Genetic Characterization of Nucleopolyhedrovirus Isolated from *Hyposidra talaca* Walker (Lepidoptera: Geometridae), a Tea Pest in Terai Region of Darjeeling Foothills, India.

A THESIS SUBMITTED TO THE UNIVERSITY OF NORTH BENGAL FOR THE AWARD OF DOCTOR OF PHILOSOPHY

In

Zoology

By

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September, 2022

Dedicated
to
All my Teachers

DECLARATION

I declare that the thesis entitled **"Genetic Characterization of Nucleopolyhedrovirus Isolated from *Hyposidra talaca* Walker (Lepidoptera: Geometridae), a Tea Pest in Terai Region of Darjeeling Foothills, India."** has been prepared by me under the guidance of Professor Min Bahadur, Department of Zoology, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

We certify that Mr. Bappaditya Ghosh has prepared the thesis entitled **"Genetic Characterization of Nucleopolyhedrovirus Isolated from *Hyposidra talaca* Walker (Lepidoptera: Geometridae), a Tea Pest in Terai Region of Darjeeling Foothills, India."** for the award of Ph.D. degree of the University of North Bengal, under our guidance. He has carried out the work at the Department of Zoology, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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Document Information

Analyzed document	Bappaditya Ghosh_Zoology.pdf (D143186448)
Submitted	2022-08-23 15:36:00
Submitted by	University of North Bengal
Submitter email	nbupig@nbu.ac.in
Similarity	0%
Analysis address	nbupig.nbu@analysis.urkund.com

Sources included in the report

W URL: https://www.researchgate.net/publication/267101495_Genomic_Sequencing_and_Analysis_of_Sucra_jujuba_Nucleopolyhedrovirus
Fetched: 2023-05-01 22:30:32 1

Entire Document

5 Chapter 1: Introduction, Review of Literature, Materials and Methods
6 Section 2: Introduction Tea cultivation in India Tea, Camellia sinensis (L.) O Kuntze, is the most common and widely consumed refreshing drink throughout the world. It is a resolutely and skilfully managed monoculture crop cultivated on large as well as small-scale between latitudes 41°N and 16°S with an annual precipitation of 1000–5000 mm and temperature of 8–35.0 C (Hazarika et al., 2008). As per 64th annual report (2017-18) of "Tea Board India", India is the World's second largest producer of tea sharing 23% (1,325.05 Million Kg) of global tea production, while China is the first producing 45%. However, India exported 14% of world tea which comes after Kenya (23%), China (20%) and Srilanka (16%). India is also one of the largest tea consumers in the world accounting 19% of global tea consumption. Almost 76% of produced tea in India is consumed within the Country itself. During 2017-18, 256.57 Million Kg of tea was exported from India with Cost, Insurance, and Freight (CIF) value of Rs. 5064.88 Crs, while 20.59 Million Kg with CIF value of Rs. 288.56 Crs was imported into India (Anonymous, 2018). Tea is the chief foliar crop in northern part of West Bengal, Assam, Sikkim, Tripura, Nilgiri of Tamil Nadu and also grown in small scale in Himachal Pradesh, Kerala, Karnataka and Orissa (Fig 1). The Assam and Darjeeling tea are very popular in India and exclusively cultivated in large number of tea plantations in the Terai-Dooars region of West Bengal and Assam. In India tea is cultivated over an area of 6,36,557.07 hectare, of which 3,37,690.35 hectare (53%) is situated in Assam and 1,48,121.74 hectare (23%) in West Bengal including both big and small growers. The economy of both of these states largely depends on the production of tea. In the northern part of West Bengal, concerning the Himalayan foothills and plains, the Terai plantation is on the Western flank of the mighty Teesta river and the Dooars plantation stretches on the eastern terrain of the river continuing upto the state of Assam. Looper pests in the Tea plantations of Terai-Dooars region of India Tea plantations of the Terai and Dooars region of West Bengal and Assam are experiencing severe attacks of different leaf eating lepidopteran pests, among which, Biston (Buzura)
7 suppressaria Guen (Lepidoptera: Geometridae) was reported as a major tea pest for several decades from Assam and Terai-Dooars region of North Bengal (Das, 1965). Recent studies reveal that a few other species of geometrid pests are attacking tea plantations among which the pest species, Hyposidra talaca Walker (Lepidoptera: Geometridae), early caterpillar of which are commonly known as 'Black Inch Looper' (Fig 2), has taken over as the major defoliating pest in tea plantations (Das et al., 2010a). Loopers of this species that primarily feed on a number of forest plants and weeds in India, Malayasia and Thiland (Das and Mukhopadhyay, 2009; Mathew et al., 2005; Winotai et al., 2005) have turned to tea as an active defoliating insect pest in plantations of Assam and Darjeeling Terai-Dooars of North Bengal (Basu Majumdar, 2004; Das et al., 2010). A substantial loss in tea production due to this defoliating lepidopteran pest has been reported from these regions (Gurusubramanian et al., 2008; Hazarika et al., 2008), that severely has affected the economy of the country (Roy and Muraleedharan, 2014). Management of Tea pest population To manage pest infestations, there are two main methods of pest control: chemical and biological. The looper pests have so far been controlled by regular application of synthetic insecticides especially (Fig 3), organophosphates and pyrethroids, but, the pests are gradually becoming less susceptible, and have developed some extent of resistance to the pesticides (Das et al., 2010b; Das and Mukhopadhyay, 2008) often resulting in their control failures (Senngrahi and Talukdar, 2005). Moreover, the application of these synthetic chemical insecticides is one of the major source of pollution of soil and water (Saravanan et al., 2009; Singh et al., 2017) and reported to be hazardous to the non-target organisms including human (Azmi et al., 2006; Mobed et al., 1992; Saravanan et al., 2009; Velmurugan et al., 2006). Because of these harmful effects, application of a number of these insecticides has been banned by the EPA of the USA and even by the Tea Research Organizations of India. The registration of many other insecticides have been reviewed (Chattopadhyay et al., 2008) and recommended under the Plant Protection Codes (PPC). Therefore, concerning the fact of development of pesticide resistance in insect pests and hazardous effects of the chemical pesticides, the organic tea has become more acceptable especially for export and health conscious tea consumers than the chemically managed conventional tea. In view of this, the

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Acknowledgement

I take this opportunity to express my deep sense of gratitude and indebtedness to those who helped me in carrying out this investigation.

My parents have been a constant source of inspiration throughout my doctoral study, without their support and encouragement it was quite impossible to finish such hard work.

I am immensely indebted and sincerely grateful to my honourable supervisor Prof. Min Bahadur, Genetics and Molecular Biology Laboratory, Department of Zoology, University of North Bengal and honourable co-supervisor Prof. Ananda Mukhopadhyay who introduced me to the exciting field of research and extended the fullest possible help and unconditional encouragement. Their invaluable suggestions, guidance and supervision throughout my work help me to solve problems and to take correct decisions during hard times of my research work.

I am thankful to Prof. Soumen Bhattacharjee, Head, Department of Zoology, University of North Bengal for ensuring the necessary facilities in the Department, particularly the instrumentation facility.

I express my thanks to my respected teachers Prof. Joydeb Pal, Prof. Sudip Barat and Prof. Tapas Kumar Chaudhury, Prof. Dhiraj Saha, Dr Tilak Saha (Associate Professor), Dr Sourav Mukherjee (Assistant Professor), Dr Ritwik Mondal (Assistant Professor), Dr Arpan Kumar Maiti (Assistant Professor), Dr Subhra Prkash Hui for their valuable guidance during my work.

I extend my thanks to Prof. Sudip Barat and Prof. Dhiraj Saha, previous Head, Department of Zoology, University of North Bengal, for providing all the necessary facilities from the Department.

I would like to sincerely acknowledge UGC for providing financial support as a fellowship under the UGC-BSR scheme, Bahadur Shah Zafar Marg, New Delhi, India (Ref. No. 4-1/2006(BSR)/7-134/2007 (BSR) dated 25.02.2013).

I am indebted to Prof. Soumen Bhattacharjee for his technical suggestions during my research work.

The contributions of my grandfather, Dr. Radhagobinda Ghosh, and my uncle Mr. Gour Chandra Ghosh, in my life, are undeniable. Without them I would never come to this stage.

I am grateful to my wife Mrs Dipa Mandal for her continuous encouragement, co-operation and moral support.

I am grateful to Dr Mahua Rudra and Mr Goutam Debnath for their initial guidance and unconditional cooperation as my elder sister and elder brother, respectively.

I am grateful to all my family members, my brothers, my sisters, and my in-laws for their continuous support.

Special thanks will go to one of my best friends, Mr Tanmoy Dutta, my sister, Ms Farha Yasmin, and my brothers, Mr Sudipta Kumar Roy, and Mr Debabrata Mandal for their continuous support and encouragement.

I am also grateful to Mr Binoy Kishore Rai for his initial guidance during my research work.

I am also thankful to Dr Susmita Dutta, Mr Akash Chowdhury, Mrs Swati Singh, Mrs Trisita Mazumdar, Mr Saugata Ghosh, Mr Debojit Dutta, Ms Arpita Ray for their untiring help and support throughout my work.

I would like to thank Mrs Jayashree Saren, Ms Ananya Das, Mr Ronit Dey, Mr Prasanta Saha, Mr Tanmoy Dutta, Dr Subhashis Paul, Mr Sourav Sarkar, Mr Debojit Dutta and Mr Saugata Ghosh, for cooperating me during sample collection.

I am thankful to Dr Sangita Khewa Subba and Dr Kumar Basnet for providing some samples.

I extend my thanks to Dr Soma Das, Dr Ritesh Biswa, Dr Sangita Khewa Subba, Dr Anjali Prasad, Dr Kumar Basnet, Mr Mrinal Ray, Ms Jayashree Saren, Dr Priyankar Dey, Dr Somit Dutta, Dr Subhrajyoti Roy, Dr Pokhraj Guha, Dr Avishek Das, Dr Rudra Prasad Roy, Ms Ruksa Nur, Dr Dawa Bhutia, Mr Sanjib Sarkar, Dr Tanmay Mukhopadhyay, Dr Sutanuka Chattaraj, Dr Uttara Dey Bhowmick, Dr Subhashis Paul, Mr Tanmoy Dutta, Ms Naznin Islam, Mr Debabrata Modak, Mr Sourav Sarkar, Mr Sona Sutradhar Dr Minu Bharti, Dr Priyanka Rai, Mr Abhishek Subba, Mr Manas Pratim Modak, Mr Sagar Sarkar, Ms Sashwati Ghosh for extending their helping hands.

Thanks are also due to the non-teaching staff of the Dept. of Zoology for their assistance in departmental facilities.

I am also thankful to all administerial staff for their help during my Ph.D. work.

In the end, I thank all those who helped me or were associated with my work directly or indirectly.

Preface

The application of synthetic chemical pesticides to control different agricultural pests is a global concern because of their adverse effects on the environment and non-target organisms including humans. Moreover, due to the irrational use of chemical pesticides, insect pests may gradually develop resistance to synthetic chemical pesticides leading to control failure. Hence, the development of a pest management strategy that is non-polluting, eco-friendly, target-specific, and sustainable will be highly acceptable. In this regard, microorganisms as biopesticides/ bioinsecticides may be an alternative to synthetic chemical pesticides.

Among several microorganisms, baculoviruses, particularly nucleopolyhedroviruses (NPVs) are well known as bioinsecticides in the integrated pest management (IPM) strategies because of their host specificity and proved to be safe to the environment, humans, other plants, and natural enemies of pests. However, as the mode of action of NPVs is slower than chemical pesticides, genetic modifications to enhance the killing efficiency of NPVs are highly required for better pest control and management. Therefore, the studies to explore the genome of pest-specific NPVs will help design an effective biopesticide.

The present study has been contemplated to **characterize the genome of NPVs isolated from *Hyposidra talaca*, a major lepidopteran tea pest in the Terai-Dooars region of the northern part of West Bengal**. As the tea plantations of the Terai-Dooars regions are facing immense defoliation by the *Hyposidra talaca* larvae resulting in huge economic loss, several synthetic chemical pesticides are regularly applied in the tea plantations of the Terai-Dooars regions to manage the pest problem. Therefore, the development of ***Hyposidra talaca* nucleopolyhedrovirus** (HytaNPV) into a potential biopesticide will be beneficial to minimize the use of chemical pesticides. In this context, the characterization of the genome and evolutionary study of HytaNPV will be very helpful in designing a virus-based pesticide.

This thesis has been divided into several chapters. A brief **introduction**, **review of literature**, **objectives,** and **materials and methods** were provided in **Chapter 1**. **Chapter 2** comprises the **results** of the survey and sampling, restriction endonuclease fragment analyses and partial restriction map of HytaNPV genome, characterization of selected genes and phylogenetic analyses and **Chapter 3** contains the detailed **discussion** of the findings of the present study, **conclusion and summary**. **Chapter 4** comprises the list of research articles, and the papers presented at different conferences under the **Appendix**, along with the **Bibliography** and **Index**.

Abstract

Introduction

The family Baculoviridae (nucleopolyhedroviruses and granuloviruses) includes a large group of DNA viruses that are pathogenic to several arthropod species, primarily to the insects of the order Lepidoptera but a few also infect members of the orders Hymenoptera and Diptera. The four genera of this virus family, Alphabaculovirus (lepidopteran-specific nucleopolyhedrovirus), Betabaculovirus (lepidopteran-specific granulovirus), Gammabaculovirus (hymenopteran-specific nucleopolyhedrovirus), and Deltabaculovirus (dipteran-specific nucleopolyhedrovirus), are host specific, highly pathogenic, and safe for industrial production and field applications. Baculoviruses, particularly nucleopolyhedroviruses (NPVs), have been considered an effective alternative to synthetic chemical pesticides to control different agricultural insect pests. An NPV (HytaNPV) isolated from a major leaf-eating lepidopteran tea pest, *Hyposidra talaca,* from the Terai-Dooars region of West Bengal and Assam in India, is found to be pathogenic against this defoliating pest and can be developed as an effective bioinsecticide against *H. talaca* in this region. Therefore, the information regarding the genome of HytaNPV and the phylogenetic relationship among different geographic isolates of HytaNPVs and the NPVs infecting other insects will be helpful to develop this virus as a biopesticide.

The present study contemplates the restriction endonuclease fragment analyses using *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I and *Xho*I, to construct the restriction profiles and to estimate the approximate genome size of nucleopolyhedrovirus isolated from *Hyposidra talaca* from Terai region of Darjeeling foothills. For comparison, a Dooars isolate has also been included in the study. The isolates collected from the two, east and west terrains of the mighty river Teesta, were designated as HytaNPV-ITK1 (Terai isolate) and HytaNPV-ID1 (Dooars isolate). Six genes (*polyhedrin, lef-8, lef-9, pif-1, pif-2* and *pif-3*) from both the isolates, those of Terai and Dooars, were sequenced. Partial restriction maps based on the obtained sequences of both the isolates were constructed and compared with the Dooars isolate of HytaNPV earlier reported by Nguyen et al. (2018). This reference isolate was designated as HytaNPV-R (Nguyen et al., 2018). Phylogenetic studies based on six gene sequences were carried out to know the taxonomic position and relationship of HytaNPVs (HytaNPV-ITK1 and HytaNPV-ID1) with the other reported baculoviruses.

The survey, sampling and maintenance of virus stock

A field survey, before the application of pesticides in tea plantations, suggested that *H. talaca* caterpillars (loopers) were found in abundance from March – November except during winter season. Adult moths become active in dimmed light during the early morning while their looper stage either perforated or defoliated the young tea leaves. NPV-infected dead looper caterpillars were noticed hanging from the tea leaves and twigs with their prolegs in a head-down position exhibiting liquefaction of the body.

NPV-infected cadavers of *Hyposidra talaca* caterpillars were collected separately from different tea plantations in the Terai region of Darjeeling foothills and also from the Dooars region of West Bengal, India for comparison. Occlusion bodies (OBs) of NPV of *H. talaca* were collected from the cadavers and purified by differential centrifugation. The stock of OBs was maintained by infecting the laboratory-reared 3^{rd}-5^{th} instar healthy larvae. OBs in large quantities were obtained from the larvae.

Viral DNA isolated from the OBs was used for restriction endonuclease fragment analyses. PCR amplification and sequencing of the target genes were carried out using gene-specific primers.

Restriction fragment analyses

Restriction fragment analysis using *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I and *Xho*I of both the Terai and the Dooars isolates, HytaNPV-ITK1 and HytaNPV-ID1 of the present study revealed 18, 8, 26, 9, 11, 7, and 20 fragments, respectively, ranging from 0.72 kb to 57.10 kb. A comparison with *in silico* restriction profiles of HytaNPV-R (Nguyen et al., 2018) revealed almost similar restriction profiles except for two restriction fragments, 3530 bp *Hin*dIII-fragment and 4857 bp *Xho*I-fragment. Such differences in restriction profiles among different geographic isolates are not uncommon, that represent a polymorphic state. Based on the restriction endonuclease fragment analyses the mean genome sizes of the HytaNPV isolates in the present study were estimated to be 138.20 kb for the Terai isolate (HytaNPV-ITK1) and 138.46 kb for the Dooars isolate (HytaNPV-ID1). The minor difference in genome sizes were either due to some undetected low molecular weight restriction fragment(s) or due to unresolved change in size(s) of restricton fragment(s) produced by observed site polymorhism, in agarose gel electrophoresis. The estimated genome size of both the isolates of the present study was found to be very similar to HytaNPV-R (Nguyen et al., 2018), which was 139.089 kb.

PCR and Sequencing of genes

Being most conserved, *polyhedrin* was sequenced along with the other five core baculoviral genes, *lef-8, lef-9, pif-1, pif-2* and *pif-3*. A total of 6.96 kb for the Terai (HytaNPV-ITK1) and 5.60 kb for the Dooars (HytaNPV-ID1) isolates of the HytaNPV genome (139.089 kb) were sequenced with some gaps. Nucleotide and protein BLAST analyses of all the gene sequences of both the isolates showed the highest sequence identity of >98% for nucleotides and >99% for amino acids with the reference HytaNPV-R (Nguyen et al., 2018).

Pairwise alignment of the obtained sequences of both the isolates (HytaNPV-ITK1 and HytaNPV-ID1) using the Dooars isolate of HytaNPV-R as a reference (Nguyen et al., 2018) revealed more than 98.99% and 99.98% sequence identity for nucleotide and amino acid, respectively. Moreover, a total of 26 non-synonymous substitutions leading to the substitution of 25 amino acids were detected among the isolates of HytaNPVs. Among the NPVs pathogenic to the specimens of other genera than *Hyposidra*, the NPV (BusuNPV) pathogenic to *Buzura suppressaria*, the old looper pest of tea in the Terai-Dooars region in India, exhibited a maximum overall amino acid sequence identity of 90.9% and nucleotide sequence identity of 85.8% with both the HytaNPV isolates of the present study for all the six genes.

In the *polyhedrin* gene, all seven (7) variable sites were synonymous suggesting conservative properties of the gene. Moreover, the overall sequence comparison among the HytaNPV isolates of the Terai-Dooars region revealed a ratio of non-synonymous to synonymous substitution <1 for *lef-8* and *pif-1* (2/6=0.33 for *lef-8* and 6/10=0.6 for *pif-1*) whereas, the ratio for *lef-9* was 16/6=2.66 and for *pif-2* was 20/12=1.66, indicating that *pif-2* and *lef-9* genes are under positive selection.

A higher proportion of variable sites for nucleotides (16 out of 1001) were detected in the *pif-2* suggesting that despite being one of the most conserved genes, the genetic variations in *pif-2* among the geographic isolates of NPV infecting the host of the same species may exist in different populations.

Partial restriction maps of the HytaNPV isolates

The partial restriction maps based on the obtained sequences of the two isolates of HytaNPV (HytaNPV-ITK1 and HytaNPV-ID1) in the present study were constructed. Two restriction sites for each of *Eco*RI and *Kpn*I in *polyhedrin* sequence, and a single site for each of *Xho*I, *Eco*RI, *Bgl*I and *Bam*HI in *lef-9, lef-8, pif-2* and *pif-1* sequences, respectively, were found to be common in both the isolates. However, no site for any of the six restriction endonucleases

analysed in the present study was detected in the *pif-3* sequences in both the isolates. Another *Bam*HI restriction site in *pif-1* which was present in HytaNPV-ID1 and HytaNPV-R was found to be absent in the Terai isolate (HytaNPV-ITK1).

Phylogenetic analysis

Both the nucleotide and amino acid substitution-based phylogenetic trees using the concatenated sequence alignment of *polyhedrin*, *lef-8*, *lef-9*, *pif-1*, *pif-2* and *pif-3* genes showed that both the group I and group II alphabaculoviruses have diverged from a common ancestor. The delta, gamma and beta- baculoviruses have diverged before the divergence of alphabaculoviruses into group I and group II. HytaNPV isolates of the present study (HytaNPV-ITK1 and HytaNPV-ID1) and the reference, HytaNPV-R reported by Nguyen et al. (2018) from the Dooars region, were phylogenetically placed under the group II alphabaculovirus. Both, HytaNPV-ITK1, and HytaNPV-ID1, appeared to be closer to the HytaNPV-R. Among the NPVs infecting the host of other genera, BusuNPV exhibited the closest relationship with the isolates of HytaNPVs. The phylogenetic analyses also revealed that the NPVs infecting the members of the family Geometridae such as BusuNPV, EcobNPV, ApciNPV and SujuNPV including HytaNPVs showed close relationships among themselves.

Conclusion

The present study revealed that though there were few genetic differences present in polymorphic conditions among the Terai and Dooars isolates of HytaNPV in West Bengal, by and large, a single variant of HytaNPV is infecting *Hyposidra talaca* in these regions. An extensive investigation using more restriction endonucleases and sequencing on a wider area may reveal more distinguishable genetic features.

Abbreviations

$\%id_N$	:	percentage of identical sites for nucleotide
$\%id_A$	:	percentage of identical sites for amino acid
acc. no.	:	accession number (NCBI)
°N	:	degree North
°S	:	degree South
µg	:	microgram
µl	:	micro liter
^0C	:	degree centigrade
AcNPV	:	*Autographa californica* NPV
AdhoNPV	:	*Adoxophyes honmai* NPV
AdorGV	:	*Adoxophyes orana* GV
AdorNPV	:	*Adoxophyes orana* NPV
AgipNPV	:	*Agrotis ipsilon* NPV
AgseGV	:	*Agrotis segetum* GV
AgseNPV	:	*Agrotis segetum* NPV
alk-exo	:	*alkaline exonuclease*
AM	:	anti meridian
AngeNPV	:	*Anticarsia gemmatalis* NPV
AnpeNPV	:	*Antheraea pernyi* NPV
ApciNPV	:	*Apocheima cinerarium* NPV
BLAST	:	Basic local alignment search tool
BLASTN	:	nucleotide BLAST
BLASTP	:	protein BLAST
BmNPV	:	*Bombyx mori* NPV
BomaNPV	:	*Bombyx mandarina* NPV
bp	:	base pair
BSA	:	bovine serum albumin
Bt	:	*Bacillus thuringiensis*
BusuNPV	:	*Buzura suppressaria* NPV
BVs	:	budded virus
CapoNPV	:	*Catopsilia pomona* NPV
cds	:	coding sequence

CfGV	:	*Choristoneura fumiferana* GV
CfNPV	:	*Choristoneura fumiferana* NPV
ChchNPV	:	*Chrysodeixis chalcites* NPV
ChmuNPV	:	*Choristoneura murinana* NPV
ChocNPV	:	*Choristoneura occidentalis* NPV
ChroNPV	:	*Choristoneura rosaceana* NPV
CIF	:	Cost, Insurance, and Freight
ClanGV-H	:	*Clostera anachoreta* GV
ClanGV-HB	:	*Clostera anastomosis* GV
ClbiNPV	:	*Clanis bilineata* NPV
c_N	:	number of conserved sites for nucleotides
CnmeGV	:	*Cnaphalocrocis medinalis* GV
CoveNPV	:	*Condylorrhiza vestigialis* NPV
CrluGV	:	*Cryptophlebia leucotreta* GV
Crs	:	Crores
CuniNPV	:	*Culex nigripalpus* NPV
CypoGV	:	*Cydia pomonella* GV
DapuNPV	:	*Dasychira pudibunda* NPV
DekiNPV	:	*Dendrolimus kikuchii Matsumura* NPV
DisaGV	:	*Diatraea saccharalis* GV
d_N	:	number of variable sites for nucleotides
d_A	:	number of variable sites for amino acids
DNA	:	deoxyribonucleic acid
dNTPs	:	deoxyribonucleotide tri-phosphates
EcobNPV	:	*Ectropis Obliqua* NPV
EDTA	:	Ethylenediaminetetraacetic acid
egt	:	ecdysteroid glucosyltransferase
EPA	:	Environmental Protection Agency
EpapGV	:	*Epinotia aporema* GV
EppoNPV	:	*Epiphyas postvittana* NPV
ErelGV	:	*Erinnyis ello* GV
et al	:	and others
EuprNPV	:	*Euproctis (=Arna) pseudoconspersa* NPV
g	:	gravity (for centrifugation)

GM	:	genetically modified
gm	:	gram
GTR	:	General time reversible (model)
GV(s)	:	granulovirus(es)
HearGV	:	*Helicoverpa armigera* GV
HearNPV	:	*Helicoverpa armigera* NPV
HycuNPV	:	*Hyphantria cunea* NPV
HytaNPV	:	*Hyposidra talaca* nucleopolyhedrovirus
HytaNPV-ID1	:	*Hyposidra talaca* nucleopolyhedrovirus Dooars isolate
HytaNPV-ITK1	:	*Hyposidra talaca* nucleopolyhedrovirus Terai isolate
HytaNPV-R	:	*Hyposidra talaca* NPV as reference
HzNPV	:	*Helicoverpa zea* NPV
in silico	:	in or on a computer: done or produced by using computer software or simulation
in vitro	:	outside the living body and in an artificial environment
IPM	:	integrated pest management
kb	:	kilo base
kDa	:	kilo dalton
Kg	:	kilogram
LafiNPV	:	*Lambdina fiscellaria* NPV
LC_{50}	:	median lethal concentration
LD_{50}	:	median lethal dose
LdNPV	:	*Lymantria dispar* NPV
Lef	:	late expression factor
LeseNPV	:	*Leucania separata* NPV
LG	:	Le Gascuel
LoobNPV	:	*Lonomia obliqua* NPV
LT_{50}	:	median lethal time
LyxyNPV	:	*Lymantria xylina* NPV
MabrNPV	:	*Mamestra brassicae* NPV
MacoNPV-A	:	*Mamestra configurata* NPV-A
MacoNPV-B	:	*Mamestra configurata* NPV-B
MaviNPV	:	*Maruca vitrata* NPV
MEGA	:	Molecular Evolutionary Genetics Analysis

$MgCl_2$	:	magnesium chloride
ml	:	milliliter
mm	:	millimetre
mM	:	millimolar
MNPV	:	multiple-nucleopolyhedrovirus
MolaGV	:	*Mocis latipes* GV
Na_2CO_3	:	Sodium carbonate
NaCl	:	Sodium chloride
NCBI	:	National Centre for Biotechnology Information
nd	:	non-synonymous mutation
NeabNPV	:	*Neodiprion abietis* NPV
NeleNPV	:	*Neodiprion lecontei* NPV
NeseNPV	:	*Neodiprion sertifer* NPV
ng	:	nanogram
NPV(s)	:	nucleopolyhedrovirus(es)
OB	:	Occlusion body
ODVs	:	Occlusion-derived virus
OpNPV	:	*Orgyia pseudotsugata* NPV
ORF	:	Open reading frame
OrleNPV	:	*Orgyia leucostigma* NPV
PCR	:	Polymerase Chain Reaction
PeluNPV	:	*Perigonia lusca* NPV
PespNPV	:	*Peridroma* sp NPV
pH	:	potential of Hydrogen
PhcyNPV	:	*Philosamia cynthia ricini* NPV
Pif	:	*per os* infectivity factor
PiraGV	:	*Pieris rapae* GV
PlxyGV	:	*Plutella xylostella* GV
PlxyNPV	:	*Plutella xylostella* NPV
PM	:	post meridian
POBs	:	polyhedral occlusion bodies
PPC	:	Plant Protection Codes
PsinNPV	:	*Chrysodeixis (=Pseudoplusia) includens* NPV
PsunGV	:	*Pseudaletia unipuncta* GV

REN	:	Restriction endonuclease
RFLP	:	Restriction fragment length polymorphism
RoNPV	:	*Rachiplusia ou* NPV
s/v	:	transition over transversion
sd	:	synonymous mutations
SDS	:	sodium dodecyl sulphate
SeNPV	:	*Spodoptera exigua* NPV
SfGV	:	*Spodoptera frugiperda* GV
SfNPV	:	*Spodoptera frugiperda* NPV
SNPV	:	single nucleopolyhedrovirus
SpliNPV-A	:	*Spodoptera litura* NPV
SpliNPV-B	:	*Spodoptera littoralis* NPV
SujuNPV	:	*Sucra jujuba* NPV
TAE	:	Tris-acetate EDTA
ThorNPV	:	*Thysanoplusia orichalcea* NPV
TnGV	:	*Trichoplusia ni* GV
TnNPV	:	*Trichoplusia ni* NPV
UrprNPV	:	*Urbanus proteus* NPV
USA	:	United States of America
UV	:	ultra violet
VLF	:	very late factor
w/v	:	weight per volume
λ DNA	:	lambda DNA

Single letter code for nucleotide

A	adenine nucleotide
G	guanine nucleotide
C	cytosine nucleotide
T	thymine nucleotide
S	guanine nucleotide/cytosine nucleotide
W	adenine nucleotide/ thymine nucleotide
Y	pyrimidine nucleotide
R	purine nucleotide
H	adenine nucleotide/ cytosine nucleotide / thymine nucleotide
M	adenine nucleotide/ cytosine nucleotide (with nitrogen base having amino group)

Single letter code for amino acid

G	Glycine	**W**	Tryptophan
P	Proline	**H**	Histidine
A	Alanine	**K**	Lysine
V	Valine	**R**	Arginine
L	Leucine	**Q**	Glutamine
I	Isoleucine	**N**	Asparagine
M	Methionine	**E**	Glutamic Acid
C	Cysteine	**D**	Aspartic Acid
F	Phenylalanine	**S**	Serine
Y	Tyrosine	**T**	Threonine

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Section 1.1: Introduction

1.1.1 Tea cultivation in India

Tea, *Camellia sinensis* (L.) O. Kuntze is the most common and widely consumed refreshing drink throughout the world. It is a resolutely and skilfully managed monoculture crop cultivated on large as well as small-scale between latitudes 41°N and 16°S with annual precipitation of 1000-5000 mm and temperature of 8-35^{0}C (Hazarika et al., 2008). As per the 64th annual report (2017-18) of "Tea Board India", India is the World's second-largest producer of tea sharing 23% (1325.05 Million Kg) of global tea production, while China is the first producing 45%. However, India exported 14% of world tea which comes after Kenya (23%), China (20%) and Srilanka (16%). India is also one of the largest tea consumers in the world accounting for 19% of global tea consumption. Almost 76% of produced tea in India is consumed within the Country itself. During 2017-18, 256.57 Million Kg of tea was exported from India with a Cost, Insurance, and Freight (CIF) value of Rs. 5064.88 Crs, while 20.59 Million Kg with a CIF value of Rs. 288.56 Crs was imported into India (Anonymous, 2018).

Tea is the chief foliar crop in the northern part of West Bengal, Assam, Sikkim, Tripura, and Nilgiri of Tamil Nadu and is also grown on a small scale in Himachal Pradesh, Kerala, Karnataka and Odissa (**Figure 1-1**). Assam tea and Darjeeling tea are very popular in India and are exclusively cultivated in a large number of tea plantations in the Terai-Dooars region of West Bengal and Assam. In India, tea is cultivated over an area of 6,36,557.07 hectare, of which 3,37,690.35 hectare (53%) is situated in Assam and 1,48,121.74 hectare (23%) in West Bengal including both big and small growers. The economy of both of these states largely depends on the production of tea. In the northern part of West Bengal, concerning the Himalayan foothills and plains, the Terai plantation is on the Western flank of the mighty Teesta river and the Dooars plantation stretches on the eastern terrain of the river continuing up to the state of Assam.

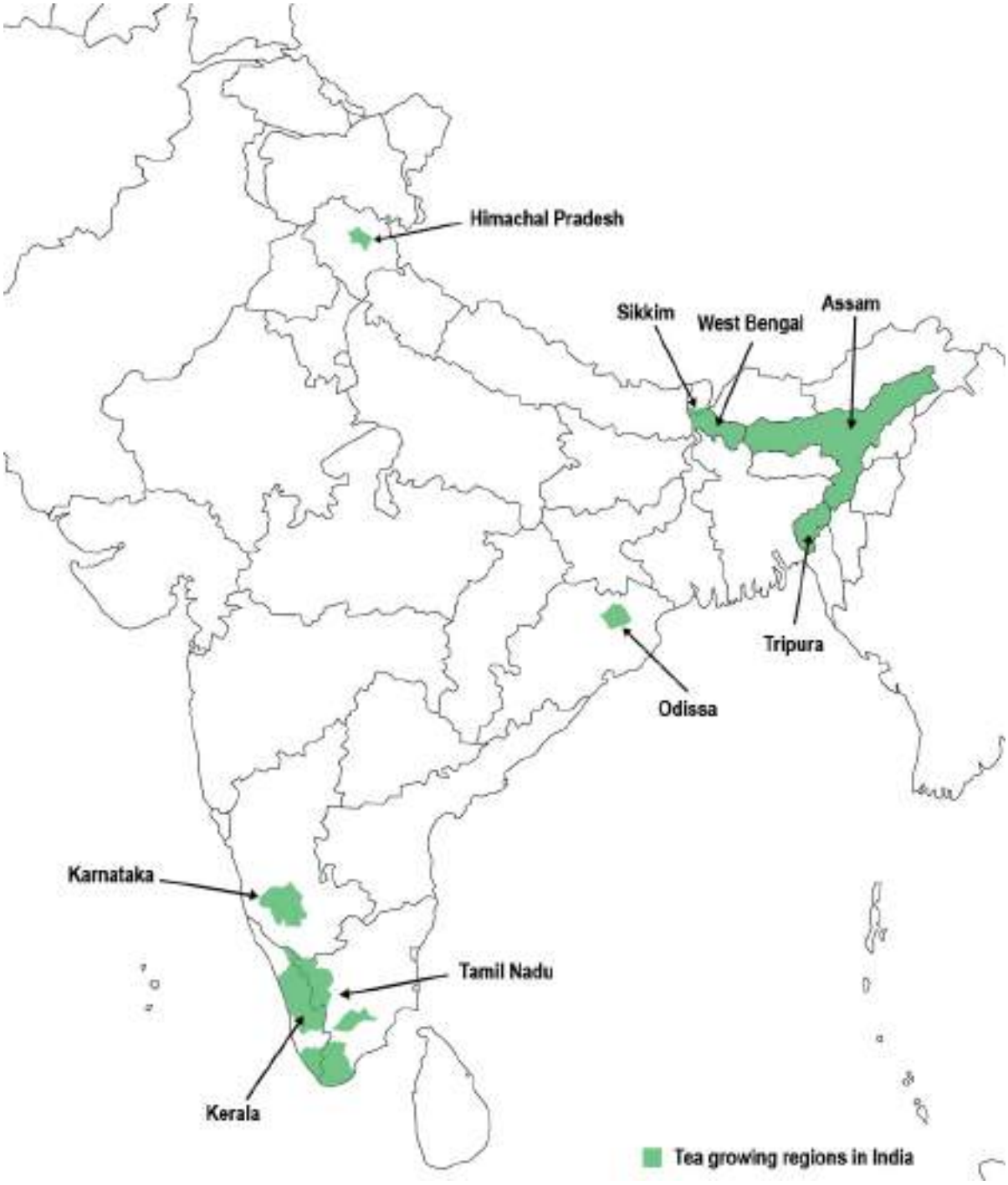


Figure 1-1: Tea growing regions in India (Map not to scale).

1.1.2 Looper pests in the Tea plantations of the Terai-Dooars region of India

Tea plantations of the Terai and Dooars region of West Bengal and Assam are experiencing severe attacks of different leaf-eating lepidopteran pests, among which, *Biston (Buzura) suppressaria* Guen (Lepidoptera: Geometridae) was reported as a major tea pest for several decades from Assam and the Terai-Dooars region of North Bengal. Recent studies reveal that a few other species of geometrid pests are attacking tea plantations among which the pest species, *Hyposidra talaca* Walker (Lepidoptera: Geometridae), the early caterpillar of which is commonly known as 'Black Inch Looper' (**Figure 1-2**), has taken over as the major defoliating pest in tea plantations (Das et al., 2010a). Loopers of this species that primarily feed on a number of forest plants and weeds in India, Malaysia and Thailand (Das and Mukhopadhyay, 2009; Mathew et al., 2005; Winotai et al., 2005) have turned to tea as an active defoliating insect pest in plantations of Assam and Darjeeling Terai-Dooars of North Bengal (Basumajumdar and Ghosh, 2004; Das et al., 2010b). A substantial loss in tea production due to this defoliating lepidopteran pest has been reported in these regions (Gurusubramanian et al., 2008; Hazarika et al., 2008), severely has affected the economy of the country (Roy and Muraleedharan, 2014).

Figure 1-2: Caterpillar of *Hyposidra talaca*.

1.1.3 Management of Tea pest population

To manage pest infestations, there are two main methods of pest control: chemical and biological. The looper pests have so far been controlled by regular application of synthetic chemical pesticides especially (**Figure 1-3**), organophosphates and pyrethroids, but, the pests are gradually becoming less susceptible, and have developed some extent of resistance to the pesticides (Das et al., 2010b; Das and Mukhopadhyay, 2008) often resulting in their control failures (Sannigrahi and Talukdar, 2003). Moreover, the application of these synthetic chemical insecticides is one of the major sources of pollution of soil and water (Saravanan et al., 2009; Singh et al., 2017) and is reported to be hazardous to non-target organisms including humans (Azmi et al., 2006; Mobed et al., 1992; Saravanan et al., 2009; Velmurugan et al., 2006). Because of these harmful effects, the application of a number of these insecticides has been banned by the Environmental Protection Agency (EPA) of the USA and even by the Tea Research Organizations of India. The registration of many other insecticides has been reviewed (Chattopadhyay et al., 2004) and recommended under the Plant Protection Codes (PPC). Therefore, concerning the fact of development of pesticide resistance to insect pests and the hazardous effects of chemical pesticides, organic tea has become more acceptable, especially for export and health-conscious tea consumers than the chemically managed conventional tea. Because of this, the protection and production of tea need to be largely dependent on non-conventional including biological control methods.

Figure 1-3: Application of Chemical pesticides in tea plantations.

The use of biological agents as biopesticides in agricultural fields is an integral part of pest management. Biocontrol is thus being considered an alternative or supplementary means for reducing the use of synthetic chemicals in agriculture (Gerhardson, 2002; Postma et al., 2003; Welbaum et al., 2010). The use of genetically modified cotton with *Bt* toxin against bollworms in China, despite initial success, is ineffective due to having no direct benefit to consumers, no substantial economic benefits to small farmers and weak intellectual property rights (Pray et al., 2001). *Bt* brinjal has also caused great controversy about its use in India (Seetharam, 2010). Recent studies in India revealed that GM plants with *Bt* endotoxin Cry1Ac have detrimental effects on the regeneration, growth and development of cotton and tobacco plants (Rawat et al., 2011). So, as an alternative option to GM crop application, baculoviruses that infect a diverse group of insects can be tried as potential biopesticides which is target specific and safe for the environment and health.

1.1.4 Baculovirus as bioinsecticide

Out of 19 virus families pathogenic to insects (**Table 1-1**), Baculoviridae is most intensely studied because of their killing efficacy to insect hosts (King et al., 2012). Baculoviridae is globally known for their application as biopesticide/bioinsecticide to control insect pest populations (Sun and Peng, 2007). The family Baculoviridae includes a large group of DNA viruses that are pathogenic to several arthropod species, primarily to the insects of the order Lepidoptera and also to the Hymenoptera and Diptera (King et al., 2012). Before the 20th century, approximately 700 baculoviruses have been identified from different lepidopteran, hymenopteran, and dipteran insects (Moscardi, 1999).

Their application to crops can be an alternative eco-friendly approach for pest management to minimize the use of synthetic chemical pesticides. Moreover, baculoviruses have also proved themselves as a powerful expression vector for expressing genes of the insect as well as vertebrate origin (Hu, 2006, 2005), and are successfully applied in the production of beneficial proteins (Shrestha et al., 2008). Being host-specific, baculoviruses are very safe for industrial production and their field applications (Sumathy et al., 1996), as microbial insecticides in integrated pest management (IPM) programs (Kunimi, 2007).

Table 1-1: List of virus families and the corresponding genera pathogenic to insects (summarized from King et al. (2012).

Sl. No.	Virus family	Genus	Host
1.	Ascoviruses	Unclassified	Lepidopteran larvae and pupae
2.	Baculoviridae	Alphabaculovirus	Lepidoptera
		Betabaculovirus	Lepidoptera
		Gammabaculovirus	Hymenoptera
		Deltabaculovirus	Diptera
3.	Iridovirus	Unclassified	Insects in aquatic and damp habitats
4.	Polydnavirus	Unclassified	Hymenoptera
5.	Poxiviridae	Entomopoxiviridae	
6.	Parvoviridae	Densoviridae	Dictyoptera, Diptera, Hemiptera, Homoptera, Lepidoptera, Odonata, Orthoptera
7.	Metaviridae	Semotivirus	Diptera
8.	Pseudoviridae	Hemivirus	Diptera
9.	Birnaviridae	Entomobirnavirus	Diptera
10.	Reoviridae	Cypoviruses	Lepidoptera
11.	Rhabdoviridae	Ephimerovirus	Diptera
		Vesiculovirus	Insect vector
12.	Orthomyxoviridae	Thogovirus	Insect vector
13.	Dicistroviridae	Cripavirus	Diptera, Lepidoptera, Orthoptera, Hemiptera and Hymenoptera
		Aparavirus	Hymenoptera, Dendrobranchiata
14.	Iflaviridae	Iflavirus	Lepidoptera
15.	Flaviviridae	Flavivirus	Diptera, Dendrobranchiata
16.	Nodaviridae	Alphanodavirus	Coleoptera, Diptera, Hemiptera etc.
17.	Tetraviridae	Betatetravirus	Lepidoptera
		Omegatetravirus	Lepidoptera
18.	Togaviridae	Aphavirus	Insect vector
19.	Tymoviridae	Marafivirus	Insect vector

1.1.5 Genome structure of the family Baculoviridae

The family Baculoviridae, possesses a circular, covalently closed, double-stranded DNA genome ranging from 80-180 kb in length, encoding for 100-200 proteins (King et al., 2012).

Baculovirus genome contains several functional genes required for oral infectivity, host-specific interaction, replication, transcription, packaging of the virus, cell cycle regulation etc. (discussed later in the "Review of literature").

1.1.6 Classification of Baculoviridae

Based on the major matrix protein present in the occlusion body, baculoviruses are of two types, nucleopolyhedrovirus (NPV) having polyhedrin as the major matrix protein and granulovirus (GV) consisting of granulin as the major matrix protein.

Based on the core genes, baculoviruses are phylogenetically divided into four genera: **Alphabaculovirus** (lepidopteran-specific NPV), **Betabaculovirus** (lepidopteran-specific Granulovirus), **Gammabaculovirus** (hymenopteran-specific NPV), and **Deltabaculovirus** (dipteran-specific NPV) (King et al., 2012). Based on the presence of the *gp67/gp64* fusion protein gene, the alphabaculoviruses have been further divided into two groups (Herniou et al., 2003):

i) Group I Alphabaculovirus - *gp67/gp64* fusion protein gene(s) is present, and
ii) Group II Alphabaculovirus - *gp67/gp64* fusion protein gene(s) is absent, instead fusion protein F is present.

1.1.7 Nucleopolyhedroviruses in IPM

Nucleopolyhedroviruses are well accommodated with integrated pest management (IPM) strategies because of their compatibility with other biological and chemical agents. Baculoviruses also have a proven safety track record if applied wisely with minimal effects on non-target beneficial insects such as honey bees and silk worms. They require an alkaline (pH 9-11) digestive system for dissolving the protein matrix to release their virions from the occlusion body to initiate infection. As the proteolytic digestive system in humans and other vertebrates are primarily functional at acidic pH, baculoviruses do not appear to cause health hazards to human and other vertebrates (Persley, 1996). Moreover, NPVs have greater potential as biopesticides because of their greater killing efficiency and their continuous production in selective insect cell lines that support a high level of their multiplication (Bonning, 2005). Therefore, microbial insect pest control with NPV can be very effective as well as eco-friendly.

1.1.8 Morphological structure of NPV

NPV contains a circular, covalently closed, double-stranded DNA genome which is surrounded by a small basic protein that neutralizes the negative charge of the DNA. This structure is protected by proteins forming a rod-shaped nucleocapsid of 30-60 nm X 250-300 nm (King et al., 2012). On the basis of nucleocapsids per virion, NPVs can further be morphologically divided into two groups. The single nucleopolyhedroviruses (SNPVs) contain a single nucleocapsid per virion, whereas the multiple-nucleopolyhedroviruses (MNPVs) contain multiple nucleocapsids per virion (Rohrmann, 2011).

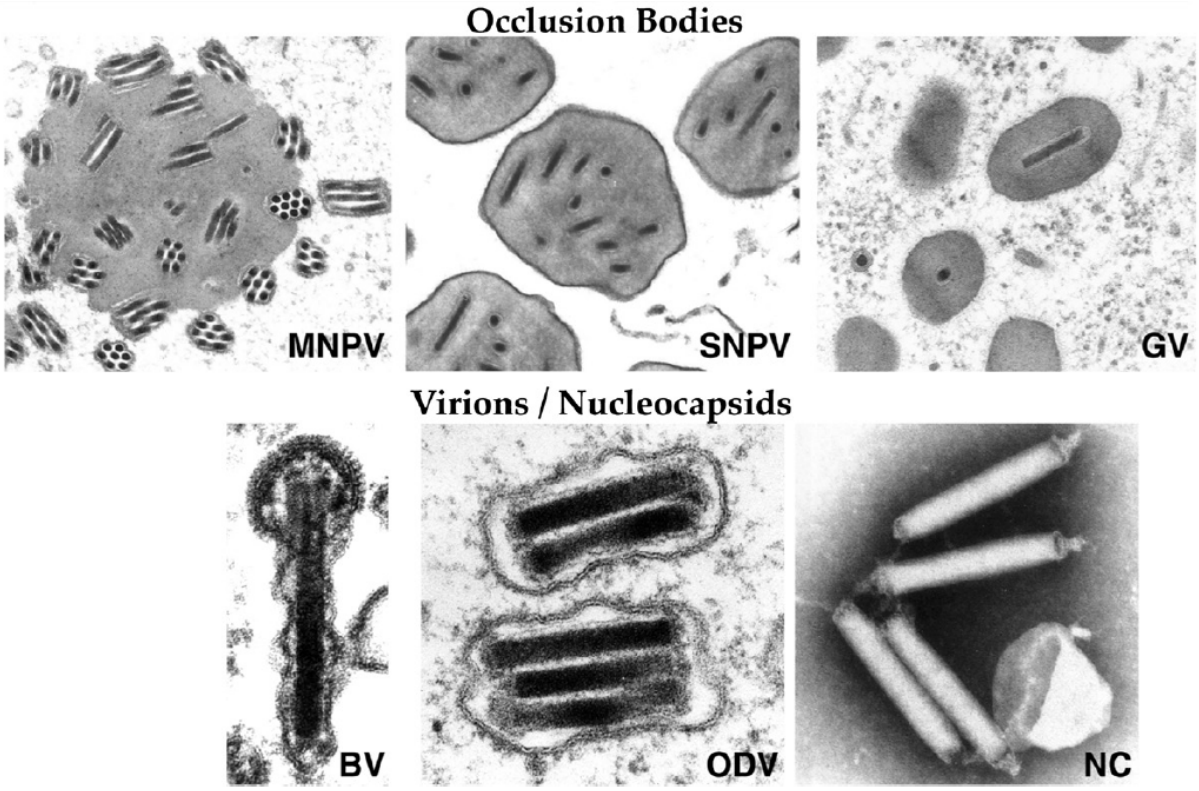


Figure 1-4: Transmission electron micrographs of occlusion bodies (MNPV, SNPV and GV), and other forms of baculoviruses, BV (budded virions), ODV (occlusion-derived virions) and nucleocapsids (NC). The picture was taken from King et al. (2012).

The virions are subsequently occluded in large protein crystals forming polyhedra or Occlusion body (OB) (**Figure 1-4**). The occluded form of NPVs is polyhedral in shape and each occlusion body is 0.15 to 15 µm in size, matures within nuclei of infected cells and characteristically contains many enveloped virions (King et al., 2012).

NPVs produce two types of virions during their infection cycle, which enable the virus to replicate efficiently within the infected body and spread its progeny among insects. Occlusion-derived viruses (ODVs) released in the environment from the dead insects

transmit infections among insects (oral infection), whereas budded viruses (BVs) within the insect body spread the infection to neighbouring cells (Keddie et al., 1989). At the late stage of infection, greatly enhanced dispersal behaviour of infected larva/ adult is observed (Goulson, 1997) followed by dramatic degradation of the host cadaver (Federici, 1997) and this pathogenicity is highly species-specific.

Occlusion-derived virus (ODV): The ODV is encapsulated or occluded in a protein crystal or matrix predominantly composed of a single protein called polyhedrin. The occluded virus is highly stable, which allows it to persist in the environment and promotes seasonal infections of susceptible insects (**Figure 1-5**).

Budded virus (BV): The budded virus is not occluded in any type of protein matrix and has an envelope distinct from ODV that facilitates systemic infection (Funk et al., 1997; King et al., 2012) (**Figure 1-5**).

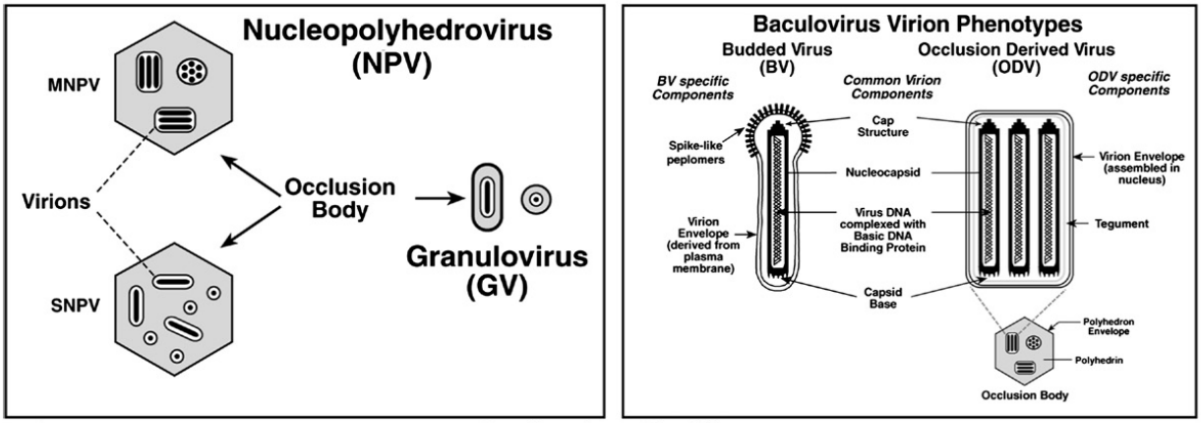


Figure 1-5: Morphology of MNPV, SNPV, Budded virus, Occlusion derived virus and Occlusion body. The Picture was taken from King et al. (2012).

1.1.9 The infection cycle of NPV

The infection cycle of NPV in nature starts when insect larvae ingest occlusion bodies (OBs) present on the contaminated foliage. When the occlusion body or polyhedron enters the mid gut of a larval host, it gets dissolved under alkaline pH (pH 9-11) releasing the virions (ODVs). These ODVs passing through the peritrophic membrane infect mid-gut cells and proceed to multiply in the nucleus. From this initial infection, budded virions (BVs) are produced which spread the infection to other body tissues such as haemocytes, tracheal cells,

fat body cells and hypodermis, where they cause a secondary infection (Tanada and Kaya, 1993).

