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Molecular and Biological Characterization of a Nucleopolyhedrovirus Isolate (Egy-*Sl*NPV) from *Spodoptera littoralis* in Egypt

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Abstract The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) is an important pest causes extensive economic losses in many cultivated crops all over the world. The viruses within the family Baculoviridae are specific pathogens of insects. The nucleopolyhedroviruses (NPVs), 1 of the 2 genera of this family, have been isolated from many insect orders, primarily from the lepidopterans. In our study, NPV was isolated from the larvae of *S. littoralis* in Egypt. The viral occlusion bodies (VOBs) were detected using a light microscope by staining thin smear of infected larvae and drop of VOBs with Giemsa stain which appear polyhedral and negative stain particles. The toxicity of this isolate against *S. littoralis* also studied, by testing five different concentrations from the occlusion bodies produced by this virus isolate against 2nd and 4th instar larvae of *S. littoralis.* and the results indicated that the 2nd instars larvae was more susceptible to all concentrations than the 4th instars larvae, evidenced by the very low LC_{25}, LC_{50}, LC_{75} and LC_{90} values. The LC_{50} calculated from toxicity experiment used in treatment 2nd and 4th insatr larvae to study the biological properties of this isolate. For the molecular identification of the virus, polyhedra (occlusion bodies [OBs]) were isolated from insects and total DNA extraction was performed. Polyhedrin gene of *S. littoralis* NPV was partially amplified by PCR and its nucleotide sequence was determined. An Open Reading Frame (ORF) of 138 nucleotides was detected. Multiple sequence alignment and phylogenetic analysis were performed to compare Egy-*Sl*NPV-*polh* gene with other 55 *polh* genes sequences from various nucleopolyhydroviruses (NPVs) and with 15 granulin genes from granuloviruses (GVs) available in GenBank. Neighbour-joining phylogeny of the nucleotide sequence and deduced amino acid sequences, clearly showed that our Egy-*Sl*NPV isolate belongs to subgroup II-B NPVs, which is defined to include the spodopteran taxa SpliNPV, SpltNPV and SpteNPV. In case of the nucleotide sequence, the Egy-*Sl*NPV isolate showed the close relationship with SpliNPV isolates, wihle in case of deduced amino acid sequence showed the close relationship with SpliNPV, SpltNPV and SpteNPV solates. The change of nucleotide of Egy-*Sl*NPV at position number 76 resulted in the difference at one amino acid position with group II-B NPVs isolates (SpliNPV, SpltNPV and SpteNPV) viz. at position number 26 [methionine (M) is present in place of valine (V) a hydrophobic amino acid]. The *polh* gene nucleotide sequence of the Egyptian isolate (Egy-*Sl*NPV) was registered under GenBank accession number KY072799. This study suggested the Egy-*Sl*NPV isolate may be useful as a potential biocontrol agent in program of integrated pest management (IPM).

Keywords *Spodoptera littoralis*, Toxicity, Molecular phylogeny, Nucleopolyhedrovirus, SpliNPV

1. Introduction

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd) is one of the most notorious and destructive phytophagous insect pests in Egypt, not only to cotton, but also to other field crops and vegetables [1]. These caterpillars are very polyphagous, causing important economic losses in both greenhouses and open field in a broad range of ornamental, industrial and vegetable crops. The problems and hazards that have arisen as a result of using conventional insecticides were incentives for the search of alternative control agents. Microbial control agents are a primary means of biological control of insect pests. The use of microbial control agents, targets for a particular pest species. The entomopathogens that have mostly been used in biological control include representatives of bacteria, fungi, viruses and nematodes [2, 3].

Baculoviruses are considered to be the largest and most broadly studied insect viruses. They are infectious for

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arthropods, particularly insects of the order Lepidoptera. Baculovirus infections have been reported in over 600 insect species of the orders Hymenoptera, Diptera, Coleoptera, Neuroptera, Trichoptera, and Thysanura, as well as in the Crustaceae order Decapoda [4]. It is believed that baculoviruses are safe and selective bioinsecticides, restricted to invertebrates. The family is divided into two genera, nucleopolyhedrovirus (NPV) and granulovirus (GV). Both genera have virions occluded in a protein matrix, the occlusion body (OBs), in the NPV called polyhedra range in size from 1 to 15 μm in diameter. The NPV has been well investigated because of its potential for use as an insect pest control agent. Polyhedrin is the most abundant protein of polyhedra and the baculoviuses produce it at very high levels in the late phase of infection; polyhedrin is a protein of about 245 to 250 amino acids. The polyhedrin has been characterized in many different viruses and shown to be highly conserved. The level of similarity observed in the polyhedrin gene and protein sequence has been used for the construction of baculoviruses phylogenetic trees [5].

Therefore, the investigation of polyhedrin gene structure is important to identify the NPV. So, the main objective of the present work is to study the toxicity and biological characterization of an Egyptian isolate to be used as effective and safe biological agent against *S. littoralis*, also obtain a sequenced highly conserved DNA fragment from our NPV isolate to be used in an easy, fast and economic prospective system for virus detection and may be useful as a potential biocontrol agent in program of integrated pest management (IPM).

2. Materials and Methods

2.1. Virus Isolate

The original virus isolate was obtained from diseased *Spodoptera littoralis* larvae collected from cotton of Agriculture Research Center, Qaha, Al-Fayom and El-Salhya. The larvae showed baculovirus infection symptoms were brought to our laboratory and examined to confirm the presence of virus by light microscope with Giemsa staining according to Mustafa, *et al*. [6], in which a thin smear of infected worm tissue was prepared on a glass slide and dried in air. The smear was immersed for 1-2 min in Giemsa, rinsed under running tap water for 5-10 sec then the smear was stained for two hours in 10% Giemsa stain (10g of Giemsa dissolved in 100ml distilled water), the dye was rinsed off in running tap water for 5-10 sec and allowed to dry in the air, then examined under a light microscope to detect the Occlusion Bodies (OBs). After the examination the diseased larvae kept at -20 ℃ until the purification of OBs (polyhedra).

2.2. Virus Propagation

The propagation of the virus isolate was performed by inoculation of the 3rd instar larvae of *S. littoralis* with *Sl*NPV Egyptian isolate which collected from the field and tested by light microscopy by surface contamination of the artificial diet. The inoculated larvae were observed daily to identify the NPV infected ones based on the sign and symptoms of disease. The tissues of dead larvae were examined as soon as possible with the naked eye and tissue smears under light microscopy as mentioned above.

