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Biological and molecular characterization of two Anticarsia gemmatalis multiple nucleopolyhedrovirus clones exhibiting contrasting virulence

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ABSTRACT

Baculovirus natural populations are known to be genetically heterogeneous and such genotypic diversity could have implications in the performance of biocontrol agents. The *Anticarsia gemmatalis nucleopolyhedrovirus* (AgMNPV) has been widely used to control the velvetbean caterpillar, *Anticarsia gemmatalis*, in Brazil. In the present work, morphological and molecular analyses as well as the biological activity of AgMNPV genotypes derived from a Brazilian field isolate (AgMNPV-79) were carried out. The existence of genotypic variants in the population was confirmed by DNA restriction analysis. Although difference in virulence was observed among the variants, the most (Ag79-01) and the least (AgL-16) virulent clones do not show any morphological and cytopathological changes when compared to the most studied isolate (AgMNPV-2D). The complete genome analysis of the two viral clones showed the presence of single open reading frames (ORFs) of the *pe-38* and *he65* genes, which contrasts with the two split ORFs present in the genome of the AgMNPV-2D isolate. The viral clone AgL-16 has many variations in the *ie-2* and *pe-38* genes, which are transcription regulatory genes responsible for the regulation of viral early gene expression during insect cell infection. Furthermore, other genes showed alterations like the *odv-e56,* which have an essential role in the maturation and envelopment of the ODVs, and *bro-a* and *bro-b genes* which were fused to form a single ORF. For the Ag79-01, although the total number of single nucleotide variants (SNVs) was more prominent in the *pe-38* gene, its genome showed very few modifications in comparison to the AgMNPV-2D genome.

1. Introduction

Baculoviruses are important insect pathogens widely used as biocontrol agents of insect pests (Grzywacz, 2017) and vectors for *in vivo* and *in vitro* recombinant protein expression (van Oers et al., 2015).

Baculoviruses are double-stranded DNA viruses having a circular genome of 80 to 180 kb which can codify 100 to 200 proteins (Theilmann et al., 2005). *Alphabaculovirus*, former *Nucleopolyhedrovirus*, produce two types of viral phenotypes: the budded viruses (BVs) and the occlusion-derived viruses (ODVs) phenotypes. Baculoviruses natural populations are known to be genetically heterogeneous and this genotypic diversity exists even inside a geographical isolate in a single infected insect (Caballero and Williams, 2008; Simón et al., 2004; Cory et al., 2005). This genotypic diversity in a baculovirus population can be the result of mutations, deletions and insertions during virus replication, or it can result from mixing and recombination of local and immigrant baculovirus strains (Muñoz et al., 1998; Simón et al., 2005; Erlandson,2009).

Deleterious mutations are usually eliminated and thus less likely to be sampled in a given population than neutral mutations (Chateigner et al., 2015). Inter and intraspecific baculovirus genome analysis found only a few strong signatures of positive selection, primarily in replication- and transcription-associated genes and several structural protein genes (Hill and Unckless, 2017). Nucleotide substitutions may lead to alterations in the activity of the encoded protein that facilitate adaptation to a new host or effectively overcome the defenses of a current host (Simón et al., 2011).

Clonal strains of baculovirus species derived either by *in vitro* or *in*

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vivo approaches have been shown to vary with respect to infectivity and virulence (Maruniak et al., 1999; Muñoz et al.,1999; Caballero and Williams, 2008; Erlandson, 2009). Other phenotypic characteristics, such as occlusion body (OB) production and insect survival time are likely to differ between variants (Simón et al., 2005). However, these genotypes can present extensive deletions in parts of their genome or only in genes essential for replication or oral infection that might interfere with host infection (López-Ferber et al., 2003; Simón et al., 2005; Simón et al., 2006).

Even though these defective genotypes lack essential genes for the establishment of primary infection, they persist in the population through the co-infection of infected cells by complete genotypes (known as helpers). The deletion variant sequesters the transcription products of the complete genotype to compensate for the missing genes, like the structural proteins, enzymes or replication regulatory factors (Simón et al., 2006).