During the later stages, the virions are retained within the nuclei of the cells of those tissues where they acquire specific proteins and are eventually embedded into the occlusion body (Tanada and Kaya, 1993)(**Figure 1-7**).

When the insect dies it ruptures to release the polyhedra to infect other insects. Insects killed by NPV infection usually contain up to 100 million OBs (Braunagel and Summers, 1994; Rohrmann, 2011).

1.1.10 Signs and symptoms

NPV-infected caterpillars show some typical symptoms 2-4 days after ingestion of the virus. The following symptoms appear sequentially (Tanada and Kaya, 1993).

i) At first, the larvae progressively cease feeding and become less active.

ii) During advanced stages of the infection as the epidermis is infected the skin becomes very fragile and ruptures easily.

iii) Subsequently, the larvae become wilted, swollen sometimes liquefied containing a mass of decomposed tissue and occlusion bodies (**Figure 1-6**).

iv) Just before death, infected larvae often climb to the highest part of the substrate on which they are located e.g., on top of the plant and attach themselves by their prolegs (Goulson, 1997). On death, they hang in a characteristic inverted V-shaped form.

Figure 1-6: NPV infected dead caterpillar of *H. talaca*.

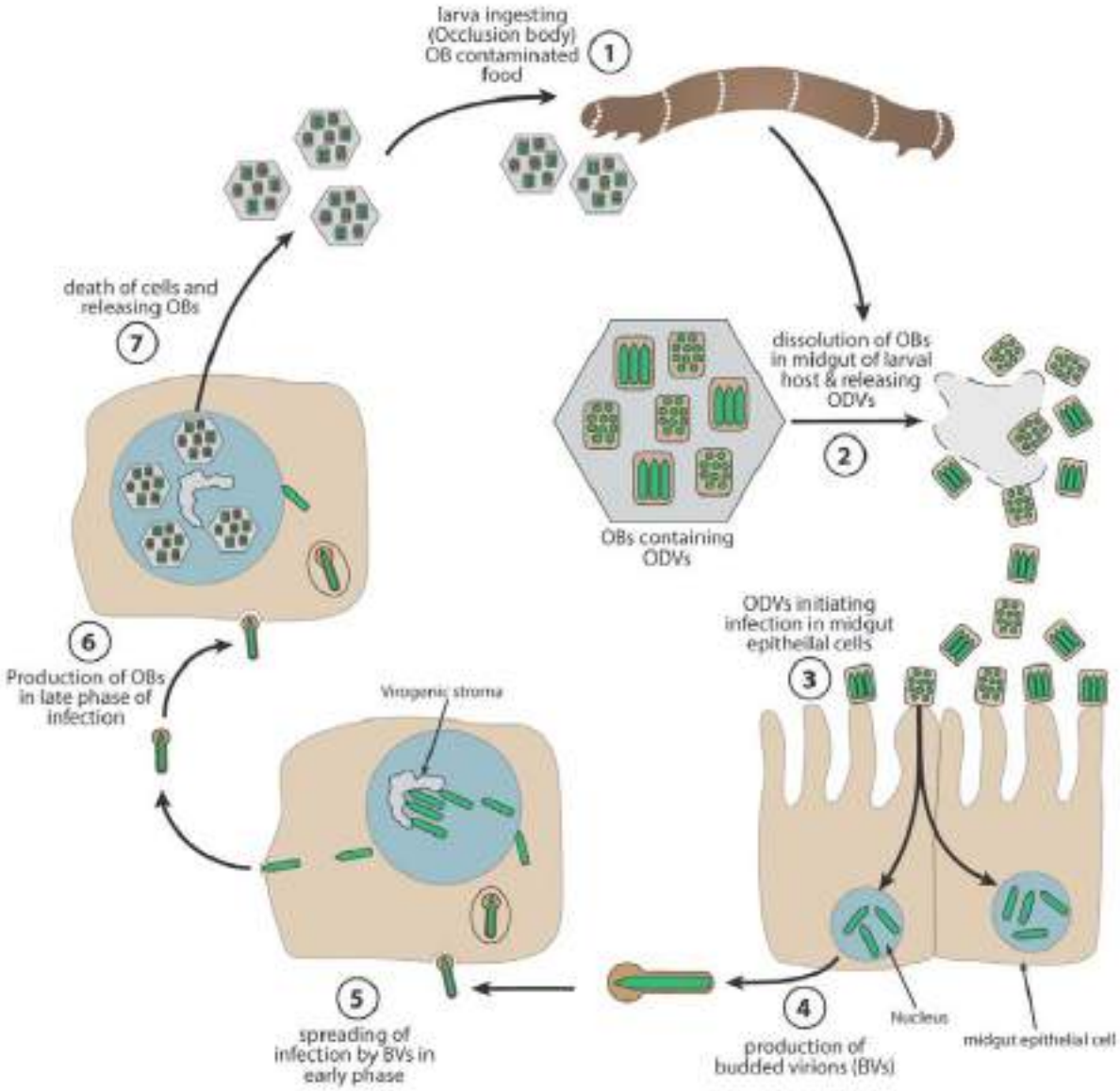


Figure 1-7: Infection cycle of nucleopolyhedrovirus through insect larva.

1.1.11 NPV as bioinsecticides

Selection of a virus adapted to a particular host or ecosystem as a bioinsecticide is done by isolating local strains of NPVs. The majority of NPVs have been isolated from the order Lepidoptera. Development of most pest-control programs utilizing baculoviruses (Cory and Bishop, 1995) is usually done by screening and determining the killing efficacy based on bioassay (LD_{50}/ LC_{50}/ LT_{50}) for their application as potential control agents. Subsequently, large-scale *in vivo* production, and suitable formulation of the virus are done for field use.

Knowledge of pest biology and behaviour is also helpful for an integrated pest management approach to pest control.

1.1.11.1 Disadvantages of the use of Natural NPVs as insecticides

Although numerous naturally occurring baculoviruses have been tested or even commercialized in a few cases, their use has not expanded as greatly as their development. Their global use has been hampered due to the slower speed of killing, narrow host range, product stability, registration and patentability.

1.1.12 Genetically modified NPVs as Bioinsecticides

Because baculoviruses are slower in killing efficiency than synthetic chemical pesticides, genetic modifications which increase the killing efficacy may be helpful for the commercialization of baculoviruses as a bioinsecticide. Introduction of insect-specific toxin gene(s) such as, exogenous toxin genes isolated from the scorpion or spiders, in the NPV genome were shown to increase the killing efficacy of that genetically modified NPV to its natural host (Bonning and Hammock, 1996; Inceoglu et al., 2001, 2006). Infection with a genetically modified NPV expressing a toxin isolated from scorpion *Androctonus australis* showed comparatively more feeding damage in infected larvae than the infection with wild-type baculovirus (Inceoglu et al., 2001). Toxin genes isolated from straw itch mite *Pyemotes tritici* (Burden et al., 2000), *Leiurus quinquestriatus hebraeus* (Froy et al., 2000), ants (Szolajska et al., 2004) or spiders (Hughes et al., 1997), have also been studied to increase the pathogenicity of the baculoviruses. NPV recombinants producing occlusion bodies with *Bacillus thuringiensis* (*Bt*) toxin, were constructed by incorporating a fusion protein consisting of polyhedrin and *Bacillus thuringiensis* (*Bt*) toxin, in the NPV genome (Chang et al., 2003).

Altering the physiology of the insect host by introducing gene(s) expressing some insect hormones or hormone-modifying enzymes into the NPV genome, can also be an approach to increase the killing efficacy of the virus. Such an approach was applied by incorporating genes like juvenile hormone esterase into the NPV genome to decrease the concentration of juvenile hormone in the host which in turn produces a signal for the caterpillar to starve and pupate (Hammock et al., 1990; Inceoglu et al., 2001). Another approach is the deletion of the

baculovirus-encoded ecdysteroid glucosyltransferase (*egt*) gene (O'reilly and Miller, 1991). Deletion of the *egt* gene from the NPV genome results in 30% faster killing of caterpillars altering the normal moulting of larva and indirectly increasing the time of feeding of infected caterpillars. The *egt* gene is not associated with viral replication and replacing it with an exogenous gene, such as any toxin gene, may increase the insecticidal activity of the recombinant virus (Sun et al., 2004).

1.1.12.1 Advantages of genetically modified NPVs as bioinsecticides

Genetically modified (GM) baculovirus can kill up to 30% faster than naturally occurring NPVs and are more stable and efficient than natural NPVs. Moreover, GM baculoviruses are non-pathogenic to vertebrate species (Sun et al., 2004) as well as to the natural enemies of larvae such as parasitoids and predators (Boughton et al., 2003).

Therefore, the genetically modified baculovirus strains can be used as potential biopesticides in future due to their eco-friendly and target-specific application.

1.1.13 *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV)

Recent studies in the tea plantations of Darjeeling foothills and Terai and the Dooars of north-eastern India revealed that the looper activities especially of *Hyposidra talaca* (Lepidoptera: Geometridae), a major defoliating insect pest in tea plantations, have greatly increased (Das et al., 2010a). A nucleopolyhedrovirus isolated from the loopers of *Hyposidra talaca* has been found pathogenic to this insect pest (Mukhopadhyay et al., 2011). Hence, *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) has the potential of being used as a bioinsecticide, especially as an alternative to synthetic chemical pesticides, both in organic as well as in bio-rationally managed plantations. So, to increase the pest control efficacy of HytaNPV and develop this NPV into a more efficient recombinant baculovirus, genetic characterization and comparison of the locally occurring isolates of HytaNPV from the Terai-Dooars belt are much required. The contemplated investigation done in this study is intended for a better understanding of variants of the viral genome.

Section 1.2: Review of literature

Because of the control failure of insect pests, their increased resistance and biohazards resulting from the overuse of chemical pesticides, baculovirus formulation are evolving as one of the promising biopesticides which is target specific, and largely non-hazardous to the environment. Its use as an efficient biopesticide is expected to reduce the application of synthetic chemical pesticides. In this regard, genome characterization, as well as modification of some baculoviruses, has been carried out globally to understand their degree of virulence and killing efficacy. This chapter of the thesis mainly consists of a review of literature on the baculovirus genome, the application of baculoviruses as bioinsecticides and the scope of genetic characterization of HytaNPV.

1.2.1 Molecular characterization of Baculovirus genome by Restriction endonuclease (REN) fragment analyses

One of the most traditional and common approaches to characterizing a virus genome and identifying, comparing and differentiating different isolates or variants of a virus is to perform a restriction endonuclease fragment analysis of its genome (Hu et al., 1998). Physical mapping of the baculovirus genome has been carried out since the 1980s. A physical map of the genome of *Autographa californica* NPV had been constructed by Miller and Dawes (1979) with *Bam*HI, *Xma*I, *Eco*RI and *Hin*dIII. A restriction map of the *Heliothis zea* single NPV (HzSNPV) genome was concocted with a set of six restriction endonucleases by Knell and Summers (1984). The Physical map of *Heliothis zea* single NPV (Hz-1) baculovirus genome by restriction mapping was studied by Chao et al. (1990). Crawford et al. (1985) reported the physical map of *Oryctes* baculovirus with four restriction endonucleases, *Bam*HI, *Eco*Rl, *Hin*dlII and *Pst*I and estimated the genome size to be 127.03 kb. A physical map of the *Mamestra configurata* NPV genome was prepared by Li et al. (1997) and the genome size was estimated to be about 156 kb.

Chen et al. (2000) characterized the genome organization of *Helicoverpa armigera* NPV (HearNPV) to study the infectivity of the virus to be used as biopesticide and reported the genome size of HearNPV to be 130.1 kb, whereas the NPV isolated from *Helicoverpa assulta*, the closest relative of HearNPV was found to have a genome size of 138 kb (Woo et al., 2006) which is 8 kb higher than that of the former. The Restriction endonuclease

fragment profile along with restriction mapping and estimation of genome size of BusuNPV isolated from another looper pest of tea, *Buzura suppressaria* has been reported in China with a mean genome size of 129 kb (Liu et al., 1993), however, Hu et al. (1998) and his collaborators documented its genome size to be 120.9 kb.

In India similar study was carried out on *Spodoptera littura* NPV where Das and Prasad (1996) reported the restriction endonuclease fragment profile of the viral genome. *S. littura* is a polyphagous insect pest of tobacco, cotton, soybean, beet etc.

Christian et al. (2001) proposed a rapid method for the identification and differentiation of nucleopolyhedroviruses pathogenic to *Helicoverpa* by RFLP analysis. Therefore, REN fragment analysis was found and established as a useful molecular tool for the identification and comparative differentiation of baculovirus isolates.

1.2.2 Molecular characterization of Baculovirus genome by sequencing

To date genomes of about 70 nucleopolyhedroviruses infecting hosts of different insect species have been sequenced (NCBI databases) and many have been analysed and published (King et al., 2012; Rohrmann, 2011).

The first NPV to be completely sequenced was *Autographa californica* NPV (AcMNPV) (Ayres et al., 1994). Each complete sequence has provided a wealth of data about the complement of genes present in each virus. A preliminary physical map of the genome of AcMNPV was constructed in the order of *Bam*HI and *Xma*I restriction sites and partial order of *Eco*RI and *Hin*dIII restriction sites (Lee and Miller, 1978).

With the advent of new and sophisticated tools and techniques in the field of molecular biology, the genomes of the different host-specific NPVs were extensively analysed and characterized. Names and genome sizes of different host-specific NPVs are presented in **Table 1-2**.

Table 1-2: Genome size of different host-specific baculovirus.

Sl. No.	Name of NPV	Abbreviation	Genome Size (bp)	References
Alphabaculovirus: Group I				
1	*Autographa californica* NPV	AcNPV	133,894	(Ayres et al., 1994)
2	*Bombyx mori* NPV	BmNPV	128,413	(Gomi et al., 1999)
3	*Orgyia pseudotsugata* MNPV	OpMNPV	131,990	(Ahrens et al., 1997)
4	*Epiphyas postvittana* MNPV	EppoMNPV	118,584	(Hyink et al., 2002)
5	*Rachiplusia ou* MNPV	RoMNPV	1315,00	(Harrison and Bonning, 2003)
6	*Choristoneura fumiferana* MNPV	CfMNPV	129,593	(de Jong et al., 2005)
7	*Choristoneura fumiferana* defective NPV	CfDEFNPV	131,160	(Lauzon et al., 2005)
8	*Hyphantria cunea* NPV	HycuNPV	132 959	(Ikeda et al., 2006)
9	*Antheraea pernyi* NPV	AnpeNPV	126,629	(Nie et al., 2007)
10	*Plutella xylostella* MNPV	PlxyMNPV	134,417	(Harrison and Lynn, 2007)
11	*Anticarsia gemmatalis* MNPV	AgMNPV	131,68	(de Brito et al., 2016)
12	*Maruca vitrata* NPV	MaviNPV	111,953	(Chen et al., 2008)
13	*Bombyx mandarina* NPV	BomaNPV	126,770	(Xu et al., 2010)
14	*Catopsilia pomona* NPV	CapoNPV	128,058	(Wang et al., 2016)
15	*Thysanoplusia orichalcea* NPV	ThorNPV	132,978	(Wang et al., 2012)
16	*Lonomia obliqua* NPV	LoobNPV	120,023	(Aragão-Silva et al., 2016)
17	*Philosamia cynthia ricini NPV*	PhcyNPV	125,376	(Qian et al., 2013)
18	*Choristoneura murinana NPV*	ChmuNPV	124,688	(Rohrmann et al., 2014)
19	*Choristoneura occidentalis NPV*	ChocNPV	128,446	(Thumbi et al., 2013)
20	*Choristoneura rosaceana NPV*	ChroNPV	129,052	(Thumbi et al., 2013)
Alphabaculovirus: Group II				
1	*Mamestra configurata* NPV	MacoNPV	155,060	(Li et al., 2002)
2	*Lymantria dispar* MNPV	LdMNPV	161,046	(Kuzio et al., 1999)
3	*Spodoptera exigua* MNPV	SeMNPV	135,610	(Ijkel et al., 1999)
4	*Helicoverpa armigera* SNPV G4 isolates	HearSNPV-G4	131,403	(Chen et al., 2001)
5	*Spodoptera litura* MNPV	SpltMNPV	139,342	(Pang et al., 2001)
6	*Helicoverpa zea* SNPV	HzSNPV	130,869	(Chen et al., 2002)
7	*Adoxophyes honmai* NPV	AdhoNPV	113,220	(Nakai et al., 2003)

Table 1-2: Genome size of different host-specific baculovirus, continued from page 25.

Sl. No.	Name of NPV	Abbreviation	Genome Size (bp)	References
8	*Chrysodeixis chalcites* NPV	ChchNPV	149,622	(Van Oers et al., 2004)
9	*Helicoverpa armigera* ANPV C1 isolates	HearSNPV-C1	130,759	(Zhang et al., 2005)
10	*Trichoplusia ni* SNPV	TnSNPV	134,394	(Willis et al., 2005)
11	*Agrotis segetum* NPV	AgseNPV-A	147,544	(Jakubowska et al., 2006)
12	*Clanis bilineata* nucleopolyhedrovirus	ClbiNPV	135,454	(Zhu et al., 2009)
13	*Leucania seperata* NPV	LsNPV	168,041	(Xiao and Qi, 2007)
14	*Spodoptera frugiperda* MNPV	SfMNPV	131,330	(Harrison et al., 2008)
15	*Agrotis ipsilon* MNPV	AgipMNPV	155,122	(Harrison, 2009)
16	*Adoxophyes orana* NPV	AdorNPV	113,220	(Hilton and Winstanley, 2008)
17	*Helicoverpa armigera* NPV NNg1 strain	HearNPV-NNg1	132,425	(Ogembo et al., 2009)
18	*Lymantria xylina* MNPV	LyxyMNPV	156,344	(Nai et al., 2010)
19	*Ectropis obliqua* NPV	EcobNPV	131,204	(Ma et al., 2007)
20	*Euproctis pseudoconspersa* NPV	EupsNPV	141,291	(Tang et al., 2009)
21	*Apocheima cinerarium NPV*	ApCiNPV	123,876	Zhang et al, 2009 (Unpublished)
22	*Sucra jujuba NPV*	SujuNPV	135,952	(Liu et al., 2014)
23	*Hyposidra talaca NPV*	HytaNPV	139,089	(Nguyen et al., 2018)
24	*Buzura suppressaria NPV*	BusuNPV	120,420	(Zhu et al., 2014)
25	*Lambdina fiscellaria* NPV	LafiNPV	157,977	(Rohrmann et al., 2015)
26	*Orgyia leucostigma* NPV	OrleNPV	157,179	(Thumbi et al., 2011)
Deltabaculovirus				
1	*Culex nigripalpus* NPV	CuniNPV	108,252	(Afonso et al., 2001)
Gammabaculovirus				
1	*Neodiprion lecontei* NPV	NeleNPV	81,755	(Lauzon et al., 2004)
2	*Neodiprion sertifer* NPV	NeseNPV	86,462	(Garcia-Maruniak et al., 2004)
3	*Neodiprion abietis* NPV	NeabNPV	84,264	(Duffy et al., 2006)

1.2.3 Phylogenetic analyses of baculoviruses

The application of baculoviruses as bioinsecticide in different agricultural fields and orchards to curb the pest populations encourages their constant study in an attempt to

understand the molecular mechanisms of their evolution and the phylogenetic relationship among them.

The genes whose orthologs are present in all baculovirus genera are considered core genes which are more conserved than the other baculovirus genes. Therefore, one of the effective and traditional markers to assess the phylogenetic relationships of baculoviruses was the amino acid or nucleotide sequence analyses of a single conserved gene such as *polyhedrin/granulin*, the envelope fusion polypeptides known as F protein and GP64, DNA polymerase or late expression factors etc. (Cowan et al., 1994; Paolo et al., 1993).

Polyhedrin is the most conserved gene present in all nucleopolyhedroviruses and is used to study the phylogenetic divergence of different NPVs (Antony et al., 2011; Bulach et al., 1999; Dasgupta et al., 2016; Paolo et al., 1993; Woo et al., 2006). Orthologs of *polyhedrin* are found in all baculoviral genomes either in the form of *polyhedrin* in nucleopolyhedroviruses (NPVs) or *granulin* in granuloviruses (GVs), except in the dipteran-specific NPVs, where it has an occlusion body protein unrelated in the primary amino acid sequence of the *polyhedrin* gene of the other NPVs and is about three times larger than the other forms (Afonso et al., 2001; Perera et al., 2006).

Dnapol is another conserved gene used to detect the phylogenetic relationships of different baculoviruses (Bulach et al., 1999). Single gene phylogenetic analysis of *Bombyx mori* NPV was conducted by Liang et al. (2013) based on *p10*, another core gene in baculoviruses. The evolutionary divergence of *Spodoptera littura* NPV was analysed based on *lef-8*, another conserved gene in baculoviruses (Wang et al., 2001).

Robust phylogenetic inferences have been obtained based on the sets of genes with homologous sequences in all baculoviruses. Therefore, most of the modern approaches were to find out the core baculovirus genes and the phylogenetic analyses based on core-protein concatemers (Jehle et al., 2006). Recently parsimonious phylogenetic inferences were procured based on the analyses of concatenated amino acid alignment of the core genes obtained from the complete genome sequences of baculoviruses (Nguyen et al., 2018; Zhu et al., 2014).

To date, 37 core genes have been identified in baculoviruses (Nguyen et al., 2018) and concatenated sequences of a combination of core genes can be used for phylogenetic

analyses. In a comparative study of 57 baculovirus strains comprising 31 core genes, Miele et al. (2011) showed that *pif-2, lef-9, lef-8* etc. were more highly conserved compared to the others.

1.2.4 Baculovirus core genes

Baculovirus contains double-stranded circular DNA genomes of 80 to 180 kb. A key feature to explore and manipulate the baculovirus genome for different biotechnological applications is to determine the core genes. The core genes have been classified according to their function such as genes involved in replication, transcription, packaging of the virus, cell cycle regulation and oral infectivity of the virus (**Table 1-3**) (Rohrmann, 2011).

Table 1-3: Core genes found in Baculoviruses [Adapted form Rohrmann, (2011)].

Genes	AcMNPV ORF	References
Matrix gene		
polyhedrin/ granulin	ORF - 8	(Rohrmann, 2011)
Envelope gene of BVs		
GP64 / Fusion protein	ORF - 128	(Herniou et al., 2003; Ijkel et al., 2000)
Replication		
lef-1	ORF - 14	(Evans et al., 1997)
lef-2	ORF - 6	(Evans et al., 1997)
DNA pol	ORF - 65	(Vanarsdall et al., 2005)
Helicase	ORF - 95	(Bideshi and Federici, 2000; McDougal and Guarino, 2000)
Transcription		
lef-4	ORF - 90	(Gross and Shuman, 1998a; Guarino et al., 1998a)
lef-8	ORF - 50	(Titterington et al., 2003)
lef-9	ORF - 62	(Iorio et al., 1998)
p47	ORF - 40	(Guarino et al., 1998b; McLachlin and Miller, 1994)
lef-5	ORF - 99	(Todd et al., 1996)
Cell cycle arrest and/or interaction with host proteins		
odv-e27	ORF - 144	(Belyavskyi et al., 1998; Braunagel et al., 1996b)
ac81	ORF - 81	(Chen et al., 2007)

***AcMNPV ORF = *Autographa californica* multiple nucleopolyhedrovirus (Genbank acc. no. L22858.1) open reading frame**

Table 1-3: Core genes found in Baculoviruses [Adapted from Rohrmann, (2011)], continued from page 28.

Genes	AcMNPV ORF	References
Packaging, assembly and release		
ODV-E25	ORF - 94	(Hong et al., 1997; Nguyen et al., 2018)
ODV-E43	ORF - 109	(Fang et al., 2003)
ODV-E18	ORF - 143	(Braunagel et al., 2003; Deng et al., 2007)
p6.9	ORF - 100	(Wang et al., 2010; Wilson et al., 1987)
vp39	ORF - 89	(Wang et al., 2010)
vlf-1	ORF - 77	(McLachlin and Miller, 1994; Mikhailov and Rohrmann, 2002a)
alk-exo	ORF - 113	(Mikhailov and Rohrmann, 2002b)
vp1054	ORF - 54	(Olszewski and Miller, 1997a)
vp91/p95	ORF - 83	(Russell and Rohrmann, 1997)
gp41	ORF - 80	(Whitford and Faulkner, 1992a, 1992b)
38k	ORF - 98	(Woo et al., 2006)
49k	ORF - 142	(Vanarsdall et al., 2007; Yang et al., 2008)
odv-nc42	ORF - 101	(Braunagel et al., 2003)
desmoplakin	ORF - 66	(Ke et al., 2008)
p33	ORF - 92	(Nie et al., 2011; Wu and Passarelli, 2010)
Oral infectivity		
pif-0/p74	ORF - 138	(Haas-Stapleton et al., 2004; Simón et al., 2005)
pif-1	ORF - 119	(Braunagel et al., 2003; Ohkawa et al., 2005)
pif-2	ORF - 22	(Braunagel et al., 2003; Simón et al., 2005)
pif-3	ORF - 115	(Braunagel et al., 2003)
pif-4/19k/odv-e28	ORF - 96	(Fang et al., 2009)
pif-5/odv-e56	ORF - 148	(Braunagel et al., 1996a; Sparks et al., 2011)
pif-6	ORF - 68	(Nguyen et al., 2018; Zhu et al., 2014)

***AcMNPV ORF = *Autographa californica* multiple nucleopolyhedrovirus (Genbank acc. no. L22858.1) open reading frame.**

1.2.5 Baculovirus Core Proteins

Several core proteins have been identified in baculoviruses involved in structural integrity and the process of infection, packaging, assembly, and release of OBs. A brief description of the core baculoviral proteins has been given below.

1.2.5.1 Matrix Protein

Polyhedrin: Polyhedrins are the major structural components of occlusion bodies found in all nucleopolyhedroviruses (NPV). NPVs produce polyhedrin protein at a very high level in the late phase of infection. Polyhedrin is about 250 amino acids long with a molecular weight of about 30kDa. The 30 kDa subunits form trimers that are arranged into dodecamers (four trimers) via disulfide bonds (Rohrmann, 2011).

1.2.5.2 Envelope proteins of budded virions

GP64: GP64 is a characteristic protein and major distinguishing feature of group I Alphabaculovirus (Herniou and Jehle, 2007; Jiang et al., 2009). It is a fatty acid acylated glycoprotein (Roberts and Faulkner, 1989), activated at low pH and functions as a fusion protein (Hohmann and Faulkner, 1983).

Fusion protein-F: Orthologs of this protein are present in Group II alphabaculovirus, betabaculovirus and deltabaculovirus, but absent in group I alphabaculovirus, where GP64 acts as an active fusion protein (Herniou and Jehle, 2007). The only exception was gammabaculovirus (hymenopteran NPVs) which lack homologs of both the F and GP64 proteins. F proteins of Group II NPVs function as low-pH envelope fusion proteins (Ijkel et al., 2000).

1.2.5.3 Oral infectivity factors/ *per os* infectivity factors (*pif*) (additional envelope proteins of ODVs)

Seven conserved *pif* genes, so far, have been identified as involved in the process of oral infection in several nucleopolyhedroviruses which are: *p74* (Kuzio et al., 1989; Yao et al., 2004), *pif-1* (Kikhno et al., 2002), *pif-2* (Fang et al., 2006; Pijlman et al., 2003), *pif-3* (Li et al., 2007; Ohkawa et al., 2005), *pif-4* (Fang et al., 2009; Huang et al., 2012; Zhu et al., 2014), *pif-5* (Zhu et al., 2014) and *pif-6* (Nie et al., 2012; Zhu et al., 2014). These genes are commonly known as *per os* infectivity factors (PIFs) and are required for oral infection of insects but superfluous for infection in cultured cells. These factors or proteins are only present in ODVs. Mutations causing lack of expression of *pif-1* (Kikhno et al., 2002), *pif-2* (Fang et al., 2006), *pif-3* (Li et al., 2007), *pif-4* (Fang et al., 2009) and *p74* (Yao et al., 2004) restrict viral oral infection prior to gene expression in midgut cells suggesting that PIFs are

important for ODV oral infection and are involved in early infection events (Fang et al., 2006). A recent study showed that the PIF1, PIF2, PIF3, PIF4 and P74 form a PIF complex during early infection events (Huang et al., 2012). Moreover, PIF1, PIF2 and P74 bring about the specific binding of ODV to the midgut cells of the host, and their involvement in virus-cell interaction in the initial step of infection (Haas-Stapleton et al., 2004; Ohkawa et al., 2005). Though PIF3 is found as an important ODV protein its role in specific binding in midgut cells was not identified instead, it is found to mediate nucleocapsid translocation along the microvilli (Ohkawa et al., 2005). PIFs have strict host specificities and are crucial for deceiving the host range of baculoviruses (Song et al., 2016).

Homologs of *Pif-6* appear to be present in all NPVs (Zhu et al., 2014). Deletion of *pif-6* in AcMNPV causes no major effect other than a longer lethal time in larvae (Li et al., 2008). Similar results were obtained when the ortholog of *pif-6* in BmNPV was deleted from a bacmid. However, the OBs produced by the mutant BmNPV bacmid were abnormal and lacked virions, indicating a role of *pif-6* in polyhedron morphogenesis (Xu et al., 2008).

1.2.5.4 Proteins involved in packaging, assembly, and release

ODV-E25: N-terminal 24 amino acids of ODV-E25 appear to contain a nuclear targeting signal (Hong et al., 1997). Its homologs are present in all baculoviruses (Nguyen et al., 2018).

ODV-EC43: Homologs of ODV-EC43 are present in all baculovirus genomes (Braunagel et al., 2003; Fang et al., 2003; Nguyen et al., 2018). Although deletion of ODV-EC43 in AcMNPV did not affect DNA replication, the virions were not infective (Fang et al., 2009).

ODV-E18: It was found to be associated with ODV by proteomic analysis (Braunagel et al., 2003; Deng et al., 2007). Homologs of ODV-E18 are present in all baculoviruses (McCarthy and Theilmann, 2008). In addition to ODV, it is also associated with BV (Wang et al., 2010).

P6.9: It is a 55 amino acid long arginine/serine/threonine-rich DNA binding protein (Wang et al., 2010; Wilson et al., 1987) which is not essential for DNA replication, but the viruses deleted for the gene are not viable (Wang et al., 2010).

VLF-1: The very late factor (VLF-1), a member of the lambda integrase family of proteins, was reported to influence the hyperexpression of very late genes (McLachlin and Miller, 1994) possibly by binding to their regulatory regions (Yang and Miller, 1999). Mutations that affect the transcription of the very late gene were not lethal, whereas, other mutations required for integrase activity produce inactive viruses (Yang and Miller, 1998).

VP39: Proteomic analysis revealed that vp39 is one of the three most abundant capsid proteins of BV (Wang et al., 2010). Homologs of vp39 are present in all baculovirus genomes (Nguyen et al., 2018).

GP41: It is present in the tegument, a structure found between the virion envelope and capsid (Whitford and Faulkner, 1992a, 1992b). Homologs of GP41 are present in all baculovirus genomes. GP41 is found to have an important role in the release of nucleocapsids from the nucleus and morphogenesis of ODVs (Olszewski and Miller, 1997b).

38K: It interacts with VP1054, VP39, and VP80 (Broyles and Moss, 1986). Deletion of this gene leads to the formation of tube-like structures devoid of DNA (Wu et al., 2006).

49k: Deletion of 49k in AcNPV and BmNPV affects nucleocapsid formation but does not found to affect DNA synthesis (McCarthy et al., 2008; Vanarsdall et al., 2007).

BV/ODV-C42: It is conserved in all baculovirus except *Culex nigripalpus* nucleopolyhedrovirus (CuNiNPV) and expresses a capsid associated proteins in both, BV and ODV (Braunagel et al., 2003).

Desmoplakin: It was reported to play an important role in releasing processes of nucleocapsids from the virogenic stroma to the cytoplasm (Ke et al., 2008).

Alk-exo: This is an alkaline nuclease having 5′-3′ exonuclease and possibly endonuclease activity also (Okano et al., 2004) and appeared to be associated with a DNA binding protein LEF-3 (Mikhailov and Rohrmann, 2002a).

Vp1054: This protein is reported as a virus structural protein required for nucleocapsid assembly (Olszewski and Miller, 1997a).

Vp91/p95: This protein was reported as a structural component in both the capsid and its surrounding envelope of ODVs (Russell and Rohrmann, 1997).

P33: It is a flavin adenine dinucleotide-linked sulfhydryl oxidase which appeared to be involved in the efficient production of virions in the nucleus of infected cells (Deng et al., 2007; Nie et al., 2011; Wu and Passarelli, 2010).

1.2.5.5 Proteins involved in replication

LEF-1: LEF-1 was reported to have a primase-like motif and is important to carry out transient DNA replication (Evans et al., 1997).

LEF-2: LEF-2 was found to interact with LEF-1 and any mutation leading to abortion of this interaction resulted in reduced levels of transient DNA replication (Evans et al., 1997).

DNA Polymerase: Baculovirus genome also expresses DNA polymerase which is conserved in all baculoviruses. Baculovirus lacking *dnapol* was unable to replicate its DNA in the host cell (Vanarsdall et al., 2005).

Helicase: The DNA helicase gene was also present in all baculovirus genomes and is one of the core genes (McDougal and Guarino, 2000).

1.2.5.6 Proteins involved in Transcription

LEF-4: It was reported as a virus-encoded RNA polymerase subunit, having RNA 5′-triphosphatase, nucleoside triphosphatase, and guanylyltransferase activities (Gross and Shuman, 1998b; Guarino et al., 1998a). It has also been shown to be a capping enzyme but the host enzyme can carry out the triphosphatase reaction during capping (Li and Guarino, 2008).

LEF-8: It is a subunit of the baculovirus late RNA polymerase complex (Crouch et al., 2007) required for transient late gene expression (Passarelli et al., 1994). Homologs are found in all baculoviruses and contain a conserved motif found in other RNA polymerases (Passarelli et al., 1994; Titterington et al., 2003).

LEF-9: It was also reported as a subunit of the baculovirus RNA polymerase complex (Guarino et al., 1998a) and was essential for the short-term expression of the late gene (Lu and Miller, 1994).

p47: It is also a subunit of the baculovirus RNA polymerase complex (Guarino et al., 1998b) and was reported as an essential factor for transient late gene transcription (Todd et al., 1995).

LEF-5: LEF-5 is another factor required for transient late gene expression. Previously, it was reported to interact with itself having a domain similar to the elongation factor TFIIS of RNA polymerase II (Harwood et al., 1998). But later experiments revealed that it acts as an initiation factor rather than an elongation factor (Guarino et al., 2002).

1.2.5.7 Proteins involved in cell cycle arrest and/or interaction with host proteins

Odv-ec27: This protein is found to act as multifunctional cyclin and is reported to be involved in the regulation of the cell cycle during virus infection (Belyavskyi et al., 1998; Braunagel et al., 1996b) and was shown to interact with p40 (ODV-C42) solely or in combination with p78/83 (Braunagel et al., 2001).

Ac81: This protein does not appear in ODVs or BVs, but interacts with Actin A3 in the cytoplasm (Chen et al., 2007).

1.2.6 Application of NPVs as bioinsecticides

One of the most important tasks in agricultural pest control is the ability to sustain pest insect population level below the economic injury threshold cost-effectively so that the cost of pest control operations justifies the income generated. The application of microorganisms pathogenic to insects is an environmentally benign method of insect pest control. In this criterion, baculoviruses have several inherent advantages as biopesticides and have so far been deemed successful. In this context, due to their inherent insecticidal activities the nucleopolyhedroviruses, a group of the Baculovirus family, have been registered and successfully used as safe and effective biopesticides for the protection of field and orchard

crops and forests in America, Europe and Asia (Black et al., 1997; Copping and Menn, 2000).

Baculoviruses have been used against insect pests since the late 1930s. *Gilpinia hercyniae*, the European spruce sawfly, after its introduction to North America in the early 20th century, became established as a major pest of spruce trees. In 1938, approximately 12,000 square miles of spruce were infested leading to serious damage. The introduction of an NPV pathogenic to this insect was reported to control the infestation and resulted in the disappearance of the pest within a few years (Balch and Bird, 1944).

Elcar™, a preparation of *Heliothis zea* Single NPV (HzSNPV), was the first baculovirus-based commercial insecticide (Ignoffo and Couch, 1981). It was pathogenic to a broad range of insect pests belonging to the genera *Helicoverpa* and *Heliothis*. HzSNPV not only controlled the pest population of cotton bollworms but also the population of pests attacking soybean, sorghum, maize, tomato and beans. The development of resistance to many chemical insecticides including pyrethroids in the pest population revived the interest in HzSNPV-based biopesticide and subsequently, the same baculovirus was registered under the name GemStar™. Another baculovirus, *Helicoverpa armigera* single NPV (HaSNPV) similar to HzSNPV was registered in China as a bioinsecticide in 1993 (Zhang et al., 1995) and has been extensively applied in cotton fields. A large range of bioinsecticides based on HaSNPV has also been used in India (Srinivasa et al., 2008).

The forests of temperate regions are frequently attacked by lepidopteran (most commonly *Lymantria dispar*, *Lymantria monacha*, *Orgiya pseudotsugata* and *Panolis flammea*) and hymenopteran (mainly *Neodiprion sertifer* and *Diprion pini*). The formulation of MNPV isolated from *L. dispar* commercialized under the trade names Gypchek, Disparivirus, Virin-ENSH, and the MNPV of *O. pseudotsugata* under the trade names TM BioControl-1 and Virtuss (Reardon et al., 1996) are sometimes used for forest protection.

Caterpillars of the genus *Spodoptera* are the major concern for agricultural fields in many countries. Two NPV-based biopesticides, SPOD-X™ containing *Spodoptera exigua* NPV to control insects on vegetable crops and Spodopterin™ containing *Spodoptera littolaris* NPV which is used to protect cotton, corn and tomatoes are commercially available. By the use of SPOD-X™ insect pest population of *Spodoptera*, commonly known as a cotton pest, was successfully controlled (Inceoglu et al., 2001). Application of *Spodoptera frugiperda* NPV over 20,000 ha in Brazil has controlled the pest population in a maize field (Moscardi, 1999), but later it has been discontinued due to technical problems in the virus production under

laboratory conditions. In Brazil, an NPV pathogenic to *Anticarsia gemmatalis*, a major caterpillar pest of soybeans, is used to curb the pest population of over a million hectares of this crop (Moscardi, 1999).

NPV isolated from *Perigonia lusca*, a tea pest in Paraguay, was used to control the insect over 900 ha of tea plantations (Sosa-Gomez et al., 1994). NPV infecting *B. suppressaria* was effectively used to control the pest in tea plantations and tung oil trees over 20,000 ha in China (Yi P. and Li Z., 1989).

NPVs pathogenic to *Trichoplusia* sp. (*Brassica* pest) and *Spodoptera litura* (cotton pest) have also been commercially produced under the name biotrol-VTN and biotrol-VSE, respectively (Nutrilite Products Inc., U.S.A.) (Dubey, 2006). NPVs isolated from *Autographa californica* and *Anagrapha falcifera* were also registered and evaluated in the field on a limited scale (Sahayaraj, 2014). The presence of NPV in geometrid loopers of Indian tea has been shown by Mukhopadhyay and workers (Mukhopadhyay et al., 2011, 2007).

1.2.7 Molecular Characterization of *Hyposidra talaca* NPV genome

Pathogenicity of *Hyposidra talaca* NPV to the respective host was reported by Mukhopadhyay et al. (2011). A dose-mortality bioassay study of OBs of HytaNPV isolated from the Terai population of loopers was conducted on the second instar caterpillars of *H. talaca*. Results showed a median lethal concentration (LC_{50}) value of 2.8 x 10^3 OBs/ml with a median lethal time (LT_{50}) of 5.45 days for 1 x 10^4 OBs/ml, 4.15 days for 1 x 10^5 OBs/ml and 4.05 days for 1 x 10^6 OBs/ml concentrations. These results encouraged us to take up a further study on the genetic characterization of HytaNPV to develop it in future as a potential bioinsecticide against *H. talaca*. The *polyhedrin* gene of HytaNPV was sequenced (Sinu et al., 2011) and based on the partial sequence of the *polyhedrin* gene, Antony et al. (2011) reported a close relation between *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) and *Biston* (=*Buzura) suppressaria* nucleopolyhedrovirus (BusuNPV), another NPV infecting the tea pest *Buzura suppressaria* mainly recorded in China (Hu et al., 1993) and was subsequently reported from the Terai-Dooars and North-East region of India (Das et al., 2010a; Dasgupta et al., 2016; Mukhopadhyay et al., 2007; Sinu et al., 2011). Preliminary RFLP analysis of the HytaNPV genome (Ghosh et al., 2015) showed that the restriction

profile and genome size of HytaNPV differ from that of BusuNPV (Hu et al., 1998). Dasgupta et al. (2016) also analyzed the phylogenetic relationship of HytaNPV based on the *polyhedrin* gene and reported it to be the same HytaNPV variant as documented by Antony et al. (2011). They concluded that the variant is predominant in the northeastern part of India having the potential of pathogenicity as an alternative to chemical pesticides to control the *H. talaca* pest population. Recently, the whole genome organization and sequence of HytaNPV of the Dooars isolate was reported by Nguyen et al. (2018). Phylogenetic analyses based on 37 core baculovirus genes revealed that the Dooars isolate of HytaNPV is a closer relative of BusuNPV of China than other NPVs infecting the other lepidopteran pest specimens.

Section 1.3: Objectives

1. To survey, sample and maintain the virus culture *in vivo* drawn from *Hyposidra talaca* looper populations of different tea plantations of Sub-Himalayan foothills and Terai region of West Bengal.

2. To isolate and purify HytaNPV polyhedral occlusion bodies (POBs) from cadavers of *Hyposidra talaca* looper using differential sucrose centrifugation method.

3. To determine the approximate genome size by restriction digestion analysis of the strains of HytaNPV.

4. To study restriction profile of the genome of HytaNPV strain(s) collected from Terai region of Darjeeling foothills.

5. To characterize HytaNPV gene(s) such as *pif* or some others, related to the pest control property of the virus.

6. To construct a phylogenetic tree of the different strains of HytaNPV based on the similarities and differences.

Section 1.4: Materials and Methods

1.4.1 Survey

Different tea plantations of Terai and the Dooars regions of West Bengal, India were surveyed around the year during 2014-2018 to observe the occurrence of the *Hyposidra talaca* caterpillars (loopers) before the spray of pesticides in the tea garden.

1.4.2 Sample collection

a) Collection of NPV-infected caterpillars

Cadavers of *Hyposidra talaca* larvae show typical symptoms of NPV infection such as caterpillars perched at the top of the tea shoots, attached and hanging head down on dying with their prolegs, with a characteristic inverted V-shape, and liquefaction of the body with light brown fluid (Federici, 1997). Such moribund specimens were collected separately for the study from different tea plantations in the Terai-Dooars region of Darjeeling foothills. The NPV-infected dead caterpillars were also collected from the Dooars region of West Bengal, India for comparison.

b) Collection of healthy caterpillar and adult moths

Healthy 3^{rd}-5^{th} instar larvae and adult moths of *Hyposidra talaca* were also collected to establish and maintain a culture in the laboratory.

1.4.2.1 Collection time

The adult moths were collected in the early morning usually between 5:30 AM to 7:00 AM as they are active in the dimmed light during this period and the larvae were collected usually in the late morning between 7:00 AM to 9:00 AM as the loopers start climbing up to the top of the tea plants during this period.

Sample collection was mainly done from March to November. The collection of caterpillars was mostly avoided during the rainy season to avoid any contamination and secondary infection.

The looper caterpillars were identified in the field and subsequently in the laboratory using the taxonomic key provided by Das and Mukhopadhyay (2009).

1.4.2.2 Sampling sites

Both NPV-infected and non-infected loopers of *H. talaca* were collected from different tea plantations in the Terai region of Darjeeling foothills. Later for better comparison, the Dooars region was also included as a collection site. Details of the sampling sites were given below (**Figure 1-8**).

a) Terai region - Matigara Tea Estate (26°42'41.1"N latitude, 88°22'30.8"E longitude), Atal Tea Estate (26°41'35.8"N latitude, 88°15'03.5"E longitude), Sathbhaiya Tea Estate (26°40'18.5"N latitude, 88°13'09.3"E longitude), and Kamalpur Tea Estate (26°42'26.3"N latitude, 88°18'24.5"E longitude) (**Figure 1-8**).

b) Dooars region - Elenbari Tea Estate (26°52'09.2"N latitude, 88°32'41.5"E longitude) and Dumdim Tea Estate (26°50'35.2"N latitude, 88°39'39.7"E longitude) (**Figure 1-8**).

Figure 1-8: Map showing the sampling sites of Terai and the Dooars region in the present study.

1.4.3 Rearing and culture of *Hyposidra talaca*

The healthy 3rd-5th instar larvae were reared in the laboratory in sterilized plastic containers (15 cm height x 8 cm diameter) with pesticide-free fresh tea leaves (*Camellia sinensis*). The pesticide-free leaves were collected from the tea plantation maintained by the Entomological Research Unit of the Department of Zoology, University of North Bengal. The cultures were maintained at room temperature with a daily change of fresh tea leaves washed in distilled water and the stalks of the twig immersed in the water-filled small 2.0 ml micro-centrifuge

tube or in the conical flask (**Figure 1-9**). The twigs were arranged in such a way that enough space was available for the free movement of larvae.

The adult moths developed from the pupa in the laboratory were reared in large plastic containers (30 cm height x 15 cm diameter) with a 2:1 male-female ratio for successful mating to maintain the culture for the next generation.

1.4.4 Isolation and purification of OBs (virus particles) from NPV-infected cadavers

Stocks of OBs were built-up separately from the cadavers of *H. talaca* following the method of (Kawarabata and Matsumoto, 1973) with some modifications. The methods were described as follows.

The cadavers were stored in distilled water and left to putrefy for about 15 days to enable the release of OBs from the larval tissues. The putrefied suspension of infected larvae was homogenized using a glass tissue homogenizer. The homogenate was filtered through four layers of cheesecloth and the filtrate was immediately centrifuged at 1000 x g for 20 minutes at room temperature. The supernatant was removed carefully by a Pasteur pipette and the sedimented OBs were suspended in 25% (w/v) sucrose dissolved in distilled water. The suspension was centrifuged at 1000 x g for 20 minutes at room temperature. The supernatant was discarded and the sedimented OBs were resuspended in 10 ml of 25% sucrose solution. The suspended OBs (1 volume) were slowly layered on 3 volumes of 50% sucrose solution and centrifuged at 1800 x g for 40 minutes at room temperature. The differential centrifugation was repeated twice and the OBs were washed 7-8 times with deionised distilled water. The pellet containing pure OBs was resuspended in distilled water and stored at 4°C until further use.

Figure 1-9: Culture of *H. talaca* larvae in the laboratory. (a) culture of healthy larvae (3rd-4th instar), (b) culture of infected larvae (3rd – 5th instar).

1.4.5 Recognition of virus by Phase contrast microscopy

The crystalline white precipitate (OBs) isolated from each cadaver was examined at 1000X magnification in a bright field compound microscope (Nikkon Eclipse 200, Nikkon, Tokyo, Japan) and photographed. The OBs were evident as bright particles under the compound microscope **Figure 2-5**.

1.4.6 Counting Polyhedra Occlusion Bodies (OBs)

The number of OBs/ml was determined by using a Neubauer haemocytometer.

1.4.7 Maintenance and Mass production of HytaNPV OBs under laboratory conditions

The prepared polyhedral OBs were used to infect the healthy *H. talaca* larvae reared in the laboratory. The tea leaves were brushed with the solution of polyhedral OBs (10^5 OBs/ml) and were used as feed for the 3rd-5th instar larvae of *H. talaca* for mass production of HytaNPV. The larvae showing symptoms of viral infection were collected for further isolation of polyhedral OBs (Virus particles).

1.4.8 Extraction and Purification of DNA from Polyhedra Occlusion Bodies of *H. talaca* NPV

Viral DNA was extracted as described by O'reilly and Miller (1991). The procedure was described below.

a) DNA extraction:

The suspension of Occlusion bodies (OBs) was centrifuged at 1800 x g for 20 min and the sedimented polyhedra were suspended in Tris-EDTA (10mM Tris, 1mM EDTA, pH 8.0). Proteinase K (10 mg/ml) was added to the suspension to a final concentration of 1mg/ml and incubated for 1 hour at 37^0C. Following proteinase k digestion, the suspension was dissolved in dissolution buffer (0.1M Na_2CO_3, 0.01M EDTA, 0.17M NaCl, pH 10.8). After dissolution 10% SDS was added into the suspension to a final concentration of 1%, mixed thoroughly and incubated for 1 hour at 37^0C. The suspension was centrifuged at 11,000 x g for 3 min and the supernatant was transferred to a fresh tube.

b) Purification of extracted DNA

An equal volume of Tris-equilibrated phenol (pH-8.0) was added to the supernatant, mixed gently for at least 5 min followed by centrifugation at 8000 x g for 10 mins at 4^0C. The top aqueous phase was transferred to a fresh tube. To it, an equal volume of a mixture of Tris-equilibrated phenol: chloroform: iso-amyl alcohol (25:24:1) was added, gently mixed and centrifuged at 8000 x g for 10 mins at 4^0C. The top aqueous phase was transferred to a fresh tube, and an equal volume of a mixture of chloroform: iso-amyl alcohol (24:1) was added and gently mixed followed by centrifugation at 8000 x g for 10 min at 4^0C. The top aqueous phase was carefully transferred to a fresh tube and twice the volume of absolute ethanol was added, incubated for 1 hour at 4^0C and centrifuged at 15,000 x g for 15 min at 4^0C. The supernatant was discarded. The sedimented DNA pellet was washed in 500µl of 70% ethanol following centrifugation at 15,000 x g for 15 min at 4^0C. The ethanol was discarded and the DNA pellet was dried. Finally, the DNA was suspended in Tris-EDTA (10mM Tris, 1mM EDTA, pH-8.0) and stored at -20^0C.

1.4.9 *In vivo* Restriction Endonuclease Digestion

HytaNPV DNA was digested by *Eco*RI, *Xho*I, *Kpn*I, *Bam*HI, *Hind*III, *Bgl*I and *Pst*I following the method of Sambrook and Russell (2001) with some modifications. 1.5-2 µg of

HytaNPV DNA was set up for digestion with 10 units of restriction enzyme in the presence of 1X restriction buffer and 1X acylated-BSA at 37°C for 4 hours for complete digestion.

The reaction mixture was set as per the manufacturer's specifications. Each component and its amount have been given below.

Substrate	**Quantity**	**Final Concentration**
DNA	1.5-2 µg	75-100ng/µl
Restriction Buffer (10X)	2 µl	1X
Acylated BSA (100X)	0.2 µl	1X (0.1mg/ml)
Restriction Endonuclease	10 units	0.5 unit/ µl
Final Volume	**20 µl (adjusted by adding Milli-Q water)**	

1.4.9.1 Electrophoresis of digested samples

The digested DNA samples were separated on 0.4-0.7% agarose gel (depending on expected fragment size) (0.4-0.7% agarose in 1x TAE buffer) at 70 volt for 4-6 hours in 1X TAE buffer (40mM Trishydroxymethylaminomethane pH 8.0, 1mM EDTA pH 8.0, 20mM acetic acid). A λ DNA *Hind*III digest molecular weight marker and a High Range DNA ruler (Thermofisher) were used to estimate the size of the digested fragments (bands). After electrophoresis, the gels were stained in ethidium bromide solution (0.5µg/ml). The gels were viewed and photographed in Gel Documentation System (SPECTROLINE BI-O-VISION, UV/WHITE Light Transilluminator) with transmitted UV light. The photographs were analyzed using the software: ImageAide: Version 3.06.

1.4.10 Construction of *in silico* Restriction map

In silico restriction map of the HytaNPV genome (Nguyen et al., 2018) and obtained sequences of the HytaNPV isolates of the present study were prepared in SnapGene viewer.

1.4.11 Designing of Primers

The set of primers to amplify the *polyhedrin* gene was used from Antony et al. (2011).