2.3. Viral Occlusion Bodies (VOBs) Purification

The method of OBs purification was done according to Sudhakar *et al.* [7] with some modification. The individually dead larvae showing symptoms of NPV were transferred to a micro centrifuge tube and homogenized in 300 μl of distilled water. The homogenates were filtered through a chees cloth. The filtrate was subjected to sucrose layer (60% wt/vol) and centrifuged for 30 min at 10.000 rpm. The band was formed on top of the layer containing OBs was collected and again subjected to sucrose layer (40% wt/vol) and centrifuged at 10.000 rpm for 30 min. The band at the bottom of the gradient containing the OBs was collected and washed with distilled water. All the above steps were carried out at 4 ℃. Pure OBs were suspended in distilled water and stored at –20 ℃. For evaluation of OBs purification method, slide of OBs was stained with Giemsa stain as mentioned above and examined under light microscopy. The number of OBs was counted by using Neubaur Hemocytometer to determine the concentration of OBs/ml. Five concentrations were prepared from the viral OBs mother suspension by serial dilution to be used in bioassay experiment.

2.4. Insect Rearing and Biological Studies

The cotton leafworm, *S. littoralis* (neonate) from the laboratory of Insect Pathogen Production unit of Plant Protection Research Institute, Agriculture Research Center were reared for three generations under highly controlled conditions to avoid any insecticide contamination. Larvae fed on artificial diet described by Shorey and Hale [8]. The insect culture was maintained at 30 ℃, 70-80% relative humidity and a 16 h photoperiod [9, 10].

The toxicity experiment was carried out using diet surface treatment procedure [11]. 2nd and 4th instars larvae of *S. littoralis* were starved for 16-20h at 30 ℃ [12], then transfer to cups with contaminated artificial diet with 20 μl of ($1x10^{10}$, $1x10^{9}$, $1x10^{8}$, $1x10^{7}$, and $1x10^{6}$ PIBs/ml) concentrations individually. After 2 days of feeding, the diet had become unpalatable, and the larvae were transferred to clean cups of diet and observed daily. Bioassays with 30 larvae per virus concentration plus 30 larvae, as control were replicated 3 times. The experiment was conducted at a constant temperature 30 ℃. Larval mortality was recorded at 2 day intervals during 10 days. The mortality percentages were corrected according to Abbott's formula [13]. Toxicity was presented graphically as log/probit regression lines, and LC_{25}, LC_{50}, and LC_{90} values as well as the slope of the probit lines were calculated [14]. LC_{50} was used in treatment the 2nd and 4th instar larvae to study the following biological parameters:

larval and pupal duration of each instar, percentage of pupation, percentage of adult emergence, longevity of moths and the fecundity and fertility of eggs/female. A control was set comprising a similar number of untreated moths.

2.5. Molecular Characterization of Nucleopolyhedrovirus Egyptian Isolate (Egy-*Sl*NPV)

2.5.1. Extraction of Viral DNA

The viral DNA was purified from the OBs according to the guanidium thiocyanate DNA extraction method described by Hammond *et al*. [15] with slight modifications. Viral OBs were treated with the extraction solution (0.5 M guanidium thicyanate and 0.1 M EDTA) and 7.5 M ammonium acetate, the mixture was made to stand on ice for 10 min and then mixed with 1:1 phenol: chloroform, this was then centrifuged at 14.000rpm for 10min in a micro centrifuge. The upper aqueous phase was recovered carefully and placed in a new eppendorf tube. 0.5 volume isopropanol was added, the DNA was precipitated and collected after centrifugation at 14.000rpm for 20min, the DNA was washed with 70% ethanol, leave to dry for 30min then resuspended in 20 µl distilled water.

2.5.2. Primer Design and PCR Amplification of Egy-*Sl*NPV Polyhedrin Gene

The universal primer sets used in PCR reaction were designed for partial Egy-*Sl*NPV-*polh* gene and synthesized in Operon, (Qiagene Co.), where the forward primer was 5′-GCCGAATACACCACTTCGTT-3′ and the reverse primer was 5′-TGTCGCTCGTGTTCAAGATC-3′. PCR amplification was performed according to Saiki *et al*. [16] with minor modification. Total reaction volume was 50 µl which contained 5 µl of 10x reaction buffer (600 mM tris HCL pH 8.3, 250 mM KCL, 1% triton X100, 100 mM B-mercaptoethanol, 2 mM $MgCl_2$), 5 µl of 1mM dNTps, 2.5 µl Taq DNA polymerase, 1 µl of each primer and 1 µl of template DNA. The amplification was carried out using UNO-Thermoblock system from Biometra. Hard denaturation of the DNA was performed at 94 ℃ for 1min followed by 35 cycles of amplification with denaturation at 94 ℃ for 30 sec, annealing at 57 ℃ for 30sec and extension at 72 ℃ for 1min. A single tailing cycle of long extension at 72 ℃ for 5min was carried out in order to ensure flush ends on the DNA molecules. The PCR product of the polyhedrin gene was determined by electrophorasis onto 1% agarose gel containing ethidium bromide (20 µg/ml) in 1x TAE buffer to examine the actual size of the PCR product. Agarose gel electropholrasis was performed in DNA mini electropholrasis sub-cell. Eight µl of PCR product and 8 µl of standard DNA marker (100 bp ladders) was mixed with 2 µl of 6x gel loading buffer. The PCR product was visualized on a UV transilluminator (wave length = 254 nm) and photographed by the camera.

2.5.3. Sequencing of Egy-*Sl*NPV-*polh* Gene

DNA fragment was purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, Cat. No. 28704). The nucleotide sequence of the PCR product of *polh* gene carried out (through Sigma Company in Egypt) by dideoxy sequencing using Capillary ABI PRISMTM BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS and performed on automated DNA Sequencer. Data were analysed using FinchTVTM version 1.4.0 software of sequencing analysis. The *polh* gene nucleotide sequence of the Egyptian isolate nucleopolyhedrovirus from *Spodoptera littoralis* (Egy-*Sl*NPV) was registered under GenBank accession number KY072799.

2.5.4. Sequence Alignments and Phylogenetic Analyses

Multiple alignments of partial Egy-*Sl*NPV-*polh* gene with other corresponding *polh/gran* gene sequences from 55 NPVs and 15 GVs (Table 1) sequences were performed using BioEdit software (Ver.7.2.5) and ClustalW (Ver.1.74) program [17]. The virus names, abbreviations and accession numbers were listed in table (1). Neighbour-joining phylogenetic trees (1000 bootstrap replicates) were inferred from the nucleotide and deduced amino acid sequences alignments by using MEGA 4.0 software [18]. Distance matrices from aligned nucleotide and deduced amino acid sequences were determined by using the Kimura 2-parameter model [19] and the JTT matrix-based model [20], respectively, for correction of superimposed substitutions with the Molecular Evolutionary Genetics Analysis (MEGA) software (Ver. 4.0) [18]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5).

