Chapter 2

Results

Section 2.1: The HytaNPV isolates in the present study

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

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

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

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

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

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

Objectives

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

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

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

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

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

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

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

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

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

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

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

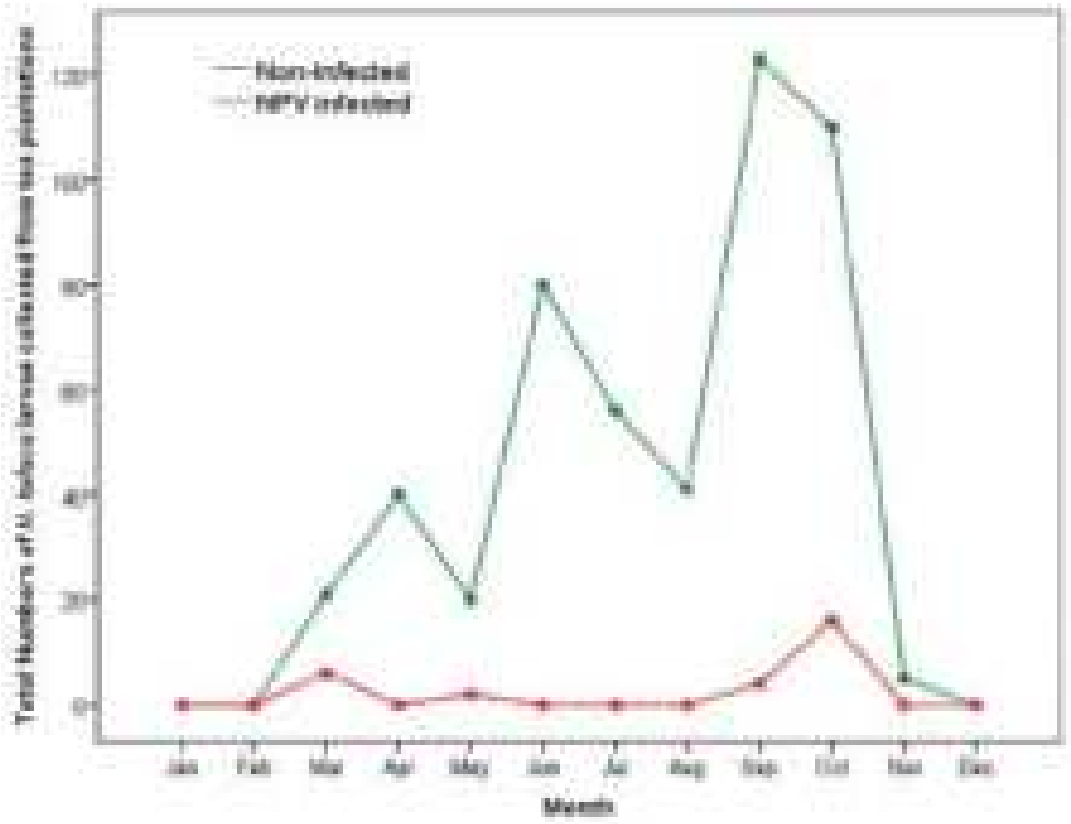


Figure 2-3: The figure showing the mean number of collected non-infected (green line) and NPV-infected (red line) *H. talaca* larvae collected from four different tea plantations in the Terai regions of Darjeeling foothills, during 2013-15.

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

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

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

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

2.2.3 Isolation of DNA from OBs

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

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

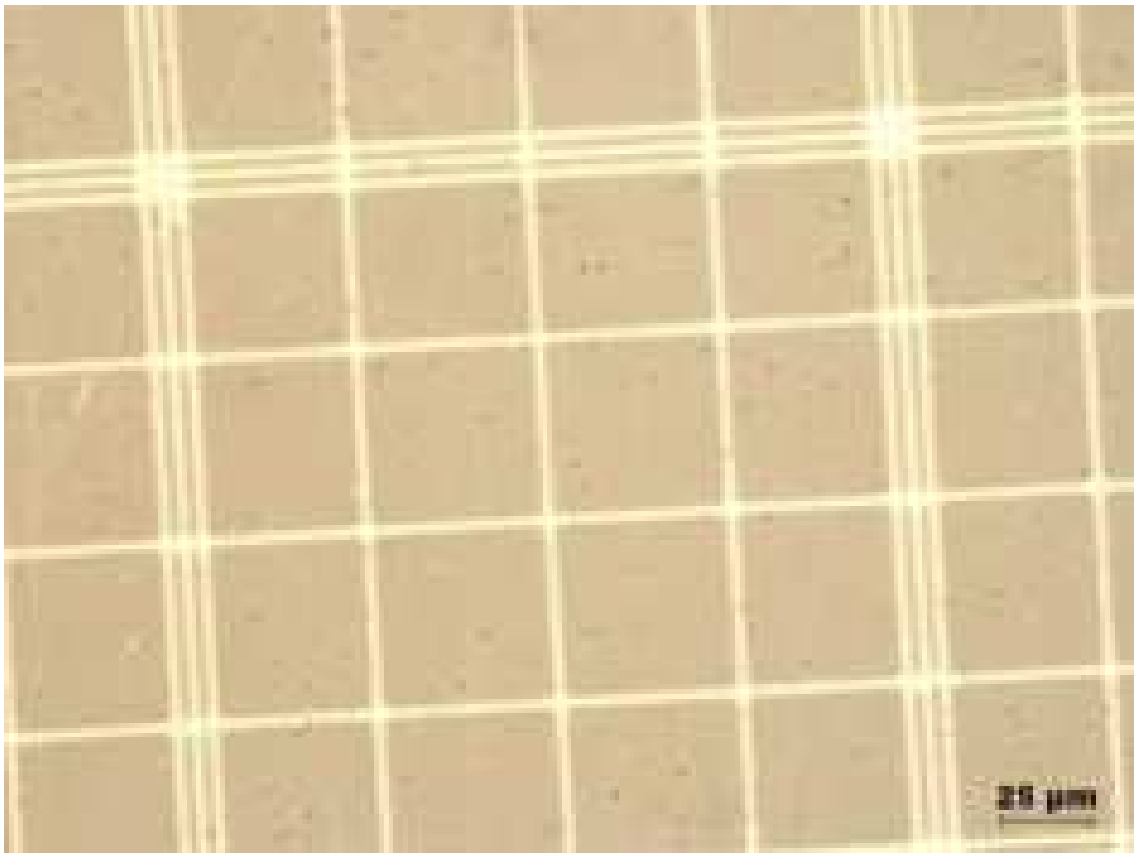


Figure 2-5: The polyhedra OBs under the compound microscope (400X).

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

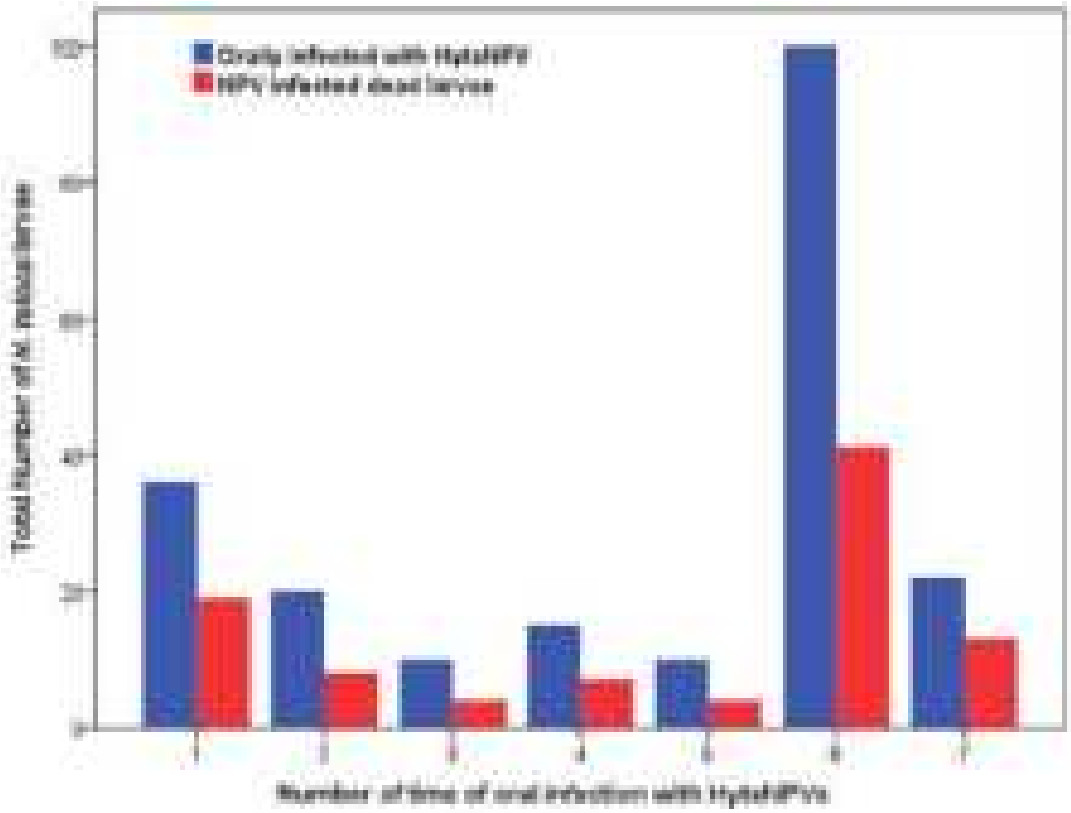


Figure 2-7: Number of *H. talaca* larvae orally infected by OBs (blue bar) in the laboratory and the number of larvae that died due to NPV infection (red bar).

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

Section 2.3: Restriction Endonuclease Fragment analysis

Objectives:

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

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

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

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

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

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

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

***RE = Restriction endonuclease**

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

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

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

Restriction Endonuclease Fragment analysis

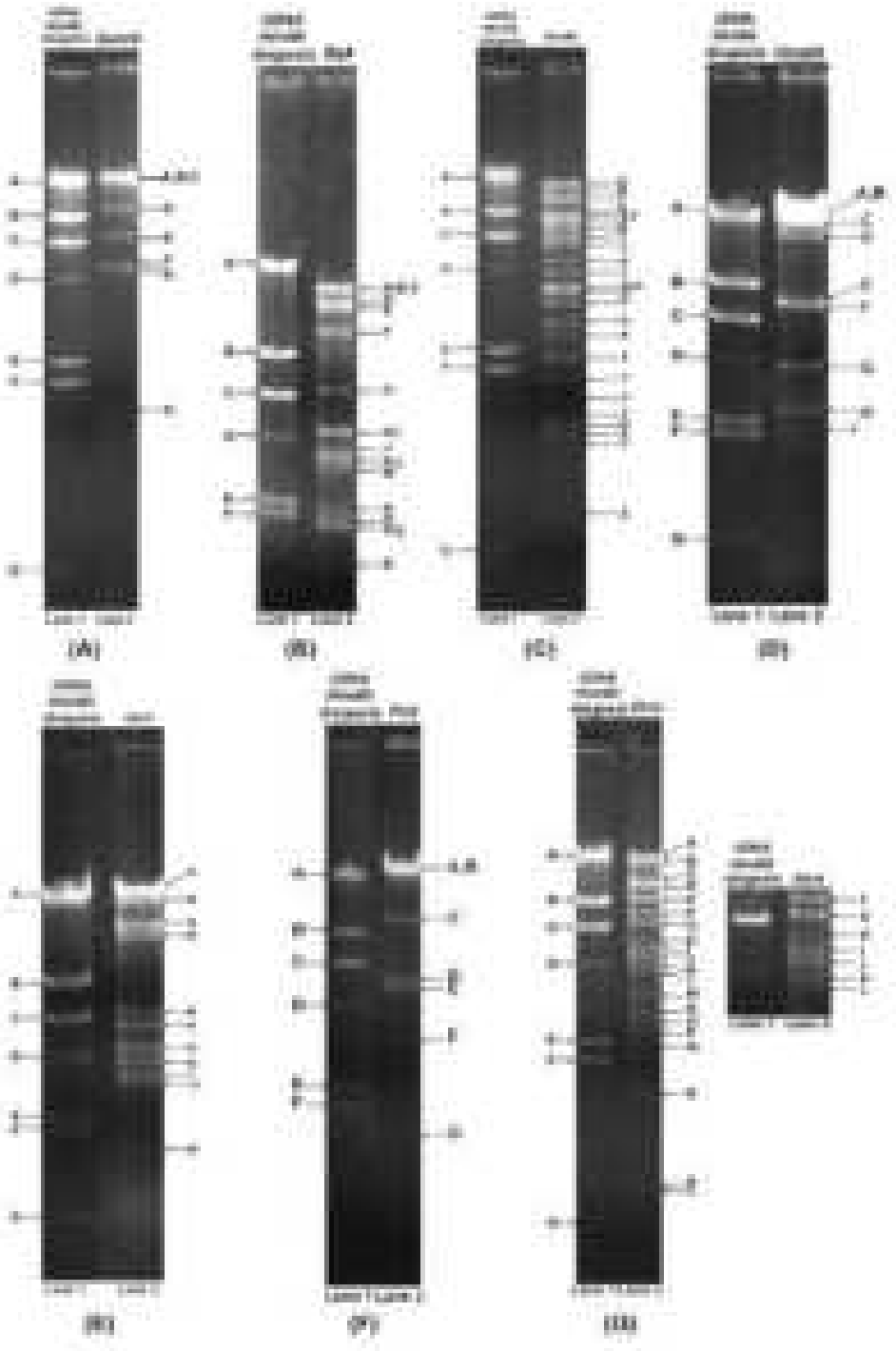


Figure 2-9: Electrophoregrams of restriction digestion of Terai isolate, HytaNPV-ITK1. A. *Bam*HI, B. *Bgl*I, C. *Eco*RI, D. *Hin*dIII, E. *Kpn*I, F. *Pst*I, and G. *Xho*I