To amplify *pif-1* and *lef-8* gene BusuNPV (KF611977.1), EcobNPV (DQ837165.1), ApciNPV (FJ914221.1), SujuNPV (KJ676450.1) and EupsNPV (FJ227128.1) were used to design the degenerate primers, during the year of 2015-2018 as there was no sequence available for those genes of HytaNPV at that time.

After the publication of the complete genome sequence of HytaNPV by Nguyen et al. (2018) the oligonucleotide primers to amplify *pif-2*, *pif-2* and *lef-9* genes were designed based on the sequence alignment of HytaNPV (MH261376.1), BusuNPV (KF611977.1), EcobNPV (DQ837165.1), ApciNPV (FJ914221.1) and SujuNPV (KJ676450.1).

Table 1-4: List of the primers used to amplify different genes and respective annealing temperature and duration of extension in each cycle of the PCR program.

Target Gene	Primer set	Primer Name	Primer sequence (5′-3′)	Annealing temperature	Extension time in Cycle
polyhedrin	SET 1	PHF1	GGACCSGGYAARAAYCAAAAA	42	50 sec
		PHR1	GCRTCWGGYGCAAAYTCYTT		
pif-1	SET 1	P1SF1	GARGGNCTNGCNAAYTGYCA	50	70 sec
		P1SR1	TNGGRTANCGNGTNGCNGG		
pif-2	SET 1	P2SF1	CTGGTCAAAAACCTACCGAC	54	60 sec
		P2SR1	GATCCGCGTTATTTTGCCG		
	SET 2	P2SF2	GGCAAAATAACGCGGATCT	54	60 sec
		P2SR2	ACGAACAACACGCAAAAATG		
pif-3	SET 1	P3SF1	CAAGAAACGTGCAGGCAAA	54.5	60 sec
		P3SR1	ATCAACAATCGCAATACGGC		
lef-8	SET 1	L8SF1	GGCACTTTCATGATHGACGG	55	70 sec
		L8SR1	CCACCGTCATTTCNCCGTG		
	SET 2	L8SF2	ACGGNGAAATGACGGTGGC	55	70 sec
		L8SR2	GGDATRTANGGRTCTTCGGC		
	SET 3	L8SF3	GCCGAAGAYCCNTAYATHCC	55	70 sec
		L8SR3	GATTGRTTDATNGTCCATTGATC		
lef-9	SET 1	L9SF1	CGCTTTCGGATTTTGTCTTCA	54.5	80 sec
		L9SR1	CCGACTTTACCGACTGGAA		
	SET 2	L9SF2	TCCAGTCGGTAAAGTCGGC	54.5	80 sec
		L9SR2	CCCGTAAATTTCGACGCTACT		

1.4.12 Polymerase Chain Reaction (PCR)

The PCR amplification of the target gene was carried out with 50ng of HytaNPV DNA in the presence of 1X GoTaq Flexi Buffer, 2mM $MgCl_2$, 250µM dNTPs mixture, 0.5µM each

of the forward and reverse primers and 2 units of GoTaq Flexi DNA Polymerase (Promega) with a PCR programme of 94ºC for 5 min (initial denaturation) followed by 35 cycles of 94ºC for 50 sec (denaturation), optimum annealing temperature for 40 sec and extension at 72ºC for the required period (**Table 1-4**); then the final extension of 7 min at 72ºC.

1.4.13 Detection and purification of PCR amplified product for sequencing

The amplified PCR products were subjected to electrophoresis in a 1% agarose gel (1% agarose in 1x TAE and ethidium bromide 0.5µg/ml). The electrophoresis was carried out at 80 volts for 1 hour in 1x TAE buffer with a 100bp DNA ladder as a molecular weight marker. After electrophoresis, the gels were observed and photographed in the Gel Documentation System (SPECTROLINE BI-O-VISION, UV/WHITE Light Transilluminator) with transmitted UV light. The amplified products of the expected size were purified with the SureExtract Spin PCR Clean up/ Gel extraction kit (Brand: Geneticx).

1.4.14 Sequencing of the amplified products

The purified PCR products were sequenced from both directions using Applied Biosystems 3730xl/ABI3730XL-15104-028 capillary sequencer.

1.4.15 Sequence alignment and BLAST search

The gene sequences obtained were aligned and joined using ClustalW in MEGAX (Kumar et al., 2018). The correct nucleotide sequences were obtained by aligning and comparing them with the reported sequences of HytaNPV (GenBank Accession No. MH261376.1; Nguyen et al., 2018) or BusuNPV (GenBank Accession No. KF611977.1; Zhu et al., 2014) using ClustalW Program with MEGAX. BLAST analyses were performed using BLASTN 2.9.0+ (Zhang et al., 2000), and BLASTX 2.9.0+ (Altschul, 1997). The representations of the aligned sequences were prepared using BioEdit version 7.2.5 (Hall, 1999) or GeneDoc (Nicholas and Nicholas, 1997). The obtained sequences were submitted to the NCBI GenBank database.

1.4.16 Sequence representations

Representations of the obtained sequences were prepared in SnapGene viewer and the alignments were prepared in BioEdit (Hall, 1999).

1.4.17 Sequence comparison

Nucleotide and amino acid sequences were compared with the Dooars isolate of HytaNPV (Accession No. MH261376.1; Nguyen et al., 2018) to detect nucleotide and amino acid substitution(s) using MEGAX (Kumar et al., 2018).

1.4.18 Sequence Divergence

The p-distance model was used to estimate the nucleotide sequence and amino acid sequence divergence by using MEGAX (Kumar et al., 2018). The ratio of transitional to transversional differences was also estimated using MEGAX. Nucleotide positions 1st, 2nd, 3rd of the codon and non-coding position were included. All positions containing gaps and missing data were eliminated.

1.4.19 Phylogenetic analysis

All the retrieved sequences were aligned using ClustalW in MEGAX applying default parameters. The phylogenetic trees were constructed by MEGAX using the maximum likelihood method (Kumar et al., 2018; Nei and Kumar, 2000). All the phylogenetic trees were tested by the bootstrap method with 1000 replicates. Positions containing gaps and missing data were not considered.

Phylogenetic analysis was carried out with nucleotide sequences of 77 baculoviruses comprising 75 sequences (1 deltabaculovirus, 3 gammabaculoviruses, 18 betabaculoviruses, 22 group I alphabaculoviruses and 31 group II alphabaculoviruses) retrieved from NCBI GenBank databases (**Table 1-5**) and the sequences of two isolates of HytaNPV of the present study, HytaNPV-ITK1 (Terai) and HytaNPV-ID1 (Dooars) based on a concatenated sequence alignment of six genes (*polyhedrin/granulin, lef-8, lef-9, pif-1, pif-2,* and *pif-3*) together. Both the amino acid and nucleotide substitution models were used for analysis. Analyses were carried out by extracting the total reading frame of each gene from the complete genome sequences of 75 baculoviruses. All the gaps and missing data were not considered for maximum likelihood analysis. A test of maximum likelihood fit was

performed for each phylogeny to find the considerably best method/model, rates and pattern of substitution to be used for constructing the trees (Nei and Kumar, 2000). Initial trees were designed by using Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated by the Maximum Composite Likelihood approach.

The General time reversible model (GTR) for nucleotide substitutions and Le Gascuel (2008) model for amino acids substitutions were used for the construction of phylogenetic trees applying non-uniformity of evolutionary rates considering discrete gamma distribution with invariant sites.

Table 1-5: List of baculovirus sequences used for phylogenetic analysis in the present study.

Sl. No.	Baculovirus	GenBank acc No.	Designation
1.	*Hyposidra talaca* NPV	MH261376.1	HytaNPV-R
2.	*Buzura suppressaria* NPV	KF611977.1	BusuNPV
3.	*Sucra jujuba* NPV	KJ676450.1	SujuNPV
4.	*Apocheima cinerarium* NPV	FJ914221.1	ApciNPV
5.	*Ectropis Obliqua* NPV	DQ837165.1	EcobNPV
6.	*Lambdina fiscellaria* NPV	KP752043.1	LafiNPV
7.	*Orgyia leucostigma* NPV	EU309041.1	OrleNPV
8.	*Euproctis (=Arna) pseudoconspersa* NPV	FJ227128.1	EuprNPV
9.	*Lymantria xylina* NPV	GQ202541.1	LyxyNPV
10.	*Lymantria dispar* NPV	AF081810.1	LdNPV
11.	*Adoxophyes orana* NPV	EU591746.1	AdorNPV
12.	*Adoxophyes honmai* NPV	AP006270.1	AdhoNPV
13.	*Mamestra configurata* NPV-A	AF539999.1	MacoNPV-A
14.	*Mamestra configurata* NPV-B	AY126275.1	MacoNPV-B
15.	*Mamestra brassicae* NPV	JQ798165.1	MabrNPV
16.	*Agrotis segetum* NPV	DQ123841.1	AgseNPV
17.	*Agrotis ipsilon* NPV	EU839994.1	AgipNPV
18.	*Spodoptera frugiperda* NPV	EF035042.2	SfNPV
19.	*Spodoptera exigua* NPV	AF169823.1	SeNPV
20.	*Spodoptera litura* NPV	AF325155.1	SpliNPV-A
21.	*Spodoptera littoralis* NPV	NC_038369.1	SpliNPV-B
22.	*Helicoverpa zea* NPV	KM596835.1	HzNPV
23.	*Helicoverpa armigera* NPV	KT013224.1	HearNPV
24.	*Leucania separata* NPV	AY394490.1	LeseNPV

Table 1-5: List of baculovirus sequences used for phylogenetic analysis in the present study, continued from page 50.

Sl. No.	Baculovirus	GenBank acc No.	Designation
25.	*Chrysodeixis chalcites* NPV	AY864330.1	ChchNPV
26.	*Chrysodeixis (=Pseudoplusia) includens* NPV	KJ631622.1	PsinNPV
27.	*Trichoplusia ni* NPV	DQ017380.1	TnNPV
28.	*Peridroma sp* NPV	KM009991.1	PespNPV
29.	*Perigonia lusca* NPV	KM596836.1	PeluNPV
30.	*Clanis bilineata* NPV	DQ504428.1	ClbiNPV
31.	*Urbanus proteus* NPV	KR011717.2	UrprNPV
32.	*Autographa californica* NPV	L22858.1	AcNPV
33.	*Plutella xylostella* NPV	DQ457003.1	PlxyNPV
34.	*Catopsilia pomona* NPV	KU565883.1	CapoNPV
35.	*Thysanoplusia orichalcea* NPV	JX467702.1	ThorNPV
36.	*Rachiplusia ou* NPV	AY145471.1	RoNPV
37.	*Bombyx mori* NPV	JQ991010.1	BmNPV
38.	*Bombyx mandarina* NPV	JQ071499.1	BomaNPV
39.	*Lonomia obliqua* NPV	NC_043520.1	LoobNPV
40.	*Antheraea pernyi* NPV	DQ486030.3	AnpeNPV
41.	*Maruca vitrata* NPV	EF125867.1	MaviNPV
42.	*Philosamia cynthia ricini* NPV	JX404026.1	PhcyNPV
43.	*Anticarsia gemmatalis* NPV	KR815455.1	AngeNPV
44.	*Epiphyas postvittana* NPV	AY043265.1	EppoNPV
45.	*Hyphantria cunea* NPV	AP009046.1	HycuNPV
46.	*Condylorrhiza vestigialis* NPV	KJ631623.1	CoveNPV
47.	*Choristoneura murinana* NPV	KF894742.1	ChmuNPV
48.	*Choristoneura occidentalis* NPV	KC961303.1	ChocNPV
49.	*Choristoneura fumiferana* NPV	AF512031.3	CfNPV
50.	*Choristoneura rosaceana* NPV	KC961304.1	ChroNPV
51.	*Dasychira pudibunda* NPV	KP747440.1	DapuNPV
52.	*Orgyia pseudotsugata* NPV	U75930.2	OpNPV
53.	*Dendrolimus kikuchii Matsumura* NPV	JX193905.1	DekiNPV
54.	*Cydia pomonella* GV	U53466.2	CypoGV
55.	*Pieris rapae* GV	JX968491.1	PiraGV
56.	*Clostera anachoreta* GV	HQ116624.1	ClanGV-H

Table 1-5: List of baculovirus sequences used for phylogenetic analysis in the present study, continued from page 50.

Sl. No.	Baculovirus	GenBank acc No.	Designation
57.	*Clostera anastomosis* GV	KC179784.1	ClanGV-HB
58.	*Adoxophyes orana* GV	AF547984.1	AdorGV
59.	*Erinnyis ello* GV	KJ406702.1	ErelGV
60.	*Choristoneura fumiferana* GV	DQ333351.1	CfGV
61.	*Agrotis segetum* GV	AY522332.4	AgseGV
62.	*Epinotia aporema* GV	JN408834.1	EpapGV
63.	*Diatraea saccharalis* GV	KP296186.1	DisaGV
64.	*Spodoptera frugiperda* GV	KM371112.1	SfGV
65.	*Helicoverpa armigera* GV	EU255577.1	HearGV
66.	*Pseudaletia unipuncta* GV	EU678671.1	PsunGV
67.	*Trichoplusia ni* GV	KU752557.1	TnGV
68.	*Mocis latipes* GV	KR011718.1	MolaGV
69.	*Plutella xylostella* GV	AF270937.1	PlxyGV
70.	*Cnaphalocrocis medinalis* GV	KP658210.1	CnmeGV
71.	*Cryptophlebia leucotreta* GV	AY229987.1	CrluGV
72.	*Neodiprion sertifer* NPV	AY430810.1	NeseNPV
73.	*Neodiprion abietis* NPV	DQ317692.1	NeabNPV
74.	*Neodiprion lecontei* NPV	AY349019.1	NeleNPV
75.	*Culex nigripalpus* NPV	AF403738.1	CuniNPV

Chapter 2

Results

Section 2.1: The HytaNPV isolates in the present study

To characterize the HytaNPV genome initially, restriction endonuclease fragment analysis and gene sequencing were carried out on the DNA of the HytaNPV collected from the Terai region of West Bengal. The HytaNPVs collected from different tea plantations in the Terai region of Darjeeling foothills (**Figure 1-8**) did not exhibit any differences in the restriction profile. Therefore, all the HytaNPVs from different tea plantations of the Terai region of West Bengal in the present study were collectively considered as a single isolate that was designated as **HytaNPV-ITK1.**

Moreover, during the study, the whole genome sequence of HytaNPV from the Dooars region was published by Nguyen et al. (2018). Therefore, for comparison HytaNPV from the Dooars region of West Bengal designated as HytaNPV-ID1, was also included for restriction fragment analysis, characterization of the genes, and phylogenetic study.

For clarity and to avoid the nomenclatural disarray and disparity of the NPV isolate pathogenic to the specimens of genus *Hyposidra talaca*, these were designated as mentioned hereunder in **Table 2-1**.

Table 2-1: HytaNPV isolates of the present study and the reference

NPV	Isolate	Designation	Reference
Hyposidra talaca NPV	Terai, West Bengal, India	HytaNPV-ITK1	Present study
Hyposidra talaca NPV	Dooars, West Bengal, India	HytaNPV-ID1	Present study
Hyposidra talaca NPV	Dooars, West Bengal, India	HytaNPV-R	MH261376.1; (Nguyen et al., 2018)

Section 2.2: Survey, sampling, preparation and maintenance of virus culture

Objectives

1. To survey, sample and maintain the virus culture *in vivo* drawn from *Hyposidra talaca* looper populations of different tea plantations of Sub-Himalayan foothills and Terai region of West Bengal.

2. To isolate and purify HytaNPV polyhedral occlusion bodies (POBs) from cadavers of *Hyposidra talaca* looper using differential sucrose centrifugation method.

2.2.1 Survey and sampling of *Hyposidra talaca* larvae (looper)

Different tea plantations in the Terai regions of West Bengal, India were surveyed round the year during 2013-2015 to observe the occurrence of caterpillars (loopers) of *Hyposidra talaca*, both NPV-infected and non-infected, before spraying pesticides in the tea garden. During the survey, both NPV-infected and non-infected loopers of *H. talaca* were collected (**Figure 2-1**).

Sampling in the Terai region of Darjeeling foothills was done from Matigara Tea Estate (26°42'41.1"N latitude, 88°22'30.8"E longitude), Atal Tea Estate (26°41'35.8"N latitude, 88°15'03.5"E longitude), Sathbhaiya Tea Estate (26°40'18.5"N latitude, 88°13'09.3"E longitude) and Kamalpur Tea Estate (26°42'26.3"N latitude, 88°18'24.5"E longitude) (**Figure 2-2**).

A total of 496 non-infected and 28 cadavers of NPV-infected loopers of *H. talaca* were collected from four tea plantations in the Terai regions of Darjeeling Foothills from August 2013 to July 2015. The mean numbers of caterpillars, both non-infected and NPV infected, collected during the survey period from different tea plantations of the Terai region have been represented in **Figure 2-3**. During the winter season (Dec-Feb) the occurrence of caterpillars was very low, whereas, it was found to increase from March and the abundance was high during May-Oct, and again decreased from November. In the winter, the tea plantations undergo a pruning phase for maintenance. As a result, there are insufficient tea leaves available for the caterpillar to feed on.

Figure 2-1: (a) The caterpillar of *Hyposidra talaca*, a leaf-eating tea pest, (b) NPV-infected dead *H. talaca* in tea plantation, (c) Non-infected *H. talaca* caterpillars collected from the tea plantation.

Figure 2-2: Map showing the sampling sites of Terai and the Dooars region in the present study.

Moreover, the activity and motility of the caterpillars were observed to be increased during the daytime in sunlight when they come to the top of the tea shoots. During the rainy season after drizzle or heavy precipitation, the caterpillars hide below the leaves or they would drop down on the ground. Therefore, the collection of caterpillars becomes difficult or more

challenging during the winter or rainy season. The looper caterpillars were mostly collected in the morning hours of the summer and autumn seasons.

Collection of NPV-infected caterpillars from the plantations should be done within a day after the death of the caterpillar before post-mortem changes such as drying of the cadavers in the sunlight or trickling with the precipitation.

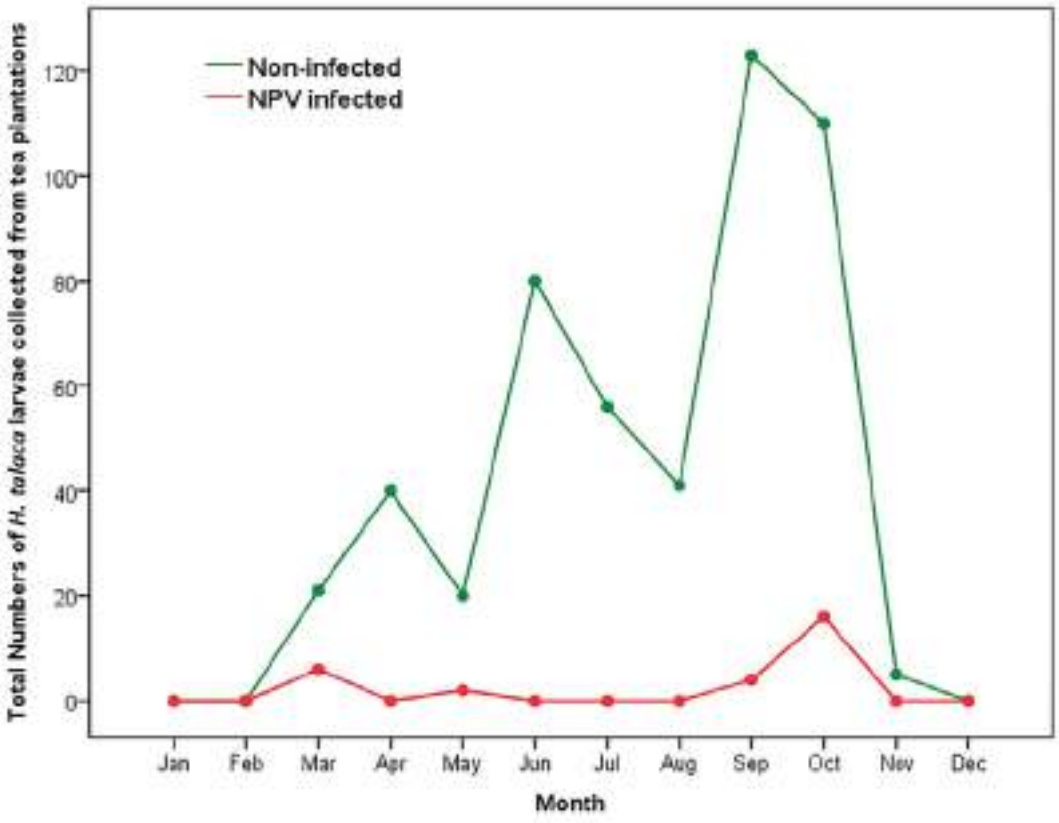


Figure 2-3: The figure showing the mean number of collected non-infected (green line) and NPV-infected (red line) *H. talaca* larvae collected from four different tea plantations in the Terai regions of Darjeeling foothills, during 2013-15.

After the publication of the complete genome sequence of HytaNPV from the Dooars region by Nguyen et al (2018), NPV isolated from *H. talaca* from the Dooars region was also included in the present study for comparison. A total of 86 non-infected and 16 cadavers of NPV-infected loopers of *H. talaca* were collected from two tea plantations of the Dooars region, Elenbari Tea Estate (26°52'09.2"N latitude, 88°32'41.5"E longitude) and Odlabari Tea Garden (26°50'35.2"N latitude, 88°39'39.7"E longitude) across the mighty Teesta river from its eastern bank of the Dooars region of West Bengal, India (**Figure 2-2**) from July 2018 to October 2018.

2.2.2 Isolation and purification of Occlusion bodies (virus particles) and maintenance of virus stock

After isolation and purification of occlusion bodies (OBs) from the NPV-infected dead cadavers (**Figure 2-4**), the OBs were identified under the compound microscope (**Figure 2-5**). The OBs were suspended to a final concentration of 10^5 OBs/ml to infect the 3^{rd}-5^{th} instar larvae of *H. talaca* reared in the laboratory. The larvae that died with symptoms of NPV infection were collected for further isolation of polyhedral OBs.

A total of 213 caterpillars of *H. talaca* were orally fed by brushing the solution of OBs (10^5 OBs/ml) on the tea leaves used for rearing and out of these 102 (Mean±SD = 47.65±7.64%) caterpillars died after getting infected with NPV (**Figure 2-6**). The number of *H. talaca* larvae orally inoculated and subsequently died due to NPV infection were represented in the bar diagram in **Figure 2-7**.

2.2.3 Isolation of DNA from OBs

DNA isolation was carried out from the viral polyhedra OB stocks prepared from NPV-infected dead cadavers of *H. talaca* collected from various tea plantations and also from NPV-infected larvae of *H. talaca* reared in the laboratory (**Figure 2-8**). The NPV suspension of 500 µl with a concentration of 10^9 OBs/ml yielded approximately 8-10 µg of DNA. The presence of baculoviral (NPV) DNA was confirmed by restriction profile analysis (**Section 2.3**) and by amplification and sequencing of the *polyhedrin* gene (**Section 2.4.2**) using the DNA stock as a template.

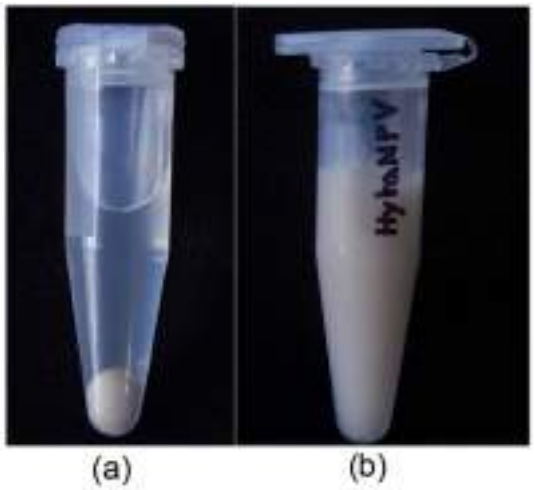


Figure 2-4: (a) The pellet of polyhedra OBs isolated from NPV infected dead cadavers of *H. talaca* after centrifugation (b) HytaNPV polyhedra OBs suspended in distilled water.

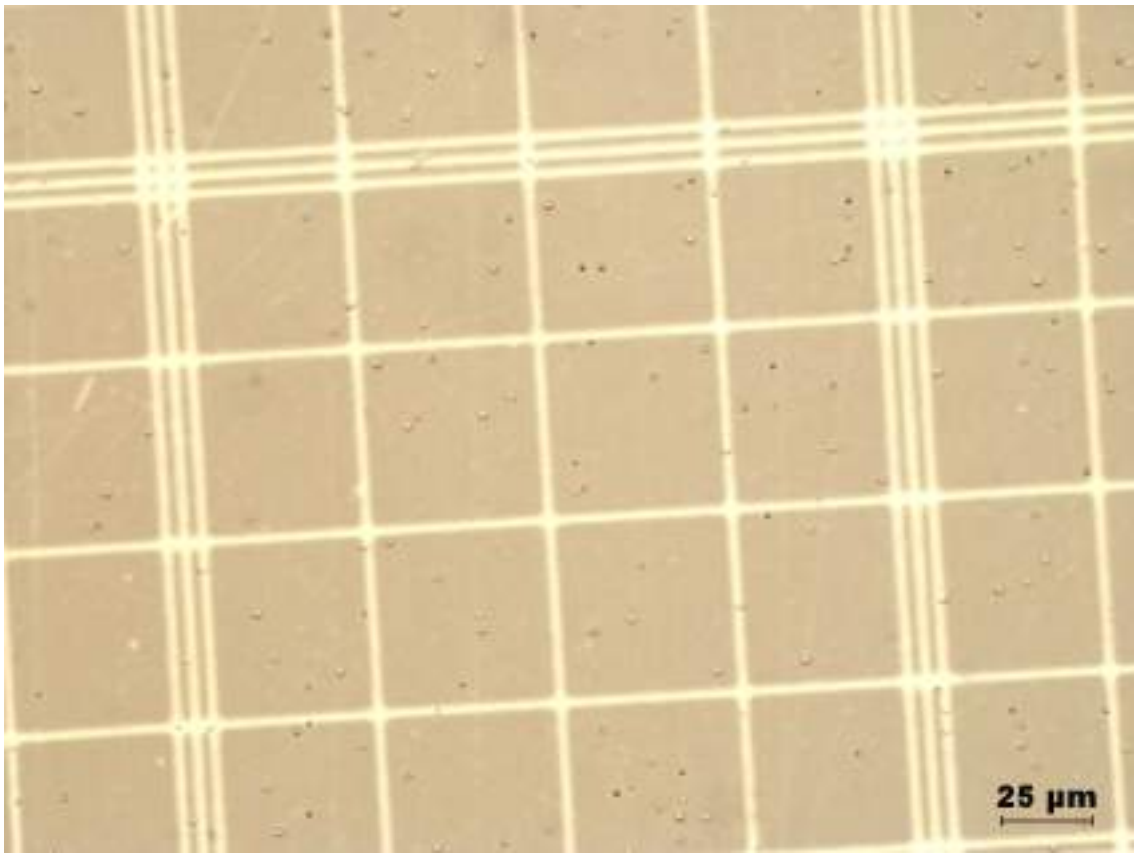


Figure 2-5: The polyhedra OBs under the compound microscope (400X).

Figure 2-6: NPV-infected dead *H. talaca* larvae obtained in the laboratory after oral inoculation with OBs (a and b).

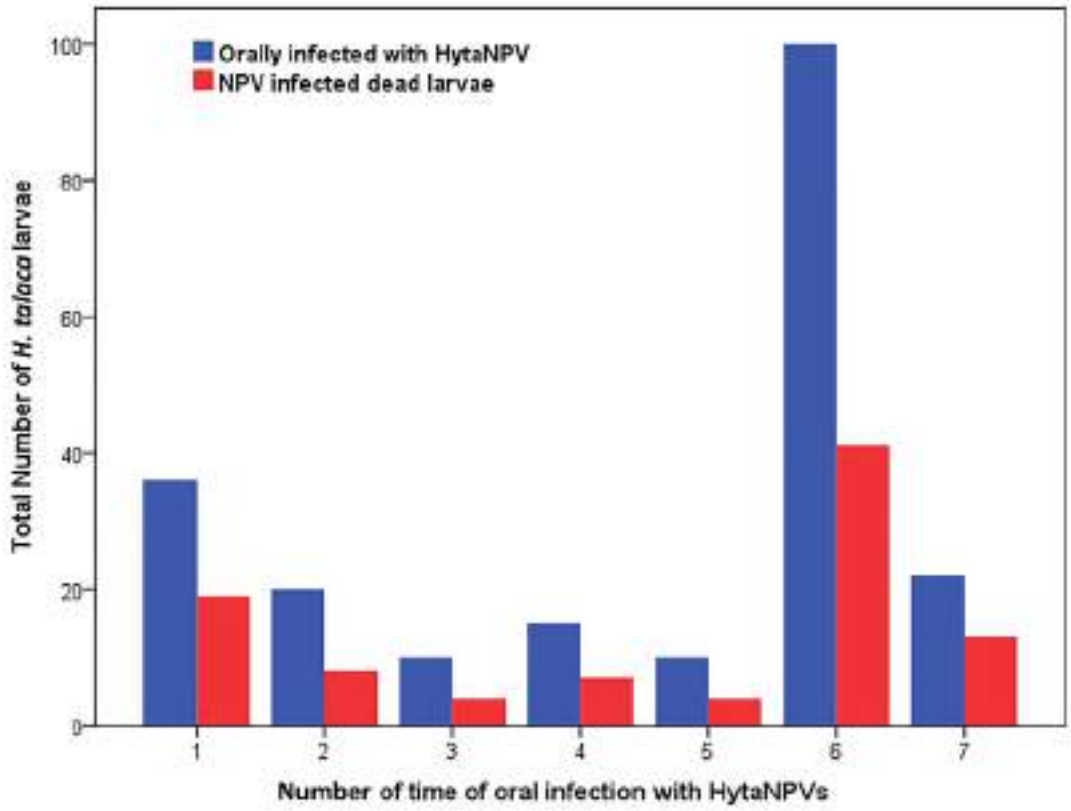


Figure 2-7: Number of *H. talaca* larvae orally infected by OBs (blue bar) in the laboratory and the number of larvae that died due to NPV infection (red bar).

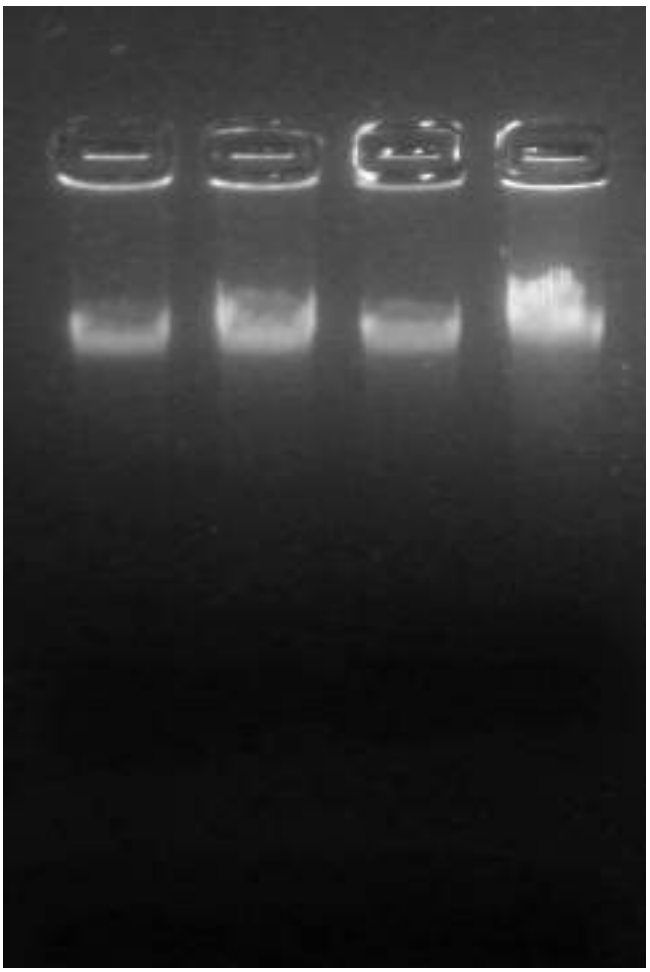


Figure 2-8: Agarose gel electrophoresis of DNA extracted from OBs isolated from *Hyposidra talaca*.

Section 2.3: Restriction Endonuclease Fragment analysis

Objectives:

3. To determine the approximate genome size by restriction digestion analysis of the strains of HytaNPV.

4. To study restriction profile of the genome of HytaNPV strain(s) collected from Terai region of Darjeeling foothills.

2.3.1 *In vitro* analysis of restriction fragment profile analysis of the HytaNPV genome from Terai and the Dooars region of West Bengal, India

For the initial characterization of the HytaNPV genome, restriction endonuclease fragment analysis was carried out on the DNA of the HytaNPV collected from the Terai and Dooars region of West Bengal. Restriction digestion of the DNA of both the isolates of HytaNPV (HytaNPV-ITK1 and HytaNPV-ID1) with *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, and *Xho*I revealed 8, 20, 26, 9, 11, 7 and 20 fragments ranging from 0.72 kb to 57.10 kb, respectively (**Table 2-2**). Restriction profiles of both the isolates of HytaNPV are shown separately in **Figure 2-9** & **Figure 2-10**. The size of the restriction fragments has been summarized in **Table 2-3**. The fragments were designated alphabetically starting with 'A' according to the size, from higher to lower as proposed by (Vlak and Smith, 1982). These numbers represent the minimum number of cleavage sites for each of the enzymes used since fragments smaller than 0.72 kb could not be detected. The molecular size of the fragments was estimated by comparing restriction fragment mobility with those of λ DNA/*Hin*dIII digest and high-range DNA ladder marker. To resolve the high molecular weight fragments (particularly more than 23 kb), the digested DNA was separated in 0.4% agarose gel and the fragments smaller than 23 kb were separated in 0.7% agarose gel.

In the present study, the exact size of the restriction fragments was estimated by comparing the relative mobility with that of the fragments of DNA molecular weight marker in agarose gel using ImageAid version 3.0 followed by a comparison with the sizes of the *in silico* digested fragments of HytaNPV-R genome (MH261376.1; Nguyen et al., 2018).

Table 2-2: Numbers and size of restriction fragments of HytaNPV DNA.

Sl No.	RE	HytaNPV-ITK1		HytaNPV-ID1	
		Nos. of fragments	Size range in Kb	Nos. of fragments	Size range in Kb
1	*Bam*HI	8	1.61 - 46.43	8	1.61 - 46.43
2	*Bgl*I	18	1.13 - 18.13	18	1.13 - 18.13
3	*Eco*RI	26	0.72 – 17.77	26	0.72 – 17.77
4	*Hin*dIII	9	**2.11** – 43.88	9	**2.62** – 43.88
5	*Kpn*I	11	1.56 – 53.82	11	1.56 – 53.82
6	*Pst*I	7	1.70 – 57.10	7	1.70 – 57.10
7	*Xho*I	20	0.74 - 23.66	20	0.74 - 23.66

***RE = Restriction endonuclease**

Both the Terai and the Dooars isolates of HytaNPV (HytaNPV-ITK1 and HytaNPV-ID1, respectively) exhibited almost similar restriction profiles **Table 2-3**. *Eco*RI digestion produced 26 fragments ranging from 0.72-17.77 kb, while 20 fragments ranging from 0.74 kb to 23.66 kb could be resolved in *Xho*I digestion (**Figure 2-9** & **Figure 2-10**). Digestion with *Bgl*I produced 18 fragments ranging from 1.13 to 18.13 kb. Other restriction enzymes, *Bam*HI, *Hin*dIII, *Kpn*I *and Pst*I produced 8, 9, 11 and 7 bands, respectively. Some fragments with high molecular weight (>25kb) were obtained in *Bam*HI, *Hin*dIII, *Kpn*I *and Pst*I digestions. *Bam*HI digestion produced fragments ranging from 1.61-46.43 kb for both the isolates, whereas, fragments ranging from 2.11-43.88 kb & 2.62-43.88 kb for Terai and the Dooars isolates, respectively were obtained from *Hin*dIII digestion. Digestions with *Kpn*I *and Pst*I produced 1.56-53.85 and 1.70-57.10 kb fragments, respectively for both the isolates (**Figure 2-9 & Figure 2-10**). The results showed that HytaNPV DNA digested with *Bam*HI and *Bgl*I have high molecular weight fragments resolved as triplets (fragments A, B & C for both the digestion), while those digested with *Hin*dIII and *Pst*I revealed doublets of high molecular weight DNA (fragments A & B for both the enzymes). Other three (3) and two (2) doublets of co-migrating fragments were detected in *Bgl*I digestion (*Bgl*I fragments: H-I, K-L & P-Q) and *Eco*RI digestion (*Eco*RI fragments: E-F & M-N), respectively. Based on the restriction endonuclease fragment analyses the mean genome sizes of the HytaNPV isolates in the present study were estimated to be 138.20 kb for the Terai isolate (HytaNPV-ITK1) and 138.46 kb for the Dooars isolate (HytaNPV-ID1) (**Table 2-3** & **Table 2-4)**.

Despite these similarities, *Hin*dIII and *Xho*I digestion of the HytaNPV-ITK1 and HytaNPV-ID1 showed a few differences between these two isolates. Though the number of restriction fragments produced by *Hin*dIII and *Xho*I digestions was similar in both the isolates, a *Hin*dIII fragment of 2.11 kb (fragments I) and an *Xho*I fragment of 4.10 kb (fragment L)

were observed in HytaNPV-ITK1 (**Figure 2-9**) which were not detected in the digestion profile of HytaNPV-ID1 (**Figure 2-10**). Moreover, the DNA of HytaNPV-ID1 showed a 4.5 kb *Xho*I restriction fragment which was absent in the *Xho*I restriction profile of HytaNPV-ITK1 (**Figure 2-9**). Similarly, a 3.53 kb *Hin*dIII restriction fragment present in HytaNPV-ID1 was not detected in HytaNPV-ITK1.

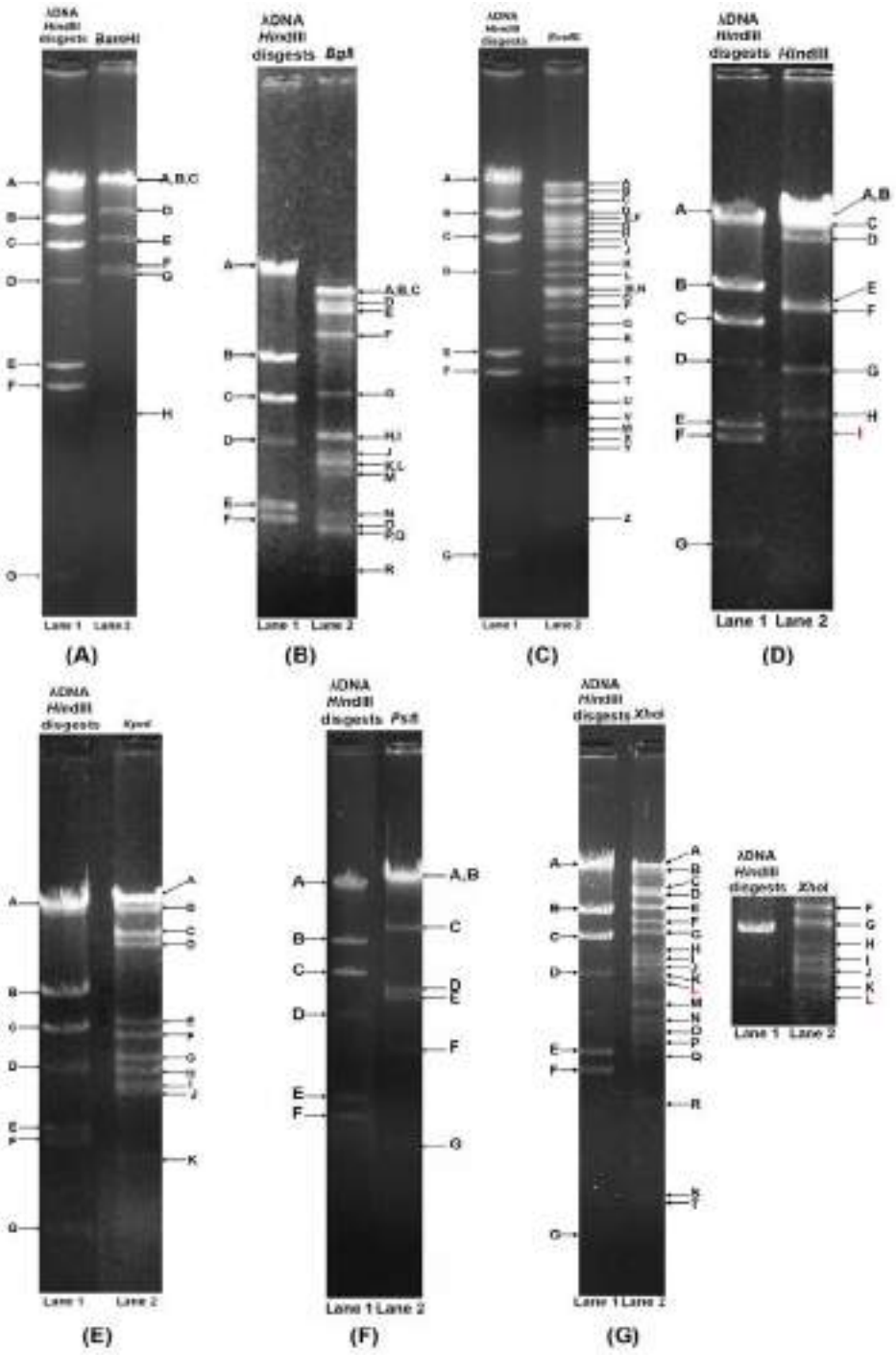


Figure 2-9: Electrophoregrams of restriction digestion of Terai isolate, HytaNPV-ITK1. A. *Bam*HI, B. *Bgl*I, C. *Eco*RI, D. *Hin*dIII, E. *Kpn*I, F. *Pst*I, and G. *Xho*I

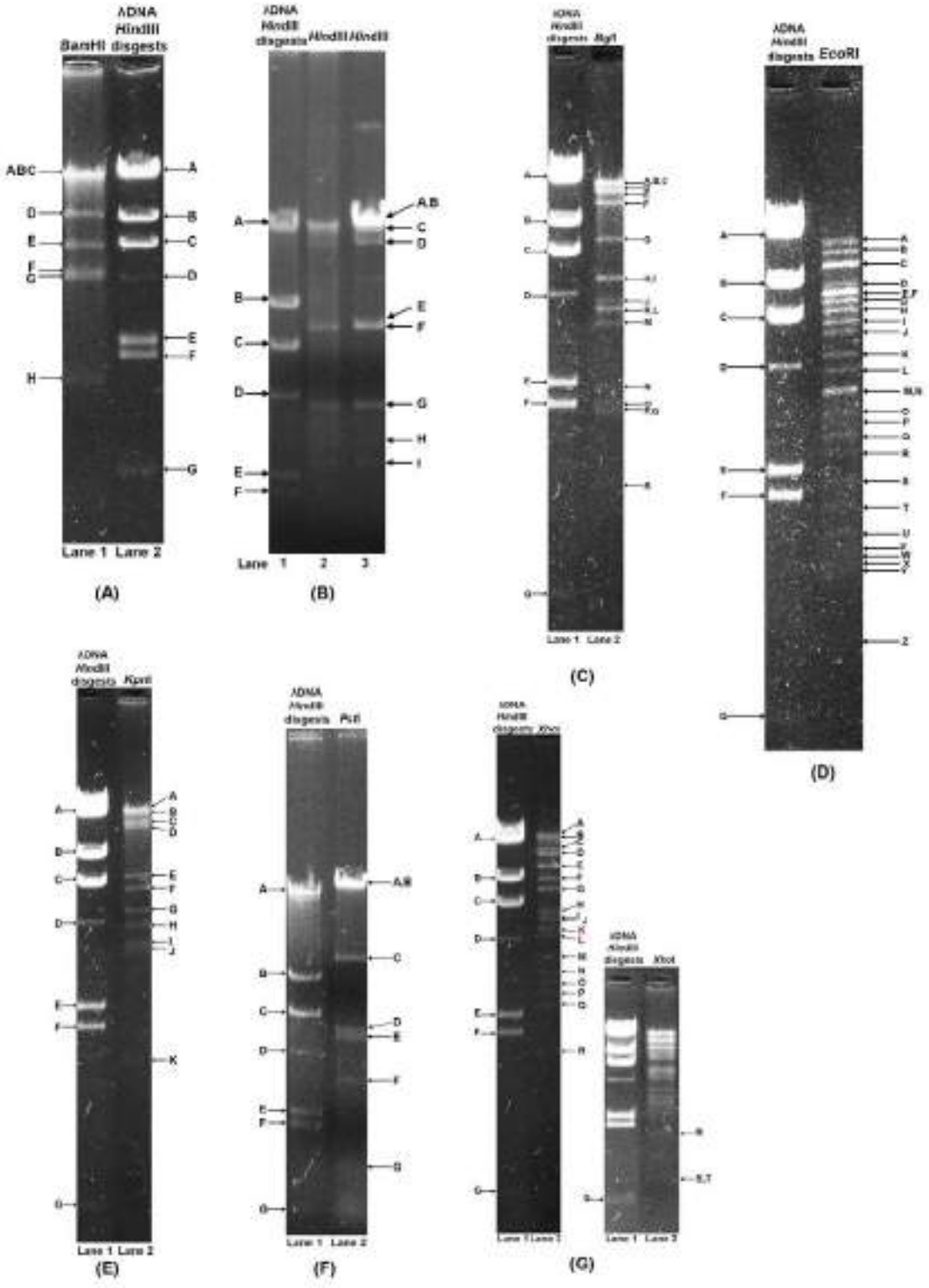


Figure 2-10: Electrophoregrams of restriction digestion of Dooars isolate, HytaNPV-ID1. A. *Bam*HI, B. *Bgl*I, C. *Eco*RI, D. *Hin*dIII, E. *Kpn*I, F. *Pst*I, and G. *Xho*I.

Table 2-3: *In vitro* restriction endonuclease fragment profile (*Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII) of HytaNPV-ITK1 (Terai) and HytaNPV-ID1 (Dooars). Fragment size was mentioned in kb.

Fragments	*Hin*dIII	*Bam*HI		*Bgl*I		*Eco*RI		*Hin*dIII	
	λ DNA	*Terai*	*Dooars*	*Terai*	*Dooars*	*Terai*	*Dooars*	*Terai*	*Dooars*
A.	23.130	46.43	46.43	18.33	18.33	17.77	17.77	43.89	43.89
B.	9.416	36.66	36.66	18.23	18.23	14.88	14.88	27.81	27.81
C.	6.557	28.07	28.07	17.99	17.99	12.03	12.03	22.77	22.77
D.	4.361	10.31	10.31	15.86	15.86	9.41	9.41	17.92	17.92
E.	2.322	6.71	6.71	14.63	14.63	8.51	8.51	8.42	8.42
F.	2.027	4.77	4.77	11.88	11.88	8.38	8.38	7.98	7.98
G.	0.564	4.52	4.52	7.14	7.14	7.73	7.73	4.16	4.16
H.		1.61	1.61	4.76	4.76	7.02	7.02	2.62	3.53
I.				4.71	4.71	6.24	6.24	2.11	2.62
J.				3.98	3.98	5.76	5.76		
K.				3.65	3.65	4.72	4.72		
L.				3.61	3.61	4.23	4.23		
M.				3.28	3.28	3.67	3.67		
N.				2.11	2.11	3.66	3.66		
O.				1.90	1.90	3.60	3.60		
P.				1.82	1.82	3.25	3.25		
Q.				1.78	1.78	2.78	2.78		
R.				1.13	1.13	2.51	2.51		
S.						2.13	2.13		
T.						1.88	1.88		
U.						1.63	1.63		
V.						1.48	1.48		
W.						1.43	1.43		
X.						1.35	1.35		
Y.						1.28	1.28		
Z.						0.72	0.72		
Total	**48.38**	**139.08**	**139.08**	**136.79**	**136.79**	**138.05**	**138.05**	**137.68**	**139.1**
Mean		**Terai (HytaNPV-ITK1) - 138.20, Dooars (HytaNPV-ID1) - 138.46**							

Table 2-4*: In vitro* restriction endonuclease fragment profile (*Kpn*I, *Pst*I, *Xho*I) of HytaNPV-ITK1 (Terai) and HytaNPV-ID1 (Dooars). Fragment size was mentioned in kb.

Fragments	*Hin*dIII	*Kpn*I		*Pst*I		*Xho*I	
	λ DNA	*Terai*	*Dooars*	*Terai*	*Dooars*	*Terai*	*Dooars*
A.	23.130	53.82	53.82	57.10	57.10	23.66	23.66
B.	9.416	23.62	23.62	53.71	53.71	19.92	19.92
C.	6.557	16.68	16.68	11.60	11.60	14.49	14.49
D.	4.361	14.73	14.73	5.69	5.69	13.13	13.13
E.	2.322	6.89	6.89	5.27	5.27	10.33	10.33
F.	2.027	5.99	5.99	3.35	3.35	8.31	8.31
G.	0.564	4.71	4.71	1.70	1.70	7.20	7.20
H.		4.22	4.22			5.52	5.52
I.		3.52	3.52			5.30	5.30
J.		3.34	3.34			4.95	4.95
K.		1.56	1.56			4.39	**4.50**
L.						**4.10**	4.39
M.						3.39	3.39
N.						2.96	2.96
O.						2.73	2.73
P.						2.50	2.50
Q.						2.28	2.28
R.						1.59	1.59
S.						0.79	0.79
T.						0.74	0.74
U.							
V.							
W.							
X.							
Y.							
Z.							
Total	**48.38**	**139.08**	**139.08**	**138.42**	**138.42**	**138.28**	**138.68**
Mean		**Terai (HytaNPV-ITK1) - 138.20, Dooars (HytaNPV-ID1) - 138.46**					

2.3.2 Comparative analyses of *in vitro* restriction endonuclease fragment profiles of HytaNPVs of the present study with *in silico* digestion profile of HytaNPV-R (Nguyen et al, 2018)

To compare the genome organization of HytaNPV-ITK1 and HytaNPV-ID1, *in silico* restriction mapping of the HytaNPV-R genome (MH261376.1; Nguyen et al, 2018) was performed in SnapGene Viewer.

2.3.2.1 *In silico* restriction map of HytaNPV-R (MH261376.1; Nguyen et al, 2018)

The restriction maps of HytaNPV-R complete genome for the restriction endonucleases, *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I and *Xho*I, used in the present study have been shown in **Figure 2-11**, **Figure 2-12** & **Figure 2-13**. *In silico* Restriction endonuclease fragment profiles of HytaNPV-R for the above-mentioned enzymes along with the position in the genome and flanking restriction sites of the fragments have been summarized in **Table 2-5, Table 2-6, Table 2-7**, and **Table 2-8**.

Restriction map of HytaNPV-R genome (139,089 bp) showed that a maximum of 34 fragments ranging from 46 - 17774 bp were produced with *Eco*RI digestion whereas, a minimum of 8 fragments ranging from 664 - 57101 bp were generated with *Pst*I. *In silico* digestion profile with *Bam*HI, *Bgl*I, *Hin*dIII, *Kpn*I, and *Xho*I revealed 9, 23, 9, 11 and 21 fragments ranging from 15 - 46431 bp, 90 - 18332 bp, 2621 – 43888 bp, 1562 - 53819 bp and 52 - 23663 bp, respectively.