Table 1. List of the 55 polyhedrin of nucleopolyhedroviruses isolates (NPVs) and 15 granulin of granuloviruses isolates (GVs) partial codons sequences used in the Neighbor-Joining analysis

Virus (Isolate)	Host	Host family	GenBank Accession No.
Nucleopolyhedrovirus			
AdhoNPV (ADN001)	*Adoxophyes honmai*	*Tortricidae*	AP006270
AmcuNPV (A8-3)	*Amorbia cuneacapsa*	*Tortricidae*	AY706685
AmalNPV	*Amsacta albistriga*	*Arctiidae*	AF118850
AnfaNPV (pRAf4.5)	*Anagrapha falcifera*	*Noctuidae*	U64896
AnpeNPV (A)	*Antheraea pernyi*	*Saturniidae*	AB062454
AgMNPV (2D)	*Anticarsia gemmatalis*	*Erebidae*	Y17753

ArceNPV	*Archips cerasivoranus*	*Tortricidae*	U40834
ArNPV (China)	*Attacus ricini*	*Saturniidae*	S68462
AcMNPV (A12-2)	*Autographa californica*	*Noctuidae*	AY706681
BusuNPV (HB)	*Buzura suppressaria*	*Geometridae*	X70844
ChroNPV	*Choristoneura rosaceana*	*Tortricidae*	U91940
DipeNPV (A3-1)	*Dirphia peruvianus*	*Saturniidae*	AY706691
EcgrNPV (S22)	*Ectropis grisescens*	*Geometridae*	AY706692
EcobNPV (A1)	*Ecotropis oblique*	*Geometridae*	DQ837165
EudiNPV (S24)	*Euproctis digramma*	*Lymantriidae*	AY706693
EupsNPV (A13-1)	*Euproctis pseudoconspersa*	*Lymantriidae*	AY706694
EppoNPV	*Epiphyas postvittana*	*Tortricidae*	AF061578
GmMNPV (A11-3)	*Galleria mellonella*	*Pyralidae*	AY706696
HearNPV (C1)	*Helicoverpa armigera*	*Noctuidae*	AF303045
HeveNPV (A24-5)	*Hemerocampa vetusta*	*Lymantriidae*	AY706699
HezeNPV	*Helicoverpa zea*	*Noctuidae*	AF334030
HycuNPV	*Hyphantria cunea*	*Arctiidae*	AF300872
LeseNPV	*Leucania seperata*	*Noctuidae*	U30302
LoobMNPV	*Lonomia oblique*	*Saturniidae*	AF232690
LdMNPV	*Lymantria dispar*	*Lymantriidae*	M23176
LymoNPV (A19-3)	*Lymantria monacha*	*Lymantriidae*	AY706702
MaamNPV (M39-4)	*Malacosoma americanum*	*Lasiocampidae*	AY706704
ManeNPV	*Malacosoma neustria*	*Lasiocampidae*	AJ277555
MabrMNPV (Oxford)	*Mamestra brassicae*	*Noctuidae*	M20927
MacoNPV (90-2)	*Mamestra confgurata*	*Noctuidae*	U59461
NephNPV (A25-5)	*Nepytia phantasmaria*	*Geometridae*	AY706709
OpMNPV	*Orgyia pseudotsugata*	*Lymantriidae*	M14885
PaflNPV	*Panolis flammea*	*Noctuidae*	D00437
PeniNPV	*Perina nuda*	*Erebidae*	U22824
PlacNPV (A14-5)	*Plusia acuta*	*Noctuidae*	AY706712
PlorNPV	*Plusia orichalcea*	*Noctuidae*	AF019882
PlmaNPV (A15-2)	*Plutella maculipennis*	*Plutellidae*	AY706713
RaouMNPV (RI)	*Rachiplusia ou*	*Noctuidae*	AF068270
SacyNPV (S36)	*Samia cynthia*	*Saturniidae*	AY706711
SpexMNPV	*Spodoptera exigua*	*Noctuidae*	NC_002169
SpexMNPV (EG)	*Spodoptera exigua*	*Noctuidae*	GQ392064
SpfrMNPV	*Spodoptera frugiperda*	*Noctuidae*	J04333
SpliNPV (A26-5)	*Spodoptera littoralis*	*Noctuidae*	AY706717
SpliNPV (AN1956)	*Spodoptera littoralis*	*Noctuidae*	JX454574
SpliNPV (3017)	*Spodoptera littoralis*	*Noctuidae*	JX454588
SpliNPV (3032)	*Spodoptera littoralis*	*Noctuidae*	JX454589
SpltNPV (GZ-1)	*Spodoptera litura*	*Noctuidae*	AF037262
SpltNPV (G2)	*Spodoptera litura*	*Noctuidae*	AF325155
SpltNPV (A17-3)	*Spodoptera litura*	*Noctuidae*	AY706714
SpltNPV (S37)	*Spodoptera litura*	*Noctuidae*	AY706715
SpteNPV (A26-1)	*Spodoptera terricola*	*Noctuidae*	AY706716
ThorNPV (A28-1)	*Thysanoplusia orichalcea*	*Noctuidae*	AY706719
TibiNPV (M50-4)	*Tineola bisselliella*	*Tineidae*	AY706720
TrniSNPV	*Trichoplusia ni*	*Noctuidae*	AF093405
WisiNPV	*Wiseana signata*	*Hepialidae*	AF016916

Table 1. Continue

Virus (Isolate)	Host	Host family	GenBank Accession No.
Granulovirus			
AdorGV (E1)	*Adoxophyes orana*	*Tortricidae*	AF337646
AgexGV (S46)	*Agrotis exclamationis*	*Noctuidae*	AY706659
AgseGV (S47)	*Agrotis segetum*	*Noctuidae*	AY706661
AnbiGV (S48)	*Andraca bipunctata*	*Bombycidae*	AY706662
ClanGV (S49)	*Clostera anachoreta*	*Notodontidae*	AY706664
CrleGV (CV3)	*Cryptophelebia leucotreta*	*Tortricidae*	AY229987
CypoGV (Mexican 1)	*Cydia pomonella*	*Tortricidae*	U53466
EpapGV	*Epinotia aporema*	*Tortricidae)*	AF473703
EuocGV (A24-1)	*Euxoa ochrogaster*	*Noctuidae*	AY706666
PemoGV (A25-3)	*Peridroma morpontora*	*Noctuidae*	AY706672
PibrGV (S54)	*Pieris brassicae*	*Pieridae*	DQ235253
PlscGV (A25-6)	*Plathypena scabra*	*Noctuidae*	AY706675
PlxyGV (K1)	*Plutella xylostella*	*Plutellidae*	AF270937
SctrGV (A26-3)	*Scotogramma trifolii*	*Noctuidae*	AY706676
XecngGV (alpha-4)	*Xestia c-nigrum*	*Noctuidae*	U70069

