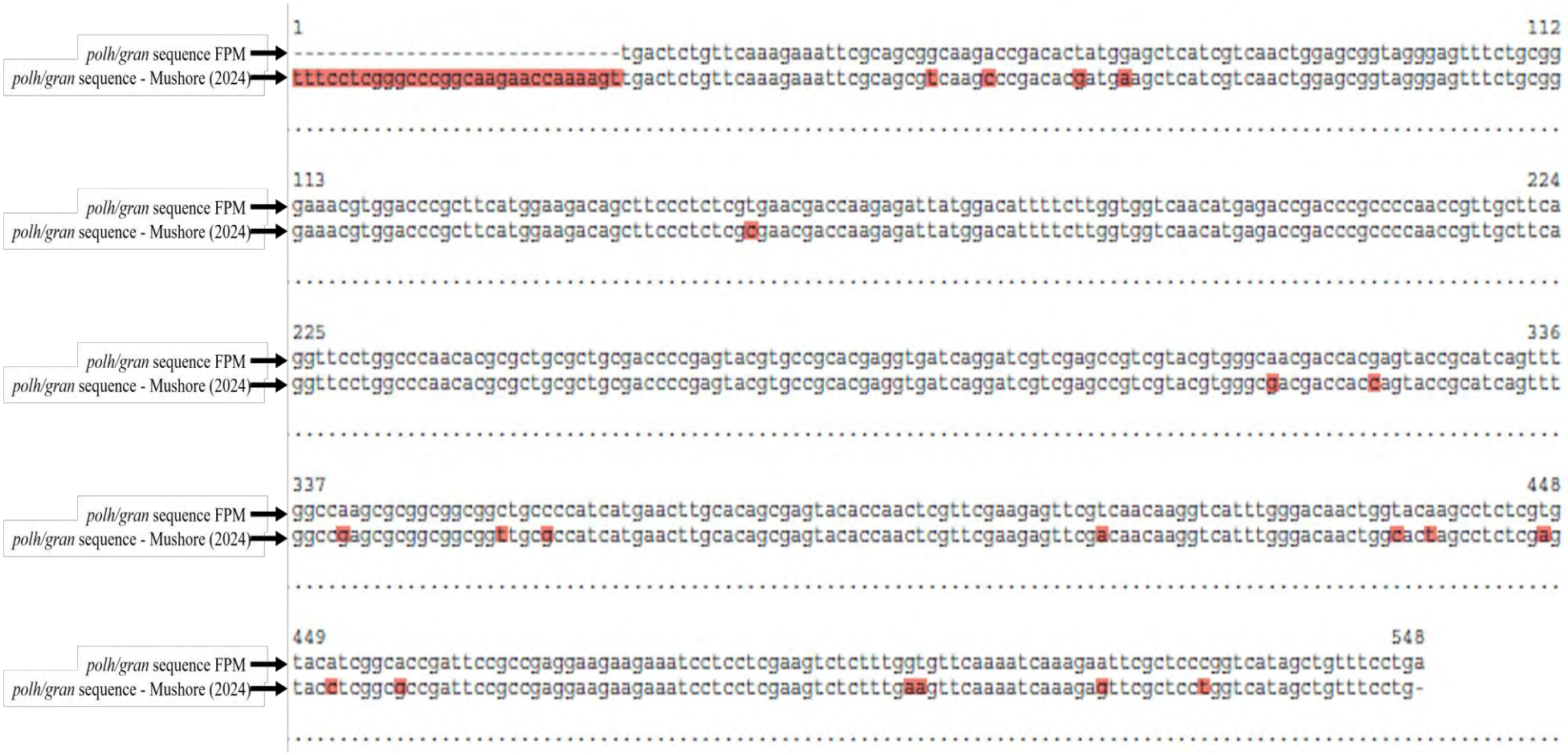

Figure S1: Sequence alignment between the FPM *polh/gran* sequence generated in this study and the *polh/gran* sequence from Mushore (2024). The sequence mismatches are highlighted in red.

Figure S2: Sequence alignment between the FPM *lef-8* sequence generated in this study and the *lef-8* sequence from Mushore (2024). The sequence mismatches are highlighted in red.

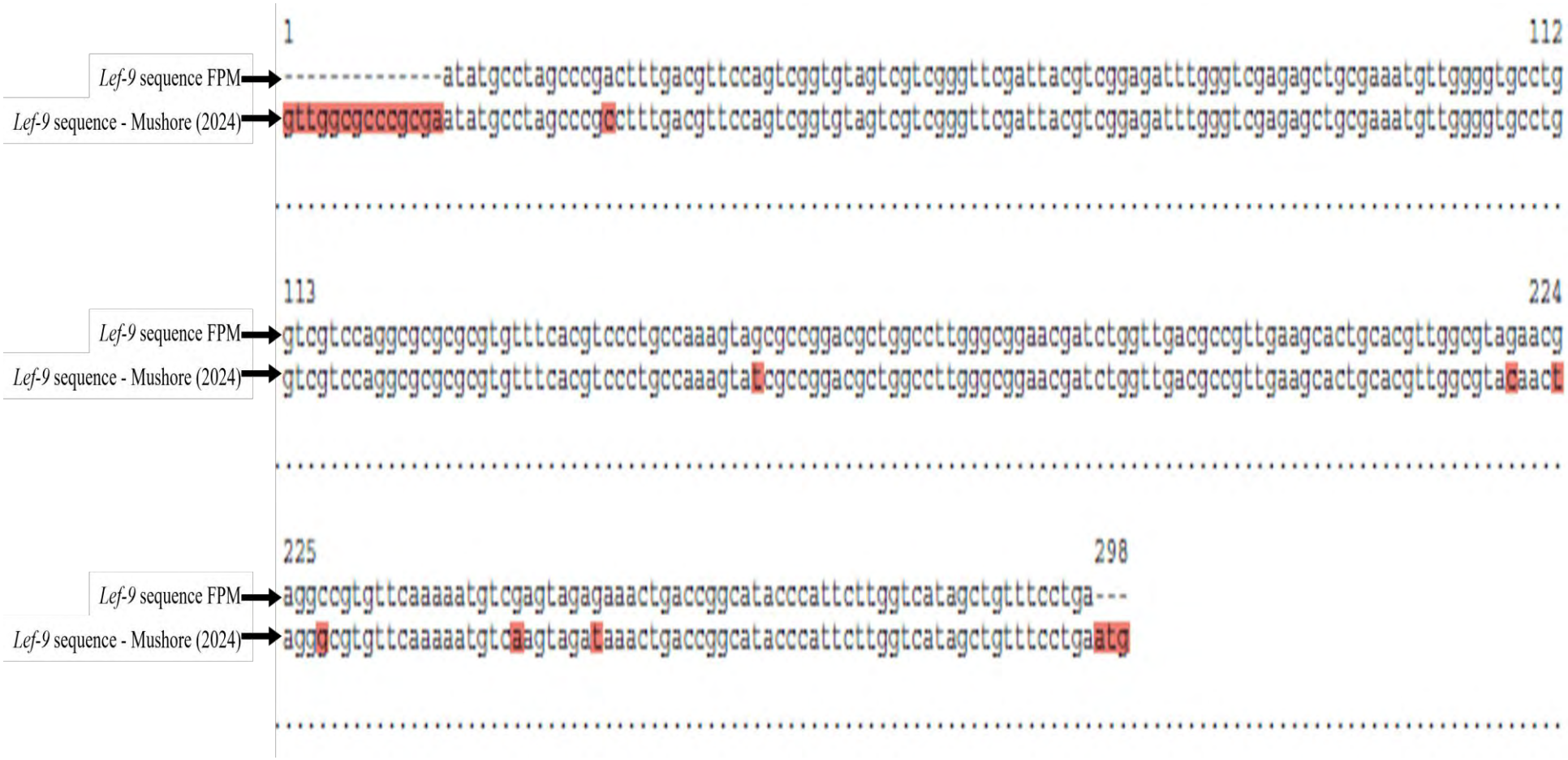

Figure S3: Sequence alignment between the FPM *lef-9* sequence generated in this study and the *lef-9* sequence from Mushore (2024). The sequence mismatches are highlighted in red.