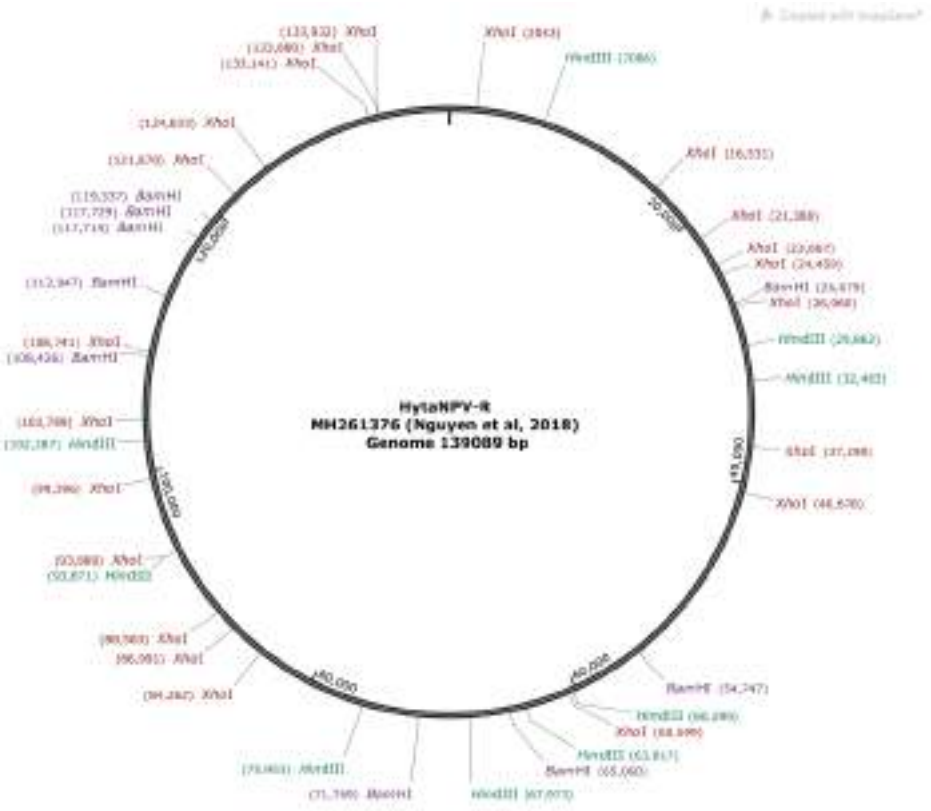


Figure 2-11: Restriction map of HytaNPV-R genome for *Bam*HI, *Hin*dIII and *Xho*I

Table 2-5: In silico *Bam*HI and *Hin*dIII fragment profiles of the complete genome of HytaNPV-R mentioning the positions of the restriction fragments along with its flanked restriction sites

	***Bam*HI**			***Hin*dIII**		
Sl. No.	**Fragment size (bp)**	**Cut positions/ coordinates**	**Flanked restriction sites**	**Fragments**	**Cut positions/ coordinates**	**Flanked restriction sites**
A.	46431	119338-26679	119337/119341 26679/26683	43888	102288-7086	102287/102291 7086/7090
B.	36657	71770-108426	71769/71773 108426/108430	27807	32484-60290	32483/32487 **60290/60294**
C.	28068	26680-54747	26679/26683 54747/54751	22776	7087-29862	7086/7090 29862/29866
D.	10313	54748-65060	54747/54751 65060/65064	17918	75954-93871	75953/75957 93871/93875
E.	6709	65061-71769	65060/65064 71769/71773	8416	93872-102287	93871/93875 102287/102291
F.	4767	112948-117714	112947/112951 117714/117718	7980	67974-75953	67973/67977 75953/75957
G.	4521	108427-112947	108426/108430 112947/112951	4156	63818-67973	**63817/63821** 67973/67977
H.	1608	117730-119337	117729/117733 119337/119341	**3527**	60291-63817	**60290/60294** **63817/63821**
I.	0015	117715-117729	117714/117718 117729/117733	2621	29863-32483	29862/29866 32483/32487
Total			**139089 bp**			

Table 2-6: *In silico Xho*I fragment profile of complete genome of HytaNPV-R mentioning the positions in the genome of the restriction fragments along with its flanked restriction sites.

		***Xho*I**	
Sl.No.	**Fragments**	**Cut positions/ coordinates**	**Flanked restriction sites**
A.	23663	60600-84262	60599/60603 84262/84266
B.	19923	40677-60599	40676/40680 60599/60603
C.	14488	2044-16531	2043/2047 **16531/16535**
D.	13129	108742-121870	108741/108745 121870/121874
E.	10330	26961-37290	26960/26964 37290/37294
F.	8308	124834-133141	124833/124837 133141/133145
G.	7200	133933-2043	133932/133936 2043/2047
H.	5516	93881-99396	93880/93884 99396/99400
I.	5297	88584-93880	88583/88587 93880/93884
J.	4952	103790-108741	103789/103793 108741/108745
K.	**4857**	16532-21388	**16531/16535** **21388/21392**
L.	4393	99397-103789	99396/99400 103789/103793
M.	3386	37291-40676	37290/37294 40676/40680
N.	2963	121871-124833	121870/121874 124833/124837
O.	2729	84263-86991	84262/84266 86991/86995
P.	2501	24460-26960	24459/24463 26960/26964
Q.	2279	21389-23667	**21388/21392** 23667/23671
R.	1592	86992-88583	86991/86995 88583/88587
S.	792	23668-24459	23667/23671 24459/24463
T.	739	133142-133880	133141/133145 133880/133884
U.	52	133881-133932	133880/133884 133932/133936

Table 2-7: *In silico Eco*RI and *Pst*I fragment profiles of the complete genome of HytaNPV-R mentioning the position in the genome of the restriction fragments along with its flanked restriction sites.

	***Eco*RI**			***Pst*I**		
	Fragments	**Cut positions/ coordinates**	**Flanked restriction sites**	**Fragments**	**Cut positions/ coordinates**	**Flanked restriction sites**
A.	17774	104931-122704	104930/104934 122704/122708	57101	138922-56933	138921/138917 56933/56929
B.	14882	38501-53382	38500/38504 53382/53386	53715	79940-133654	79939/79935 133654/133650
C.	12032	122705-134736	122704/122708 134736/134740	11600	66642-78241	66641/66637 78241/78237
D.	9415	93004-102418	93003/93007 102418/102422	5691	56934-62624	56933/56929 62624/62620
E.	8510	2015-10524	2014/2018 10524/10528	5267	133655-138921	133654/133650 138921/138917
F.	8379	63335-71703	63334/63338 71703/71707	3353	62625-65977	62624/62620 65977/65973
G.	7729	20016-27744	20015/20019 27744/27748	1698	78242-79939	78241/78237 79939/79935
H.	7025	56310-63334	56309/56313 63334/63338	664	65978-66641	65977/65973 66641/66637
I.	6245	73835-80079	73834/73838 80079/80083			
J.	5756	14260-20015	14259/14263 20015/20019			
K.	4725	134945-580	134944/134948 580/584			
L.	4229	34272-38500	34271/34275 38500/38504			
M.	3675	85225-88899	85224/85228 88899/88903			
N.	3660	81565-85224	81564/81568 85224/85228			
O.	3597	10525-14121	10524/10528 14121/14125			
P.	3247	27745-30991	27744/27748 30991/30995			
Q.	2777	53533-56309	53532/53536 56309/56313			
R.	2512	102419-104930	102418/102422 104930/104934			
S.	2131	71704-73834	71703/71707 73834/73838			
T.	1885	89836-91720	89835/89839 91720/91724			
U.	1627	31298-32924	31297/31301 32924/32928			
V.	1485	80080-81564	80079/80083 81564/81568			
W.	1434	581-2014	580/584 2014/2018			
X.	1347	32925-34271	32924/32928 34271/34275			
Y.	1283	91721-93003	91720/91724 93003/93007			

Table 2-7 *In silico Eco*RI and *Pst*I fragment profiles of the complete genome of HytaNPV-R mentioning the position in the genome of the restriction fragments along with its flanked restriction sites, continued from page 75.

		*Eco*RI		*Pst*I		
Z.	Fragments	Cut positions/ coordinates	Flanked restriction sites	Fragments	Cut positions/ coordinates	Flanked restriction sites
A	215	88900-89114	88899/88903 89114/89118			
BI	208	134737-134944	134736/134740 134944/134948			
C(	150	53383-53532	53382/53386 53532/53536			
DI	63	31235-31297	31234/31238 31297/31301			
EI	46	14122-14167	14121/14125 14167/14171			
FI	46	14168-14213	14167/14171 14213/14217			
G(	46	14214-14259	14213/14217 14259/14263			

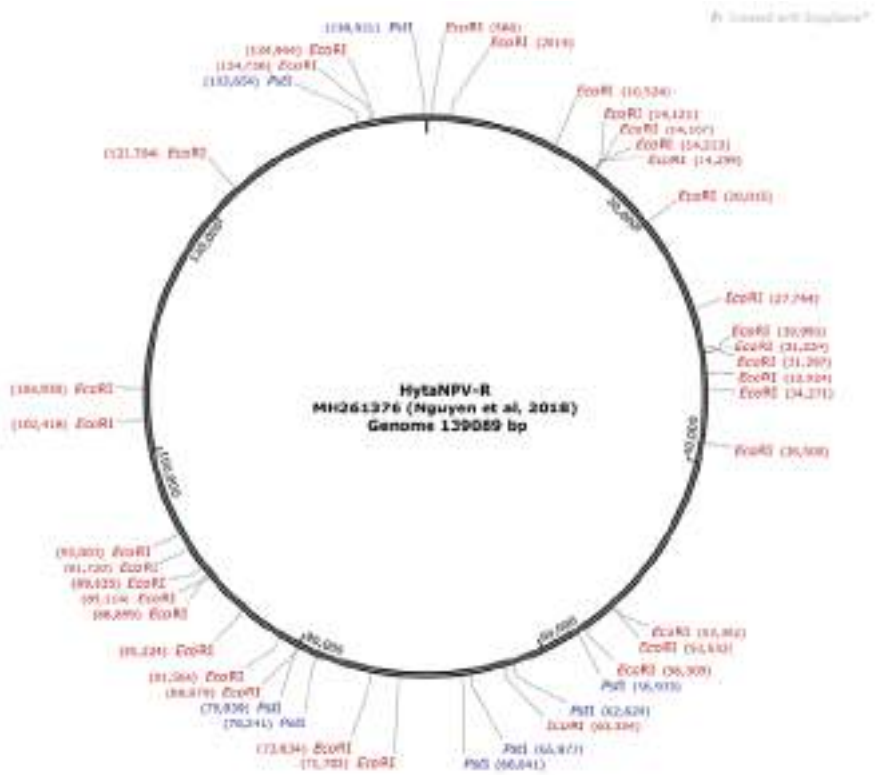


Figure 2-12: Restriction map of HytaNPV-R genome for *Eco*RI and *Pst*I

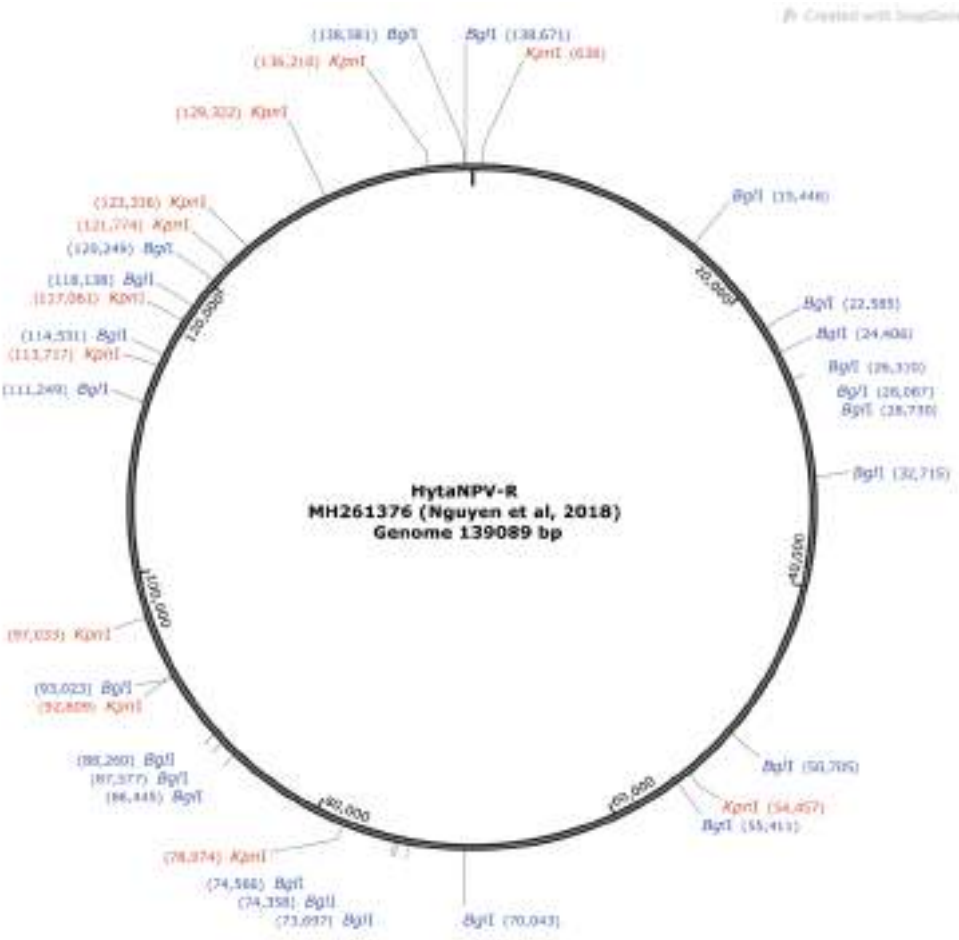


Figure 2-13: Restriction map of HytaNPV-R genome for *Bgl*I and *Kpn*I

Table 2-8: *In silico Kpn*I and *Bgl*I fragment profile of complete genome of HytaNPV-R mentioning the position in the genome of the restriction fragments along with its flanked restriction sites.

Fragments sizes (in bp) for respective restriction endonuclease digestion						
	***Kpn*I**			***Bgl*I**		
Sl. No.	**Fragments**	**Cut positions/ coordinates**	**Flanked restriction sites**	**Fragments**	**Cut positions/ coordinates**	**Flanked restriction sites**
A.	53819	639-54457	638/634 54457/54453	18332	120250-138581	120249/120246 138581/138578
B.	23617	54458-78074	54457/54453 78074/78070	18226	93024-111249	93023/93020 111249/111246
C.	16684	97034-113717	97033/97029 113717/113713	17990	32716-50705	32715/32712 50705/50702
D.	14735	78075-92809	78074/78070 92809/92805	15864	138672-15446	138671/138668 15446/15443
E.	6888	129323-136210	129322/129318 136210/136206	14632	55412-70043	55411/55408 70043/70040
F.	5986	123337-129322	123336/123332 129322/129318	11879	74567-86445	74566/74563 86445/86442
G.	4713	117062-121774	117061/117057 121774/121770	7139	15447-22585	15446/15443 22585/22582

Table 2-8: *In silico Kpn*I and *Bgl*I fragment profile of complete genome of HytaNPV-R mentioning the position in the genome of the restriction fragments along with its flanked restriction sites, continued from page 77.

Fragments sizes (in bp) for respective restriction endonuclease digestion						
	***Kpn*I**				***Bgl*I**	
H.	4224	92810-97033	92809/92805 97033/97029	4763	88261-93023	88260/88257 93023/93020
I.	3517	136211-638	136210/136206 638/634	4706	50706-55411	50705/50702 55411/55408
J.	3344	113718-117061	113717/113713 117061/117057	3985	28731-32715	28730/28727 32715/32712
K.	1562	121775-123336	121774/121770 123336/123332	3654	70044-73697	70043/70040 73697/73694
L.				3607	114532-118138	114531/114528 118138/118135
M.				3282	111250-114531	111249/111246 114531/114528
N.				2111	118139-120249	118138/118135 120249/120246
O.				1904	24407-26310	24406/24403 26310/26307
P.				1821	22586-24406	22585/22582 24406/24403
Q.				1777	26311-28087	26310/26307 28087/28084
R.				1.132	86446-87577	86445/86442 87577/87574
S.				683	87578-88260	87577/87574 88260/88257
T.				661	73698-74358	73697/73694 74358/74355
U.				643	28088-28730	28087/28084 28730/28727
V.				208	74359-74566	74358/74355 74566/74563
W.				90	138582-138671	138581/138578 138671/138668

2.3.2.2 Comparison of *in vitro* and *in silico* restriction endonuclease fragment profiles of HytaNPV isolates

The comparison of the number and the size-range of the restriction fragments between *in silico* analysis of HytaNPV-R complete genome and *in vitro* digestions of HytaNPV isolates (HytaNPV-ITK1 and HytaNPV-ID1) of the present study have been summarized in **Table 2-9**, **Table 2-10** & **Table 2-11**. The Tables show the comparative analysis of restriction endonuclease fragment profiles. In the case of *in vitro* digestion, low molecular weight fragments (<0.70 kb/<700 bp) were not resolved in agarose gel electrophoresis. The size of the fragments larger than the largest fragment of DNA marker is difficult to estimate

properly. The size of such bands was estimated from the total size estimated by *Eco*RI, *Bgl*I and *Xho*I digestion and also by considering the reported size of the HytaNPV genome by Nguyen et al. (2018). Crook et al. (1985) have estimated the heavier restriction fragments of *Sma*I, *Apa*I and *Hin*dIII profiles based on the total genome size estimated from restriction profiles of *Eco*RI, *Bam*HI and *Xho*I (having REN fragments a maximum of 34.7 kb).

Table 2-9: Comparisons of the number and size of restriction fragments between in silico digestion of HytaNPV-R complete genome and in vitro digestions of HytaNPV-ITK1 and HytaNPV-ID1

		***In silico* study**		***In vitro* study**			
Sl No.	**RE**	**HytaNPV-R**		**HytaNPV-ITK1**		**HytaNPV-ID1**	
		Nos. of fragments	**Size range in Kb**	**Nos. of fragments**	**Size range in Kb**	**Nos. of fragments**	**Size range in Kb**
1	*Bam*HI	9	0.015 - 46.431	8	1.61 - 46.43	8	1.61 - 46.43
2	*Bgl*I	23	0.090 - 18.332	18	1.13 - 18.13	18	1.13 - 18.13
3	*EcoR*I	34	0.046 – 17.774	26	0.72 – 17.77	26	0.72 – 17.77
4	*Hin*dIII	9	2.621 – 43.888	9	**2.11** – 43.88	9	**2.62** – 43.88
5	*Kpn*I	11	1.562 – 53.819	11	1.56 – 53.82	11	1.56 – 53.82
6	*Pst*I	8	0.664 – 57.101	7	1.70 – 57.10	7	1.70 – 57.10
7	*Xho*I	21	0.052 - 23.663	20	0.74 - 23.66	20	0.74 - 23.66

***RE = Restriction endonuclease**

Restriction endonuclease profiles obtained by *Kpn*I (both *in silico* and *in vitro*) in HytaNPV isolates were identical. The *in silico* restriction fragments, *Bam*HI-I (15 bp), *Bgl*I-S to *Bgl*I-W (683 bp - 90 bp), *Eco*RI-AA to EcoRI-HH (243 bp – 46 bp) and *Pst*I-H (664 bp), were absent in *in vitro* analyses as these low molecular weight fragments (<700 bp) could not be resolved in agarose gel electrophoresis.

HytaNPV-ID1 and HytaNPV-R showed a similar *Hin*dIII profile, whereas, the 3527 bp *Hin*dIII fragment was absent in HytaNPV-ITK1. Moreover, an additional *Hin*dIII fragment of 2110 bp was found in HytaNPV-ITK1, which was absent in HytaNPV-ID1 and HytaNPV-R (**Table 2-10**). Moreover, the total number of restriction fragments in HytaNPV-ITK1 was found to be the same as HytaNPV-R and HytaNPV-ID1, and no unique *Hin*dIII fragment of 1417 bp (3527-2110bp) was found in the *Hin*dIII profile of HytaNPV-ITK1.

The number of *Xho*I sites in all the three isolates of HytaNPV was found to be almost similar, except a 4857 bp *Xho*I fragment of HytaNPV-R (**Table 2-10**) was found to be absent in both the Terai (HytaNPV-ITK1) and Dooars (HytaNPV-ID1) isolates of HytaNPV of the present study (**Table 2-4**). Instead, for HytaNPV-ITK1, a 4100bp and in HytaNPV-ID1, a 4500bp unique *Xho*I fragment was detected (**Table 2-10**).

Table 2-10: Comparisons of *in silico* and in vitro restriction endonuclease fragment profiles (*Bam*HI, *Bgl*I, *Eco*RI & *Hin*dIII) of HytaNPV isolates (HytaNPV-ITK1, HytaNPV-ID1 and HytaNPV-R)

*Bam*HI fragments (in bp)	HytaNPV-R (*in silico*)	HytaNPV –ITK1(*in vitro*)	HytaNPV –ID1 (*in vitro*)	*Bgl*I fragments (in bp)	HytaNPV-R (*in silico*)	HytaNPV –ITK1(*in vitro*)	HytaNPV –ID1 (*in vitro*)	*Eco*RI fragments (in bp)	HytaNPV-R (*in silico*)	HytaNPV –ITK1(*in vitro*)	HytaNPV –ID1 (*in vitro*)	*Hin*dIII fragments (in bp)	HytaNPV-R (*in silico*)	HytaNPV –ITK1(*in vitro*)	HytaNPV –ID1 (*in vitro*)
46431	+	+	+	18332	+	+	+	17774	+	+	+	43888	+	+	+
36657	+	+	+	18226	+	+	+	14882	+	+	+	27807	+	+	+
28068	+	+	+	17990	+	+	+	12032	+	+	+	22776	+	+	+
10313	+	+	+	15864	+	+	+	9415	+	+	+	17918	+	+	+
6709	+	+	+	14632	+	+	+	8510	+	+	+	8416	+	+	+
4767	+	+	+	11879	+	+	+	8379	+	+	+	7980	+	+	+
4521	+	+	+	7139	+	+	+	7729	+	+	+	4156	+	+	+
1608	+	+	+	4763	+	+	+	7025	+	+	+	**3527***	+	-	+
15	+	?	?	4706	+	+	+	6245	+	+	+	2621	+	+	+
				3985	+	+	+	5756	+	+	+	**2110***	-	+	-
				3654	+	+	+	4725	+	+	+				
				3607	+	+	+	4229	+	+	+				
				3282	+	+	+	3675	+	+	+				
				2111	+	+	+	3660	+	+	+				
				1904	+	+	+	3597	+	+	+				
				1821	+	+	+	3247	+	+	+				
				1777	+	+	+	2777	+	+	+				
				1132	+	+	+	2512	+	+	+				
				683	+	?	?	2131	+	+	+				
				661	+	?	?	1885	+	+	+				
				643	+	?	?	1627	+	+	+				
				208	+	?	?	1485	+	+	+				
				90	+	?	?	1434	+	+	+				
								1347	+	+	+				
								1283	+	+	+				
								721	+	+	+				
								243	+	?	?				
								215	+	?	?				
								208	+	?	?				
								150	+	?	?				
								63	+	?	?				
								46	+	?	?				
								46	+	?	?				

Similar fragment +
Fragment absent -
Unique fragment
Undetected fragment ?

Table 2-11: Comparisons of *in silico* and in vitro restriction endonuclease fragment profiles (*Kpn*I, *Pst*I, *Xho*I) of HytaNPV isolates (HytaNPV-ITK1, HytaNPV-ID1 and HytaNPV-R).

*Kpn*I fragments (in bp)	HytaNPV-R (*in silico*)	HytaNPV –ITK1(*in vitro*)	HytaNPV –ID1 (*in vitro*)	*Pst*I fragments (in bp)	HytaNPV-R (*in silico*)	HytaNPV –ITK1(*in vitro*)	HytaNPV –ID1 (*in vitro*)	*Xho*I fragments (in bp)	HytaNPV-R (*in silico*)	HytaNPV –ITK1(*in vitro*)	HytaNPV –ID1 (*in vitro*)
53819	+	+	+	57101	+	+	+	23663	+	+	+
23617	+	+	+	53715	+	+	+	19923	+	+	+
16684	+	+	+	11600	+	+	+	14488	+	+	+
14735	+	+	+	5691	+	+	+	13129	+	+	+
6888	+	+	+	5267	+	+	+	10330	+	+	+
5986	+	+	+	3353	+	+	+	8308	+	+	+
4713	+	+	+	1698	+	+	+	7200	+	+	+
4224	+	+	+	664	+	?	?	5516	+	+	+
3517	+	+	+					5297	+	+	+
3344	+	+	+					4952	+	+	+
1562	+	+	+					**4857***	+	-	-
								4500*	-	-	+
								4393	+	+	+
								4100*	-	+	-
								3386	+	+	+
								2963	+	+	+
								2729	+	+	+
								2501	+	+	+
								2279	+	+	+
								1592	+	+	+
								792	+	+	+
								739	+	+	+
								52	+	?	?

Similar fragment present	+
Fragment absent	-
Unique fragment	
Undetected fragment	?

Section 2.4: Sequencing and characterization of HytaNPV genes

Objectives

5. To characterize HytaNPV gene(s) such as *pif* or some others, related to the pest control property of the virus.

2.4.1 PCR amplification of selected genes

Polyhedrin, the most conserved gene in all nucleopolyhedroviruses, has been used for phylogenetic studies of baculoviruses. In Betabaculoviruses (granuloviruses), instead of the *polyhedrin* gene, an ortholog, *granulin*, is present. Along with *polyhedrin* five other core genes, *lef-8*, *lef-9*, *pif-1*, *pif-2* and *pif-3*, were included for analyses in the present study. Two of them, *lef-8* and *lef-9*, the late expression factors, encode RNA polymerases and are expressed in the late phase of infection. The rest of the three, *pif-1*, *pif-2* and *pif-3*, are *per os* infectivity factor genes, the products of which are expressed in the occlusion bodies and bring about the host-specific binding in the peritrophic membrane of the insect host. The position and size of these six genes in the HytaNPV-R genome (Nguyen et al., 2018) have been summarized in **Table 2-12**. The size of the genes and their product vary among the baculoviruses (King et al., 2012).

Table 2-12: Position and size of the six genes (used for analyses in the present study) in the HytaNPV-R genome (Nguyen et al., 2018).

Genes	Position in Genome (nucleotide position)	Length of the gene (nucleotide)	Length of the protein product (amino acids)
polyhedrin	1..741	741 bp	247
lef-8	50,958..53,597	2640 bp	880
lef-9	36,624..38,141	1518 bp	506
pif-1	1,16,956..1,18,542	1587 bp	529
pif-2	1,10,655..1,11,806	1152 bp	384
pif-3	1,01,064..1,01,693	630 bp	210

PCR products amplified with appropriate primers (**Table 2-13**) using the DNA of NPVs isolated from *Hyposidra talaca* (HytaNPV) were sequenced (**Figure 2-16, Figure 2-17**). Details of the primers and the length of the PCR products have been summarized in **Table 2-13,** and the binding sites of the primers using the HytaNPV-R (MH261376.1; Nguyen et al., 2018) as a reference template have been presented in **Figure 2-14**. The photographs of agarose gel electrophoresis of the PCR products have been documented in **Figure 2-15.**

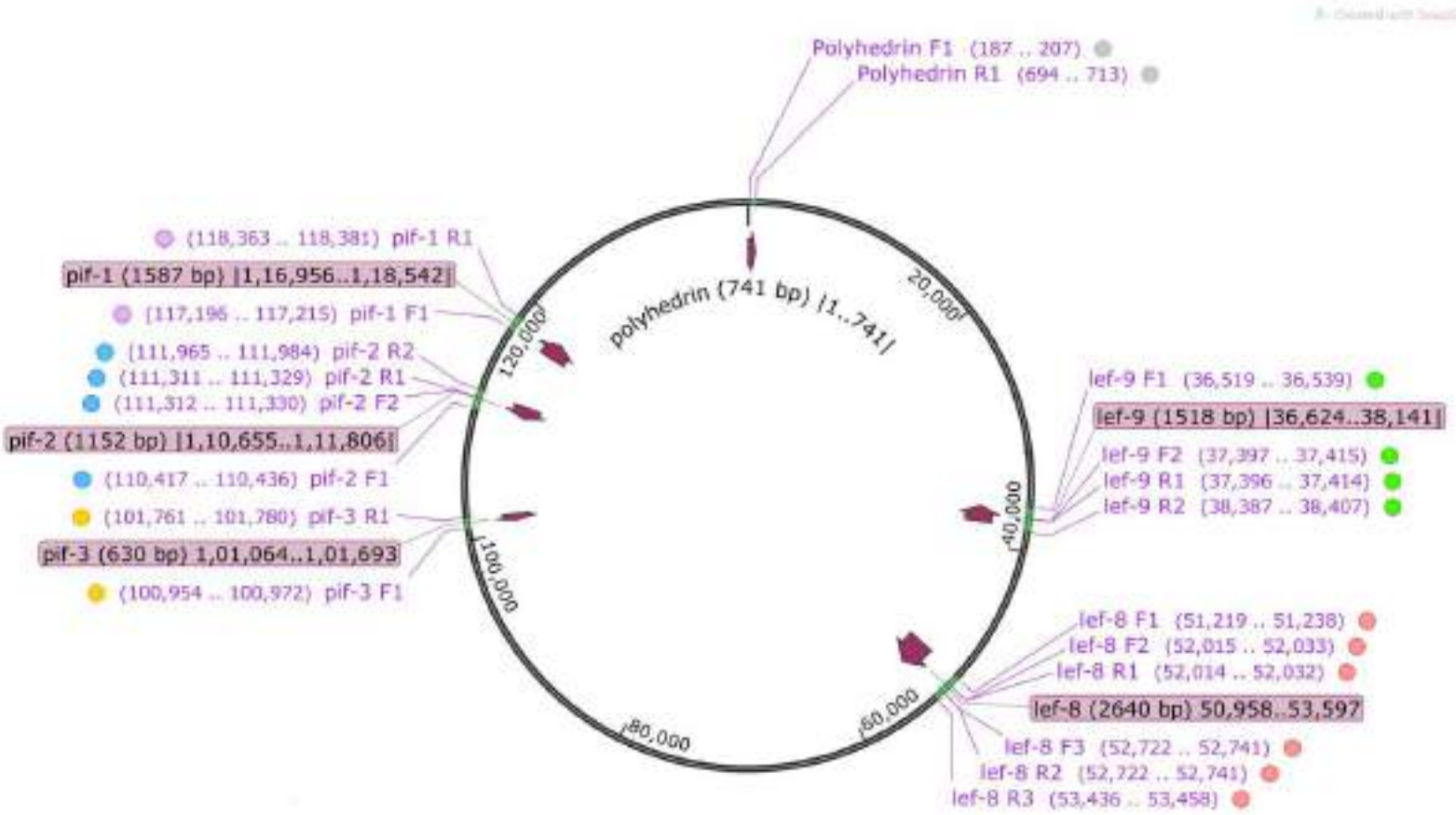


Figure 2-14: Position and details of the six genes used in the present study (purple arrow) and the binding sites of the primers used to amplify respective genes of the HytaNPV genome. HytaNPV-R 139.089 kb (MH261376.1; Nguyen et al., 2018) was used as a reference template. Details of the genes were highlighted in purple boxes and details of the primers have been represented without any highlighted box. Coloured circles were used to distinguish the primers used for different genes.

Table 2-13: Details of sequences, binding sites (nucleotide position) of primers used to amplify specific genes of HytaNPV and length of the respective PCR products. Binding sites of the primers were shown using HytaNPV-R (MH261376.1; Nguyen et al., 2018) as a reference.

Primer	Direction	Primer Sequence	length (nt)	Binding site	
				Start	Stop
		***polyhedrin* Primers**			
Polh F1	Forward	5'-GGACCSGGYAARAAYCAAAAA-3'	21	187	207
Polh R1	Reverse	5'-GCRTCWGGYGCAAAYTCYTT-3'	20	694	713
Product length	*527 bp*	*Fragment A (Primer set: Polh F1 & Polh R1)*			
		***lef-8* Primers**			
lef-8 F1	Forward	5'-GGCACTTTCATGATHGACGG-3'	20	51,219	51,238
lef-8 R1	Reverse	5'-CCACCGTCATTTCNCCGTG-3'	19	52,014	52,032
Product length	*813 bp*	*Fragment A (Primers set: lef-8 F1& lef-8 R1)*			
lef-8 F2	Forward	5'-ACGGNGAAATGACGGTGGC-3'	19	52,015	52,033
lef-8 R2	Reverse	5'-GGDATRTANGGRTCTTCGGC-3'	20	52,722	52,741
Product length	*726 bp*	*Fragment B (Primer set: lef-8 F2& lef-8 R2)*			
lef-8 F3	Forward	5'-GCCGAAGAYCCNTAYATHCC-3'	20	52,722	52,741
lef-8 R3	Reverse	5'-GATTGRTTDATNGTCCATTGATC-3'	23	53,436	53,458
Product length	*736 bp*	*Fragment C (Primer set: lef-8 F3& lef-8 R3)*			
Product length	*1539 bp*	*Fragment AB (Primer set: lef-8 F1& lef-8 R2)*			
Product length	*1462 bp*	*Fragment ABC (Primer set: lef-8 F1& lef-8 R3)*			
		***lef-9* Primers**			
lef-9 F1	Forward	5'- CGCTTTCGGATTTTGTCTTCA -3'	21	36,519	36,539
lef-9 R1	Reverse	5'- CCGACTTTACCGACTGGAA -3'	19	37,396	37,414
Product length	*813 bp*	*Fragment A (Primer set: lef-9 F1 & lef-9 R1)*			
lef-9 F2	Forward	5'- TCCAGTCGGTAAAGTCGGC -3'	19	37,397	37,415
lef-9 R2	Reverse	5'- CCCGTAAATTTCGACGCTACT -3'	21	38,387	38,407
Product length	*726 bp*	*Fragment B (Primer set: lef-9 F2 & lef-9 R2)*			
Product length	*1462 bp*	*Fragment AB (Primer set: lef-9 F1 & lef-9 R2)*			
		***pif-1* Primers**			
pif-1 F1	Forward	5'-GARGGNCTNGCNAAYTGYCA-3'	20	1,17,196	1,17,215
pif-1 R1	Reverse	5'-TNGGRTANCGNGTNGCNGG-3'	19	1,18,363	1,18,381
Product length	*1186 bp*	*Fragment A (Primer set: pif-1 F1 & pif-1 R1)*			
		***pif-2* Primers**			
pif-2 F1	Forward	5'-CTGGTCAAAAACCTACCGAC-3'	20	1,10,417	1,10,436
pif-2 R1	Reverse	5'-GATCCGCGTTATTTTGCCG-3'	19	1,11,311	1,11,329
Product length	*913 bp*	*Fragment A (Primer set: pif-2 F1 & pif-2 R1)*			
pif-2 F2	Forward	5'-GGCAAAATAACGCGGATCT-3'	19	1,11,312	1,11,330
pif-2 R1	Reverse	5'-ACGAACAACACGCAAAAATG-3'	20	1,11,965	1,11,984
Product length	*673 bp*	*Fragment B (Primer set: pif-2 F2 & pif-2 R2)*			
Product length	*1,587 bp*	*Fragment AB (Primer set: pif-2 F1 & pif-2 R2)*			
		***pif-3* Primers**			
pif-3 F1	Forward	5'-CAAGAAACGTGCAGGCAAA-3'	19	1,00,954	1,00,972
pif-3 R1	Reverse	5'-ATCAACAATCGCAATACGGC'3'	20	1,01,761	1,01,780
Product length	*827 bp*	*Fragment A (Primer set: pif-3 F1 & pif-3 R1)*			

PCR amplification of selected genes

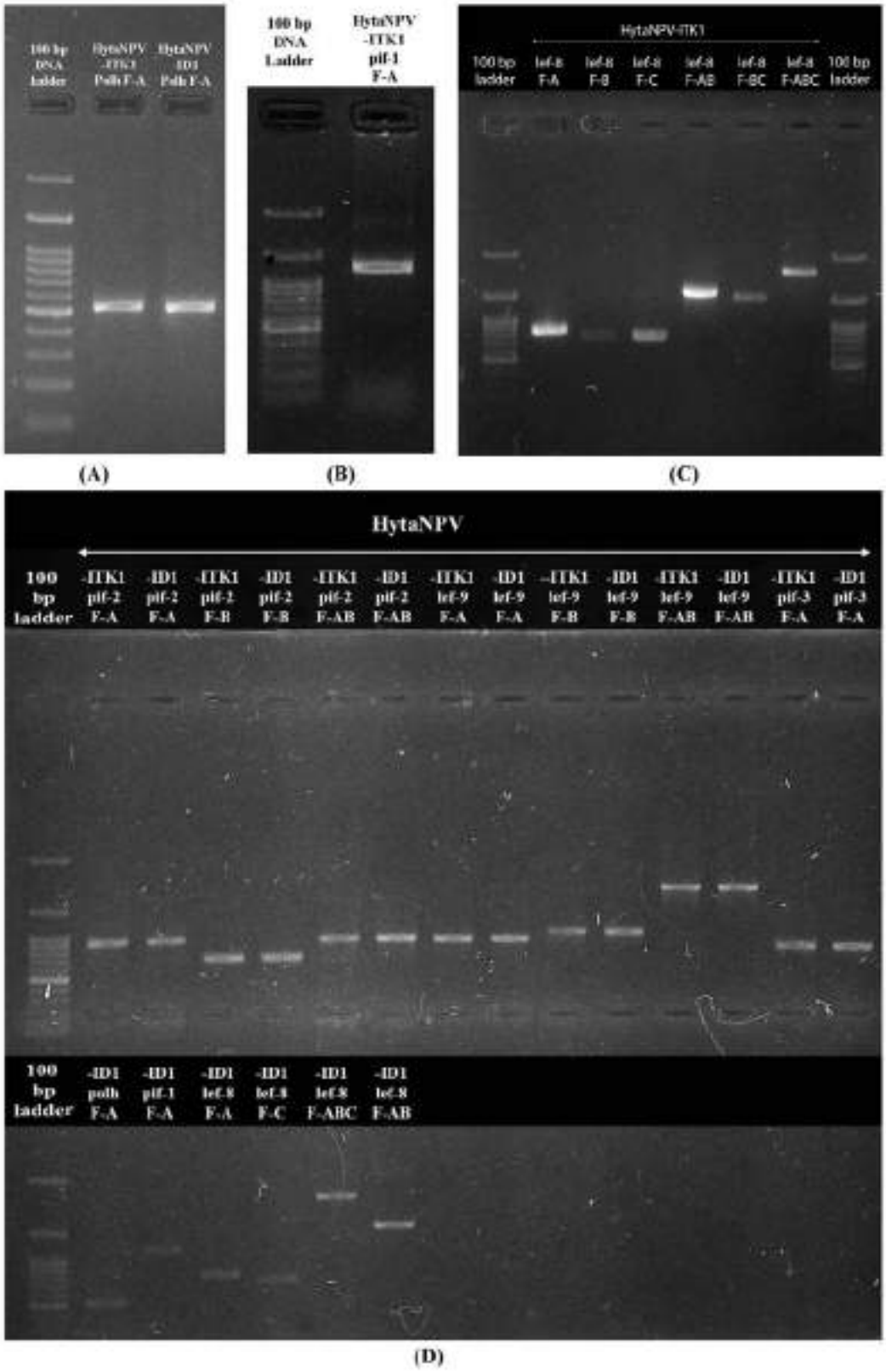


Figure 2-15: Electrophoregrams showing PCR products of HytaNPV DNA. HytaNPV-ITK1 and HytaNPV-ID1 represent the Terai and Dooars isolate, respectively and F stands for 'fragment' (see Table 2-13).

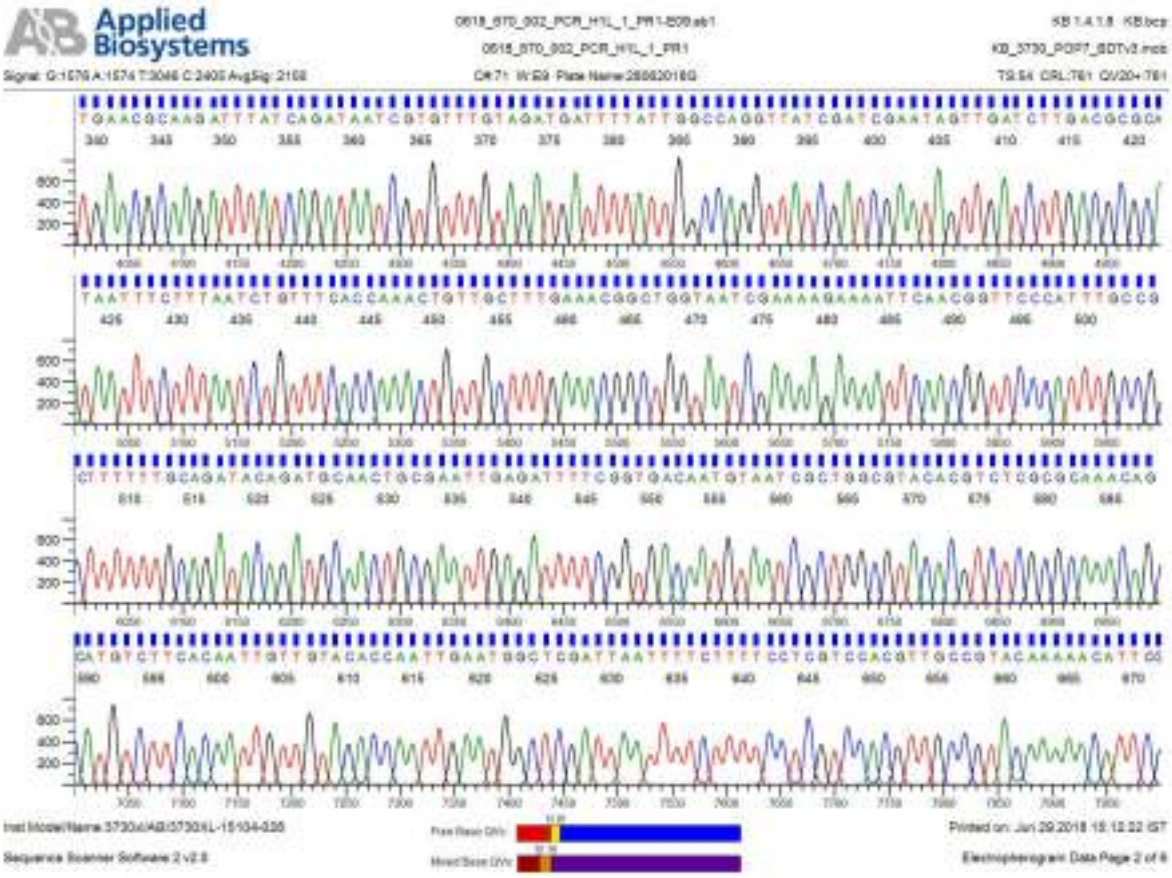


Figure 2-16: Electrophoregram of sequencing of *lef-8* gene.

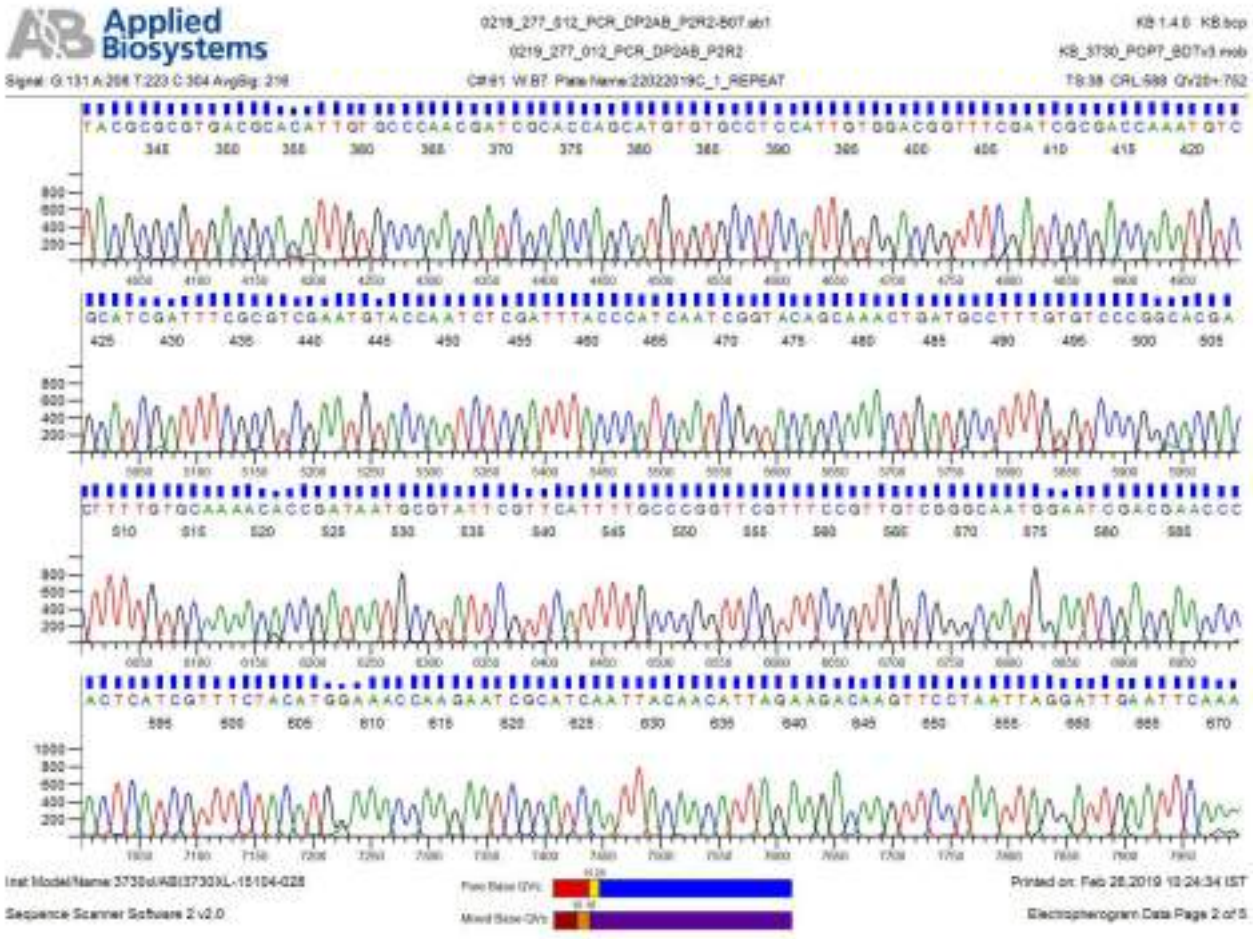


Figure 2-17: Electrophoregram of sequencing of *pif*-2 gene.

All the sequences obtained for individual genes of HytaNPVs (Terai isolate: HytaNPV-ITK1 and Dooars isolate: HytaNPV-ID1) in the present study were submitted to the NCBI GenBank database with the following details (**Table 2-14**).

Table 2-14: Details of gene sequences submitted to NCBI GenBank database. The location of each sequence has been shown using HytaNPV-R (MH261376.1; Nguyen et al, 2018) as reference.

Sl. No.	Isolate	Country: Region/ Location	Accession No. (submission date)	Sequence length (bp)	Ref location (MH261376.1) nucleotide position
		polyhedrin			
1	HytaNPV-ITK1	India: Terai, Darjeeling, West Bengal	KX665534.1 (04-AUG-2016)	527	187 .. 713
2	HytaNPV-ID1	India: Dooars, West Bengal	MN153042.2 (03-JUL-2019)	524	190 .. 713
		lef-8			
3	HytaNPV-ITK1	India: Terai, Darjeeling, West Bengal	MH558670.1 (01-JUL-2018)	2210	51,237 .. 53,446
4	HytaNPV-ID1	India: Dooars, West Bengal	MN153043.1 (03-JUL-2019)	722	52,722 .. 53,443
		lef-9			
5	HytaNPV-ITK1	India: Terai, Darjeeling, West Bengal	MN117909.1 (28-JUN-2019)	1518	36,624 .. 38,141
6	HytaNPV-ID1	India: Dooars, West Bengal	MN117910.2 (28-JUN-2019)	1518	36,624 .. 38,141
		pif-1			
7	HytaNPV-ITK1	India: Terai, Darjeeling, West Bengal	KU050704.1 (05-NOV-2015)	510	1,17,205 .. 1,17,714
8	HytaNPV-ITK1	India: Terai, Darjeeling, West Bengal	MH558671.1 (01-JUL-2018)	930	1,17,205 .. 1,18,134
9	HytaNPV-ID1	India: Dooars, West Bengal	MN153041.1 (03-JUL-2019)	1117	1,17,235 .. 1,18,351
		pif-2			
10	HytaNPV-ITK1	India: Terai, Darjeeling, West Bengal	MN153040.1 (03-JUL-2019)	1152	1,11,806 .. 1,10,655
11	HytaNPV-ID1	India: Dooars, West Bengal	MT642700.1 (17-JUN-2020)	1098	1,11,752 .. 1,10,655
		pif-3			
12	HytaNPV-ITK1	India: Terai, Darjeeling, West Bengal	MT642701.1 (17-JUN-2020)	630	1,01,693 .. 1,01,064
13	HytaNPV-ID1	India: Dooars, West Bengal	MT642702.1 (17-JUN-2020)	630	1,01,693 .. 1,01,064

2.4.2 Sequencing of the *polyhedrin* gene

In the present study, 527 and 524 bp sequences of *polyhedrin* gene were obtained for HytaNPV-ITK1 and HytaNPV-ID1, respectively (**Table 2-14**, **Figure 2-18**).

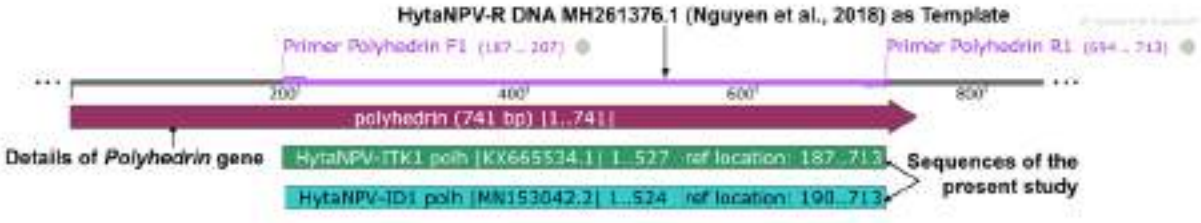


Figure 2-18: Primer binding, amplicon and sequence details of polyhedrin gene using HytaNPV-R as reference (the amplified portion was shown in purple colour on the template DNA).

2.4.2.1 Blast analysis

In NCBI blastn search, partial sequence of *polyhedrin* gene of HytaNPV-ITK1 and HytaNPV-ID1 showed a maximum identity of 98.86% and 98.66% with HytaNPV-R from India, respectively, while 100% similarity was found for protein (Protein ID: AWW14361.1) in Blastx results (**Table 2-15**). HytaNPV-ITK1 and HytaNPV-ID1 exhibited maximum nucleotide similarity with EcobNPV (86.148% and 86.069%, respectively) and maximum amino acid sequence identity with BusuNPV (98.286% and 98.276%, respectively) among the NPVs infecting the specimens of other genera than *Hyposidra*. NPVs infecting the hosts of the different families other than Geometridae showed less similarity with HytaNPV-ITK1 and HytaNPV-ID1 for both nucleotide as well as protein.

2.4.2.2 Alignment

Clustal W alignment of the partial sequences of *polyhedrin* gene of HytaNPV-ITK1 and HytaNPV-ID1 using HytaNPV-R as a template revealed coverage of 71.12% and 70.72% of the total reading frame of *polyhedrin* gene, respectively (**Figure 2-19**). A total of 520 conserved and 7 variable sites for nucleotide and 175 conserved without any variable sites for amino acid were found, respectively (**Figure 2-19**).

Table 2-15: Blast results of *polyhedrin* sequence.