The molecular mechanisms by which baculoviruses execute their transcriptional program and discriminate between early and late genes are of considerable interest (Friesen, 1997). Early gene expression by definition precedes DNA replication and occurs in the presence of DNA synthesis inhibitors. The early genes encode all proteins needed for viral DNA replication and to reprogram the host cells towards virus replication (Rohrmann, 2013). The early gene expression phase can also be further divided into immediate early and delayed early phases. In this context, the immediate early genes products (e.g. IE-0, IE-1, IE-2 and PE-38) transactivate delayed early genes (Milks et al., 2003). The late phase of gene expression can also be divided into late and very late phases and corresponds to the expression of mainly structural protein genes (Lu et al., 1997). Two baculovirus genes *polyhedrin/granulin* and *p10*, involved in the OB formation and release, fall into the very late class of genes and require also late genes for their expression (Todd et al.,1995; Todd et al., 1996). Also, during the late gene expression phase, genes encoding structural proteins of the nucleocapsid, *vp39*, and basic core protein, *p6.9*, are abundantly transcribed (Morgado et al., 2017).

Analyses of genomic sequences of different baculovirus have shown a great intragenic and intergenic diversity (Chen et al., 2002; Ferreira et al., 2014; Craveiro et al., 2016; Brito et al., 2015; Ardisson-Araújo et al., 2016; Brito et al., 2018) and comparison among these genomic sequences has been very helpful not only for the identification of the common core genes, but also for the elucidation of evolutionary relationships among the members of the *Baculoviridae* family (Miele et al., 2011).

The *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (AgMNPV) was successfully used to control the velvetbean caterpillar, *A. gemmatalis*, in Brazil for more than two decades (Moscardi 1999, Moscardi et al., 2011). Implementation of this program began in the 1982/1983 soybean season, when approx. 2000 ha of soybean were treated with the virus. Initially, small amounts of the virus were produced in *A. gemmatalis* at Embrapa Soybean facilities with artificial diet. Frozen killed larvae were then distributed to extension officers to the farmers and the resulting inocula were used to treat other areas in the same season or to collect and store dead larvae for the subsequent season. A great advance was achieved with the development of a wettable powder formulation of the virus in 1986. As a result of its commercialization by private companies, the use of the AgMNPV increased from about 1.0 million ha in 1990 to approx. 1.5 million ha in 1995. It then reached 2.0 million hectares of soybeans in 2003/2004 season. Subsequently the use of this virus experienced a sharp decline to 300,000 ha, due to changes in agricultural practices by soybean growers. As a consequence of the environmental imbalance, the soybean looper (*Chrysodeixis includens*) became the most severe lepidoptera pest in soybean in Brazil and *A. gemmatalis* became a secondary pest (Sosa-Gómez, 2017).

The complete genomic sequence of the AgMNPV prototype, AgMNPV-2D, was described around a decade ago (Oliveira et al., 2006). The virus entire genome was reported containing a total of 132,239 bp distributed in 152 open reading frames (ORFs). More recently, a full genome comparison among 17 wild isolates of AgMNPV was reported and the pangenome of this virus showed to contain at least 167 hypothetical genes in which 151 are shared by all genomes (Brito et al., 2015). Besides, the analysis showed that events of gene fission and fusion were common, as four genes (*pe-38* and *he-65, ag144, bro-c* genes) were observed as single or split open reading frames. Gains and losses of genomic fragments were also observed within tandem repeats, such as in eight direct repeats and four homologous regions. The evolution of AgMNPV is mainly driven by small indels, substitutions, gain and loss of nucleotide stretches or entire coding regions, resulting in relevant phenotypic alterations, which can probably affect AgMNPV infectivity.

In order to study the diversity of the AgMNPV population, several clones of a wild type preparation (AgMNPV-79) were isolated and their DNAs analyzed by restriction enzyme digestion profile. Besides, virulence tests in *A. gemmatalis* larvae were carried out showing a very distinct degree of infectivity among the different clones. The two viral clones presenting the lowest and highest LC_{50}, (Ag79-01 and AgL-16, respectively), were chosen to perform genomic analyses. The genome sequence of the virus prototype AgMNPV-2D, was used for comparison of the variants with extremely distinct pattern of virulence.