Table S1: K-2-P distance matrix results

Agrotis_segetum_NP	Agrotis_ipsilon_MNP	Adoxophyes_orana_N	Adoxophyes_honmai_	SepaNPV	
0,50921	0,44852	0,63362	0,63218		SepaNPV
0,55321	0,54793	0,05197		0,02669	Adoxophyes_honmai_NPV
0,55351	0,56717		0,00369	0,02709	Adoxophyes_orana_NPV
0,20083		0,02337	0,02300	0,01739	Agrotis_ipsilon_MNPV
	0,00911	0,02327	0,02354	0,01992	Agrotis_segetum_NPV-A
0,01029	0,00562	0,02314	0,02302	0,01953	Agrotis_segetum_NPV-B
0,02342	0,02163	0,03178	0,03160	0,02246	Antheraea_pernyi_NPV
0,02365	0,02531	0,02592	0,02723	0,02304	Anticarsia_gemmatalis_MNPV
0,02367	0,02527	0,02598	0,02731	0,02305	Anticarsia_gemmatalis_NPV
0,02123	0,02331	0,01846	0,01851	0,02584	Apocheima_cinerarium_NPV
0,06687	0,06943	0,06352	0,06643	0,07535	Autographa_californica_NPV
0,02421	0,02528	0,02428	0,02439	0,02502	Bombyx_mandarina_NPV
0,06708	0,07065	0,06209	0,06685	0,07381	Bombyx_mori_NPV
0,02050	0,02070	0,01976	0,01991	0,02279	Buzura_suppressaria_NPV
0,02281	0,02352	0,02385	0,02472	0,02487	Catopsilia_pomona_NPV
0,02317	0,02424	0,02616	0,02672	0,02263	Choristoneura_fumiferana_DEF_MN
0,02197	0,02125	0,02954	0,02934	0,02053	Choristoneura_fumiferana_MNPV
0,02131	0,02053	0,03033	0,03046	0,02028	Choristoneura_murinana_NPV
0,02254	0,02202	0,02896	0,02942	0,02067	Choristoneura_rosaceana_NPV
0,01886	0,01852	0,02168	0,02161	0,02411	Chrysodeixis_chalcites_NPV
0,02372	0,02544	0,02152	0,02211	0,02538	Clanis_bilineata_NPV
0,02336	0,02623	0,02512	0,02630	0,02426	Condylorrhiza_vestigialis_MNPV
0,02141	0,02129	0,01754	0,01748	0,02273	Cryptophlebia_peltastica_NPV
0,22368	0,17309	0,27071	0,28210	0,18010	Culex_nigripalpus_NPV
0,02386	0,02393	0,02525	0,02479	0,02438	Cyclophragma_undans_NPV
0,03894	0,03735	0,04709	0,04769	0,04158	Cydia_pomonella_GV
0,02259	0,02092	0,02880	0,02923	0,01998	Dione_juno_NPV
0,01977	0,02059	0,01971	0,02002	0,02345	Ecotropis_obliqua_NPV
0,17758	0,16970	0,12927	0,13445	0,17880	Epiphyas_postvittana_NPV
0,01908	0,01987	0,02061	0,02046	0,02046	Euproctis_digramma_NPV
0,02077	0,02148	0,02128	0,02132	0,02107	Euproctis_pseudoconspersa_NPV
0,07720	0,08074	0,07143	0,07187	0,08095	Helicoverpa_armigera_NPV
0,02011	0,01918	0,02210	0,02140	0,02095	Hemileuca_sp._NPV
0,02381	0,02426	0,02751	0,02708	0,02347	Hyphantria_cunea_NPV
0,01849	0,01860	0,02063	0,02020	0,01989	Hyposidra_talaca_NPV
0,01905	0,01837	0,02084	0,02067	0,01937	Lambdina_fiscellaria_NPV
0,01693	0,01556	0,02695	0,02559	0,01816	Leucania_separata_NPV
0,05237	0,05023	0,04732	0,04775	0,05337	Lonomia_obliqua_MNPV
0,06410	0,05681	0,09095	0,09067	0,05273	Lymantria_dispar_MNPV
0,01879	0,01666	0,02839	0,02781	0,01713	Lymantria_xylina_MNPV
0,01931	0,01891	0,02100	0,02087	0,02151	Malacosoma_neustria_NPV
0,01401	0,01370	0,02034	0,02074	0,02269	Mamestra_brassicae_MNPV
0,02871	0,02822	0,04021	0,04131	0,04205	Mamestra_configurata_NPV-A
0,01403	0,01357	0,02037	0,02077	0,02238	Mamestra_configurata_NPV-B
0,02525	0,02487	0,02290	0,02342	0,02550	Maruca_vitrata_MNPV
0,01332	0,01149	0,02459	0,02413	0,01900	Mythimna_unipuncta_NPV
0,11281	0,13653	0,07376	0,07694	0,13408	Neodiprion_lecontei_NPV
0,02338	0,02332	0,02526	0,02525	0,02640	Operophtera_brumata_NPV
0,01882	0,01856	0,02091	0,02071	0,02090	Orgyia_leucostigma_NPV
0,07302	0,07142	0,08740	0,08861	0,06330	Orgyia_pseudotsugata_MNPV
0,03186	0,03482	0,02241	0,02357	0,03444	Oxyplax_ochracea_NPV
0,01074	0,00965	0,02444	0,02324	0,01701	Peridroma_sp._NPV
0,01977	0,02012	0,02104	0,02011	0,02248	Perigonia_lusca_SNPV
0,02360	0,02375	0,02467	0,02429	0,02550	Plutella_xylostella_MNPV
0,02255	0,02199	0,02322	0,02370	0,02660	Rachiplusia_nu_NPV
0,01126	0,01011	0,02461	0,02390	0,01927	Spodoptera_eridania_NPV
0,01269	0,01228	0,02201	0,02223	0,02128	Spodoptera_exempta_NPV
0,02712	0,02630	0,04424	0,04451	0,03975	Spodoptera_exigua_NPV
0,01330	0,01382	0,02207	0,02243	0,02341	Spodoptera_frugiperda_MNPV
0,02200	0,02089	0,02621	0,02567	0,02206	Spodoptera_litura_NPV
0,01145	0,01041	0,02415	0,02362	0,01976	Spodoptera_litura_NPV_II
0,02183	0,02155	0,02111	0,02098	0,02395	Sucra_jujuba_NPVT

Autographa_californic	Apocheima_cinerariu	Anticarsia_gemmatali	Anticarsia_gemmatali	Antheraea_pernyi_NP	Agrotis_segetum_NP	
1,48580	0,62687	0,59352	0,59480	0,57655	0,49754	SepaNPV
1,36094	0,44562	0,66619	0,66351	0,73604	0,55407	Adoxophyes_honmai_NPV
1,31216	0,44097	0,65132	0,64965	0,74550	0,56236	Adoxophyes_orana_NPV
1,43211	0,55488	0,63031	0,62870	0,54683	0,09348	Agrotis_ipsilon_MNPV
1,39182	0,52012	0,60039	0,59885	0,57129	0,22868	Agrotis_segetum_NPV-A
1,42757	0,53762	0,63221	0,63240	0,57476		Agrotis_segetum_NPV-B
1,37212	0,71831	0,28336	0,28246		0,02338	Antheraea_pernyi_NPV
1,21803	0,62610	0,00273		0,01272	0,02570	Anticarsia_gemmatalis_MNPV
1,22928	0,62318		0,00081	0,01277	0,02564	Anticarsia_gemmatalis_NPV
1,26369		0,02400	0,02415	0,03013	0,02160	Apocheima_cinerarium_NPV
	0,05812	0,05604	0,05553	0,06800	0,06950	Autographa_californica_NPV
0,03093	0,02232	0,01617	0,01614	0,01941	0,02546	Bombyx_mandarina_NPV
0,00446	0,06203	0,05007	0,04959	0,06309	0,06854	Bombyx_mori_NPV
0,06476	0,01609	0,02349	0,02362	0,02660	0,02007	Buzura_suppressaria_NPV
0,05099	0,02134	0,01749	0,01754	0,02014	0,02309	Catopsilia_pomona_NPV
0,05714	0,02642	0,00416	0,00417	0,01275	0,02481	Choristoneura_fumiferana_DEF_MN
0,06470	0,02962	0,01256	0,01263	0,01192	0,02252	Choristoneura_fumiferana_MNPV
0,06280	0,02942	0,01202	0,01197	0,01163	0,02160	Choristoneura_murinana_NPV
0,06258	0,02895	0,01267	0,01255	0,01173	0,02361	Choristoneura_rosaceana_NPV
0,05834	0,01852	0,02459	0,02463	0,02794	0,01887	Chrysodeixis_chalcites_NPV
0,07367	0,02218	0,02713	0,02703	0,02913	0,02479	Clanis_bilineata_NPV
0,05405	0,02414	0,00537	0,00532	0,01330	0,02614	Condylorrhiza_vestigialis_MNPV
0,05593	0,01755	0,02459	0,02455	0,02723	0,02135	Cryptophlebia_peltastica_NPV
0,70250	0,33152	0,18356	0,18333	0,17784	0,19311	Culex_nigripalpus_NPV
0,05547	0,02437	0,01840	0,01839	0,01760	0,02342	Cyclophragma_undans_NPV
0,12346	0,04698	0,04266	0,04254	0,04483	0,03976	Cydia_pomonella_GV
0,06010	0,02725	0,01152	0,01160	0,01202	0,02303	Dione_juno_NPV
0,06400	0,01600	0,02568	0,02570	0,02703	0,01992	Ecotropis_obliqua_NPV
0,02726	0,14395	0,09142	0,09127	0,13807	0,16181	Epiphyas_postvittana_NPV
0,06457	0,01655	0,02341	0,02356	0,02559	0,02001	Euproctis_digramma_NPV
0,07416	0,01688	0,02434	0,02436	0,02525	0,02154	Euproctis_pseudoconspersa_NPV
0,02658	0,06597	0,07622	0,07694	0,10333	0,07320	Helicoverpa_armigera_NPV
0,06311	0,01747	0,02508	0,02528	0,02712	0,01968	Hemileuca_sp._NPV
0,05882	0,02529	0,01114	0,01108	0,01327	0,02410	Hyphantria_cunea_NPV
0,06241	0,01654	0,02325	0,02332	0,02455	0,01813	Hyposidra_talaca_NPV
0,06540	0,01842	0,02132	0,02136	0,02301	0,01848	Lambdina_fiscellaria_NPV
0,07692	0,02459	0,02538	0,02548	0,02350	0,01689	Leucania_separata_NPV
0,18138	0,04096	0,03878	0,03842	0,04320	0,04949	Lonomia_obliqua_MNPV
0,02907	0,10841	0,07546	0,07544	0,07187	0,05977	Lymantria_dispar_MNPV
0,07704	0,02702	0,02538	0,02539	0,02179	0,01873	Lymantria_xylina_MNPV
0,05984	0,01858	0,02486	0,02511	0,02933	0,01941	Malacosoma_neustria_NPV
0,05854	0,01937	0,02615	0,02613	0,02638	0,01466	Mamestra_brassicae_MNPV
0,15235	0,03903	0,04715	0,04675	0,05143	0,03048	Mamestra_configurata_NPV-A
0,05802	0,01942	0,02623	0,02620	0,02627	0,01467	Mamestra_configurata_NPV-B
0,03830	0,02226	0,01685	0,01694	0,02034	0,02577	Maruca_vitrata_MNPV
0,06283	0,02223	0,02512	0,02509	0,02228	0,01293	Mythimna_unipuncta_NPV
0,20676	0,07552	0,09903	0,09858	0,15272	0,12279	Neodiprion_lecontei_NPV
0,07852	0,02125	0,03234	0,03215	0,03004	0,02284	Operophtera_brumata_NPV
0,06215	0,01538	0,02353	0,02373	0,02441	0,01877	Orgyia_leucostigma_NPV
0,02039	0,09137	0,05229	0,05249	0,05162	0,07535	Orgyia_pseudotsugata_MNPV
0,05708	0,02292	0,02131	0,02135	0,02541	0,03179	Oxyplax_ochracea_NPV
0,06635	0,02363	0,02401	0,02415	0,02048	0,01113	Peridroma_sp._NPV
0,06040	0,01683	0,02337	0,02341	0,02530	0,02036	Perigonia_lusca_SNPV
0,02497	0,02178	0,01696	0,01697	0,01971	0,02470	Plutella_xylostella_MNPV
0,06196	0,02181	0,02686	0,02674	0,03138	0,02353	Rachiplusia_nu_NPV
0,06538	0,02252	0,02462	0,02474	0,02309	0,01089	Spodoptera_eridania_NPV
0,05835	0,01969	0,02496	0,02494	0,02582	0,01276	Spodoptera_exempta_NPV
0,18062	0,04133	0,04675	0,04657	0,04217	0,02748	Spodoptera_exigua_NPV
0,05961	0,01937	0,02567	0,02560	0,02501	0,01395	Spodoptera_frugiperda_MNPV
0,06952	0,02286	0,02769	0,02779	0,02717	0,02170	Spodoptera_litura_NPV
0,06550	0,02248	0,02432	0,02442	0,02313	0,01110	Spodoptera_litura_NPV_II
0,05932	0,01637	0,02554	0,02577	0,02785	0,02094	Sucra_jujuba_NPVT