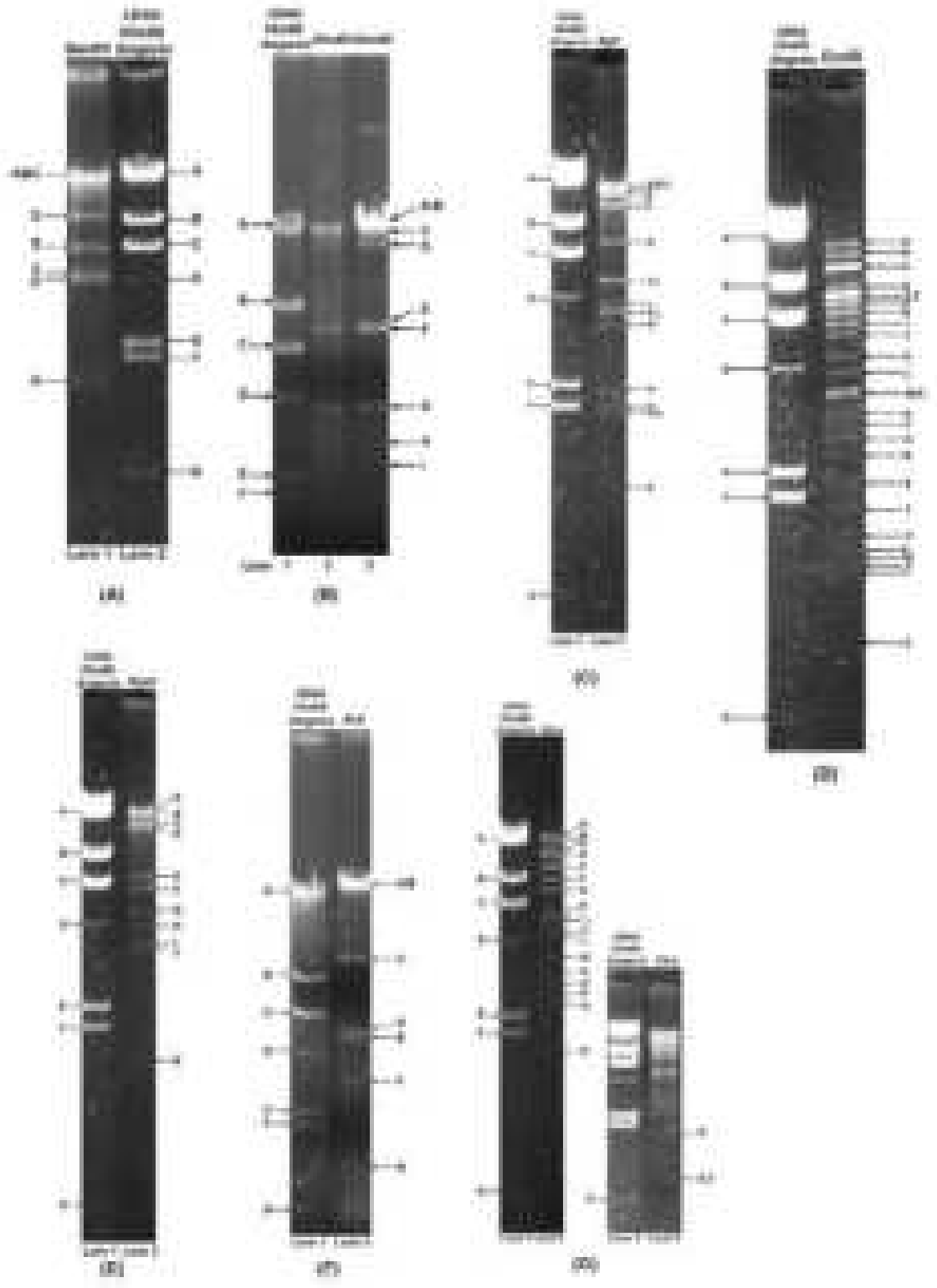


Figure 2-10: Electrophoregrams of restriction digestion of Dooars isolate, HytaNPV-ID1. A. *Bam*HI, B. *Bgl*I, C. *Eco*RI, D. *Hin*dIII, E. *Kpn*I, F. *Pst*I, and G. *Xho*I.

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

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

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

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

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

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

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

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

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

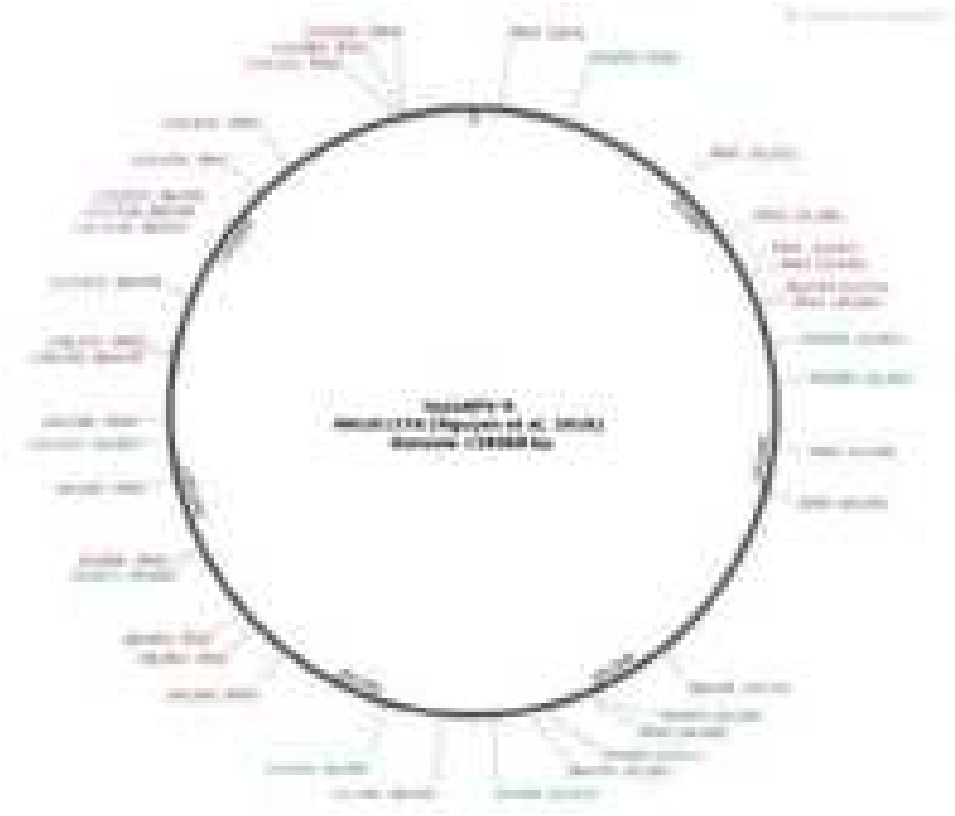


Figure 2-11: Restriction map of HytaNPV-R genome for *Bam*HI, *Hin*dIII and *Xho*I

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

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

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

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

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

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

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

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

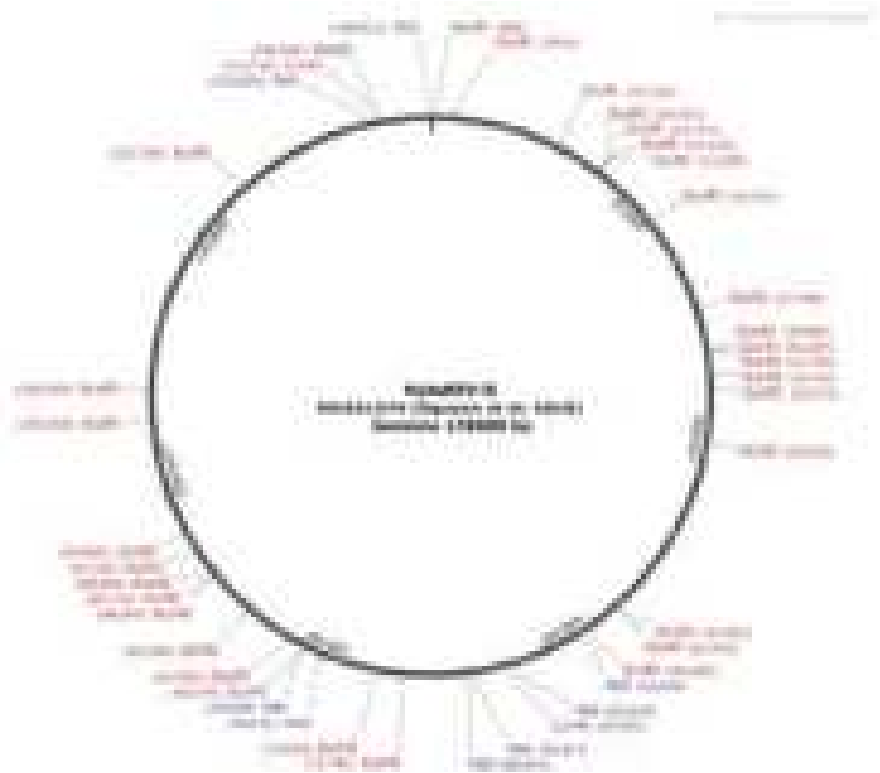


Figure 2-12: Restriction map of HytaNPV-R genome for *Eco*RI and *Pst*I

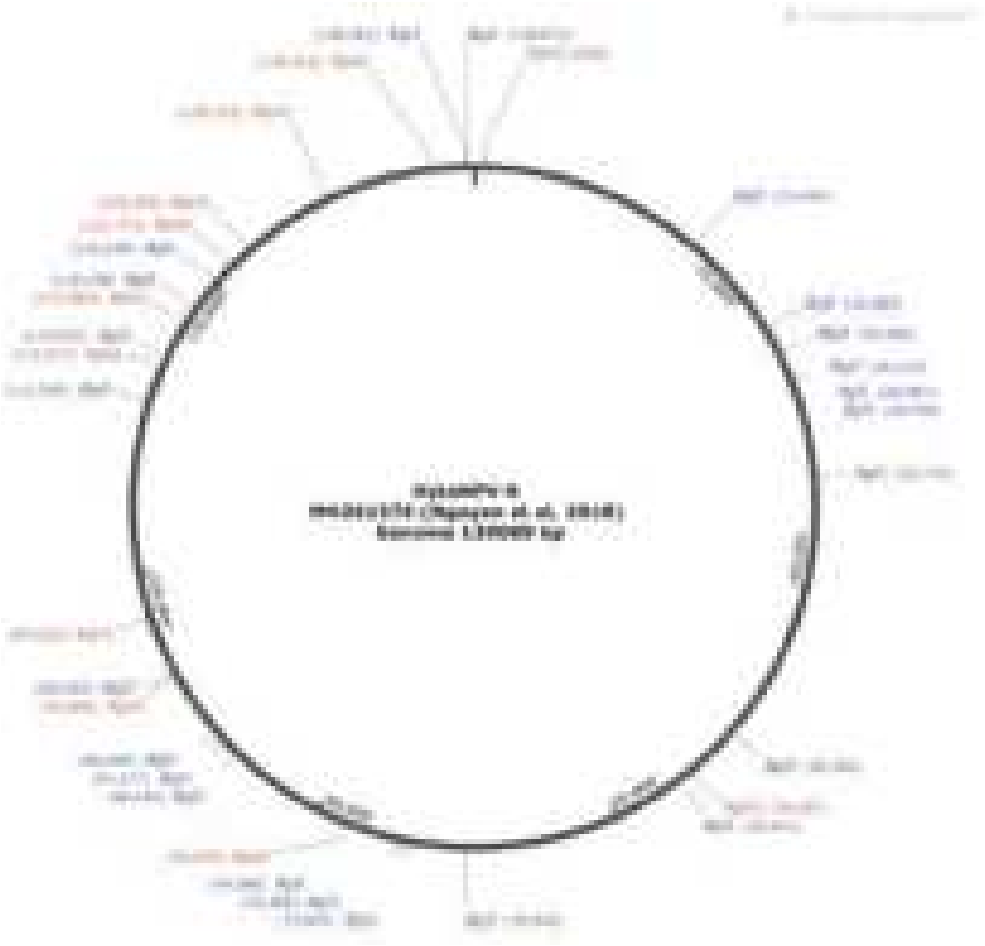


Figure 2-13: Restriction map of HytaNPV-R genome for *Bgl*I and *Kpn*I

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

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

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

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

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

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

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

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

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

***RE = Restriction endonuclease**

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

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

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

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

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

Similar fragment	+
Fragment absent	-
Unique fragment	
Undetected fragment	?

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

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

Section 2.4: Sequencing and characterization of HytaNPV genes

Objectives

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

2.4.1 PCR amplification of selected genes

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

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

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

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

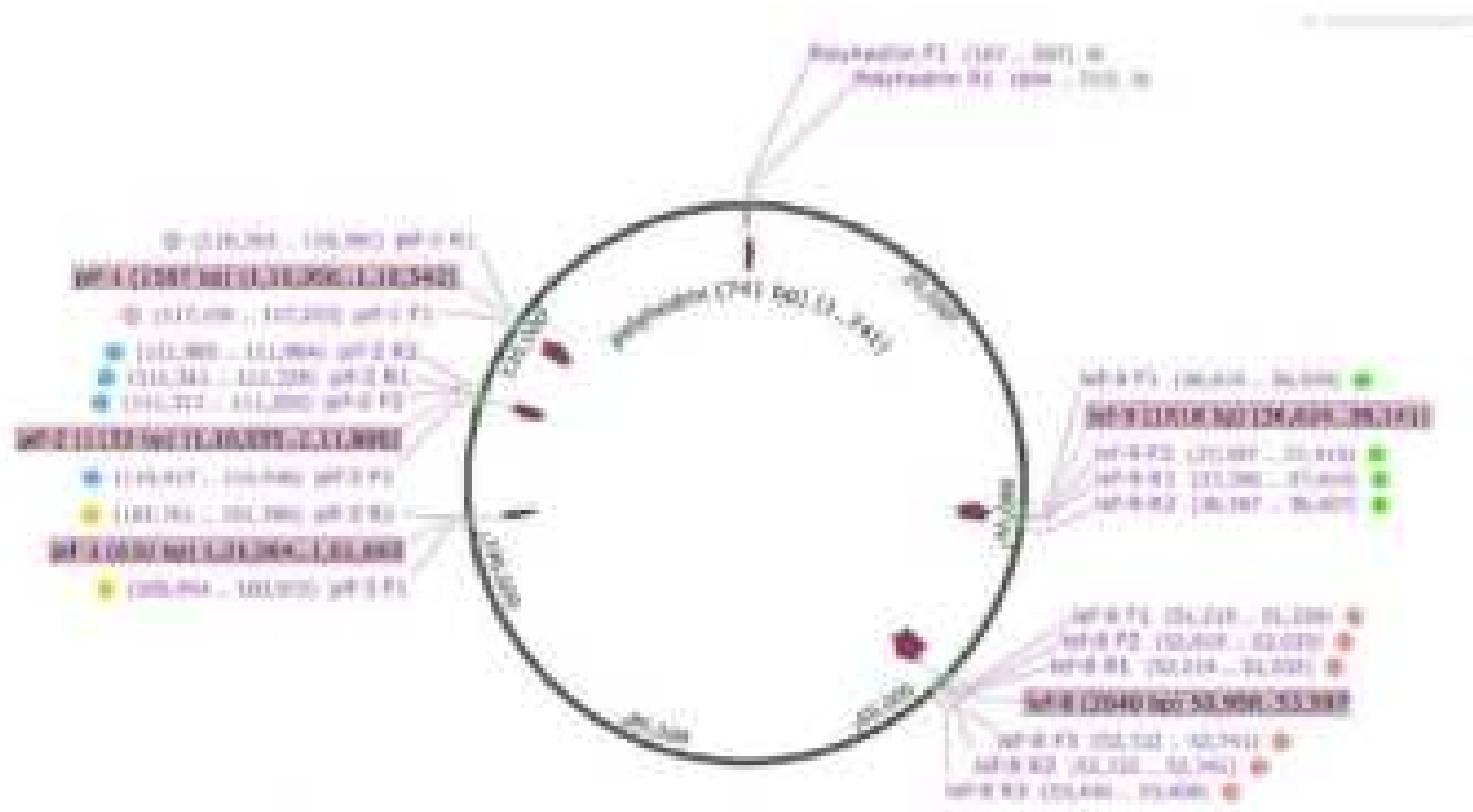


Figure 2-14: Position and details of the six genes used in the present study (purple arrow) and the binding sites of the primers used to amplify respective genes of the HytaNPV genome. HytaNPV-R 139.089 kb (MH261376.1; Nguyen et al., 2018) was used as a reference template. Details of the genes were highlighted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