3. Results

3.1. Sample Collection and Light Microscopy Examination

The virus isolate was obtained from diseased *Spodoptera littoralis* larvae (showed symptoms of viral infection) collected from cotton of Agriculture Research Center, Qaha, Al-Fayom and El-Salhya. Viral symptoms of infected *S. littoralis* which collected from the field can be summarized in: slow motion larvae (Fig. 1-A), cuticle showing red color (Fig. 1-B), hanging larvae (Fig. 1-C) and liquefied larval body (Fig. 1-D). The virus was propagated in a *S. littoralis* laboratory colony, purified and kept at -80 ℃ for further studies. The viral occlusion bodies (VOBs) were detected using a light microscope by staining thin smear of infected larvae and drop of VOBs with Giemsa stain which appear polyhedral and negative stain particles (Fig. 2).

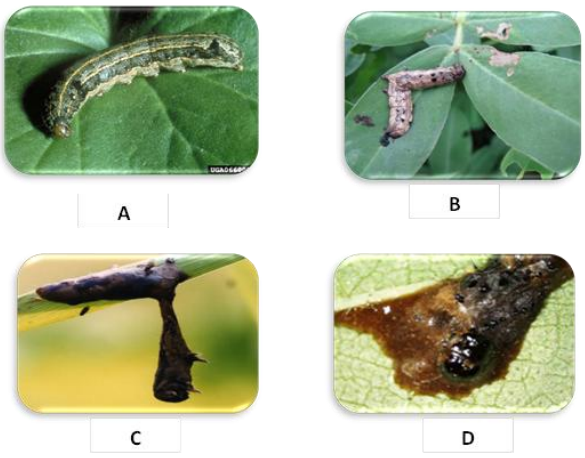

Figure 1. Viral symptoms of infected *Spodoptera littoralis* showing slow motion larvae (A), red color cuticle (B), hanging larvae (C) and liquefied larval body (D)

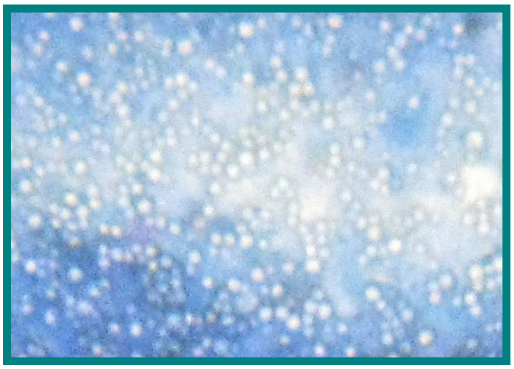

Figure 2. Viral occlusion bodies polyhedra of *Sl*NPV under light microscopy

3.2. Biological Studies of Nucleopolyhedrovirus Egyptian Isolate (Egy-SlNPV) against Spodoptera Littoralis

The results of bioassay studies of Egy-*Sl*NPV against *S. littoralis* are presented in table (2). The data showed that, the mortality percentage in the two tested instar larvae increased with increasing the concentrations and time elapsed post treatment. The NPV concentration $1x10^{10}$ PIB/ml ingested by larvae produces approximately 96% mortality percentage at 10 days post inoculation for *S. littoralis* 2nd instar larvae and 83% of the same concentration for 4th instar larvae. The remaining concentrations produce mortality percentages ranged from 90 to 48% and 75 to 45% of the two tested instars larvae used, respectively.

The data in table (3) showed that, the 2nd instar larvae was more susceptible to all concentrations than the 4th instar larvae, evidenced by the very low LC_{25}, LC_{50}, LC_{75} and LC_{90} values. Slopes for concentration mortality relationship for larvae of various ages are shown in Fig. (3), the shift in the position of the probit lines reflects the increase in LC_{25}, LC_{50}, LC_{75} and LC_{90} with age.

Biological study's results revealed a decrease in the mean larval, pupal, and adult durations for the *S. littoralis* 2nd and 4th instar larvae treated with LC_{50} of $1.28x10^6$ and $2.53x10^7$ (PIB/ml) for the two tested instar larvae, respectively. Treatment also caused a reduction in the pupation and adult emergence percentage, as well as a great reduction in the mean number of eggs/female and the mean number of hatched eggs (Tables 4, 5 and 6).

Table 2. Efficacy of SlNPV against 2nd and 4th instar larvae of Spodoptera littoralis after 10 days post treatment

Concentrations PIBs/ml	Mortality %	
	2nd instar	4th instar
$1x10^{10}$	96	83
$1x10^{9}$	90	75
$1x10^{8}$	86	65
$1x10^{7}$	61.6	52.3
$1x10^{6}$	48.3	45

Table 3. Susceptibility of *Spodoptera littoralis* 2nd and 4th instars larvae to *Sl*NPV

Instars	LC_{90} (PIB/ml)	LC_{75} (PIB/ml)	LC_{50} (PIB/ml)	LC_{25} (PIB/ml)	Slope ± SE
2nd instars	8.66E+09	3.95E+08	1.28E+06	4.15E+05	0.453 ± 0.052
4th instars	1.82E+12	9.12E+09	2.53E+07	70358.87	0.264 ± 0.043

Table 4. Effect of *Sl*NPV on larval duration, pupation rate and duration of *Spodoptera littoralis*

Tested Compounds	Mean larval duration (days) ± SE		% Pupation		Mean pupal duration (days) ± SE	
	2nd	4th	2nd	4th	2nd	4th
***Sl*NPV**	14.5 ± 0.1*	11.0 ± 1.7**	51.0	47.0	11.3 ± 1.5***	9.3 ± 1.3***
Control	15.0 ± 0.2	12.3 ± 1.1	100	100	13.6 ± 0.5	14.0 ± 1.7

*: Significant at P> 0.05 **: highly significant at P> 0.01 ***: Very highly significant at P> 0.001