Choristoneura_fumife	Choristoneura_fumife	Catopsilia_pomona_N	Buzura_suppressaria_	Bombyx_mori_NPV	Bombyx_mandarina_	
0,53613	0,57870	0,60755	0,56861	1,49363	0,63343	SepaNPV
0,68470	0,66190	0,60256	0,48105	1,38938	0,61420	Adoxophyes_honmai_NPV
0,69697	0,65839	0,59450	0,47296	1,32498	0,60626	Adoxophyes_orana_NPV
0,55624	0,61005	0,58670	0,51994	1,46364	0,59853	Agrotis_ipsilon_MNPV
0,56910	0,58819	0,57919	0,51259	1,41333	0,58825	Agrotis_segetum_NPV-A
0,57801	0,61618	0,57805	0,50701	1,42836	0,60218	Agrotis_segetum_NPV-B
0,26953	0,27192	0,48375	0,66101	1,29564	0,45959	Antheraea_pernyi_NPV
0,28727	0,06102	0,43921	0,60078	1,15035	0,40127	Anticarsia_gemmatalis_MNPV
0,28668	0,06156	0,43802	0,59889	1,16065	0,40177	Anticarsia_gemmatalis_NPV
0,71966	0,65972	0,54926	0,39509	1,35189	0,56334	Apocheima_cinerarium_NPV
1,32953	1,24581	1,16003	1,33097	0,07279	0,77691	Autographa_californica_NPV
0,44410	0,40567	0,31392	0,58029	0,58978		Bombyx_mandarina_NPV
1,27575	1,15433	1,11761	1,37834		0,02278	Bombyx_mori_NPV
0,62785	0,62207	0,55237		0,06721	0,02330	Buzura_suppressaria_NPV
0,44177	0,43962		0,02211	0,04862	0,01325	Catopsilia_pomona_NPV
0,26406		0,01771	0,02521	0,05007	0,01610	Choristoneura_fumiferana_DEF_MN
	0,01161	0,01803	0,02479	0,06099	0,01877	Choristoneura_fumiferana_MNPV
0,00525	0,01149	0,01903	0,02624	0,06049	0,01924	Choristoneura_murinana_NPV
0,00618	0,01156	0,01844	0,02454	0,05700	0,01840	Choristoneura_rosaceana_NPV
0,02671	0,02553	0,02252	0,02033	0,05940	0,02199	Chrysodeixis_chalcites_NPV
0,03013	0,02801	0,02519	0,02243	0,06958	0,02629	Clanis_bilineata_NPV
0,01305	0,00535	0,01686	0,02361	0,04881	0,01590	Condylorrhiza_vestigialis_MNPV
0,02582	0,02488	0,02039	0,01855	0,05633	0,02117	Cryptophlebia_peltastica_NPV
0,18250	0,18117	0,22222	0,24068	0,65350	0,22866	Culex_nigripalpus_NPV
0,01778	0,01771	0,01368	0,02484	0,05040	0,01473	Cyclophragma_undans_NPV
0,04370	0,04185	0,04119	0,04307	0,12129	0,04438	Cydia_pomonella_GV
0,00955	0,01164	0,01631	0,02448	0,05603	0,01769	Dione_juno_NPV
0,02565	0,02589	0,01922	0,01626	0,06094	0,02228	Ecotropis_obliqua_NPV
0,11444	0,08780	0,12900	0,12013	0,02744	0,09953	Epiphyas_postvittana_NPV
0,02244	0,02392	0,02059	0,01583	0,06513	0,02205	Euproctis_digramma_NPV
0,02363	0,02519	0,02117	0,01566	0,06944	0,02306	Euproctis_pseudoconspersa_NPV
0,09103	0,07831	0,07248	0,06708	0,02522	0,07105	Helicoverpa_armigera_NPV
0,02634	0,02582	0,02288	0,01730	0,06536	0,02420	Hemileuca_sp._NPV
0,01074	0,01127	0,01788	0,02427	0,05478	0,01626	Hyphantria_cunea_NPV
0,02325	0,02446	0,02024	0,00903	0,06124	0,02217	Hyposidra_talaca_NPV
0,02264	0,02134	0,02001	0,01758	0,06255	0,02072	Lambdina_fiscellaria_NPV
0,02450	0,02478	0,02525	0,02428	0,07086	0,02443	Leucania_separata_NPV
0,04228	0,03956	0,03169	0,04399	0,16892	0,03384	Lonomia_obliqua_MNPV
0,06571	0,07037	0,08615	0,09594	0,02905	0,07768	Lymantria_dispar_MNPV
0,02075	0,02510	0,02560	0,02375	0,07138	0,02544	Lymantria_xylina_MNPV
0,02593	0,02517	0,02118	0,01907	0,06072	0,02262	Malacosoma_neustria_NPV
0,02530	0,02621	0,02151	0,01903	0,06143	0,02248	Mamestra_brassicae_MNPV
0,04869	0,04998	0,04032	0,03850	0,16409	0,04200	Mamestra_configurata_NPV-A
0,02542	0,02635	0,02163	0,01898	0,06117	0,02267	Mamestra_configurata_NPV-B
0,01920	0,01725	0,01336	0,02327	0,03201	0,00643	Maruca_vitrata_MNPV
0,02267	0,02474	0,02243	0,02030	0,06503	0,02332	Mythimna_unipuncta_NPV
0,14435	0,10863	0,07788	0,08910	0,20104	0,08978	Neodiprion_lecontei_NPV
0,03218	0,03179	0,02736	0,02368	0,08534	0,02789	Operophtera_brumata_NPV
0,02325	0,02460	0,02137	0,01553	0,06106	0,02216	Orgyia_leucostigma_NPV
0,04499	0,04667	0,06943	0,08436	0,02013	0,06503	Orgyia_pseudotsugata_MNPV
0,02418	0,02148	0,01632	0,02309	0,05194	0,01594	Oxyplax_ochracea_NPV
0,02034	0,02332	0,02421	0,02126	0,06729	0,02413	Peridroma_sp._NPV
0,02409	0,02403	0,02060	0,01796	0,06437	0,02279	Perigonia_lusca_SNPV
0,01910	0,01727	0,01384	0,02262	0,02969	0,00421	Plutella_xylostella_MNPV
0,02773	0,02749	0,02523	0,02304	0,06339	0,02437	Rachiplusia_nu_NPV
0,02182	0,02326	0,02281	0,02106	0,06368	0,02430	Spodoptera_eridania_NPV
0,02436	0,02536	0,02101	0,01902	0,05908	0,02196	Spodoptera_exempta_NPV
0,04532	0,04724	0,04267	0,04041	0,17943	0,04483	Spodoptera_exigua_NPV
0,02525	0,02485	0,02234	0,01969	0,06070	0,02398	Spodoptera_frugiperda_MNPV
0,02630	0,02783	0,02324	0,02415	0,07105	0,02405	Spodoptera_litura_NPV
0,02233	0,02297	0,02260	0,02135	0,06429	0,02390	Spodoptera_litura_NPV_II
0,02654	0,02592	0,02110	0,01527	0,06167	0,02271	Sucra_jujuba_NPVT

Cryptophlebia_peltast	Condylorrhiza_vestigi	Clanis_bilineata_NPV	Chrysodeixis_chalcite	Choristoneura_rosace	Choristoneura_murina	
0,56420	0,59935	0,60548	0,58341	0,53625	0,52068	SepaNPV
0,42828	0,64742	0,54088	0,51407	0,67928	0,69988	Adoxophyes_honmai_NPV
0,43201	0,62985	0,52266	0,51838	0,68742	0,70354	Adoxophyes_orana_NPV
0,51043	0,64030	0,58247	0,45870	0,55567	0,53679	Agrotis_ipsilon_MNPV
0,51806	0,59393	0,56598	0,45709	0,57290	0,55941	Agrotis_segetum_NPV-A
0,50681	0,64102	0,57898	0,47361	0,58501	0,56579	Agrotis_segetum_NPV-B
0,67843	0,29171	0,69328	0,68705	0,26814	0,26112	Antheraea_pernyi_NPV
0,62552	0,09114	0,66314	0,62650	0,27859	0,27236	Anticarsia_gemmatalis_MNPV
0,62802	0,09173	0,66465	0,62400	0,28096	0,27373	Anticarsia_gemmatalis_NPV
0,43589	0,61524	0,50832	0,45747	0,70137	0,71412	Apocheima_cinerarium_NPV
1,22491	1,19650	1,45853	1,27041	1,31493	1,31048	Autographa_californica_NPV
0,53932	0,39792	0,62170	0,57352	0,44818	0,46901	Bombyx_mandarina_NPV
1,25375	1,12793	1,42755	1,31420	1,24277	1,29807	Bombyx_mori_NPV
0,45561	0,59650	0,51361	0,50603	0,61502	0,64286	Buzura_suppressaria_NPV
0,54906	0,41704	0,62445	0,58682	0,44609	0,46593	Catopsilia_pomona_NPV
0,62965	0,08886	0,66945	0,65262	0,24996	0,25584	Choristoneura_fumiferana_DEF_MN
0,64729	0,29237	0,71078	0,67876	0,11334	0,08938	Choristoneura_fumiferana_MNPV
0,64998	0,27996	0,72148	0,68412	0,12096		Choristoneura_murinana_NPV
0,65494	0,27568	0,68391	0,65994		0,00674	Choristoneura_rosaceana_NPV
0,46458	0,60258	0,50705		0,02587	0,02749	Chrysodeixis_chalcites_NPV
0,49523	0,64534		0,02117	0,02887	0,03066	Clanis_bilineata_NPV
0,60847		0,02632	0,02412	0,01275	0,01229	Condylorrhiza_vestigialis_MNPV
	0,02390	0,02048	0,01833	0,02542	0,02603	Cryptophlebia_peltastica_NPV
0,27169	0,19096	0,23216	0,27295	0,18297	0,18027	Culex_nigripalpus_NPV
0,02367	0,01773	0,02792	0,02523	0,01770	0,01949	Cyclophragma_undans_NPV
0,04284	0,04407	0,04625	0,04223	0,04333	0,04396	Cydia_pomonella_GV
0,02627	0,01185	0,03017	0,02490	0,00948	0,00969	Dione_juno_NPV
0,01770	0,02358	0,02115	0,01914	0,02658	0,02570	Ecotropis_obliqua_NPV
0,12108	0,09176	0,15663	0,13264	0,10434	0,12412	Epiphyas_postvittana_NPV
0,01828	0,02237	0,02091	0,01961	0,02329	0,02416	Euproctis_digramma_NPV
0,01921	0,02398	0,02101	0,02099	0,02323	0,02344	Euproctis_pseudoconspersa_NPV
0,06405	0,07666	0,07137	0,06559	0,08644	0,09205	Helicoverpa_armigera_NPV
0,01896	0,02484	0,02240	0,02000	0,02484	0,02488	Hemileuca_sp._NPV
0,02543	0,01133	0,02839	0,02696	0,01084	0,01090	Hyphantria_cunea_NPV
0,01833	0,02245	0,02053	0,01960	0,02358	0,02376	Hyposidra_talaca_NPV
0,01850	0,02116	0,02002	0,02036	0,02316	0,02266	Lambdina_fiscellaria_NPV
0,02422	0,02690	0,02711	0,02252	0,02448	0,02396	Leucania_separata_NPV
0,04617	0,03684	0,05032	0,04592	0,04249	0,04275	Lonomia_obliqua_MNPV
0,09264	0,07584	0,09665	0,08151	0,06236	0,06371	Lymantria_dispar_MNPV
0,02298	0,02559	0,02619	0,02467	0,02260	0,02190	Lymantria_xylina_MNPV
0,01812	0,02435	0,02047	0,01818	0,02472	0,02698	Malacosoma_neustria_NPV
0,01902	0,02600	0,02254	0,01802	0,02575	0,02623	Mamestra_brassicae_MNPV
0,03593	0,04909	0,04168	0,03386	0,04612	0,04906	Mamestra_configurata_NPV-A
0,01897	0,02604	0,02242	0,01803	0,02574	0,02599	Mamestra_configurata_NPV-B
0,02266	0,01767	0,02606	0,02320	0,01917	0,02019	Maruca_vitrata_MNPV
0,02043	0,02537	0,02389	0,01848	0,02270	0,02220	Mythimna_unipuncta_NPV
0,07736	0,10040	0,07940	0,09400	0,13607	0,15294	Neodiprion_lecontei_NPV
0,02121	0,03099	0,02505	0,02321	0,03136	0,03257	Operophtera_brumata_NPV
0,01877	0,02289	0,02172	0,02163	0,02377	0,02441	Orgyia_leucostigma_NPV
0,08696	0,05041	0,09772	0,08169	0,04017	0,04245	Orgyia_pseudotsugata_MNPV
0,02197	0,01994	0,02814	0,02577	0,02476	0,02537	Oxyplax_ochracea_NPV
0,02135	0,02430	0,02289	0,01810	0,02084	0,01931	Peridroma_sp._NPV
0,01722	0,02324	0,01819	0,01753	0,02403	0,02458	Perigonia_lusca_SNPV
0,02104	0,01648	0,02623	0,02083	0,01903	0,01915	Plutella_xylostella_MNPV
0,02166	0,02589	0,02303	0,01868	0,02913	0,02959	Rachiplusia_nu_NPV
0,02138	0,02507	0,02437	0,01945	0,02188	0,02141	Spodoptera_eridania_NPV
0,01948	0,02506	0,02190	0,01660	0,02500	0,02475	Spodoptera_exempta_NPV
0,04143	0,05092	0,04360	0,03994	0,04473	0,04674	Spodoptera_exigua_NPV
0,01984	0,02438	0,02337	0,01713	0,02473	0,02517	Spodoptera_frugiperda_MNPV
0,02263	0,02688	0,02629	0,02382	0,02721	0,02717	Spodoptera_litura_NPV
0,02068	0,02492	0,02408	0,01909	0,02249	0,02226	Spodoptera_litura_NPV_II
0,01803	0,02381	0,02163	0,02090	0,02566	0,02725	Sucra_jujuba_NPVT