Sl. No.	Subject	subject acc. no.	% identity	alignment length	mismatches
blastn (nucleotide blast)					
Query acc. no. KX665534.1 (HytaNPV-ITK1)					
1	HytaNPV-R	MH261376.1	98.861	527	6
2	EcobNPV	DQ837165.1	86.148	527	73
3	BusuNPV	KF611977.1	83.871	527	85
4	SujuNPV	KJ676450.1	81.973	527	95
5	ApciNPV	FJ914221.1	81.784	527	96
6	EupsNPV	FJ227128.1	80.645	527	102
7	AcMNPV	L22858.1	78.558	527	113
8	BmNPV	JQ991010.1	74.194	527	136
Query acc. no. MN153042.2 (HytaNPV-ID1)					
1	HytaNPV-R	MH261376.1	98.664	524	7
2	EcobNPV	DQ837165.1	86.069	524	73
3	BusuNPV	KF611977.1	83.969	524	84
4	SujuNPV	KJ676450.1	81.679	524	96
5	ApciNPV	FJ914221.1	81.679	524	96
6	EupsNPV	FJ227128.1	80.534	524	102
7	AcMNPV	L22858.1	78.244	524	114
8	BmNPV	JQ991010.1	74.046	524	136
blastx (protein blast)					
Query acc. no. KX665534.1 (HytaNPV-ITK1)					
1	HytaNPV-R	AWW14361.1	100	175	0
2	BusuNPV	YP_009001778.1	98.286	175	3
3	EcobNPV	YP_874194.1	96.571	175	6
4	ApciNPV	YP_006607771.1	94.286	175	10
5	EupsNPV	YP_002854611.1	94.286	175	10
6	SujuNPV	YP_009186692.1	93.714	175	11
7	AcMNPV	AAA46736.1	93.143	175	12
8	BmNPV	AFJ06797.1	88	175	21
Query Acc. no. MN153042.2 (HytaNPV-ID1)					
1	HytaNPV-R	AWW14361.1	100	174	0
2	BusuNPV	YP_009001778.1	98.276	174	3
3	EcobNPV	YP_874194.1	96.552	174	6
4	ApciNPV	YP_006607771.1	94.253	174	10
5	EupsNPV	YP_002854611.1	94.253	174	10
6	SujuNPV	YP_009186692.1	93.678	174	11
7	AcMNPV	AAA46736.1	93.103	174	12
8	BMNPV	AFJ06797.1	87.931	174	21

Figure 2-19: Nucleotide and translated amino acid sequence alignment of the *polyhedrin* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background.

2.4.2.3 Restriction sites on *polyhedrin* sequence

The partial sequences of *polyhedrin* gene of HytaNPV-ITK1 and HytaNPV-ID1 showed restriction sites for *Eco*RI and *Kpn*I, each homologous to the restriction sites of *Eco*RI (position: 580) and *Kpn*I (position: 638) of the genome of HytaNPV-R (**Figure 2-20**).

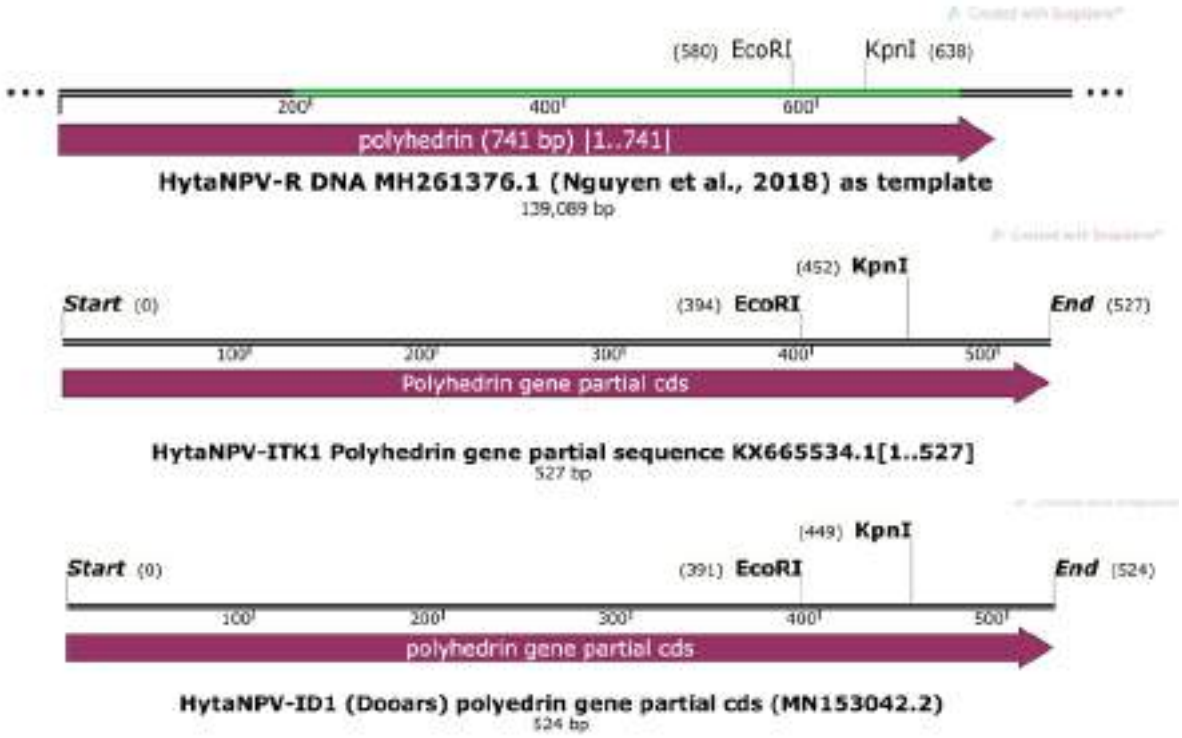


Figure 2-20: Restriction sites present in the *polyhedrin* of different HytaNPV isolates.

2.4.3 Sequencing of *lef-8* gene

In the present study, PCR with the primers specific to the *lef-8* gene (**Table 2-13**) using the DNA of HytaNPV-ITK1 and HytaNPV-ID1 produced amplicons of 813bp (Fragment A), 726bp (Fragment B), 736bp (Fragment C), 1539bp (Fragment AB), 1462bp (Fragment BC) and 2275bp (Fragment ABC) (**Figure 2-14**, **Figure 2-15**). After sequencing of the amplified products 2210 bp (Product: 736 amino acids) sequence representing partial cds of the *lef-8* gene was obtained for HytaNPV-ITK1, whereas, a 722 bp partial cds was obtained for that of HytaNPV-ID1. Both of the sequences were submitted to the NCBI GenBank database with the following details (**Table 2-14, Figure 2-21**).

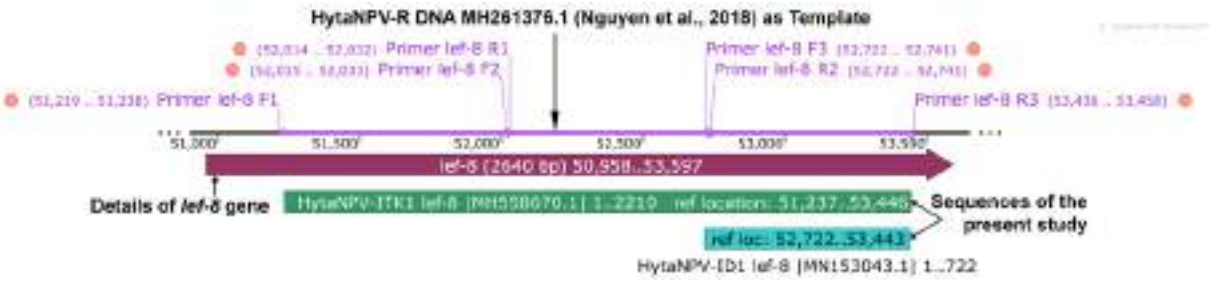


Figure 2-21: Primer binding, amplicon and sequence details of the *lef-8* gene using HytaNPV-R as reference.

2.4.3.1 Blast analysis

In NCBI blastn search, partial sequence of *lef-8* gene of HytaNPV-ITK1 and HytaNPV-ID1 showed a maximum identity of 99.77% and 99.44% with HytaNPV-R (MH261376.1) from India, respectively, whereas, among the NPVs pathogenic to the specimens of different genus BusuNPV (KF611977.1) from China showed higher nucleotide sequence homology of 83.44% and 84.77%, respectively.

Blastx results showed a similarity of 99.45% for HytaNPV-ITK1 and 99.58% for HytaNPV-ID1 with HytaNPV-R (Protein ID: AWW14417.1) (**Table 2-16**) whereas, a comparatively lower similarity of 90.22% and 92.50% were obtained for HytaNPV-ITK1 and HytaNPV-ID1, respectively when compared with BusuNPV (YP_009001888.1).

2.4.3.2 Alignments

Clustal W alignment of the partial sequences of the *lef-8* gene of HytaNPV-ITK1 and HytaNPV-ID1 using HytaNPV-R as a reference template revealed coverage of 83.71% and

27.34% of the total reading frame of *lef-8*, respectively (**Figure 2-22**). A total of 9 and 5 variable sites were found in the 2210 nucleotides and 736 amino acids long alignments, respectively (**Figure 2-22**).

Table 2-16: Blast results *lef-8* sequence

Sl. No.	Subject Details	subject acc. no.	% identity
blastn (nucleotide blast)			
Query acc. no. MH558670.1 (HytaNPV-ITK1)			
1	HyaNPV	MH261376.1	99.77
2	BusuNPV	KF611977.1	83.44
3	ApciNPV	FJ914221.1	70.85
4	SujuNPV	KJ676450.1	75.92
5	EcobNPV	DQ837165.1	70.32
6	EupsNPV	FJ227128.1	73.78
7	BmNPV	JQ991010.1	67.81
8	AcMNPV	L22858.1	67.68
Query acc. no. MN153043.1 (HytaNPV-ID1)			
1	HyaNPV-R	MH261376.1	99.45
2	BusuNPV	KF611977.1	84.77
3	ApciNPV	FJ914221.1	71.65
4	SujuNPV	KJ676450.1	77.41
5	EcobNPV	DQ837165.1	70.92
6	EupsNPV	FJ227128.1	75.49
7	BmNPV	JQ991010.1	69.90
8	AcMNPV	L22858.1	71.38
blastx (protein blast)			
Query acc. no. MH558670.1 (HytaNPV-ITK1)			
1	HyaNPV-R	AWW14483.1	99.46
2	BusuNPV	YP_009001888.1	90.22
3	SujuNPV	YP_009186820.1	75.46
4	ApciNPV	YP_006607789.1	72.19
5	EcobNPV	YP_874291.1	72.36
6	EupsNPV	YP_002854725.1	74.87
7	AcMNPV	NP_054149.1	62.74
8	BmNPV	AFN21074.1	62.87
Query acc. no. MN153043.1 (HytaNPV-ID1)			
1	HyaNPV-R	AWW14483.1	99.58
2	BusuNPV	YP_009001888.1	92.50
3	SujuNPV	YP_009186820.1	82.92
4	ApciNPV	YP_006607789.1	74.17
5	EcobNPV	YP_874291.1	75.52
6	EupsNPV	YP_002854725.1	76.67
7	AcMNPV	NP_054149.1	67.50
8	BmNPV	AFN21074.1	67.92

Sequencing of lef-8 gene

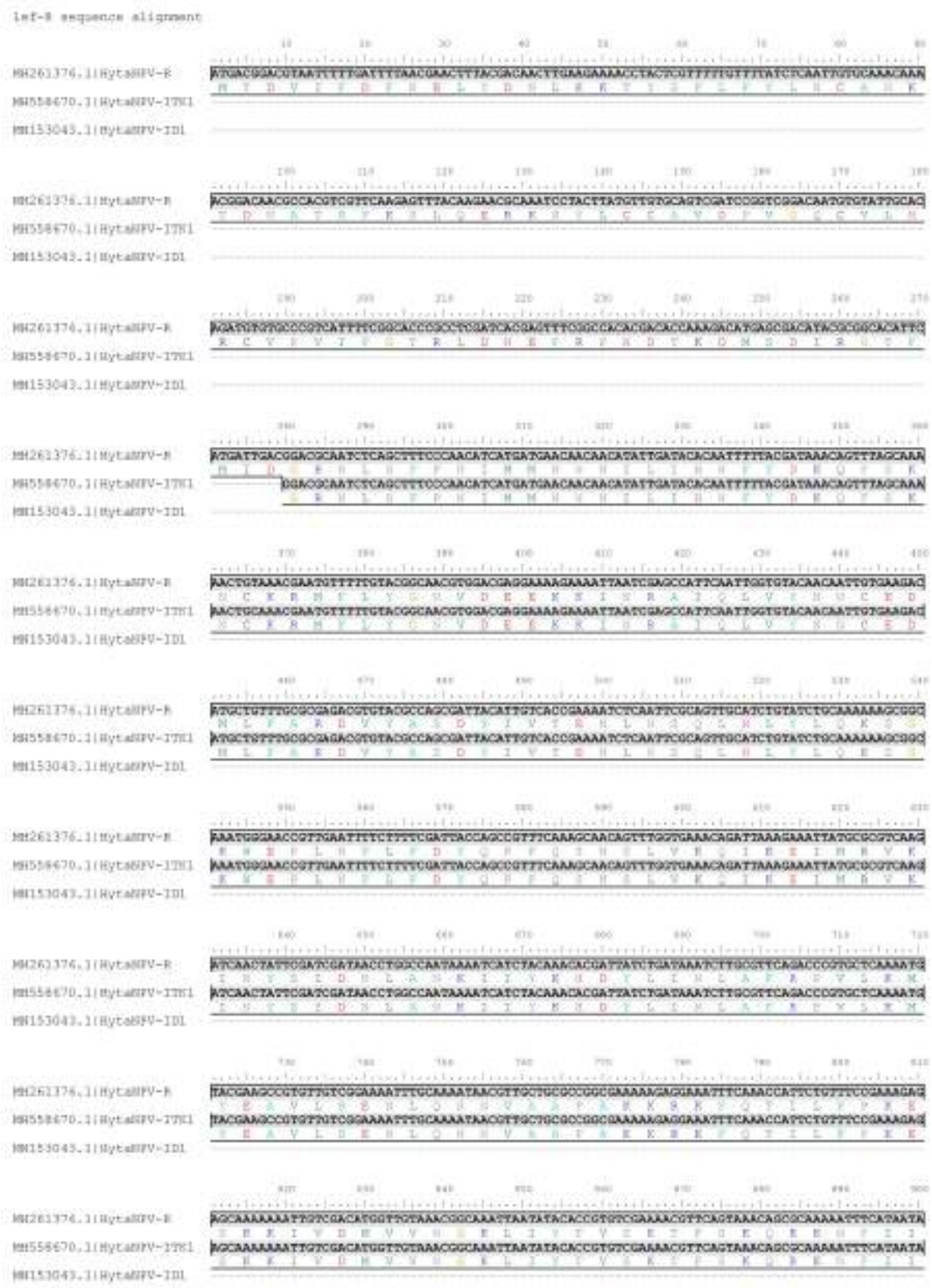


Figure 2-22: Nucleotide and translated amino acid sequence alignment of the *lef-8* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background.

Figure 2-22: Nucleotide and translated amino acid sequence alignment of the *lef-8* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background [Continued…].

Sequencing of lef-8 gene

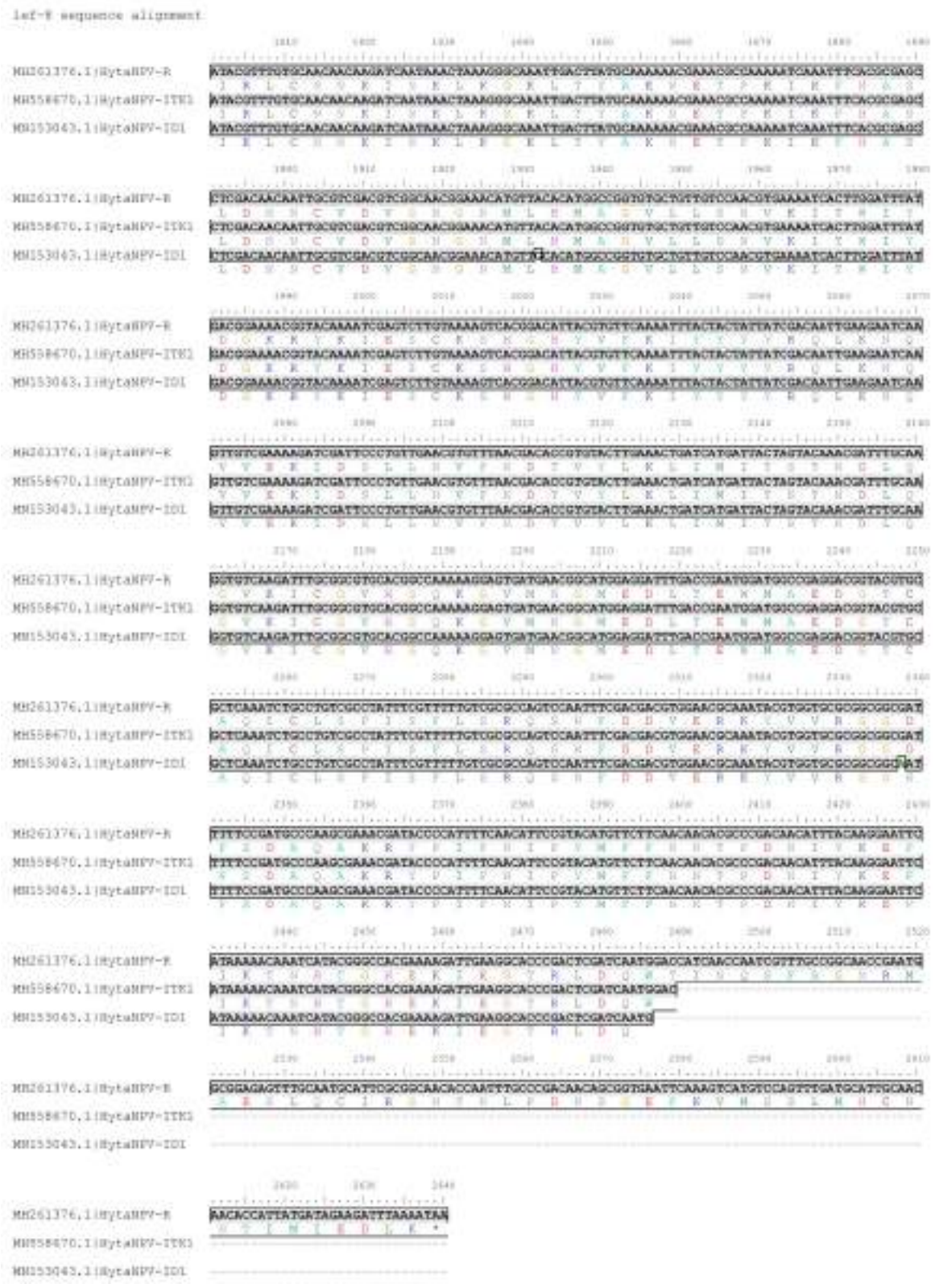


Figure 2-22: Nucleotide and translated amino acid sequence alignment of the *lef-8* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background [Continued…].

2.4.3.3 Restriction sites on *lef-8* sequence

Both of the partial sequences of the *lef-8* gene of HytaNPV-ITK1 and HytaNPV-ID1 had a restriction site for *Eco*RI at nucleotide position 2146 and 661, respectively, equivalent to the *Eco*RI site at nucleotide position 53382 of the genome of HytaNPV-R (**Figure 2-23**).

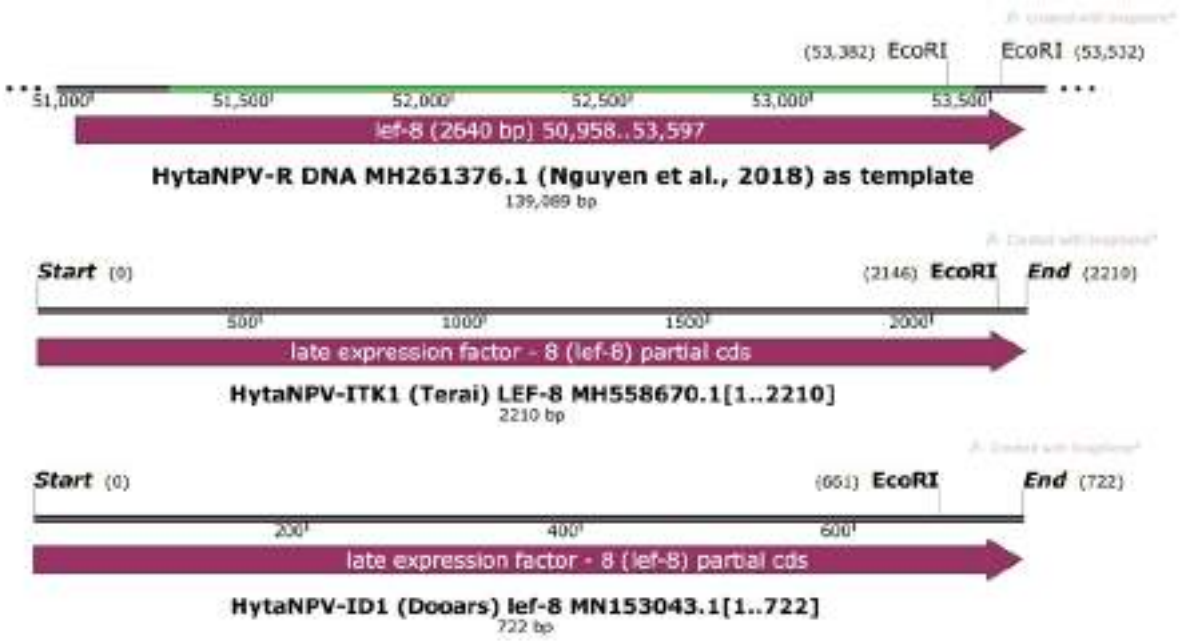


Figure 2-23: Restriction sites present in the *lef-8* of different HytaNPV isolates.

2.4.4 Sequencing of *lef-9* gene

PCR amplification of *lef-9* gene (primers are shown in **Table 2-13**) using the DNA of NPVs from *Hyposidra talaca* as template produced amplicons of 895bp (Fragment A), 1010bp (Fragment B), and 1905bp (Fragment AB) (as shown in **Figure 2-14, Figure 2-15**). After sequencing of the amplified products, a 1518 bp (Product: 506 amino acids) sequence representing the complete cds of the *lef-9* gene was obtained for HytaNPV-ITK1 and HytaNPV-ID1, respectively consisting of an intermediate gap of 55 bp for HytaNPV-ITK1 and three intermediate gaps of 8bp, 4 bp and 2 bp for HytaNPV-ID1. Both the sequences were submitted to the NCBI GenBank database (already shown in **Table 2-14). Figure 2-24** represents the primer binding sites, amplicon and sequence details of *lef-9*.

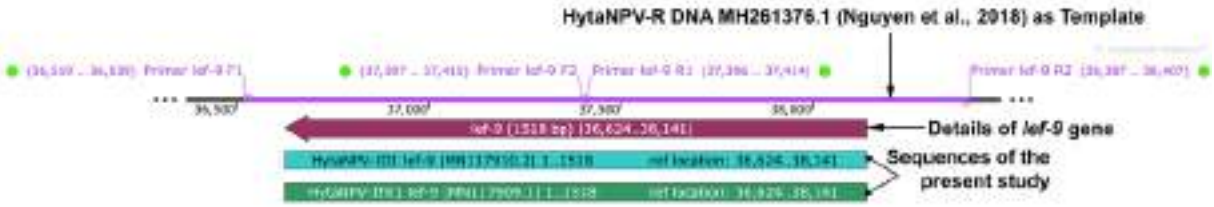


Figure 2-24: Primer binding, amplicon and sequence details of the *lef-9* gene using HytaNPV-R as a template.

2.4.4.1 Blast analysis

In NCBI blastn search, the partial sequences of the *lef-9* gene of HytaNPV-ITK1 and HytaNPV-ID1 showed a maximum identity of 99.52% and 99.33% with HytaNPV-R from India (MH261376.1), respectively. In compared to BusuNPV (KF611977.1) a nucleotide identity of 85.01% and 85.07% were found for HytaNPV-ITK1 and HytaNPV-ID1, respectively (**Table 2-17**).

Blastx results showed that HytaNPV-ITK1 and HytaNPV-ID1 had the highest similarity of 98.97% and 98.79% with the reference HytaNPV-R (Protein ID: AWW14399.1), respectively, while the maximum similarity of 92.37% (for HytaNPV-ITK1) and 92.17% (for HytaNPV-ID1) was revealed with BusuNPV (YP_009001888.1) among the other NPVs infecting the hosts of different genera.

2.4.4.2 Alignments

Clustal W alignment of the partial sequences of the *lef-9* gene of HytaNPV-ITK1 and HytaNPV-ID1 using HytaNPV-R as a template revealed a complete coverage of the total

reading frame of *lef-9* (**Figure 2-25**). A total of 11 variable sites were detected in the 1518 bp long alignment, while seven (7) variable sites were found out of 506 amino acid long alignments (**Figure 2-25**).

Table 2-17: Blast results lef-9 sequence

Sl. No.	Subject Details	subject acc. no.	% identity
blastn (nucleotide blast)			
Query acc. no. MN117909.1 (HytaNPV-ITK1)			
1	HytaNPV-R	MH261376.1	99.52
2	BusuNPV	KF611977.1	85.01
3	ApciNPV	FJ914221.1	74.38
4	SujuNPV	KJ676450.1	74.63
5	EcobNPV	DQ837165.1	75.79
6	EupsNPV	FJ227128.1	74.49
7	BmNPV	JQ991010.1	71.98
8	AcMNPV	L22858.1	70.19
Query acc. no. MN117910.2 (HytaNPV-ID1)			
1	HyaNPV-R	MH261376.1	99.33
2	BusuNPV	KF611977.1	85.07
3	ApciNPV	FJ914221.1	74.43
4	SujuNPV	KJ676450.1	74.64
5	EcobNPV	DQ837165.1	75.87
6	EupsNPV	FJ227128.1	74.58
7	BmNPV	JQ991010.1	72.20
8	AcMNPV	L22858.1	70.18
blastx (protein blast)			
Query acc. no. MN117909.1 (HytaNPV-ITK1)			
1	HyaNPV-R	AWW14483.1	98.97
2	BusuNPV	YP_009001888.1	92.37
3	SujuNPV	YP_009186820.1	83.093
4	ApciNPV	YP_006607789.1	82.34
5	EcobNPV	YP_874291.1	79.79
6	EupsNPV	YP_002854725.1	78.22
7	AcMNPV	NP_054149.1	68.87
8	BmNPV	AFN21074.1	68.44
Query acc. no. MN117910.2 (HytaNPV-ID1)			
1	HyaNPV-R	AWW14483.1	98.80
2	BusuNPV	YP_009001888.1	92.17
3	SujuNPV	YP_009186820.1	82.70
4	ApciNPV	YP_006607789.1	81.99
5	EcobNPV	YP_874291.1	79.71
6	EupsNPV	YP_002854725.1	77.85
7	AcMNPV	NP_054149.1	68.97
8	BmNPV	AFN21074.1	68.55

Sequencing of lef-9 gene

Figure 2-25: Nucleotide and translated amino acid sequence alignment of *lef-9* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as reference. Similar sequences were highlighted in grey background.

Figure 2-25: Nucleotide and translated amino acid sequence alignment of *lef-9* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as reference. Similar sequences were highlighted in grey background [Continued…].

2.4.4.3 Restriction sites on *lef-9* sequence

One *Xho*I restriction site at nucleotide position, 847, was detected in both of the partial sequences of the *lef-9* in HytaNPV-ITK1 and HytaNPV-ID1 homologous to the *Xho*I restriction site at nucleotide position 37,290 of HytaNPV-R genome, taken as the reference (**Figure 2-26**).

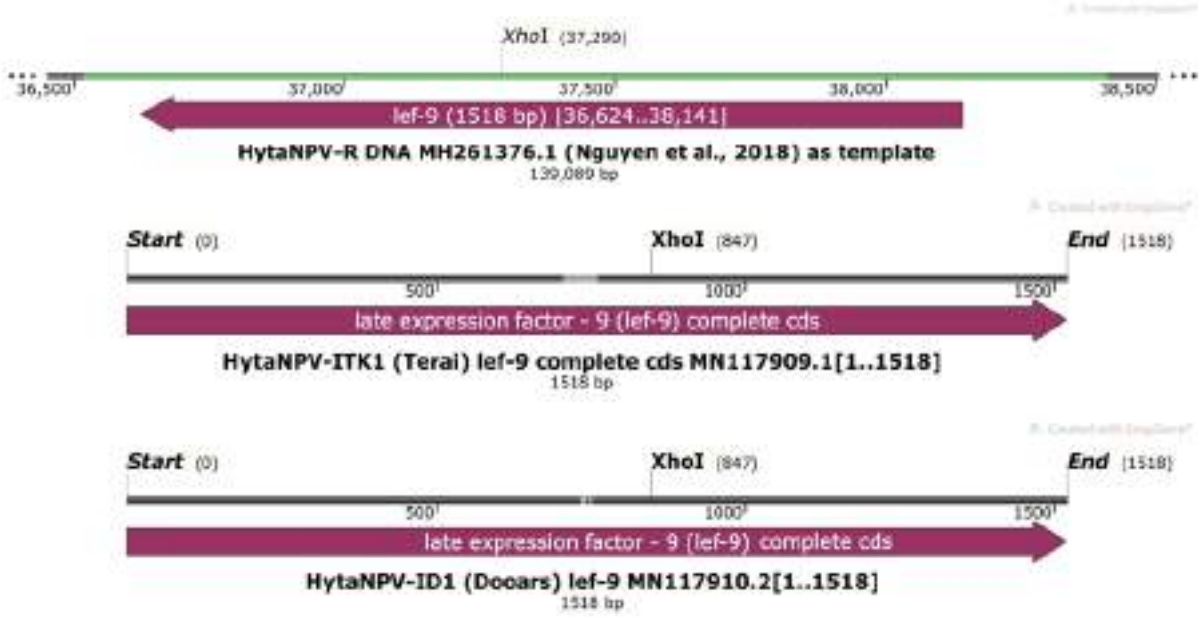


Figure 2-26: Restriction sites present in the *lef-9* of different HytaNPV isolates.

2.4.5 Sequencing of *pif-1* gene

PCR amplification of *pif-1* gene with specific primers (**Table 2-13**) using the DNA of two NPV isolates from *Hyposidra talaca* (HytaNPV-ITK1 and HytaNPV-ID1) as template produced an amplicon of 1186 bp (Fragment A) (as shown in **Figure 2-14, Figure 2-15**). After sequencing the amplified product, 930 bp (Product: 310 amino acids) and 1117 bp (Product: 372 amino acids) sequences were obtained for HytaNPV-ITK1 and HytaNPV-ID1, respectively. Both the sequences were submitted to the NCBI GenBank database (**Table 2-14).** **Figure 2-27** shows the graphical representation of primer binding, amplicon size and sequence details of *pif-1*.

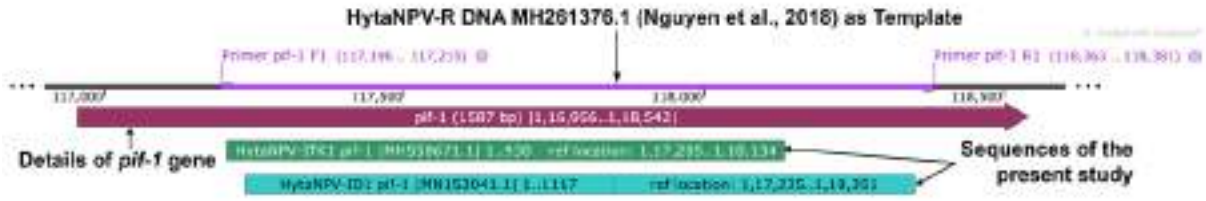


Figure 2-27: Primer binding, amplicon and sequence details of the *pif-1* gene using HytaNPV-R as a template.

2.4.5.1 Blast analysis

In NCBI blastn search, partial sequence of *pif-1* gene of HytaNPV-ITK1 and HytaNPV-ID1 revealed a maximum identity of 99.25% and 99.73% with HytaNPV-R (MH261376.1) from India, respectively. Among the NPVs infecting the hosts of different genera, BusuNPV (KF611977.1) from China exhibited the highest similarity of 80.65% with HytaNPV-ITK1 and 81.11% with HytaNPV-ID1, respectively.

Similarly, both the isolates, HytaNPV-ITK1 and HytaNPV-ID1 had an amino acid similarity of 99.36% and 99.73% with HytaNPV-R (Protein ID: AWW14483.1) and 83.23% and 85.48% with BusuNPV (YP_009001888.1) for *pif-1* gene, respectively in BlastX analysis (**Table 2-18**).

2.4.5.2 Alignments

Clustal W alignment of *pif-1* partial sequences of HytaNPV-ITK1 and HytaNPV-ID1 using HytaNPV-R as a template revealed coverage of 58.60% and 70.38% of the total reading

frame of *pif-1*, respectively (**Figure 2-28**). A total of 10 and 3 variable sites were detected in the 1137 nucleotide alignment and 382 amino acid alignments, respectively (**Figure 2-28**).

Table 2-18: Blast results *pif-1* sequence

Sl. No.	Subject Details	subject acc. no.	% identity
blastn (nucleotide blast)			
Query acc. no. MH558671.1 (HytaNPV-ITK1)			
1	HyaNPV-R	MH261376.1	99.25
2	BusuNPV	KF611977.1	80.65
3	ApciNPV	FJ914221.1	70.37
4	SujuNPV	KJ676450.1	71.19
5	EcobNPV	DQ837165.1	70.94
6	EupsNPV	FJ227128.1	72.04
7	BmNPV	JQ991010.1	70.11
8	AcMNPV	L22858.1	69.90
Query acc. no. MN153041.1 (HytaNPV-ID1)			
1	HyaNPV-R	MH261376.1	99.73
2	BusuNPV	KF611977.1	81.11
3	ApciNPV	FJ914221.1	68.37
4	SujuNPV	KJ676450.1	69.60
5	EcobNPV	DQ837165.1	68.46
6	EupsNPV	FJ227128.1	73.15
7	BmNPV	JQ991010.1	70.32
8	AcMNPV	L22858.1	70.88
blastx (protein blast)			
Query acc. no. MH558671.1 (HytaNPV-ITK1)			
1	HyaNPV-R	AWW14483.1	99.36
2	BusuNPV	YP_009001888.1	83.23
3	SujuNPV	YP_009186820.1	74.60
4	ApciNPV	YP_006607789.1	70.87
5	EcobNPV	YP_874291.1	68.39
6	EupsNPV	YP_002854725.1	66.12
7	AcMNPV	NP_054149.1	54.31
8	BmNPV	AFN21074.1	54.55
Query acc. no. MN153041.1 (HytaNPV-ID1)			
1	HyaNPV-R	AWW14483.1	99.73
2	BusuNPV	YP_009001888.1	85.48
3	SujuNPV	YP_009186820.1	73.33
4	ApciNPV	YP_006607789.1	69.25
5	EcobNPV	YP_874291.1	68.27
6	EupsNPV	YP_002854725.1	60.70
7	AcMNPV	NP_054149.1	53.58
8	BmNPV	AFN21074.1	53.05

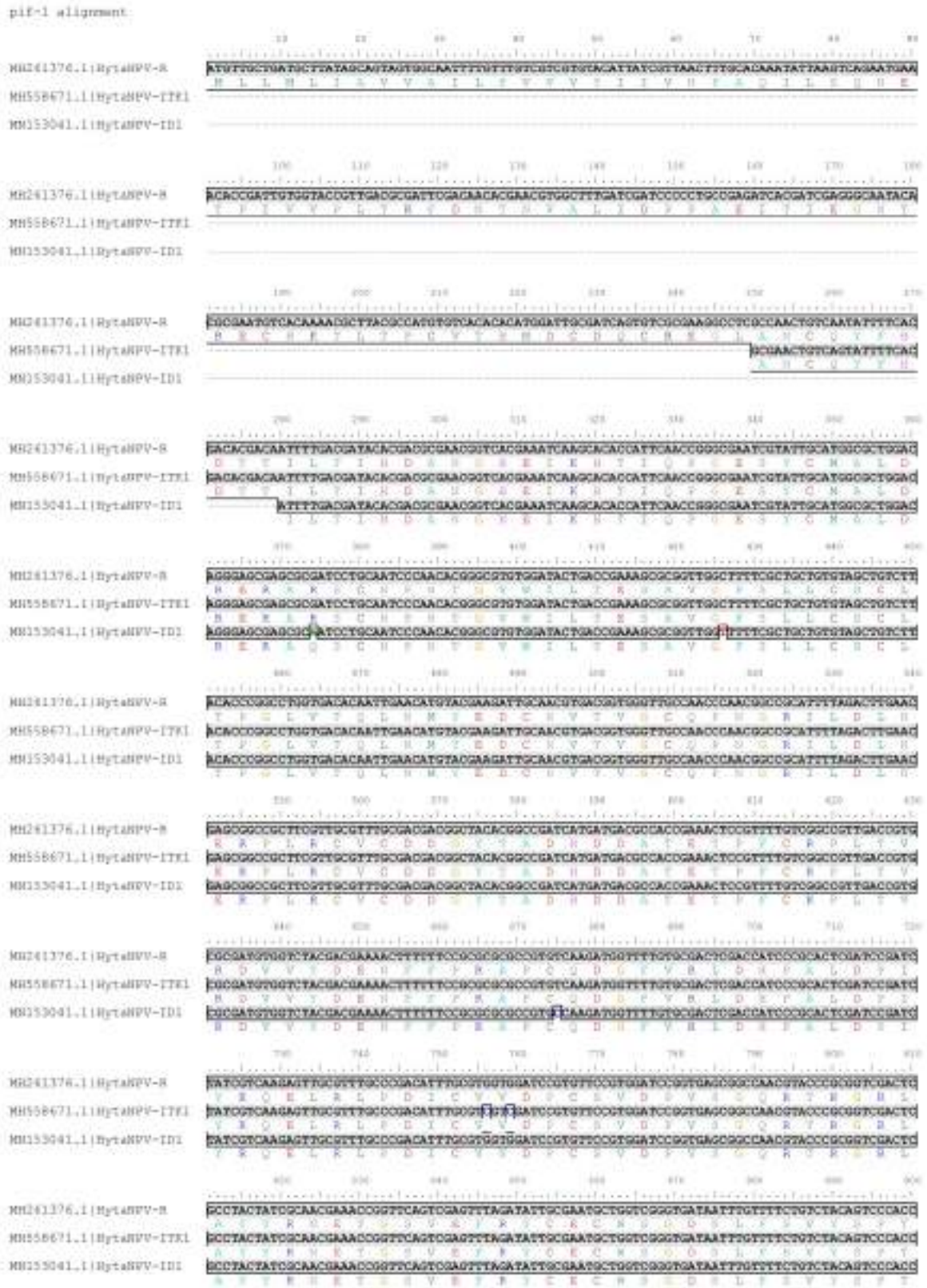


Figure 2-28: Nucleotide and translated amino acid sequence alignment of *pif-1* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as reference. Similar sequences were highlighted in grey background.

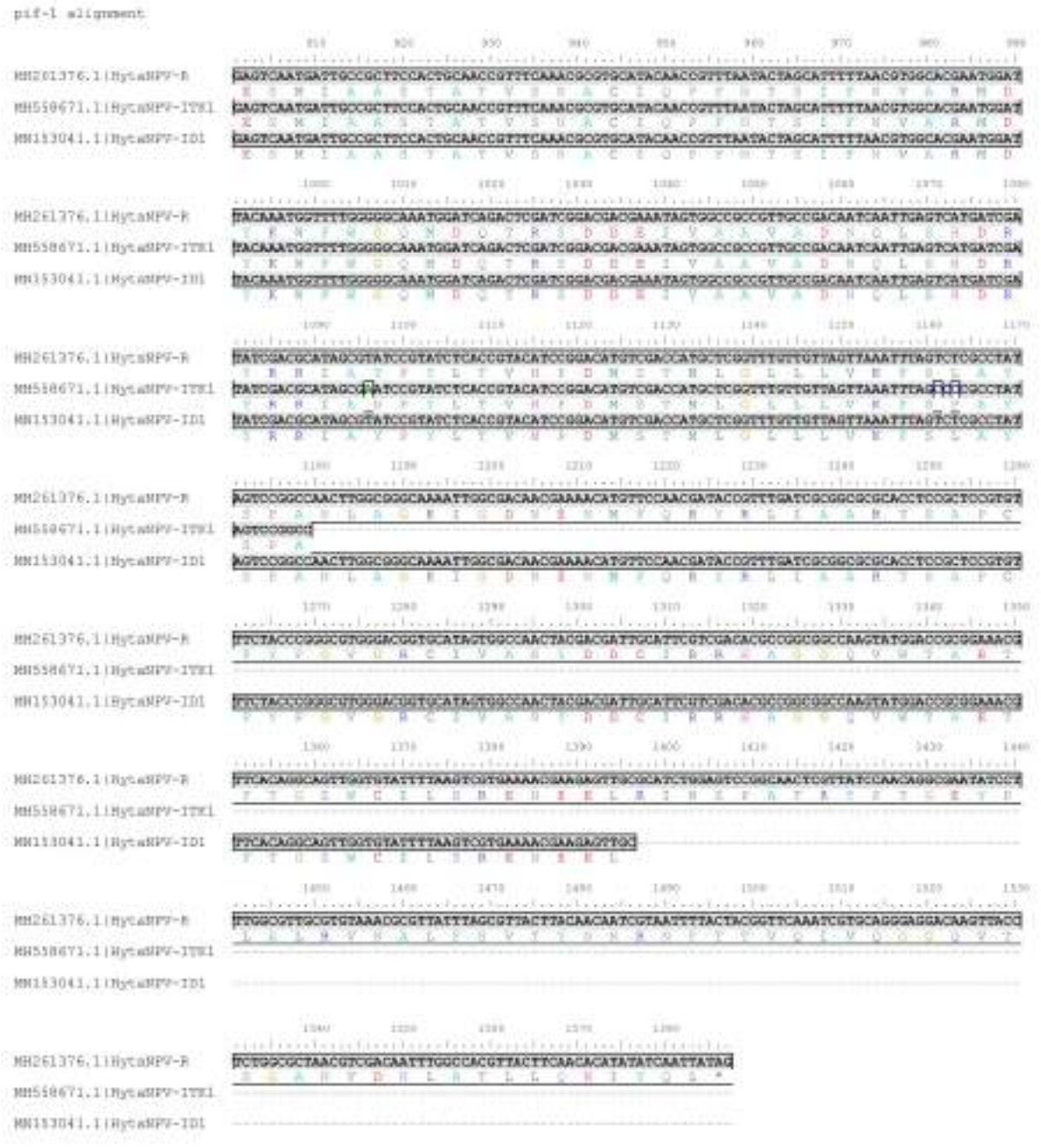


Figure 2-28: Nucleotide and translated amino acid sequence alignment of *pif-1* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as reference. Similar sequences were highlighted in grey background [Continued…].

2.4.5.3 Restriction sites on *pif-1* sequence

The partial sequence of the *pif-1* gene of HytaNPV-ID1 had two restriction sites for *Bam*HI at nucleotide positions 480 and 495 and one restriction site for *Bgl*I at position 904, which were homologous to the *Bam*HI sites of HytaNPV-R at nucleotide positions 117,714 and 117,729, and *Bgl*I restriction site of the same present at 118,138 (**Figure 2-29**).

The *pif-1* partial sequence of HytaNPV-ITK1, revealed only one *Bam*HI site at the nucleotide position 525 which was homologous to the *Bam*HI site at 495 of HytaNPV-ID1, while the other restriction site for *Bam*HI (117,729 of HytaNPV-R) was absent. The *Bgl*I site (118,138) of HytaNPV-R was beyond the sequence coverage of the *pif-1* gene of HytaNPV-ITK1 (**Figure 2-29**).

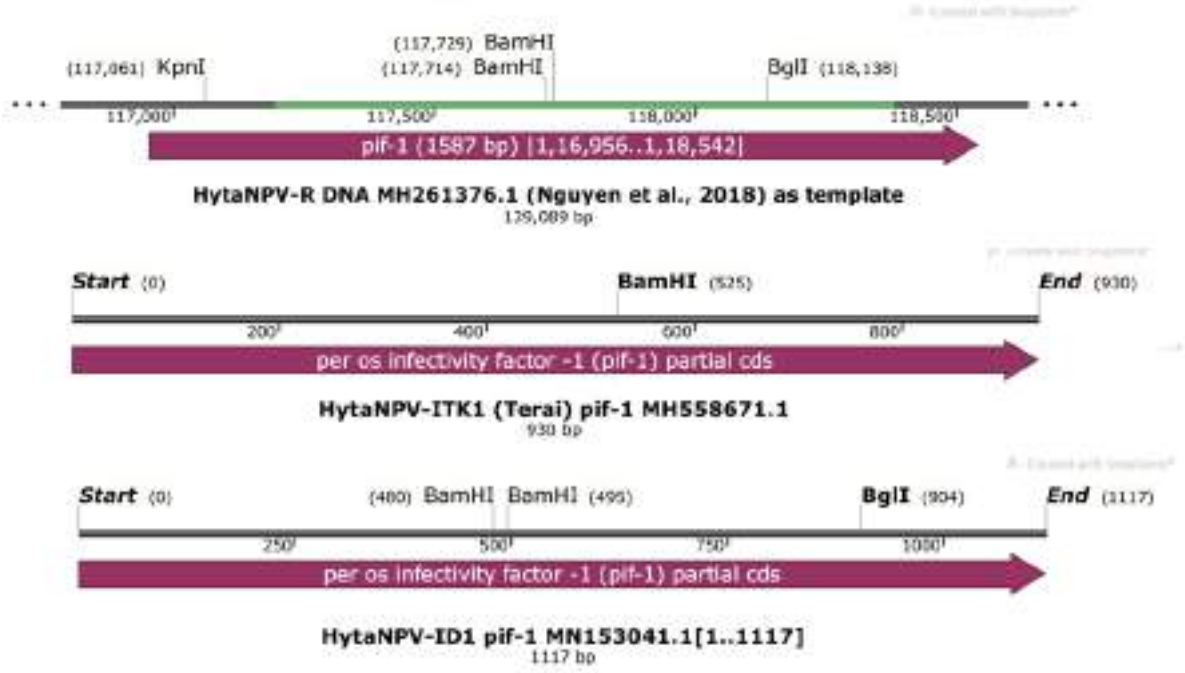


Figure 2-29: Restriction sites present in the *pif-1* of different HytaNPV isolates.

2.4.6 Sequencing of *pif-2* gene

In the present study, PCR with *pif-2* gene-specific primers (shown in **Table 2-13**) using the DNA of HytaNPV as a template produced amplicons of 913 bp (Fragment A), 673 bp (Fragment B) and 1587 bp (Fragment AB) (as shown in **Figure 2-14, Figure 2-15**). After sequencing of the amplified products, a sequence of 1152 bp (Product: 384 amino acids) complete coding sequence (cds) of *pif-2* gene was obtained having a 22 nucleotide long continuous intermediate gap for HytaNPV-ITK1 and a sequence of 1098 bp (Product: 366 amino acids) having incomplete 5′-end partial cds of *pif-2* gene consisting three intermediate gaps (5, 3, 87 nucleotides) was obtained for HytaNPV-ID1. Both the sequences were submitted to the NCBI GenBank database (**Table 2-14). Figure 2-30** represents primer binding, amplicon and the sequence size details of the *pif-2* gene.

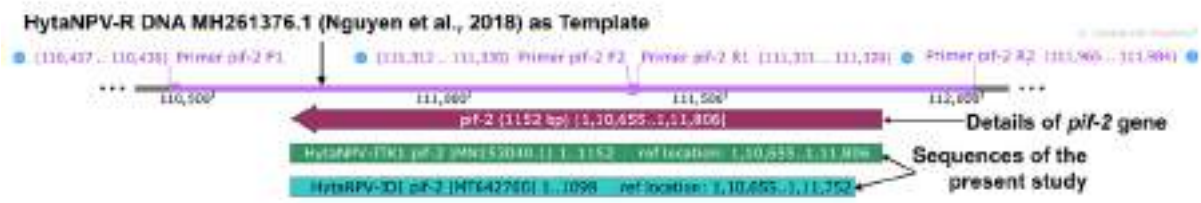


Figure 2-30: Primer binding, amplicons and sequence details of the *pif-2* gene using HytaNPV-R as a template.

2.4.6.1 Blast analysis

In NCBI blastn search, the complete sequence of *pif-2* gene of HytaNPV-ITK1 and the partial sequence of HytaNPV-ID1 showed a maximum identity of 99.38% and 98.87% with HytaNPV-R (MH261376.1) from India, respectively. Among the NPVs pathogenic to the specimen of different genera BusuNPV (KF611977.1) exhibited the nucleotide sequence identity of 84.12% with HytaNPV-ITK1 and 87.96% with HytaNPV-ID1, respectively.

In Blastx search, HytaNPV-ITK1 and HytaNPV-ID1 showed an amino acid sequence similarity of 98.04% and 97.83% with HytaNPV-R (Protein ID: AWW14472.1), and 84.12% and 87.96% with BusuNPV (Protein ID: YP_009001878.1), respectively (**Table 2-19**).

2.4.6.2 Alignments

Clustal W alignment of the sequences of the HytaNPV-ITK1 and HytaNPV-ID1 *pif-2* gene using HytaNPV-R as template revealed that the HytaNPV-ITK1 *pif-2* sequence covered the

complete reading frame, while *pif-2* of HytaNPV-ID1 covered 95.31% of total reading frame of *pif-2* gene (**Figure 2-31**). A total of 16 and 10 variable sites were detected in the 1152 nucleotide long alignment and the 384 amino acid long translated alignment, respectively (**Figure 2-31**).

Table 2-19: Blast results *pif-2* sequence

Sl. No.	Subject Details	subject acc. no.	% identity
blastn (nucleotide blast)			
Query acc. no. MN153040.1 (HytaNPV-ITK1)			
1	HyaNPV-R	MH261376.1	99.38
2	BusuNPV	KF611977.1	84.12
3	ApciNPV	FJ914221.1	72.14
4	SujuNPV	KJ676450.1	71.53
5	EcobNPV	DQ837165.1	71.76
6	EupsNPV	FJ227128.1	71.037
7	BmNPV	JQ991010.1	70.43
8	AcMNPV	L22858.1	70.15
Query acc. no. MT642700 (HytaNPV-ID1)			
1	HyaNPV-R	MH261376.1	98.87
2	BusuNPV	KF611977.1	83.66
3	ApciNPV	FJ914221.1	71.34
4	SujuNPV	KJ676450.1	71.13
5	EcobNPV	DQ837165.1	72.22
6	EupsNPV	FJ227128.1	70.76
7	BmNPV	JQ991010.1	71.39
8	AcMNPV	L22858.1	71.56
blastx (protein blast)			
Query acc. no. MN153040.1 (HytaNPV-ITK1)			
1	HyaNPV-R	AWW14472.1	98.04
2	BusuNPV	YP_009001878.1	87.96
3	SujuNPV	YP_009186804.1	77.37
4	ApciNPV	YP_006607853.1	72.82
5	EcobNPV	YP_874299.1	72.75
6	EupsNPV	YP_002854731.1	73.67
7	AcMNPV	NP_054051.1	64.05
8	BmNPV	AFN21127.1	62.92
Query acc. no. MT642700 (HytaNPV-ID1)			
1	HyaNPV-R	AWW14472.1	97.83
2	BusuNPV	YP_009001878.1	86.99
3	SujuNPV	YP_009186804.1	75.62
4	ApciNPV	YP_006607853.1	69.70
5	EcobNPV	YP_874299.1	71.12
6	EupsNPV	YP_002854731.1	72.76
7	AcMNPV	NP_054051.1	61.49
8	BmNPV	AFN21127.1	60.87

Sequencing of pif-2 gene

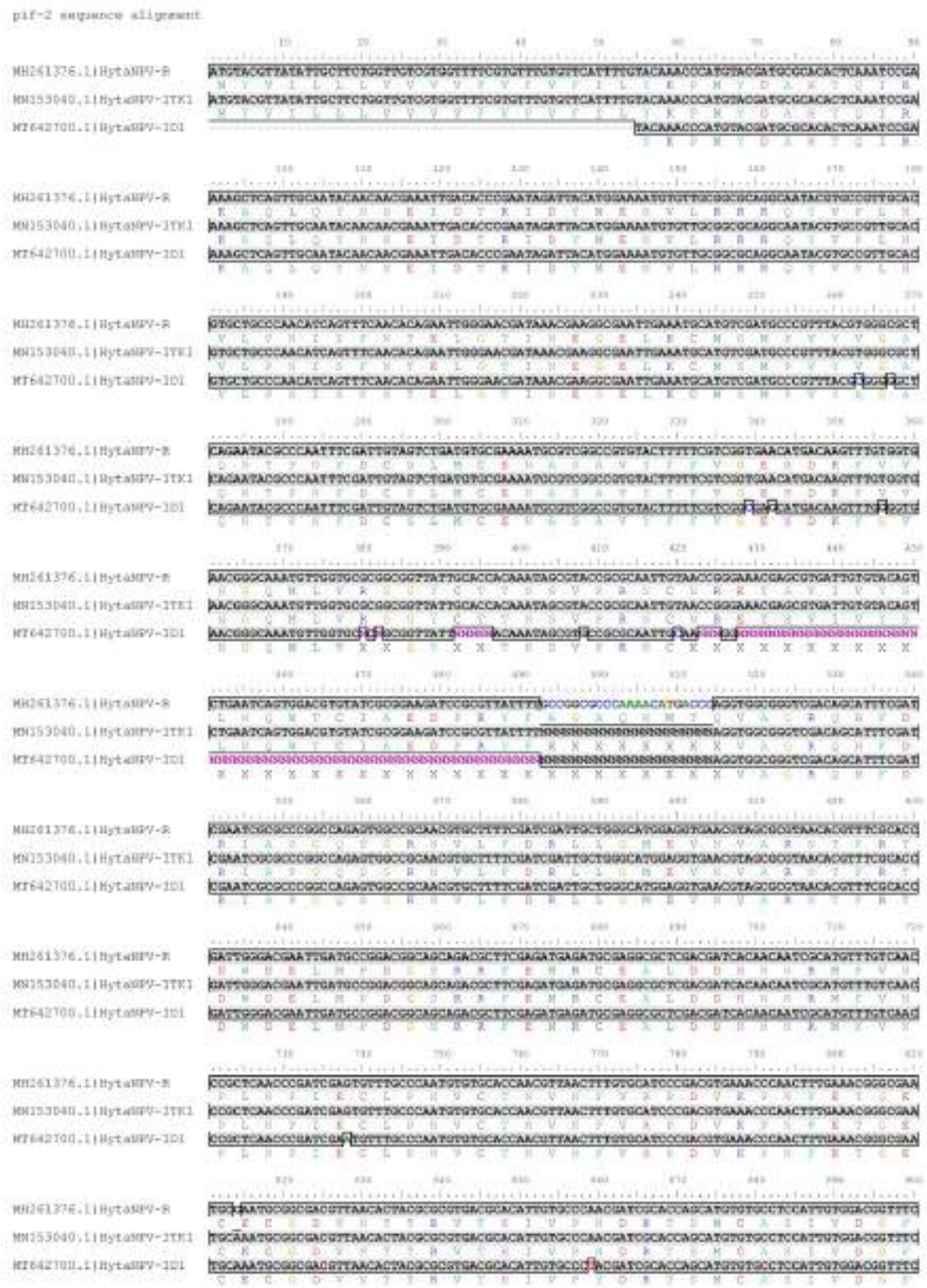


Figure 2-31: Nucleotide and translated amino acid sequence alignment of the *pif-2* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background.