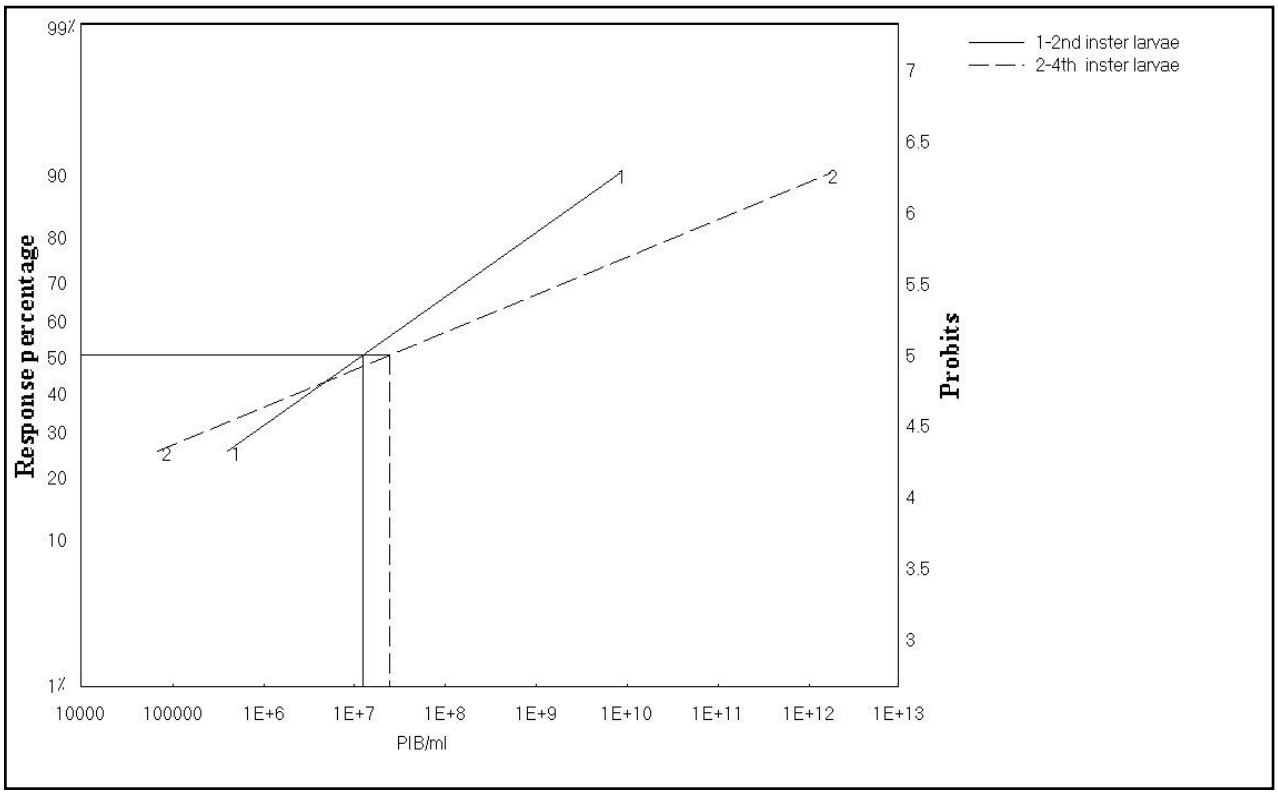

Figure 3. Toxicity lines of *Sl*NPV on 2nd and 4th instars larvae of *Spodoptera littoralis*

Table 5. Effect of *Sl*NPV on adult emergence percentage and adult longevity of *Spodoptera littoralis*

Tested compounds	% Adult emergence		Mean adult longevity (days) ± SE			
	2nd	4th	2nd		4th	
			♂	♀	♂	♀
***Sl*NPV**	94.40	94.20	8.3 ± 1.15***	9.3 ± 0.57***	14.3 ± 1.2**	12.0 ± 1.7***
Control	100.00	100.00	13.6 ± 1.15	14.6 ± 0.58	17.0 ± 1.0	15.3 ± 0.57

: highly significant at P> 0.01 *: Very highly significant at P> 0.001

Table 6. Effect *Sl*NPV of on mean oviposition rate/female and egg hatchability of *Spodoptera littoralis*

Tested compounds	Mean no. of eggs/female ± SE		Mean no. hatched eggs/female ± SE	
	2nd	4th	2nd	4th
***Sl*NPV**	576 ± 9.1***	712 ± 13.38***	415 ± 3.6***	530 ± 21.3***
Control	2215 ± 60.6	2072 ± 15.1	2103 ± 4.04	1957 ± 12.11

***: Very highly significant at P> 0.001

3.3. Molecular Characterization of the Egy-*Sl*NPV-*polh* Gene

The partial codons sequence of the polyhedrin (*polh*) gene was amplified using specific PCR primers. Electrophoresis analysis of the PCR product showed a single fragment at ~138 bp of the test naturally infected *S. littoralis* (Fig. 4). The sequence obtained for the *polh* gene was 138 bp long, representing 0.55/3 of the complete *polh* open reading frame (ORF), which comprised 1425 bases of nucleotides potentially coding for 46 amino acids.

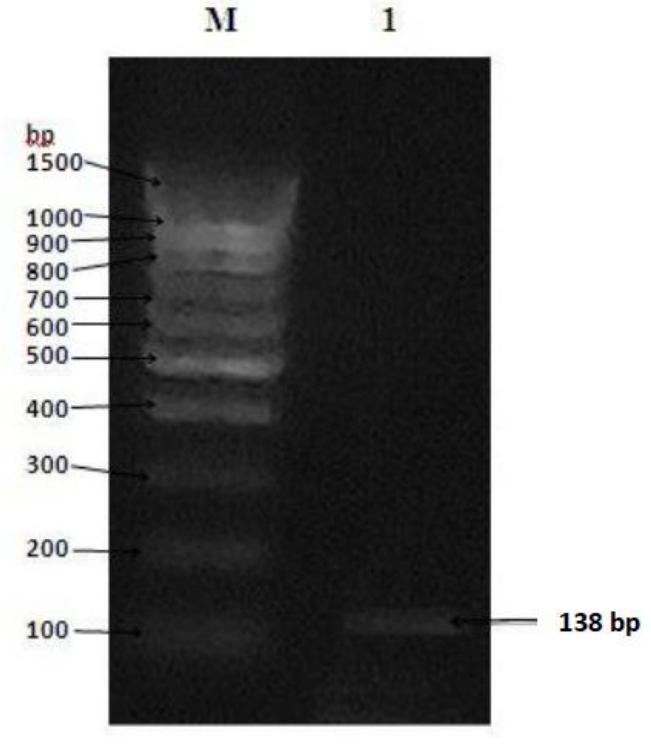

Figure 4. 1 % agarose gel showing an electrophoresis analysis of the PCR product amplification using Egy-*Sl*NPV-*polh* gene specific primers; M = molecular weight marker (100 bp ladder); lane 1: PCR product from *S. littoralis* infected with NPV

3.3.1. Nucleotide and Deduced Amino Acid Sequences Analysis

Multiple sequence alignment and phylogenetic analysis were performed to compare Egy-*Sl*NPV-*polh* gene with other 55 *polh* genes sequences from various nucleopolyhydroviruses (NPVs) and with 15 granulin genes from granuloviruses (GVs) available in GenBank (Table 1).