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

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

PCR amplification of selected genes

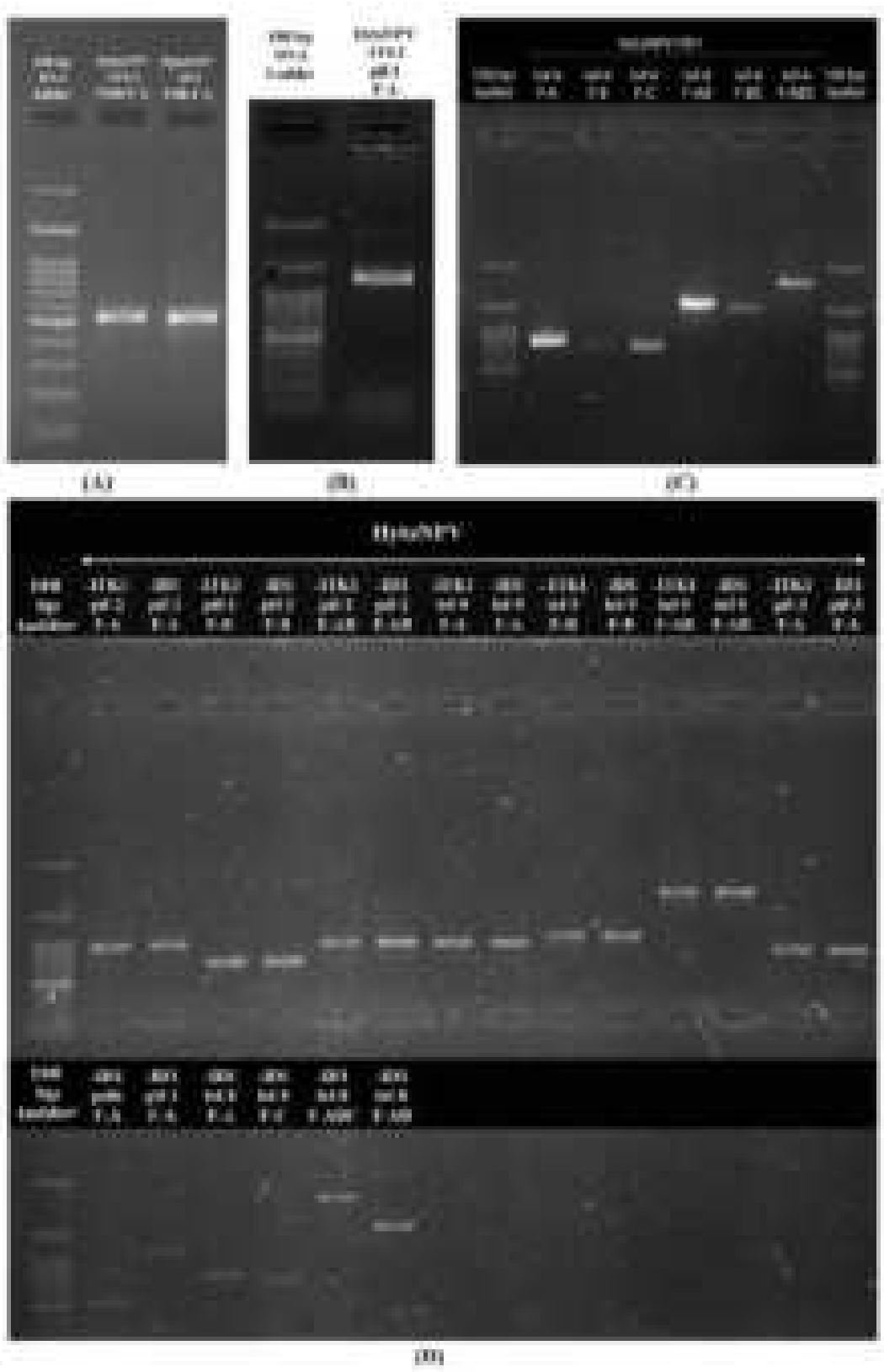


Figure 2-15: Electrophoregrams showing PCR products of HytaNPV DNA. HytaNPV-ITK1 and HytaNPV-ID1 represent the Terai and Dooars isolate, respectively and F stands for 'fragment' (see Table 2-13).

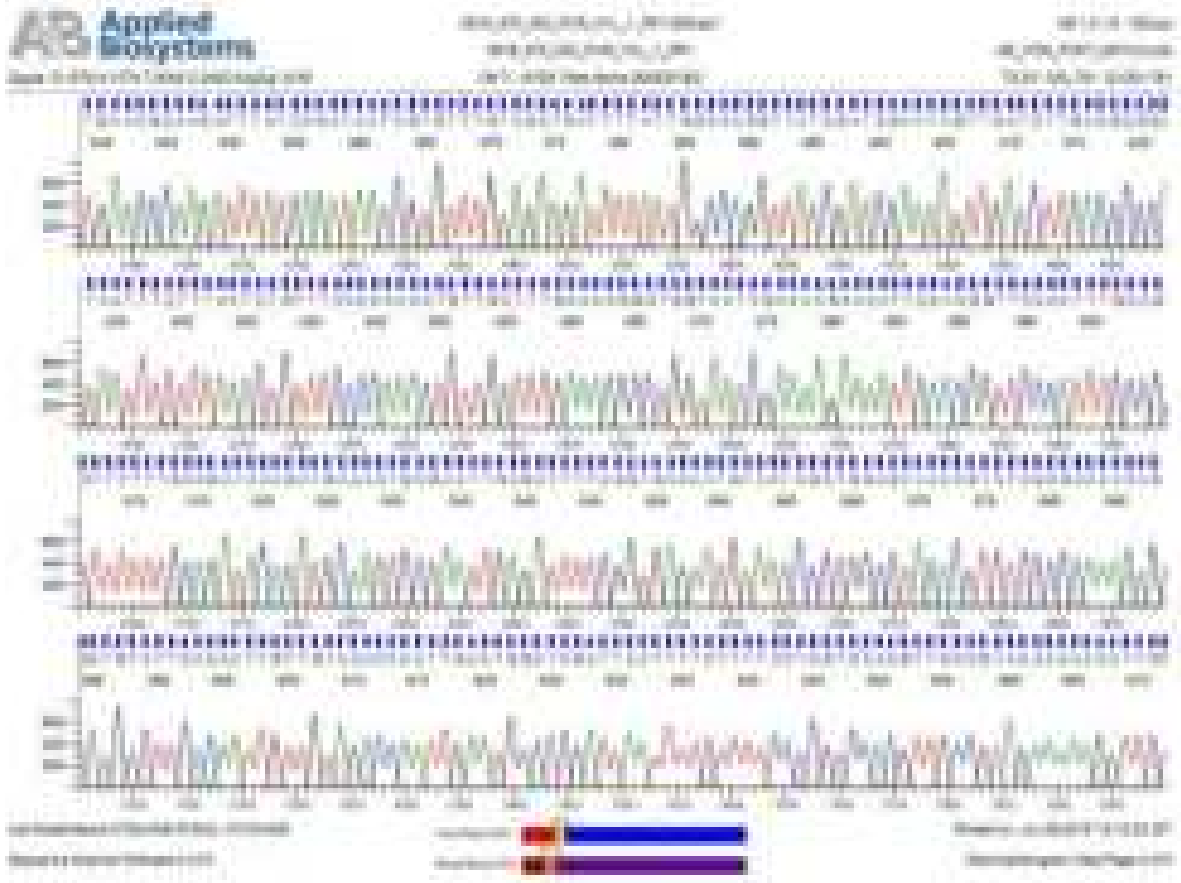


Figure 2-16: Electrophoregram of sequencing of *lef-8* gene.

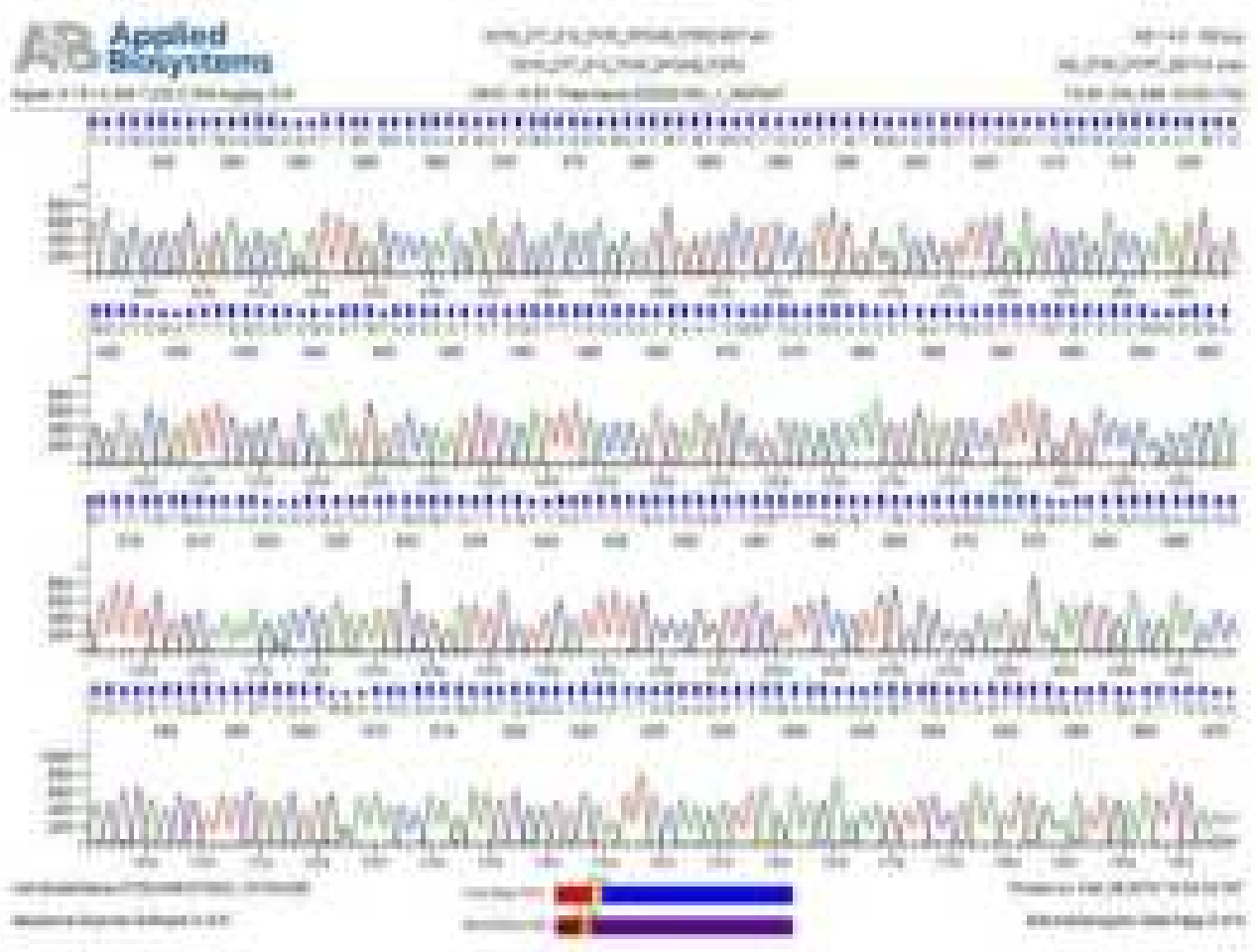


Figure 2-17: Electrophoregram of sequencing of *pif*-2 gene.

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

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

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

2.4.2 Sequencing of the *polyhedrin* gene

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

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

2.4.2.1 Blast analysis

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

2.4.2.2 Alignment

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

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

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

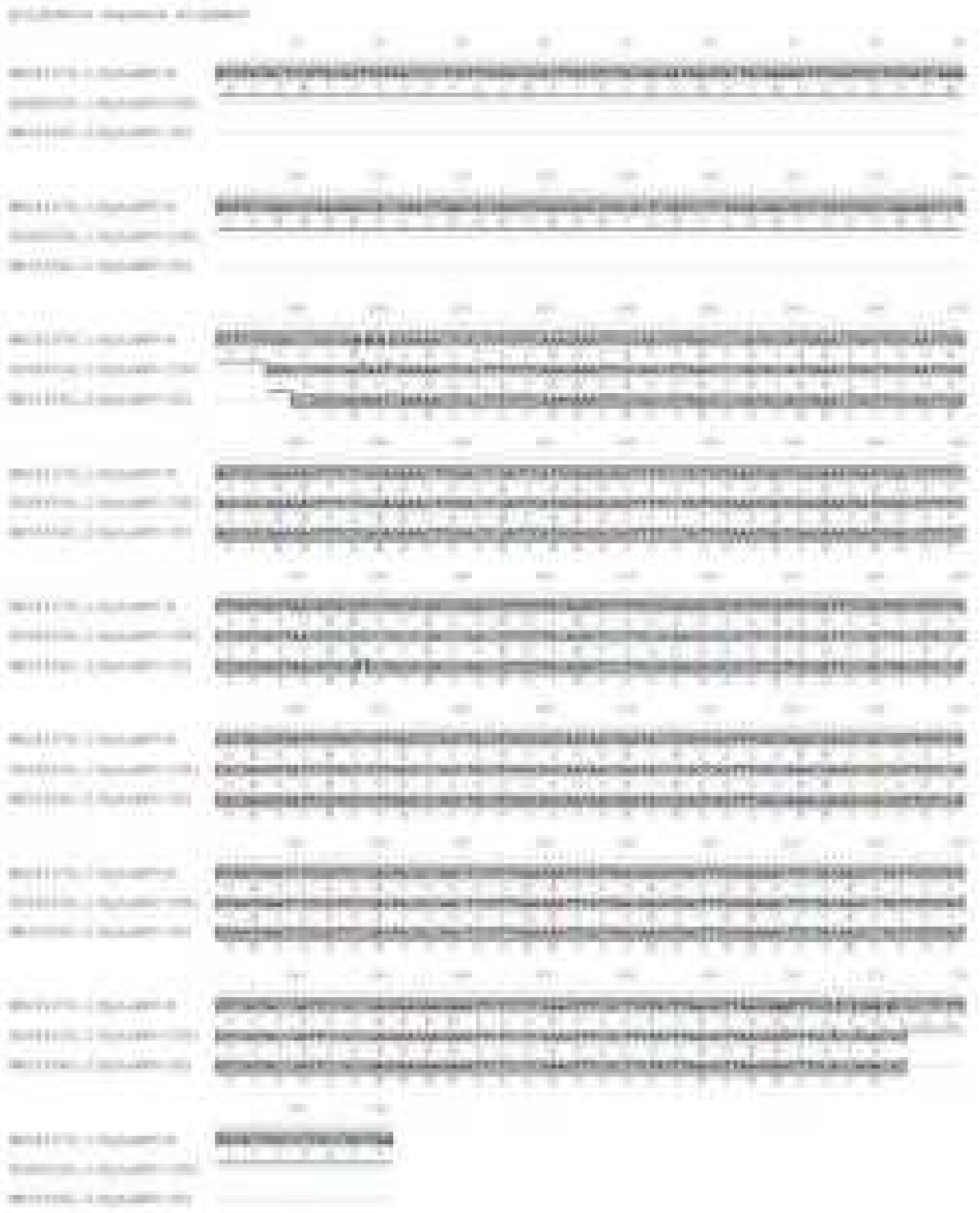


Figure 2-19: Nucleotide and translated amino acid sequence alignment of the *polyhedrin* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background.