Epiphyas_postvittana	Ecotropis_obliqua_N	Dione_juno_NPV	Cydia_pomonella_GV	Cyclophragma_undan	Culex_nigripalpus_N	
2,52240	0,56874	0,51697	0,96884	0,58564	2,46075	SepaNPV
2,16751	0,49606	0,67800	1,05496	0,61125	3,19948	Adoxophyes_honmai_NPV
2,11753	0,48489	0,67956	1,04068	0,62496	3,13600	Adoxophyes_orana_NPV
2,41245	0,50304	0,52422	0,89658	0,58503	2,43596	Agrotis_ipsilon_MNPV
2,43213	0,48739	0,56940	0,92268	0,59149	2,77406	Agrotis_segetum_NPV-A
2,35647	0,49970	0,56541	0,92380	0,57786	2,55089	Agrotis_segetum_NPV-B
2,09724	0,66198	0,27560	1,01023	0,43256	2,42057	Antheraea_pernyi_NPV
1,66663	0,62911	0,26215	0,99098	0,43829	2,54128	Anticarsia_gemmatalis_MNPV
1,66767	0,63023	0,26019	0,99067	0,43641	2,54376	Anticarsia_gemmatalis_NPV
2,23146	0,39789	0,66987	1,05992	0,62610	3,32712	Apocheima_cinerarium_NPV
0,64718	1,30763	1,26624	2,11671	1,23825	5,38971	Autographa_californica_NPV
1,82405	0,55867	0,42373	1,01135	0,34812	2,76104	Bombyx_mandarina_NPV
0,64815	1,30330	1,20446	2,11304	1,14960	5,18821	Bombyx_mori_NPV
2,05184	0,39703	0,59547	0,98663	0,60838	2,85658	Buzura_suppressaria_NPV
2,11651	0,50923	0,40112	0,94585	0,33384	2,77579	Catopsilia_pomona_NPV
1,65722	0,62903	0,26005	0,97513	0,42194	2,50541	Choristoneura_fumiferana_DEF_MN
1,90048	0,64131	0,20641	1,01221	0,44320	2,46547	Choristoneura_fumiferana_MNPV
1,96244	0,64545	0,20971	1,00162	0,46780	2,46805	Choristoneura_murinana_NPV
1,82030	0,65155	0,19869	0,98576	0,44002	2,51507	Choristoneura_rosaceana_NPV
2,17888	0,48474	0,63750	0,97222	0,63503	3,10874	Chrysodeixis_chalcites_NPV
2,38047	0,50744	0,70327	1,03169	0,67029	2,89307	Clanis_bilineata_NPV
1,66469	0,59616	0,25886	1,00844	0,42728	2,57601	Condylorrhiza_vestigialis_MNPV
2,07467	0,44112	0,64519	0,99401	0,60573	3,06998	Cryptophlebia_peltastica_NPV
5,79416	2,96324	2,27392	3,19436	2,68193		Culex_nigripalpus_NPV
2,02221	0,57775	0,41152	0,99501		0,21059	Cyclophragma_undans_NPV
3,15057	0,94301	0,94466		0,04337	0,27320	Cydia_pomonella_GV
1,91400	0,62282		0,04079	0,01653	0,15321	Dione_juno_NPV
2,08701		0,02549	0,03996	0,02349	0,25983	Ecotropis_obliqua_NPV
	0,13256	0,11352	0,25167	0,11621	0,87065	Epiphyas_postvittana_NPV
0,13365	0,01581	0,02099	0,03969	0,02178	0,21933	Euproctis_digramma_NPV
0,14146	0,01576	0,02341	0,04394	0,02221	0,21283	Euproctis_pseudoconspersa_NPV
0,03991	0,06829	0,09223	0,11855	0,07660	1,46348	Helicoverpa_armigera_NPV
0,13961	0,01656	0,02481	0,04719	0,02524	0,22074	Hemileuca_sp._NPV
0,10013	0,02451	0,01046	0,04405	0,01779	0,18118	Hyphantria_cunea_NPV
0,12355	0,01574	0,02090	0,04018	0,02369	0,20728	Hyposidra_talaca_NPV
0,14190	0,01630	0,02132	0,03738	0,02090	0,18889	Lambdina_fiscellaria_NPV
0,15736	0,02255	0,02086	0,03835	0,02438	0,17265	Leucania_separata_NPV
0,95716	0,04366	0,04112	0,06777	0,03542	0,61377	Lonomia_obliqua_MNPV
0,04407	0,08433	0,06252	0,12484	0,07724	0,56091	Lymantria_dispar_MNPV
0,17243	0,02348	0,02082	0,03923	0,02356	0,17685	Lymantria_xylina_MNPV
0,13175	0,01822	0,02293	0,04226	0,02306	0,22201	Malacosoma_neustria_NPV
0,13140	0,01883	0,02432	0,04150	0,02566	0,22986	Mamestra_brassicae_MNPV
0,85256	0,03622	0,04611	0,05568	0,04778	0,41975	Mamestra_configurata_NPV-A
0,13377	0,01863	0,02416	0,04118	0,02577	0,23241	Mamestra_configurata_NPV-B
0,10304	0,02309	0,01739	0,04487	0,01486	0,21770	Maruca_vitrata_MNPV
0,15298	0,01991	0,02111	0,03786	0,02463	0,16970	Mythimna_unipuncta_NPV
0,42160	0,07614	0,14140	0,13089	0,10455	0,58873	Neodiprion_lecontei_NPV
0,18128	0,02273	0,03029	0,03969	0,02752	0,25277	Operophtera_brumata_NPV
0,12849	0,01563	0,02184	0,04520	0,02345	0,23475	Orgyia_leucostigma_NPV
0,02226	0,08894	0,04362	0,12074	0,06447	0,81680	Orgyia_pseudotsugata_MNPV
0,11850	0,02281	0,02439	0,05248	0,01860	0,28863	Oxyplax_ochracea_NPV
0,16987	0,01978	0,01946	0,03769	0,02308	0,14132	Peridroma_sp._NPV
0,13960	0,01923	0,02272	0,04125	0,02323	0,23307	Perigonia_lusca_SNPV
0,10030	0,02218	0,01738	0,04461	0,01535	0,22522	Plutella_xylostella_MNPV
0,15458	0,02289	0,03095	0,04604	0,02839	0,34157	Rachiplusia_nu_NPV
0,15818	0,01993	0,02124	0,03834	0,02351	0,18023	Spodoptera_eridania_NPV
0,12871	0,02025	0,02216	0,03960	0,02392	0,21958	Spodoptera_exempta_NPV
1,28253	0,04001	0,03806	0,05329	0,04735	0,44835	Spodoptera_exigua_NPV
0,14657	0,01971	0,02454	0,03922	0,02526	0,20977	Spodoptera_frugiperda_MNPV
0,14708	0,02159	0,02567	0,04070	0,02402	0,22121	Spodoptera_litura_NPV
0,16469	0,01985	0,02085	0,04030	0,02354	0,17704	Spodoptera_litura_NPV_II
0,12644	0,01613	0,02431	0,04289	0,02364	0,25101	Sucra_jujuba_NPVT