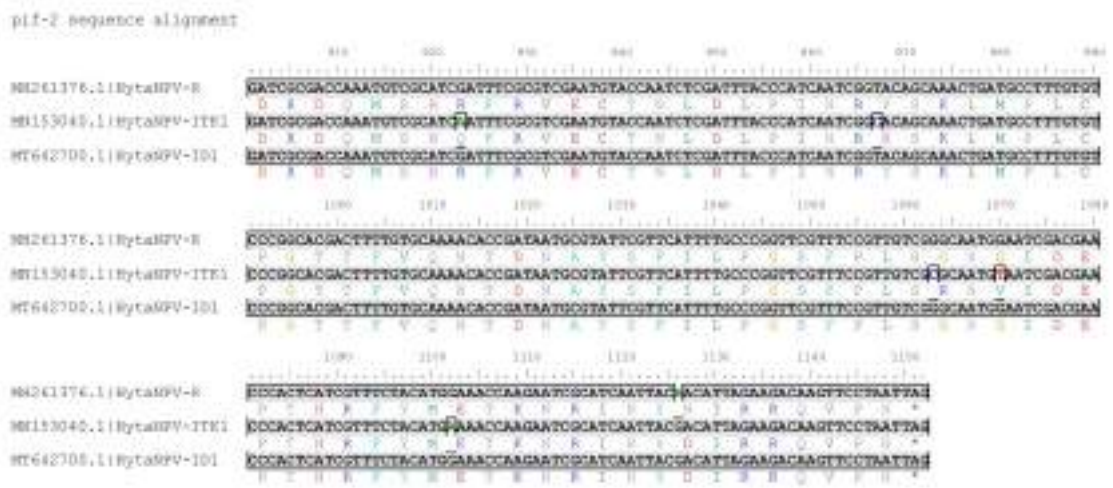


Figure 2-31: Nucleotide and translated amino acid sequence alignment of *pif-2* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as reference. Similar sequences were highlighted in grey background [Continued…].

2.4.6.3 Restriction sites on *pif-2* sequence

The *pif-2* sequences of HytaNPV-ITK1 and HytaNPV-ID1 contained a restriction site for *Bgl*I at the nucleotide position of 560 and 506, respectively, both homologous to the restriction site of *Bgl*I at position 111249 of HytaNPV-R (**Figure 2-32**).

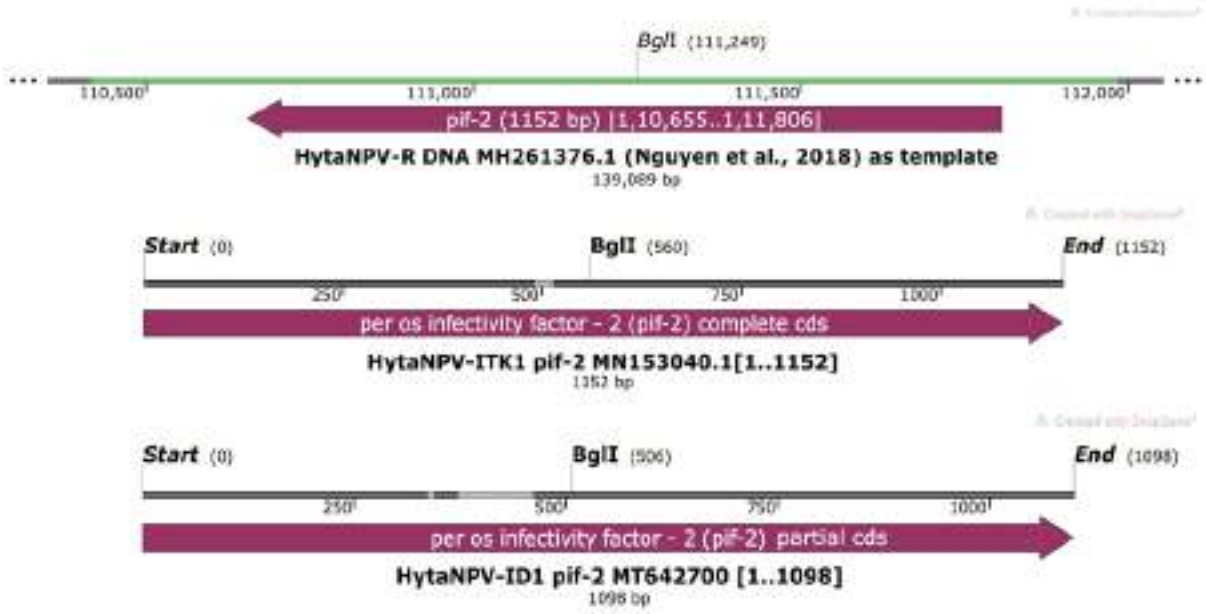


Figure 2-32: Restriction sites present in the *pif-2* of different HytaNPV isolates.

2.4.7 Sequencing of *pif-3* gene

PCR amplification of HytaNPV DNA using *pif-3* gene-specific primers (**Table 2-13**) produced an amplicon of 827 bp (Fragment A) (shown in **Figure 2-14, Figure 2-15**). After sequencing the amplified products, a sequence of 630 bp (Product: 210 amino acids) containing stretches of intermediate gaps of 160 bp, and 18 bp, was obtained for HytaNPV-ITK1. Whereas, a sequence of 630 bp (Product: 210 amino acids) complete coding sequence (cds) of *pif-3* gene was obtained for HytaNPV-ID1without any gap. Both the sequences were submitted to the NCBI GenBank database (**Table 2-14). Figure 2-33** depicts the primer binding, amplicon size and sequence details of the *pif-3* gene.

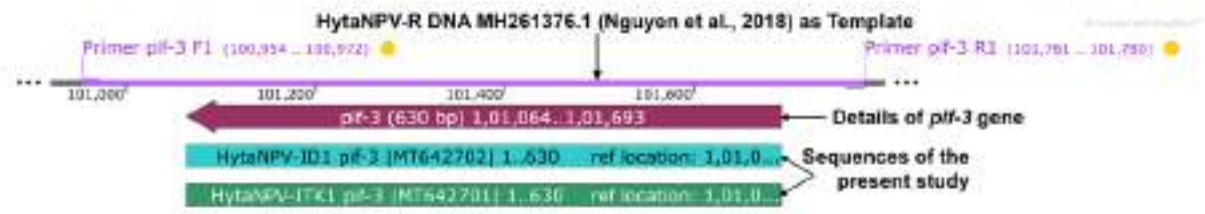


Figure 2-33: Primer binding, amplicon and sequence details of the *pif-3* gene using HytaNPV-R as a template.

2.4.7.1 Blast analysis

In the NCBI blastn search, the *pif-3* sequence of HytaNPV-ITK1 and HytaNPV-ID1 isolates showed 100% identity with HytaNPV-R (MH261376.1) from India (**Table 2-20**).

2.4.7.2 Alignments

On Clustal W alignment of the sequences of *pif-3* gene of HytaNPV-ITK1 and HytaNPV-ID1 using HytaNPV-R as reference template, the *pif-3* sequence covered 69.52% (deficiency due to intermediate gaps) and 100% of the total reading frame (**Figure 2-34**), respectively. Not a single variable site was found in the nucleotide sequence alignments of the *pif-3* (**Figure 2-34**).

Table 2-20: Blast results *pif-3* sequence.

Sl. No.	Subject Details	subject acc. no.	% identity
blastn (nucleotide blast)			
Query acc. no. MT642701 (HytaNPV-ITK1)			
1	HyaNPV-R	MH261376.1	100
2	BusuNPV	KF611977.1	80.49
3	ApciNPV	FJ914221.1	64.31
4	EcobNPV	DQ837165.1	72.53
Query acc. no. MT642702 (HytaNPV-ID1)			
1	HyaNPV-R	MH261376.1	100
2	BusuNPV	KF611977.1	77.71
3	ApciNPV	FJ914221.1	69.56
5	EcobNPV	DQ837165.1	72.53
blastx (protein blast)			
Query acc. no. MT642701 (HytaNPV-ITK1)			
1	HyaNPV-R	AWW14472.1	100
2	BusuNPV	YP_009001867.1	76.923
3	SujuNPV	YP_009186791.1	56.863
4	ApciNPV	YP_006607848.1	56.731
5	EcobNPV	YP_874304.1	57.843
6	EupsNPV	YP_002854712.1	52.475
8	BmNPV	AFN21211.1	56.322
Query acc. no. MT642702 (HytaNPV-ID1)			
1	HyaNPV-R	AWW14472.1	100
2	BusuNPV	YP_009001867.1	78.453
3	SujuNPV	YP_009186791.1	60.109
4	ApciNPV	YP_006607848.1	61.878
5	EcobNPV	YP_874304.1	58.152
6	EupsNPV	YP_002854712.1	59.649
8	BmNPV	AFN21211.1	47.541

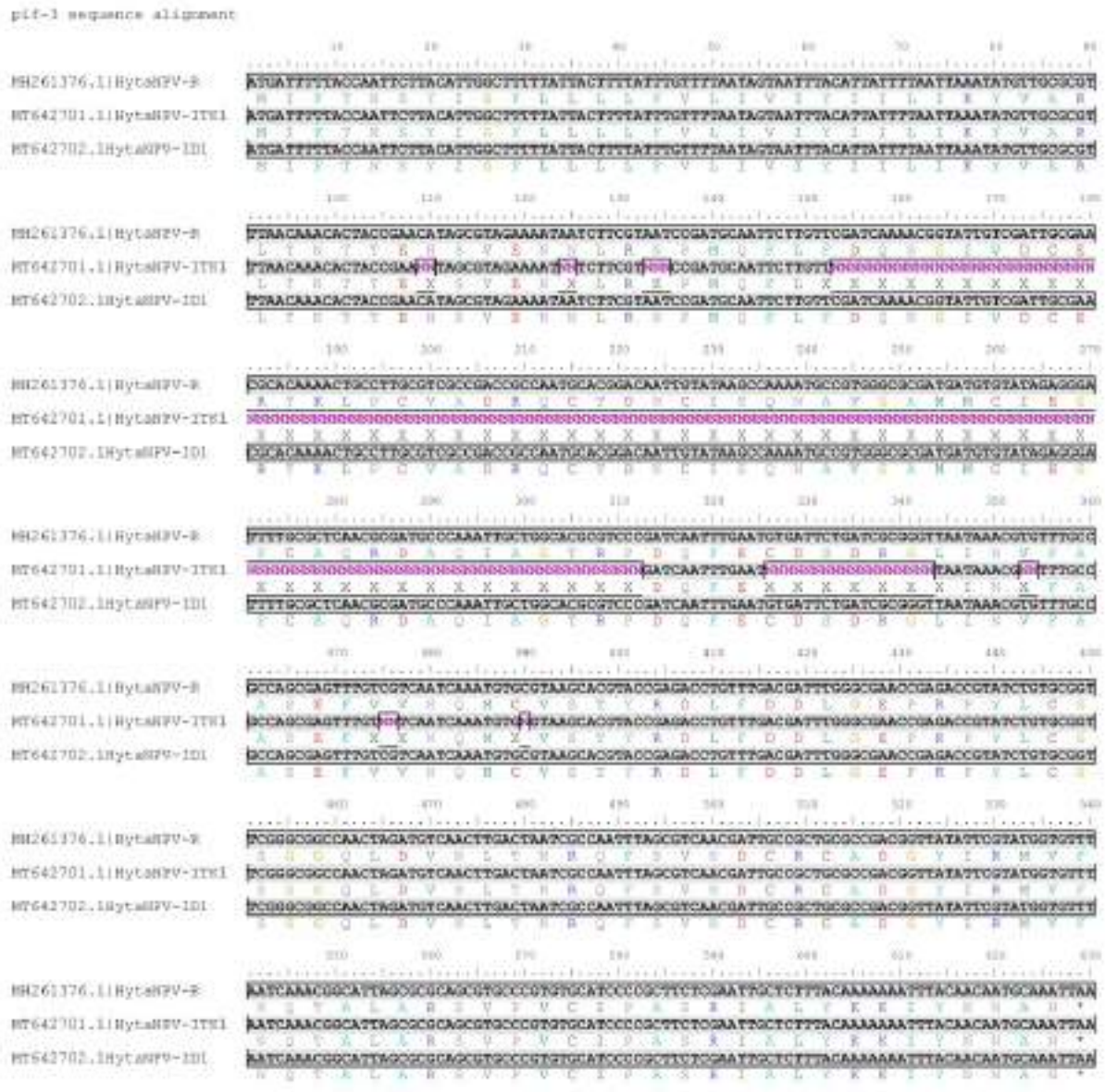


Figure 2-34: Nucleotide and translated amino acid sequence alignment of the *pif-3* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background.

2.4.7.3 Restriction sites on *pif-3* sequence

No restriction site was found in the *pif-3* sequences of HytaNPV isolates for any of the seven restriction endonucleases used in the present study (**Figure 2-35**).

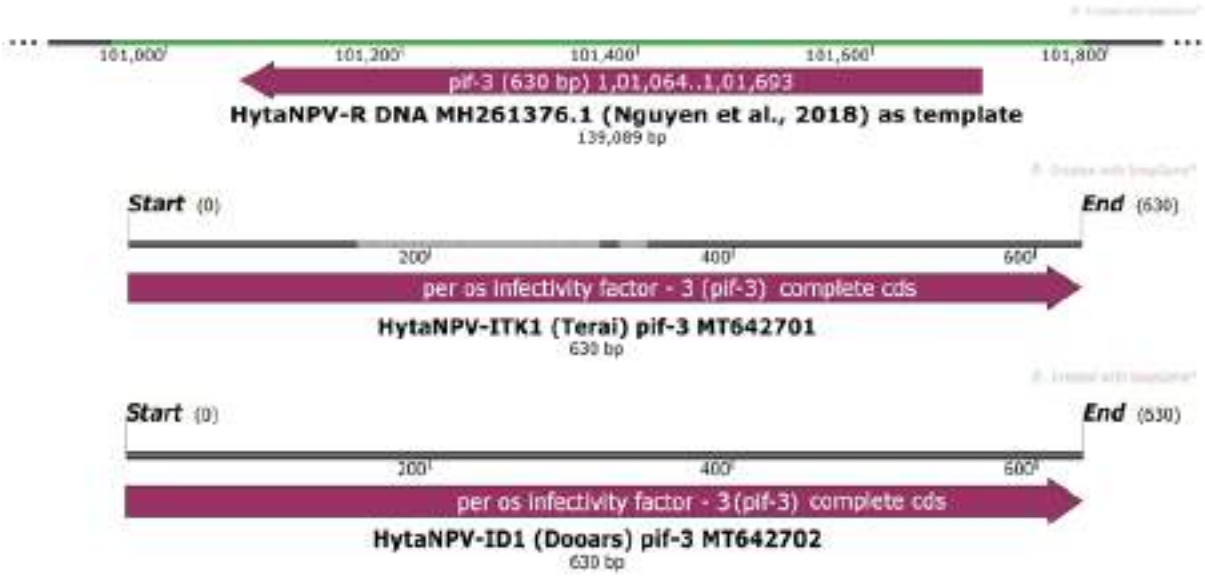


Figure 2-35: Restriction sites present in the *pif-3* of different HytaNPV isolates.

2.4.8 Sequence alignment analysis

Results of the pairwise and overall comparisons of gene sequences (*polyhedrin, lef-8, lef-9, pif-1, pif-2, pif-3*) of the HytaNPV isolates (ITK1, and ID1) with HytaNPV-R (Nguyen et al., 2018) as reference have been presented in **Table 2-21**. Overall comparisons of the gene sequences in the present study showed a total of 46 variable sites (d_N) (**Table 2-21 cluster IV**), whereas the d_N was 32 in both the pairwise comparison of the HytaNPV-ITK1 (**Table 2-21 cluster I**) and HytaNPV-ID1 (**Table 2-21 cluster II**) with HytaNPV-R, while it was 35 between two isolates of the present study, HytaNPV-ITK1 and HytaNPV-ID1 (**Table 2-21 cluster III**). For all the genes the percentage of identical sites ($\%id_N$), the number of transitions/transversions (s/v) and the number of synonymous mutations/ number of non-synonymous mutations (sd/nd) were also estimated (**Table 2-21**). The $\%id_N$ for different genes varied from lowest 98.86 (*polyhedrin*) to highest 100 (*pif-3*) in HytaNPV-ITK1 (**Table 2-21 cluster I**) and lowest $\%id_N$ of 98.66 (*polyhedrin*) to highest 100 (*pif-3*) in HytaNPV-ID1 (**Table 2-21 cluster II**) when compared with HytaNPV-R. The $\%id_N$ varied from 98.60 for *pif-2* to 100 for *pif-3* with a mean value of 99.31 between the two isolates, HytaNPV-ITK1 and HytaNPV-ID1 (**Table 2-21 cluster III**). The overall comparison revealed the range of $\%id_N$ = 98.40 (*pif-2*) to 100 (*pif-3*) with mean $\%id_N$ of 99.09 (**Table 2-21 cluster IV**).

In all comparisons, no mutation was found for *pif-3* being identical to the reference HytaNPV-R as well as between the two isolates of the present study. The overall sequence comparison among the HytaNPV isolates revealed that all the mutations found in *polyhedrin* were synonymous, whereas, the ratio of non-synonymous to synonymous mutations (nd/sd) was comparatively high in *lef-9* and *pif-2*, with nd/sd of 16/6=2.67 and 20/12=1.67, respectively than that in *lef*-8 and *pif-1* (2/6 for *lef-8* and 6/10 for *pif-1*).

The position and details of the non-synonymous substitutions have been summarized in **Table 2-22**.

Table 2-21: Pairwise and overall comparisons of the gene sequences of HytaNPV isolates using HytaNPV (MH261376.1) as reference. c_N = number of conserved sites for nucleotides, d_N = number of variable sites for nucleotides, $\%id_N$ = % of identical sites for nucleotide, nd = non-synonymous substitution, sd = synonymous substitution, d_A = number of variable sites for amino acids, $\%id_A$ = % of identical sites for amino acids.

Gene	Pairwise comparisons between **HytaNPV-ITK1** and HytaNPV-R						Pairwise comparisons between **HytaNPV-ID1** and HytaNPV-R					
	Cluster-I						**Cluster-II**					
	c_N	**d_N**	**$\%id_N$**	**nd/sd**	**d_A**	**$\%id_A$**	**c_N**	**d_N**	**$\%id_N$**	**nd/sd**	**d_A**	**$\%id_A$**
polyhedrin	521	6	98.86	0/6	0	100.00	517	7	98.66	0/7	0	100.00
lef-8	2205	5	99.77	4/1	4	99.46	718	4	99.45	1/3	1	99.58
lef-9	1456	7	99.52	6/1	5	98.97	1496	7	99.53	4/3	4	99.20
pif-1	923	7	99.25	2/5	2	99.36	1114	3	99.73	1/2	1	99.73
pif-2	1123	7	99.38	7/0	7	98.13	990	11	98.90	5/6	5	98.48
pif-3	440	0	100.00	0/0	0	100.00	630	0	100.00	0/0	0	100.00
Total	6668	32	99.52	19/13	18	99.20	5465	32	99.42	11/21	11	99.40
	Pairwise comparisons between HytaNPV-ITK1 and **HytaNPV-ID1**						**Overall comparisons among HytaNPV-ITK1, HytaNPV-ID1 & HytaNPV-R** (without gap or missing data)					
	Cluster-III						**Cluster-IV**					
	c_N	**d_N**	**$\%id_N$**	**nd/sd**	**d_A**	**$\%id_A$**	**c_N**	**d_N**	**$\%id_N$**	**nd/sd**	**d_A**	**$\%id_A$**
polyhedrin	523	1	99.81	0/1	0	100.00	520	7	98.66	0/14	0	100.00
lef-8	718	4	99.45	1/3	1	99.58	718	4	99.45	2/6	1	99.58
lef-9	1454	8	99.45	6/2	5	98.97	1451	11	99.25	16/6	7	98.59
pif-1	892	8	99.11	3/5	3	99.00	892	8	99.11	6/10	3	99.00
pif-2	987	14	98.60	8/6	8	97.56	985	16	98.40	20/12	10	97.00
pif-3	440	0	100.00	0/0	0	100.00	440	0	100.00	0/0	0	100.00
Total	5014	35	99.31	18/17	17	98.99	5006	46	99.09	44/48	21	98.76

Table 2-22: Details of the non-synonymous nucleotide substitutions and respective amino acid substitutions using HytaNPV-R as reference.

Sl. No.	**Non-synonymous nucleotide substitutions** Substitution position for nucleotide in the reading frame/size of total reading frame of the gene (base position in the codon; substitution detail)	**Amino acid substitutions** Substitution position for the amino acid in polypeptide sequence/total nos. of amino acid in the gene (substitution detail)	**Isolate(s)**	**Gene** Transition (s), Transversion (v)
		lef-8		
1.	1307/2640 (2nd base; T – G)	436/880 (M – R)	HytaNPV-ITK1	*v*
2.	1349/2640 (2nd base; T – G)	450/880 (V – G)	HytaNPV-ITK1	*v*
3.	1358/2640 (2nd base; A – C)	453/880 (H – P)	HytaNPV-ITK1	*v*
4.	1402/2640 (1st base; A – C)	468/880 (T – P)	HytaNPV-ITK1	*v*
5.	2338/1587 (1st base; G – A)	780/880 (D – N)	HytaNPV-ID1	*s*
		lef-9		
6.	183/1518 (3rd base; A – C)	61/506 (R – S)	Both	*v*
7.	482/1518 (2nd base; G – A)	161/506 (G – D)	HytaNPV-ITK1	*s*
8.	646/1518 (1st base; G – A)	216/506 (V – K)	HytaNPV-ITK1	*s*
9.	647/1518 (2nd base; T – A)		HytaNPV-ITK1	*v*
10.	650/1518 (2nd base; A – G)	217/506 (D – G)	HytaNPV-ITK1	*s*
11.	1138/1518 (1st base; G – C)	380/506 (M – L)	HytaNPV-ID1	*v*
12.	1145/1518 (2nd base; G – T)	382/506 (G – V)	HytaNPV-ID1	*v*
13.	1483/1518 (1st base; T – G)	495/506 (L – V)	Both	*v*
		pif-1		
14.	374/1587 (1st base; G – A)	125/529 (R – Q)	HytaNPV-ID1	*s*
15.	1096/1587 (1st base; T – A)	366/529 (Y – N)	HytaNPV-ITK1	*v*
16.	1163/1587 (2nd base; T – C)	388/529 (L – P)	HytaNPV-ITK1	*s*
		pif-2		
17.	263/1152 (2^{nd} base; T – C)	88/384 (V – A)	HytaNPV-ID1	*s*
18.	356/1152 (2^{nd} base; T – G)	119/384 (V – G)	HytaNPV-ID1	*v*
19.	814/1152 (1^{st} base; G – A)	272/384 (E – K)	Both	*s*
20.	859/1152 (1^{st} base; A – T)	287/384 (N – Y)	HytaNPV-ID1	*v*
21.	923/1152 (2^{nd} base; G – A)	308/384 (R – Q)	HytaNPV-ITK1	*s*
22.	967/1152 (1^{st} base; T – C)	323/384 (Y – H)	HytaNPV-ITK1	*s*
23.	1063/1152 (1^{st} base; G – C)	355/384 (G – R)	HytaNPV-ITK1	*v*
24.	1070/1152 (2^{nd} base; G – T)	357/384 (G – V)	HytaNPV-ITK1	*v*
25.	1102/1152 (1^{st} base; G – A)	368/384 (E – K)	HytaNPV-ITK1	*s*
26.	1126/1152 (1^{st} base; A – G)	376/384 (N – D)	Both	*s*

2.4.9 Partial restriction maps of HytaNPV isolates

The *in silico* restriction maps of both HytaNPV-ITK1, and HytaNPV-ID1 with the seven restriction endonucleases (*Eco*RI, *Bam*HI, *Kpn*I, *Bgl*I, *Hind*III, *Pst*I, *Xho*I) used for restriction profile analyses in the present study, were constructed by aligning the obtained gene sequences (*polyhedrin, lef-8, lef-9, pif-1, pif-2 and pif-3*) using HytaNPV-R (Nguyen et al., 2018) as a template (**Figure 2-36**, **Figure 2-37**).

The partial restriction maps based on the obtained sequences of the two isolates of HytaNPV (HytaNPV-ITK1 and HytaNPV-ID1) in the present study were constructed. Two restriction sites each for *Eco*RI and *Kpn*I in *polyhedrin* sequence whereas, single sites for *Xho*I, *Eco*RI, *Bgl*I and *Bam*HI in *lef-9*, *lef-8*, *pif-2* and *pif-1* sequences, respectively, were found to be common in both the isolates. However, no sites for any of the six restriction endonucleases analysed in the present study were detected in the *pif-3* sequences in both the isolates. The only difference was that another *Bam*HI restriction site in *pif-1* was found to be absent in the Terai isolate (HytaNPV-ITK1).

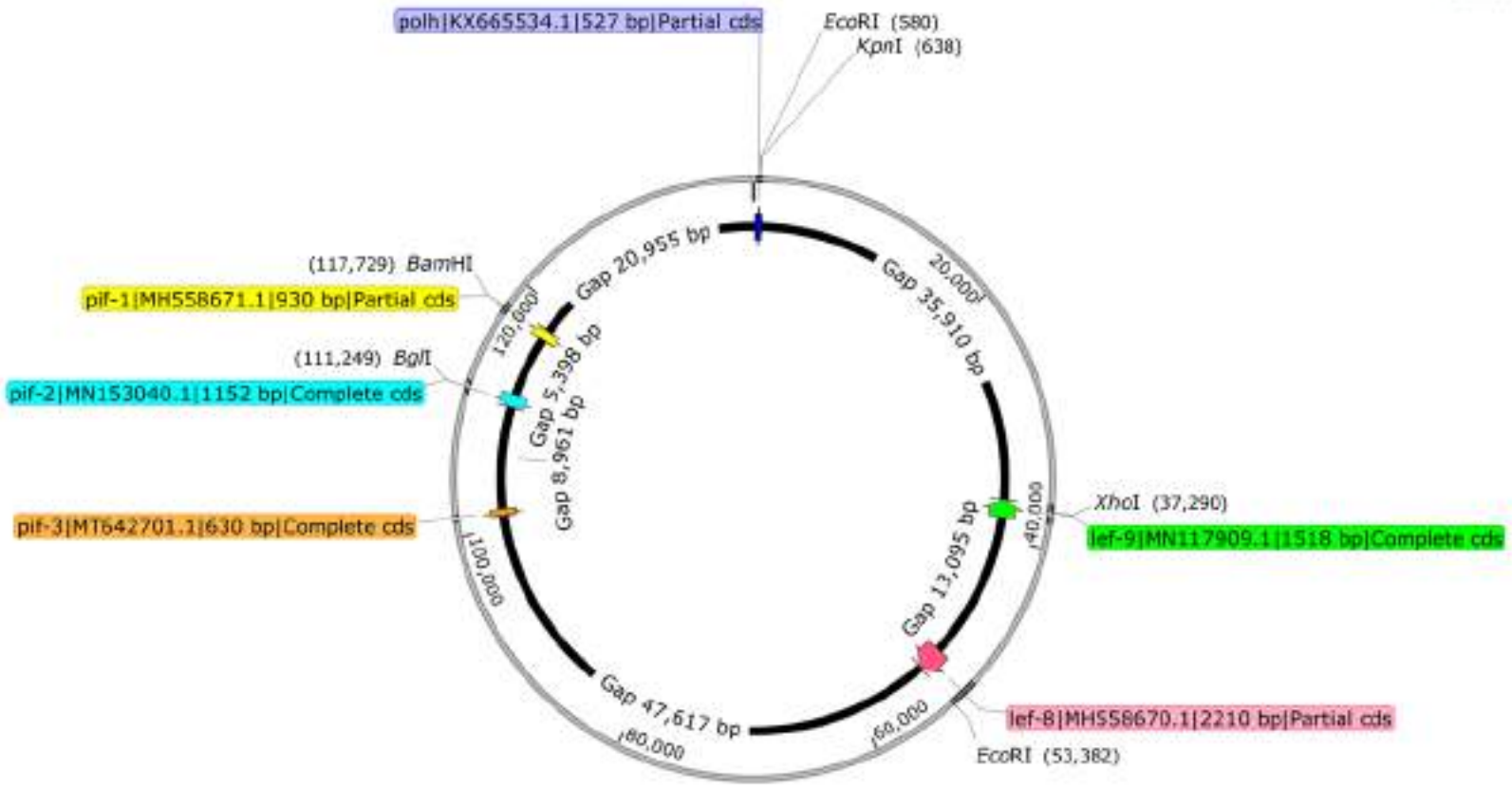


Figure 2-36: Partial restriction map of HytaNPV-ITK1 (Terai isolate) using HytaNPV-R (Nguyen et al., 2018) as a template.

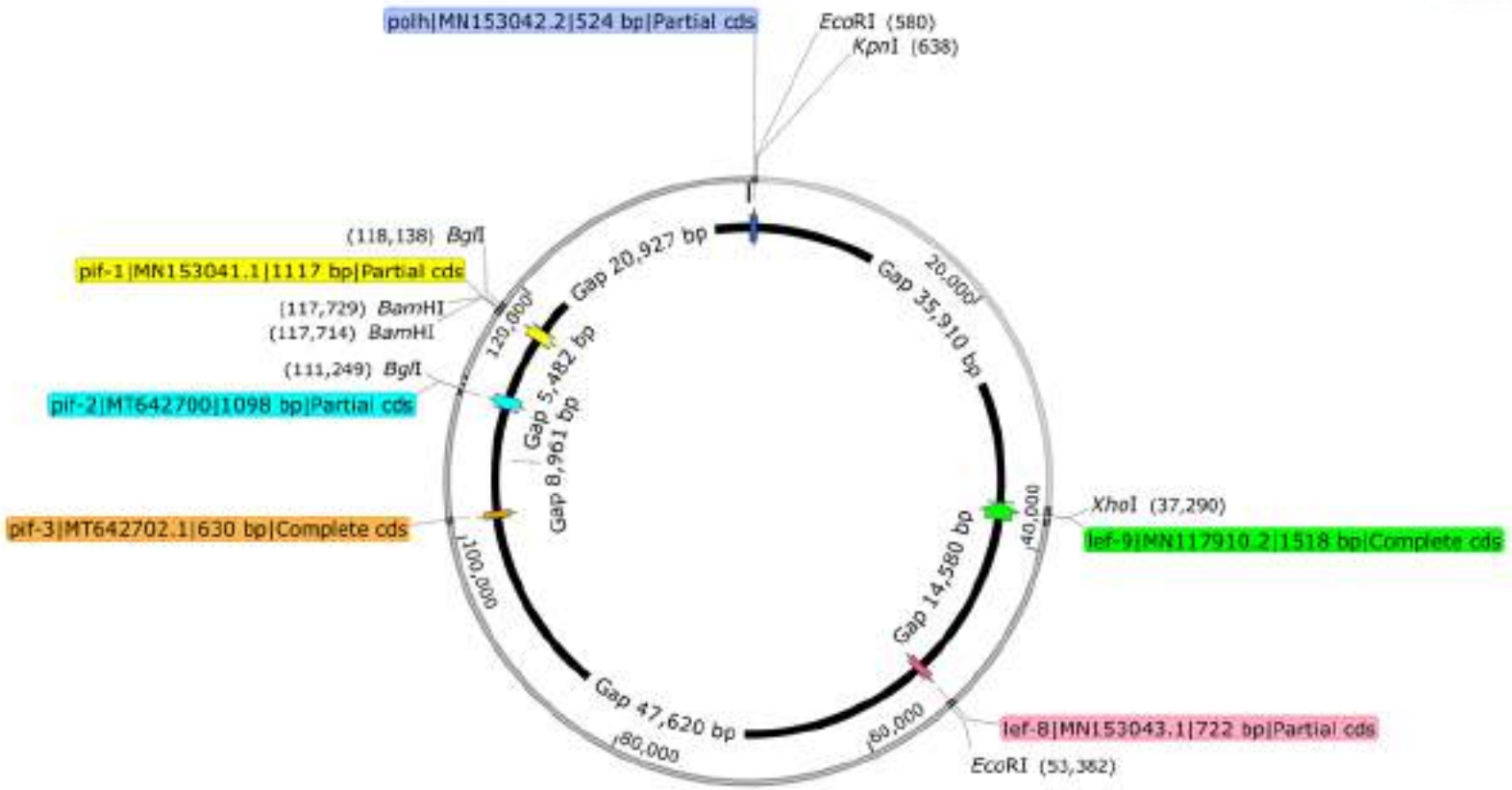


Figure 2-37: Partial restriction map of HytaNPV-ID1 (Dooars isolate) using HytaNPV-R (Nguyen et al., 2018) as a template.

Section 2.5: Phylogenetic Analysis

Objective

6. To construct a phylogenetic tree of the different strains of HytaNPV based on the similarities and differences.

2.5.1 Phylogenetic analyses with 77 baculoviruses

Because no different strains of HytaNPV were found in the Terai-Dooars region of West Bengal, different geographic isolates of HytaNPV representing the Terai (HytaNNPV-ITK1) and the Dooars (HytaNNPV-ID1) regions were used in the present study for phylogenetic analysis. The phylogenetic trees based on concatenated sequences of six genes together showed that two isolates of HytaNPVs (HytaNPV-ITK1 and HytaNPV-ID1) of the present study formed a cluster with the HytaNPV-R from the Dooars, India (MH261376.1; Nguyen et al., 2018), under group II alphabaculoviruses. The cluster of HytaNPVs showed close relationships with BusuNPV, SujuNPV, EcobNPV, ApciNPV, EupsNPV and OrleNPV (**Figure 2-38 A & B, Figure 2-39 A & B**).

Both the phylogenetic trees based on concatenated sequences of above mentioned six genes together using nucleotide (**Figure 2-38**) and amino acid (**Figure 2-39**) substitutions showed that delta, gamma and beta- baculoviruses have diverged before the divergence of alphabaculoviruses into group I and group II. All the gammabaculoviruses, betabaculoviruses, and group I alphabaculoviruses were found to have a monophyletic origin (**Figure 2-38 A & B**, **Figure 2-39 A & B**). The group II alphabaculoviruses also showed a monophyletic origin as per nucleotide-based phylogeny (**Figure 2-38A**), while the amino acid-based phylogeny suggested a paraphyletic origin of group II alphabaculoviruses (**Figure 2-39A**).

The phylogenetic trees indicated an early divergence of deltabaculovirus (CuniNPV) and appeared as an outgroup. The ancestor of deltabaculovirus diverged into the progenitor of gammabaculovirus on one hand and progenitor of the beta- and alpha-baculoviruses on the other hand with a bootstrap value of 100. Group I and Group II alphabaculoviruses appeared as sister groups that diverged separately from their recent common ancestor with a bootstrap value of 100.

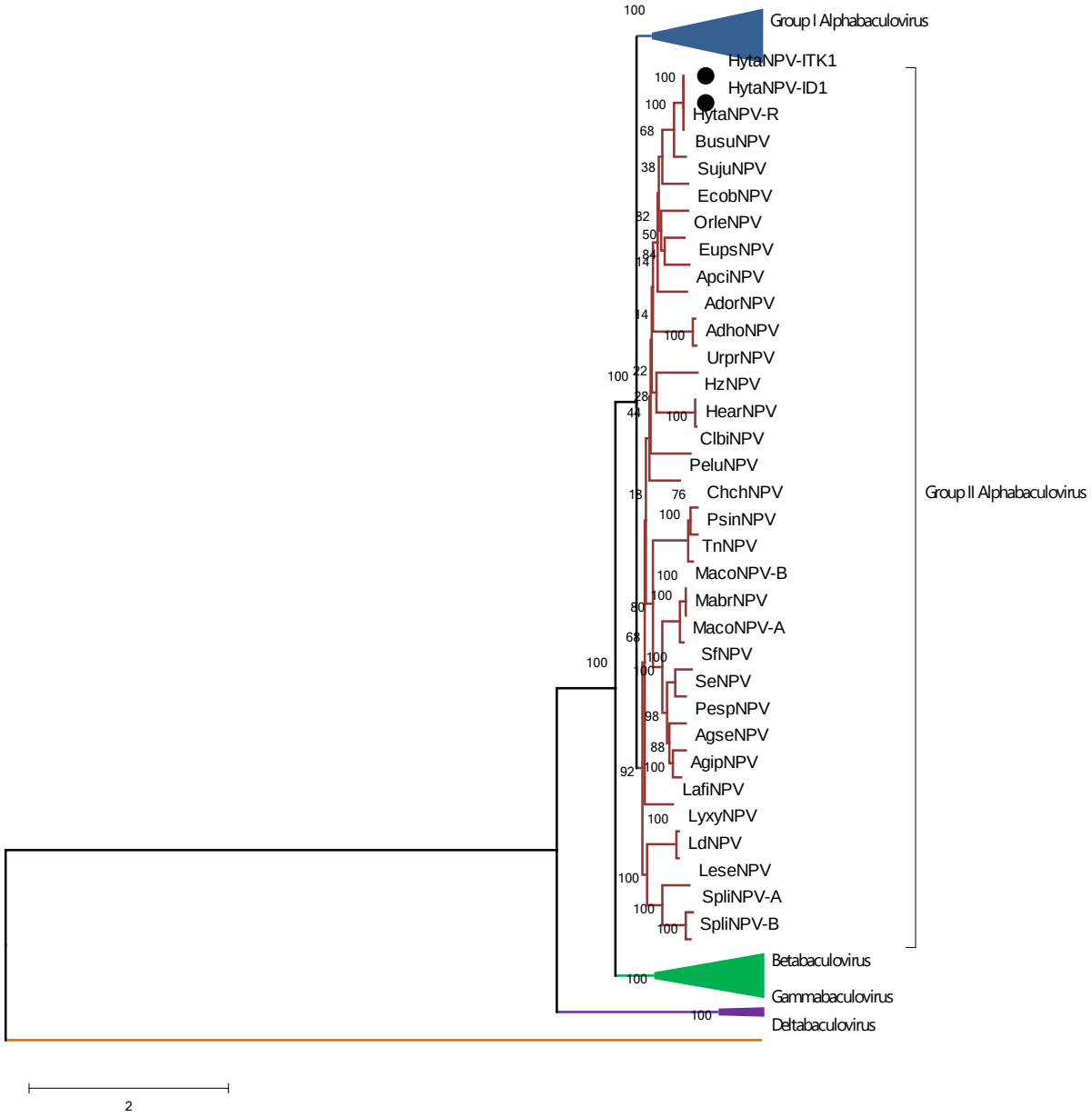


Figure 2-38A: Maximum likelihood tree based on concatenated sequence alignment of six genes together using nucleotide substitution. Bootstrap values were shown at each node. The Bar/scale represents the number of substitutions per site.

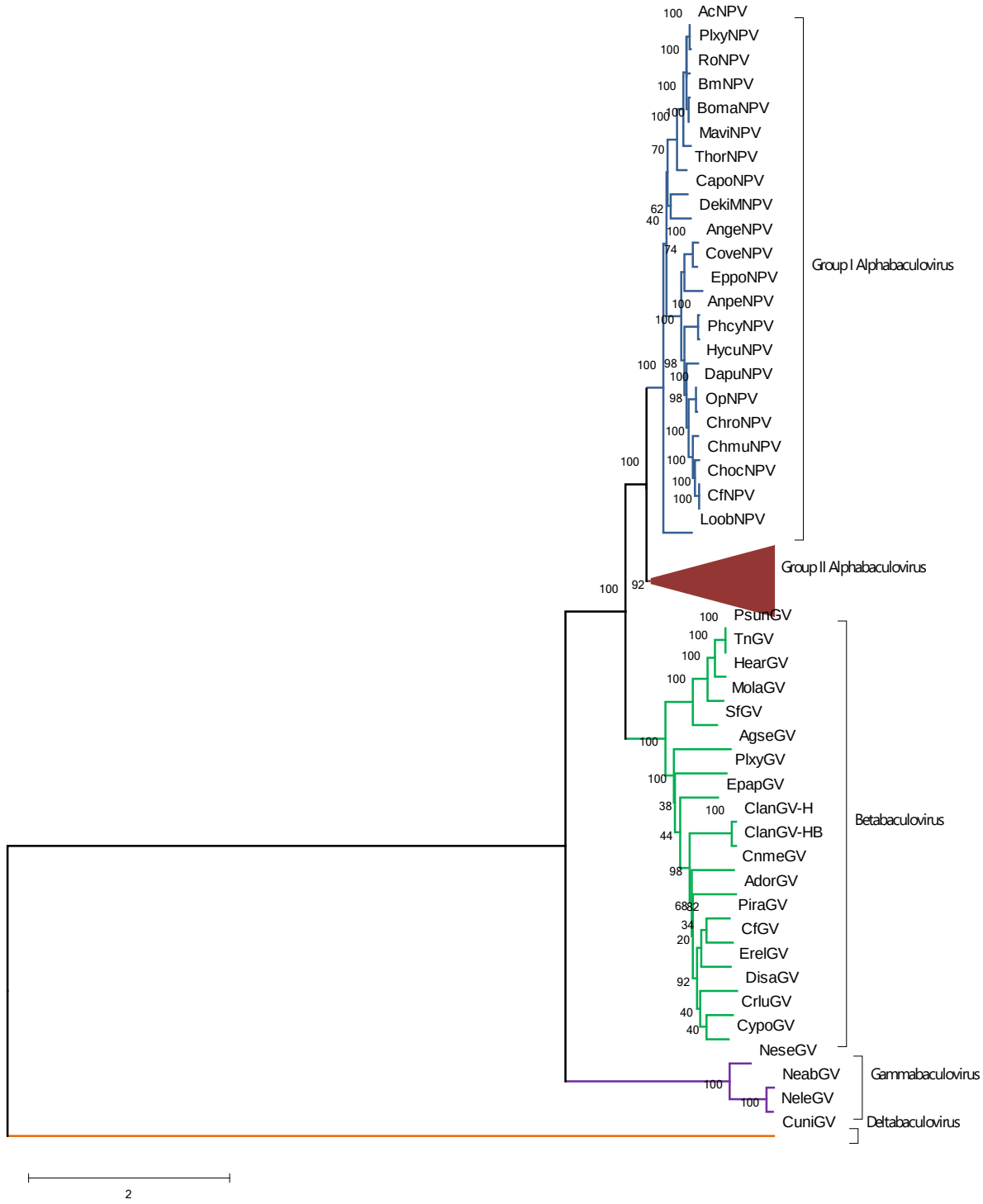


Figure 2-38B: Maximum likelihood tree based on concatenated sequence alignment of six genes together using nucleotide substitution. Bootstrap values were shown at each node. The Bar/scale represents the number of substitutions per site.

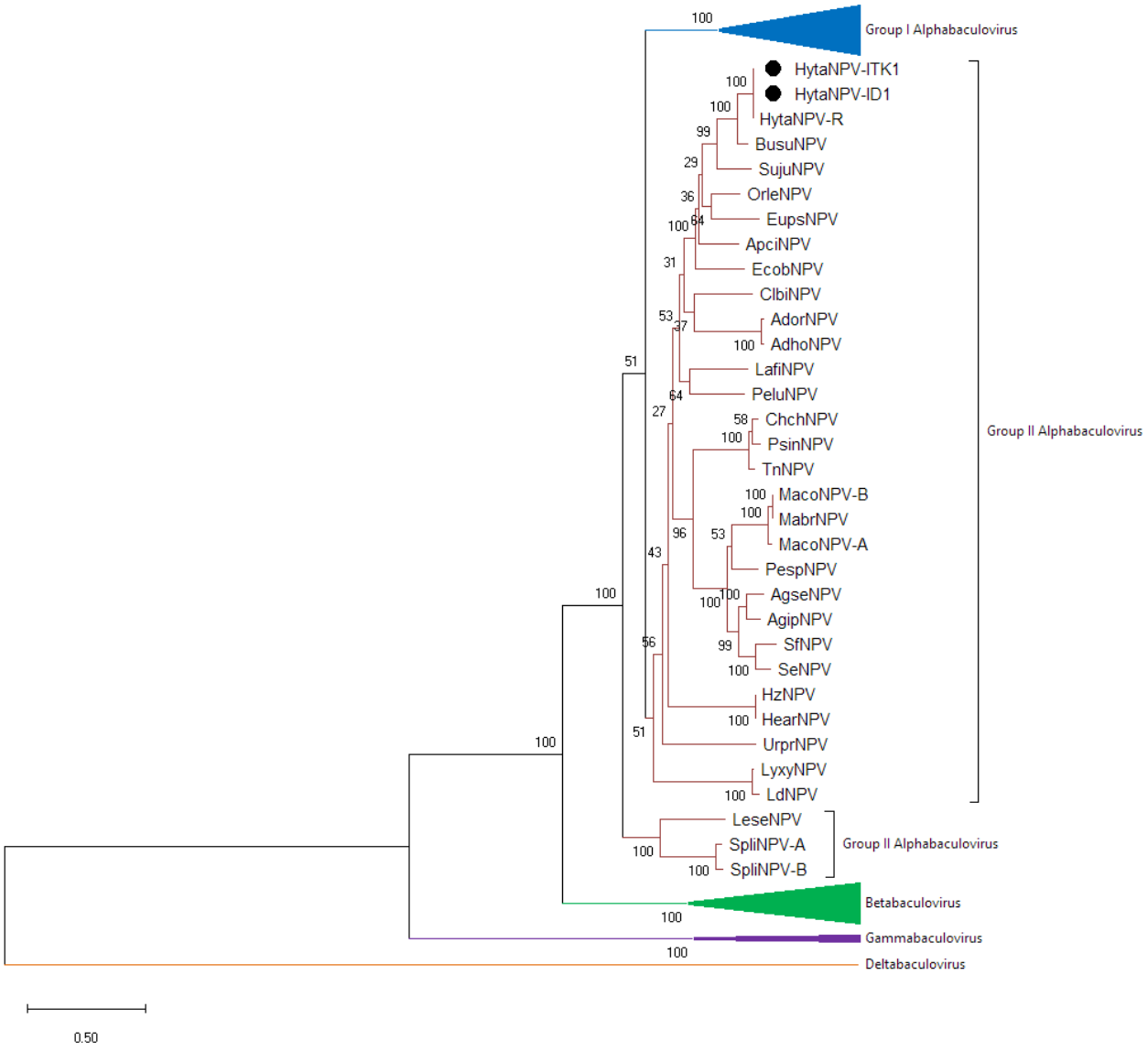


Figure 2-39A: Maximum likelihood tree based on concatenated sequence alignment of six genes together using amino acid substitution. Bootstrap values were shown at each node. The Bar/scale represents the number of substitutions per site.

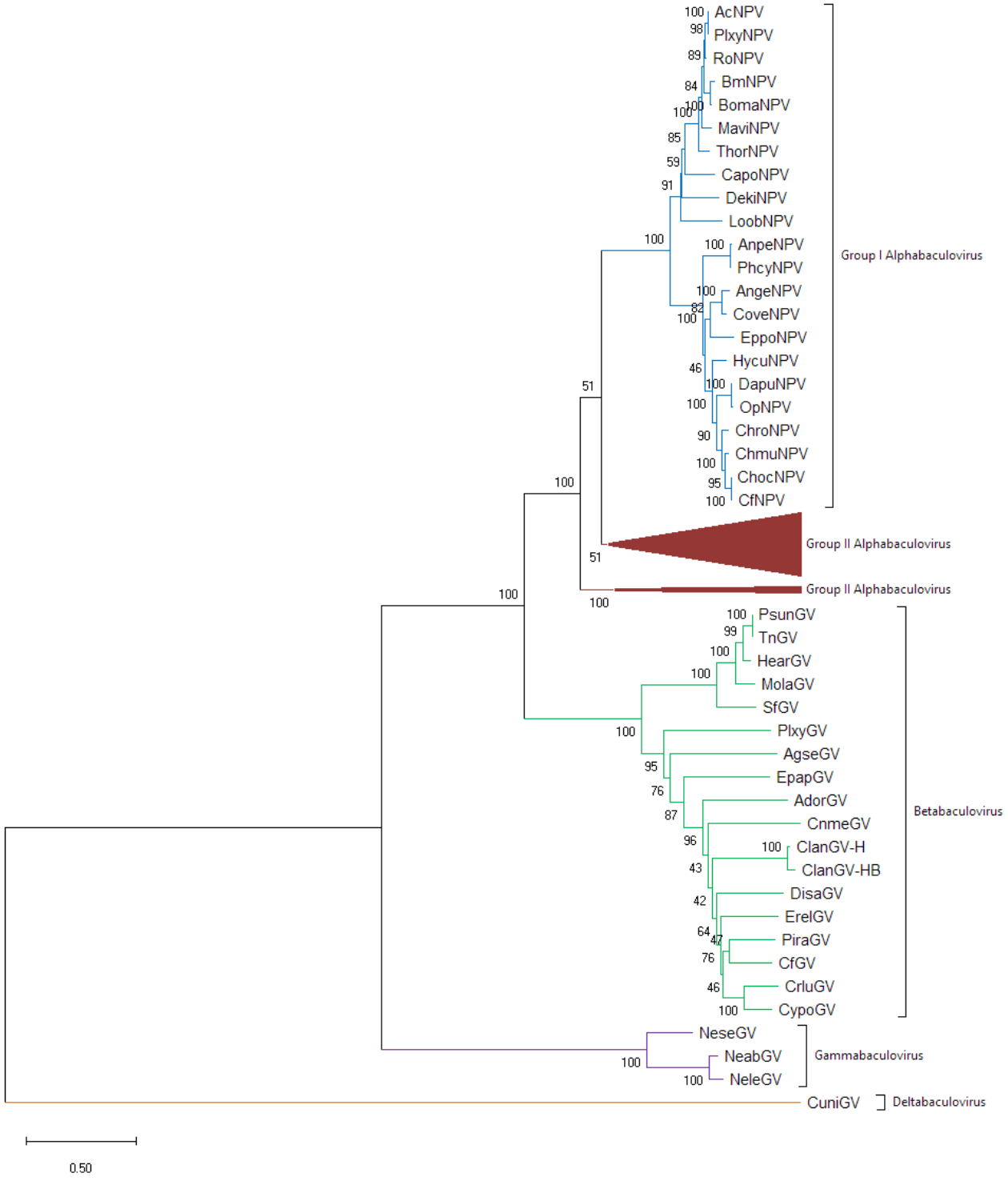


Figure 2-39B: Maximum likelihood tree based on concatenated sequence alignment of six genes together using amino acid substitution. Bootstrap values were shown at each node. The Bar/scale represents the number of substitutions per site.