2. Material and methods

2.1. Virus, cells and insects

The viral clones were purified by plaque assay and serial dilution methods (O'Reilly et al., 1992), in IPLB-SF21 (Vaughn et al., 1977) and BTI-Tn-5B1-4 or Tn-5BI-4 (Granados et al., 1994) cells respectively. In the case of plaque assay, serial dilutions of virus containing solutions were used to infect insect cells for 1 h and after that, the virus solution was removed and an agarose overlay was added to the infected cells in order to restrict viral spread, preventing indiscriminate infection through the liquid growth medium. After 5–7 days p.i., live cells were stained with a dye (neutral red) and dead infected cells appeared as non-stained group of infected cells known as a plaque. For the end-point dilution assay, serial dilutions of virus containing solutions were used to infect insect cells in 96 well plates. After 5–7 days p.i., the cells were observed by light microscopy for the presence or absence of virus infection. The AgMNPV 79 is a wild type isolate collected in Brazil in 1979. It was then plaque purified and initially resulted in six different variants. One of these variants, AgMNPV-2D, is considered the *Anticarsia gemmatalis* baculovirus prototype because it represented approximately 40% of the variants obtained (Johnson and Maruniak, 1989; Maruniak, 1989; Maruniak et al., 1999). The AgMNPV obtained by plaque assay were named AgL-06, AgL-16, AgL-34 and AgL-37 while the clones purified by serial dilution were named Ag79-01, Ag79-02, Ag79-03 and Ag79-04.

Anticarsia gemmatalis larvae were reared on artificial diet (Greene et al., 1976) modified by Hoffmann-Campo et al. (1985)), at 27 °C to 28 °C, under laboratory conditions in an Insect Breeding Platform. Third-instar larvae were then infected in order to produce virus polyhedra and to perform virulence assays.

2.2. Budded virus (BVs) DNA extraction and restriction endonuclease analysis

Tn-5BI-4 cells were infected with all AgMNPV clones BVs according to O'Reilly et al. (1992). Initially, cells were grown in T-25 (Cornning) flasks containing complete TNMFH medium with a 1×10^6 cells/flask. After cell seeding, the supernatant was removed and the viral solution (MOI = 10) of all AgMNPV clones BVs was added. Following 1 h adsorption period, the inoculum was withdrawn and complete media was added to the cells, which were incubated at 27 °C for five days. The supernatants of infected cells containing BVs were loaded on to a 25%

sucrose cushion and purified by ultracentrifugation as described by O'Reilly et al. (1992). The purified samples were submitted to two cycles of phenol:chlorophorm:isoamyl alcohol (25:24:1) with centrifugations at 12,000*g* for 5 min at 4 °C between each cycle. The DNA was precipitated overnight in a solution containing 2.5X the initial volume of 100% ethanol and 1/10 of the initial volume of 3 M sodium acetate, centrifuged as described above, washed with 70% ethanol, suspended in Milli-Q® H_2O and stored at 4 °C for further analysis. The viral DNAs were cleaved with *Pst*I (Jena Bioscience) and *Hin*dIII (Pharmacia) restriction endonucleases following the manufacturer's instructions. The restricted DNA pattern was subjected to electrophoresis in 1% agarose gels and photographed using a LAB TRADE photodocumentation system.

2.3. Cytopathology of virus infected cells

Tn-5BI-4 cells were separately infected with Ag79-01, AgL-16 and AgMNPV-2D BVs as described above and monitored for cytopathic effects by phase-contrast light microscopy for 5 days. They were photographed at 48 h post-infection under an inverted light microscope (Axiovert 135 M – Zeiss).

