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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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Authors Info

B. Ghosh[1], A. Mukhopadhyay[2] and M. Bahadur[1]*

[1]Genetics and Molecular Biology Laboratory, Department of Zoology, University of North Bengal, Darjeeling-734 013, India

[2]Entomology Research Unit, Department of Zoology, University of North Bengal, Darjeeling-734 013, India

***Corresponding Author Email :** bahadurmin@rediffmail.com

Edited by

Dr. Sumati Gaumat

Reviewed by

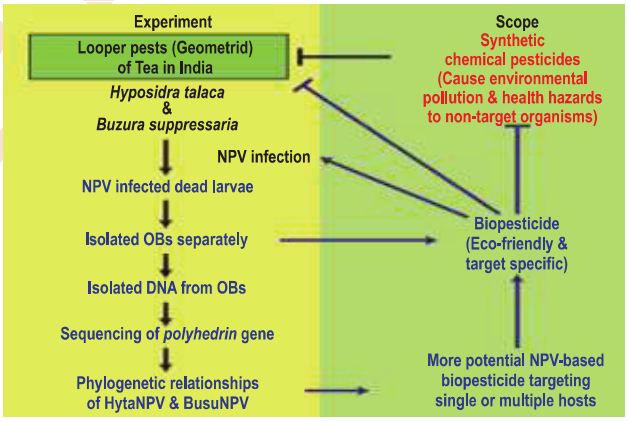
Dr. R. Varatharajan
Dr. R. B. Raizada

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 (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

