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Biological activity and genome composition of a Tunisian isolate of Spodoptera littoralis nucleopolyhedrovirus (SpliNPV-Tun2)

Saoussen Ben Tiba[1,2,3], Asma Laarif[3], Jörg T. Wennmann[1], Thameur Bouslama[3] and Johannes A. Jehle[1*]

Abstract

Background: The baculovirus Spodoptera littoralis nucleopolyhedrovirus (SpliNPV) is an entomopathogenic virus utilized as a biological control agent of the Egyptian cotton leaf worm, *Spodoptera littoralis*. Several studies have focused on the identification of different SpliNPV isolates from a biological and molecular point of view, but few of them conducted in-depth analyses of the genomic composition of these isolates.

Results: Identification of a novel isolate of SpliNPV, termed Tun2, which was purified from infected *S. littoralis* larvae from Tunisia was reported. This isolate was propagated in vivo and its median lethal concentration (LC_{50}) was determined to be 1.5×10^4 occlusion bodies (OBs)/ml for third instar *S. littoralis* larvae at 7 days of post-infection. OB production in late fourth instar larvae was estimated to be at least 2.7×10^9 OBs/g larval weight. The completely sequenced genome of SpliNPV-Tun2 was 137,099 bp in length and contained 132 open reading frames (ORF). It showed a 98.2% nucleotide identity to the Egyptian isolate SpliMNPV-AN1956, with some striking differences; between both genomes, insertion and deletion mutations were noticed in 9 baculovirus core genes, and also in the highly conserved *polyhedrin* gene. The homologs of ORF 106 and ORF 107 of SpliNPV-AN1956 appeared to be fused to a single ORF 106 in SpliNPV-Tun2, similar to the homologous ORF 110 in SpltNPV-G2.

Conclusion: SpliNPV-Tun2 is proposed as a new variant of SpliNPV and a potential candidate for further evaluation as a biocontrol agent for *S. littoralis* and probably other *Spodoptera* species.

Keywords: Egyptian cotton leaf worm, *Spodoptera littoralis*, *Baculoviridae*, *Alphabaculovirus*, Bioassays, Survival time analysis, Illumina sequencing, Genome annotation, Phylogeny, Biological control

Background

The Egyptian cotton leaf worm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) is considered one of the major pests of cotton, tobacco, and corn in the Mediterranean Area and Asia. Larvae of *S. littoralis* are polyphagous, causing substantial economic losses in both greenhouse and open field crops on a broad range of ornamental, industrial, and vegetable crops (Martins et al. 2005). Due to the severe damage to various crops, controlling this pest is an important issue for integrated pest management. Up to now, *S. littoralis* management has mainly focused on chemical insecticides. However, numerous studies have been carried out on the possibility of biological control of the pest. Insect viruses and entomopathogenic bacteria, fungi, and nematodes have been investigated as biological control agents of *S. littoralis* (Hajek and Shapiro-Ilan, 2018). The *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV) is a baculovirus that has been evaluated, registered, and applied for control of *S. littoralis*, as well as the fall armyworm, *Spodoptera frugiperda*, and the tobacco cutworm *Spodoptera litura* in Africa, America and Japan (Abdel-Khalik

*Correspondence: johannes.jehle@julius-kuehn.de

[1] Federal Research Centre for Cultivated Plants, Institute for Biological Control, Julius Kühn Institute (JKI), Heinrichstr 243, 64287 Darmstadt, Germany
Full list of author information is available at the end of the article

et al.2017; El-Sheikh 2015; Takatsuka et al. 2016). Baculoviruses comprise a large group of double-stranded, circular DNA viruses that infect insects from the orders Lepidoptera, Hymenoptera, and Diptera. Many of these viruses have been investigated because of their potential as biological control agents against agricultural and forest pests (Moscardi 1999). Based on phylogenetic analysis, the *Baculoviridae* family is separated into 4 genera: *Alphabaculovirus* (lepidopteran-specific nucleopolyhedroviruses, NPVs), *Betabaculovirus* (lepidopteran-specific granuloviruses, GVs), *Gammabaculovirus* (hymenopteran-specific NPVs) and *Deltabaculovirus* (dipteran-specific NPVs) (Jehle et al. 2006). SpliNPV belongs to the species *Spodoptera littoralis nucleopolyhedrovirus* of the genus *Alphabaculovirus* (Harrison et al. 2018).

Different SpliNPV variants have been isolated from cotton leaf worm populations in different countries, and intra-specific variation between isolates was identified by restriction endonuclease or partial gene sequencing (Breitenbach et al. 2013; Cherry and Summers 1985; Kislev and Edelman 1982; Martins et al. 2005;). So far, only the Egyptian isolate SpliMNPV-AN1956 has been fully sequenced; its genome is 137,998 bp in length, harbours 132 ORFs, and 15 homologous repeat regions (*hrs*), and is closely related to the nucleopolyhedrovirus G2 (SpltNPV-G2). Comparisons of the genome sequence of SpliMNPV-AN1956 and SpltNPV-G2 revealed an average of 85% amino acid identity across all genes and high collinearity of the 2 genomes, despite the lack/gain of 16 ORFs (Pang et al. 2001). It was reported that NPVs isolated from *Spodoptera* spp. have a rather narrow host range (Jakubowska et al. 2010). For example, the Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) infects only larvae of its host *S. exigua* (Jakubowska et al. 2010), whereas SpliNPV was shown to be infectious also to *S. frugiperda, S. exigua,* and *S. litura* (Takatsuka et al. 2016). Recently, a Tunisian isolate, named SpliMNPV-Tun, was detected in 2008 from infected cotton leaf worm caterpillars collected in Tunisian tomato greenhouses and identified as a SpliNPV variant based on the partial *polyhedrin (polh)* gene sequence (Laarif et al. 2011). Here, the identification of a further SpliNPV isolate, termed Tun2, which was obtained from a *S. littoralis* colony that was established from collected caterpillars from tomato fields in 2013 is reported. This isolate was tested for its activity towards third instar *S. littoralis* larvae, and its complete genome was determined to study its relationship to other SpliNPV variants.

Methods

Insects and virus detection

Larvae of *S. littoralis* were collected in 2013 from tomato fields (Monastir, Central-East, Tunisia) to establish a laboratory colony at the laboratory of entomology at Regional Research Centre in Horticulture and Organic Agriculture (CRRHAB). For colony maintenance, larvae were fed on a semi-artificial diet (Shorey and Gaston 1965) and kept at a temperature of 28 ± 2 °C and 60 ± 5% relative humidity (RH). Adults were kept in cylinders, and egg deposits were collected on filter papers. The filter papers were transferred to Petri dishes until larval hatching. A piece of artificial diet was added to the Petri dishes where larvae were kept until pupation. Occasionally, larvae from the rearing showed symptoms of nucleopolyhedrovirus infection indicating activation of a covert infection of the *S. littoralis* population. Diseased larvae were removed from the rearing and stored individually at − 20 °C.

Occlusion body purification

Baculoviral occlusion bodies (OB) were purified from pooled infected cadavers according to El-Salamouny et al. (2003). Briefly, the cadavers were homogenized in sterile distilled water and the homogenate was filtered through a muslin cloth. The obtained crude OB suspension was washed twice with 0.1% sodium dodecyl sulphate (SDS) and pelleted by low centrifugation. The pellet was resuspended in 50 mM Tris/HCl (pH 8), transferred to a 2-ml Eppendorf reaction vial, and HCl (0.1 M) or Na_2Co_3 (0.1 M) was added to adjust its pH to 7. Then, the OB suspension was centrifuged through a sucrose gradient and resuspended in H_2O. OB concentration was counted using a Neubauer Cell Counting Chamber (0.1 mm depth) and phase contrast light microscopy (Leica DMRBE, Leica, Wetzlar, Germany) (Eberle et al. 2012).