2.4. Transmission electron microscopy

For ultrastructural analyses cells were processed for electron microscopy according to Bozzola & Russell (1992). Initially, cells were infected with AgMNPV-2D, Ag79-01 and AgL-16 as described above and incubated at 27 °C, as well as the mock infected cells. At 48 h p.i., cells were centrifuged at 3000*g*/3min and the pellet was washed in phosphate buffered saline (PBS) and immersed in a fixative (2.5% glutaraldehyde in 0,1 M sodium cacodylate, pH 7.3) at 4 °C, overnight. Secondary fixation was carried out for 1 h with 1% osmium tetroxide. After fixation, the cells were dehydrated through an ascending acetone series followed by a sequence of acetone: Spurr resin (3:1, 2:1: 1:1 and 5 ml of Spurr). The samples were kept in Spurr at 37 °C for 72 h. Ultrathin sections obtained with a Leica ultramicrotome using a diamond knife were stained with 2% uranyl acetate and visualized at the Zeiss TEM 109 electron microscope.

2.5. Virulence assays in Anticarsia gemmatalis larvae

Virulence assays were carried out using purified polyhedra of infected larvae with AgMNPV-79 wt, AgMNPV-2D, plaque assay selected clones (AgL-06, AgL-16, AgL-34 e AgL-37) and end point dilution selected clones (Ag79-01, Ag79-02, Ag79-03 e Ag79-04). In brief, dead infected larvae were macerated in buffer containing 1% ascorbic acid, 2% SDS, 0.01 M Tris-HCl pH 7.8, 0.001 EDTA, followed by filtration in cheese cloth. The filtrate was washed twice with 1 M NaCl and 0.1% SDS followed by centrifugation at 10,000*g* for 10 min. The pellet containing OBs was then suspended in TE buffer and counted in a hemocytometer. The clones were divided in Group A, selected by plaque assay, and Group B, selected by serial dilution.

Bioassays were performed according to Morales and Moscardi (1993). The OBs were incorporated to the free of preservatives artificial diet and offered to the larvae. A total of six treatments were prepared, with two repetitions for each viral clone, using the following concentrations: 4.8×10^3; 1.6×10^3; 5.4×10^2; 1.8×10^2; 6.0×10 and 2.0×10 OBs/ml of artificial diet. Three 3rd instar *A. gemmatalis* larvae were placed in a 50 ml plastic cup with artificial diet. For each treatment a total of 60 larvae were tested. For negative control, the virus solution was not incorporated into the artificial diet. Larvae were maintained in an environmental chamber at 27 °C and a 14 h photoperiod. For mortality analysis, daily observations were carried out from the fourth inoculation day until pupation. The obtained data was analyzed using R Statistical Software (R Development Core Team, 2012) to determine LC_{50} – average Lethal Concentration (virus concentration to kill 50% of tested insects) with package 'Ecotox' (Finney, 1971, 1978). Superposition of confidence intervals of 84% was used to test statistical differences in LC_{50s} (Payton et al., 2003). The MicroProbit 3.0 software was used to determine LT_{50} – average Lethal Time (time to kill 50% of tested insects) using confidence intervals of 95%.

2.6. Sequencing and genome analysis

In order to sequence the entire genome of both Ag79-01 and AgL-16, dead larvae infected with 3.0×10^5 OB/ml from each clone were used to extract the DNA from polyhedra according to Maruniak (1986). The OBs were incubated in 1 M sodium carbonate pH 10 followed by proteinase K (100 µg/ml) digestion for 16 h at 37 °C pH 8.0. The DNA was extracted by phenol: chloroform followed by ethanol precipitation. The genomic DNA of both clones (Ag79-01 and AgL-16) were quantified in a spectrophotometer (M5, Molecular Devices) and submitted to genome sequencing throughout the pyrosequencing technique performed by the 454 Genome Sequencer (GS) FLX™ Standard (Roche) at Macrogen Inc (Seoul, Republic of Korea). The resulting reads (11,492 and 13,302, respectively) were trimmed to remove sequencing adaptors and low quality regions ($Q \geq 20$) using Geneious 6.0.4 software (Biomatters Limited). Genome assembly was also performed with Geneious 6.0.4 using AgMNPV-2D (Genbank accession number, DQ813662) genome as a reference. The genome coverage was approximately 20x for both clones. The ORFs were predicted with Find ORF tool (Geneious 6.0.4) and annotated using AgMNPV-2D genome as reference. In order to identify polymorphism in coding and non-coding regions the genome of both clones and AgMNPV-2D were compared. A phylogenetic tree was inferred based on the complete genome alignment of 20 genomes using FastTree algorithm (Price et al., 2010). The genome synteny analysis was performed with Mauve (Darling et al., 2004) and plotted using genoPlotR (Guy et al., 2010) package in R. The genome sequence of Ag79-01 e AgL-16 has been deposited in GenBank under the accession no MG746625 and MG746626, respectively.

