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).

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.