Bioassays

For testing the biological activity of SpliNPV-Tun2, *per os* infection experiments were conducted with third instar larvae of *S. littoralis.* For this, 25 larvae were fed with 1.5–2.5 g artificial diet plugs prepared with final concentrations of 10^3–10^8 OBs/ml (Shaurub et al. 2014). Untreated control groups consisted of 75 larvae. Each treatment consisted of 3 independent replicates. The mortality data were corrected with untreated control mortality using the formula of Abbott (1925). Calculation of the median lethal concentration (LC_{50}) at 7 days post-infection (dpi) was estimated by Probit analysis using linear regression implemented in the ToxRat 3.2.1 software package (ToxRat Solutions GmbH, Germany). From the same experiment, larval mortality was determined

for each concentration at least at 5 different time points within the time range of 1–14 dpi. Statistical analysis was done with *R* (version 4) and RStudio (version 1.1393). Survival analysis was conducted with *R* packages "Survival" (version 2.38) and "Survminer" (version 0.4.3). A test of significant variance between Kaplan–Meier curves was performed by a log-rank test (level of significance, $P < 0.05$).

OB productivity of *S. littoralis* larvae

An OB dose of 10^4 OBs of SpliNPV-Tun2 was pipetted onto cubes of diet of 5 mm^3 each and individually offered to early fourth instar larvae of *S. littoralis* (Grzywacz et al. 1998). When the doses were completely ingested within 2 days, a non-contaminated diet was added every second day until 12 dpi. Larvae were harvested at 14 dpi. Three different methods for OB purification were compared; low-speed centrifugation (LSC) (Harrison 2008), sucrose gradient ultracentrifugation (SGU) (El-Salamouny et al. 2003), and sucrose cushion centrifugation (SCC) (Wennmann and Jehle 2014). Purified OBs were counted as described above. OB counting was performed 3 times for each treatment; the obtained concentrations were multiplied with the volume (5 ml), and then normalized with the larva weight. Results were expressed as OBs/g of larval tissue and were used to compute the arithmetic mean of OB/g of each experiment. Differences in the mean number of OB/g were statistically evaluated for a significance value of $P \leq 0.05$ using analysis of variance (ANOVA) and the Tukey's Honestly Significant Difference test (Tukey-HSD) comparison of means with standard R code (R version 3.3.1 in RStudio 3.4.0).

Viral DNA extraction

Viral DNA was extracted according to Bernal et al. (2013) with some modification. Occlusion derived virions (ODVs) were released from OBs by mixing 100 µl of the OB suspension (containing about 10^9 OBs/ml) with 100 µl Na_2CO_3 (0.5 M), 50 µl SDS (10%, w/v) in a final volume of 500 µl. After incubation at 60 °C for 10 min, the suspension was neutralized to pH 7 by adding 0.1 M HCl. Undissolved OBs and other debris were pelleted by centrifugation at 3800 g for 5 min. The supernatant containing the released ODVs was transferred to a fresh vial and treated with 25 µl Proteinase K (10 mg/ml) for 1 h at 50 °C. Viral DNA was extracted twice with Tris/HCl-saturated phenol and once with chloroform by using Phase Lock gel tubes (all purchased from, Carl Roth GmbH + Co., KG, Karlsruhe, Germany), followed by standard ethanol precipitation (Eberle et al. 2012). The DNA pellet was dissolved in 100 µl distilled H_2O.

PCR amplification and sequencing of the polyhedrin gene

The PCR amplification of the *polh* gene was chosen according to the specific primers designed by Martins et al. (2005) to amplify a complete SpliNPV *polh* gene (750 bp): 5′-ATG TAT AGT CGC TAC AGT GCC TAC-3′ (forward primer) and 5′-TTA GTA CGC GGG ACC GGT GT-3′ (reverse primer). The PCR mix comprised 34.5 µl of water, 10 µl Green buffer (Promega), 1 µl dNTPs (10 µM each), 1.5 µl Go Taq DNA polymerase (Promega), and 1 µl of each primer (10 µM). Finally, 1 µl of DNA was added to obtain a final volume of 50 µl for each reaction. PCR was initiated at 95 °C for 1 min of denaturation followed by 35 cycles at 95 °C for 30 s, 46 °C for 30 s, 72 °C for 45 s and the final extension at 72 °C for 5 min. The amplification product was visualized by 1% agarose gel electrophoresis at 90 V for 40 min in 1 × TAE buffer after staining with Midori Green DNA strain (NIPPON Genetics Europe). The PCR product was purified with DNA clean and concentrator kit (Zymo Research) according to the manufacturer's instruction, and both strands were Sanger sequenced. The *polh* sequencing was done for different single infected larvae randomly chosen. Sequences were compiled and then aligned with the complete *polh* gene sequences available in GenBank.

Genome sequencing

Sequencing and raw data processing

About 50 ng purified DNA was subjected to commercial NexteraXT library preparation and Illumina NextSeq500 sequencing at StarSEQ GmbH company (Mainz, Germany). In total, more than 1.76 million reads of 151 nt in length were obtained. Raw reads were processed by adapter trimming and quality filtering excluding reads with an average phred quality score below 30 (Gueli Alletti et al. 2017). Quality filtered reads with a length shorter than 50 nt were excluded from the analysis for paired reads and 51 nt for unpaired reads. Paired and unpaired reads were kept after all steps of filtering and quality control.

Genome sequence assembly

The remaining set of reads was used for de novo sequence assembly as well as mapping against the whole genome sequence of SpliMNPV-AN1956 (GenBank accession number JX454574) (Breitenbach et al. 2013). CLC de novo assembly resulted in multiple contigs (> 1000 bp). Contigs were mapped and fit together to a single contig comprising the whole genome. This contig was considered as a first consensus (cons1). In a second approach, all reads were mapped against the SpliMNPV-AN1956 genome using BWA-MEM. From here, a second consensus (cons2) was extracted applying a majority rule (> 99%). Both consensus sequences were then aligned

to each other and checked for differences, which mainly occurred in repeated as well as homologous repeat regions (*hrs*). The alignment was then checked manually for ambiguities and sequence discrepancies. The correction was based on the read coverage supporting one ambiguous region per contig generated by CLC. The cut-off of the adopted corrections was coverage of 20 reads per ambiguous region. One final genome sequence of SpliNPV-Tun2 was generated based on the majority of read coverage and submitted to GenBank (Accession number MG958660).

Phylogenetic reconstruction

The 38 core genes of SpliNPV-Tun2 were translated to amino acid sequence, then aligned with core gene amino acid sequences from 88 group II NPVs, 39 group I NPVs, and from CpGV-M and SpliGV-K1 as outgroups using MUSCLE alignment tool v3.8.425 as implemented in Geneious Prime® v11 (Biomatters Ltd., Auckland, New Zealand) (Edgar 2004). The concatenated alignments of the amino acid sequences of the 38 baculovirus core genes (Wennmann et al. 2018) were then used to infer a phylogenetic tree using the Minimum Evolution method implemented in MEGA.7 (Kumar et al. 2016).

Comparison of the SpliNPV-Tun2 genome to other NPVs

All of the 132 SpliNPV-Tun2 ORFs were tested for sequence similarity using BlastX. A detailed comparison of the similarity with genomes of SpliNPV-AB1956 and SpltNPV-G2 was made. The genome characteristics were compared in terms of length, GC%, ORF number, presence of genes.

Results

In 2013, a laboratory colony of *S. littoralis* collected from tomato fields in Monastir (Tunisia) was established. In the reared colony, an occasional occurrence of moribund larvae with symptoms of a nucleopolyhedrosis infection was observed. The purification of viral OBs and DNA, PCR amplification using *polh* specific primers and sequence analysis (data not shown) indicated that the infective agent was a SpliNPV isolate, which was eventually termed SpliNPV-Tun2.

Virulence and OB yield of SpliNPV-Tun2

Concentration mortality bioassays with third instar larvae were performed to determine the virulence of SpliNPV-Tun2. The LC_{50} value at 7 dpi was estimated to 1.5×10^4 OB/ml with a 95% confidence interval of $0.2–5.6 \times 10^4$ OB/ml ($n = 525$, slope probit line = 0.42, $Chi^2 = 8.81$). The survival rates determined at various time points after infection were inversely proportional to the applied OB concentration of $10^3–10^8$ OB/ml (Fig. 1). In the uninfected control, a slight decrease in the survival probability with 84% was observed at 14 dpi [95% Cl (76.1–92.7%)]. A concentration-dependent decrease in the survival probability was observed in the treatment groups starting from 4 dpi with 96.7% [95% Cl (96.0–97.4%)] and reached 7.81% [95% Cl (6.48–9.40%)] at 14 dpi. The median mortality was obtained between 7 dpi for applied concentrations of 10^7 and 10^8 OB/ml and 10 dpi for the lowest concentrations10^3 and 10^6 OB/ml of SpliNPV-Tun2. To estimate the survival covariance by time and by treatment, the survival was presented by percentage and survival data were normalized with lambda = 0.57 (Table 1). The survival time was statistically different depending on the applied virus concentrations. By using the different concentrations of SpliNPV-Tun2 OBs, all treatments produced different survival percentages depending on the time ($F = 7.78$, P value < 0.01).