The two HytaNPV isolates of the present study (HytaNPV-ITK1 and HytaNPV-ID1) appeared to be similar to each other forming a branch with a bootstrap value of 78. All three HytaNPV isolates (HytaNPV-ITK1, HytaNPV-ID1 & HytaNPV-R) were found to be most close forming a single cluster with a bootstrap value of 100. Moreover, the HytaNPVs showed a close relationship with BusuNPV having bootstrap values of 100 and 99 for nucleotide (**Figure 2-38 A & B**) and amino acid (**Figure 2-39 A & B**) substitution-based trees, respectively. Five NPVs, namely HytaNPV, BusuNPV, SujuNPV, EcobNPV, and ApciNPV, infecting the hosts of the family: Geometridae, along with OrleNPV and EupsNPV were found to be more closely related with each other that formed a clade with a bootstrap value of 84 and 100 for nucleotide and amino acid-based trees, respectively, compared to other members of group II alphabaculoviruses, such as AdorNPV, HearNPV, PeluNPV, TnNPV, MacoNPV, SfNPV, LdNPV, SpliNPV etc. However, a group II alphabaculovirus, LafiNPV, pathogenic to the host of the family: Geometridae exhibited a distant relationship with other NPVs infecting the host of the same family and formed a separate cluster (**Figure 2-38A**). The amino acid-based tree suggested that LafiNPV is more closely related to PeluNPV forming a branch with a bootstrap value of 64. Moreover, the amino acid-based phylogeny revealed a comparatively closer relationship among the NPVs pathogenic to the host of family Geometridae, HytaNPV, BusuNPV, SujuNPV, EcobNPV, ApciNPV and LafiNPV compared to the nucleotide-based tree forming a cluster along with some other NPVs (AdhoNPV, AdorNPV, ClbiNPV, OrleNPV, EupsNPV and PeluNPV) with a bootstrap value of 53 (**Figure 2-39**). Both the nucleotide and amino acid substitution-based phylogeny revealed a close relationship between OrleNPV and EupsNPV, the members of group II alphabaculoviruses, with HytaNPV, BusuNPV, EcobNPV, SujuNNPV and ApciNPV.

As, representing and explaining the distance matrices of 77 baculoviruses are very difficult, a detailed phylogenetic analysis emphasizing other important factors (nucleotide and amino acid divergence, transition/transversion ratio) was performed by taking some representative baculoviruses for each group.

2.5.2 Comprehensive phylogenetic analyses with 19 baculoviruses representing each group

A comprehensive phylogenetic analysis was conducted with 19 baculoviruses to construct a phylogenetic tree considering a few baculoviruses representing each group along with the closely related NPVs infecting the hosts of family Geometridae only (**Table 2-23**). The obtained trees were compared with the phylogenetic tree based on 77 baculoviruses. 77 NPVs and representative NPVs were tested for sequence divergence, and phylogenetic tree construction and were found to be similar.

Table 2-23: list of NPVs considered for detailed phylogenetic analysis.

Baculovirus genus/ group	Representing NPVs
Group I Alphabaculovirus	AcNPV, BmNPV, CfNPV
Group II Alphabaculovirus (infecting the hosts of family Geometridae)	HytaNPV, BusuNPV, SujuNPV, EcobNPV, ApciNPV
Group II Alphabaculovirus (infecting the hosts of the families other than Geometridae)	HearNPV, SfNPV
Betabaculovirus	CypoGV, CfGV and HearGV
Gammabaculovirus	NeleNPV, NeabNPV, NeseNPV
Delta baculovirus	CuniNPV

Table 2-24 represents the nucleotide sequence divergence (π_N = p-distance based on nucleotide sequence) (lower half) and the transition vs transversion ratio (s/v) (upper half), whereas, **Table 2-25** represents the amino acid sequence divergence (π_P = p-distance based on amino acid sequence).

A sequence divergence of π_N = 0.007, π_P = 0.009 and s/v = 1.818, was found between HytaNPV-ITK1 and HytaNPV-ID1, while HytaNPV-R revealed π_N = 0.005, π_P = 0.007 and s/v = 1.625 with HytaNPV-ITK1 and π_N = 0.007, π_P = 0.006 and s/v = 1.727 with HytaNPV-ID1. Among all the NPVs infecting other than *Hyposidra talaca*, BusuNPV showed the lowest sequence divergence (π_N ranging from 0.160 to 0.162 and the π_P ranging from 0.086 to 0.091) and highest s/v (ranging from 2.448 to 2.527) with the three isolates of HytaNPV. The other three NPVs, SujuNPV, EcobNPV, and ApciNPV, under group II Alphabacurovirus infecting the hosts of family Geometridae, revealed a higher sequence divergence of π_N = 0.264-0.282 and π_P = 0.182-0.224 with the HytaNPVs compared to BusuNPV. These three NPVs on the other hand showed a slightly lower s/v ratio, 1.045-

1.116, with HytaNPVs. Other representatives of group II alphabaculovirus namely, HearNPV and SfNPV, infecting the host of family Noctuidae have higher sequence divergence than the NPVs infecting the hosts of family Geometridae showing a π_N = 0.320-0.323 and π_P = 0.293-0.300 but had a lower s/v values ranging from 0.917 to 0.958. AcNPV, BmNPV and CfNPV, the representatives of group I alphabaculovirus have showed a π_N = 0.333-0.346, π_P = 0.312-0.348 and s/v = 0.857-1.022 with the isolates of HytaNPV. The beta-, gamma- and delta- baculoviruses have increasingly higher sequence divergence with HytaNPVs. The π_N, π_P and s/v values ranged from 0.427-0.449, 0.486-0.503 and 0.720-0.781, respectively between betabaculoviruses and isolates of HytaNPVs, while the evolutionary divergence in terms of π_N, π_P and s/v were 0.510-0.518, 0.601-0.606 and 0.668-0.699, respectively between gammabaculoviruses and HytaNPVs. Highest evolutionary divergence of π_N = 0.556-0.601, π_P = 0.711-0.732 and lowest s/v ratio ranging from 0.576 to 0.648 were noted between deltabaculovirus, CuniNPV and other groups of baculoviruses.

Table 2-24: Pairwise distance matrix representing p-distance based on nucleotide substitutions (lower half) and transition/transversion ratio (upper half) of a concatenated sequence alignment of six genes together.

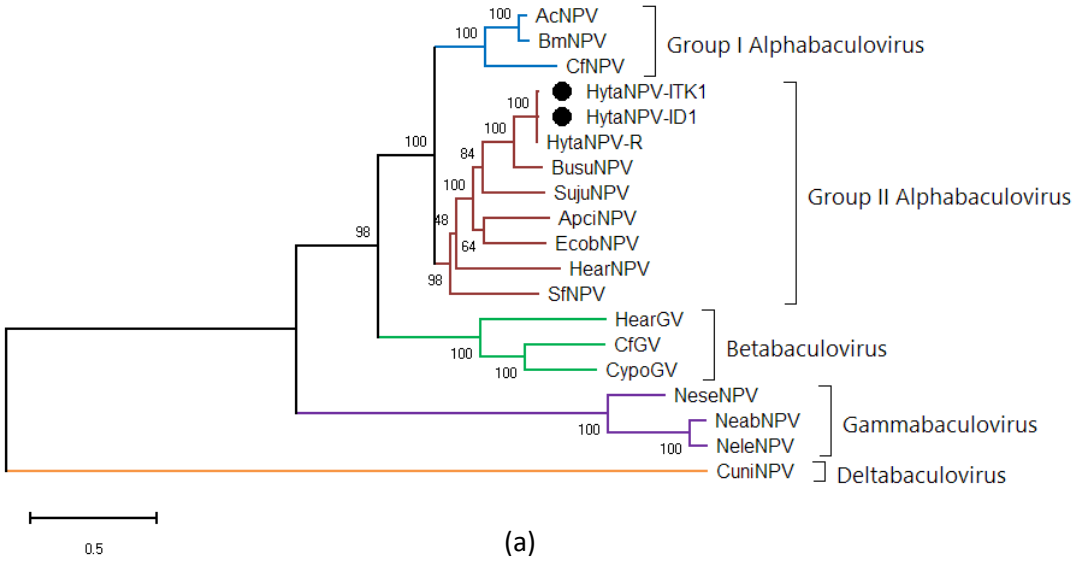
	Baculoviruses	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	HytaNPV-ITK1	*	1.818	1.625	2.527	1.063	1.045	1.109	0.948	0.928	1.022	0.986	0.862	0.747	0.781	0.722	0.668	0.697	0.678	0.577
2	HytaNPV-ID1	0.007	*	1.727	2.448	1.048	1.045	1.116	0.958	0.922	1.007	0.970	0.863	0.740	0.777	0.722	0.668	0.695	0.681	0.576
3	HytaNPV-R	0.005	0.007	*	2.483	1.056	1.052	1.104	0.944	0.917	1.007	0.969	0.857	0.742	0.772	0.720	0.668	0.699	0.682	0.578
4	BusuNPV	0.162	0.162	0.160	*	1.121	1.059	1.053	0.970	0.991	0.976	0.948	0.910	0.764	0.734	0.736	0.675	0.692	0.645	0.614
5	SujuNPV	0.265	0.266	0.264	0.268	*	0.994	1.051	0.831	0.851	0.898	0.862	0.915	0.730	0.705	0.741	0.650	0.656	0.678	0.604
6	EcobNPV	0.276	0.277	0.276	0.280	0.283	*	1.106	0.878	0.869	0.907	0.865	0.893	0.703	0.705	0.779	0.646	0.667	0.634	0.590
7	ApciNPV	0.282	0.282	0.280	0.276	0.279	0.279	*	0.885	0.971	1.020	0.990	1.042	0.744	0.668	0.735	0.630	0.634	0.608	0.617
8	HearNPV	0.321	0.322	0.320	0.319	0.331	0.333	0.326	*	0.953	0.926	0.851	0.947	0.778	0.743	0.743	0.643	0.652	0.639	0.639
9	SfNPV	0.323	0.323	0.321	0.331	0.322	0.322	0.325	0.346	*	0.849	0.842	0.809	0.702	0.751	0.701	0.665	0.678	0.707	0.580
10	AcNPV	0.335	0.335	0.333	0.332	0.336	0.339	0.338	0.362	0.351	*	2.261	1.293	0.783	0.740	0.723	0.632	0.622	0.706	0.615
11	BmNPV	0.341	0.340	0.339	0.341	0.343	0.337	0.350	0.359	0.358	0.064	*	1.271	0.784	0.751	0.737	0.634	0.622	0.700	0.622
12	CfNPV	0.345	0.346	0.343	0.355	0.366	0.364	0.384	0.386	0.368	0.277	0.277	*	0.732	0.758	0.761	0.676	0.684	0.784	0.540
13	CypoGV	0.429	0.427	0.427	0.436	0.438	0.441	0.458	0.450	0.442	0.445	0.446	0.446	*	1.224	0.840	0.664	0.665	0.653	0.572
14	CfGV	0.445	0.444	0.443	0.429	0.436	0.435	0.420	0.437	0.451	0.436	0.440	0.476	0.313	*	0.852	0.611	0.607	0.570	0.599
15	HearGV	0.448	0.449	0.446	0.447	0.449	0.442	0.454	0.462	0.451	0.456	0.456	0.467	0.380	0.384	*	0.621	0.647	0.615	0.597
16	NeleNPV	0.510	0.511	0.512	0.512	0.509	0.504	0.500	0.510	0.518	0.519	0.522	0.544	0.547	0.518	0.525	*	2.669	0.911	0.626
17	NeabNPV	0.515	0.516	0.516	0.516	0.510	0.509	0.502	0.515	0.522	0.522	0.524	0.551	0.545	0.514	0.529	0.107	*	0.852	0.643
18	NeseNPV	0.518	0.518	0.518	0.507	0.504	0.507	0.495	0.500	0.511	0.522	0.521	0.546	0.536	0.509	0.516	0.304	0.299	*	0.648
19	CuniNPV	0.556	0.557	0.556	0.566	0.575	0.567	0.578	0.579	0.569	0.568	0.568	0.556	0.569	0.594	0.591	0.601	0.604	0.601	*

Table 2-25: Pairwise distance matrix representing p-distance based on amino acid substitution of a concatenated sequence alignment of six genes together.

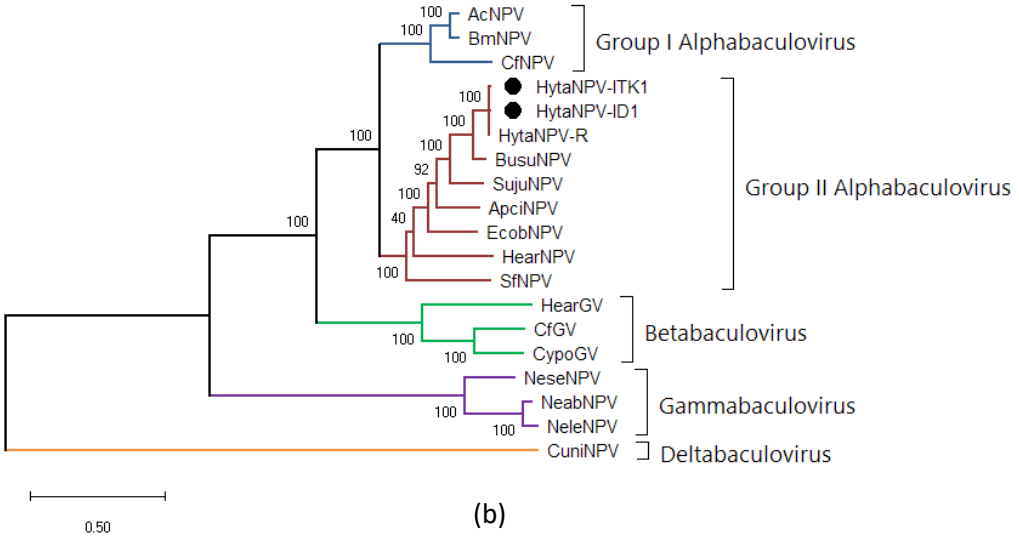
	Baculoviruses	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	HytaNPV-ITK1	*																	
2	HytaNPV-ID1	0.009	*																
3	HytaNPV-R	0.007	0.006	*															
4	BusuNPV	0.091	0.091	0.086	*														
5	SujuNPV	0.185	0.186	0.182	0.174	*													
6	EcobNPV	0.224	0.224	0.220	0.226	0.228	*												
7	ApciNPV	0.215	0.217	0.213	0.210	0.213	0.221	*											
8	HearNPV	0.295	0.296	0.293	0.287	0.306	0.300	0.294	*										
9	SfNPV	0.298	0.300	0.296	0.295	0.295	0.289	0.293	0.321	*									
10	AcNPV	0.316	0.315	0.312	0.310	0.318	0.320	0.320	0.355	0.355	*								
11	BmNPV	0.322	0.321	0.318	0.321	0.326	0.323	0.324	0.359	0.358	0.058	*							
12	CfNPV	0.348	0.350	0.346	0.348	0.357	0.362	0.362	0.393	0.384	0.232	0.228	*						
13	CypoGV	0.487	0.488	0.486	0.491	0.493	0.489	0.503	0.494	0.503	0.489	0.488	0.507	*					
14	CfGV	0.497	0.496	0.495	0.499	0.503	0.487	0.502	0.500	0.498	0.489	0.488	0.518	0.248	*				
15	HearGV	0.502	0.503	0.501	0.498	0.493	0.489	0.505	0.500	0.497	0.500	0.499	0.515	0.384	0.388	*			
16	NeleNPV	0.601	0.602	0.602	0.599	0.601	0.600	0.606	0.600	0.609	0.617	0.618	0.625	0.634	0.622	0.623	*		
17	NeabNPV	0.605	0.606	0.606	0.604	0.608	0.598	0.608	0.606	0.614	0.618	0.618	0.625	0.632	0.627	0.625	0.079	*	
18	NeseNPV	0.605	0.606	0.606	0.604	0.601	0.600	0.602	0.603	0.602	0.610	0.615	0.617	0.629	0.626	0.630	0.287	0.282	*
19	CuniNPV	0.711	0.715	0.711	0.709	0.713	0.711	0.711	0.722	0.717	0.711	0.707	0.709	0.722	0.731	0.732	0.727	0.728	0.730

Both the phylogenetic trees based on nucleotide (**Figure 2-40A**) and amino acid (**Figure 2-40B**) substitutions considering representative members from each group of baculoviruses along with the closely related NPVs infecting the host of the family Geometridae showed almost similar tree topology. HytaNPV-ITK1 and HytaNPV-ID1 of the present study formed a single cluster with HytaNPV-R having a bootstrap value of 100. The isolates of HytaNPVs showed the closest relationship with BusuNPV forming a cluster with a bootstrap value of 100. Moreover, the NPVs infecting the hosts of family Geometridae, BusuNPV, SujuNPV, EcobNPV, and ApciNPV, formed a clade with a bootstrap value of 100.

Further, the phylogenetic trees constructed taking the representative members (**Figure 2-40**) and the phylogenetic trees of 77 baculoviruses (**Figure 2-38 A & B, Figure 2-39 A & B**) exhibited similar branching patterns and tree topology regarding the divergence of delta-, gamma-, beta-, group I and group II alpha- baculoviruses. In both cases **(Figure 2-38, Figure 2-39, Figure 2-40)**, the deltabaculovirus diverged before the divergence of gammabaculoviruses. Then betabaculoviruses diverged as a sister group of alphabaculoviruses followed by the divergence of alphabaculoviruses into two clusters, the group I and group II.



(a)



(b)

Figure 2-40: Maximum likelihood tree based on concatenated sequence alignment of six genes together using nucleotide (a) and amino acid (b) substitution. Bootstrap values were shown at each node. The Bar/scale represents the number of substitutions per site.

Chapter 3

Discussion, Conclusion, & Summary

Section 3.1: Discussion

India is the second largest tea producer sharing 23% of global production and one of the largest tea consumers accounting for 19% of global consumption (Anonymous, Report Tea Board, 2017-18). Tea is grown in different parts of India, that include Darjeeling and its Terai-Dooars region of West Bengal, Assam, Sikkim, Tripura, Nilgiri of Tamil Nadu, Himachal Pradesh, Kerala, Karnataka and Orissa.

Among the different arthropod pests damaging tea plantations, *Hyposidra talaca* Walker, (Lepidoptera: Geometridae), the caterpillar stages of which are commonly known as loopers, has emerged as a major defoliating pest (Das et al., 2010a; Sinu et al., 2011). This pest migrated from forest to tea causing a serious pest problem to the Darjeeling and Assam tea plantations resulting in a huge economic loss in these regions (Roy and Muraleedharan, 2014). Though the looper pests can be effectively controlled by regular applications of synthetic chemical pesticides, especially, organophosphate and pyrethroids, nevertheless the tea pests in general, are gradually becoming less susceptible to the pesticides and may develop higher tolerance in different ways (D. Roy et al., 2021; S. Roy et al., 2021; Saha, 2016). Several detoxification enzymes are reported to play a key role in developing tolerance against commonly used insecticides in major tea pests (Saha, 2016). Different workers reported variations of susceptibility against several organophosphates and pyrethroids in *H. talaca* populations from different sub-Himalayan regions of India and suggested the role of different enzymes in the development of resistance mechanisms (Das, 2014; Das et al., 2010a; Dasgupta et al., 2016; D. Roy et al., 2021; S. Roy et al., 2021). This often results in control failures (Majumder et al., 2012; Sannigrahi and Talukdar, 2003) leading to a great economic loss.

Synthetic chemical pesticides have severe detrimental effects on the environment as well as non-target organisms including humans (Azmi et al., 2006; Mobed et al., 1992; Saravanan et al., 2009). Studies have shown that people associated with tea industries (mainly workers) have increased levels of various genetic damage (Dutta and Bahadur, 2016). Aquatic organisms including fish are also at high risk of developing genetic and physiological abnormalities (Singh

et al., 2017). Therefore, the use of a safe biopesticide has been unequivocally advocated worldwide and a large number of researches are underway to produce biopesticides including viruses that are safe and target-specific (Chandler et al., 2011; Kumar et al., 2021). The use of Bt toxin against insect pests and genetically modified host plant has some controversies (Rawat et al., 2011), therefore, baculovirus as a biopesticide holds great promise.

Initially, the occurrence of NPV was reported from *Hyposidra talaca* collected from the Terai-Dooars regions of North Bengal (Mukhopadhyay et al., 2011). Its dose-mortality bioassays revealed a median lethal concentration (LC_{50}) value of 2.8 x 10^3 OBs/ml on the second instar larvae of *H. talaca*, whereas, the median lethal time (LT_{50}) was reported to be 5.45, 4.15 and 4.05 days for 1 x 10^4, 1 x 10^5 and 1 x 10^6 OBs/ml, respectively. At the time of the initiation of the present work in 2014, there was no information available about the genome size or genome organization of HytaNPV except for a 527 bp partial sequence of *polyhedrin* gene (Antony et al., 2011). The PCR conjugated RFLP analysis is one of the advanced techniques to detect genetic polymorphism (Christian et al., 2001; Ojha et al., 2017), but designing primers requires comprehensive information about the genome of the species and that also cannot detect the polymorphism throughout the genome. Therefore, without such primary information on the genome, Restriction endonuclease (REN) fragment profile analysis of the whole genome appears to be a useful technique to find the genetic polymorphism (Goto et al., 1992; Redman et al., 2010; Rowley et al., 2010; Smith and Summers, 1979; Williams et al., 2011). Hence, the REN fragment profile analysis was adopted to explore and compare the genome of different HytaNPV isolates in the present study.

3.1.1 Restriction endonuclease (REN) fragment analysis

Restriction endonuclease (REN) fragment analysis is one of the conventional preliminary approaches to determine the genome organization and the approximate genome size of a virus (Chen et al., 2000; Hu et al., 1998; Lin et al., 2012) and also is an effective and stable tool to detect genetic polymorphism among different geographic isolates of NPV (Redman et al., 2010; Shapiro et al., 1991; Stiles and Himmerich, 1998; Williams et al., 2011). Restriction fragment

analyses have been widely used for the characterization of genomes from different populations of baculoviruses (Cory et al., 2005; Williams et al., 2011).

Crook et al. (1985) analyzed GVs from *Artogeia (=Pieris) rapae* and *Pieris brassicae* by restriction analysis and reported no significant differences among the isolates for *Xho*I, *Sma*I and *Bgl*I restriction profiles, but 11 of them produced distinguishable *Eco*RI, *Bst*I and *Hin*dlII restriction profiles. They could resolve 14 REN fragments for *Eco*RI, *Bam*HI and *Xho*I, ranging from 0.8 to 34.7 kb and two *Hin*dIII fragments of 92.5kb and 33.5 kb. Crook et al. (1985) studied the physical maps of three variants of *Cydia pomonella* GV from seven different regions in Europe using *Eco*RI, *Bam*HI, *Hin*dIII, *Sma*I and *Apa*I. Stiles and Himmerich, (1998) used restriction analysis and reported changes in the restriction pattern of the isolates and depicted the biological activities in *Autographa californica* NPVs. Shapiro et al. (1991) used REN fragment analyses to successfully distinguish 11 of 15 isolates of *Spodoptera fiugiperda* nucleopolyhedrovirus (SfNPV) from Louisiana using *Bam*HI, *Hin*dIII, and *Eco*RI. They reported a great variation in the *Eco*RI profile only having 9 common fragments out of 20 among all isolates, whereas the variations in 3 and 1 fragments were present in *Bam*HI and *Hin*dIII profiles.

Our results of restriction analyses in two isolates using *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I and *Xho*I revealed fragment numbers, 8, 18, 26, 9, 11, 7 and 20, respectively in HytaNPV-ITK1 and HytaNPV-ID1. The restriction fragment size was found to be identical in both the isolates, which corroborated the *in silico* restriction fragment profiles of HytaNPV-R (Nguyen et al., 2018). However, fragments less than 700 bp could not be detected on agarose gel after electrophoresis of digested fragments. A number of studies have also reported such limitations of agarose gel electrophoresis-based RFLP studies. The *Eco*RI fragments smaller than 3.3 kb could not be scored in the SfNPV genome (Shapiro et al., 1991). An earlier study on SfMNPV using *Bam*HI, *Eco*RI and *Pst*I yielded almost identical REN fragment patterns in different isolates with slight variations in a single *Bam*HI fragment (5.1 kb) which was found to be present in only one of the five isolates (Rowley et al., 2010). Such studies also support our results of almost identical restriction patterns in the present study of HytaNPV isolates. Such

studies are very important for initial characterization where genome information or genome organization is lacking.

By analyzing the *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I and *Xho*I restriction profiles, the mean genome sizes of both the HytaNPV isolates of the present study, were approximately estimated to be 138.20 kb for HytaNPV-ITK1 (Terai) and 138.46 for HytaNPV-ID1 (Dooars) (**Table 2-3, Table 2-4**). The minor difference in genome sizes were either due to some undetected low molecular weight restriction fragment(s) or due to unresolved change in size of restricton fragment(s) produced by observed site polymorhism(s), in agarose gel electrophoresis. The size of the baculoviral genome ranges from 80 kb to 180 kb (King et al., 2012). The genome size of HytaNPV estimated in the present study comes within the above range.

In a preliminary study, we reported an approximate genome size of 176 kb for HytaNPV by restriction profile analysis (Ghosh et al., 2015), which was higher than the genome size estimated finally. Because in the earlier study of 2015 there was no reported restriction profile for the HytaNPV genome available for comparison, some partially digested fragments were erroneously considered to estimate the genome size. Moreover, resolving whether an agarose gel electrophoretic band was a singlet, doublet or triplet of fragments (particularly for those bands which were >23 kb), based on the relatively small quantity of DNA present in those bands was possibly not very accurate. As a consequence, higher approximate genome size was reported which was precisely estimated to be approximately 138 kb in the final analyses. Such a variation in the estimation of genome sizes between two analyses is not unforeseen. Liu et al. (1993) reported the restriction endonuclease fragment profile along with the genome size of 129 kb for BusuNPV, pathogenic to old looper pest of tea, *Biston(=Buzura) suppressaria* in China, however, the genome size of 120.9 kb of BusuNPV was documented by Hu et al. (1998). Later on, Zhu et al. (2014) reported a more precise genome size of 120.42 kb by sequencing the complete genome of BusuNPV.

The final estimated genome size of 138.20 kb for HytaNPV-ITK1 and 138.46 for HytaNPV-ID1 in the present study are closely comparable to the genome size reported by Nguyen et al. (2018) for the Dooars isolate, HytaNPV-R (139.089 kb) using complete genome sequencing. Such a little variation between the estimated genome sizes using two different techniques is not

unusual, as some small restriction fragments were not detectable in agarose gel electrophoresis. Comparing the *in vitro* restriction profiles of HytaNPV-ITK1 and HytaNPV-ID1, with *in silico* restriction profiles of HytaNPV-R revealed that some low molecular weight fragments of *Bam*HI, *Bgl*I, *Eco*RI, *Pst*I were not detected as these fragments could not be resolved in agarose gel electrophoresis. This is the reason that the total genome size estimated from the restriction profiles of above mentioned four endonucleases was slightly lower than the reported genome size of HytaNPV-R by (Nguyen et al., 2018). A little variation in genome size among the closely related virus strains was also evident. The genome size of *Helicoverpa armigera* NPV (HearNPV) was reported to be 130.1 kb (Chen et al., 2000), whereas, the genome size of *Helicoverpa assulta*, a close relative of HearNPV, was found to be 138 kb (Woo et al., 2006). This suggests that genome size may vary among the strains or isolates of a closely related species, based on techniques used. Sequencing is the ultimate strategy for detecting the exact genome size.

The River Teesta acts as a major geographical barrier between the sub-Himalayan terrains of Terai and the Dooars plantations of North Bengal. As the present study was initiated to characterize the Terai isolates of HytaNPV, the Dooars isolate was also included for comparison.

No significant differences were found among the *Bam*HI, *Bgl*I, *Eco*RI, *Pst*I and *Kpn*I restriction profiles of HytaNPV-ITK1 (Terai isolate), HytaNPV-ID1 (Dooars isolate) and the reference HytaNPV-R. In the case of *Hin*dIII and *Xho*I digestions, only some variations were detected among the isolates of HytaNPVs. Such similarities in restriction profiles with few variations appear to be common among different geographic isolates of NPVs (Graham et al., 2004; Li et al., 2005; Redman et al., 2010).

Our results of restriction analyses showed almost a similar restriction profile between the HytaNPV-ID1, and HytaNPV-ITK1 (present study) when compared with HytaNPV-R reported by Nguyen et al. (2018), however a 3527 bp (3.53 kb) *Hin*dIII fragment was absent in HytaNPV-ITK1. On the other hand in HytaNPV-ITK1, an additional 2110 bp (2.11 kb) HindIII fragment was detected which was found to be absent in HytaNPV-ID1 (present study) and

HytaNPV-R (Nguyen et al., 2018) isolates. But the total number of restriction fragments in HytaNPV-ITK1 was similar to HytaNPV-ID1 and HytaNPV-R. A fragment of 1417 bp (1.42 kb) resulting from a 3527 bp fragment (3527-2110bp) theoretically assumed to be present, is missing. This result may be interpreted with the assumption that the 3527 bp (3.52 kb) *Hin*dIII fragment (Fragment H) (**Section 2.3: Table 2-5**) is flanked by a 4156 bp *Hin*dIII fragment (Fragment G) (**Section 2.3: Table 2-5**) having restriction site 63817/63821 in one end and by 27807 bp *Hin*dIII fragment on the other end with restriction site 60290/60294. As a 4156 bp fragment was present in all the HytaNPV isolates, the *Hin*dIII restriction site 63817/63821 cannot be mutated, however, a mutation in *Hin*dIII site 60290/60294 may produce a larger fragment of 29224 bp (27807+1417 bp), instead of 27807 bp fragment.

The number of *Xho*I sites in the two HytaNPV isolates of the present study was found to be the same which was in concurrence with the HytaNPV-R (Nguyen et al., 2018) as produced *in silico* study. However, a 4857 bp *Xho*I fragment (position: 16532-21388 bp) in the genome of HytaNPV-R (**Table 2-6**) by *in silico* study was absent in both the Terai and the Dooars isolates. Instead, a unique 4100 bp *Xho*I fragment was detected in HytaNPV-ITK1, whereas, for HytaNPV-ID1 it was of 4500bp, but none of these fragments was present in the HytaNPV-R. *In silico* analysis of HytaNPV-R revealed that the 4857 bp *Xho*I fragment (fragment K) is flanked by a 14,488 bp *Xho*I fragment (Fragment C), (**Section 2.3: Table 2-6**) and 2279 bp fragment (Fragment Q) (**Section 2.3: Table 2-6**). *Xho*I fragments, 14.49 kb and 2.28 kb observed in HytaNPV-ITK1 and HytaNPV-ID1, respectively correspond with the 14488 bp and 2279 bp *Xho*I fragment of HytaNPV-R, indicating a new *Xho*I site inside 4857 bp fragment, because neither a 757 bp fragment (4857-4100 = 757 bp) nor a 357 bp fragment (4857-4500 = 357 bp) could be detected (**Figure 2-9**, **Figure 2-10**, **Table 2-4**). Such variations in REN fragments were also reported among different geographical isolates of SfMNPV (Rowley et al., 2010). Extensive study and sequencing may resolve the exact nature of such polymorphisms.

The *in silico* restriction map of *polyhedrin, lef-8, lef-9, pif-1, pif-2* and *pif-3* sequences obtained in the present study showed that restriction sites for any of the restriction endonucleases used were found to be present in all the gene sequences (complete/partial) except *pif-3*. The restriction maps for all the five gene sequences of the two isolates, HytaNPV-ITK1 and

HytaNPV-ID1 are in complete agreement with the HytaNPV-R (Nguyen et al, 2018) except *pif-1*. A single *Bam*HI site in the *pif-1* sequence present in HytaNPV-ID1 and HytaNPV-R was absent in the HytaNPV-ITK1 isolates from Terai. This mutation resulted in the absence of a 15bp *Bam*HI fragment in HytaNPV-ITK1, which could possibly be perceived through sequencing, but was not detectable in agarose gel. Rowley et al. (2010) reported the presence of a single *Eco*RI fragment (6.1 kb) in three of the five geographical isolates of SfMNPV. Studies of genetic diversity of *Spodoptera exempta* NPV from Tanzania revealed that few isolates were also found to be distinguishable by the presence of one or two unique restriction fragments (Redman et al., 2010), indicating the presence of new mutations.

But the *in vitro* restriction analyses of the two isolates of HytaNPV in the present study showed almost similar restriction profiles when compared with the *in silico* restriction profiles of HytaNPV-R (MH261376.1; Nguyen et al., 2018) (**Table 2-10, Table 2-11**).

Genetic polymorphisms in the restriction sites have also been reported by Rowley et al. (2010) among different isolates of *Spodoptera frugiperda* NPV. Williams et al. (2011) performed RFLP analysis conjugated with southern blot and documented variations in the restriction profiles among nucleopolyhedroviruses isolated from OpNPV in the Western United States. Our results of REN fragment analyses strongly indicate that the isolates of HytaNPV from the Terai and the Dooars regions of West Bengal have some genetic variations despite similarities. Genetic Variation among different wild populations of baculoviruses may play a key role in the process of baculovirus epizootiology (Erlandson, 2009).

3.1.2 Analyses of gene sequences

Sequencing and characterization of genes of a virus is the most useful way to know the molecular and phenotypic properties of the virus, such as stability, the pattern of interaction with the host, killing efficacy and also the evolutionary relationships with the other viruses. Information about the gene and genome sequences also helps to detect the co-evolutionary pattern of the virus with its host (Pappas et al., 2021). This helps to produce genetically modified viruses to combat pests of different plants.

Discussion

Six genes (*polyhedrin, lef-8, lef-9, pif-1, pif-2* and *pif-3*) from both the isolates, HytaNPV-ITK1 (Terai) and HytaNPV-ID1 (Dooars), were sequenced. Complete cds with some intermediate gaps were obtained for *pif-3* and *lef-9* of both the isolates and *pif-2* of HytaNPV-ITK1, whereas, partial cds (incomplete 5′ and 3′ ends) were obtained for *polyhedrin*, *pif-1* and *lef-8* of both the isolates and *pif-2* of HytaNPV-ID1.

Both nucleotide and protein BLAST of all the six genes (complete or partial) of HytaNPV-ITK1 and HytaNPV-ID1 showed maximum similarity with HytaNPV-R (Nguyen et al., 2018). Among the NPVs infecting the specimens of other genera than *Hyposidra*, BusuNPV of *Biston (=Buzura) suppressaria*, an old Geometrid (looper) pest of tea, showed the maximum overall amino acid sequence identity of 90.9% and nucleotide sequence identity of 85.8% with both the isolates of the present study for all the six genes. However, in the case of the *polyhedrin* gene, higher nucleotide sequence similarity was found with EcobNPV (~86%) than BusuNPV (~84%). Although for the amino acid sequence of *polyhedrin* gene, BusuNPV showed a higher sequence similarity (98.3%) than EcobNPV (96.5%) with the HytaNPV. Different workers have also showed a close relationship of HytaNPV with BusuNPV based on partial sequence of *polyhedrin* gene (Antony et al., 2011; Dasgupta et al., 2016). The complete genome sequence of HytaNPV-R also revealed the closest relationship between HytaNPV-R and BusuNPV (Nguyen et al., 2018). Our sequence alignment studies have shown less similarity in decreasing order with other NPVs infecting the host caterpillars of other genera.

Pairwise sequence comparison among HytaNPV-ITK1, HytaNPV-ID1 and HytaNPV-R revealed that the nucleotide sequence identity ranged from 99.31-99.52% (results: **Table 2-21**), whereas, 98.99-99.40% identity was found for amino acid sequence. The overall nucleotide and amino acid sequence identity was estimated to be 99.09% and 98.76%, respectively among the three isolates of HytaNPV. These results corroborate the comparative studies based on the *polyhedrin* gene among the isolates of NPVs infecting species of *Hyposidra* as reported by Antony et al. (2011) and Dasgupta et al. (2016). Such a similarity among the geographic isolates of NPVs infecting the same host of the same genus was very common. Craveiro et al. (2013) reported a high level of similarity (>98%) for *polyhedrin, lef-8, lef-9* and *pif-2* genes among different isolates of *Pseudoplusia includens* SNPV from Brazil.

The overall sequence comparison among the three isolates revealed that for the *polyhedrin* gene, though, 7 variable sites were found yet all were synonymous, and no non-synonymous substitution was detected, suggesting and supporting the conservative properties of the *polyhedrin* gene as reported by Paolo et al. (1993) and Rohrmann (2011).

The pairwise comparison revealed comparatively a higher ratio of non-synonymous (nd) to synonymous (sd) substitution between the HytaNPV isolates of the Terai and the Dooars region, than the isolates within the Dooars as evident from *lef-8, lef-9* and *pif-2* (**Table 2-21**; cluster I, II & III). The *lef-8* and *lef-9* are involved in transcription whereas, the *pif-2* gene plays a key role in oral infectivity. As the environmental component of these two regions (the Terai and Dooars) does not differ significantly, such a higher ratio of nd/sd between these two geographic regions suggested that either these mutations do not significantly affect the functioning of the genes, or these genes possibly play a key role in the evolution of the viruses. Moreover, the overall comparison showed a ratio of nd/sd <1 for *lef-8*, and *pif-1* (2/6=0.33 for *lef-8* and 6/10=0.6 for *pif-1*) whereas, the ratio (nd/sd) for *lef-9* (16/6=2.66), and *pif-2* (20/12=1.66) were >1 (**Table 2-21**; cluster IV), indicating that *pif-2* and *lef-9* genes are under positive selection (Yang, 2001). Moreover, a higher proportion of variable sites (d_N) for nucleotides (16 out of 1001) were detected in the *pif-2* sequence (results: Cluster IV, **Table 2-21**). These findings suggested that though *pif-2* is one of the most conserved *per os* infectivity genes, the genetic variations among the geographic isolates of NPV infecting the host of the same species may exist in different populations possibly indicating local environmental stress on a gene. Such variability in the *pif-2* gene among close geographic isolates of NPV corroborates the findings of Craveiro et al. (2013) among the different isolates of NPV from *Pseudoplusia includens*. In the present study, no variable sites were found in the *pif-3* sequence. However, our results of sequence comparison showed that the *pif-1*, *pif-3* and *lef-8* genes had comparatively lower variability than other genes among the isolates of HytaNPV. Overall sequence comparison among the isolates of HytaNPVs indicated the existence of differences in sequence variations, among different genes studied.

3.1.3 Phylogenetic analysis

Different molecular data have been widely used to determine the phylogenetic relationship among closely related organisms, including viruses. To use baculovirus as biopesticide it needs to be ascertained that the field applications will not affect any non-target beneficial arthropods, such as mulberry and non-mulberry silk moths, especially in the northern part of West Bengal and Assam. Therefore, it is essential to know the phylogenetically close relatives of HytaNPV isolates of the Dooars and Terai region of West Bengal.

Three complementary approaches: i) the concatenated sequences of the core genes (ii) gene order, and iii) the gene content of the genomes, were used to infer the phylogeny of several virus families including Baculoviridae (Khan and Ahmad, 2019; Montague and Hutchison, 2000). By applying these approaches using seven of the core genes of baculoviruses including *pif-1*, *pif-2*, *lef-8* and *lef-9*, Herniou et al. (2003, 2001) reported that the phylogenetic tree based on six of the core genes was similar to the phylogeny based on all the reported core genes of baculoviruses. In the present study six core genes, *polyhedrin, lef-8, lef-9, pif-1, pif-2* and *pif-3,* were used for phylogenetic analysis.

Both the nucleotide and amino acid substitution-based phylogenetic trees using concatenated sequence alignment of *polyhedrin, lef-8, lef-9, pif-1, pif-2* and *pif-3* genes showed that *Hyposidra talaca* NPVs were phylogenetically placed under the group II alphabaculovirus. The two isolates of HytaNPV (HytaNPV-ITK1 and HytaNPV-ID1) appeared to be very close to the reference HytaNPV-R. By comparing the partial *polyhedrin* sequence Antony et al. (2011) and Dasgupta et al. (2016) also reported the similarity of the NPV isolates infecting *Hyposdita talaca* from Terai and the Dooars regions. Among the NPVs infecting the host of other genera, BusuNPV was shown to have the closest relationship with the isolates of HytaNPVs (Ghosh et al., 2019) corroborating the findings of Antony et al. (2011) and Sinu et al. (2011). The phylogenetic tree based on 77 baculoviruses also revealed that the NPVs infecting the hosts of family Geometridae such as BusuNPV, EcobNPV, ApciNPV and SujuNPV have a close relationship with the HytaNPVs. Moreover, EupsNPV (from China) and OrleNPV (from Canada), pathogenic to the host of family Erebidae, also exhibited closeness with HytaNPV.

These results are in accordance with the phylogeny of HytaNPV reported by Nguyen et al. (2018) based on the amino acid sequence of 37 core baculovirus genes. Such a phylogenetic closeness among the NPVs infecting the hosts of the family Geometridae is also evident in the evolutionary studies done by a number of workers (Liu et al., 2014; Zhu et al., 2014). However, LafiNPV, another alphabaculovirus pathogenic to the host of the family Geometridae included in the present study showed comparatively a distant relationship with HytaNPV than the other members infecting the host of the same family. This may be due to the inclusion of fewer gene sequences for the phylogenetic studies in the present study as also reasoned by Herniou et al. (2003, 2001). Herniou and his collaborators (Herniou et al., 2001) first compared the phylogenetic trees based on complete genomes with those based on single genes. They also reported that *polh* phylogenies showed significant differences with phylogenies based on other genes (Herniou et al., 2003). Phylogenetic trees based on *polh* portrayed AcMNPV as a sister group of the rest of the group I NPVs including BmNPV, while other phylogenetic trees cluster AcMNPV and BmNPV together. The phylogeny based on amino acid, and nucleotide sequences have topological differences. The phylogeny based on amino acid sequence showed a relatively closer relationship of LafiNPV with the other member of alphabaculoviruses infecting Geometridae than the phylogeny based on the nucleotide sequence. Such variations in the phylogeny based on DNA and protein data have been shown by Li et al. (2016) and the preference of proteome data over nucleotide has also been suggested for viral phylogeny.

Our analyses showed that the NPVs, namely, AcNPV, BmNPV, CfNPV, OpNPV etc., infecting the different specimens of lepidopteran hosts (caterpillars) clustered together to form a monophyletic clade of group I Alphabaculovirus. The phylogeny showed that both the group I, and group II alphabaculoviruses diverged from a common ancestor. The analyses also revealed an early divergence of delta-, gamma- and beta- baculoviruses before the divergence of alphabaculoviruses into group I and group II (**Figure 2-39**). This topology of the phylogenetic trees of our study corroborates with several reported phylogenetic trees based on the concatenated sequences of all baculovirus core genes (Afonso et al., 2001; de Jong et al., 2005; Duffy et al., 2006; Ikeda et al., 2006; Nguyen et al., 2018; Xu et al., 2010; Zhu et al., 2014).

Discussion

For comprehensive understanding, a precise phylogenetic analysis was conducted which also revealed a similar divergence pattern of baculoviruses as shown in the phylogenetic tree (**Figure 2-40**). Transition bias over transversion has been detected in different eukaryotic species (Brown et al., 1986; Kocher and Wilson, 1991; Matson and Baker, 2001) including viruses (Lyons and Lauring, 2017) at the population level. The transition has been shown at an initial level of saturation over transversions among the widely diverged species/populations in mammals (Rosenberg et al., 2003) which can also be true in the case of slow-evolving DNA viruses (Duchêne et al., 2015). Our results also corroborate the same with a higher transition/transversion ratio (s/v) of approximately 2.00 with the closely related groups with a declining trend in widely diverged baculoviruses. Moreover, the divergence matrix showed a continuous decline in the s/v ratio over time or divergence supporting the reliability of the phylogenetic tree in the present study. Duchêne et al. (2015) showed that the s/v ratio declines with time in the virus evolution under the time reversal model of nucleotide substitution.

As the application of Bt formulations to curb the tea pest problems may potentially affect the silk industry in the Northern part of West Bengal and Assam. The Mulberry silk industry largely depends on infection-free silkworm, *Bombyx mori*, so pest management through the use of *Bacillus thuringiensis* is largely restricted in the tea-silkworm ecosystem (Dashora et al., 2017). The present study revealed that HytaNPV, group II alphabaculovirus, and BmNPV, a group I alphabaculovirus, were phylogenetically distant from each other suggesting that there is hardly a chance of cross-infectivity. So, NPV being highly species-specific in their host range (Rohrmann, 2011), the application of HytaNPV as a biopesticide in these regions can be safe for other non-target economically beneficial organisms, including silk worms.

Section 3.2: Conclusion

The comparative analyses of restriction endonuclease fragment profiles using *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I and *Xho*I, and gene sequences (*polyhedrin*, *lef-8*, *lef-9*, *pif-1*, *pif-2* and *pif-3*) suggest that all the *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) isolates, HytaNPV-ITK1 (Terai isolate), HytaNPV-ID1 (Dooars isolate) of the present study, and HytaNPV-R (Dooars isolate; Nguyen et al., 2018) used as reference appear to be similar with few polymorphic sites.

The phylogenetic trees with 77 baculoviruses as well as 19 baculoviruses representing each group based on concatenated sequence alignment of the above-mentioned six genes showed that the delta, gamma and beta- baculoviruses have diverged before the divergence of alphabaculoviruses into group I and group II. HytaNPVs of the present study and the reference (HytaNPV-R) were phylogenetically placed under the group II alphabaculovirus. Both the isolates, HytaNPV-ITK1 and HytaNPV-ID1, appear to be very close to the Dooars isolate of HytaNPV-R reported by Nguyen et al. (2018). *Buzura suppressaria* nucleopolyhedrovirus (BuzuNPV) was found to be the closest relative of HytaNPVs. Moreover, the NPVs infecting the host species of family Geometridae such as BusuNPV, EcobNPV, ApciNPV and SujuNPV including HytaNPVs showed close relationships among themselves forming a single cluster.

Moreover, the topology of phylogenetic trees of our study based on concatenated sequence alignment of *polyhedrin*, *lef-8*, *lef-9*, *pif-1*, *pif-2* and *pif-3* are comparable with the phylogenetic trees of baculoviruses based on concatenated sequences of all baculovirus core genes reported by several researchers (Afonso et al., 2001; de Jong et al., 2005; Duffy et al., 2006; Ikeda et al., 2006; Nguyen et al., 2018; Xu et al., 2010; Zhu et al., 2014) suggesting that this set of core genes can also be considered for the phylogenetic studies of baculoviruses.

These findings suggested that till date a single variant of HytaNPV is infecting the tea pest, *Hyposidra talaca* in the Terai and the Dooars region of West Bengal, indicating that if the HytaNPV is used as a bioinsecticide, a single variant of that can be effective in the tea plantations of the entire Terai-Dooars region of West Bengal and also expectedly in Assam. Moreover, as the HytaNPV is phylogenetically distant from *Bombyx mori* nucleopolyhedrovirus

(BmNPV), hardly there will be any chances of cross-infectivity. An extensive investigation using more restriction endonucleases and sequencing on a wider area may reveal more distinguishable genetic features.

Section 3.3: Summary

Survey

- A field survey, before the application of pesticides in tea plantations, suggested that *H. talaca* caterpillars (loopers) were found in abundance from March – November except winter season.
- The activity of the caterpillars is very high during the daytime. After a rain, either they are found to hide below the leaves or they would drop down to the ground. Adult moths become active in dimmed light during the early morning.
- NPV-infected dead caterpillars were noticed hanging from the leaves with their prolegs in a head-down position exhibiting liquefaction of the body.

Sampling and isolation of NPV OBs

- NPV-infected cadavers of *Hyposidra talaca* larvae were collected separately from different tea plantations in the Terai region of Darjeeling foothills along with the Dooars region of West Bengal, India. NPV occlusion bodies (OBs) from the cadavers of *H. talaca* were collected and purified by differential centrifugation.

Restriction fragment analysis

- Viral DNA was isolated from the OBs followed by restriction endonuclease fragment using *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I and *Xho*I of both the Terai and Dooars isolates, HytaNPV-ITK1 and HytaNPV-ID1, respectively.
- The mean genome size of both the HytaNPV isolates of the present study, HytaNPV-ITK1 (Terai) and HytaNPV-ID1 (Dooars) were approximately estimated to be 138 kb which are comparable to the genome size reported by Nguyen et al. (2018) for the Dooars isolate, HytaNPV-R (139.089 kb).

Summary

- No significant differences were found among the *Bam*HI, *Bgl*I, *Eco*RI, *Pst*I and *Kpn*I restriction profiles of HytaNPV-ITK1 (Terai isolate), HytaNPV-ID1 (Dooars isolate) and the reference HytaNPV-R.
- In the case of *Hin*dIII and *Xho*I digestions, few polymorphisms in the restriction profile were detected among the isolates of HytaNPVs.
- Such similarities in restriction profiles with little variations are common among different geographic isolates of NPVs.

Sequencing of genes

- PCR amplification and sequencing of the target genes were carried out using gene-specific primers.
- Being most conserved, *polyhedrin* was sequenced along with the other five core baculoviral genes, *lef-8*, *lef-9*, *pif-1*, *pif-2* and *pif-3*. A total of 6.96 kb for the Terai (HytaNPV-ITK1) and 5.60 kb for the Dooars (HytaNPV-ID1) isolate of the HytaNPV genome (139.089 kb) were sequenced with some gaps.
- Both nucleotide and translated protein BLAST of all the six genes (complete or partial) of HytaNPV-ITK1 and HytaNPV-ID1 showed maximum similarity with HytaNPV-R (Nguyen et al., 2018).
- Among the NPVs infecting the specimens of other genera than *Hyposidra*, BusuNPV of *Biston (=Buzura) suppressaria*, an old Geometrid looper pest of tea, showed the maximum overall amino acid sequence identity of 90.9% and nucleotide sequence identity of 85.8% with both the isolates of the present study for all the six genes.
- In the *polyhedrin* gene, all seven (7) variable sites were synonymous suggesting conservative properties of the gene.
- The pairwise comparison revealed a higher ratio of non-synonymous to synonymous substitution between the HytaNPV isolates of the Terai and the Dooars region, than the isolates within the Dooars as evident from *lef-8, lef-9* and *pif-2*.

- The overall sequence comparison among the HytaNPV isolates of the Terai-Dooars region revealed a ratio of non-synonymous to synonymous substitution (nd/sd) <1 for *lef-8* and *pif-1* whereas, the ratio (nd/sd) for *lef-9* and *pif-2* was >1, indicating that *lef-9* and *pif-2* genes are under positive selection.
- A higher proportion of variable sites (d_N) for nucleotides (16 out of 1001) were detected in the *pif-2* suggesting that though *pif-2* is one of the most conserved genes, the genetic variations among the geographic isolates of NPV infecting the host of the same species may exist in different populations possibly indicating local environmental stress on a gene.