The pairwise similarity of the nucleotide and deduced amino acid sequences of the *polh*/*gran* gene of nucleopolyhedrovirus Egyptian isolate (Egy-*Sl*NPV) obtained from naturally infected *S. littoralis* showed 71.0–97.8% and 76.1-97.8, respectively, homology with reported isolates of NPVs in NCBI GenBank as compared to 58.0-68.1% and 52.2-58.7% with nucleotide and amino acid sequences of the out group member (GVs), respectively. The nucleotide sequence of the *polh* gene of Egy-*Sl*NPV showed maximum homology (97.8%) with the four nucleopolyhedrovirus isolates (SpliNPV) from *Spodoptera littoralis* (A26-5, AN1956, 3017 and 3032 under Accession Nos. AY706717, JX454574, JX454588 and JX454589, respectively), and 94.2% with nucleopolyhedrovirus isolates (SpltNPV) from *S. litura* (GZ-1, G2, A17-3 and S37 under Accession Nos. AF037262, AF325155, AY706714 and AY706715, respectively). On the other hand, the deduced amino acid sequence of the *polh* gene of Egy-*Sl*NPV showed maximum homology (97.8%) with 9 nucleopolyhedrovirus isolates [4 SpliNPV, 4 SpltNPV and 1 isolate from *S. terricola* (SpteNPV A26-1 under Accession No. AY706716)].

3.3.2. Phylogenetic Analysis

Phylogenetic analyses of the partial nucleotide and deduced amino acid sequences of Egy-*Sl*NPV-*polh* gene was used to consider whether the Egy-*Sl*NPV is consistent with viral infective evolution, a conservative test of whether they are likely to represent nucleopolyhedrovirus infect *Spodoptera littoralis*. Neighbour-joining phylogeny of the Egy-*Sl*NPV (Fig. 5) indicated the presence of two distinct

NPVs groups (I & II), where group II NPVs includes three subgroups (II-A, II-B & II-C), almost all of which are supported by high bootstrap scores in the phylogeny of the *polh* gene alignment (Fig. 5-A & B).

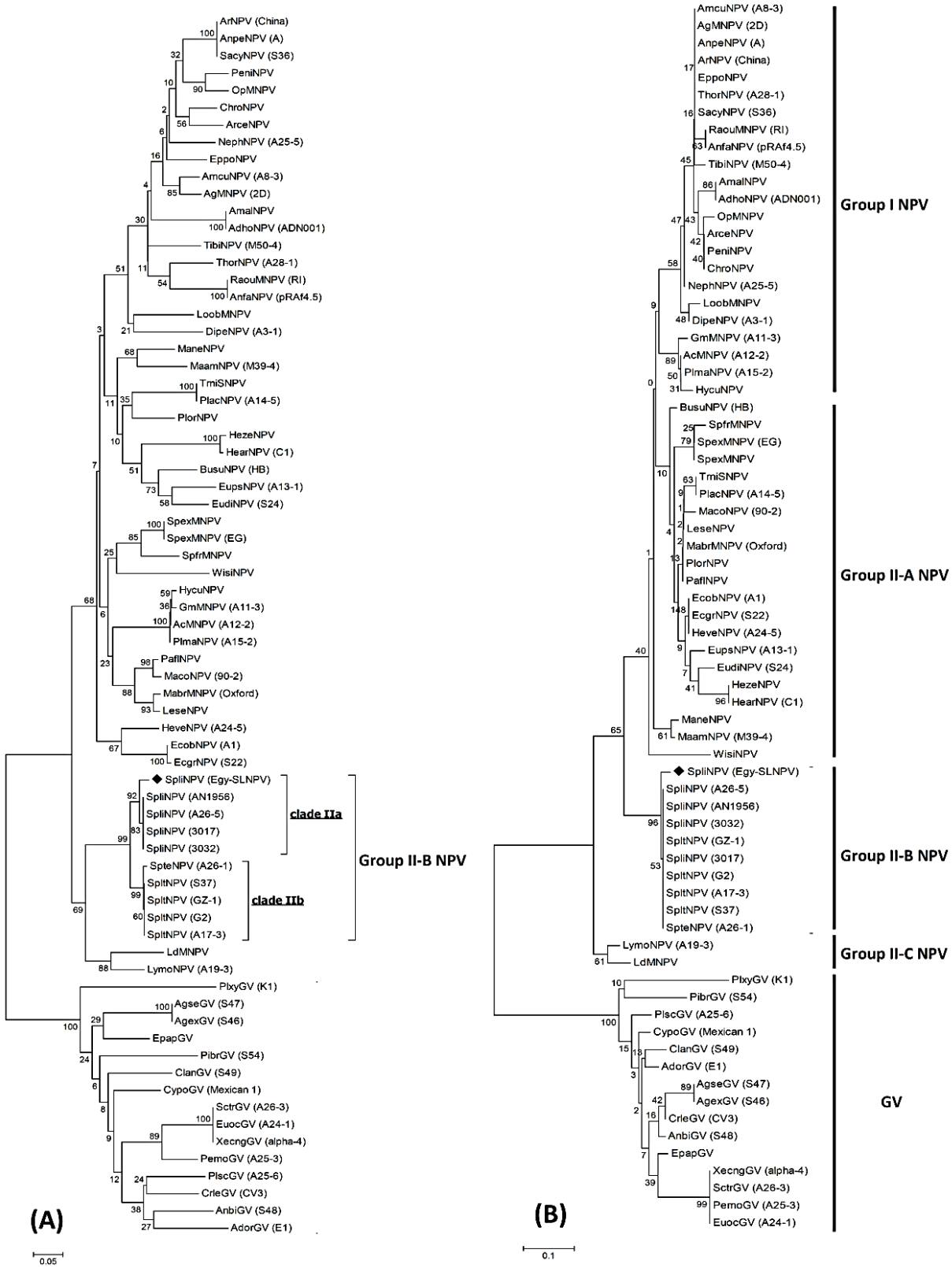

Figure 5. Neighbour-joining tree of Egy-*Sl*NPV strain based on nucleotide (A) and deduced amino acid (B) sequences with the corresponding partial *polh* gene of 55 nucleopolyhydroviruses (NPVs) and partial *gran* gene of granuloviruses (GVs). The numbers represent bootstrap percentage values based on 1000 replicates. The optimal tree with the sum of branch length = 5.18580891 for nucleotide sequence and 2.35130621 for protein sequence are shown. The tree is indicate into two NPVs groups (I & II) and a group of GVs

Within the subgroup II-B NPVs in a phylogenetic tree of the nucleotide sequences, two monophyletic clades were identified (Fig. 5-A). The first clade (IIa) included the five SpliNPV isolates (Egy-*Sl*NPV, A26-5, AN1956, 3017 and 3032). The second clade (IIb) included the SpteNPV isolate (A26-1) and four isolates of SpltNPV (GZ-1, G2, A17-3 and S37). On the other hand, the subgroup II-B NPVs in a phylogenetic tree of the deduced amino acid sequences contains one monophyletic clade (Fig. 5-B). It was included 5 SpliNPV isolates, 4 SpltNPV isolates and SpteNPV isolate. From the phylogenetic analyses of the nucleotide and deduced amino acid sequences, that clearly showed that our Egy-*Sl*NPV isolate belongs to subgroup II-B NPVs. On the other hand, granuloviruses (GVs) was positioned as a separated group (out group member).