Hyposidra_talaca_NP	Hyphantria_cunea_N	Hemileuca_sp._NPV	Helicoverpa_armigera	Euproctis_pseudocons	Euproctis_digramma_	
0,50822	0,59100	0,54706	1,53970	0,53247	0,50506	SepaNPV
0,49652	0,65722	0,52645	1,43773	0,50134	0,49431	Adoxophyes_honmai_NPV
0,49271	0,66158	0,53824	1,43705	0,49318	0,49210	Adoxophyes_orana_NPV
0,47017	0,60981	0,48366	1,58985	0,51403	0,47649	Agrotis_ipsilon_MNPV
0,46643	0,59660	0,51009	1,50840	0,49061	0,46253	Agrotis_segetum_NPV-A
0,46594	0,60740	0,49095	1,50414	0,50457	0,49173	Agrotis_segetum_NPV-B
0,60707	0,28806	0,65318	1,79787	0,62080	0,61166	Antheraea_pernyi_NPV
0,58911	0,25217	0,63676	1,52396	0,60373	0,58538	Anticarsia_gemmatalis_MNPV
0,58645	0,25209	0,63349	1,52048	0,60133	0,58139	Anticarsia_gemmatalis_NPV
0,40274	0,63743	0,41300	1,36866	0,41474	0,40225	Apocheima_cinerarium_NPV
1,33004	1,27191	1,35923	0,62902	1,45901	1,34911	Autographa_californica_NPV
0,55267	0,40714	0,58516	1,47995	0,57268	0,54568	Bombyx_mandarina_NPV
1,33617	1,21290	1,40902	0,62056	1,43392	1,39164	Bombyx_mori_NPV
0,19285	0,60304	0,41591	1,38609	0,38090	0,38450	Buzura_suppressaria_NPV
0,51924	0,43027	0,57307	1,46752	0,54173	0,52361	Catopsilia_pomona_NPV
0,61413	0,24212	0,62829	1,53845	0,62315	0,59125	Choristoneura_fumiferana_DEF_MN
0,60585	0,22801	0,65997	1,68514	0,59931	0,57497	Choristoneura_fumiferana_MNPV
0,61116	0,22617	0,63384	1,70732	0,61014	0,59684	Choristoneura_murinana_NPV
0,60333	0,22022	0,63086	1,64601	0,58001	0,57917	Choristoneura_rosaceana_NPV
0,49723	0,65661	0,49836	1,35841	0,49645	0,48493	Chrysodeixis_chalcites_NPV
0,49080	0,68226	0,51953	1,43562	0,48913	0,49810	Clanis_bilineata_NPV
0,57873	0,24421	0,62135	1,51623	0,60218	0,56470	Condylorrhiza_vestigialis_MNPV
0,45729	0,63599	0,47512	1,35598	0,45864	0,44488	Cryptophlebia_peltastica_NPV
2,64168	2,53908	2,70398	7,54600	2,72207	2,79717	Culex_nigripalpus_NPV
0,58125	0,43406	0,60231	1,52830	0,55213	0,55507	Cyclophragma_undans_NPV
0,91396	1,01115	1,05955	2,12624	1,00526	0,94468	Cydia_pomonella_GV
0,53870	0,21928	0,61540	1,70231	0,58909	0,54513	Dione_juno_NPV
0,38007	0,60711	0,39937	1,41339	0,38045	0,38900	Ecotropis_obliqua_NPV
2,10651	1,77181	2,25862	0,91711	2,23312	2,15573	Epiphyas_postvittana_NPV
0,36485	0,56477	0,37633	1,39154	0,27576		Euproctis_digramma_NPV
0,36940	0,58921	0,39704	1,44258		0,01221	Euproctis_pseudoconspersa_NPV
1,40963	1,65033	1,45393		0,07211	0,06715	Helicoverpa_armigera_NPV
0,40117	0,62244		0,07059	0,01673	0,01589	Hemileuca_sp._NPV
0,56821		0,02517	0,08774	0,02332	0,02204	Hyphantria_cunea_NPV
	0,02235	0,01661	0,06768	0,01564	0,01532	Hyposidra_talaca_NPV
0,01670	0,02227	0,02044	0,07045	0,01714	0,01609	Lambdina_fiscellaria_NPV
0,02088	0,02566	0,02181	0,07466	0,02117	0,02095	Leucania_separata_NPV
0,04728	0,03854	0,04614	0,21851	0,04494	0,04473	Lonomia_obliqua_MNPV
0,07434	0,06949	0,07539	0,02778	0,08907	0,07129	Lymantria_dispar_MNPV
0,01895	0,02445	0,02180	0,08924	0,02138	0,01930	Lymantria_xylina_MNPV
0,01817	0,02476	0,02004	0,06251	0,01810	0,01708	Malacosoma_neustria_NPV
0,01914	0,02620	0,01941	0,06877	0,01991	0,01836	Mamestra_brassicae_MNPV
0,03783	0,04847	0,03748	0,26250	0,04064	0,03712	Mamestra_configurata_NPV-A
0,01902	0,02660	0,01941	0,06901	0,01995	0,01829	Mamestra_configurata_NPV-B
0,02265	0,01715	0,02366	0,07636	0,02374	0,02159	Maruca_vitrata_MNPV
0,01853	0,02439	0,01958	0,08523	0,02064	0,01933	Mythimna_unipuncta_NPV
0,08436	0,12369	0,09126	0,19880	0,08314	0,09374	Neodiprion_lecontei_NPV
0,02241	0,03125	0,02383	0,08798	0,02336	0,02292	Operophtera_brumata_NPV
0,01523	0,02214	0,01599	0,06983	0,01472	0,01394	Orgyia_leucostigma_NPV
0,07453	0,04520	0,08521	0,03170	0,08653	0,07547	Orgyia_pseudotsugata_MNPV
0,02502	0,02160	0,02661	0,07255	0,02255	0,02343	Oxyplax_ochracea_NPV
0,01888	0,02306	0,01906	0,07768	0,02089	0,01890	Peridroma_sp._NPV
0,01740	0,02439	0,01808	0,06453	0,01761	0,01607	Perigonia_lusca_SNPV
0,02147	0,01689	0,02310	0,07573	0,02419	0,02175	Plutella_xylostella_MNPV
0,02304	0,02867	0,02357	0,07337	0,02514	0,02411	Rachiplusia_nu_NPV
0,02015	0,02331	0,01881	0,07808	0,02164	0,02038	Spodoptera_eridania_NPV
0,01842	0,02524	0,01970	0,06664	0,02018	0,01912	Spodoptera_exempta_NPV
0,04004	0,04339	0,04118	0,25469	0,03760	0,03985	Spodoptera_exigua_NPV
0,01909	0,02528	0,02013	0,07060	0,02182	0,02134	Spodoptera_frugiperda_MNPV
0,02327	0,02758	0,02342	0,07209	0,02301	0,02126	Spodoptera_litura_NPV
0,01993	0,02374	0,01937	0,07882	0,02111	0,02001	Spodoptera_litura_NPV_II
0,01426	0,02445	0,01748	0,06502	0,01734	0,01708	Sucra_jujuba_NPVT

Malacosoma_neustria	Lymantria_xylina_M	Lymantria_dispar_M	Lonomia_obliqua_M	Leucania_separata_N	Lambdina_fiscellaria_	
0,51486	0,43335	1,20228	1,17518	0,47150	0,47607	SepaNPV
0,50650	0,64180	1,66809	1,06327	0,62204	0,50818	Adoxophyes_honmai_NPV
0,51692	0,66345	1,68652	1,06381	0,64658	0,51066	Adoxophyes_orana_NPV
0,47046	0,42416	1,27899	1,09407	0,39576	0,45190	Agrotis_ipsilon_MNPV
0,48439	0,45520	1,36190	1,16020	0,43149	0,44684	Agrotis_segetum_NPV-A
0,48926	0,47875	1,32794	1,07467	0,44075	0,45204	Agrotis_segetum_NPV-B
0,68119	0,55631	1,46279	0,98344	0,57690	0,58002	Antheraea_pernyi_NPV
0,60831	0,64425	1,50889	0,89801	0,63531	0,55271	Anticarsia_gemmatalis_MNPV
0,60546	0,64407	1,51347	0,90308	0,63277	0,55099	Anticarsia_gemmatalis_NPV
0,45534	0,61857	1,86913	1,00557	0,60152	0,45173	Apocheima_cinerarium_NPV
1,28508	1,52155	0,66773	2,69962	1,48345	1,37278	Autographa_californica_NPV
0,55618	0,62317	1,53906	0,79359	0,63233	0,54518	Bombyx_mandarina_NPV
1,30844	1,48295	0,67548	2,55507	1,44252	1,36486	Bombyx_mori_NPV
0,47733	0,56776	1,70007	1,02440	0,60252	0,43177	Buzura_suppressaria_NPV
0,54308	0,61087	1,66626	0,77632	0,64027	0,51695	Catopsilia_pomona_NPV
0,61265	0,63532	1,46485	0,91058	0,61761	0,55622	Choristoneura_fumiferana_DEF_MN
0,63793	0,56013	1,40954	0,95111	0,60860	0,57686	Choristoneura_fumiferana_MNPV
0,64487	0,56873	1,37443	0,96536	0,60468	0,57795	Choristoneura_murinana_NPV
0,60756	0,56180	1,34768	0,94729	0,59850	0,58078	Choristoneura_rosaceana_NPV
0,46830	0,60293	1,59971	1,04350	0,55587	0,48814	Chrysodeixis_chalcites_NPV
0,49326	0,62429	1,74261	1,08846	0,62988	0,48416	Clanis_bilineata_NPV
0,60034	0,64445	1,50809	0,86338	0,64945	0,55021	Condylorrhiza_vestigialis_MNPV
0,45162	0,57620	1,69012	1,02954	0,58801	0,45562	Cryptophlebia_peltastica_NPV
2,75058	2,46604	4,72391	4,91799	2,49173	2,52395	Culex_nigripalpus_NPV
0,57481	0,58150	1,55010	0,83733	0,62778	0,53992	Cyclophragma_undans_NPV
0,94867	0,91986	2,17333	1,37837	0,89401	0,87997	Cydia_pomonella_GV
0,56198	0,52328	1,36089	0,94837	0,52563	0,52978	Dione_juno_NPV
0,45000	0,57027	1,58373	1,02449	0,55431	0,40144	Ecotropis_obliqua_NPV
2,19094	2,49299	0,94417	6,30088	2,41111	2,22784	Epiphyas_postvittana_NPV
0,42150	0,47102	1,47492	1,04445	0,52132	0,37818	Euproctis_digramma_NPV
0,43524	0,51315	1,65387	1,02841	0,53247	0,40774	Euproctis_pseudoconspersa_NPV
1,30765	1,68938	0,65931	3,03626	1,50633	1,44402	Helicoverpa_armigera_NPV
0,48413	0,54316	1,51307	1,04188	0,56312	0,47466	Hemileuca_sp._NPV
0,60488	0,60047	1,44119	0,89400	0,62981	0,56258	Hyphantria_cunea_NPV
0,44985	0,47357	1,48921	1,06450	0,53233	0,40768	Hyposidra_talaca_NPV
0,41807	0,47366	1,39119	1,07319	0,50878		Lambdina_fiscellaria_NPV
0,52950	0,47946	1,34286	1,15384		0,02015	Leucania_separata_NPV
1,07596	1,19907	4,33417		0,05279	0,04550	Lonomia_obliqua_MNPV
1,45625	0,79921		0,43900	0,06122	0,06773	Lymantria_dispar_MNPV
0,50444		0,03127	0,05536	0,01871	0,01915	Lymantria_xylina_MNPV
	0,02003	0,07091	0,04771	0,02186	0,01713	Malacosoma_neustria_NPV
0,01878	0,02320	0,07113	0,04502	0,02082	0,01927	Mamestra_brassicae_MNPV
0,04047	0,04273	0,25796	0,02399	0,03920	0,03603	Mamestra_configurata_NPV-A
0,01881	0,02308	0,07104	0,04538	0,02066	0,01918	Mamestra_configurata_NPV-B
0,02315	0,02721	0,08385	0,03409	0,02619	0,02242	Maruca_vitrata_MNPV
0,02002	0,01878	0,06070	0,05299	0,01662	0,01945	Mythimna_unipuncta_NPV
0,09375	0,13812	0,50970	0,15471	0,12309	0,09406	Neodiprion_lecontei_NPV
0,02245	0,02446	0,09556	0,04942	0,02173	0,02269	Operophtera_brumata_NPV
0,01885	0,01946	0,07988	0,04559	0,02197	0,01679	Orgyia_leucostigma_NPV
0,07270	0,06506	0,01958	0,31270	0,07073	0,06578	Orgyia_pseudotsugata_MNPV
0,02696	0,03449	0,13338	0,03258	0,03300	0,02674	Oxyplax_ochracea_NPV
0,01789	0,01690	0,04857	0,05400	0,01470	0,01797	Peridroma_sp._NPV
0,01728	0,02113	0,07529	0,04426	0,02025	0,01756	Perigonia_lusca_SNPV
0,02125	0,02499	0,07748	0,03526	0,02465	0,02100	Plutella_xylostella_MNPV
0,02341	0,02955	0,10280	0,04489	0,02701	0,02600	Rachiplusia_nu_NPV
0,01879	0,01928	0,05940	0,04936	0,01731	0,01884	Spodoptera_eridania_NPV
0,01748	0,02044	0,06766	0,04761	0,01760	0,01823	Spodoptera_exempta_NPV
0,04007	0,03821	0,26345	0,02546	0,03638	0,03858	Spodoptera_exigua_NPV
0,01954	0,02247	0,07338	0,04733	0,02010	0,02002	Spodoptera_frugiperda_MNPV
0,02132	0,02212	0,07499	0,04991	0,01638	0,02088	Spodoptera_litura_NPV
0,01929	0,01934	0,06323	0,04748	0,01795	0,01929	Spodoptera_litura_NPV_II
0,01970	0,02174	0,08201	0,04730	0,02342	0,01845	Sucra_jujuba_NPVT

