Molecular Characterization of Nucleopolyhedrovirus of Three Lepidopteran Pests Using Late Expression Factor-8 Gene

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Abstract A PCR based method for detection of viral DNA in nucleopolyhedrovirus of three lepidopterans, *Spodoptera litura*, *Amsacta albistriga* and *Helicoverpa armigera*, was developed by employing the late expression factor-8 (*lef-8*) gene of three NPV using specific primers. The amplicons of 689, 699 and 665 bp were amplified, respectively, and the nucleotide sequences were submitted to GenBank and the accession numbers were obtained. The sequences of *lef-8* gene of *S. litura* NPV and *H. armigera* NPV matched with those of their respective references in the GenBank database, thereby confirming their identity, however, the sequence of *A. albistriga* NPV was the first sequence submitted to the GenBank database. The sequence similarity analysis between the three *lef-8* gene of NPV sequenced in the present study revealed that there was no significant similarity between them, however *A. albistriga* NPV and *S. litura* NPV were found to be closely

related. CLUSTAL alignment of the sequences generated revealed general relatedness among NPVs *lef-8* gene. The study confirmed that *lef-8* gene can be used for quick and correct discriminatory identification of insect viruses.

Keywords Nucleopolyhedrovirus · Late expression gene · Lepidopterans

Introduction

Nucleopolyhedrosis viruses (NPVs) comprise a genus in a family of occluded viruses (Baculoviridae) which have high specificity to insects, particularly Lepidoptera [14]. The NPV belong to subgroup ‘A’ of this family of viruses, are double stranded DNA viruses having rod shaped capsids, which multiply in the nuclei of the host cells. During late stages of infection, polyhedral inclusion bodies that are responsible for transmission of virus are formed [6]. The baculovirus has two distinct phenotypes in its contagion cycle; the budded virus that provides the dissemination of infection within insect tissues and occlusion virus spreading infection to other larvae. The infection leads to change of color and luster and ultimately rupture of cuticle and liquefaction of the larval body [19]. The earliest records of baculoviruses date back to the sixteenth century [22].

Gene expression in baculoviruses has been extensively studied in *Autographa californica* (Speyer) and four phases of expression classified were—immediate early, delayed early, late and very late [7]. Several studies have been conducted to identify the NPVs through molecular characterization around the world in which DNA fragment patterns from restriction endonuclease (REN) digestion of viral DNA have been used to demonstrate genetic variation

The nucleotide sequence data reported in this paper are available in GenBank Database under the accession numbers JF276035 for *Spodoptera litura lef-8*, JF290436 for *Amsacta albistriga lef-8* and JN591550 for *Helicoverpa armigera lef-8*.

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among different isolates. Polymerase chain reaction-based approaches that are more sensitive and less time consuming than REN analysis have been developed to classify baculoviruses [5, 10, 12, 24]. These approaches use degenerate oligonucleotides to amplify conserved regions from the genomes and allow for the use of freely available software to determine the extent of similarity and the translated protein sequence from amplified sequences.

There are several genes such as *egt*, *ac17*, *lef-2*, *polh*, *p35* and *16 s RNA*, which can be used for identification of the baculovirus [13, 18, 23], however, the late expression factor-8 gene (*lef-8*) of nucleopolyhedrovirus encodes the largest subunit of the virally encoded DNA-directed RNA polymerase specific for the transcription of late and very late viral genes,

In our study, hot-start PCR, a technique that reduces non-specific amplification during the initial set up stage of the PCR, was adopted to amplify conserved region of the late expression gene, *lef-8*. The *lef-8* gene has been amplified from baculovirus that infect many insect species as a tool for identification of viruses. The *lef-8* is a highly conserved region which encodes the largest subunit of the virally encoded DNA-direct RNA polymerase specific for the transcription of late and very late viral genes and is used to characterize in most of the lepidopteran NPVs [1, 4, 21].