2.4.2.3 Restriction sites on *polyhedrin* sequence

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

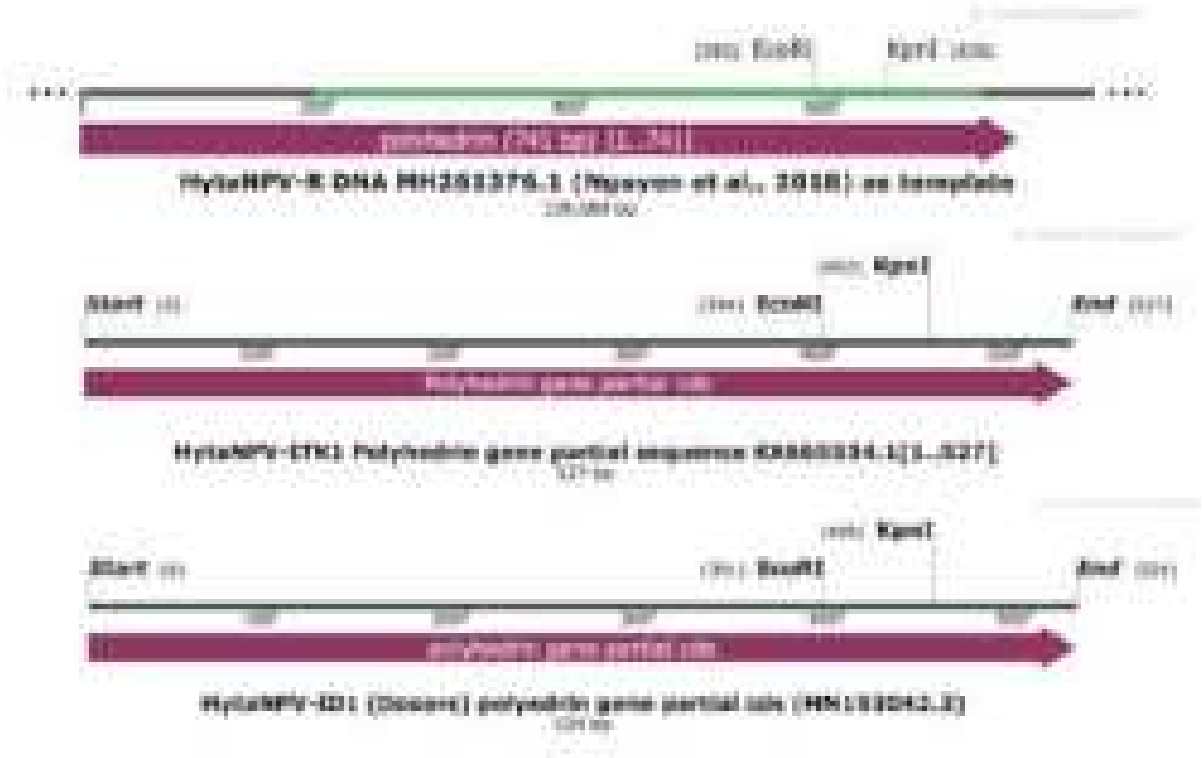


Figure 2-20: Restriction sites present in the *polyhedrin* of different HytaNPV isolates.

2.4.3 Sequencing of *lef-8* gene

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

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

2.4.3.1 Blast analysis

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

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

2.4.3.2 Alignments

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

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

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

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

Sequencing of lef-8 gene

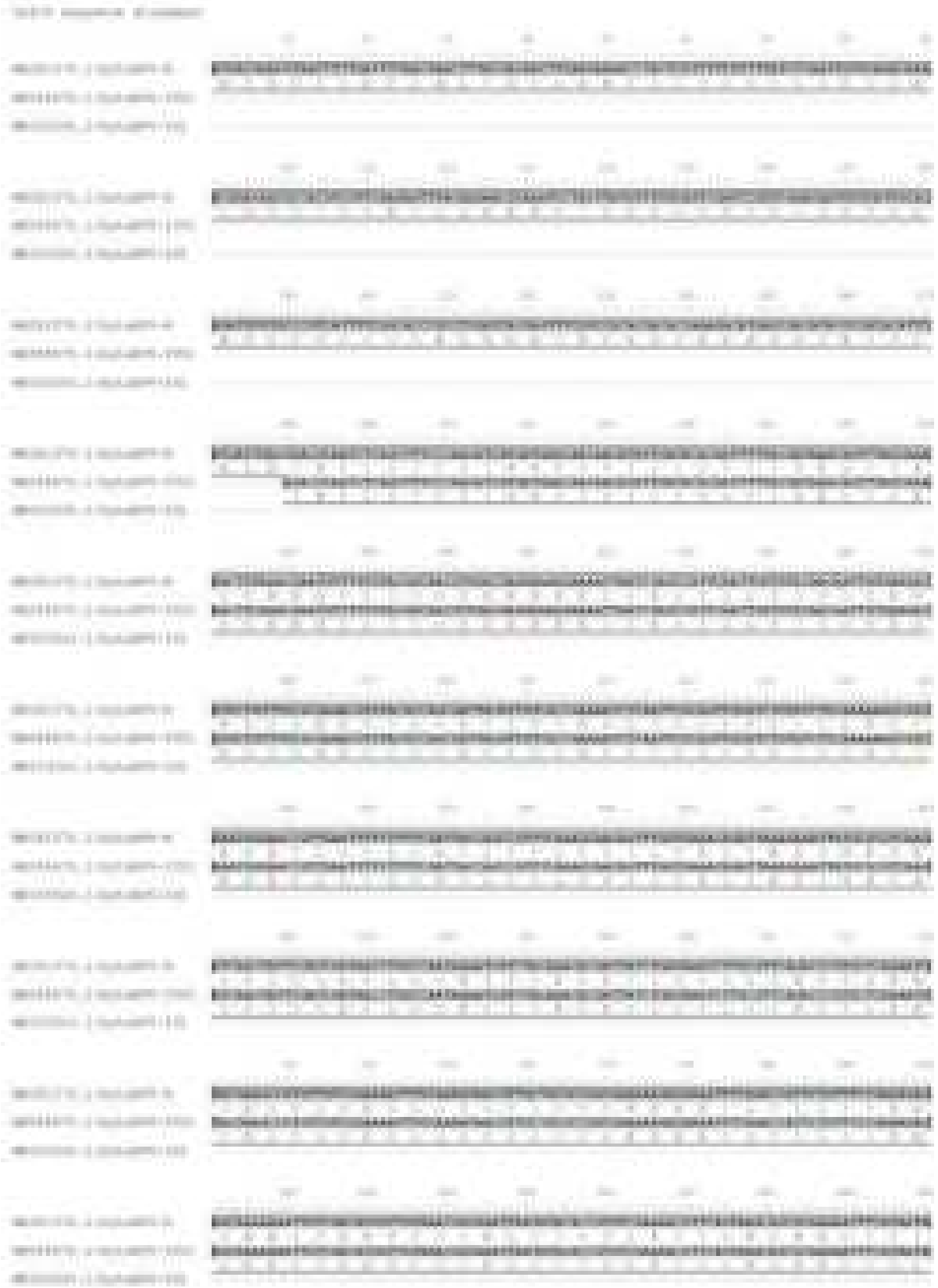


Figure 2-22: Nucleotide and translated amino acid sequence alignment of the *lef-8* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background.

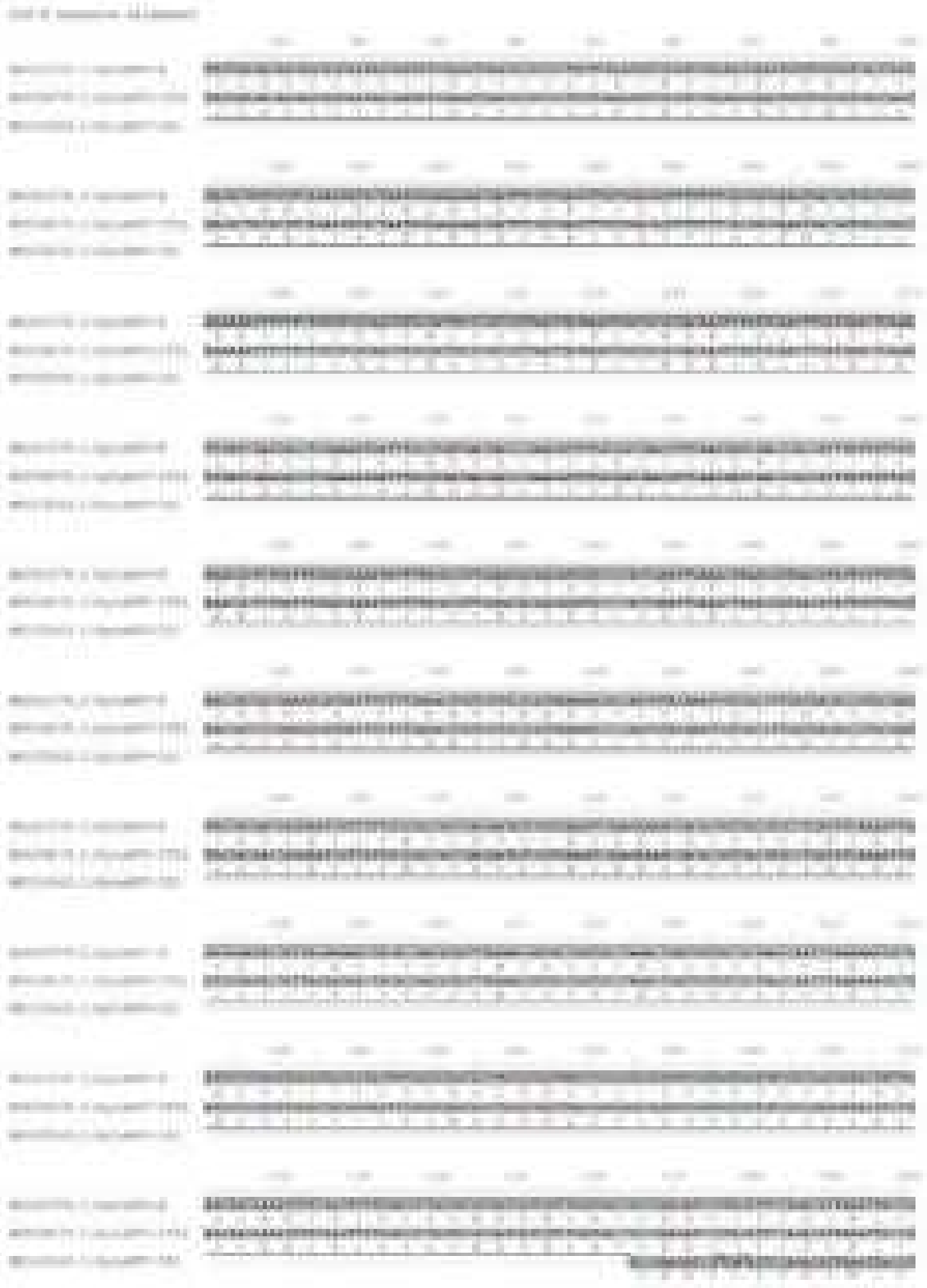


Figure 2-22: Nucleotide and translated amino acid sequence alignment of the *lef-8* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background [Continued…].

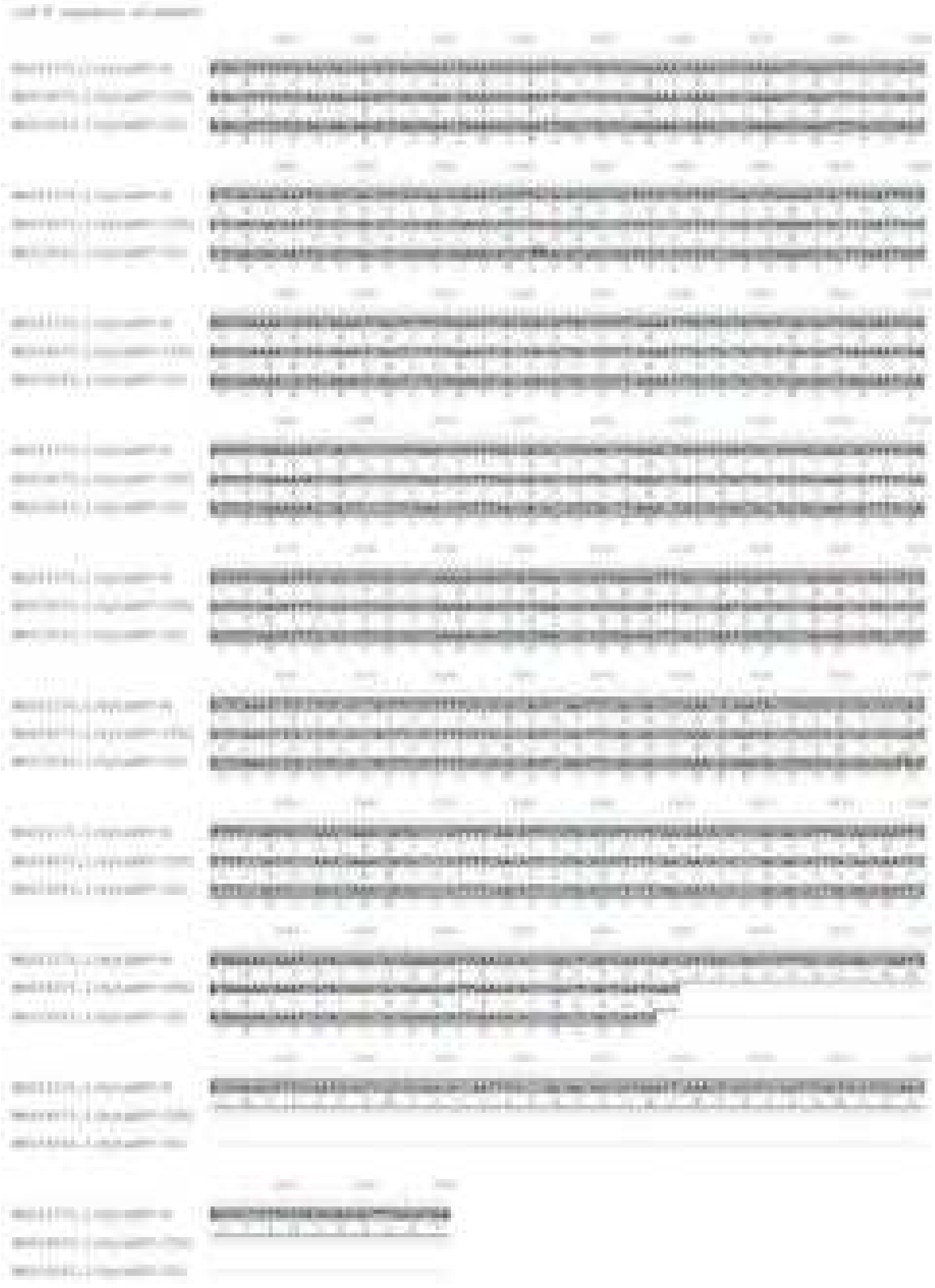


Figure 2-22: Nucleotide and translated amino acid sequence alignment of the *lef-8* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background [Continued…].