Neodiprion_lecontei_	Mythimna_unipuncta	Maruca_vitrata_MNP	Mamestra_configurata	Mamestra_configurata	Mamestra_brassicae_	
2,12862	0,48249	0,63523	0,54881	0,95659	0,55416	SepaNPV
1,53849	0,56312	0,59622	0,50668	0,92481	0,50728	Adoxophyes_honmai_NPV
1,50428	0,57331	0,58328	0,49470	0,92268	0,49602	Adoxophyes_orana_NPV
2,15241	0,27392	0,60960	0,32764	0,70213	0,32959	Agrotis_ipsilon_MNPV
1,93169	0,31056	0,61765	0,33676	0,71566	0,33765	Agrotis_segetum_NPV-A
2,04003	0,30743	0,61715	0,35390	0,73852	0,35368	Agrotis_segetum_NPV-B
2,25636	0,57010	0,46909	0,65363	1,11383	0,65755	Antheraea_pernyi_NPV
1,82795	0,63065	0,41236	0,65965	1,08953	0,65833	Anticarsia_gemmatalis_MNPV
1,82890	0,62955	0,41097	0,65946	1,09576	0,65814	Anticarsia_gemmatalis_NPV
1,50168	0,53349	0,55408	0,48852	0,90918	0,48780	Apocheima_cinerarium_NPV
2,86603	1,33429	0,89753	1,28551	2,47816	1,29067	Autographa_californica_NPV
1,70766	0,59979	0,11686	0,56423	0,99966	0,56395	Bombyx_mandarina_NPV
2,85177	1,40431	0,79081	1,33385	2,66043	1,34360	Bombyx_mori_NPV
1,70227	0,50256	0,58081	0,48149	0,92295	0,48220	Buzura_suppressaria_NPV
1,57594	0,57373	0,31408	0,55240	0,95728	0,54930	Catopsilia_pomona_NPV
1,90709	0,61695	0,41959	0,66173	1,12923	0,65946	Choristoneura_fumiferana_DEF_MN
2,19822	0,58958	0,45490	0,65986	1,10988	0,65777	Choristoneura_fumiferana_MNPV
2,29187	0,58059	0,47790	0,65262	1,10787	0,65785	Choristoneura_murinana_NPV
2,11084	0,57039	0,46447	0,63827	1,06348	0,63881	Choristoneura_rosaceana_NPV
1,75070	0,45478	0,58432	0,44679	0,82760	0,44629	Chrysodeixis_chalcites_NPV
1,58228	0,54903	0,61585	0,54227	0,97343	0,54387	Clanis_bilineata_NPV
1,81399	0,62996	0,41376	0,65478	1,13007	0,65441	Condylorrhiza_vestigialis_MNPV
1,54394	0,49922	0,57355	0,47215	0,84731	0,47118	Cryptophlebia_peltastica_NPV
4,70339	2,44297	2,71953	2,82411	4,14098	2,80971	Culex_nigripalpus_NPV
1,83197	0,60385	0,35834	0,63441	1,07033	0,63260	Cyclophragma_undans_NPV
2,13489	0,89846	1,02799	0,95363	1,20381	0,95947	Cydia_pomonella_GV
2,17982	0,54639	0,41515	0,61459	1,06737	0,61742	Dione_juno_NPV
1,55301	0,48901	0,57036	0,46487	0,85587	0,46599	Ecotropis_obliqua_NPV
4,38410	2,36419	1,87109	2,14485	5,91902	2,12609	Epiphyas_postvittana_NPV
1,75915	0,48284	0,54154	0,45489	0,88584	0,45709	Euproctis_digramma_NPV
1,62319	0,50269	0,58570	0,48450	0,93403	0,48423	Euproctis_pseudoconspersa_NPV
2,88804	1,63813	1,51332	1,42017	3,26647	1,41864	Helicoverpa_armigera_NPV
1,66225	0,49768	0,58667	0,48918	0,90638	0,49035	Hemileuca_sp._NPV
2,00591	0,60881	0,42058	0,64631	1,08012	0,64109	Hyphantria_cunea_NPV
1,66737	0,45587	0,56628	0,47855	0,88727	0,47925	Hyposidra_talaca_NPV
1,77170	0,48064	0,57023	0,46404	0,84730	0,46584	Lambdina_fiscellaria_NPV
2,06140	0,40381	0,66092	0,51972	0,91689	0,52139	Leucania_separata_NPV
2,35794	1,14772	0,78425	1,01530	0,58828	1,01153	Lonomia_obliqua_MNPV
4,50800	1,34907	1,65507	1,42507	3,26582	1,42858	Lymantria_dispar_MNPV
2,18610	0,46966	0,65301	0,55710	0,97864	0,56049	Lymantria_xylina_MNPV
1,72625	0,48860	0,56125	0,47803	0,90813	0,47750	Malacosoma_neustria_NPV
1,71424	0,33449	0,59316	0,00410	0,40365		Mamestra_brassicae_MNPV
2,36442	0,70691	1,05360	0,40513		0,01542	Mamestra_configurata_NPV-A
1,72110	0,33163	0,59469		0,01556	0,00098	Mamestra_configurata_NPV-B
1,75208	0,62344		0,02369	0,04595	0,02357	Maruca_vitrata_MNPV
1,94072		0,02467	0,01419	0,02910	0,01429	Mythimna_unipuncta_NPV
	0,11507	0,09463	0,08905	0,15592	0,08834	Neodiprion_lecontei_NPV
0,10668	0,02333	0,02776	0,02338	0,03889	0,02353	Operophtera_brumata_NPV
0,09244	0,01910	0,02334	0,01965	0,03865	0,01964	Orgyia_leucostigma_NPV
0,52767	0,06841	0,06814	0,07713	0,24407	0,07759	Orgyia_pseudotsugata_MNPV
0,07669	0,03089	0,01637	0,02524	0,04898	0,02531	Oxyplax_ochracea_NPV
0,12506	0,01089	0,02554	0,01363	0,02873	0,01360	Peridroma_sp._NPV
0,08834	0,01937	0,02325	0,01902	0,03638	0,01925	Perigonia_lusca_SNPV
0,09208	0,02294	0,00696	0,02209	0,03920	0,02188	Plutella_xylostella_MNPV
0,08649	0,02168	0,02588	0,02142	0,03815	0,02119	Rachiplusia_nu_NPV
0,10880	0,01242	0,02552	0,01537	0,02986	0,01543	Spodoptera_eridania_NPV
0,09919	0,01381	0,02355	0,01351	0,02733	0,01367	Spodoptera_exempta_NPV
0,18329	0,02862	0,04757	0,02932	0,01416	0,02935	Spodoptera_exigua_NPV
0,09169	0,01483	0,02493	0,01481	0,02948	0,01484	Spodoptera_frugiperda_MNPV
0,09245	0,01996	0,02403	0,02292	0,04582	0,02304	Spodoptera_litura_NPV
0,10811	0,01225	0,02520	0,01495	0,02871	0,01501	Spodoptera_litura_NPV_II
0,08284	0,02129	0,02205	0,01970	0,03700	0,01954	Sucra_jujuba_NPVT

