

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.

Section 4.2: Bibliography

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Section 4.3: Index

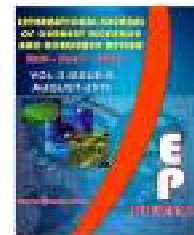
- Alphabaculovirus, 14, 15, 25, 130, 148,
 Baculoviridae, 13, 14
 Baculovirus core genes, 28
 Betabaculovirus, 7, 14, 15, 130
 BioEdit, 48
 bioinsecticide, 13, 19, 20, 21
Biston (Buzura) suppressaria, 11
 Black Inch Looper, 11
 BmNPV, 145
Bombyx mori, 145
Bt toxin, 13
 Budded virus, 17
Camellia sinensis, 9
 CIF, 9
 degenerate primers, 46
 Deltabaculovirus, 7, 14, 15, 26
 Dooars, 9
 Gammabaculovirus, 7, 14, 15, 26, 130
 Genetically modified NPVs, 20
 GM crop, 13
 GM plants, 13
gp67/gp64, 15
Hyposidra talaca, 11
Hyposidra talaca nucleopolyhedrovirus, 21
 integrated pest management, 13
lef-8, 28, 33
lef-9, 28, 34
 lethal concentration, 36, 140
 Matrix Protein, 1, 30
 Mulberry silk, 148, 150
 Nucleopolyhedrovirus, 15
 Occlusion body, 16, 17
 Occlusion-derived virus, 16
 organophosphates, 12
 phylogenetic tree, 125
pif, 30
 Plant Protection Codes, 12
 Polyhedrin, 29
 positive selection, 147
 pyrethroids, 12
 Restriction endonuclease (REN) fragment
 analyses, 23
 RFLP, 24, 36, 140, 145
 synthetic chemical insecticides, 12
 Teesta river, 9
 Terai, 9
 transition, 49, 118, 130, 133, 150
 transversion, 49, 118, 130, 133, 150



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Restriction endonuclease fragment analysis of *Hyposidra talaca* nucleopolyhedrovirus genome

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KEYWORDS

Hyposidra talaca,
Biopesticide,
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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 *EcoRI*, *XhoI*, *KpnI*, *BamHI* and *PstI* produced 29, 21, 13, 10 and 9 fragments, respectively ranging from 0.62 to 62.00 Kb. *PstI* 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), Betabaculovirus (lepidopteran-specific granulovirus), Gammabaculovirus (hymenopteran-specific nucleopolyhedro-virus), and Deltabaculovirus (dipteran-specific nucleopolyhedrovirus) (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 Na₂CO₃, 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 *EcoRI*, *XhoI*, *KpnI*, *BamHI* and *PstI* 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 *EcoRI*, *XhoI*, *KpnI*, *BamHI* and *PstI* 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 *HindIII* 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.

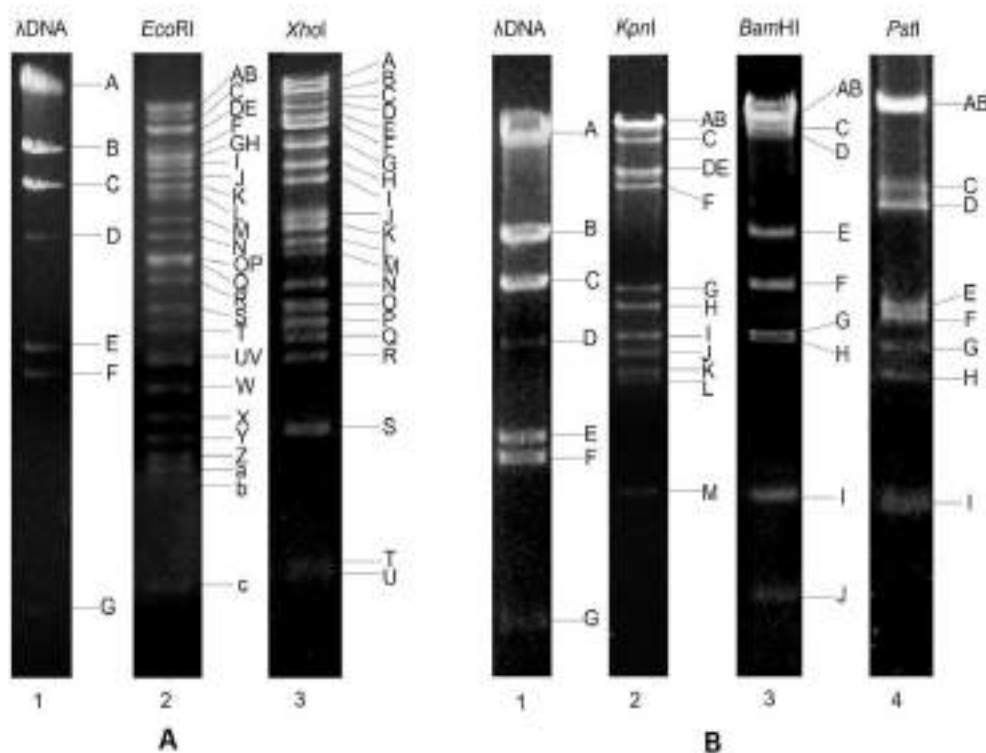
To resolve the high molecular weight fragments (particularly more than 23 kb), the digested DNA were separated in 0.4% agarose gel and to detect the fragments smaller than 23 kb, the digested fragments were separated in 0.7% agarose gel. *EcoRI* digestion produced 29 fragments ranging from 0.62-19.53 kb while 21 fragments could be resolved ranging from 0.67 kb to 25.56 kb with *XhoI* digestion (Figure 1A). Digestion with *KpnI* produced 13 fragments ranging from 1.46 to 37.50 kb. Other restriction digestions with *BamHI* and *PstI* produced 10 and 9 bands, respectively ranging from 0.71 to 48 kb for *BamHI* and from 1.68 kb to 62 kb for *PstI* (Figure 1B). The results showed that HytaNPV DNA digested with *KpnI*, *BamHI* and *PstI* have high molecular weight DNA above 23 kb which were resolved as doublet. The fragments AB in *KpnI*, *PstI* and *BamHI* are more than 23 kb in size and appear to co-migrate (Figure 1B). The fragments AB in *EcoRI* and DE in *KpnI* digestion were resolved by running the digests in 0.4% agarose gel for 6 hours (data not shown). Another doublet DE in *EcoRI* was confirmed by double digestion with *EcoRI* and *PstI* (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 (*HindIII* digested λ DNA as molecular weight marker has been shown also)