3. Results

3.1. Restriction endonuclease analysis (REN)

Restriction endonuclease analysis was carried out to verify the heterogeneity of the clones selected by plaque assay (group A viruses, AgL-06, AgL-16, AgL-34 and AgL-37) or by serial dilution (group B viruses, Ag79-01, Ag79-02, Ag79-03, and Ag79-04). The DNA patterns after the digestion with *Pst* I and *Hind* III (data not shown) were compared to the AgMNPV-2D digested with the same enzymes. Regardless of the enzyme, a different polymorphism pattern was observed for each clone independent of the purification system used.

3.2. Insect bioassays

Bioassays were carried out to determine the virulence of the viral clones of Group A and group B, in comparison with the wild-type AgMNPV-79 and AgMNPV-2D. Table 1 and 2 show the results of the average lethal concentration and the respective confidence intervals (84%). Among group A, the AgL-06 clone had the lowest value (792 OBs/ml) and the AgL-16 had the highest (1221 OBs/ml). Group B clones showed differences in the LC_{50} ranging from 73 OBs/ml (Ag79-01) to 425 OBs/ml (Ag79-02), i.e., the Ag79-01 was capable of killing 50% of the population with a viral concentration 6 times lower than the Ag79-02. Therefore, the Ag79-01 clone was considered the most virulent clone in this study while the AgL-16 was the least virulent, based on statistical differences to the reference clone and wild type isolate. Independently of the Group (A or B) the AgMNPV-79 wild type and the AgMNPV-2D clone had similar LC_{50} values.

Those two viruses with distinct virulence were compared regarding their average lethal time (LT_{50}), which was determined based on the

Table 1
Average lethal concentration (LC_{50}) of the viral clones (Group A) in third instar *Anticarsia gemmatalis larvae*.

Virus	LC_{50} (OBs/ml)	Fiducial Limits (84%)	Slope	X^2
AgMNPV-79	395a	291–540	1.51	7.60
AgMNPV-2D	585ab	406–870	1.12	7.25
AgL-06	792b	652–974	1.37	3.03
AgL-16	1221c	976–1564	1.22	2.35
AgL-34	1110bc	860–1470	1.03	4.37
AgL-37	838bc	683–1041	1.29	2.23

*Concentration of each viral clone used: 20, 60, 180, 540, 1600 and 4860 OBs/ml and water.
**LC_{50}: number of occlusion bodies (OBs) / mL of insect diet. Different letters after LC_{50} values for each isolate are significantly different (P = 0.05).

Table 2
Average lethal concentration (LC_{50}) of the viral clones (Group B) in third instar *Anticarsia gemmatalis* larvae.

Virus	LC_{50} (OBs/ml)	Fiducial Limits (84%)	Slope	X^2
AgMNPV-79	439c	248–805	1.24	19.30
AgMNPV-2D	391c	332–460	1.79	5.32
Ag79-01	73a	47–104	1.23	7.29
Ag79-02	425c	280–655	1.22	10.4
Ag79-03	130ab	78–204	1.43	15.3
Ag79-04	285bc	194–419	1.49	11.4

*Concentration of each viral clone used: 20, 60, 180, 540, 1600 and 4860 OBs/ml and water.
**LC_{50}: number of occlusion bodies (OBs) / mL of insect diet. Different letters after LC_{50} values for
each isolate are significantly different (P = 0.05).

Table 3
Average lethal time (TL_{50}) of selected viral clones in third instar *Anticarsia gemmatalis* larvae.