3.3.3. Evolutionary Divergence between Egy-*Sl*NPV Isolate and Subgroup II-B NPVs Isolates

The *polh* gene nucleotide and deduced amino acid sequences alignment were analyzed to identify amino acid mutations that occurred on multiple branches basal to the defined subgroup II-B NPVs (Figs. 6 & 7). All of these sequences were multiple aligned using the ClustalW program with minor manual adjustments, where the number of base and amino acid substitutions per site from between sequences are shown in table (7). Standard error estimate (s) are shown above the diagonal. The nucleotide sequence analyses were conducted using the Kimura 2-parameter (K-2-P) model where the rate nucleotide sequence variation among sites was modeled with a gamma distribution (shape parameter = 5), while the amino acid sequence analyses were conducted using the JTT matrix-based model with a gamma distribution (shape parameter = 5).

The analysis involved 10 nucleotides and amino acid sequences of subgroup II-B NPVs, including Egy-*Sl*NPV isolate. All ambiguous positions were removed for each sequence pair. There were a total of 138 and 46 positions in the final dataset for nucleotide and amino acid sequences, respectively. A total of 9 and 1 variable sites were found in subgroup II-B NPVs nucleotide and amino acids, respectively, including the gaps where 5 and 0 were parsimoniously informative nucleotide and amino acids, respectively. As well as, 4 and 1 were singleton sites, respectively (Figs. 6 & 7). The differences in nucleotides of Egy-*Sl*NPV with other subgroup II-B NPVs isolates were also present at various nucleotide positions due to replacement of either purines with pyrimidines (one place), purine to purine (one place) or pyrimidines with purines (at one place) viz. at positions number 72 (G is replaced by C), 76 (G is replaced by A) and 84 (C is replaced by A), respectively. The change of nucleotide at position number 76 resulted in the difference at one amino acid position with group II-B NPVs isolates viz. at position number 26 [methionine (M) is present in place of valine (V) a hydrophobic amino acid] (Fig. 7).

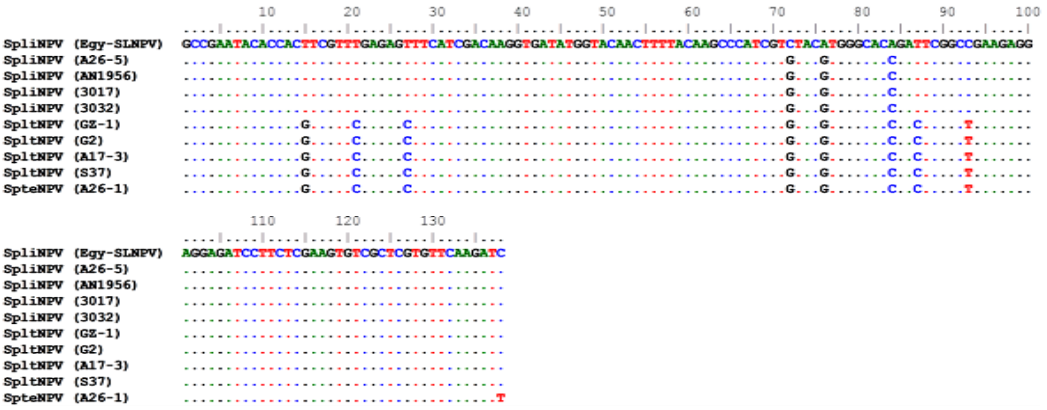

Figure 6. Multiple nucleotide sequence alignment of the partial *polh* gene of the studied Egy-*Sl*NPV strain with the corresponding amino acid sequence of four SpliNPV isolates (A26-5, AN1956, 3017 and 3032), four isolates of SpltNPV (GZ-1, G2, A17-3 and S37) and the SpteNPV isolate (A26-1)

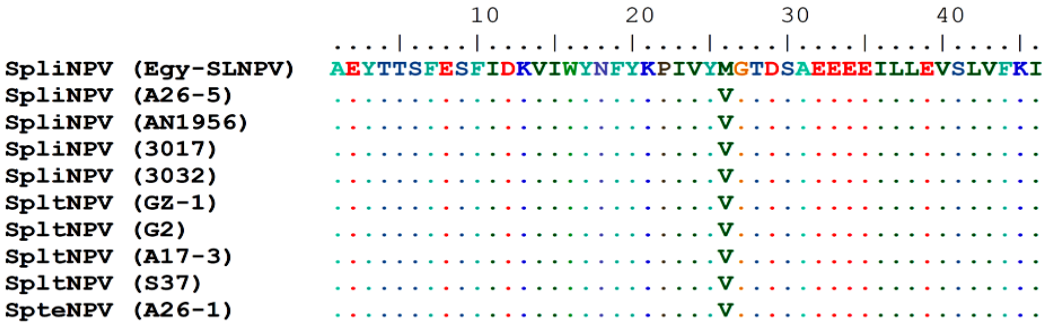

Figure 7. Multiple deduced amino acid sequence alignment of the partial *polh* gene of the studied Egy-*Sl*NPV strain with the corresponding amino acid sequence of four SpliNPV isolates (A26-5, AN1956, 3017 and 3032), four isolates of SpltNPV (GZ-1, G2, A17-3 and S37) and the SpteNPV isolate (A26-1)

Table 7. Pairwise distances of nucleotide and amino acid sequences of partial *polh* gene of the Egy-*Sl*NPV/SpliNPV/SpltNPV/SpteNPV cluster