Fragments	λ DNA	<i>EcoRI</i>	<i>XhoI</i>	<i>KpnI</i>	<i>BamHI</i>	<i>PstI</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) *EcoRI* (lane 2) and *XhoI* (lane 3) in 0.7% agarose gel (B) *KpnI* (lane 2), *BamHI* (lane 3) and *PstI* (lane 4) in 0.4% agarose gel. *HindIII* digests of λ DNA 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.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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

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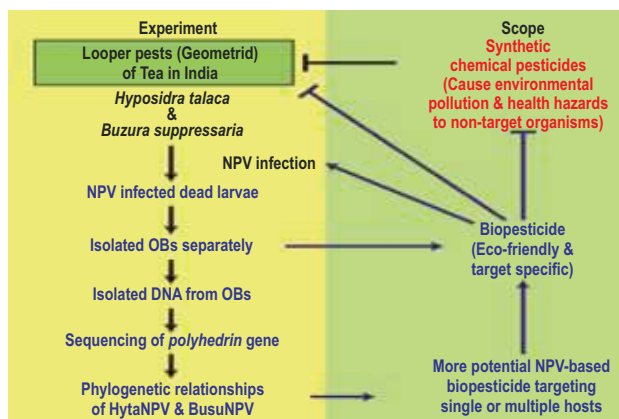
 Dr. R. Varatharajan
 Dr. R. B. Raizada

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

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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 (HyaNPV) 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, HyaNPV 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 (Rohrman, 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 (Rohrman, 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 (HyaNPV) 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 HyaNPV 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 Na₂CO₃, 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 HyaNPV and BusuNPV DNA in separate reactions in the presence of 1x GoTaq Flexi Buffer, 2 mM MgCl₂, 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 94°C for 5 min followed by 35 cycles of 94°C for 50 sec, 51°C for 40 sec and 72°C for 50 sec; then final extension of 7 min at 72°C. The amplified products of ≈ 527 bp were purified with the Sure Extract Spin PCR Clean up/ Gel extraction kit (Genetix 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), HytaNPV-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