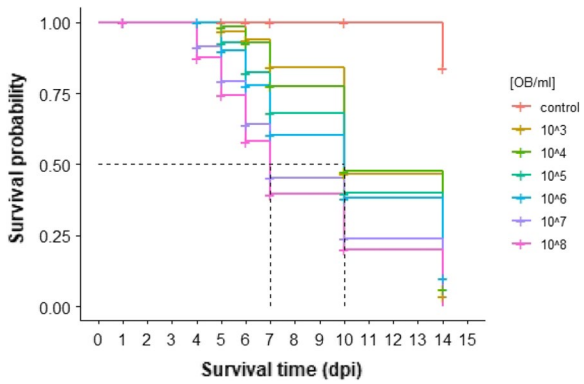

Fig. 1 Kaplan–Meier survival analysis of *Spodoptera littoralis* L3 larvae infected with different concentrations of SpliNPV-Tun2, ranging from 10^3 to 10^8 OB/ml. The untreated control is given as an orange line. Each line contains three independent replicates with 25 larvae each. Survival time is given in days post-infection (dpi). Dash lines represent the median survival time (ST_{50}) for high (10^7 and 10^8 OB/ml) and low OB (10^6 OB/ml or less) concentrations

Table 1 Analysis of variance comparison of survival percentage of third instar larvae of *Spodoptera littorals* infected with SpliNPV-Tun2 isolate

Source	DF	MS	*F* value	*P* value*
Time	6	28,840.5	402.41	< 0.001
Treatments	11	8333.6	116.28	< 0.001
Time × treatments	66	557.3	7.78	< 0.001
Residual SD	168	71.7		
Error	8.466			

DF Degree of freedom, *MS* Mean square

*Two-way factorial ANOVA at $\alpha = 0.05$

OB productivity of late fourth and fifth instar larvae was quantified. The mean weight of larvae with virus infection symptoms was 1548 mg with a standard deviation (s.d.) of 82.5 mg. The OBs were harvested at 14 dpi when infected larvae were seen as highly moribund. Three different standard methods for OB purification were compared, i.e. LSC, SCC, and SGU (Wennmann and Jehle 2014). OB yield was found to be significantly different among LSC, SCC and SGU purification methods (ANOVA, $P \leq 0.05$) [$F(2,6) = 88.11$, $p < 0.001$]. LSC yielded 2.7×10^9 OB/g larvae weight, followed by SCC 1.3×10^9 OB/g larvae weight, whereas SGU yielded only 5×10^8 OB/g larvae weight. (Fig. 2).

Genome sequence of SpliNPV-Tun2

A total of 1,597,175 filtered reads amounting to (90.6%) of the total raw reads were used for the analysis. From the total of the filtered reads, 1,508,620 paired reads and 88,555 unpaired reads could be mapped to the reference genome of SpliNPV-AN1956, whereas about 13,500 reads did not map to SpliNPV-AN1956 but gave BLAST hits with insect or bacterial DNA sequences.

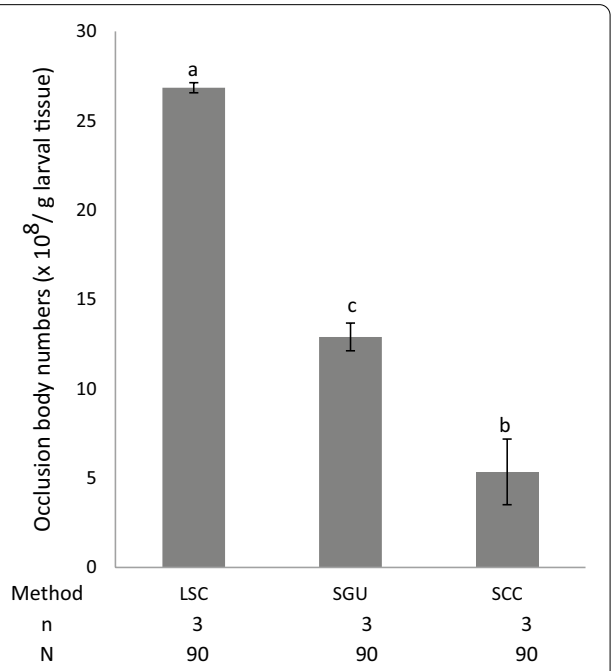

Fig. 2 Occlusion body yield (OB/g) from infected early L4 larvae harvested at 14 days post-infection depending on three purification methods low-speed centrifugation (LSC), sucrose gradient ultracentrifugation (SGU), and sucrose cushion centrifugation (SCC); *n* and *N* give the number of independent repetition the number of the tested larva, respectively. Vertical bars indicate the 95% confidential limits. Comparison of means using Student *t* test: Means with different letters (**a**, **b** and **c**) are significantly different at $P < 0.05$

The obtained genome consensus sequence of SpliNPV-Tun2 (MG958660) was supported by an average of 720-fold sequencing depth (s.d. = 316). It had a length of 137,099 bp and a GC content of 44.7% (Table 2). It contained 132 open reading frames (ORF) and 15 homologous repeat regions (*hrs*). Based on the nucleotide sequences, the genomes of SpliNPV-Tun2 and SpliMNPV-AN1956 were 98.2% identical and the *hrs* in both genomes were at the same location. The genome of SpliNPV-Tun2 was 899 bp shorter than that of SpliMNPV-AN1956 through alignment of the 2 genomes revealed the same number of ORFs, but with some differences. Sixty-nine ORF had a 100% predicted amino acid identity to SpliMNPV-AN1956 ORFs. The rest of the ORFs' amino acid identities ranged between 90 and 99%, whereas ORF 37 had only 97% amino acid (aa) sequence identity (Table 2). The sequences encoding putative proteins accounted for 88.4% for SpliNPV-Tun2, while the coding density was 87.9% in SpliMNPV-AN1956. The number of intergenic regions is 101 for SpliNPV-Tun2 and 102 in SpliMNPV-AN1956, with mean distances of 124 bp and 121 bp, respectively. Some of the intergenic regions consisted of palindromic sequences; both isolates contained 15 *hrs* at the same genome location (Table 2).

Phylogenetic reconstruction and genetic distance

A minimum evolution phylogenetic tree based on the concatenated amino acid sequences of 38 baculovirus core genes of group I and group II NPVs was inferred (Fig. 3). It corroborated the close relationship between SpliNPV-Tun2 and -AN1956. The next neighbour to both isolates was SpltNPV-G2. The SpliNPV isolates and SpltNPV-G2 are only distantly related to other *Spodoptera*-specific NPVs, such as SeMNPV, SpltNPV-II and Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) (Fig. 3).

For baculovirus species demarcation, the Kimura-2-Parameter (K2P) distance of the 38 baculovirus core genes can be used as criterion, according to which, 2 isolates are considered to belong to the same species if their K2P distance is smaller < 0.021 and to different species if the K2P distance is > 0.072 (Wennmann et al. 2018). With a K2P distance of 0.001, SpliNPV-Tun2 and -AN1956 are 2 isolates belonging to the same species *Spodoptera littoralis nucleopolyhedrovirus*. In contrast, SpltNPV-G2 is distant enough (0.099 from the two viruses to constitute a separate species *Spodoptera litura nucleopolyhedrovirus* (Wennmann et al. 2018). The other known *Spodoptera*-specific NPV isolates constitute several other discrete species (Fig. 3).