2.4.3.3 Restriction sites on *lef-8* sequence

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

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

2.4.4 Sequencing of *lef-9* gene

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

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

2.4.4.1 Blast analysis

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

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

2.4.4.2 Alignments

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

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

Table 2-17: Blast results lef-9 sequence

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

Sequencing of lef-9 gene

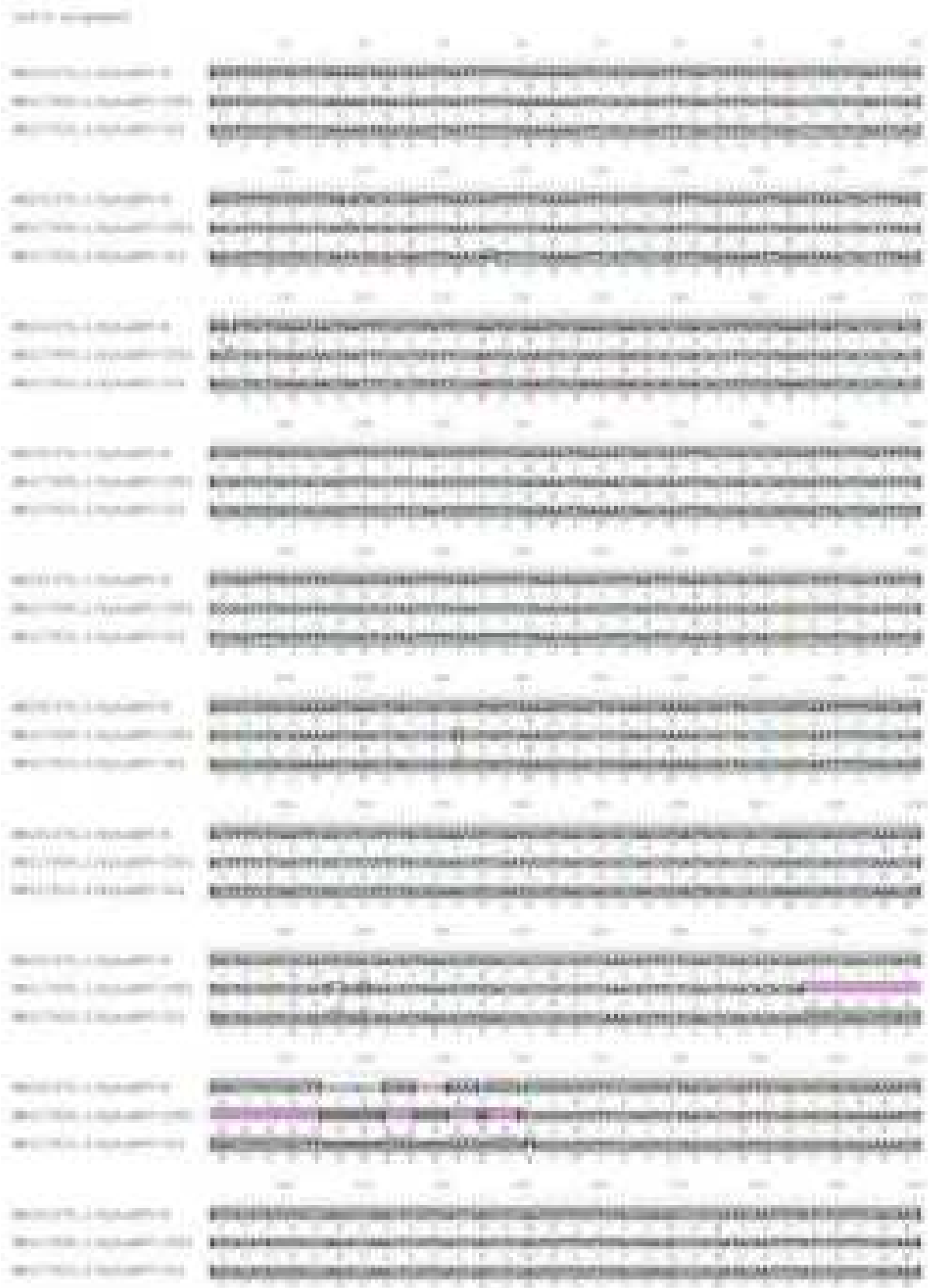


Figure 2-25: Nucleotide and translated amino acid sequence alignment of *lef-9* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as reference. Similar sequences were highlighted in grey background.

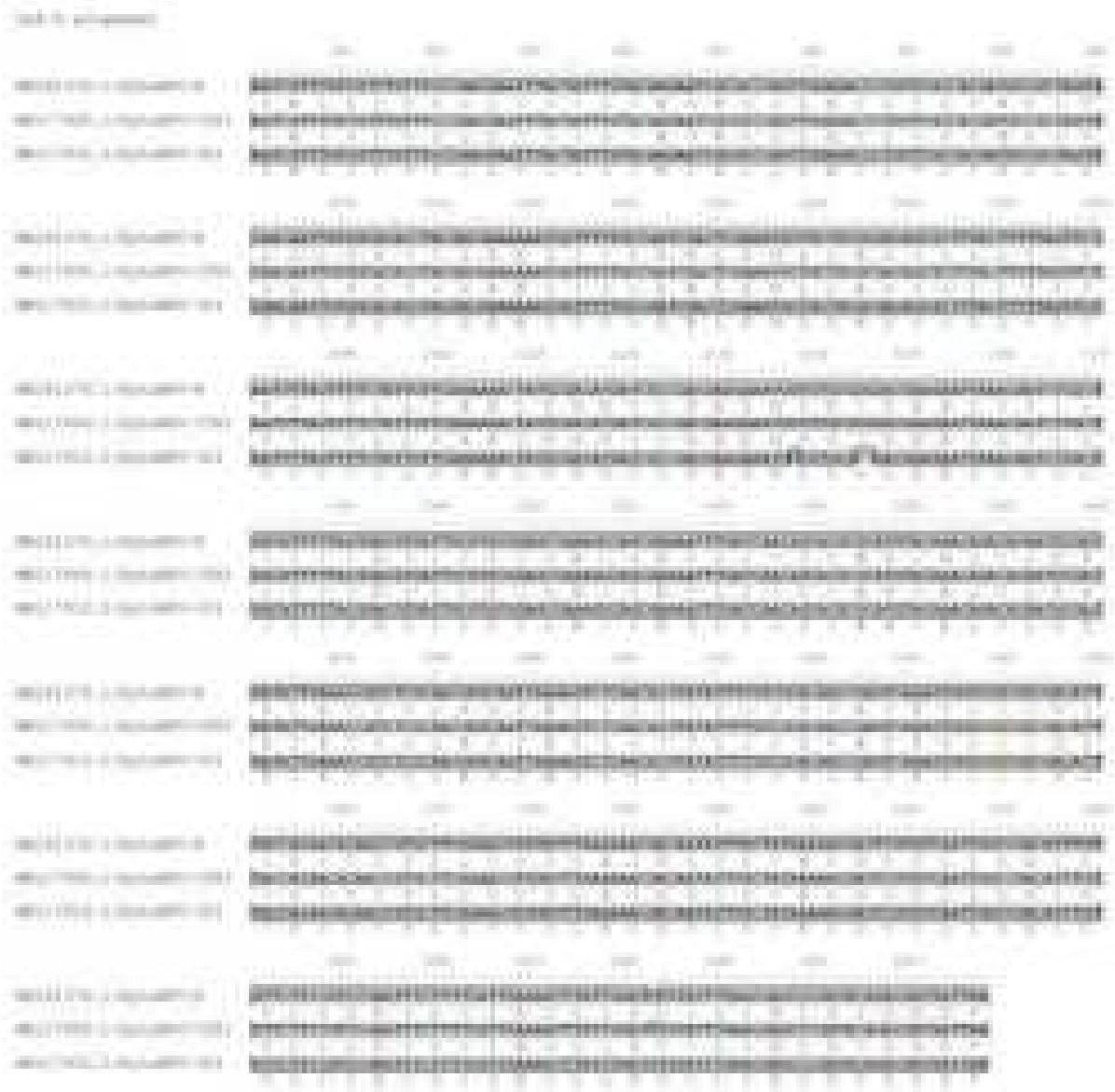


Figure 2-25: Nucleotide and translated amino acid sequence alignment of *lef-9* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as reference. Similar sequences were highlighted in grey background [Continued…].

2.4.4.3 Restriction sites on *lef-9* sequence

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

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

2.4.5 Sequencing of *pif-1* gene

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

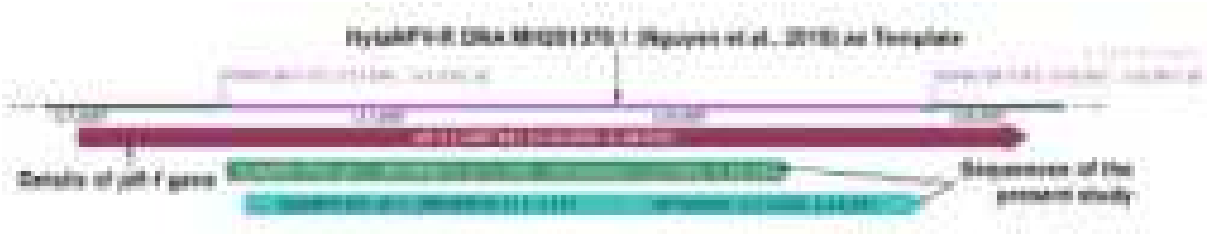


Figure 2-27: Primer binding, amplicon and sequence details of the *pif-1* gene using HytaNPV-R as a template.

2.4.5.1 Blast analysis

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

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

2.4.5.2 Alignments

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

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

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

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

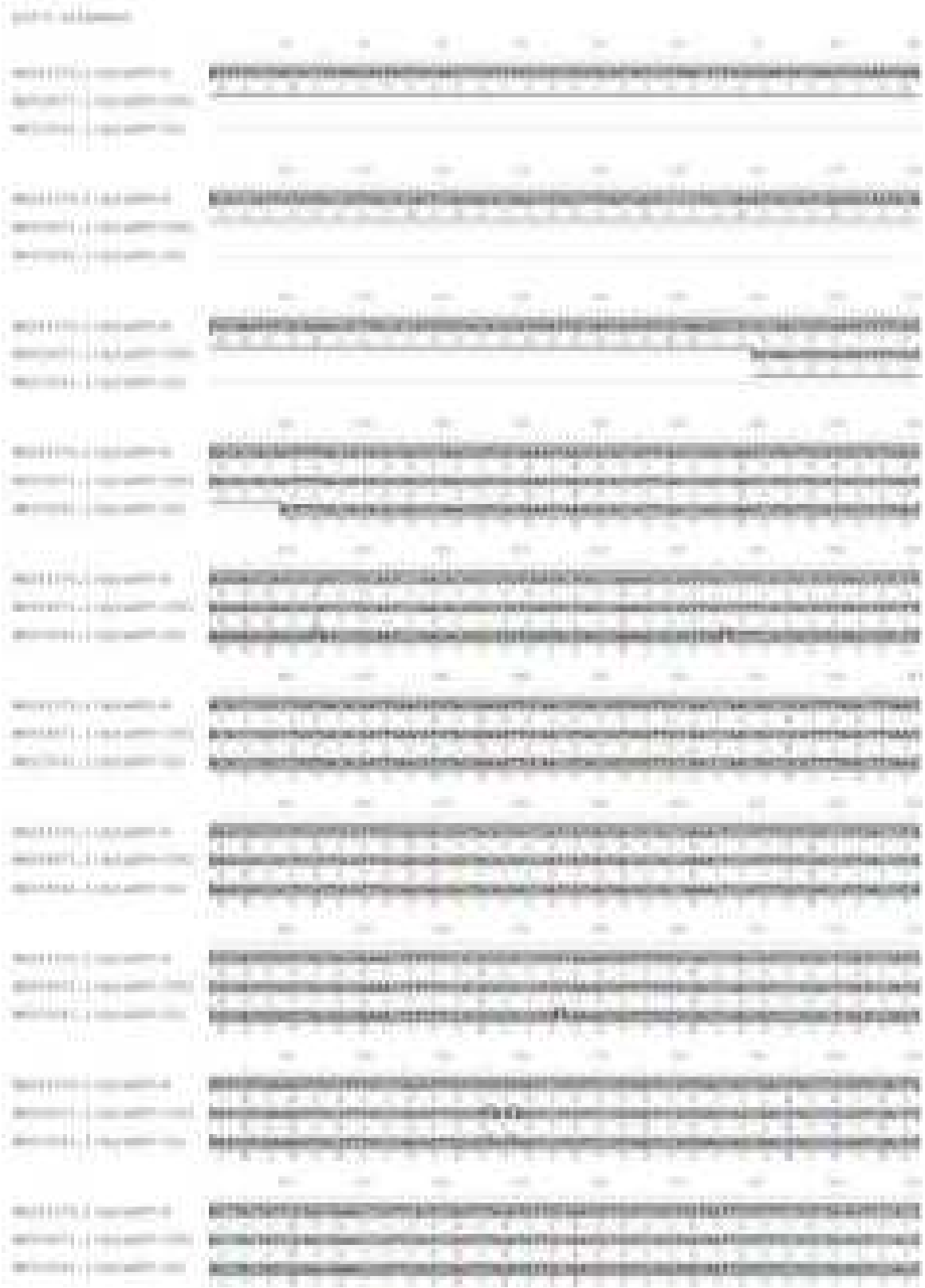


Figure 2-28: Nucleotide and translated amino acid sequence alignment of *pif-1* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as reference. Similar sequences were highlighted in grey background.

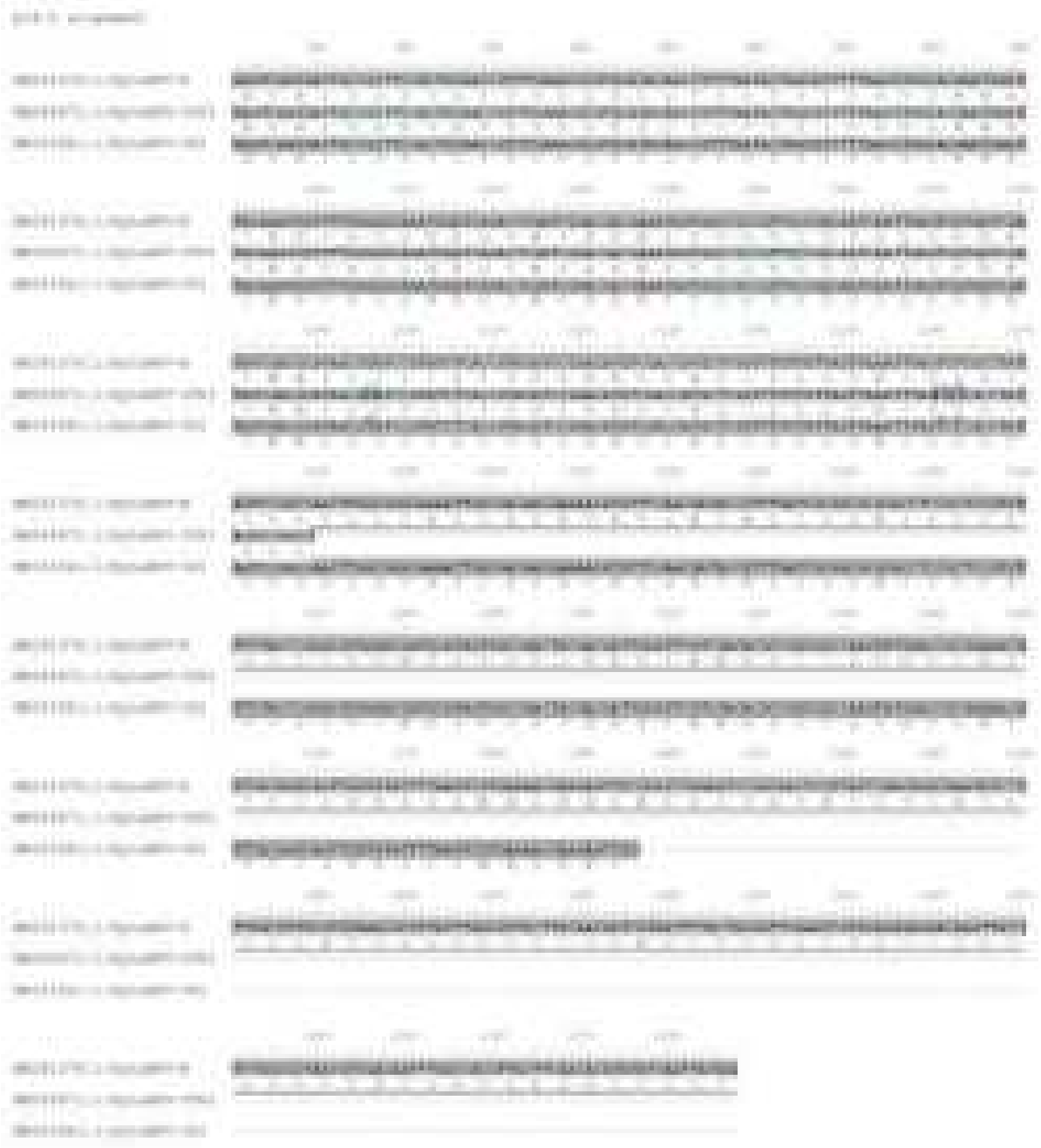


Figure 2-28: Nucleotide and translated amino acid sequence alignment of *pif-1* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as reference. Similar sequences were highlighted in grey background [Continued…].