Perigonia_lusca_SNP	Peridroma_sp._NPV	Oxyplax_ochracea_N	Orgyia_pseudotsugata	Orgyia_leucostigma_	Operophtera_brumata	
0,52759	0,41113	0,79592	1,33178	0,52293	0,63928	SepaNPV
0,48747	0,55844	0,59181	1,62503	0,50808	0,59233	Adoxophyes_honmai_NPV
0,50136	0,57690	0,56432	1,63122	0,50793	0,59462	Adoxophyes_orana_NPV
0,48364	0,21163	0,78099	1,42277	0,47451	0,56673	Agrotis_ipsilon_MNPV
0,47209	0,24559	0,73951	1,41582	0,47002	0,56933	Agrotis_segetum_NPV-A
0,48755	0,24842	0,72005	1,48246	0,48103	0,56592	Agrotis_segetum_NPV-B
0,62512	0,52007	0,60300	1,09713	0,62022	0,74065	Antheraea_pernyi_NPV
0,59214	0,61401	0,51039	1,11881	0,60026	0,75702	Anticarsia_gemmatalis_MNPV
0,59113	0,61295	0,50979	1,11656	0,59754	0,75676	Anticarsia_gemmatalis_NPV
0,42280	0,54410	0,57493	1,65929	0,37088	0,53810	Apocheima_cinerarium_NPV
1,28804	1,36238	1,23497	0,48523	1,30187	1,54205	Autographa_californica_NPV
0,55871	0,59720	0,38507	1,33069	0,55022	0,67156	Bombyx_mandarina_NPV
1,35288	1,41406	1,13859	0,47582	1,31044	1,64519	Bombyx_mori_NPV
0,45857	0,53001	0,59025	1,59636	0,37503	0,56456	Buzura_suppressaria_NPV
0,52914	0,60452	0,37363	1,39160	0,53120	0,66267	Catopsilia_pomona_NPV
0,60996	0,58633	0,52237	1,04024	0,61256	0,75286	Choristoneura_fumiferana_DEF_MN
0,59749	0,54094	0,57444	0,99631	0,60567	0,77043	Choristoneura_fumiferana_MNPV
0,60314	0,52296	0,59345	0,96519	0,61482	0,78173	Choristoneura_murinana_NPV
0,59333	0,53689	0,58353	0,94117	0,59598	0,76281	Choristoneura_rosaceana_NPV
0,44262	0,43825	0,62325	1,56104	0,51041	0,56185	Chrysodeixis_chalcites_NPV
0,43816	0,54341	0,66221	1,72008	0,50146	0,60389	Clanis_bilineata_NPV
0,58573	0,60900	0,48558	1,08370	0,58362	0,74066	Condylorrhiza_vestigialis_MNPV
0,42540	0,50949	0,56468	1,61245	0,45349	0,54728	Cryptophlebia_peltastica_NPV
2,81687	2,18819	3,18351	5,60719	2,80854	3,00242	Culex_nigripalpus_NPV
0,58195	0,56868	0,44740	1,32323	0,57691	0,66983	Cyclophragma_undans_NPV
0,95910	0,87042	1,14300	2,11248	1,00360	0,91469	Cydia_pomonella_GV
0,57975	0,49904	0,57854	0,98393	0,55511	0,72872	Dione_juno_NPV
0,46323	0,47686	0,57268	1,61458	0,38045	0,54826	Ecotropis_obliqua_NPV
2,21063	2,41212	2,04849	0,51559	2,07744	2,57634	Epiphyas_postvittana_NPV
0,42186	0,44234	0,59050	1,48589	0,33234	0,55494	Euproctis_digramma_NPV
0,42556	0,48784	0,58616	1,58076	0,35246	0,57585	Euproctis_pseudoconspersa_NPV
1,32743	1,52081	1,43953	0,72215	1,43244	1,64514	Helicoverpa_armigera_NPV
0,45149	0,48191	0,63861	1,62809	0,39083	0,58533	Hemileuca_sp._NPV
0,58893	0,58160	0,52449	1,00554	0,56861	0,74411	Hyphantria_cunea_NPV
0,43407	0,46738	0,63062	1,49360	0,36658	0,55723	Hyposidra_talaca_NPV
0,43315	0,43156	0,64777	1,38074	0,40503	0,54607	Lambdina_fiscellaria_NPV
0,50667	0,37540	0,78302	1,43036	0,54404	0,54522	Leucania_separata_NPV
1,00319	1,14174	0,80169	3,51273	1,05460	1,10662	Lonomia_obliqua_MNPV
1,47836	1,14589	2,12203	0,49281	1,50410	1,74350	Lymantria_dispar_MNPV
0,51437	0,40516	0,79701	1,36827	0,48575	0,62579	Lymantria_xylina_MNPV
0,44033	0,45195	0,64449	1,47551	0,45728	0,54560	Malacosoma_neustria_NPV
0,48065	0,32860	0,63627	1,51018	0,49255	0,57092	Mamestra_brassicae_MNPV
0,87203	0,70825	1,09284	3,12713	0,89896	0,91413	Mamestra_configurata_NPV-A
0,47666	0,32728	0,63340	1,50641	0,49168	0,56498	Mamestra_configurata_NPV-B
0,56371	0,62752	0,38558	1,36051	0,56559	0,66984	Maruca_vitrata_MNPV
0,48556	0,25916	0,72646	1,40608	0,47772	0,55428	Mythimna_unipuncta_NPV
1,68603	2,05395	1,52800	4,63939	1,69715	1,88610	Neodiprion_lecontei_NPV
0,53667	0,57448	0,72148	1,89458	0,54116		Operophtera_brumata_NPV
0,43740	0,46476	0,63661	1,51053		0,02195	Orgyia_leucostigma_NPV
1,53740	1,29138	1,72403		0,07919	0,10911	Orgyia_pseudotsugata_MNPV
0,58230	0,80424		0,10076	0,02574	0,03054	Oxyplax_ochracea_NPV
0,45759		0,03661	0,06242	0,01900	0,02397	Peridroma_sp._NPV
	0,01852	0,02363	0,08189	0,01812	0,02197	Perigonia_lusca_SNPV
0,02087	0,02287	0,01724	0,06505	0,02258	0,02601	Plutella_xylostella_MNPV
0,02248	0,02251	0,02708	0,09677	0,02404	0,02690	Rachiplusia_nu_NPV
0,01890	0,01092	0,03079	0,06846	0,01908	0,02336	Spodoptera_eridania_NPV
0,01722	0,01374	0,02610	0,07539	0,01933	0,02297	Spodoptera_exempta_NPV
0,03826	0,02752	0,05357	0,23809	0,03784	0,04023	Spodoptera_exigua_NPV
0,01886	0,01421	0,02801	0,07973	0,02009	0,02332	Spodoptera_frugiperda_MNPV
0,02154	0,02035	0,02856	0,07896	0,02291	0,02219	Spodoptera_litura_NPV
0,01857	0,01093	0,03116	0,06899	0,01889	0,02338	Spodoptera_litura_NPV_II
0,01793	0,02153	0,02426	0,08623	0,01699	0,02306	Sucra_jujuba_NPVT

Spodoptera_frugiperd	Spodoptera_exigua_N	Spodoptera_exempta_	Spodoptera_eridania_	Rachiplusia_nu_NPV	Plutella_xylostella_M	
0,56457	0,92340	0,52570	0,49065	0,64584	0,62768	SepaNPV
0,55727	0,98397	0,53211	0,56709	0,57283	0,60304	Adoxophyes_honmai_NPV
0,54812	0,99040	0,52941	0,58154	0,56436	0,59930	Adoxophyes_orana_NPV
0,31503	0,63707	0,28077	0,23154	0,54297	0,57699	Agrotis_ipsilon_MNPV
0,31435	0,66760	0,30233	0,26966	0,55277	0,58649	Agrotis_segetum_NPV-A
0,33145	0,66603	0,29465	0,24890	0,56448	0,59512	Agrotis_segetum_NPV-B
0,62008	1,00164	0,63339	0,57668	0,73617	0,47284	Antheraea_pernyi_NPV
0,63105	1,07509	0,61621	0,62516	0,67555	0,41928	Anticarsia_gemmatalis_MNPV
0,63177	1,07871	0,61691	0,62356	0,67574	0,41980	Anticarsia_gemmatalis_NPV
0,48023	0,95724	0,48233	0,54660	0,51551	0,54487	Apocheima_cinerarium_NPV
1,31192	2,71696	1,28281	1,35310	1,31165	0,63226	Autographa_californica_NPV
0,58785	1,01588	0,56408	0,59217	0,61703	0,06634	Bombyx_mandarina_NPV
1,35394	2,76467	1,32629	1,36293	1,37143	0,75603	Bombyx_mori_NPV
0,49857	0,93167	0,49319	0,52433	0,56687	0,55574	Buzura_suppressaria_NPV
0,56508	0,98273	0,54226	0,56383	0,63267	0,33023	Catopsilia_pomona_NPV
0,62338	1,07482	0,63468	0,59968	0,67401	0,42628	Choristoneura_fumiferana_DEF_MN
0,62775	1,02708	0,61150	0,56509	0,68759	0,45042	Choristoneura_fumiferana_MNPV
0,63913	1,06585	0,61865	0,56645	0,71321	0,45843	Choristoneura_murinana_NPV
0,62109	1,01310	0,62541	0,55982	0,69466	0,46234	Choristoneura_rosaceana_NPV
0,43436	0,91459	0,42943	0,48054	0,43854	0,54161	Chrysodeixis_chalcites_NPV
0,54365	0,97849	0,52202	0,56403	0,56147	0,62170	Clanis_bilineata_NPV
0,61112	1,13395	0,61691	0,62552	0,65178	0,41494	Condylorrhiza_vestigialis_MNPV
0,49492	0,91785	0,46372	0,52212	0,52418	0,53161	Cryptophlebia_peltastica_NPV
2,62907	4,12573	2,68177	2,49579	3,46619	2,74234	Culex_nigripalpus_NPV
0,62036	1,05990	0,59039	0,58448	0,68550	0,37174	Cyclophragma_undans_NPV
0,91794	1,15686	0,91820	0,90086	1,04304	1,00063	Cydia_pomonella_GV
0,62712	0,93702	0,56462	0,54055	0,74155	0,42584	Dione_juno_NPV
0,48829	0,89802	0,49122	0,48920	0,55237	0,55067	Ecotropis_obliqua_NPV
2,27731	7,06185	2,10452	2,34264	2,33365	1,84847	Epiphyas_postvittana_NPV
0,51103	0,91933	0,47247	0,50522	0,56690	0,53740	Euproctis_digramma_NPV
0,52144	0,86869	0,47973	0,52007	0,59185	0,58415	Euproctis_pseudoconspersa_NPV
1,43775	3,24696	1,40620	1,54791	1,44651	1,51185	Helicoverpa_armigera_NPV
0,50720	0,94219	0,48189	0,48444	0,56688	0,56077	Hemileuca_sp._NPV
0,62483	1,02752	0,62254	0,59663	0,69317	0,42448	Hyphantria_cunea_NPV
0,47747	0,90996	0,46196	0,49281	0,56053	0,54456	Hyposidra_talaca_NPV
0,48917	0,89670	0,45179	0,46620	0,61089	0,54042	Lambdina_fiscellaria_NPV
0,51215	0,84528	0,45692	0,44221	0,67169	0,62737	Leucania_separata_NPV
1,06631	0,61391	1,06801	1,07928	1,04887	0,82073	Lonomia_obliqua_MNPV
1,51107	3,35174	1,41903	1,32420	1,81532	1,53601	Lymantria_dispar_MNPV
0,55175	0,90009	0,51421	0,47595	0,69650	0,60963	Lymantria_xylina_MNPV
0,48162	0,91572	0,44074	0,46980	0,56150	0,52920	Malacosoma_neustria_NPV
0,36374	0,73309	0,33749	0,36990	0,50400	0,55416	Mamestra_brassicae_MNPV
0,73648	0,34632	0,69614	0,73718	0,90634	0,94812	Mamestra_configurata_NPV-A
0,36166	0,72770	0,33605	0,36895	0,50507	0,55845	Mamestra_configurata_NPV-B
0,60900	1,04592	0,58727	0,61438	0,64231	0,13442	Maruca_vitrata_MNPV
0,34592	0,70950	0,33261	0,29319	0,52885	0,57688	Mythimna_unipuncta_NPV
1,70502	2,55562	1,78177	1,90414	1,65088	1,71938	Neodiprion_lecontei_NPV
0,56767	0,91035	0,55374	0,56186	0,62984	0,63745	Operophtera_brumata_NPV
0,48161	0,88803	0,47541	0,47866	0,57348	0,55143	Orgyia_leucostigma_NPV
1,52234	3,04617	1,49116	1,38832	1,72603	1,34987	Orgyia_pseudotsugata_MNPV
0,67371	1,14562	0,64871	0,71780	0,65571	0,41302	Oxyplax_ochracea_NPV
0,34058	0,69407	0,32961	0,27043	0,54874	0,57256	Peridroma_sp._NPV
0,46797	0,87977	0,43152	0,47686	0,54370	0,52087	Perigonia_lusca_SNPV
0,56168	0,99895	0,54518	0,57824	0,58655		Plutella_xylostella_MNPV
0,51914	0,95310	0,53668	0,56172		0,02375	Rachiplusia_nu_NPV
0,20936	0,46198	0,28438		0,02317	0,02373	Spodoptera_eridania_NPV
0,28359	0,68514		0,01200	0,02217	0,02129	Spodoptera_exempta_NPV
0,60763		0,02855	0,01848	0,04193	0,04351	Spodoptera_exigua_NPV
	0,02552	0,01197	0,00994	0,02122	0,02271	Spodoptera_frugiperda_MNPV
0,02111	0,03866	0,01962	0,02058	0,02596	0,02283	Spodoptera_litura_NPV
0,01001	0,01879	0,01164	0,00261	0,02232	0,02334	Spodoptera_litura_NPV_II
0,01984	0,03773	0,02005	0,02112	0,02152	0,02110	Sucra_jujuba_NPVT