In the present study, three insects, *Helicoverpa armigera* (Hübner), *Spodoptera litura* (Fabricius) and *Amsacta albistriga* (Walker) were collected on tomato, beetroot and peanut fields in Bangalore, India and Pavgada in Tumkur, India. These insects cause extensive crop loss and require extensive insecticide application [2, 17]. Baculoviruses have great potential as biological insecticides, causing epizootics in insect populations to give substantial/regional protection [20]. The NPV were isolated from these insects and used in the study.

In this study, comparison of *lef-8* sequences and subsequent translated protein sequences and a technique using hot-start PCR to identify the viruses could be used for quick and correct identification of insect viruses.

Materials and Methods

Insect Culture and Maintenance

The larvae of three species of insects, viz, *H. armigera* from tomato field in Malur (Kolar district) 13°01'16"N, 77°56' 17"E during October 2011; *S. litura* from beetroot field in Malur (Kolar district) during October 2011 and *A. albistriga* from groundnut field in Pavgada (Tumkur district) 14°06'16"N, 77°16'E during August–September 2011, were collected. The colonies of the first two species were maintained on artificial diet [15], while *A. albistriga*

was maintained on bouquets of groundnut plants that were surface sterilized with 1 % sodium hypochlorite solution. The entire rearing protocol was done under controlled conditions in Biological Oxygen Demand (BOD) incubators maintained at 27 ± 0.5 °C and 60 % RH. All the three species were reared for two generations before multiplying virus on them. The wild-type virus, isolated from diseased larvae of these species, was multiplied on larvae collected from the field.

Virus Purification, Isolation and Production

The original virus isolated was obtained from diseased larvae collected from the fields. The Occlusion bodies (polyhedra) from the infected larvae were purified by centrifugation at 2,500 rpm once for 1 min. The debris was pelleted whereas the OB remained in the supernatant. For the isolation of virus, the suspension was again subjected to centrifugation at 10,000 rpm twice for 5 min each time. The OB settled at the sides of the walls of the centrifuge vial. This process was repeated to isolate only the OBs leaving behind the contaminants. This pellet was then suspended in 1 ml of double distilled water and stored at -20 °C for further use. The virus obtained from the field collected diseased larvae were further confirmed by infecting them on respective hosts in order to determine their specificity. None of the three viruses were cross infecting.

Isolation of Viral DNA

DNA extractions were performed using the traditional 0.1 M sodium carbonate solution. The final suspension included 0.5 mg ml^{-1} of proteinase K and 1 % SDS. This was followed by phenol chloroform: isoamylalcohol extraction with the final ethanol precipitation (Woo, 2001). The DNA was resuspended in a TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The extracted DNA was visualized in 0.4 % agarose gel.

PCR Amplification and Sequencing

The coding region of the late expression factor gene, the *lef-8* gene, which is highly conserved among the NPVs, was targeted as template DNA. Primer sets for the amplification of *lef-8* gene were designed as species-specific primers according to the *HaNPV* and *SINPV* sequences in the Genbank database of National Centre for Biotechnology Information. Each PCR mixture consisted of a 50 ng DNA sample, 0.25 U of *Taq* DNA polymerase, $10\times$ *Taq* buffer, 2.5 mM MgCl_2 , 2.5 mM each of four dNTPs and 1 μl of each forward and reverse primer in a final volume of 50 μl . Since a hot-start PCR approach was made, *Taq* DNA polymerase was added to the final volume after the

denaturation temperature in the PCR cycle. The cycling parameters for PCR were as follows: cycle 1, 4 min at 95 °C, cycle 2–35, 2 min at 95 °C, 2 min 46 °C and 1 min at 72 °C, Cycle 36, 2 min at 72 °C [20]. Amplification was accomplished using a DNA thermal cycler (Biorad C1000 machine). The PCR products were analyzed by a 0.9 % agarose gel along with the MassRuler™ Low Range DNA Ruler (Fermentas). After the PCR, the gel was stained with ethidium bromide and photographed using UV light.