2.4.5.3 Restriction sites on *pif-1* sequence

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

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

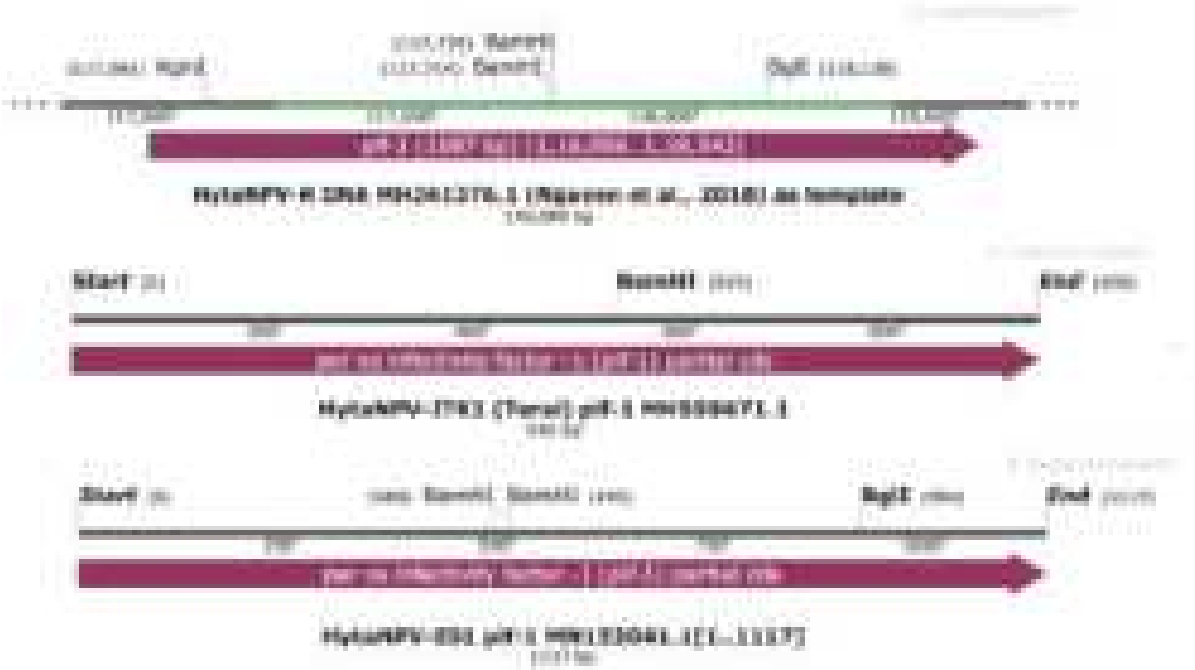


Figure 2-29: Restriction sites present in the *pif-1* of different HytaNPV isolates.

2.4.6 Sequencing of *pif-2* gene

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

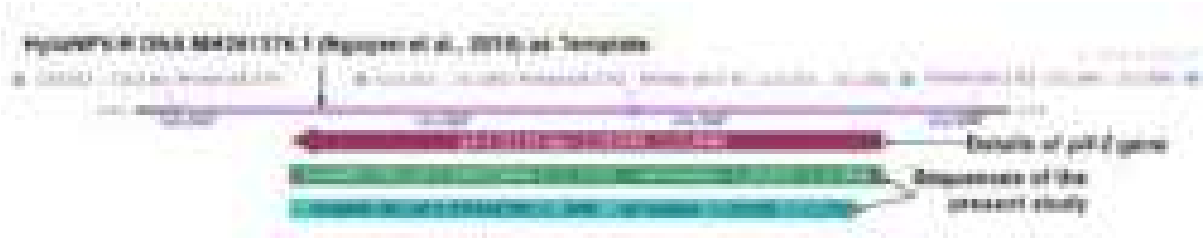


Figure 2-30: Primer binding, amplicons and sequence details of the *pif-2* gene using HytaNPV-R as a template.

2.4.6.1 Blast analysis

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

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

2.4.6.2 Alignments

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

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

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

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

Sequencing of pif-2 gene

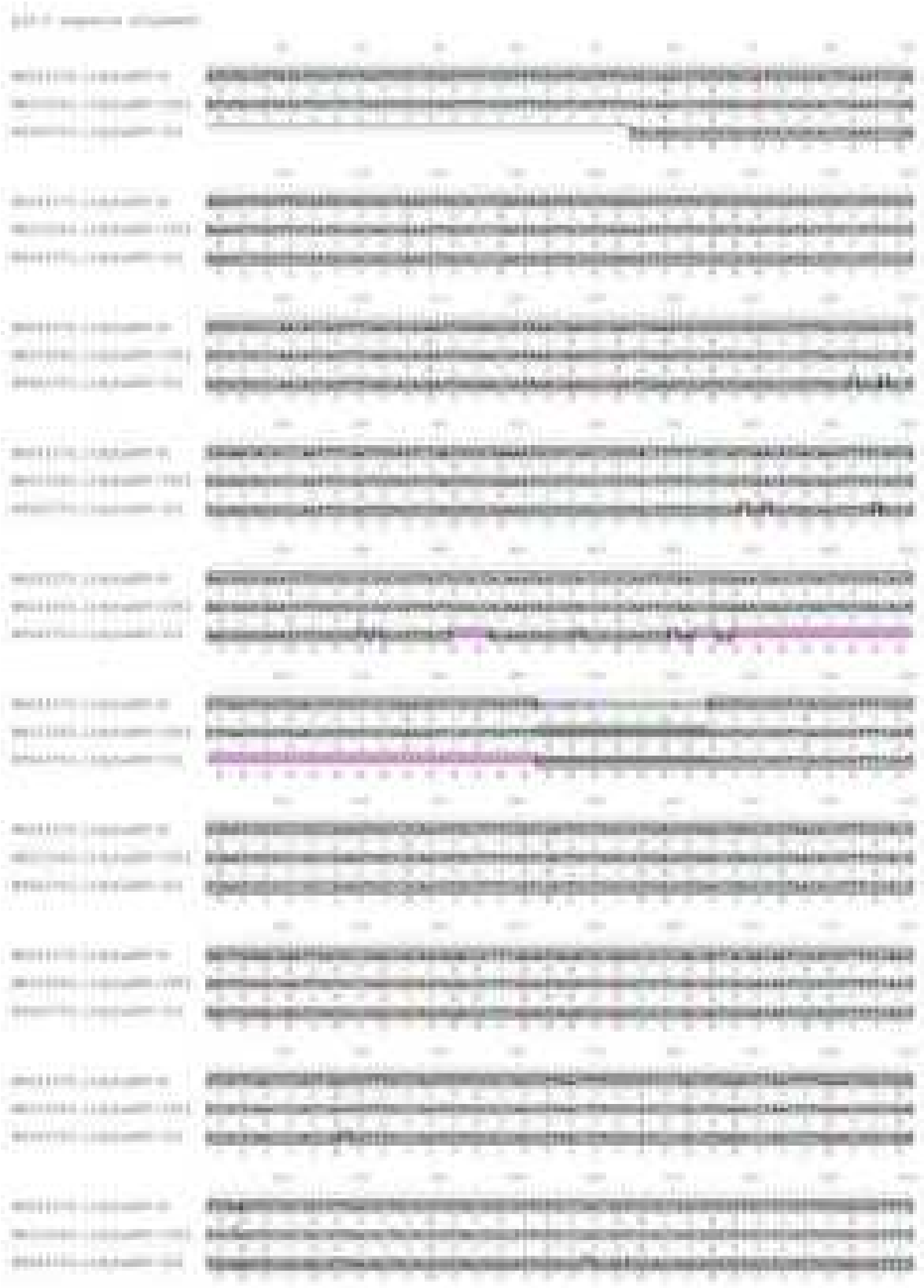


Figure 2-31: Nucleotide and translated amino acid sequence alignment of the *pif-2* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background.

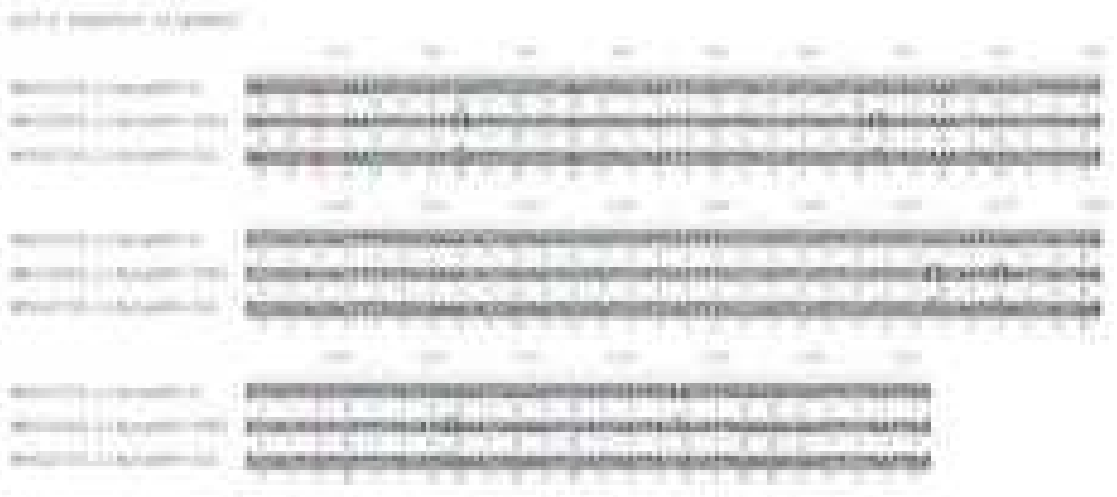


Figure 2-31: Nucleotide and translated amino acid sequence alignment of *pif-2* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as reference. Similar sequences were highlighted in grey background [Continued…].

2.4.6.3 Restriction sites on *pif-2* sequence

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

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

2.4.7 Sequencing of *pif-3* gene

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

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

2.4.7.1 Blast analysis

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

2.4.7.2 Alignments

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

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

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

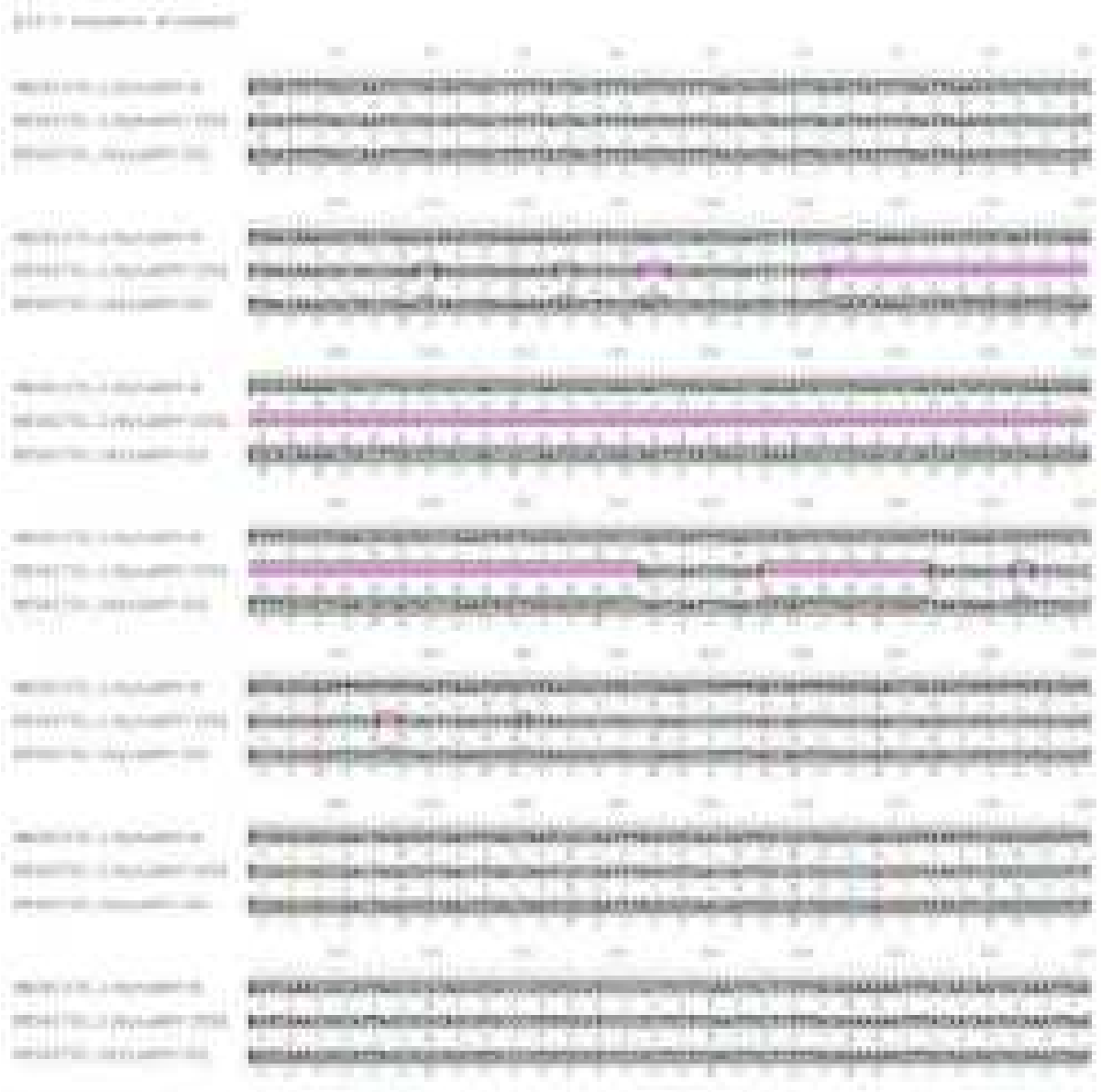


Figure 2-34: Nucleotide and translated amino acid sequence alignment of the *pif-3* gene of HytaNPV-ITK1 and HytaNPV-ID1 with HytaNPV-R as a reference. Similar sequences were highlighted in grey background.