Urbanus_proteus_NP	Trichoplusia_ni_SNP	Sucra_jujuba_NPV	Spodoptera_litura_NP	Spodoptera_litura_NP	
0,66477	0,58605	0,57853	0,49782	0,55088	SepaNPV
0,50802	0,51729	0,50318	0,55853	0,61407	Adoxophyes_honmai_NPV
0,50640	0,52778	0,50045	0,57561	0,63088	Adoxophyes_orana_NPV
0,62788	0,45896	0,53119	0,23872	0,50050	Agrotis_ipsilon_MNPV
0,61729	0,45657	0,53081	0,27434	0,51778	Agrotis_segetum_NPV-A
0,64459	0,47796	0,52382	0,25890	0,51924	Agrotis_segetum_NPV-B
0,75887	0,65634	0,67798	0,57995	0,64357	Antheraea_pernyi_NPV
0,67647	0,59355	0,64476	0,62094	0,67125	Anticarsia_gemmatalis_MNPV
0,67821	0,59877	0,64269	0,61935	0,67093	Anticarsia_gemmatalis_NPV
0,47264	0,47100	0,41381	0,54716	0,55957	Apocheima_cinerarium_NPV
1,35660	1,19212	1,27264	1,36601	1,45171	Autographa_californica_NPV
0,60160	0,56161	0,55277	0,58733	0,60315	Bombyx_mandarina_NPV
1,34275	1,27587	1,31947	1,37961	1,47628	Bombyx_mori_NPV
0,53413	0,50647	0,35973	0,53359	0,59688	Buzura_suppressaria_NPV
0,58473	0,56526	0,54165	0,56640	0,59305	Catopsilia_pomona_NPV
0,69921	0,61570	0,65310	0,59632	0,66479	Choristoneura_fumiferana_DEF_MN
0,71306	0,65780	0,65852	0,57734	0,64472	Choristoneura_fumiferana_MNPV
0,70784	0,66158	0,67646	0,57931	0,64901	Choristoneura_murinana_NPV
0,70737	0,62564	0,63908	0,56376	0,66399	Choristoneura_rosaceana_NPV
0,57096	0,13028	0,51511	0,46992	0,57959	Chrysodeixis_chalcites_NPV
0,56345	0,50281	0,51218	0,56015	0,60726	Clanis_bilineata_NPV
0,65040	0,59486	0,60431	0,62273	0,65880	Condylorrhiza_vestigialis_MNPV
0,50748	0,47020	0,45355	0,50567	0,56120	Cryptophlebia_peltastica_NPV
3,09946	3,12274	2,91060	2,47247	2,75842	Culex_nigripalpus_NPV
0,62695	0,62040	0,59534	0,58832	0,60345	Cyclophragma_undans_NPV
1,00263	0,97564	0,98348	0,91659	0,91336	Cydia_pomonella_GV
0,69625	0,61860	0,62571	0,53807	0,62750	Dione_juno_NPV
0,50724	0,47929	0,39582	0,48900	0,53874	Ecotropis_obliqua_NPV
2,25033	2,16626	2,13163	2,40227	2,28148	Epiphyas_postvittana_NPV
0,52238	0,49732	0,40988	0,50080	0,52373	Euproctis_digramma_NPV
0,52054	0,51446	0,41618	0,51343	0,55736	Euproctis_pseudoconspersa_NPV
1,35854	1,35097	1,36969	1,55373	1,44668	Helicoverpa_armigera_NPV
0,54285	0,49962	0,42477	0,49125	0,56642	Hemileuca_sp._NPV
0,66541	0,63330	0,61302	0,60031	0,66380	Hyphantria_cunea_NPV
0,52762	0,49155	0,34094	0,49379	0,56350	Hyposidra_talaca_NPV
0,53527	0,47962	0,44753	0,47822	0,52373	Lambdina_fiscellaria_NPV
0,67283	0,55560	0,57255	0,45170	0,40717	Leucania_separata_NPV
1,12870	1,04426	1,08721	1,05320	1,12083	Lonomia_obliqua_MNPV
1,80912	1,55372	1,60402	1,37349	1,50367	Lymantria_dispar_MNPV
0,65873	0,59291	0,53876	0,48165	0,53639	Lymantria_xylina_MNPV
0,51920	0,46265	0,49511	0,47398	0,51848	Malacosoma_neustria_NPV
0,58231	0,44240	0,49522	0,36264	0,54944	Mamestra_brassicae_MNPV
1,03146	0,80154	0,89131	0,72332	1,01309	Mamestra_configurata_NPV-A
0,58013	0,44132	0,49740	0,36077	0,54817	Mamestra_configurata_NPV-B
0,64059	0,58102	0,55263	0,61288	0,60111	Maruca_vitrata_MNPV
0,62455	0,44692	0,51747	0,29284	0,48575	Mythimna_unipuncta_NPV
1,55834	1,70250	1,62001	1,89248	1,75450	Neodiprion_lecontei_NPV
0,62815	0,55606	0,56230	0,56964	0,54700	Operophtera_brumata_NPV
0,49584	0,50006	0,39852	0,47482	0,56384	Orgyia_leucostigma_NPV
1,71608	1,62625	1,63433	1,39752	1,53584	Orgyia_pseudotsugata_MNPV
0,61709	0,62368	0,60672	0,71672	0,68372	Oxyplax_ochracea_NPV
0,62036	0,44127	0,52311	0,27191	0,49091	Peridroma_sp._NPV
0,50073	0,44450	0,45110	0,46585	0,54482	Perigonia_lusca_SNPV
0,60369	0,51648	0,51969	0,57473	0,57472	Plutella_xylostella_MNPV
0,61217	0,43329	0,51918	0,54836	0,62422	Rachiplusia_nu_NPV
0,61418	0,46798	0,52154	0,02985	0,49655	Spodoptera_eridania_NPV
0,58026	0,43021	0,50236	0,27923	0,47426	Spodoptera_exempta_NPV
1,03536	0,87139	0,90269	0,46324	0,90368	Spodoptera_exigua_NPV
0,56669	0,42470	0,48732	0,21595	0,50990	Spodoptera_frugiperda_MNPV
0,59533	0,55453	0,60876	0,50288		Spodoptera_litura_NPV
0,61328	0,46575	0,52930		0,02091	Spodoptera_litura_NPV_II
0,54059	0,50066	0,02027	0,02125	0,02495	Sucra_jujuba_NPVT

Appendix A

Ethics Approval Letters

RHODES UNIVERSITY
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Rhodes University Animal Research Ethics Committee
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e: s.nunqele@ru.ac.za
NHREC Registration number: AREC-251114-018

https://www.ru.ac.za/researchgateway/ethics/

9 February 2022

DR Iain Paterson

Email: I.Paterson@ru.ac.za

Review Reference: 2022-3842-6516

Dear DR Iain Paterson

Re: Animal ethics application: Centre for Biological Control

Principal Investigator:

Collaborators: ,

This letter confirms that the above Annual Report has been reviewed and **APPROVED** by the Rhodes University Animal Research Ethics Committee (RU-AREC). Your Approval number is: **2022-3842-6516.**

Approval has been granted for one year from the date of this letter. **An annual progress report** will be required in order to renew approval for an additional period of one year. Ethics approval for a project can be renewed twice for a project (i.e. the maximum number of years that a project can receive ethics approval for is 3 years). Thereafter, the project will need to undergo a full application should you require ethics clearance for longer than 3 years.

Sincerely

Dr. Shelley Edwards

Chair: Rhodes University Animal Research Ethics Committee, RU-AREC

cc: Ms Danielle de Vos - Ethics Coordinator

Page 1 of 1

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NHREC Registration number: AREC-251114-018

https://www.ru.ac.za/researchgateway/ethics/

1 November 2022

DR Iain Paterson

Email: I.Paterson@ru.ac.za

Review Reference: 2022-3842-7223

Dear DR Iain Paterson

Re: Animal ethics application: Centre for Biological Control Nov

Principal Investigator:

Collaborators: As per original application

This letter confirms that the above Annual Report has been reviewed and **APPROVED** by the Rhodes University Animal Research Ethics Committee (RU-AREC). Your Approval number is: 2022-3842-7223.

Approval has been granted for one year from the date of this letter. **An annual progress report** will be required in order to renew approval for an additional period of one year. Ethics approval for a project can be renewed twice for a project (i.e. the maximum number of years that a project can receive ethics approval for is 3 years). Thereafter, the project will need to undergo a full application should you require ethics clearance for longer than 3 years.

Sincerely

Dr. Roman Tandlich

Chair: Rhodes University Animal Research Ethics Committee, RU-AREC

cc: Ethics Coordinator

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NHREC Registration number: AREC-251114-018

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6 October 2023

DR Iain Paterson

Email: I.Paterson@ru.ac.za

Review Reference: 2023-3842-8102

Dear DR Iain Paterson

Re: Animal ethics application: Centre for Biological Control Oct

Principal Investigator:

Collaborators: ,

This letter confirms that the above Annual Report has been reviewed and **APPROVED** by the Rhodes University Animal Research Ethics Committee (RU-AREC). Your Approval number is: 2023-3842-8102.

Approval has been granted for one year from the date of this letter. **An annual progress report** will be required in order to renew approval for an additional period of one year. Ethics approval for a project can be renewed twice for a project (i.e. the maximum number of years that a project can receive ethics approval for is 3 years). Thereafter, the project will need to undergo a full application should you require ethics clearance for longer than 3 years.

Sincerely

Dr. Roman Tandlich

Chair: Rhodes University Animal Research Ethics Committee, RU-AREC

cc: Ethics Coordinator

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8 September 2024

DR Iain Paterson

Email: I.Paterson@ru.ac.za

Review Reference: 2024-8120-9068

Dear DR Iain Paterson

Re: Animal ethics application: Centre for Biological Control (2024)

Principal Investigator: Prof Iain Paterson

Collaborators: As specified in the application

This letter confirms that the above research proposal has been reviewed and **APPROVED** by the Rhodes University Animal Research Ethics Committee (RU-AREC). Your Approval number is 2024-8120-9068.

Your approval is depending on the following conditions:

The applicants and the CBC staff are requested to document the major activities of the centre through regular collection of photographic or video evidence. This evidence is requested to be collected throughout the period of approval and submitted to RUAREC with the next annual progress report.

Approval has been granted for one year from the date of this letter. **An annual progress report** will be required in order to renew approval for an additional period of one year.

Please ensure that the RU-AREC committee is notified should any substantive change(s) be made, for whatever reason, during the research process. This includes changes in investigators. Please also ensure that a brief report is submitted to the ethics committee on the completion of the research. The purpose of this report is to indicate whether the research was conducted successfully, if any aspects could not be completed, or if any problems arose that the RU-AREC should be aware of. If a thesis or dissertation arising from this research is submitted to the library's electronic theses and dissertations (ETD) repository, please notify the committee of the date of submission and/or any reference or cataloging number allocated.

Sincerely

Dr. Roman Tandlich

Chair: Rhodes University Animal Research Ethics Committee, RU-AREC

cc: Ethics Coordinator