Gene Sequence and Protein Sequence Comparison

The PCR products were purified before sequencing and DNA sequencing was performed by Scigenome Laboratory, India. The sequences obtained through our studies were compared with those of the existing GenBank database. DNA and protein comparisons with entries in the sequence databases were performed using selected BLAST programs. While comparing for sequence homology, the *Ha*NPV *lef-8* and *S*NPV *lef-8* sequences obtained from our study were compared with the existing *Ha*NPV *lef-8* sequences and *S*NPV *lef-8* sequences in the GenBank database. However, *A. albistriga* NPV *lef-8* sequence, being the first ever to be submitted did not have any similar database to be compared with, although there are GenBank files for egt, ac17, *lef-2* and *polh* genes. Comparison was also made among the three sequences (*Ha* NPV *lef-8*, *Sl* NPV *lef-8* and *Aa* NPV *lef-8*) from our study to analyze the extent of similarity for which, multiple sequence alignments were performed with a DNA analysis program, LASERGENE (DNASTAR). In all these comparison tools, percentage identity indicates the percentage of identical residues between the multiple sequences. The comparative table for the three *lef-8* sequences was made by analyzing the extent of similarity each sequence had with its respective database.

Phylogenetic Analysis

Phylogenetic analysis of *lef-8* was performed to study taxonomical relationship among the three species. This was determined by the Mega 4 (DNASTAR) tool using the neighbor joining method.

Results

Sequence Analysis of *Lef-8* Gene

The species-specific primer designed using the FastPCR online tool amplified a fragment size of 650–700 bp in all the three species, viz., *H. armigera*, *S. litura* and *A. albistriga* (Fig. 1). The nucleotide sequence of *lef-8* and its deduced amino acid sequence were submitted to the

GenBank with the accession numbers JF276035, JF290436 and JN591550, respectively, for *S. litura*, *A. albistriga* and *H. armigera*. Single open reading frames (ORF) that could encode polypeptides of 247, 261 and 240 amino acids were detected for the respective sequences. No stop codon was observed all over the amino acid sequence.

BLAST analysis indicated that *H. armigera* NPV *lef-8* (JN591550) and *S. litura* NPV *lef-8* (JF276035) sequences obtained in our studies were similar to the GenBank databases with an identity value of 99–98 % and an expect value of 0. When the sequences, of *H. armigera* NPV *lef-8* and *S. litura* NPV *lef-8* were compared to each other, there was no similarity observed indicating that the sequence is highly conserved across the species. Although *A. albistriga* NPV *lef-8* could not be analyzed for similarity in the GenBank, the sequences were aligned with the *H. armigera* NPV *lef-8* and *S. litura* NPV *lef-8* sequences obtained from our isolates. One striking feature observed was that *A. albistriga* NPV *lef-8* gene was highly similar to the nucleotide sequence of *S. litura* NPV but not to the *H. armigera* NPV *lef-8* gene.

Base pair size of *S. litura* NPV *lef-8* sequence was similar to the sequences with accession numbers AY706581.1 (743 bp), AB326103.1 (700 bp) and AY706580.1 (677 bp) (Table 1) including the sequences with the accession numbers AB451186.1 (227 bp) and AB451187.1 (446 bp), which had even more partial sequence. All these sequences were similar to our sequence in the range of 99–98 %. As for the *H. armigera* NPV *lef-8* sequence, the comparison was done with the complete sequence (Accession no. AY118080.1) which is of 2,705 bp, yet had 99 % identity. The other sequences (FJ157290.1, FJ157289.1, FJ157288.1 and FJ157287.1) of 744 bp size were similar to our sequence with 98 % similarity.

The precise alignment among the three *lef-8* sequences of the insect species under study clearly indicates that the *S. litura* NPV *lef-8* and *A. albistriga* NPV *lef-8* gene have a very high percentage of identity with each other but *H. armigera* NPV *lef-8* gene aligns only at few bases with the other two species (Fig. 2).