Table 2 Feature of the SpliNPV-Tun2 genome compared to those of SpliNPV-AN1956 and SpltNPV-G2

ORF name	SpliNPV-Tun2			SpliNPV-AN1956			SpliNPV-Tun2 vs SpliNPV-AN1956	SpltNPV-G2			SpliNPV-Tun2 vs SpltNPV-G2
	No	Start → end	Size (aa)	No	Start → end	Size (aa)	Range (%identity)	No	Start → end	Size (aa)	Range (%identity)
polh	1	1 → 750	249	1	1 → 747	248	248/249 (99%)	1	1 → 750	249	249/249 (100%)
pp78/83	2	747 ← 2,396	549	2	744 ← 2,378	544	51/542 (95%)	2	747 ← 2,393	548	245/345 (71%)
pk-1	3	2,398 → 3,228	276	3	2,380 → 3,216	278	274/278 (99%)	3	2,392 → 3,204	270	237/270 (88%)
hoar	4	3,590 ← 5,980	796	4	3,578 ← 5,956	792	790/797 (99%)	4	3,519 ← 5,714	731	244/300 (81%)
	5	6,638 ← 6,444	64	5	6,420 ← 6,614	64	64/64 (100%)	5	6,027 ← 6,230	67	26/35 (74%)
	6	6,764 → 8,982	756	6	6,742 → 9,006	754	616/659 (93%)	7	6,447 → 8,600	717	338/443 (76%)
Ie-0	7	9,274 → 10,149	291	7	9,297 → 10,172	291	291/291 (100%)	8	8,851 → 9,720	289	259/290 (89%)
dutpase	8	10,186 → 10,701	171	8	10,211 → 10,726	171	171/171 (100%)	10	9,774 → 10,268	164	105/131 (80%)
(5 P-I repeats)	hr1	10,769…11,040		hr1	10,794…11,070			hr1	10,301…10,800		
	9	11,113 → 12,54	476	9	11,097 → 12,518	473	470/470 (100%)	11	10,801 → 12,210	469	456/470 (97%)
odv-e18	10	12,566 → 12,817	83	10	12,544 → 12,795	83	83/83 (100%)	12	12,234 → 12,485	83	83/83 (100%)
odv-ec27	11	12,841 → 13,695	284	11	12,819 → 13,673	284	284/284 (100%)	13	12,517 → 13,368	283	271/284 (95%)
	12	13,723 → 14,004	93	12	13,701 → 13,982	93	93/93 (100%)	14	13,400 → 13,681	93	87/93 (94%)
Ac146	13	14,094 ← 14,706	203	13	14,073 ← 14,687	204	202/204 (99%)	15	13,726 ← 14,328	200	163/206 (79%)
i.e.-1	14	14,748 → 16,997	749	14	14,729 → 16,978	749	730/730 (100%)	16	14,501 → 16,567	688	501/696 (72%)
odv-e56	15	17,058 → 18,167	369	15	17,040 → 18,152	370	351/351 (100%)	17	16,693 → 17,808	371	328/351 (93%)
	16	18,182 → 18,733	183	16	18,167 → 18,718	183	183/183 (100%)	18	17,823 → 18,374	183	174/183 (95%)
p10	17	18,787 → 19,101	104	17	18,771 → 19,085	104	80/80 (100%)	19	18,439 → 18,756	105	80/80 (100%)
	18	19,079 → 20,053	324	18	19,063 → 20,034	324	323/324 (99%)	20	18,734 → 19,678	314	315/324 (97%)
p74	19	20,088 ← 22,064	658	19	20,068 ← 22,044	658	658/658 (100%)	21	19,706 ← 21,679	657	658/658 (100%)
rr1	20	22,166 ← 24,640	824	20	22,146 ← 24,632	828	802/803 (99%)	23	22,066 ← 24,378	770	758/760 (99%)
	21	24,690 → 25,904	404	21	24,682 → 25,902	406	402/406 (99%)	24	24,586 → 25,785	399	236/412 (57%)
(10 P-I and 6 P-II-like repeats)	hr2	26,028…26,311		hr2	26,117…27,290			hr2	26,751…27,151		
	22	27,116 → 28,123	335	22	27,305 → 28,309	334		26	27,162 → 28,055	297	271/304 (89%)
me53	23	28,136 ← 29,05	304	23	28,322 ← 29,236	304	303/304 (99%)	27	28,076 ← 28,981	301	284/301 (94%)
	24	29,138 → 29,344	68	24	29,324 → 29,530	68	68/68 (100%)	28	29,013 → 29,273	86	52/68 (76%)
lef-6	25	29,355 ← 29,813	152	25	29,541 ← 30,002	153	149/153 (97%)	29	29,287 ← 29,724	145	107/158 (68%)
dbp	26	29,872 ← 30,732	286	26	30,061 ← 30,921	286	286/286 (100%)	30	29,788 ← 30,660	290	234/290 (81%)
	27	30,856 → 31,257		27	31,049 → 31,450	133	133/133 (100%)	31	30,718 → 31,167	149	120/133 (90%)
ubi/gp37	28	31,283 → 32,296	337	28	31,476 → 32,489	337	336/337 (99%)	32	31,136 → 32,191	351	309/337 (92%)
39 k/PP3	29	32,351 ← 33,343	330	29	32,541 ← 33,533	330	330/330 (100%)	33	32,246 ← 33,214	322	258/340 (76%)
lef-11	30	33,207 ← 33,638	143	30	33,397 ← 33,828	143	143/143 (100%)	34	33,075 ← 33,509	144	123/144 (85%)
Ac38	31	33,608 ← 34,285	225	31	33,798 ← 34,478	226	221/226 (98%)	35	33,479 ← 34,141	220	194/225 (86%)

Table 2 (continued)

ORF name	SpliNPV-Tun2			SpliNPV-AN1956			SpliNPV-Tun2 vs SpliNPV-AN1956	SpltNPV-G2			SpliNPV-Tun2 vs SpltNPV-G2
	No	Start → end	Size (aa)	No	Start → end	Size (aa)	Range (%identity)	No	Start → end	Size (aa)	Range (%identity)
p47	32	34,362 ← 35,627	421	32	34,555 ← 35,820	421	421/421 (100%)	36	34,211 ← 35,479	422	371/422 (88%)
lef-12	33	35,66 → 36,256	198	33	35,853 → 36,449	198	198/198 (100%)	37	35,506 → 36,111	201	168/198 (85%)
(1 P-1 repeat)	hr3	36,321…36,372		hr3	36,506…36,547			hr3	36,102…36,301		
lef-8	34	36,433 ← 39,168	911	34	36,618 ← 39,353	911	910/911 (99%)	38	36,316 ← 39,072	918	830/920 (90%)
bjdp	35	39,167 → 40,078	303	35	39,352 → 40,263	303	303/303 (100%)	39	39,071 → 39,979	302	303/303 (100%)
	36	40,101 → 40,67	189	36	40,286 → 40,855	189	189/189 (100%)	40	40,001 → 40,570	189	189/189 (100%)
	37	40,691 ← 40,891	66	37	40,877 ← 41,071	64	55/66 (83%)	41	40,590 ← 40,754	54	54/66 (82%)
chitA	38	40,906 ← 42,705	599	38	41,086 ← 42,885	599	598/599 (99%)	42	40,767 ← 42,461	564	562/563 (99%)
(5 P-I repeats)	hr4	42,602…43,004		hr4	42,837…43,091			hr4	42,502…43,051		
	39	43,126 ← 43,728	200	39	43,305 ← 43,907	200	198/200 (99%)	43	43,021 ← 43,635	204	130/203 (64%)
	40	43,945 ← 44,583	212	40	44,073 ← 44,711	212	210/212 (99%)	44	43,779 ← 44,405	208	167/212 (79%)
	41	44,653 → 45,099	148	41	44,783 → 45,229	148	148/148 (100%)	45	44,475 → 44,888	137	123/148 (83%)
	42	45,133 ← 46,407	424	42	45,265 ← 46,533	422	410/425 (96%)	46	44,926 ← 46,194	422	241/257 (94%)
	43	46,412 ← 46,639	75	43	46,538 ← 46,765	75	75/75 (100%)	47	46,199 ← 46,426	75	71/75 (95%)
Lef-10	44	46,599 → 46,85	83	44	46,725 → 46,973	82	75/83 (90%)	48	46,386 → 46,640	84	71/84 (85%)
vp1054	45	46,687 → 47,727	346	45	46,813 → 47,850	345	330/330 (100%)	49	46,474 → 47,532	352	306/337 (91%)
	46	47,856 → 48,071	71	46	47,979 → 48,194	71	71/71 (100%)	50	47,653 → 47,868	71	57/71 (80%)
	47	48,355 → 48,855	166	47	48,478 → 48,978	166	166/166 (100%)	51	48,172 → 48,693	173	154/166 (93%)
	48	48,906 ← 49,472	188	48	49,032 ← 49,601	189	170/172 (99%)	52	48,722 ← 49,246	174	104/152 (68%)
	49	49,489 ← 49,737	82	49	49,618 ← 49,866	82	66/66 (100%)	53	49,271 ← 49,519	82	57/66 (86%)
cathepsin	50	49,784 → 50,794	336	50	49,913 → 50,923	336	334/336 (99%)	54	49,566 → 50,579	337	313/337 (93%)
p49	51	50,843 → 52,183	446	51	50,972 → 52,312	446	446/446 (100%)	55	50,628 → 51,947	439	376/446 (84%)
fp25k	52	52,291 ← 52,884	197	52	52,420 ← 53,013	197	197/197 (100%)	57	52,075 ← 52,668	197	197/197 (100%)
lef-9	53	53,042 ← 54,538	498	53	53,171 ← 54,667	498	498/498 (100%)	59	52,839 ← 54,335	498	479/498 (96%)
(8 P-I repeats)	hr5	54,548…55,204		hr5	54,677…55,304			hr5	54,401…55,642		
	54	55,105 → 55,392	95	54	55,352 → 55,648	98	76/82 (93%)	_	_	_	_
	55	55,424 → 55,732	102	55	55,681 → 55,989	102	80/80 (100%)	_	_	_	_
(3 P-I and 10 P-II-like repeats)	hr6	55,715…56,710		hr6	56,009…57,005			hr6	55,992…56,166		
	56	56,760 ← 57,793	346	56	57,057 ← 58,097	346	342/346 (99%)	62	56,221 ← 57,324	367	210/371 (57%)
	57	57,944 → 58,948	334	57	58,250 → 59,254	334	333/334 (99%)	63	57,477 → 58,478	333	302/335 (90%)
	58	59,434 ← 59,030	134	58	59,335 ← 59,742	134	131/134 (98%)	64	58,556 ← 58,966	136	98/136 (72%)
	59	59,030 ← 59,434	313	59	59,675 ← 60,616	313	313/313 (100%)	65	58,899 ← 59,840	313	262/310 (85%)