Nucleotide distance and standard error										
	1	2	3	4	5	6	7	8	9	10
1		0.012	0.012	0.012	0.012	0.021	0.021	0.021	0.021	0.023
2	0.022		0.000	0.000	0.000	0.017	0.017	0.017	0.017	0.018
3	0.022	0.000		0.000	0.000	0.017	0.017	0.017	0.017	0.018
4	0.022	0.000	0.000		0.000	0.017	0.017	0.017	0.017	0.018
5	0.022	0.000	0.000	0.000		0.017	0.017	0.017	0.017	0.018
6	0.061	0.038	0.038	0.038	0.038		0.000	0.000	0.000	0.007
7	0.061	0.038	0.038	0.038	0.038	0.000		0.000	0.000	0.007
8	0.061	0.038	0.038	0.038	0.038	0.000	0.000		0.000	0.007
9	0.061	0.038	0.038	0.038	0.038	0.000	0.000	0.000		0.007
10	0.069	0.046	0.046	0.046	0.046	0.007	0.007	0.007	0.007	
Amino acid distance and standard error										
	1	2	3	4	5	6	7	8	9	10
1		0.023	0.023	0.023	0.023	0.023	0.023	0.023	0.023	0.023
2	0.022		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.022	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	0.022	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000
5	0.022	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000
6	0.022	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
7	0.022	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
8	0.022	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000
9	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000
10	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

The nucleotide and amino acid distances between isolates ranged from 0.000 to 0.069 and from 0.000 to 0.022, respectively (Table 7). The nucleotide distance of the Egy-*Sl*NPV recorded lower value with four SpliNPV isolates, while in the case of the amino acid distance, lower value was recorded with all isolates in subgroup II-B NPVs (SpliNPV, SpltNPV and SpteNPV isolates). The genetic diversity value of all nucleotide and amino acid sequences of subgroup II-B NPVs population, representing the average number of nucleotide and amino acid substitutions per site between pairs of sequences, where the average evolutionary divergence overall nucleotide and amino acid sequence pairs were 0.027 ± 0.010, and 0.004 ± 0.004, respectively.

4. Discussion

The Egyptian cotton leafworm, *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) is one of the most notorious and destructive phytophagous insect pests in Egypt, not only to cotton, but also to other field crops and vegetables [1]. The new nature baculovirus isolates with better insecticidal characteristics is safer and has not the risks of releasing genetically engineered product in nature. Seufi [21] reported that baculoviruses have been isolated from Upper as well as Lower Egypt, in which the cultivation was washed by chemical insecticides, it would be expected to find more and more isolates in virgin regions where no insecticides were used. So, the main objective of the present work is to study the biological and molecular characterization of an Egyptian isolate to be used as effective and safe biological agent against *S. littoralis*, where it become may be useful as a potential biocontrol agent in program of integrated pest management (IPM).

The virus isolate was obtained from diseased *Spodoptera littoralis* larvae exhibited viral-like symptoms collected from cotton of Agriculture Research Center, Qaha, Al-Fayom and El-Salhya. Viral symptoms of infected *S. littoralis* which collected from the field are slow motion larvae, cuticle showing red color, hanging larvae and liquefied larval body, where the similar results were reported by Toprak *et al.* [22].

The viral occlusion bodies (VOBs) of the nucleopolyhedrovirus Egyptian isolate (Egy-*Sl*NPV) were detected using a light microscope by staining thin smear of infected larvae and drop of VOBs with Giemsa stain which appear polyhedral and negative stain particles. The occlusion bodies of NPVs are specifically identified as polyhedral, where baculoviruses occlude their virions in large, proteinaceous occlusion bodies which help the virus to remain viable outside the host for years [23].

The infectivity of Egy-*Sl*NPV against *S. littoralis* larvae revealed that 2nd instar larvae were more sensitive than 4th instar larvae. The sensitivity of young instar was reported by Gomez*et et al.* [24]. Different susceptibility levels of *S. littoralis* larvae to NPV were observed by several authors [21, 25, 26]. Such difference in susceptibility may be due to the difference in larval age, the number of virions contained in occlusion bodies and the feeding habit of the insect [26, 27]. The results were agreeable to that of Stairs [28], who found that susceptibility decreased markedly as larvae of *Malacosoma disstria* grew older. In parallel, Duan and Otvos [29] reported that mortality was higher when younger larvae of Choristonura fumiferana were used.

The LC_{50} values and un parallel toxicity lines also proved the susceptibility of younger instar than older one, and this agree with finding of Whitlock [30] in *Heliothis armigera* and differ from those of Gitanjali *et al.* [31] and Monobrullah and Nagata [32]. In the later, it was concluded that the responses of larvae of all instars were similar since the line of different instars were parallel.

On the basis of biological assays there is a great reduction of all biological parameters studied and these results have agreed with those of Abdel-Salam *et al.* [33] who treated *S. littoralis* larvae with Viroset (*Sl*NPV); and with those of Yasmein [34], who treated larvae of *S. littoralis* with Littovir (*Sl*NPV).

The Egy-*Sl*NPV isolate infected *S. littoralis* was detected and identified by PCR using polyhedrin gene primers. The polyhedrin gene proved to be suitable for the development of the generic amplification technique, the polyhedrin gene is highly conserved between NPVs, making it the preferred choice for phylogeny studies [35]. Although the length and molecular mass of polyhedrin are somewhat different, depending on the species, the high similarity could build the phylogenetic trees on the basis of polyhedrin sequences. The full length of the polyhedrin gene from lepidopteran NPVs ranged from 483 bp to 747 bp, where in case of polyhedrin gene from *Spodoptera* sp. NPVs, its full length ranges from 510 bp to 747 bp [36].

The partial polyhedrin (*polh*) gene sequence (138 bp) of the PCR-amplified fragment of the nucleopolyhedrovirus Egyptian isolate (Egy-*Sl*NPV) was done to determine the sequence information and variability with other corresponding partial 55 *polh* genes sequences from various nucleopolyhydroviruses (NPVs) and 15 granulin genes from granuloviruses (GVs) available in GenBank. Consequently, these partial sequences allowed the identification and classification of the newly analyzed Egy-*Sl*NPV isolate. Neighbour-joining phylogeny of the partial *polh* gene sequence of Egy-*Sl*NPV indicated the presence of two distinct NPVs groups (I & II), where NPVs taxa from Lepidoptera form a closely related clade, while the branch lengths of the GV clade are generally longer. These results in agreement with Zanotto *et al.* [37] and Cowan *et al.* [38], who reported that based on the reconstruction of the phylogenetic relationships of polyhedrin genes and on diferential relative rate of evolution, classed NPVs into groups I and II. Also, from the phylogenetic analyses of the nucleotide and deduced amino acid sequences, that clearly showed that our Egy-*Sl*NPV isolate belongs to subgroup II-B NPVs, where group II NPVs includes three subgroups (II-A, II-B & II-C). Subgroup II-B is defined to include the the spodopteran taxa SpliNPV, SpltNPV and SpteNPV, where these results are in agreement with Bulach *et al.* [35].

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