The nucleotide sequence of *A. albistriga* NPV is the first ever sequence submitted to the GenBank and thus, our isolate was identified as “*Amsacta albistriga* NPV *lef-8* like gene” in the NCBI database.

Fig. 1 Hot- start PCR detection based on *lef-8* gene at 700 bp (M = Marker (M):100 bp ladder, lane 1 = *S. litura lef-8* NPV gene; Lane 2 = *A. albistriga lef-8* NPV gene; Lane 3 = *H. armigera lef-8* NPV gene; Lane 4 = negative)

Table 1 Analysis of % identity of the obtained sequences with their respective database in GenBank (the accession numbers are listed according to the extent of similarity with our results)

Species	Base	Homologous database in GenBank with their base pair size and % identity		
	Size (bp)	Accession numbers	Base pair size	% identity
<i>Spodoptera litura</i>	689	AB326103.1	700	99
NPV <i>lef-8</i>		AB451186.1	277	99
		AY706580.1	677	98
		AB451187.1	446	98
<i>Helicoverpa armigera</i>	665	AY118080.1	2,705	99
NPV <i>lef-8</i>		FJ157290.1	744	98
		FJ157289.1	744	98
		FJ157288.1	744	98
		FJ157286.1	744	98
<i>Amsacta albistriga</i>	699	Closely related to <i>Spodoptera litura</i> NPV <i>lef-8</i> Sequence		

Protein Sequence Analysis of *Lef-8* Gene

The nucleotide sequences were translated to their respective amino acid sequences using JustBio, a suite of online tools for analysis of DNA/RNA, proteins and arrays. This tool translates the nucleotide sequence to its respective amino acid sequence in three different frame shifts. The frame having sequence with no stop codon was chosen for subsequent analysis. These sequences, devoid of any stop codons were also subjected to the same protocol of comparisons with protein databases in the GenBank. The results were similar to that of the genetic data and analysis showed clearly that the protein encoded by the *H. armigera* NPV *lef-8* gene is different from those encoded by *S. litura* NPV *lef-8* and *A. albistriga* NPV *lef-8* gene, which are similar (Fig. 3).

Appropriate searches of protein databases showed that the *lef-8* proteins were also highly conserved among all baculoviruses whose genomes have been sequenced so far and thereby supported the generic data obtained. High identities between these isolates suggested that *lef-8* was well conserved in baculovirus genomes and an ideal candidate gene for molecular identification.

Sequence Analysis of *Lef-8* Gene

The phylogenetic analysis resulted in clustering the *lef-8* genes into two distinct clades wherein *Sl* and *Aa* *lef-8* genes belong to the same clade with *Ha* *lef-8* gene in different clade (Fig. 4). The analysis is supported by the bootstrap values for the branches as indicated in the figure. This

phylogenetic tree showed that *Spodoptera litura* and *Amsacta albistriga* NPV *lef-8* are closely related but not *Helicoverpa armigera* NPV *lef-8*.

Discussion

Molecular methods based on PCR and DNA sequences of PCR products can greatly reduce the amount of time needed for identifying unknown baculovirus isolates. According to De Moraes et al. [5], PCR is a highly sensitive technique that amplifies target DNA sequences, and viral DNA obtained from infected larvae is a good source of DNA for PCR amplification. The sensitivity of the PCR for baculovirus amplification has been also reported [3].

The *lef-8* gene that encodes the largest subunit of the virally encoded DNA-directed RNA polymerase specific for the transcription of late and very late viral genes is an integral part of the NPV genome and *lef-8* has been identified in all sequenced baculoviruses [10], which makes it an ideal gene. The good conservation of *lef-8* in baculoviruses was also reported according to the sizes of *lef-8* and the high identity values detected between some of the NPV [23]. Hefferon [9] reviewed the baculovirus *lefs* and their roles in viral replication, late gene expression and determination of baculovirus host range.