Table 2 (continued)

ORF name	SpliNPV-Tun2			SpliNPV-AN1956			SpliNPV-Tun2 vs SpliNPV-AN1956	SpltNPV-G2			SpliNPV-Tun2 vs SpltNPV-G2
	No	Start → end	Size (aa)	No	Start → end	Size (aa)	Range (%identity)	No	Start → end	Size (aa)	Range (%identity)
	60	59,367 ← 60,308	138	60	60,558 ← 60,974	138	138/138 (100%)	66	59,800 ← 60,198	132	123/135 (91%)
	61	60,25 ← 60,666	349	61	60,979 → 62,028	349	346/349 (99%)	67	60,203 → 61,276	357	314/357 (88%)
	62	60,671 → 61,720	800	62	62,263 ← 64,665	800	799/800 (99%)	68	61,552 ← 63,924	790	698/805 (87%)
	63	62,026 ← 64,428	1020	63	64,667 → 67,729	1020	1019/1020 (99%)	69	63,926 → 66,994	1022	924/1027 (90%)
	64	67,446 ← 67,633	36	64	67,683 ← 67,871	62	16/16 (100%)	_	_	_	_
	hr7	67,605…67,800		hr7	67,916…68,117			hr7	67,362…76,581		
	65	67,907 → 69,049	380	65	68,224 → 69,366	380	380/380 (100%)	70	67,509 → 68,651	380	309/380 (81%)
	66	69,204 ← 69,827	207	66	69,444 ← 70,067	207	207/207 (100%)	71	68,727 ← 69,353	208	160/213 (75%)
	67	69,899 ← 70,282	127	67	70,139 ← 70,522	127	127/127 (100%)	72	69,412 ← 69,795	127	123/127 (97%)
	68	70,305 ← 70,559	84	68	70,545 ← 70,799	84	68/68 (100%)	73	69,814 ← 70,068	84	67/68 (99%)
lef-1	69	70,624 ← 71,772	382	69	70,864 ← 72,015	383	358/359 (99%)	74	70,133 ← 71,287	384	331/340 (97%)
vlf-1	70	71,793 ← 72,155	120	70	72,036 ← 72,398	120	119/120 (99%)	75	71,307 ← 71,669	120	84/124 (68%)
gp41	71	72,152 ← 73,132	326	71	72,395 ← 73,375	326	311/311 (100%)	76	71,666 ← 72,658	330	307/313 (98%)
	72	73,107 ← 73,820	237	72	73,350 ← 74,063	237	236/237 (99%)	77	72,633 ← 73,331	232	206/238 (87%)
tlk-20	73	73,681 ← 74,268	195	73	73,924 ← 74,505	193	179/195 (92%)	78	73,210 ← 73,803	197	160/203 (79%)
vp90	74	74,237 → 76,807	856	74	74,474 → 77,044	856	850/856 (99%)	79	73,772 → 76,357	861	739/862 (86%)
(6 P-I repeats)	hr8	76,989…77,270		hr8	77,112…77,422			hr8	76,362…76,581		
cg30	75	77,320 ← 78,099	259	75	77,534 ← 78,313	259	259/259 (100%)	80	76,639 ← 77,391	250	188/257 (73%)
vp39	76	78,128 ← 79,036	302	76	78,342 ← 79,250	302	302/302 (100%)	81	77,450 ← 78,358	302	296/302 (98%)
lef-4	77	79,038 → 80,504	488	77	79,252 → 80,721	489	479/489 (98%)	82	78,360 → 79,787	475	413/488 (85%)
p33	78	80,535 ← 81,302	255	78	80,751 ← 81,518	255	255/255 (100%)	83	79,833 ← 80,600	255	248/255 (97%)
	79	81,301 → 81,831	176	79	81,517 → 82,050	177	174/177 (98%)	84	80,599 → 81,147	182	167/182 (92%)
odv-e25	80	81,828 → 82,508	226	80	82,047 → 82,727	226	226/226 (100%)	85	81,144 → 81,827	227	215/227 (95%)
DNA helicase	81	82,613 ← 86,368	1251	81	82,832 ← 86,587	1251	1251/1251 (100%)	86	81,918 ← 85,625	1235	1155/1251 (92%)
	82	86,337 → 86,852	171	82	86,556 → 87,071	171	171/171 (100%)	87	85,594 → 86,106	170	165/171 (96%)
38 k	83	86,859 ← 87,776	305	83	87,079 ← 87,996	305	304/305 (99%)	88	86,114 ← 87,028	304	298/305 (97%)
lef-5	84	87,672 → 88,568	298	84	87,892 → 88,785	297	296/298 (99%)	89	86,924 → 87,832	302	254/305 (83%)
p6.9	85	88,589 ← 88,858	89	85	88,806 ← 89,069	87	10/10 (100%)	90	87,850 ← 88,104	84	10/10 (100%)
p40	86	88,919 ← 90,022	367				345/346 (99%)	91	88,162 ← 89,253	363	331/367 (90%)
(8 P-I repeats)	hr9	90,022 ← 88,919		hr9	90,305…90,732			hr9	89,262…89,651		
p12	87	90,546 ← 90,914	122	86	89,130 ← 90,233	367	122/122 (100%)	92	89,657 ← 90,022	121	97/122 (80%)
p45	88	90,911 ← 92,038	375	87	90,770 ← 91,138	122	374/375 (99%)	93	90,019 ← 91,140	373	356/373 (95%)

Table 2 (continued)