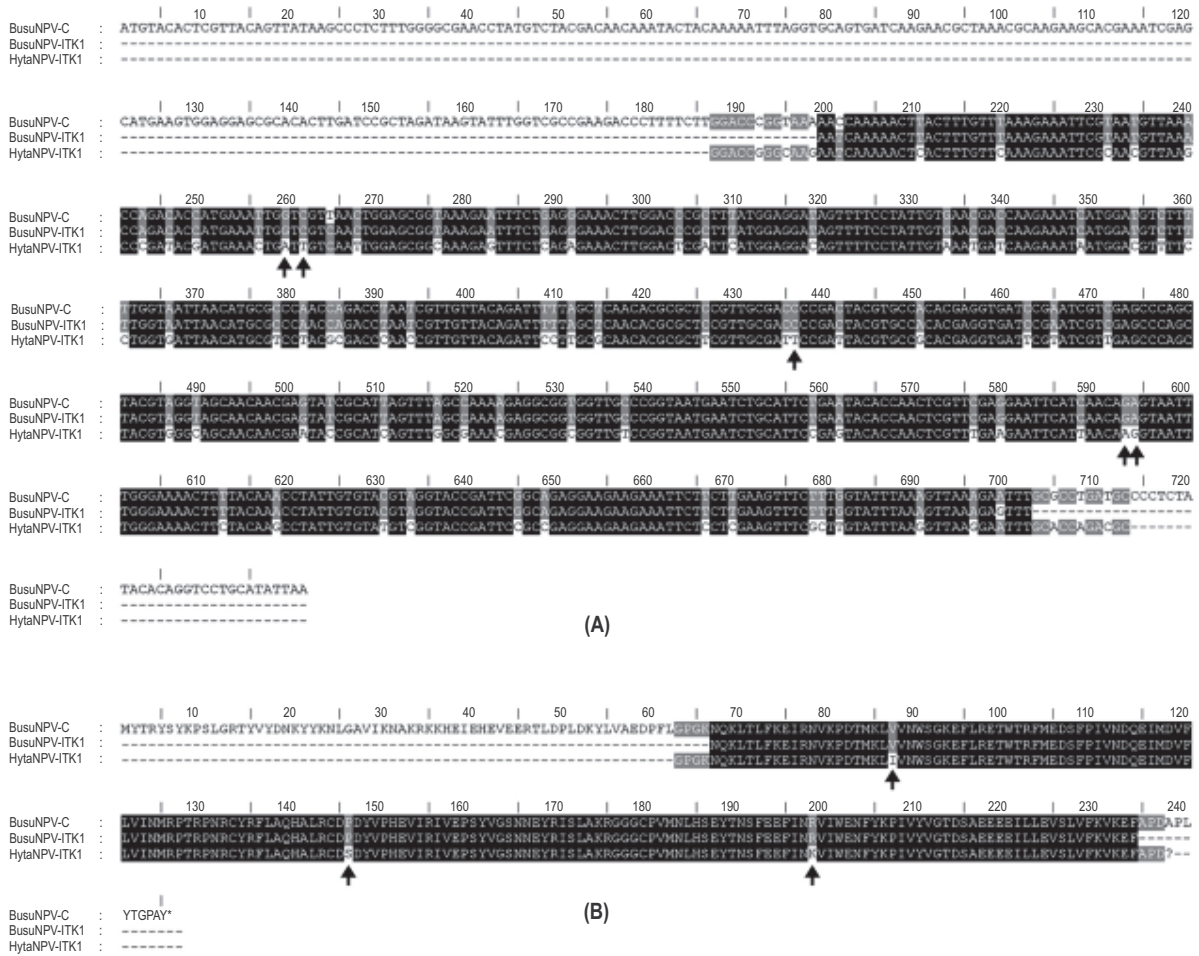


Fig. 1 : Nucleotide (A) and translated amino acid (B) sequence alignment of *polyhedrin* gene of BusuNPV-C, BusuNPV-ITK1 and HytaNPV-ITK1. Identical sites for any two sequences and conserved sites were shaded in grey and black, respectively. Arrow denotes the non-synonymous substitutions producing unique 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 Philippines. EcobNPV, another Chinese isolate, showed higher sequence divergence with NPVs infecting the specimens of genus *Hyposidra* and *Buzura* with NSD, 0.167-