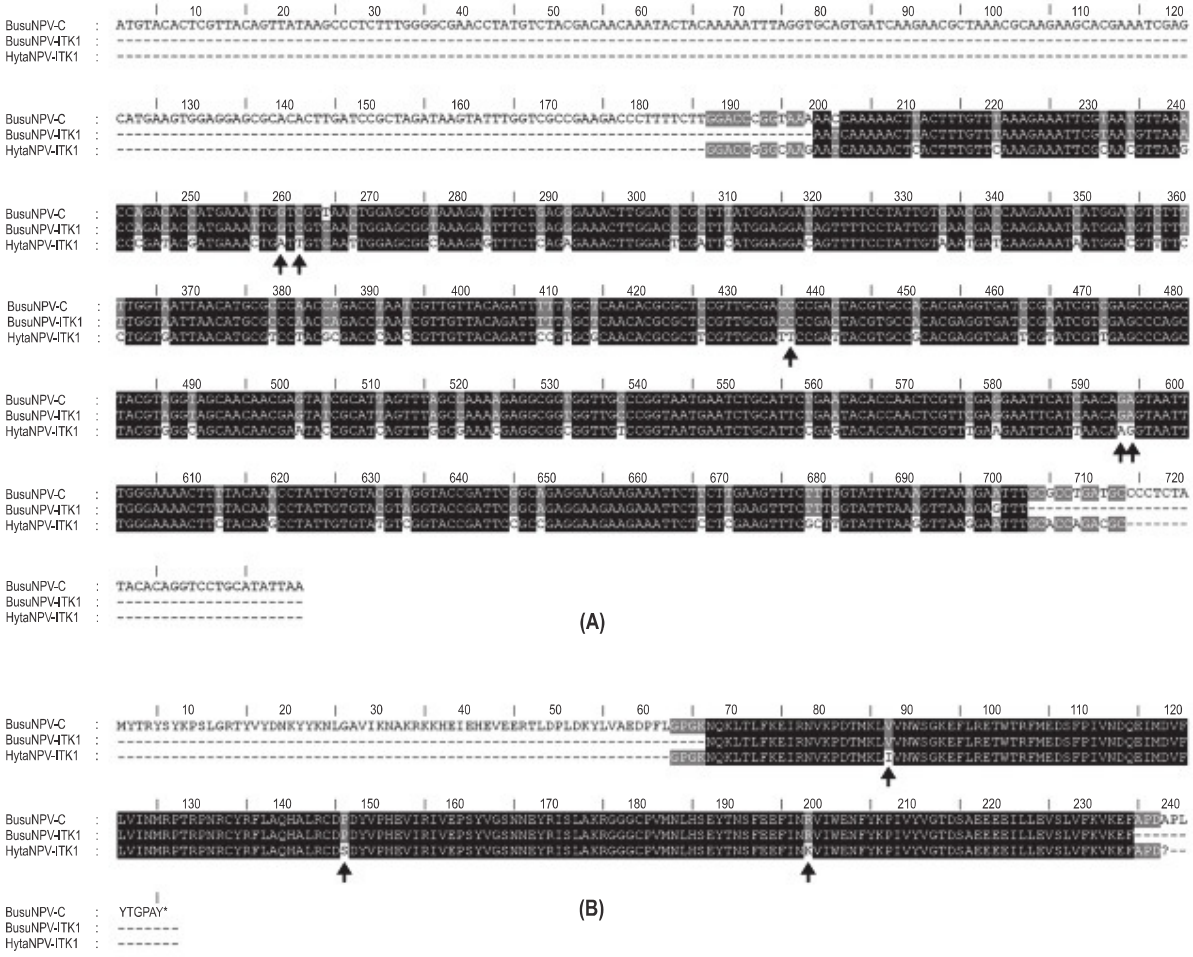

Fig. 1 : Nucleotide (A) and translated amino acid (B) sequence alignment of *polyhedrin* gene of BusuNPV-C, BusuNPV-ITK1 and HytaNPV-ITK1. Identical sites for any two sequences and conserved sites were shaded in grey and black, respectively. Arrow denotes the non-synonymous substitutions producing unique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