ORF name	SpliNPV-Tun2			SpliNPV-AN1956			SpliNPV-Tun2 vs SpliNPV-AN1956	SpltNPV-G2			SpliNPV-Tun2 vs SpltNPV-G2
	No	Start → end	Size (aa)	No	Start → end	Size (aa)	Range (%identity)	No	Start → end	Size (aa)	Range (%identity)
vp80	89	92,058 → 93,992	644	88	91,135 ← 92,262	375	638/648 (98%)	94	91,167 → 93,101	644	548/630 (87%)
	90	93,989 → 94,156	55	89	92,282 → 94,228	648	55/55 (100%)	95	93,101 → 93,268	55	54/55 (98%)
odv-ec43	91	94,182 → 95,267	361	90	94,421 → 95,506	361	361/361 (100%)	96	93,300 → 94,385	361	357/361 (99%)
	92	95,358 → 95,696	112	91	95,599 → 95,937	112	112/112 (100%)	97	94,466 → 94,804	112	103/112 (92%)
odv-e66	93	95,686 ← 97,836	716	92	95,927 ← 98,074	715	715/716 (99%)	98	94,794 ← 96,872	692	633/679 (93%)
p13	94	97,779 ← 98,636	285	93	98,017 ← 98,874	285	285/285 (100%)	99	96,875 ← 97,744	289	262/289 (91%)
(8 P-I repeats)	hr10	98,699…99,009		hr10	98,878…99,348			hr10	97,753…98,049		
	95	99,224 ← 99,748	174	94	99,458 ← 99,982	174	171/174 (98%)	127	125,316 ← 125,834	172	87/149 (58%)
	96	100,205 → 101,173	322	95	100,441 → 101,409	322	320/322 (99%)	100	98,090 → 99,055	321	242/322 (75%)
	97	101,215 ← 101,913	232	96	101,451 ← 102,149	232	232/232 (100%)	101	99,106 ← 99,816	236	206/240 (86%)
	98	101,933 ← 103,342	469	97	102,169 ← 103,578	469	468/469 (99%)	102	99,836 ← 101,209	457	374/470 (80%)
	99	103,357 ← 103,89	177	98	103,593 ← 104,126	177	176/177 (99%)	103	101,236 ← 101,775	179	157/179 (88%)
	100	103,972 ← 104,169	65	99	104,211 ← 104,408	65	65/65 (100%)	104	101,855 ← 102,055	66	45/66 (68%)
	101	104,256 → 105,551	431	100	104,495 → 105,790	431	431/431 (100%)	105	102,193 → 103,449	418	372/420 (89%)
(7 P-I and 24 P-II-like repeats)	hr11	105,681…105,729		hr11	104,511…107,449						
	102	106,726 ← 107,649	518	101	107,481 ← 108,530	349	212/212 (100%)	107	105,624 → 106,226		140/209 (67%)
pif-3	103	107,678 → 108,28	317	102	108,559 → 109,161	200	200/200 (100%)	108	106,234 → 106,587	200	184/199 (92%)
	104	108,395 → 108,63	237	103	109,276 → 109,512	78	78/78 (100%)	112	107,915 ← 109,045	208	181/208 (87%)
alk-exo	105	108,655 ← 109,902	835	104	109,541 ← 110,788	415	413/415 (99%)	109	109,007 → 109,165	408	339/411 (82%)
	106	109,998 ← 111,158	387	106/107	111,873 ← 112,064@112,210 ← 112,605	131/63	232/232 (100%)@60/62 (97%)	110	109,168 ← 109,551	376	172/230 (75%)
	107	111,207 → 111,408	67	_	_	_	33/33 (100%)	111	109,553 → 110,758	52	30/33 (91%)
	108	111,307 ← 111,702	131	108	112,607 → 113,839	410	395/395 (100%)	112	110,817 ← 111,449	127	106/131(81%)
	109	111,704 → 112,936	410	109	113,893 ← 114,669	258	256/259 (99%)	113	111,433 ← 111,777	401	331/395 (84%)
lef-2	110	112,992 ← 113,771	259	110				114	105,624 → 106,226	210	189/209 (90%)
	111	113,608 ← 114,033	141	111	114,509 ← 114,931	140	14/116 (98%)	115	106,234 → 106,587	114	82/97 (85%)
p24 capsid	112	114,02 → 114,739	239	112	114,918 → 115,637	239	238/239 (99%)	116	111,825 → 112,559	244	220/241 (91%)
(7 P-I repeats)		114,740…115,157		hr12				hr12	112,542…112,961		
	113	115,35 → 118,139	929	113	116,248 → 119,034	928	918/929 (99%)	118	113,090 → 115,849	918	762/908 (84%)
	114	118,156 ← 118,875	239	114	119,051 ← 119,782	243	239/243 (98%)	119	115,866 ← 116,585	239	239/242 (99%)
bro-a	115	118,941 ← 119,489	182	115	119,836 ← 120,390	184	180/185 (97%)	120	116,653 → 117,213	186	179/185 (97%)
egt	116	119,733 ← 121,331	532	116	120,636 ← 122,234	532	532/532 (100%)	121	117,465 → 119,033	522	505/505 (100%)

Table 2 (continued)

ORF name	SpliNPV-Tun2			SpliNPV-AN1956			SpliNPV-Tun2 vs SpliNPV-AN1956	SpltNPV-G2			SpliNPV-Tun2 vs SpltNPV-G2
	No	Start → end	Size (aa)	No	Start → end	Size (aa)	Range (%identity)	No	Start → end	Size (aa)	Range (%identity)
fgf	117	121,458 → 122,189	243	117	122,363 → 123,094	243	242/243 (99%)	122	119,165 → 119,905	246	242/243 (99%)
	118	122,214 ← 122,447	77	118	123,121 ← 123,354	77	77/77 (100%)	123	119,928 → 120,161	77	77/77 (100%)
pif-1	119	122,452 ← 124,029	525	119	123,359 ← 124,936	525	523/525 (99%)	124	120,184 → 121,764	526	505/507 (99%)
(2 P-I repeats)				hr13	(2 P-Irepeats)			hr13	121,771…121,926		
38.7 k	120	124,29 ← 125,327	345	120	125,203 ← 126,240	345	343/345 (99%)	128	125,891 ← 126,919	342	287/343 (84%)
lef-1	121	125,314 ← 126,009	231	121	126,227 ← 126,922	231	231/231 (100%)	129	126,906 ← 127,601	231	214/231 (93%)
	122	125,99 ← 126,37	126	122	126,903 ← 127,289	128	126/128 (98%)	130	127,582 ← 127,950	122	110/127 (87%)
	123	126,367 ← 126,897	176	123	127,286 ← 127,816	176	176/176 (100%)	131	127,947 ← 128,477	176	159/176 (90%)
calyx/pep	124	126,907 ← 127,968	353	124	127,826 ← 128,887	353	353/353 (100%)	132	128,481 ← 129,515	344	155/168 (92%)
pkip	125	128,057 → 128,596	179	125	128,976 → 129,515	179	179/179 (100%)	133	129,544 → 130,161	205	157/180 (87%)
arif-1	126	128,63 ← 129,358	242	126	129,549 ← 130,277	242	238/242 (98%)	134	130,199 ← 130,936	245	215/246 (87%)
pif-2	127	129,386 → 130,651	421	127	130,305 → 131,570	421	421/421 (100%)	135	130,910 → 132,187	425	371/404 (92%)
(2P-I and 6 P-II-like repeats)		130,679…131,114		hr14	(2 P-I and 6P-II-like repeats)			hr14	132,105…133,451		
Ac23	128	131,200 → 133,242	680	128	132,119 → 134,155	678	670/681 (98%)	136	133,451 → 135,499	682	608/683 (89%)
	129	133,276 ← 134,004	242	129	134,189 ← 134,917	242	241/242 (99%)	137	135,545 ← 136,240	231	150/190 (79%)
	130	134,109 → 134,885	258	130	135,022 → 135,798	258	258/258 (100%)	138	136,338 → 137,117	259	223/259 (86%)
	131	134,972 → 135,394	90	131	135,885 → 136,295	136	58/58 (100%)	_	_	_	_
(6 P-II-like repeats)		135,610…135,811		hr15				hr15	137,619…138,104		
	132	135,866 ← 136,750	294	132	136,765 ← 137,649	294	294/294 (100%)	140	138,104 ← 138,952	282	138/295 (47%)

Given are the names of open reading frames (ORF), ORF number (No.), start and end position of the ORFs including direction of transcription (arrow), the translated amino acid (aa) length of the ORF, and the compared range and percent identity of the ORFs