Several studies have been reported wherein *lef-8* gene has been the subject of study for the characterization as well as for examining the relationships in the insect species. Zhang et al. [25] designed a gene-specific primer and amplified the *lef-8* gene in *Lymantria dispar* and deduced its amino acid sequence. They were compared with the GenBank database and the phylogenetic relationship was studied. Jehle et al. [11] reported that the PCR amplification of highly conserved regions of late expression factor 8 (*lef-8*) provides a powerful tool to identify lepidopteran-specific baculoviruses and to study their diversity.

A very recent study on nucleopolyhedroviruses associated with Antler moth *Cerapteryx graminis* also proved that concatenated sequences of baculovirus *lef-8* are suitable markers to mirror viral speciation [8, 16] used *lef-8* to characterize, analyze the genetic variation and examine the relationships of 90 isolates of NPVs from heliothine pests.

Our PCR studies resulted in sequencing amplicons of 689, 699 and 665 bp region for *S. litura*, *A. albistriga* and *H. armigera* *lef-8* genes, respectively, which is similar to the work done by Toprak and Gurkan [20], where they were able to amplify 714 bp region of *S. littoralis* (Boisd.) partial *lef-8* gene. The BLAST analysis showed that *S. litura* NPV *lef-8* sequence exhibits sequence homology with “*Spodoptera litura* NPV isolate S37 *lef-8* gene, partial cds” (Acc. No. AY706581.1), with an identity value of 99 %. *A. albistriga* *lef-8* sequence was closely related to “*Spodoptera litura* NPV

Fig. 2 Similarity between the sequence alignments of the *lef-8* gene of the *S. litura* NPV, *A. albistriga* NPV and *H. armigera* NPV using LASERGENE (DNASTAR) program. The shaded region indicates

the aligned region among the three sequences. The alignment is clearly visible in *S. litura* and *A. albistriga* NPV sequences with very few scattered alignments in *H. armigera* NPV.

isolate S37 *lef-8* gene, partial cds" (Acc. No. AY706581.1) also. Similarly, *H. armigera lef-8* sequence, with *Helicoverpa armigera* nuclear polyhedrosis virus late expression factor 8, Orf39-like protein, Orf40-like protein, and chitinase genes, complete cds (Acc. No. AY118080.1), with an identity value of 99 %. Since this was a complete cds, we further compared with the other partial cds in the GenBank database and observed 98 % identity with *Helicoverpa armigera* NPV strain Jodhan putative DNA directed RNA polymerase subunit (*lef-8*) gene, partial cds (Acc. No. FJ157289.1).

Our protein sequences were compared with the translated protein queries of the same nucleotide GenBank database with which our nucleotide sequences were identical. The results supported the genomic data in terms of their percentage similarity. Hence, there was 99, 98 and 98 % similarity in the *S. litura* NPV *lef-8*, *A. albistriga lef-8* and *H. armigera lef-8* sequences, respectively. Furthermore, Lange et al. [12] highlighted that the virus DNA used for PCR amplification was obtained by complete DNA isolation of virus-infected larvae, indicating that insect

Fig. 3 Similarity between the deduced amino acid sequences of the *lef-8* gene of *A. albistriga*, *S. litura* NPV and *H. armigera* NPV using LASERGENE (DNASTAR) program. The shaded region indicates

the aligned region among the three amino acid sequences. The alignment is clearly visible in *S. litura* and *A. albistriga* NPV sequences with very few scattered alignments in *H. armigera* NPV

Fig. 4 Phylogeny of the *lef-8* gene using Mega 4 tool. Branch lengths were determined by the neighbour-joining method. SI 1, Ha1 and Aa1 with the accession numbers are the *lef-8* gene sequences obtained in our study whereas SI 2, SI 3, Ha 2 and Ha 3 with accession numbers are the *lef-8* sequences retrieved from the GenBank database.

DNA as a background neither disturbed the reaction nor resulted in false-positive amplification products.

As a result of this study, the procedure based on PCR and nucleotide sequences of *lef-8* gene proved to be an easy and reliable way for identifying baculovirus isolates and revealed that *lef-8* gene can be used for quick and correct identification of insect viruses.

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