Phylogenetic analysis

- The phylogenetic trees revealed an early divergence of delta-, gamma- and beta-baculoviruses before the divergence of alphabaculoviruses into group I and group II which corroborates the other findings based on the concatenated sequences of all baculovirus core genes.
- A higher transition over transversion ratio (s/v) of approximately 2.00 with the closely related groups was found with a declining trend in widely diverged baculoviruses.
- Both the nucleotide and amino acid substitution-based phylogenetic trees showed that *Hyposidra talaca* NPVs were phylogenetically placed under the group II alphabaculovirus.
- Both the isolates, HytaNPV-ITK1 and HytaNPV-ID1, appeared to be very closer to the reference HytaNPV-R forming a single cluster.
- NPVs infecting the hosts of family Geometridae such as BusuNPV, EcobNPV, ApciNPV and SujuNPV have a close relationship with the HytaNPVs.
- Being present under the group I alphabaculovirus, *Bombyx mo*ri NPV (BmNPV) was found to be phylogenetically distant from HytaNPV.
- The topology of phylogenetic trees of the present study based on concatenated sequence alignment of *polyhedrin*, *lef-8*, *lef-9*, *pif-1*, *pif-2* and *pif-3* are comparable

with the phylogenetic trees of baculoviruses based on all baculovirus core genes reported by several researchers suggesting that this set of core genes can also be considered for the phylogenetic studies of baculoviruses.

Conclusion

- The present study revealed that though there were few genetic differences present in polymorphic conditions among the Terai and Dooars isolates of HytaNPV in West Bengal, a single variant of HytaNPV is infecting *Hyposidra talaca* in these regions.
- HytaNPV, group II alphabaculovirus, and BmNPV (NPV pathogenic to *Bombyx mori*, silk moth), a group I alphabaculovirus, were phylogenetically distant from each other.

Chapter 4

Appendix, Bibliography, Index

Section 4.1: Appendix

4.1.1 Publications

1. Bappaditya Ghosh, Ananda Mukhopadhyay, Ananya Das and Min Bahadur. 2015. Restriction Endonuclease Fragment Analysis of Hyposidra talaca Nucleopolyhedrovirus Genome. *International Journal of Current Research and Academic Review*, 3(8): 81-87. (ISSN: 2347-3215)

2. B. Ghosh, A. Mukhopadhyay and M. Bahadur. 2019. Comparison of Polyhedrin Partial Sequence of NPV Variants Pathogenic to Tea Pests (Geometridae) in India and their Phylogeny. *Journal of Environmental Biology*. 40: 9-16. (pISSN: 0254-8704, eISSN: 2394-0379) DOI: http://doi.org/10.22438/jeb/40/1/MRN-826

4.1.2 Papers presented at conferences

1. Bappaditya Ghosh, Ananda Mukhopadhyay, Min Bahadur (2015 Jan-Feb). Estimation of the Genome Size of Nucleopolyhedrovirus isolated from Hyposidra talaca Walker (Lepidoptera: Geometridae), a Major Tea Pest in North Bengal Region. Paper presented at "National Conference on Applied Zoology in Sustainable Development: An Update", 2nd February, Department of Zoology, University of North Bengal, Darjeeling, West Bengal, India.

2. Bappaditya Ghosh, Ananda Mukhopadhyay, Min Bahadur (2018 April). Phylogenetic relationship of Nucleopolyhedroviruses isolated from Geometrid caterpillars infesting tea plants in India. Presented at "National Conference on Recent Trends in Zoological Research in North East India", April 19th-20th, 2018, Jointly organized by Department of Zoology North-Eastern Hill University, Shillong-793022, Meghalaya, India and Zoological Society (Kolkata).

3. Bappaditya Ghosh, Ananda Mukhopadhyay, Min Bahadur (2019 April). Evolutionary divergence of nucleopolyhedrovirus isolates pathogenic to geometrid tea pests of Terai region of Darjeeling foothills, India. Presented at "Interdisciplinary National seminar 'Vistas in Life Science Research" 29th March, 2019, Jointly organized by Department of Biotechnology, Department of Microbiology, Department of Tea Science & Department of Bioinformatics, North Bengal University.

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Section 4.3: Index

International Journal of Current Research and Academic Review
ISSN: 2347-3215 Volume 3 Number 8 (August-2015) pp. 81-87
www.ijcrar.com

Restriction endonuclease fragment analysis of *Hyposidra talaca* nucleopolyhedrovirus genome

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KEYWORDS

Hyposidra talaca, Biopesticide, Nucleo polyhedrovirus, Restriction endonuclease, *Camellia sinensis*

A B S T R A C T

A nucleopolyhedrovirus isolated from *Hyposidra talaca*, a major defoliating pest of Tea in Northern region of West Bengal, India, is pathogenic to this pest. Restriction digestion of *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) genome with *Eco*RI, *Xho*I, *Kpn*I, *Bam*HI and *Pst*I produced 29, 21, 13, 10 and 9 fragments, respectively ranging from 0.62 to 62.00 Kb. *Pst*I produced the largest fragment of 62 kb. Based on the restriction analysis the mean size of the HytaNPV genome was estimated to be 174.76 kb. The present study will help to develop this virus as a potential biopesticide in the Integrated Pest Management strategy of Tea.

Introduction

Hyposidra talaca Walker (Lepidoptera: Geometridae), commonly known as 'Black Inch Looper' is a major leaf-eating lepidopteran pest of tea (*Camellia sinensis*) in Terai-Dooars region of Eastern Himalaya (Das *et al.*, 2010). A number of synthetic chemical pesticides, especially organophosphates and pyrethroids, which are regularly applied in the tea gardens for controlling the looper pests including *H. talaca*, proved to be hazardous to the environment and human health (Mobed et al., 1992). Studies have shown that gradually the pests have developed tolerance to these synthetic chemical pesticides and become less susceptible, often resulting in control failures (Gurusubramanian *et al.*, 2008; Roy *et al.*, 2010). To minimize the use of synthetic pesticides, alternative approaches of pest management in agriculture have been contemplated, among these microbial pesticides based management appears to be more eco-friendly and effective.

An effective biopesticide that has been successfully applied in the management of many crops, orchard and forest are prepared based on baculoviruses (nucleopolyhedrovirus and granulovirus). Baculoviridae is a large family of pathogens that infect insects, particularly the order: Lepidoptera (Blissard *et al.,* 2000). Baculoviruses have a large circular, super-coiled and double-stranded DNA genome ranging between 80-180 kb packaged into rod-shaped virions (King *et al.,* 2011). More than 700 baculoviruses have been identified from the insects of the orders Lepidoptera, Hymenoptera, and Diptera (Moscardi, 1999).

Baculoviruses as microbial insecticides are ideal tools in integrated pest management (IPM) programs as they are usually highly specific to their insect hosts, thus, they are safe to the environment, humans, other plants, and natural enemies (Yasuhisa, 2007). Based on 30 core baculovirus genes they are phylogenetically divided into four genera: Alphabaculovirus (lepidopteran-specific nucleopolyhedrovirus), Betabaculo-virus (lepidopteran-specific granulovirus), Gammabaculovirus (hymenopteran-specific nucleopolyhedro-virus), and Deltabaculo-virus (dipteran-specific nucleopolyhedro-virus) (King *et al.,* 2011).

Alphabaculoviruses can be further subdivided into group I and II nucleopolyhedroviruses (NPVs) (Herniou *et al.,* 2003). In nucleopolyhedroviruses (NPVs) nucleocapsids are occluded in large protein crystals forming Occlusion body (OB). NPV produces two types of viruses during their infection cycle: occlusion-derived viruses (ODVs), which transmit infections among insects (oral infection), whereas budded viruses (BVs) spread infection to neighbouring cells (Keddie *et al.,* 1989). At the late stage of infection, the infected larvae show enhanced dispersal behaviour (Goulson, 1997), followed by dramatic degradation of the host cadavers by liquefaction (Federici, 1997) and this pathogenicity is highly species-specific.

A strain of *Hyposidra talaca* NPV (HytaNPV) has been found pathogenic to the concerned pest in laboratory condition (Mukhopadhyay *et al.,* 2011). Therefore, HytaNPV can be developed as an alternative to the synthetic chemical pesticides to control the *H. talaca* in the tea gardens. The present study has been initiated to characterize the HytaNPV genome by restriction endonuclease analysis.

Materials and Methods

Stocks of HytaNPV OBs were built-up from cadavers of NPV infected *H. talaca* larvae following the method of Kawarabata and Matsumoto (1973) with some modifications. Cadavers of *H. talaca* showing typical symptoms of NPV infection were collected and stored in 1 ml of distilled water at room temperature for putrefaction to enable the release of OBs from the infected tissues. The putrefied suspension was homogenized, the homogenate was then filtered through double layers of cheese cloth and the filtrate was centrifuged at 1000x g for 20 min at 20°C. The supernatant was removed and the sedimented polyhedra were suspended in 25% (w/v) sucrose dissolved in distilled water and centrifuged at 1000x g for 20 min at 20°C. The pelleted polyhedra were re-suspended in 25% sucrose solution in a volume of 10ml and were layered on 30 ml of 50% sucrose solution and centrifuged at 1800xg for 40 min at 20°C. The last step was repeated twice and the polyhedra were washed several times with de- ionized distilled water and stored at -20°C for future use.

OBs of HytaNPV were suspended in Tris-EDTA (10mM Tris, 1mM EDTA, pH-8.0) and then were dissolved by adding dissolution buffer (0.1M Na_2CO_3, 0.01M EDTA, 0.17M NaCl, pH 10.8). Viral DNA was extracted by proteinase K (1 mg/ml) digestion in presence of 1% SDS followed by phenol-chloroform purification as described by O'Reilly *et al.* (1994). The DNA was then ethanol precipitated, dried, dissolved in Tris-EDTA (pH 8.0) and stored at -20°C. Restriction digestion of HytaNPV DNA by *Eco*RI, *Xho*I, *Kpn*I, *Bam*HI and *Pst*I were carried out following the method of Sambrook and Russell (2001). About 1.5–2 µg of HytaNPV DNA was set up for digestion with 10 units Restriction enzyme in presence of 1x restriction buffer and 1x acetylated-BSA at 37°C for overnight for complete digestion. The digested fragments were separated in 0.4-0.7% agarose gel at 70 V for 6 hours and gels were viewed and photographed in Gel Documentation System (SPECTROLINE BI-O-VISION, UV/WHITE Light Transilluminator) and analyzed using the software: ImageAide: Version 3.06.

Results and Discussion

Restriction digestion of DNA of HytaNPV with *Eco*RI, *Xho*I, *Kpn*I, *Bam*HI and *Pst*I produced 29, 21, 13, 10 and 9 fragments, respectively ranging between 0.62 kb to 62 kb. Restriction profiles of these digestions are shown in figure 1 and size of the restriction endonuclease fragments is summarized in table 1. The size of the each fragment was determined comparing restriction endonuclease fragment mobilities with those of *Hin*dIII digested λDNA as molecular weight standard. The fragments were designated alphabetically starting with 'A' according to the size from higher to lower as proposed by Vlak and Smith (1982). This number represents the minimum number of cleavage sites for each of these five enzymes, since fragments smaller than 0.62 kb were not detectable.

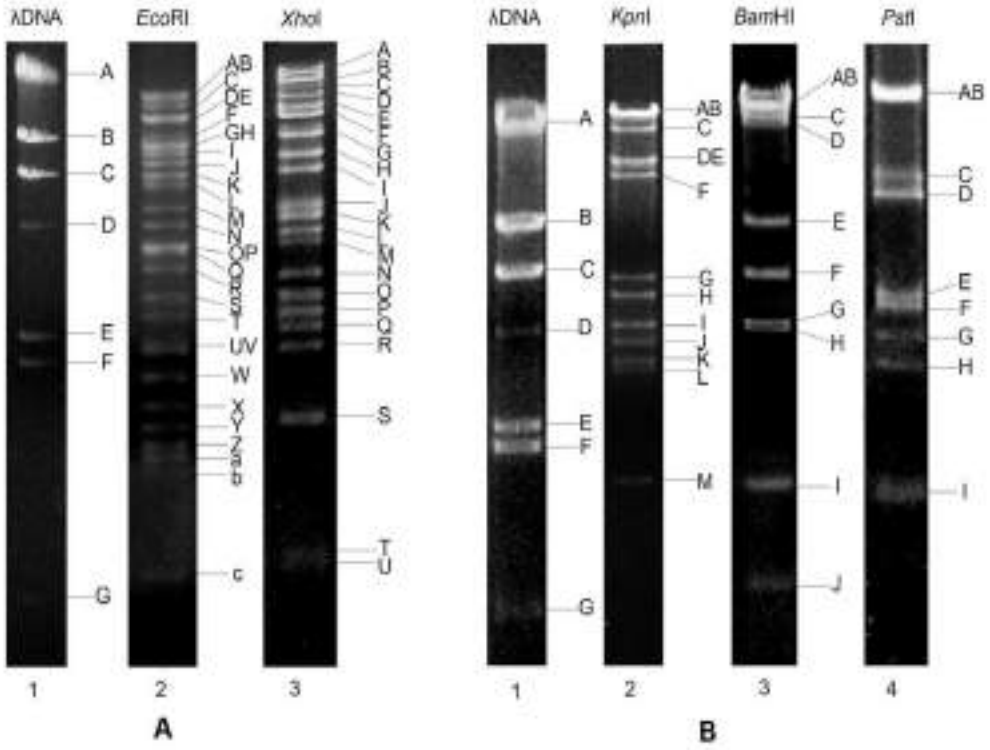
To resolve the high molecular weight fragments (particularly more than 23 kb), the digested DNA were separated in 0.4% agarose gel and to detect the fragments smaller than 23 kb, the digested fragments were separated in 0.7% agarose gel. *Eco*RI digestion produced 29 fragments ranging from 0.62-19.53 kb while 21 fragments could be resolved ranging from 0.67 kb to 25.56 kb with *Xho*I digestion (Figure 1A). Digestion with *Kpn*I produced 13 fragments ranging from 1.46 to 37.50 kb. Other restriction digestions with *Bam*HI and *Pst*I produced 10 and 9 bands, respectively ranging from 0.71 to 48 kb for *Bam*HI and from 1.68 kb to 62 kb kb for *Pst*I (Figure 1B). The results showed that HytaNPV DNA digested with *Kpn*I, *Bam*HI and *Pst*I have high molecular weight DNA above 23 kb which were resolved as doublet. The fragments AB in *Kpn*I, *Pst*I and *Bam*HI are more than 23 kb in size and appear to co-migrate (Figure 1B). The fragments AB in *Eco*RI and DE in *Kpn*I digestion were resolved by running the digests in 0.4% agarose gel for 6 hours (data not shown). Another doublet DE in *Eco*RI was confirmed by double digestion with *Eco*RI and *Pst*I (data not shown). The mean size of the HytaNPV genome was estimated to be 174.76 kb (Table 1).

Restriction endonuclease fragment analysis of different NPVs has been carried out to estimate the genome size (Hu *et al.*, 1998; Chen *et al.*, 2000; Lin *et al.*, 2012) and has been shown to be relatively stable and is often used as an effective tool to differentiate closely related NPVs (Woo *et al.*, 2006).

Table.1 Size of HytaNPV DNA fragments (in kb) after restriction digestion with respective enzyme (*Hind*III digested λ DNA as molecular weight marker has been shown also)

Fragments	λ DNA	*Eco*RI	*Xho*I	*Kpn*I	*Bam*HI	*Pst*I
A	23.130	19.53	25.56	37.50	48.00	62.00
B	9.416	18.13	22.39	37.00	47.50	61.00
C	6.557	15.62	20.31	21.57	27.50	14.74
D	4.361	12.87	18.01	17.68	22.85	13.57
E	2.322	12.72	15.38	16.87	10.48	6.08
F	2.027	9.28	13.89	15.17	6.92	5.66
G	0.564	8.53	10.42	6.75	4.97	4.17
H		8.30	8.36	5.89	4.68	3.49
I		7.89	7.18	4.66	1.40	1.68
J		7.11	5.21	4.17	0.71	
K		6.34	4.83	3.49		
L		5.89	4.36	3.24		
M		4.82	4.06	1.46		
N		4.25	3.33			
O		3.76	2.85			
P		3.60	2.64			
Q		3.57	2.42			
R		3.35	2.20			
S		2.89	1.44			
T		2.60	0.68			
U		2.18	0.67			
V		2.10				
W		1.90				
X		1.63				
Y		1.47				
Z		1.36				
a		1.27				
b		1.17				
c		0.62				
Total		174.75	176.19	175.45	175.01	172.39
Mean				174.76		

Figure.1 Restriction fragment profiles of HytaNPV DNA. (A) *Eco*RI (lane 2) and *Xho*I (lane 3) in 0.7% agarose gel (B) *Kpn*I (lane 2), *Bam*HI (lane 3) and *Pst*I (lane 4) in 0.4% agarose gel. *Hin*dIII digests of λDNA was used as molecular weight marker (lane 1). Restriction fragments of individual restriction profiles are designated alphabetically starting with 'A' for the largest fragment



Though the isolation and bioassay study related to the lethal concentrations and lethal time of HytaNPV from Terai-Dooars tea plantations were carried out by Mukhopadhyay *et al.* (2011), no information on the total size of the genome or restriction digestion analysis is available till date. So the present study is the first report on the restriction profiling of HytaNPV. Genome size of baculoviruses ranges from 80 to 180 kb (King *et al.*, 2011). The size of HytaNPV genome estimated in this study falls within the above range. The Restriction endonuclease fragment profile along with restriction mapping and estimation of genome size of BusuNPV isolated from another looper pest of tea *Biston(=Buzura) suppressaria*, has been reported in China with a mean genome size of 129 kb (Liu *et al.*, 1993), however Hu *et al.* (1998) documented a genome size of 120.9 kb. Recently the whole genome organization and sequence of BusuNPV was reported by Zhu *et al.* (2014) with a genome size of 120.42 kb. BusuNPV is reported as a close relative of HytaNPV (Antony *et al.*, 2011) but the estimated genome size of HytaNPV in the present study is higher by about 55 kb than BusuNPV. It appears that such genome size variation is not unusual between closest relatives of NPV. Chen *et al.* (2000) reported the genome size of *Helicoverpa armigera* NPV (HearNPV) to be 130.1 kb but the NPV isolated from *Helicoverpa assulta*, a closest relative of HearNPV was shown to have a genome size of 138 kb (Woo *et al.*, 2006) which is 8 kb higher than that of the former. The present

study will provide some idea about the genome of HytaNPV broadly revealing the size and the cleavage sites of the five restriction endonucleases. Further studies are contemplated to determine the exact size, restriction map and constitution of the HytaNPV genome by restriction digestions using different endonucleases and subsequent sequencing.

Acknowledgement

Authors are thankful to the Head, Department of Zoology for providing the Departmental Central laboratory facility which is supported by the Fund for Improvement of Science and Technology Infrastructure programme, Department of Science and Technology, New Delhi, India and the Special Assistance Programme of the University Grants Commission, New Delhi. The fellowship to Mr. Bappaditya Ghosh under the Basic Scientific Research support of University Grants Commission, Bahadur Shah Zafar Marg, New Delhi, India (Ref. No. 4-1/2006(BSR)/7-134/2007 (BSR) dated 25.02.2013) is sincerely acknowledged.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Journal of Environmental Biology

JEB™ p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/40/1/MRN-826

Comparison of polyhedrin partial sequence of NPV variants pathogenic to tea pests (Geometridae) in India and their phylogeny

Paper received: 04.01.2018 *Revised received*: 28.04.2018 *Re-revised received*: 21.05.2018 *Accepted*: 09.06.2018

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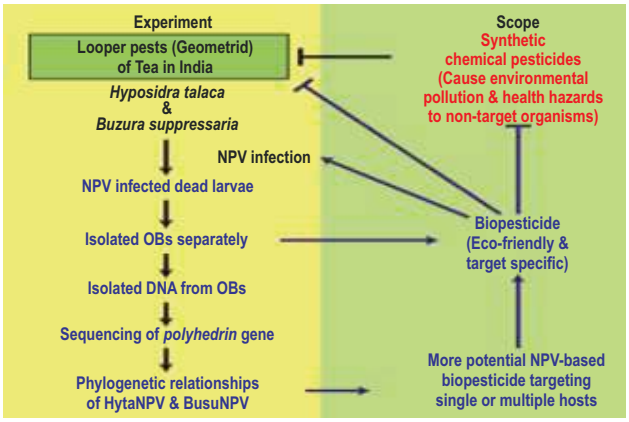
Abstract

Aim : The present study was undertaken to understand the phylogenetic relationships of NPVs (Nucleopolyhedroviruses) isolated from two pest species, *Hyposidra talaca* and *Biston* (=*Buzura*) *suppressaria.* The phylogenetic analyses based on the polyhedrin gene were assessed.

Methodology : Occlusion bodies (OBs) were isolated separately from NPV infected dead *Hyposidra talaca* and *Buzura suppressaria* larvae, and DNA was isolated from OBs. The *polyhedrin* gene was amplified and sequenced followed by sequence divergence and phylogenetic analyses using MEGA5.

Results : The phylogenetic analyses based on the *polyhedrin* gene revealed that the NPV isolated from *Hyposidra talaca* (HytaNPV-ITK1) formed a single cluster with the isolates of NPVs infecting *Hyposidra* specimens in India sharing 99% nucleotide identity, whereas the NPV isolated from *Buzura suppressaria* (BusuNPV-ITK1) showing 99% nucleotide homology with the NPV isolate of *B. suppressaria* reported from China formed a different cluster. A nucleotide identity of 85% was found between HytaNPV-ITK1 and BusuNPV-ITK1.



Interpretation : Phylogenetic analyses, based on the polyhedrin sequence of 47 baculoviruses, revealed that these two variants of NPVs (HytaNPV-ITK1 and BusuNPV-ITK1) infecting *Hyposidra talaca* and *Buzura suppressaria* were comparatively closer to each other than those infecting specimens of other lepidopteran genera.

Key words: *Buzura suppressaria, Hyposidra talaca,* Nucleopolyhedrovirus, Phylogeny, Tea pest

How to cite: Ghosh, B., A. Mukhopadhyay and M. Bahadur: Comparison of polyhedrin partial sequence of NPV variants pathogenic to tea pests (Geometridae) in India and their phylogeny. *J. Environ. Biol.*, **40,** 9-16 (2019).

Introduction

Tea, *Camellia sinensis* (L.) O.Kuntze, is an intensively managed monoculture plantation crop cultivated on large and small-scale. It is the chief foliar crop in the Terai-Dooars region of Darjeeling foothills and Assam of North-Eastern India. One of the conditional problems of loss in tea production is damage caused by insect pests. Recent studies in the tea plantations of this area, revealed an increased activity of *Hyposidra talaca* (Lepidoptera: Geometridae), a common defoliating insect pest of tea plantations (Das *et al.,* 2010). Regular application of synthetic chemical pesticides, especially organophosphates and pyrethroids to curb the pest population including *H. talaca,* is a major cause of environmental pollution and has also proved to be hazardous to human (Azmi *et al.,* 2009) and other non-target organisms (Saravanan *et al.,* 2009; Velmurugan *et al.,* 2006).

During field survey, an NPV isolated from *Hyposidra talaca* has been found pathogenic to this defoliating tea pest (Mukhopadhyay *et al.,* 2011). Several bioassay studies (Mukhopadhyay *et al.,* 2011; Sinu *et al.,* 2011, Dasgupta *et al.,* 2016) with *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) suggest that this can be developed into a potential biopesticide to control the tea pest, *H. talaca.* Being specific to its host, baculoviruses in general are very safe for industrial production and field application (Sumathy *et al.,* 1996). The virus, HytaNPV collected in this study is an Alphabaculovirus, one of the four genera of family Baculoviridae (King *et al.,* 2011) and it is lepidopteran-specific nucleopolyhedrovirus (NPV). Understanding the phylogenetic interrelationship of the viruses and their host may help to determine the nature of coadaptation and coevolution in long term association.

As the interaction of virus with their host occurs at molecular level, phylogenetic study on the basis of molecular data is highly significant (Zanotto *et al.,* 1993). Comparative analysis of more than 50 baculovirus genomes have revealed a set of 31 conserved genes of which *polyhedrin* is the most conserved (Rohrmann, 2013). Orthologs of *polyhedrin* are found in all baculoviral genomes either in the form of *polyhedrin* in nucleopolyhedroviruses (NPVs) or granulin in granuloviruses (GVs), except in the dipteran-specific NPVs, where it has an occlusion body protein unrelated in the primary amino acid sequence of the *polyhedrin* gene of other NPVs and is about three times as large (Perera, 2006; Afonso, 2001). *Polyhedrin,* that encodes a major structural protein of OBs, is expressed at a very high level at the late phase of infection to produce large numbers of OBs (Rohrmann, 2013). This gene has been used as an effective marker to study the phylogenetic relationships among different NPVs (Zanotto *et al.,* 1993; Woo *et al.,* 2006).

Based on the partial sequence of *polyhedrin* gene, Antony *et al.* (2011) reported a close relation between *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) and *Biston* (=*Buzura*) *suppressaria* nucleopolyhedrovirus (BusuNPV), mainly found in China (Hu *et al.,* 1993), this NPV was subsequently reported from Terai-Dooars and North-East region of India (Mukhopadhyay *et al.,* 2007; Antony *et al.,* 2011). Studies to determine the geographic variability among baculoviruses of same or different variants or isolates and to find out the significance of genetic variations in the biology of baculoviruses are the important areas of current research. Such studies may provide insight into the evolution of baculoviruses and their hosts, which may help in the development of more effective virus strains for the eco-friendly microbial control of insect pests. The present study contemplated to investigate and revisit the phylogenetic relationships of HytaNPV and BusuNPV isolated from Darjeeling Terai region of India based on *polyhedrin* gene, while taking into consideration other Indian isolates of NPVs reported from tea plantations as well as some other exotic baculoviruses.

Materials and Methods

Isolation and purification of OBs from NPV infected cadavers: Moribund cadavers of *Hyposidra talaca* and *Biston (=Buzura) suppressaria* larvae showing typical symptoms of NPV infection were collected separately for study from the tea plantations of Terai regions of Darjeeling foothills, West Bengal, India.

Stocks of OBs were built-up separately from the cadavers of *H. talaca* and *B. suppressaria* following the method of Kawarabata and Matsumoto (1973) with some modifications. The cadavers were stored in 1 ml of distilled water at room temperature for putrefaction to enable the release of OBs from the infected tissues. The putrefied suspension was homogenized and the homogenate was filtered through double layers of cheese cloth followed by centrifugation of the filtrate at 1000xg for 20 min at 20°C. The supernatant was removed and the sedimented polyhedra were suspended in 25% (w/v) sucrose dissolved in distilled water and centrifuged at 1000xg for 20 min at 20°C. Subsequently, the sedimented polyhedra were resuspended in 10 ml of 25% sucrose solution and were layered on 30 ml of 50% sucrose solution, centrifuged at 1800xg for 40 min at 20°C. The last step was repeated twice and the polyhedra were washed several times with de-ionized distilled water. Finally, the OBs were suspended in Tris-EDTA (10mM Tris, 1mM EDTA, pH 8.0) and stored at -20°C for future use. Isolated polyhedra (OBs) from the cadavers of infected larvae were examined under light microscope at 1000x resolution.

Viral DNA isolation, PCR amplification and sequencing of *polyhedrin* : The OBs were dissolved by adding dissolution buffer (0.1M Na_2CO_3, 0.01M EDTA, 0.17M NaCl, pH 10.8) and the viral DNA was extracted by proteinase K (1 mg ml^{-1}) digestion in the presence of 1% SDS, followed by phenol-chloroform purification as described by O'Reilly *et al.* (1994). The PCR amplification of *polyhedrin* gene was carried out with 50 ng of HytaNPV and BusuNPV DNA in separate reactions in the presence of 1x GoTaq Flexi Buffer, 2 mM $MgCl_2$, 250 µM dNTPs mixture, 0.5 µM each of forward and reverse primers (Forward: 5'-GGA CCS GGY AAR AAY CAA AAA-3'; Reverse: 5'-GCR TCW GGY GCA AAY TCY TT-3') (Antony *et al.,* 2011) and 2 units of

GoTaq Flexi DNA Polymerase (Promega) in a PCR programme of 94ºC for 5 min followed by 35 cycles of 94ºC for 50 sec, 51ºC for 40 sec and 72ºC for 50 sec; then final extension of 7 min at 72ºC. The amplified products of ≈ 527 bp were purified with the Sure Extract Spin PCR Clean up/ Gel extraction kit (Geneticx Brand) and were sequenced from both directions using Applied Biosystems 3730xl/ABI3730XL-15104-028 capillary sequencer.

Sequence alignment, BLAST search, Phylogenetic analysis and Sequence Divergence : The partial sequences were aligned and joined using MEGA5 (Tamura *et al.,* 2011) taking BusuNPV (Zhu *et al.,* 2014) as reference and were BLAST searched using BLASTN 2.5.0+ and BLASTX 2.5.0+ (Altschul *et al.,* 1997). Multiple sequence alignment was prepared in Gene Doc version 2.7.000 (Nicholas *et al.,* 1997). Partial sequence of *polyhedrin* gene of HytaNPV (527 bp) and BusuNPV (504 bp) were submitted in the GenBank database with the accession number KX665534 and KX665535, respectively.

The phylogenetic trees were constructed by MEGA5 using maximum likelihood method (Tamura *et al.,* 2011) based on JTT matrix-based model (Jones *et al.,* 1992). Trees were tested by bootstrap method with 1000 replicates. Maximum Composite Likelihood model and JTT matrix based model were used to estimate the nucleotide sequence divergence (NSD) and amino acid sequence divergence (ASD), respectively, by using MEGA5. The gaps and missing data were not considered. To avoid the nomenclature disarray and disparity of the NPV isolates, pathogenic to the specimens of genus *Hyposidra* and *Buzura,* these NPV isolates were designated and is shown in Table 1.

Results and Discussion

Being the most conserved gene, the *polyhedrin* gene sequence has been widely used to detect phylogenetic relationships among the baculoviruses (Zanotto *et al.,* 1993; Rohrmann, 2013). In the present study, the partial sequences of *polyhedrin* gene of HytaNPV-ITK1 and BusuNPV-ITK1 were found to cover 71.12% and 68.02% of the total reading frame of *polyhedrin* gene of BusuNPV-C, respectively (Fig. 1).

In NCBI blastn search, 527 bp partial sequence of *polyhedrin* gene of HytaNPV-ITK1 showed 99% identity with HytaNPV-IW1, HytaNPV-IW2, HyinNPV and BusuNPV-IA from India, while a similarity of 86% and 84% were found with *Ectropis obliqua* NPV (EcobNPV) and BusuNPV-C from China, respectively. Blastx results revealed that HytaNPV-ITK1 *polyhedrin* gene had 100% homology with HytaNPV-IW1 (Protein ID: AEK86285.1), HytaNPV-IW2 (AJN00735.1) and HyinNPV, 97% and 98% with EcobNPV (YP_874194.1) and BusuNPV-C (YP_009001778.1) with a difference of six and three amino acids, respectively. Higher sequence homology of *polyhedrin* (99% at nucleotide and 100% at amino acid level) among different isolates of NPV infecting the specimens of same genus from close geographic regions is corroborated with the findings of Liang *et al.* (2013) among the isolates of *Bombyx mori* NPV from Guangxi Zhuang Autonomous Region of China. In Blastn search, the 504 bp partial sequence of *polyhedrin* gene of BusuNPV-ITK1 showed 99% identity with BusuNPV-C, 85% with HytaNPV-IW1, HytaNPV-ITK1 and BusuNPV-IA, 84% with HyinNPV and HytaNPV-IW2 and 83% with that of EcobNPV. Moreover, Blastx search showed 100% similarity with BusuNPV-C (Protein ID: YP_009001778.1) having no amino acid difference, 98% with HytaNPV-IW1 (AEK86285.1), HytaNNPV-IW2 (AJN00735.1) and HytaNPV-ITK1 with a difference of three amino acids and 97% with EcobNPV (YP_874194.1) with a difference of five amino acids. Ashika *et al.* (2017) reported a very high *polyhedrin* sequence homology of *Helicoverpa armigera* NPV from India with that from Spain, Kenya and China, which strongly supports the results of this study between the isolates of NPV infecting *Buzura suppressaria* in India and China.

Our results showing a nucleotide similarity of 85% and amino acid similarity of 98% between HytaNPV-ITK1 and BusuNPV-ITK1 were slightly different from the results reported by Antony *et al.* (2011). A sequence identity of 98% and 100% was found between the NPVs isolated from *H. talaca* and *B. suppressaria* (HytaNPV-IW1 and BusuNPV-IA, respectively) from the Dooars region of India and were suggested as same variant of NPVs. Present results using Terai specimens corroborate the above with a slight difference (2%) at amino acid level between these two isolates of NPV, however, a nucleotide homology of 85% in the present study compared to 98% reported by Antony *et al.* (2011) reflect that the two isolates, HytaNPV-ITK1 and BusuNPV-ITK1, are likely different variant of NPVs.

Clustal W alignment of HytaNPV-ITK1 and BusuNPV-ITK1 *polyhedrin* sequence in the present study, using BusuNPV-C as template (Fig. 1), revealed a total of 441 conserved and 86 variable sites for nucleotide and 172 conserved and 3 variable sites for amino acid, respectively. This suggested that most of the nucleotide substitutions were synonymous, except five which were found to be non-synonymously fixed in HytaNPV-ITK1 only producing three amino acid changes at positions 259, 261 (87), 436(146) and 593-594 (198) (position of amino acid substitutions are mentioned in the parenthesis) and were similar to the substitutions as shown by Antony *et al.* (2011) and Dasgupta *et al.* (2016) in the NPVs isolated from the specimens of genus *Hyposidra* (HytaNPV-IW1, HytaNPV-IW2 and HyinNPV) in India.

Sequence divergence analysis based on *polyhedrin* gene among the NPV isolates pathogenic to the genus *Hyposidra* and *Buzura* was carried out using *Ectropis obliqua* as a close group, and *Neodiprion sertifer* (Hymenopteran-specific NPV) as an outgroup. The results of the nucleotide and amino acid sequence divergence are shown in Table 2 and 3, respectively, and are comparable. HytaNPV-ITK1 (present study), HytaNPV-IW1, HytaNPV-IW2, HyinNPV and BusuNPV-IA, all from India, showed a nucleotide sequence divergence (NSD) ranging from 0.000-0.008 and amino acid sequence divergence (ASD) of 0.000 reflecting closeness among them, while these five isolates showed a higher NSD ranging from 0.187-0.193 and an ASD of

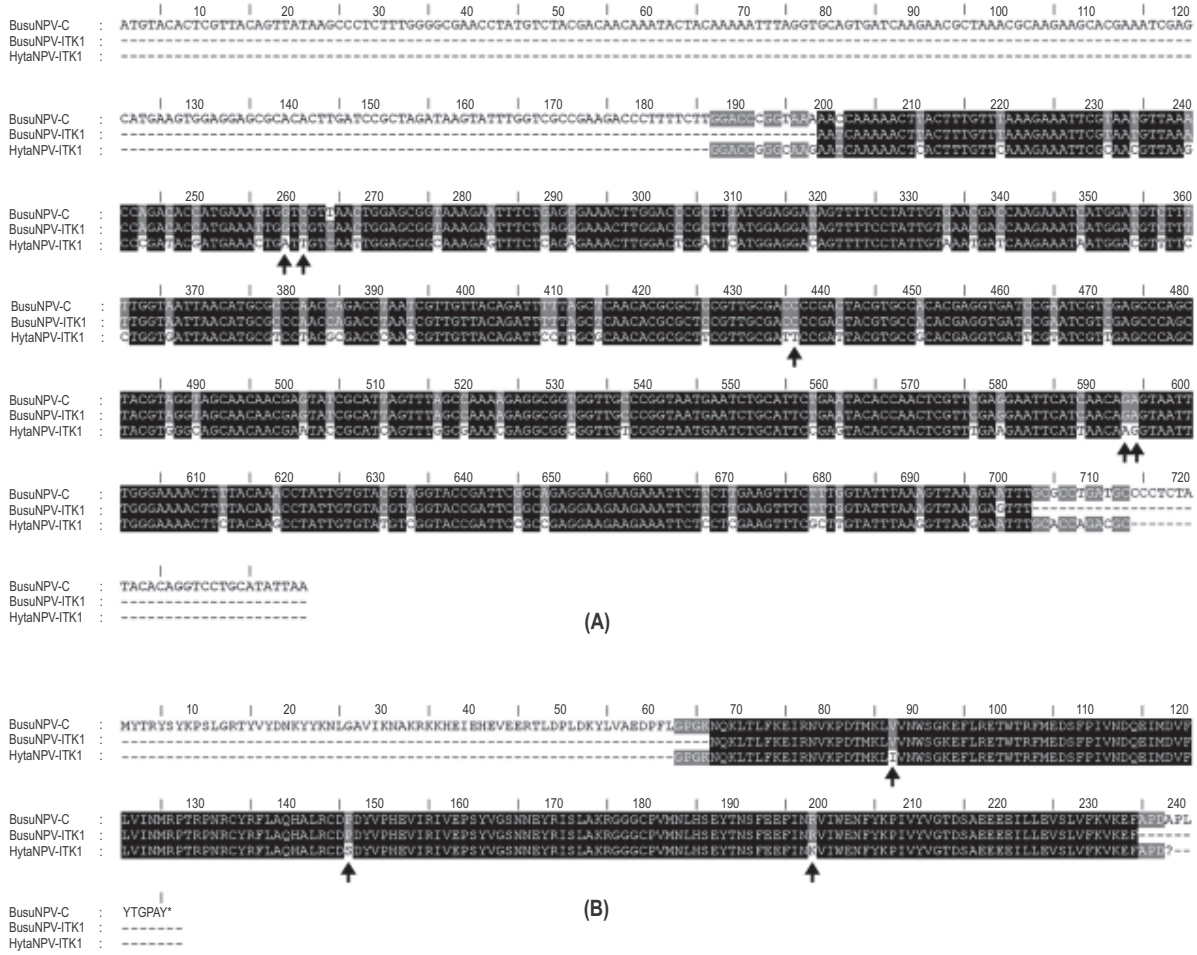


Fig. 1 : Nucleotide (A) and translated amino acid (B) sequence alignment of *polyhedrin* gene of BusuNPV-C, BusuNPV-ITK1 and HytaNPV-ITK1. Identical sites for any two sequences and conserved sites were shaded in grey and black, respectively. Arrow denotes the non-synonymous substitutions producing unique amino acid substitutions in HytaNPV-ITK1.

0.018 with BusuNPV-ITK1 (India) of the present study and BusuNPV-C of China. The results also revealed that BusuNPV-ITK1 and BusuNPV-C were more close to each other with an NSD of 0.008 and ASD of 0.000. The phylogenetic tree based on the maximum likelihood method using *polyhedrin* gene of 47 NPV isolates (Fig. 2) revealed that both the isolates of present study, HytaNPV-ITK1 and BusuNPV-ITK1, were placed in the same cluster wherein HytaNPV-ITK1 (India) shared the branch with other Indian isolates of NPVs infecting the specimens of genus *Hyposidra* (HyinNPV, HytaNPV-IW1 and HytaNPV-IW2) and also with *Buzura* (BusuNPV-IA) with a bootstrap value of 94, while BusuNPV-ITK1 from India shared the same branch with BusuNPV-C (China) with a bootstrap value of 86.

To resolve the phylogenetic relationships between and among the NPV isolates infecting tea pests of family Geometridae in India and China, a radiation tree based on maximum likelihood method was constructed using NPV specific to *Neodiprion sertifer* as outgroup (Fig. 3). This study revealed that the HytaNPV-ITK1 was embedded in the same branch with a bootstrap value of 96 (Cluster 2, Fig. 3) including all the documented Indian isolates infecting the specimens of genus *Hyposidra* and *Buzura,* except BusuNPV-ITK1, which on the other hand formed a separate branch with the Chinese isolate, BusuNPV-C having bootstrap value of 90 (Cluster 1, Fig. 3). Such close relationship between BusuNPV-ITK1 and BusuNPV-C isolates infecting the specimens of same genus from different geographic locations is in concurrence with the findings of Laviña-Caoili *et al.* (2001) who reported geographic variants of *Spodoptera litura* NPV from China and Philipines. EcobNPV, another Chinese isolate, showed higher sequence divergence with NPVs infecting the specimens of genus *Hyposidra* and *Buzura* with NSD, 0.167-

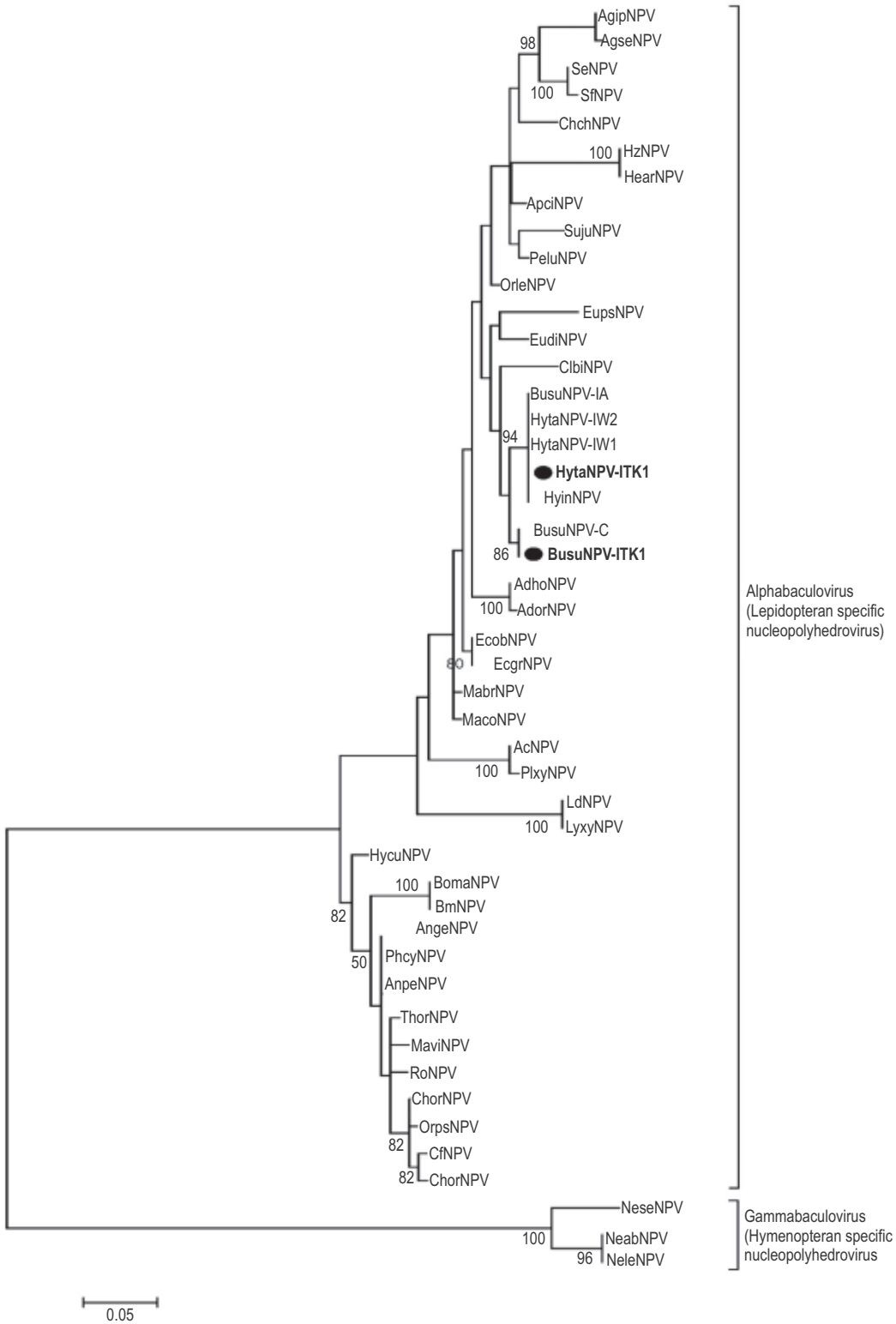


Fig. 2 : Maximum Likelihood tree based on *polyhedrin* gene for 47 baculoviruses. Bootstrap values more than 50% were shown at each node.

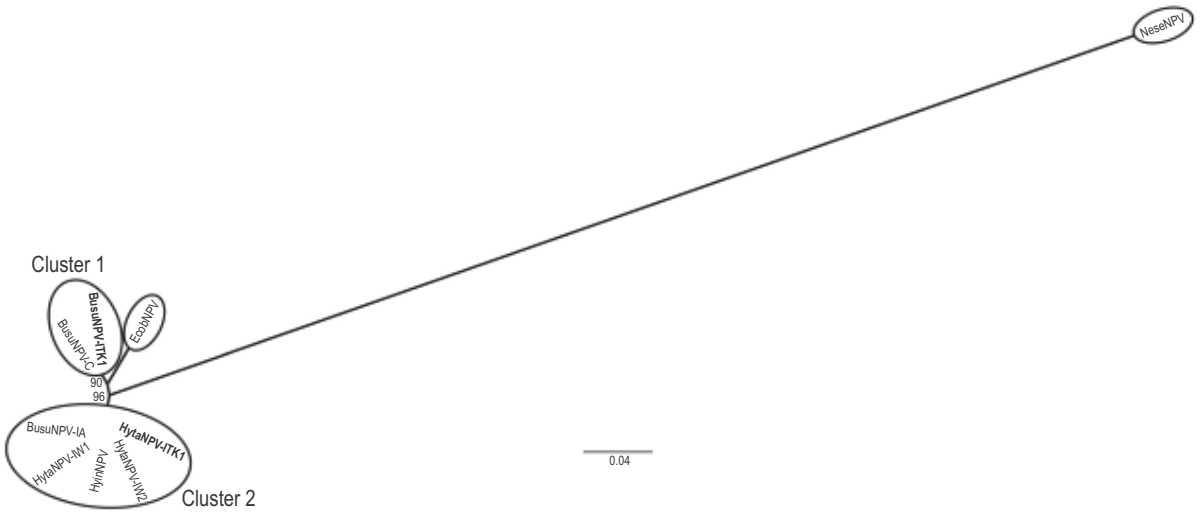


Fig. 3 : Maximum Likelihood tree based on *polyhedrin* gene for nine baculoviruses. Bootstrap values more than 50% were shown at each node.

0.228 and ASD, 0.031-0.037 (Table 2, 3), forming a separate branch (Fig. 3). From the sequence divergence (Table 2, 3) and phylogenetic analyses (Fig. 2, 3), it was evident that all the Indian isolates, infecting the genus *Hyposidra* and *Buzura*, except BusuNPV-ITK1 of the present study appeared to be similar showing the nucleotide and amino acid identity of 99% (NSD=0.006) and 100% (ASD=0.000), whereas BusuNPV-ITK1 was found to be similar to the Chinese isolate of BusuNPV-C with the nucleotide and amino acid identity of 99% (NSD=0.004) and 100% (ASD=0.000), respectively.

This phenomenon of phylogenetic closeness among the NPV isolates infecting the specimens of same genus is also supported by the phylogenetic studies carried out by Woo *et al.* (2006) on *Helicoverpa assulta* NPV based on *polyhedrin* gene. The studies showing close relatedness between *Spodoptera*

Table 1 : List of isolates of NPVs infecting the specimens of genus *Hyposidra* and *Buzura* used in this study for analysis

NPV	Isolate	GenBank Acc Number	Designation	Reference
Buzura suppressaria NPV	Hubei, China	KF611977.1	BusuNPV-C	Zhu *et al.,* 2014
Buzura suppressaria NPV	Assam, India	JF510034.1	BusuNPV-IA	Antony *et al.,* 2011
***Buzura suppressaria* NPV**	**Terai, West Bengal, India**	**KX665535.2**	**BusuNPV-ITK1**	**Present study**
Hyposidra talaca NPV	West Bengal, India	JF510035.1	HytaNPV-IW1	Sinu *et al.,* 2011
Hyposidra talaca NPV	Terai, India	KP027542.1	HytaNPV-IW2	Dasgupta *et al.,* 2016
***Hyposidra talaca* NPV**	**Terai, West Bengal, India**	**KX665534.1**	**HytaNPV-ITK1**	**Present study**
Hyposidra infixaria NPV	Assam, India	JF510036.1	HyinNPV	Antony *et al.,* 2011

Table 2 : Estimation of evolutionary divergence based on nucleotide sequence of *polyhedrin* gene in pairwise comparisons between different isolates of NPVs

	1	2	3	4	5	6	7	8
HytaNPV-ITK1								
HytaNPV-IW1	**0.008**							
HytaNPV-IW2	**0.000**	**0.008**						
HyinNPV	**0.006**	**0.002**	**0.006**					
BusuNPV-IA	**0.006**	**0.006**	**0.006**	**0.004**				
BusuNPV-ITK1	**0.190**	**0.190**	**0.190**	**0.193**	**0.187**			
BusuNPV-C	**0.190**	**0.190**	**0.190**	**0.193**	**0.193**	**0.008**		
EcobNPV	**0.167**	**0.178**	**0.167**	**0.175**	**0.175**	**0.236**	**0.228**	
NeseNPV	**7.720**	**7.718**	**7.720**	**7.719**	**7.719**	**7.714**	**7.616**	**7.438**

Table 3 : Estimation of evolutionary divergence based on amino acid sequence of *polyhedrin* gene in pairwise comparisons between different isolates of NPVs

	1	2	3	4	5	6	7	8
HytaNPV-ITK1								
HytaNPV-IW1	**0.000**							
HytaNPV-IW2	**0.000**	**0.000**						
HyinNPV	**0.000**	**0.000**	**0.000**					
BusuNPV-IA	**0.000**	**0.000**	**0.000**	**0.000**				
BusuNPV-ITK1	**0.018**	**0.018**	**0.018**	**0.018**	**0.018**			
BusuNPV-C	**0.018**	**0.018**	**0.018**	**0.018**	**0.018**	**0.000**		
EcobNPV	**0.037**	**0.037**	**0.037**	**0.037**	**0.037**	**0.031**	**0.031**	
NeseNPV	**0.636**	**0.636**	**0.636**	**0.636**	**0.636**	**0.630**	**0.630**	**0.637**

frugiperda NPV and *Spodoptera exigua* NPV (Harrison *et al.,* 2008) and between *Lymantria xylina* NPV and *Lymantria dispar* NPV (Nai *et al.,* 2010) also corroborate our results. Complete genome sequence of NPVs isolated from *B. suppressaria* (BusuNPV-C, Table 1) (Zhu *et al.,* 2014) and *E. obliqua* (EcobNPV) (Ma *et al.,* 2007) from China revealed that these were two different variant of NPVs. The results of this study showed a high nucleotide and amino acid sequence divergence (NSD=0.225 and ASD=0.031) between the aforesaid strains with respect to *polyhedrin* gene which also indicate that they were different NPVs. On the other hand, HytaNPV-ITK1 and BusuNPV-ITK1 from India with an NSD=0.187 and ASD=0.018 appeared to be different variants of NPV. The phylogenetic analysis showing HytaNPV-ITK1 arrayed in Cluster 2 and BusuNPV-ITK1 in Cluster 1 with bootstrap values of 96 and 90 (Fig. 3), respectively, also corroborates the above view. Moreover, RFLP analyses with different restriction endonucleases of the genome of BusuNPV from China (Hu *et al.,* 1998) and HytaNPV from Terai, India (Ghosh *et al.,* 2015) showed that the restriction profiles of HytaNPV differ from that of the BusuNPV. However, cross infectivity cannot be over ruled as suggested by Antony *et al.* (2011), that a single variant of NPV can infect specimen of both the genus *Hyposidra* and *Buzura.* Further, phylogenetic analysis based on *polyhedrin* sequence of 47 baculoviruses revealed that these two variants (NPVs infecting *H. talaca* and *B. suppressaria*) were comparatively closer to each other than those infecting other genera which is also supported by the phylogenetic relationships shown by Antony *et al.* (2011), Sinu *et al.* (2011) and Dasgupta *et al.* (2016).

From the phylogenetic analyses, based on *polyhedrin* sequences, it can be concluded that HytaNPV-ITK1 and BusuNPV-ITK1 isolated from *Hyposidra talaca* and *Buzura suppressaria,* were two different variants which are comparatively closer to each other than those infecting specimens of other lepidopteran genera.

Acknowledgments

The financial support as fellowship to Mr. Bappaditya Ghosh under UGC-BSR scheme, Bahadur Shah Zafar Marg, New Delhi, India (Ref. No. 4-1/2006(BSR)/7-134/2007 (BSR) dated 25.02.2013) is sincerely acknowledged. Authors are thankful to the Head, Department of Zoology, University of North Bengal for providing Departmental Instrumentation Facilities under UGC-SAP and DST-FIST Programmes, New Delhi, India.

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