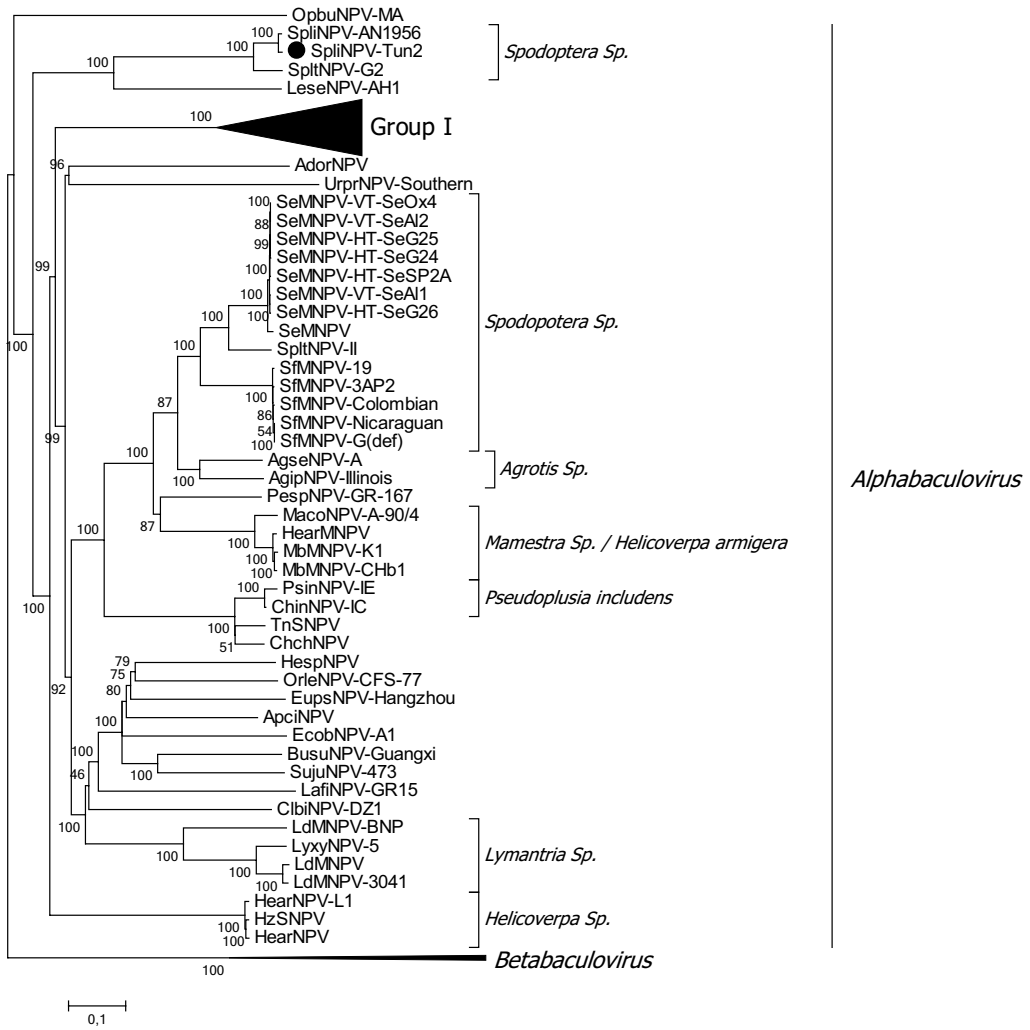

Fig. 3 Baculovirus phylogeny based on Minimum Evolution (ME) method of amino acid sequences of 38 core genes of different NPV. Translated amino acid sequences of each core gene were separately aligned, and alignments were concatenated using Geneious 8. The consensus tree was obtained by a heuristic search with 500 bootstrap replicates. The Model is the JTT model (Jones et al. 1992) with a uniform rate among site and without invariant site. Bootstrap values (> 50%) are shown at each node. Only *Alphabaculovirus* group II is given in detail. *Alphabaculovirus* group I and *Betabaculovirus* were reduced. Betabaculoviruses represented with only CpGV-M and SpliGV-K1 for this analysis were used as outgroup

Main genome differences between SpliNPV-Tun2 and -AN1956

Compared to SpliNPV-AN1956, the new isolate SpliNPV-Tun2 showed insertion and deletion mutations in 62 ORFs, of which 37 ORFs are with predicted function. ORFs with significant changes caused by deletions and insertions are illustrated in (Fig. 4). These differences affect ORFs coding for predicted virus proteins related to virus structure, such as the structural protein PP78/81, the capsid-associated protein VP80 and VP1054, the OB matrix protein (Polyhedrin, POLH), the nucleotide metabolism (Ribonucleotide Reductase, RR1), proteins involved in viral DNA replication (Late Expression Factor 2 (LEF-2) and LEF-10, Protein kinase 1 (PK-1), LEF-5, and the group II *Alphabaculovirus*-specific HOAR and the BRO-a. Furthermore, a considerable number of amino acid changes were found but will not be further detailed here.

A notable difference is the presence of a tyrosine residue close to the N-terminus fifth amino acid position of the predicted POLH of SpliNPV-Tun2, a residue which is missing in the POLH of SpliNPV-AN1956 (Fig. 4). Another difference between the genome sequences of SpliNPV-Tun2 and -AN1956 is related to ORFs 106 and

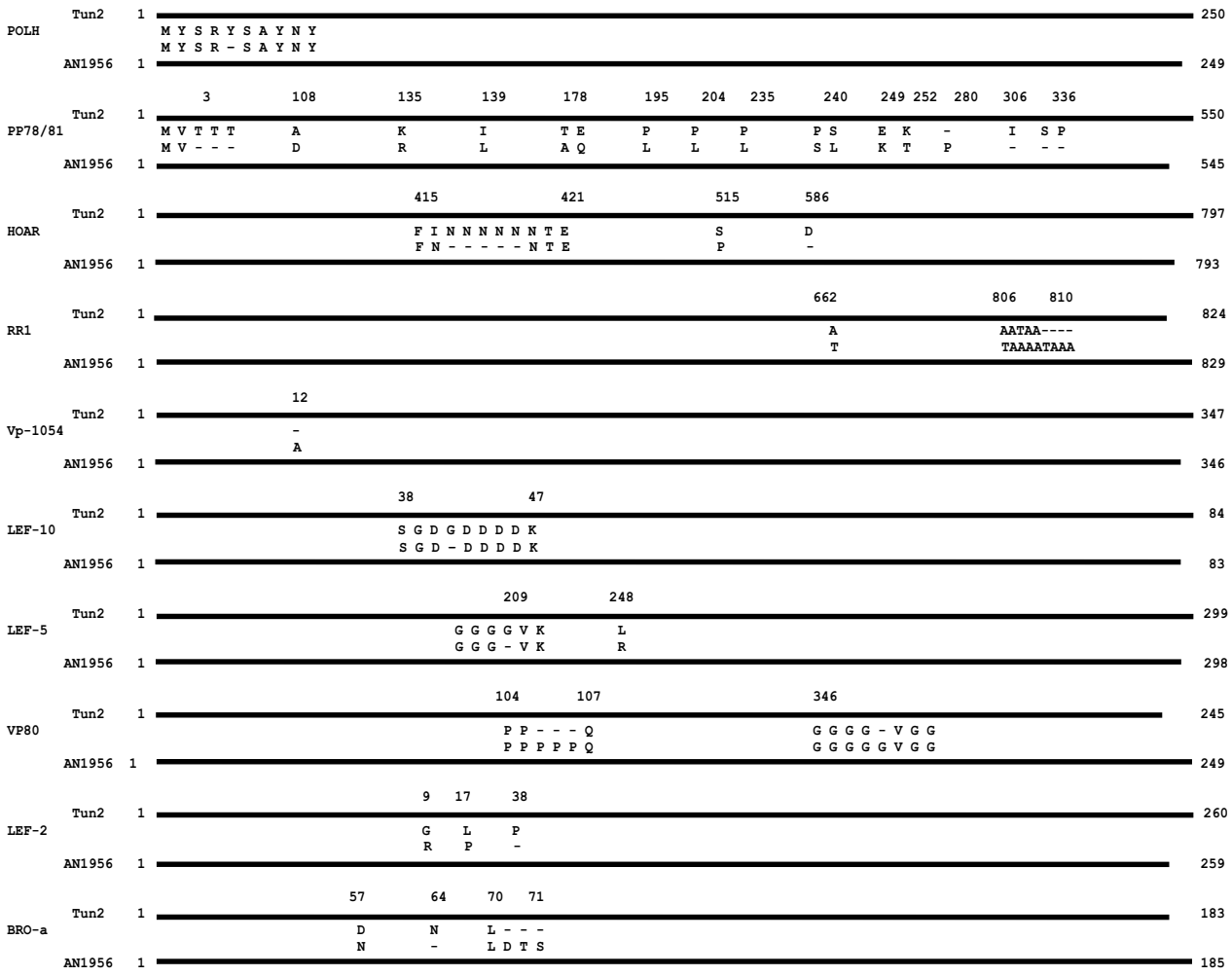

Fig. 4 Graphic representation of selected open reading frames with insertion and deletion (indel) mutations and amino acid changes of SpliNPV-Tun2 and -AN1956 (JX454574). The total protein length of each open reading frame (ORF) is shown to the right, while the numbers above each insertion/deletion region represent the corresponding amino acids from the aligned reference sequence. Deletions/insertions/substitution are illustrated between the bars