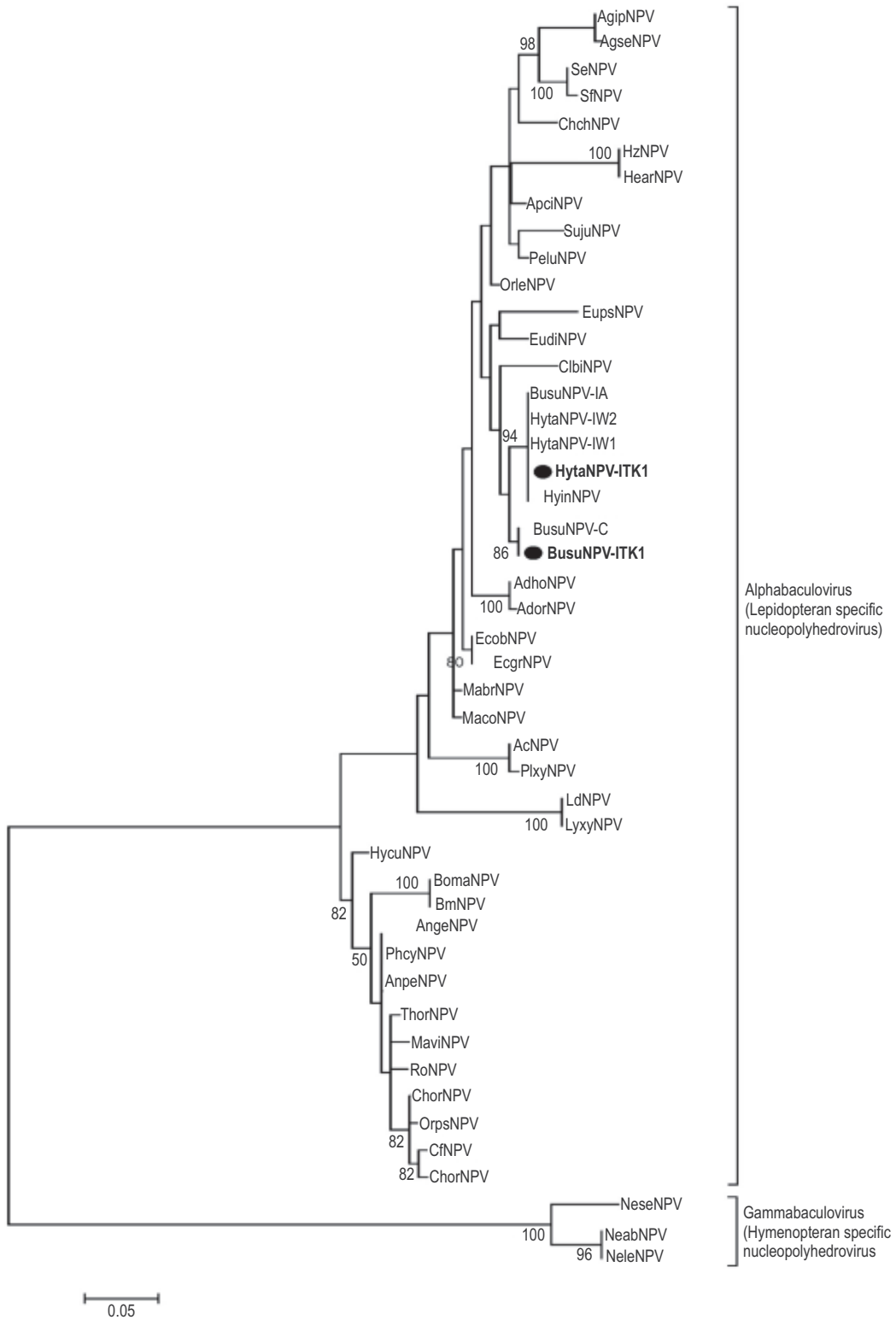


Fig. 2 : Maximum Likelihood tree based on *polyhedrin* gene for 47 baculoviruses. Bootstrap values more than 50% were shown at each node.

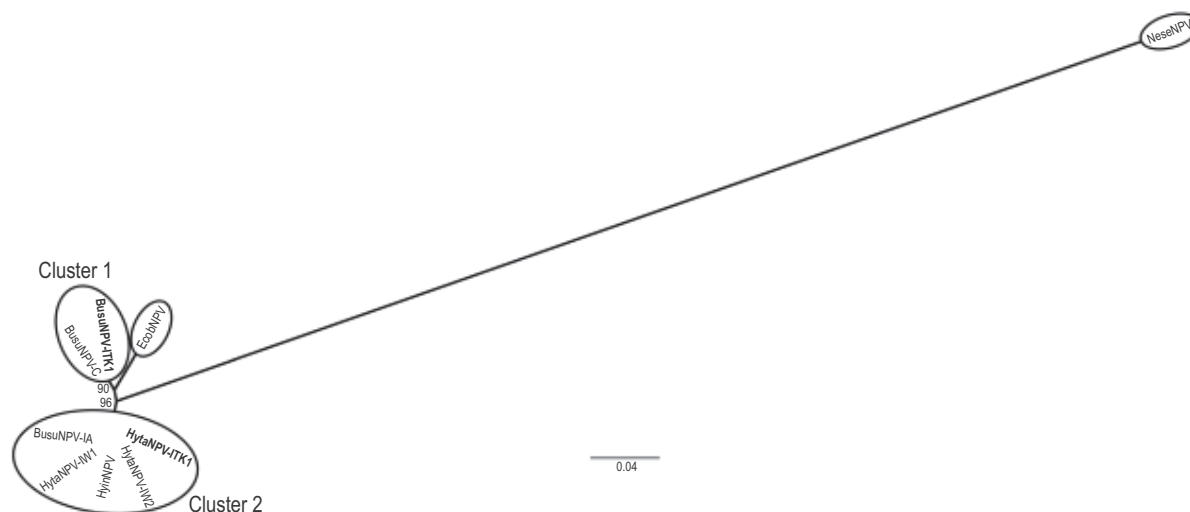


Fig. 3 : Maximum Likelihood tree based on *polyhedrin* gene for nine baculoviruses. Bootstrap values more than 50% were shown at each node.

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

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