2.4.7.3 Restriction sites on *pif-3* sequence

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

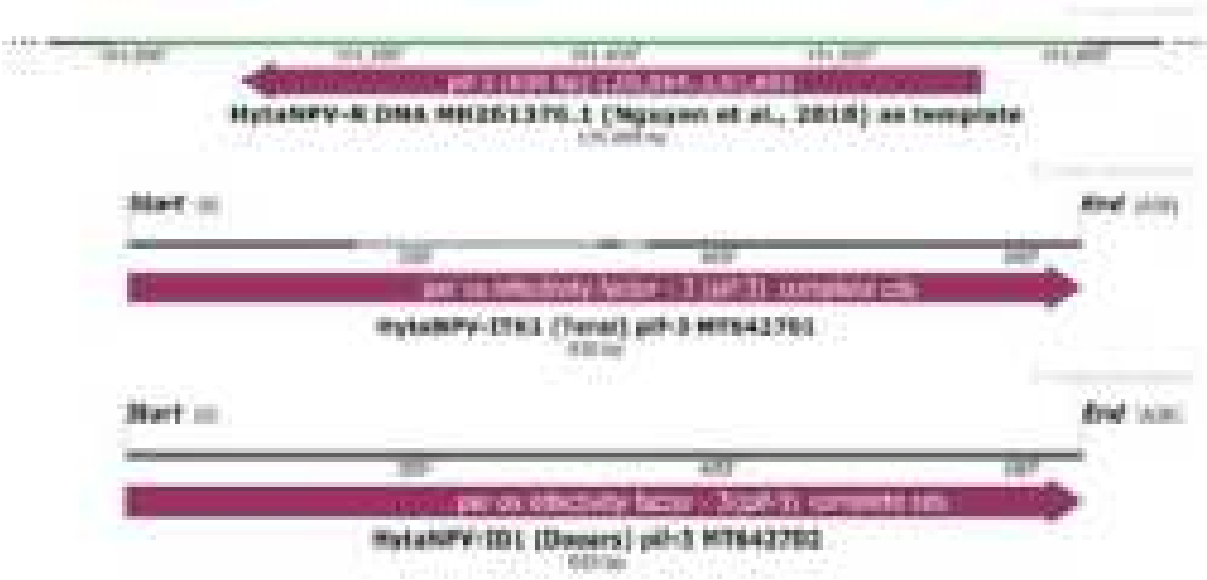


Figure 2-35: Restriction sites present in the *pif-3* of different HytaNPV isolates.

2.4.8 Sequence alignment analysis

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

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

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

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

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

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

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

2.4.9 Partial restriction maps of HytaNPV isolates

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

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

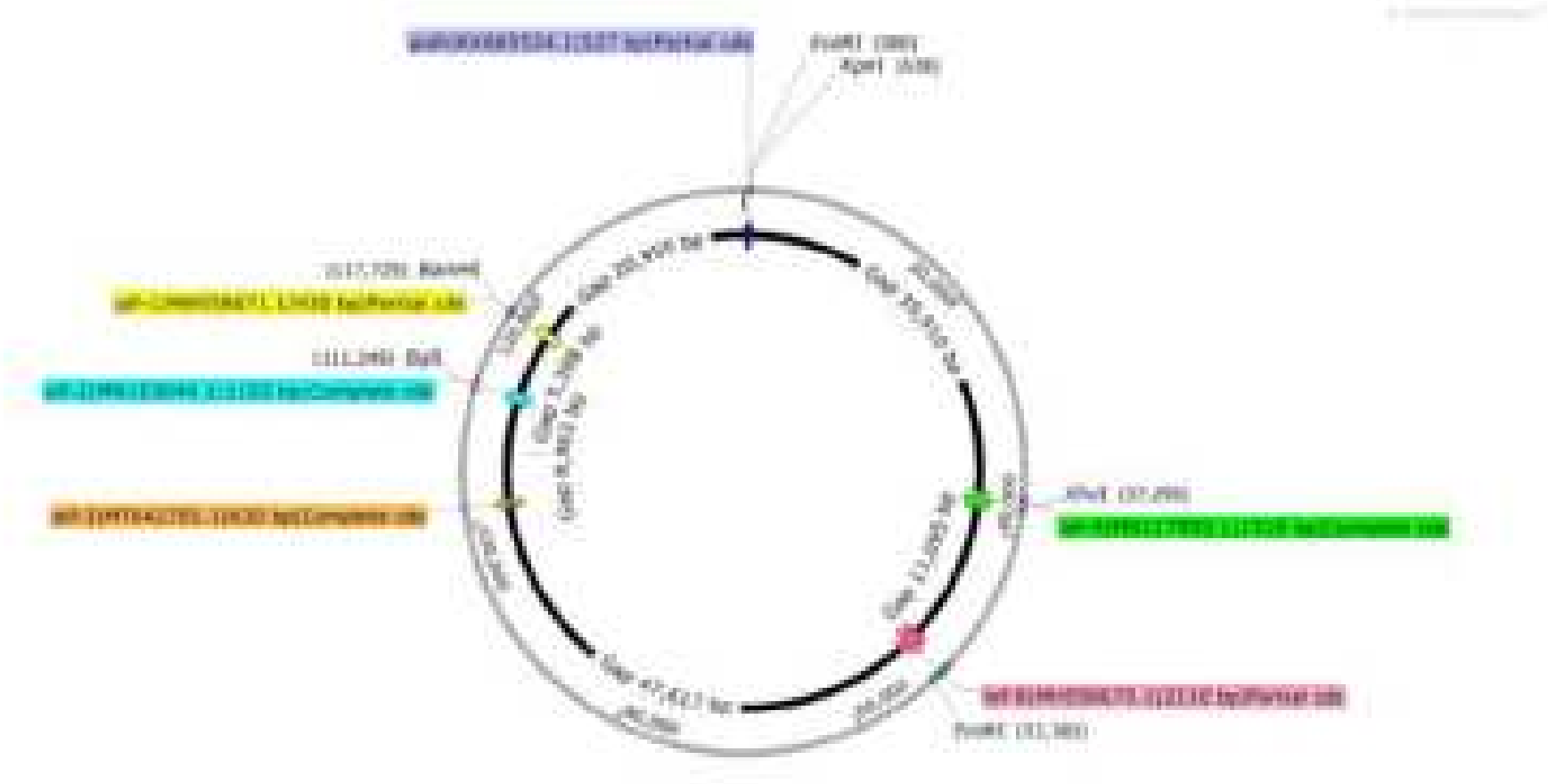


Figure 2-36: Partial restriction map of HytaNPV-ITK1 (Terai isolate) using HytaNPV-R (Nguyen et al., 2018) as a template.

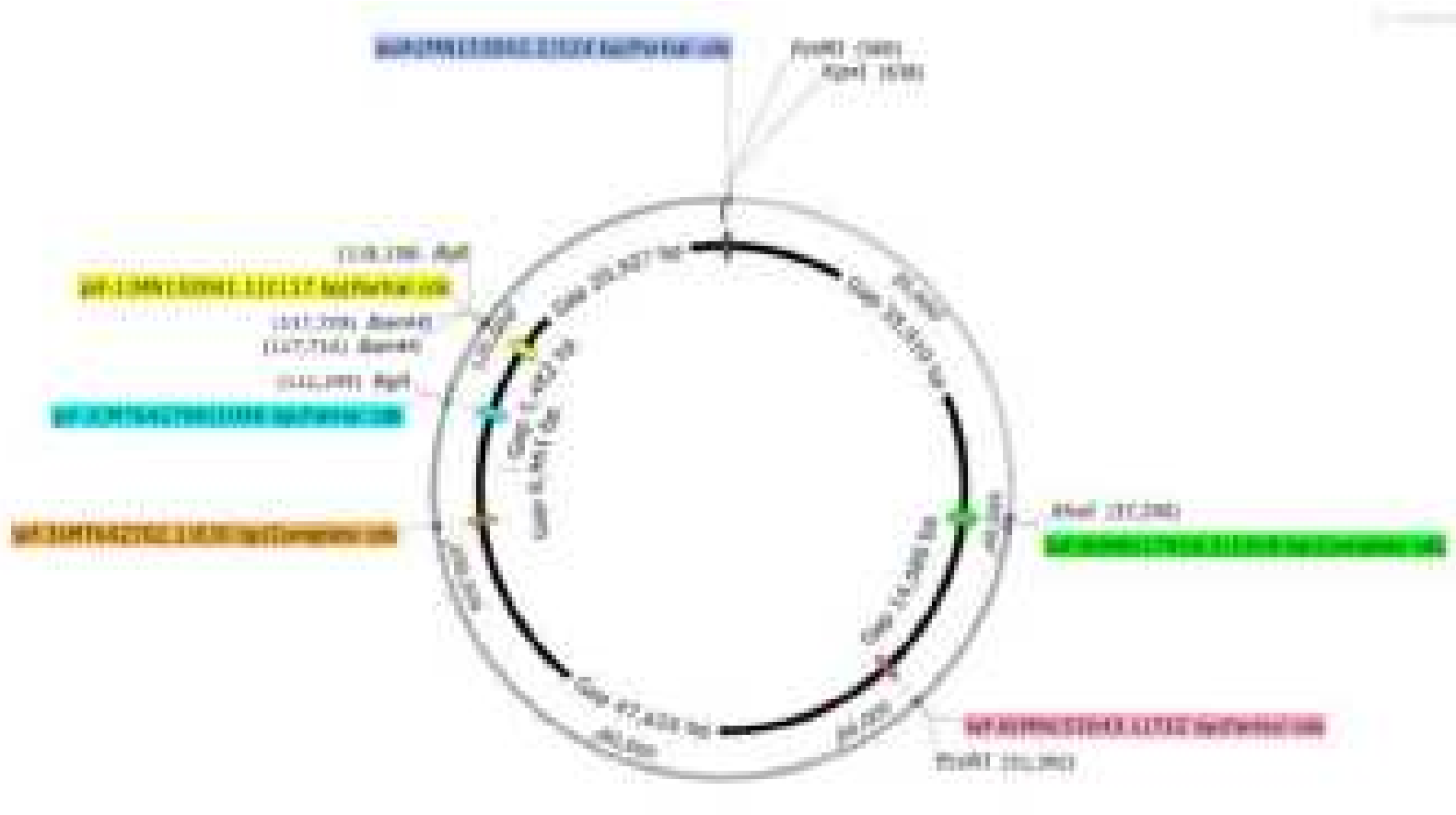


Figure 2-37: Partial restriction map of HytaNPV-ID1 (Dooars isolate) using HytaNPV-R (Nguyen et al., 2018) as a template.

Section 2.5: Phylogenetic Analysis

Objective

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

2.5.1 Phylogenetic analyses with 77 baculoviruses

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

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

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

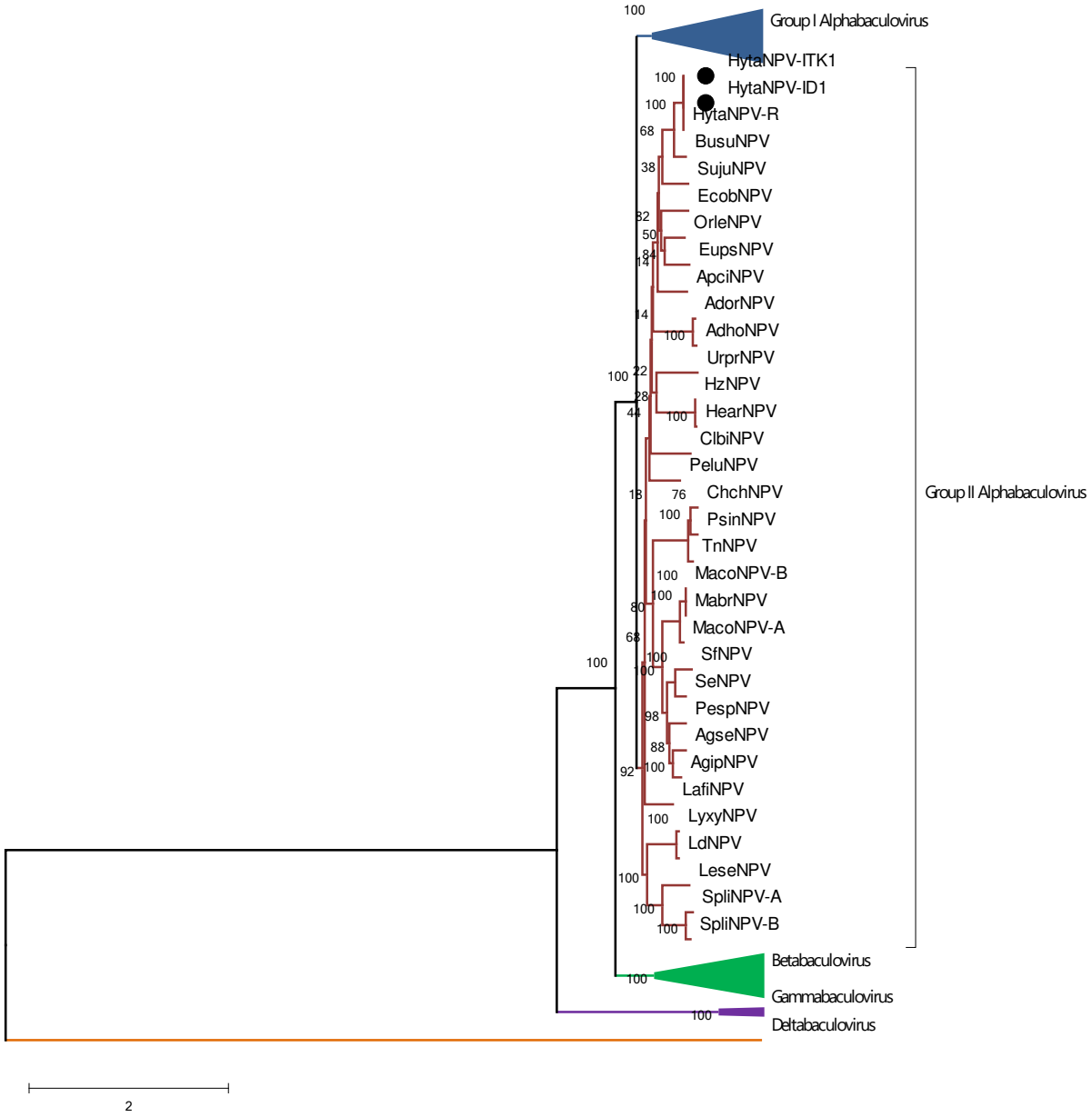


Figure 2-38A: Maximum likelihood tree based on concatenated sequence alignment of six genes together using nucleotide substitution. Bootstrap values were shown at each node. The Bar/scale represents the number of substitutions per site.