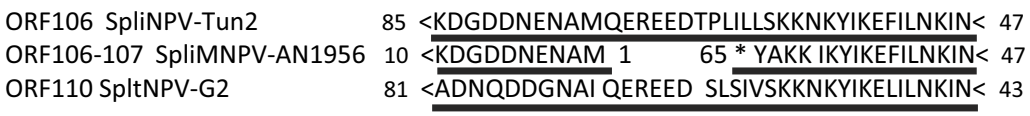

Fig. 5 Alignment of the translated ORF 106 in SpliNPV-Tun2 to the homologous ORF 106–107 *of* SpliMNPV-AN1956 and ORF 110 *in* SpltNPV-G2. Numbers and arrows on both sides of the lines indicate the amino acid position and ORF orientation in the corresponding translated gene. *indicates the stop codon of ORF 107 of SpliMNPV-AN1956

107. Whereas in SpliNPV-AN1956 two ORF 106 and ORF 107 were located from genome position 110,884 < 111,843 (319 aa) and 111,873 < 112,064 (63 aa), respectively, these two ORFs were identified as one single ORF in SpliNPV-Tun2 (ORF 106, genome position 109,998 < 111,161, (387 aa)). The split of the ORF 106 homolog of SpliNPV-Tun2 into two ORFs 106 and 107 in SpliNPV-AN1956 is caused by a missing thymidine residue at genome position 110,991 of SpliNPV-AN1956, causing a frameshift and separation into two ORFs (Fig. 5). Interestingly, a similar homologous ORF 110 (genome position

107,915 < 109,045, 376 aa) is present in the narrowly related SpltNPV-G2, which may suggest a variation in SpliNPV-AN1956 or a sequencing error at this position in the original sequence of SpliNPV-AN1956. Compared to SpliNPV-AN1956, SpliNPV-Tun2 encodes an additional ORF 107 (111,207 > 111,408, 67 aa), which 5′ region overlaps with the 5′ region of the adjacent ORF 108. In SpltNPV-G2, the 5′ region of ORF 111 (109,007 > 109,165, 52 aa) would be homologous to the 3′ region of ORF 107 of –Tun2 (Fig. 5).

Discussion

A new variant of SpliNPV, termed Tun2, was isolated and characterized by bioassays and genome sequencing. SpliNPV-Tun2 was found in a *S. littoralis* colony that was derived from larvae collected in tomato fields in Central-East Tunisia, in 2013. Another natural SpliNPV-Tun isolate was found in 2008 from tomato field in Chott-Mariem (Sousse) (Laarif et al. 2011), suggesting that SpliNPV is present in wild populations of *S. littoralis* in Tunisia. Though the conditions of bioassays performed with SpliNPV-Tun2 were not fully identical to the bioassays carried out with SpliNPV-Tun, both the LC_{50} and the ST_{50} values were similar, suggesting that both isolates may not have significant biological differences.

OB productivity was quantified in the fourth instar larvae, as this instar was identified to be optimal for virus production (Grzywacz et al. 1998). Three different methods for OB purification were tested, of which the low-speed centrifugation (LSC) method (Harrison 2008) producing the highest yields of polyhedral OBs, which corresponded to an OB yield of about 4.2×10^9 OB/larvae. The superiority of LSC for polyhedral OB purification compared to sucrose gradient ultracentrifugation (SGU) and sucrose cushion centrifugation (SCC) was previously noted for isolation of Agrotis segetum nucleopolyhedrovirus by Wennmann and Jehle (2014). LSC and SCC are methods typically used for OB purification from NPVs (Harrison 2008), whereas SGU appears to counterselect for NPV polyhedra but favours purification of GV granules yielding about five times less NPV OBs than the other 2 methods (Wennmann and Jehle 2014).

Whole genome sequencing of SpliNPV-Tun2 revealed its close relationship to SpliNPV-AN1956 (Breitenbach et al. 2013), another isolate of SpliNPV from North Africa, which originated from Egypt and was first described by Abul Nasr (1956). Other isolates of SpliNPV from North Africa and the Mediterranean area were reported by Laarif et al. (2011). Only a few differences between the genome sequence of SpliNPV-Tun2 SpliNPV-AN1956 were noted: (i) the genome of SpliNPV-Tun2 is little shorter, (ii) both genomes contain the same number of ORFs and *hrs* and are fully collinear to each other, (iii) minor indel mutations could be identified in 34 ORFs as well as in intergenic regions, (iv) genetic changes were noticed in nine baculovirus core genes, and also in the highly conserved *polh* gene, and (v) the ORFs 106 and 107 of SpliNPV-AN1956 appeared to be fused to a single ORF 106 in SpliNPV-Tun2 but an additional ORF 107 was identified.

Phylogenetic analyses based on the 38 baculovirus core genes have been shown to reflect isolate and species phylogeny of baculovirus evolution and are considered as the most reliable method to infer the phylogenetic position of a given baculovirus (Wennmann et al. 2018). Our Minimum Evolution phylogenetic analysis revealed SpliNPV-AN156 and SpltNPV-G2 as closest neighbours of SpliNPV-Tun2. SpltNPV-G2 is an in vivo cloned genotype of an isolate separated from cadavers of *S. litura,* cotton leaf worm, in the area of Guangzhou, China (Pang et al.2001). Breitenbach et al. (2013) found that SplitNPV-AN1956 and SpltNPV-G2 share a highly collinear genome and form a distantly related clade to other NPVs specific for *Spodoptera* species, such as SeMNPV, SfMNPV, and SpltNPV-II. Our phylogenetic analyses confirm that SplitNPV-Tun2, -AN1956, and SpltNPV-G2 form a clade of *Spodoptera*-specific NPVs which is separate from other group II alphabaculoviruses isolated from *Spodoptera* species, such as SeMNPV, SpltNPV-II, and SfMNPV. K2P distances of the 38 core genes clearly indicate that SpliNPV-Tun2 and -AN1956 should be considered as isolates from the same species, whereas SpltNPV-G2 belongs to a separate alphabaculovirus species, as well as all other even more distant NPVs isolated from *Spodoptera* sp. (Wennmann et al. 2018; Escasa et al.2019).

Conclusions

Identification and genome sequence of the new isolate SpliNPV-Tun2 originating from Tunisia extended the present knowledge related to the genetic diversity of SpliNPV. With the detailed characterization of its genome, SpliNPV-Tun2 is proposed to be further evaluated as a biological agent for control of *S. littoralis* and potentially for the fall armyworm, *S. frugiperda*, and tobacco cutworm *S. litura.*

Abbreviations

aa: Amino acid; dpi: Days post-inoculation; hrs: Homologous repeat regions; OB: Occlusion body; ODV: Occlusion derived virion; ORF: Open reading frame; PCR: Polymerase chain reaction; LC_{50}: Median lethal concentration; LSC: Low-speed centrifugation; SCC: Sucrose cushion centrifugation; SGU: Sucrose gradient ultracentrifugation; ST_{50}: Median survival time.

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Author contributions
SBT contributed to study conception and design, data collection, data analysis and interpretation, draft manuscript preparation, AL contributed to study conception and design, securing funding, data analysis and interpretation, JTW contributed to data analysis and interpretation, TB contributed to data analysis and interpretation, and JAJ contributed to data analysis and interpretation, manuscript conception and major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Author details
[1]Federal Research Centre for Cultivated Plants, Institute for Biological Control, Julius Kühn Institute (JKI), Heinrichstr 243, 64287 Darmstadt, Germany. [2]Faculty of Sciences of Bizerte, University of Carthage, 7021 Jarzouna, Tunisia. [3]LR21AGR03-Production and Protection for a Sustainable Horticulture, Regional Research Centre On Horticulture and Organic Agriculture, University of Sousse, BO. 57, 4042 Chott-Mariem, Tunisia.

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