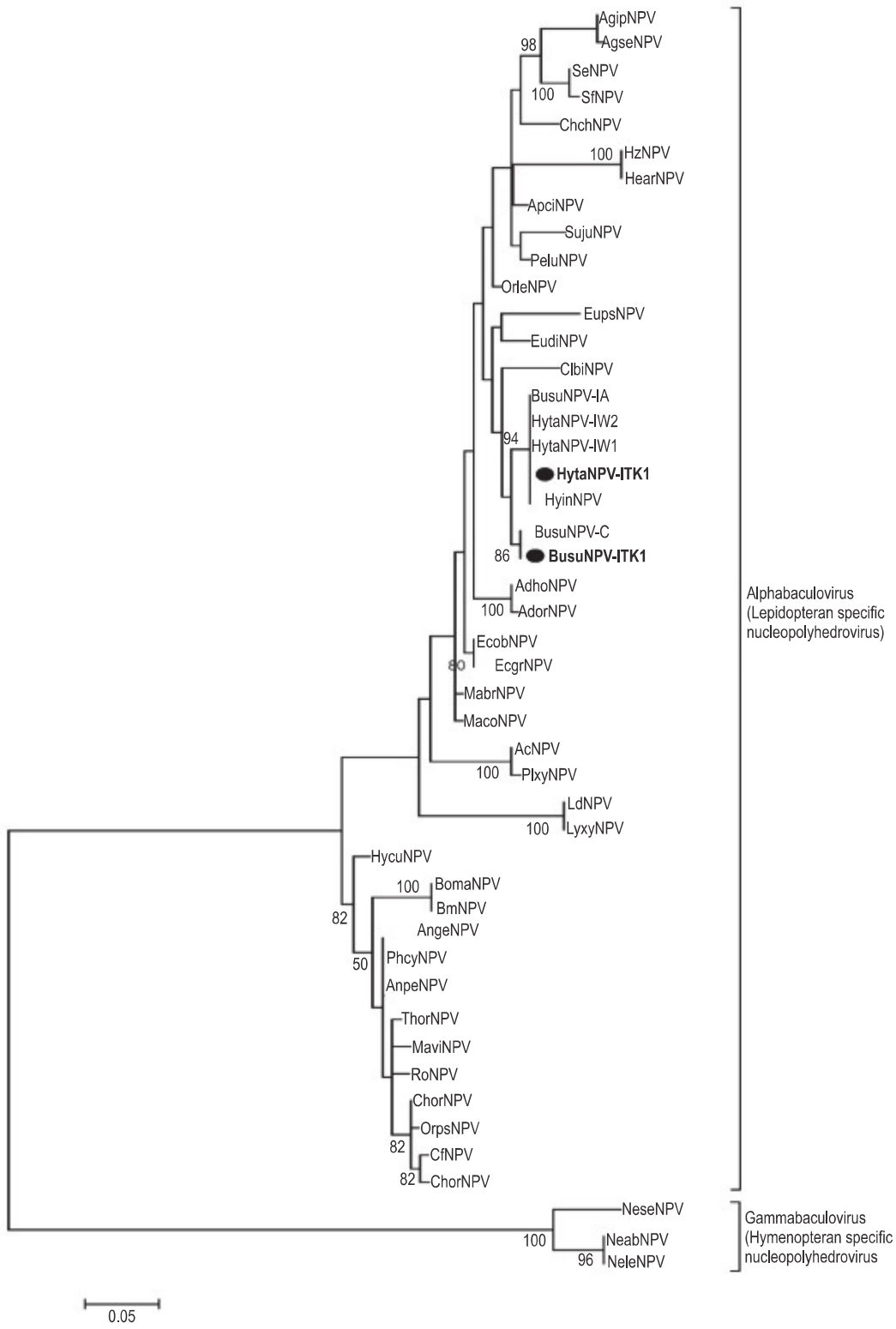

Fig. 2 : Maximum Likelihood tree based on *polyhedrin* gene for 47 baculoviruses. Bootstrap values more than 50% were shown at each node.

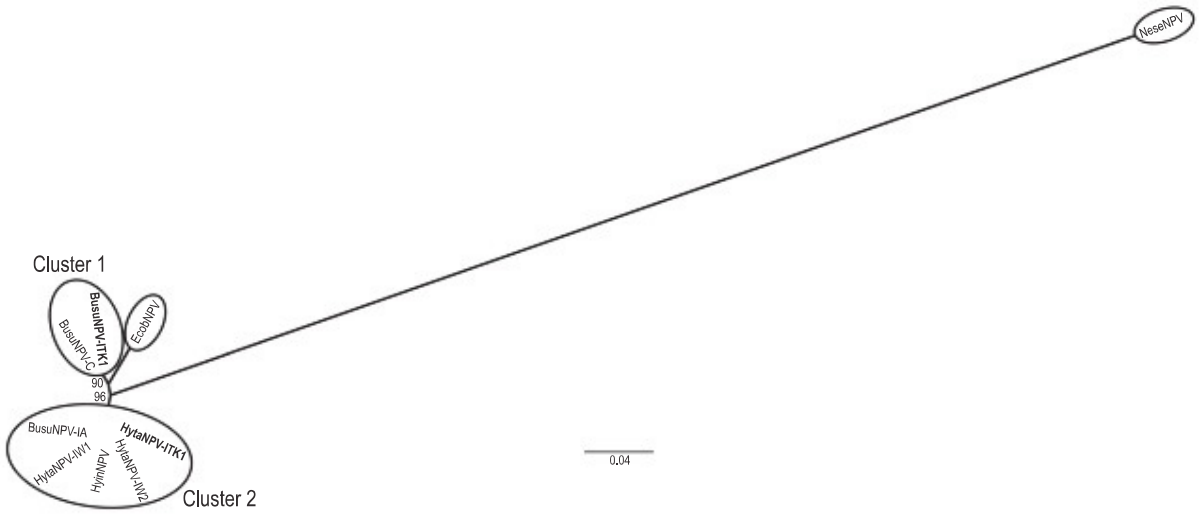

Fig. 3 : Maximum Likelihood tree based on *polyhedrin* gene for nine baculoviruses. Bootstrap values more than 50% were shown at each node.

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	78
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.

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