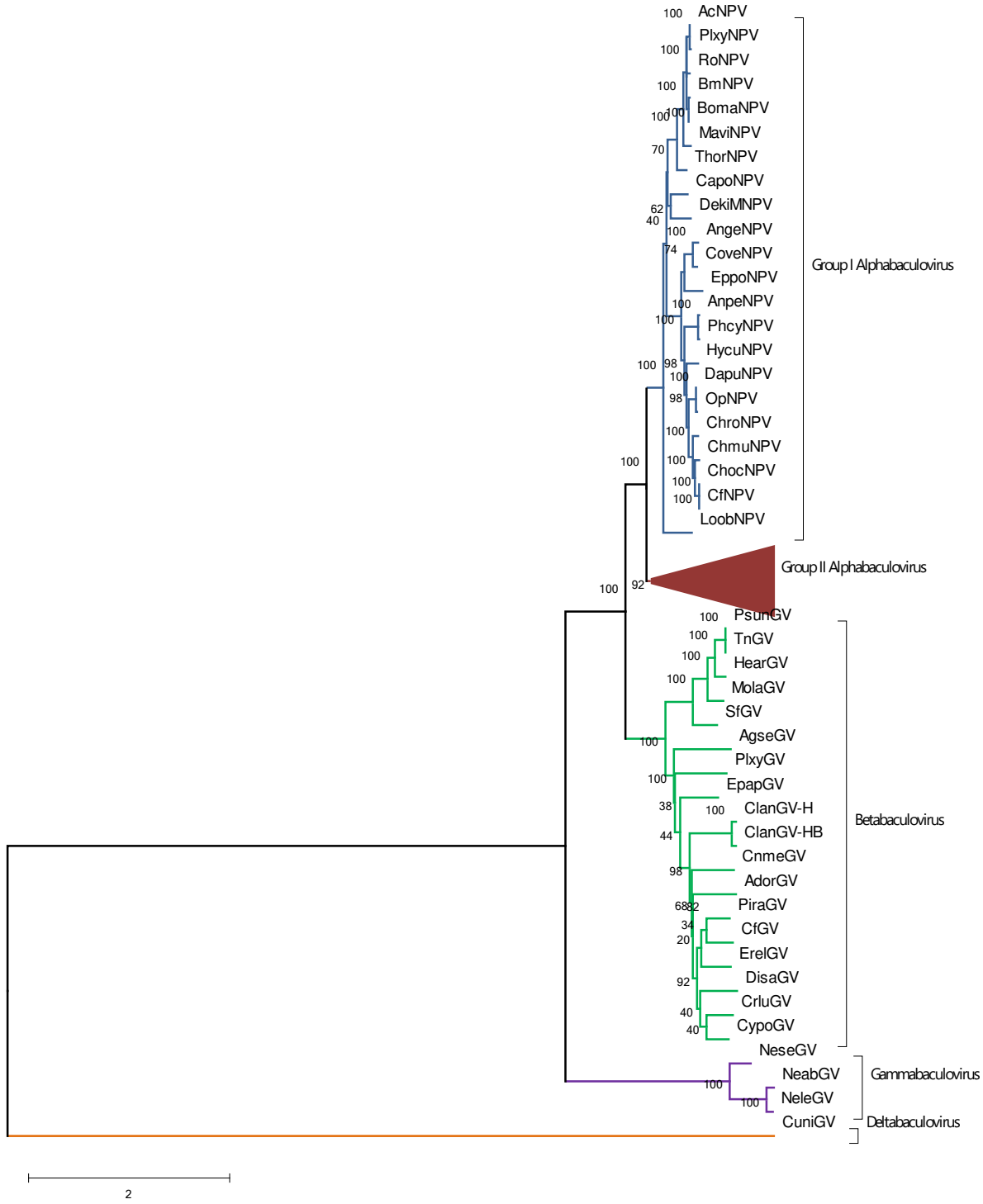


Figure 2-38B: Maximum likelihood tree based on concatenated sequence alignment of six genes together using nucleotide substitution. Bootstrap values were shown at each node. The Bar/scale represents the number of substitutions per site.

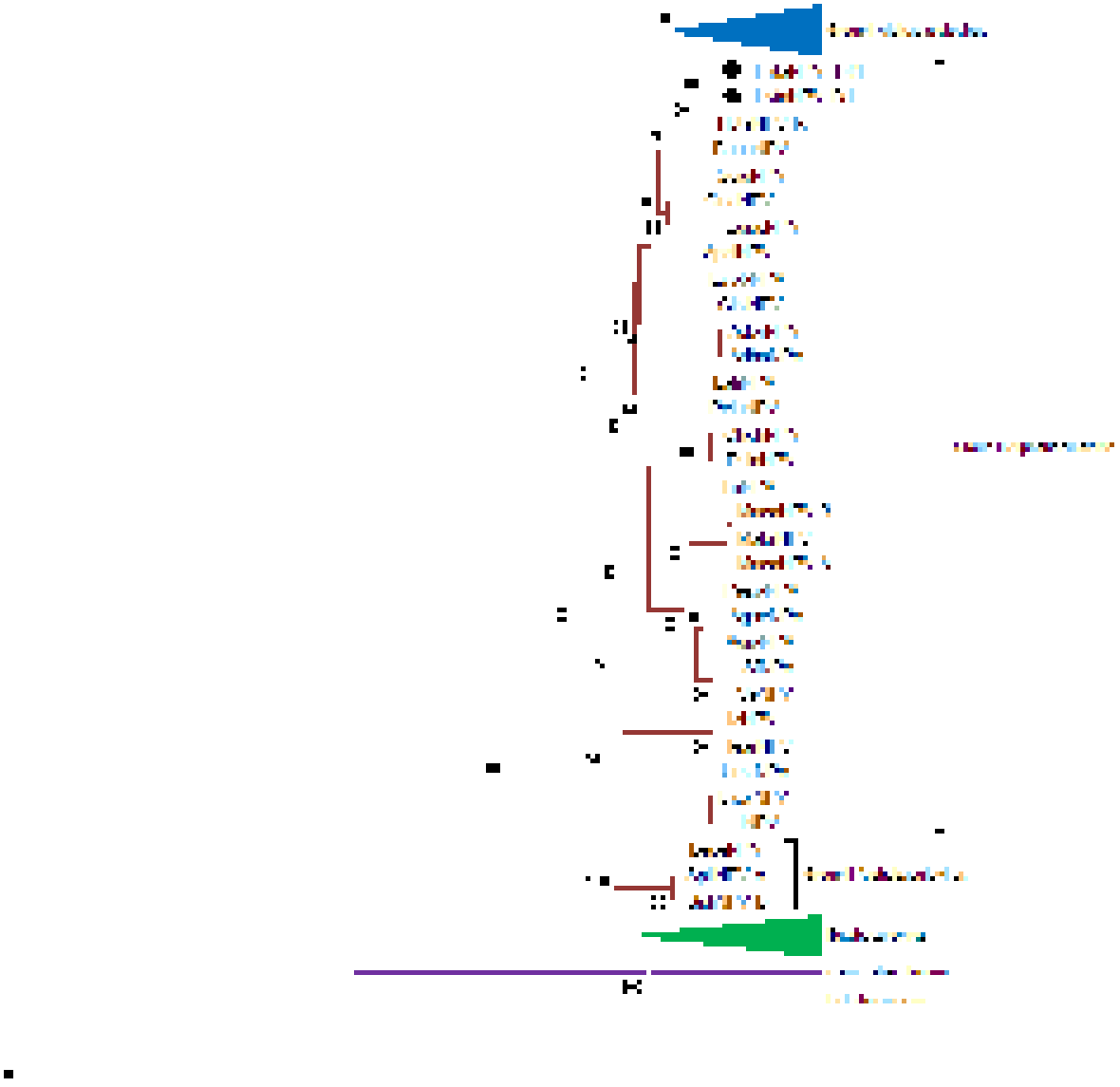


Figure 2-39A: Maximum likelihood tree based on concatenated sequence alignment of six genes together using amino acid substitution. Bootstrap values were shown at each node. The Bar/scale represents the number of substitutions per site.

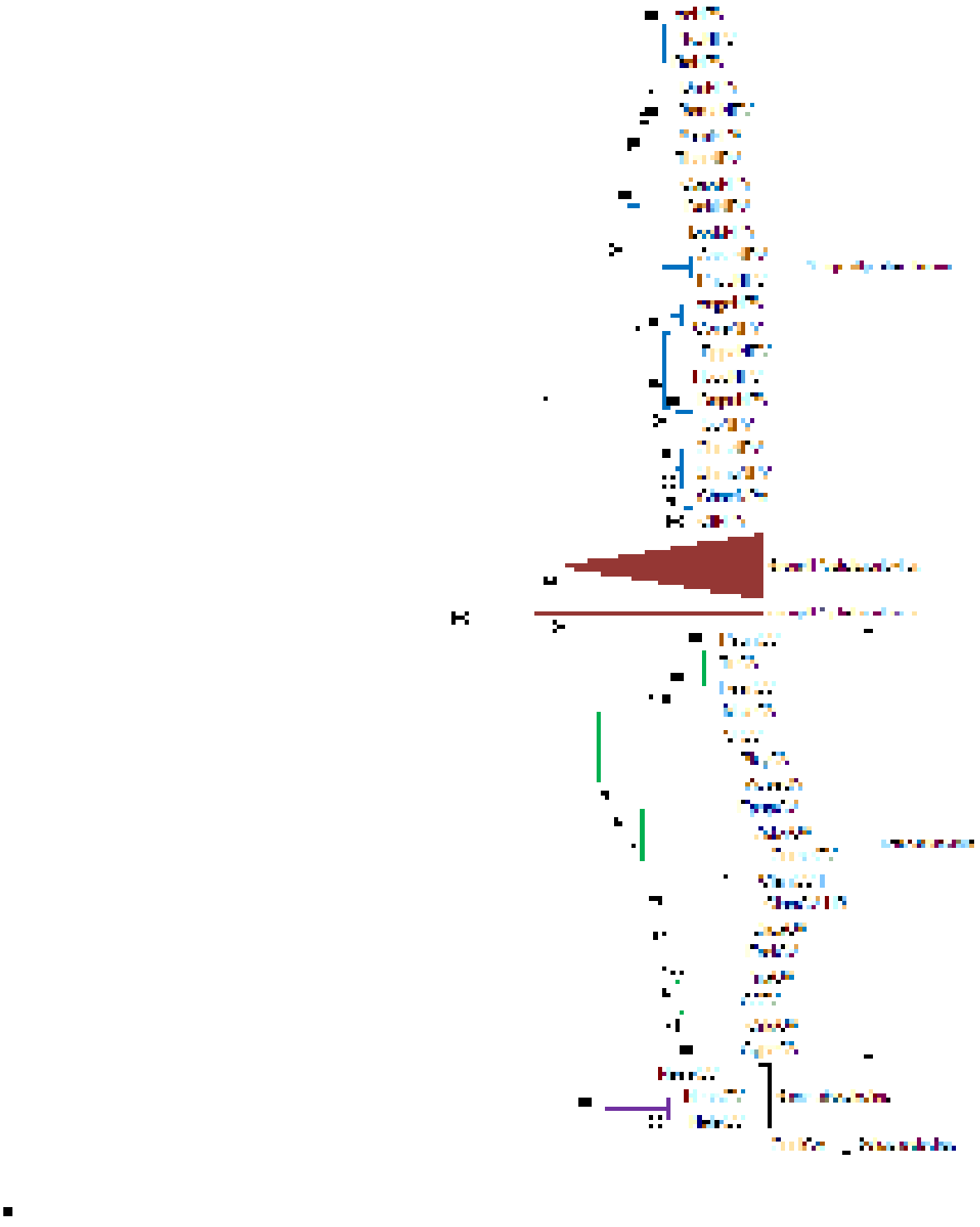


Figure 2-39B: Maximum likelihood tree based on concatenated sequence alignment of six genes together using amino acid substitution. Bootstrap values were shown at each node. The Bar/scale represents the number of substitutions per site.

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

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

2.5.2 Comprehensive phylogenetic analyses with 19 baculoviruses representing each group

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

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

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

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

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

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

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

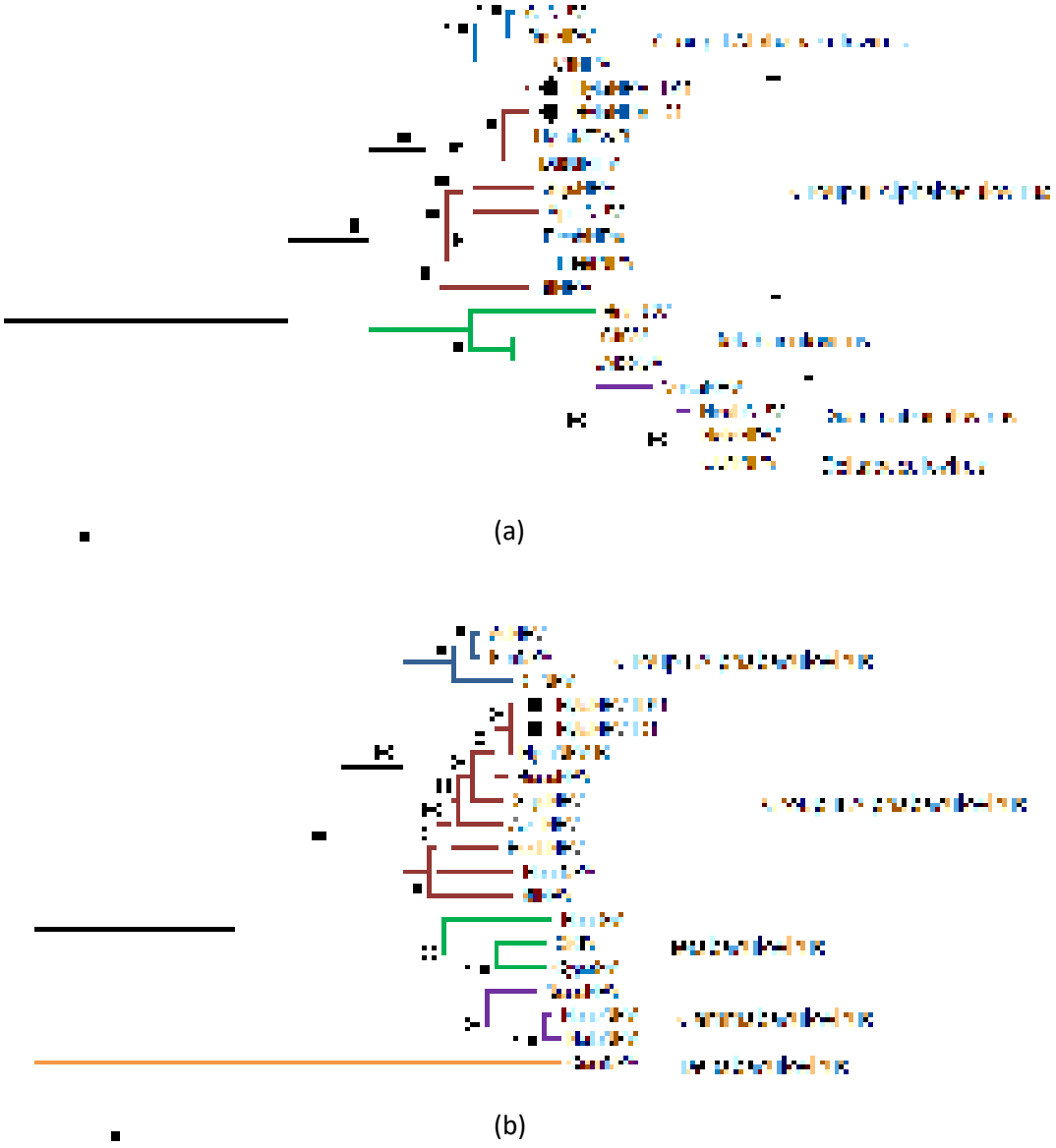
	Baculoviruses	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	HytaNPV-ITK1	*	1.818	1.625	2.527	1.063	1.045	1.109	0.948	0.928	1.022	0.986	0.862	0.747	0.781	0.722	0.668	0.697	0.678	0.577
2	HytaNPV-ID1	0.007	*	1.727	2.448	1.048	1.045	1.116	0.958	0.922	1.007	0.970	0.863	0.740	0.777	0.722	0.668	0.695	0.681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

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

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

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

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



(a)

(b)

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