Virus	Concentration (OBs/ml)	LT_{50}* (days)	Fiducial Limits (95%)	Slope	X^2
AgMNPV-79	540	10.32b	9.44–11.72	9.01	17.83
	4860	6.81c	6.38–7.4	36.2	29.2
AgMNPV-2D	540	10.28b	9.5–11.6	12.12	23.61
	4860	8.29d	7.71–9.10	13	28.07
Ag79-01	540	7.57a	7.13–8.16	15.7	22.08
	4860	6.31c	6.14–6.49	16.2	5.27
AgL-16	540	N.A.	N.A.	N.A.	N.A.
	4860	8.22d	7.56–9.24	13.14	38.71

Different letters after LT_{50} values for each concentration are significantly different (P = 0.05).
N.A. = Not applied at this concentration as it caused less than 50% mortality observed in the bioassays.
* LT_{50}: Average of time (days) necessary to kill 50% of the larvae population in the test.

lowest (540 OB/ml) and highest (4860 OB/ml) viral concentration. The LT_{50} (Table 3) for AgL-16 was not evaluated at lowest concentration (540 OBs/ml) since it caused less than 50% mortality in infected insects. In the highest concentration, AgL-16 took 8.22 days to kill 50% of the experimental insect population. Whereas for the Ag79-01 clone (Table 3), the LT_{50} with the 540 OB/ml viral concentration required 7.57 days to kill 50% of the population. This was the fastest mortality and statistically different from the wild type AgMNPV-79 and from the reference clone AgMNPV-2D.

3.3. Cytopathology of virus infected cells

Tn-5BI-4 cells infected with Ag79-01, AgL-16 clones, and AgMNPV-2D (48 h p.i.) showed typical cytopathic effects induced by baculoviruses such as nuclear hypertrophy, presence of occlusion bodies and cell rounding (Fig. 1 A-D). As expected, no cytopathic effects were observed in the mock infected cells. In the ultrastructural analysis of virus infected cells, no morphological differences were observed in cells infected with any of the viruses (Fig. 2A–D).

3.4. Viral genome analysis

The genome of the viral clones showing the highest (AgL-16) and the lowest (Ag79-01) LC_{50} towards *A. gemmatalis* larvae were sequenced. The entire genome sequence analysis was based on 11,492 reads with length varying from 25 to 871 nucleotides and 13,305 with length varying from 26 to 984 nucleotides, respectively. The reads were obtained after sequences of low quality were trimmed and used for the genome assembly. The A + T and G + C content found in both genomes were of 55.5% and 44.5%, respectively, in which there was a predominant A + T content characteristic of members from the *Baculoviridae* family.

When both clones were aligned against the genome of reference (AgMNPV-2D) no large indels (> 1 kb) were found among the three genomes and overall the pairwise identity was > 95%. However, while the AgMNPV-2D has a size of 132,239 bp and contains **158** predicted ORFs, the genomes of Ag79-01 and AgL-16 showed **156** predicted ORFs and 131,897 bp and 130,956 bp, respectively. Analysis of the clones sharing genes showed that the inconsistency in the number of ORFs in both clones was due to the fusion of *pe-38* and *he65* genes into single ORFs. These genes are split into two ORFs in the AgMNPV-2D genome (Brito et al., 2015). Similarly, the *he65* gene was fused into a single ORF in both Ag79-01 and AgL16 clones. A nonsynonymous substitution from a T → C position changed the termination codon to a glutamine amino acid resulting in the fusion of the *he65* ORF. Moreover, AgL-16 presented a small hypothetical ORF (5136 - > 4999) and a fusion of *bro-a* and *bro-b* gene, with a 734 bp deletion in *bro-a*. Both genes were previously described in geographical isolates of AgMNPV (Brito et al., 2015).

The genomes of both clones were aligned against the genome of reference (AgMNPV-2D) and the number of single nucleotide variant (SNV) was analyzed to verify if these changes would occur in genes that are essential for infection. Intragenic polymorphic analysis indicated a total of 308 and 907 total SNVs when the genomes of the Ag79-01 and the AgL-16 were aligned against the genome of the AgMNPV-2D, respectively (Fig. 3). While for the AgL-16 we found 183 nonsynonymous and 724 synonymous polymorphisms, for the Ag79-01 we only found 37 nonsynonymous and 271 synonymous mutations. However, the majority of these changes were distributed into two or less polymorphisms per gene. The analyses indicated these polymorphisms were concentrated in the *pe-38*, *bro-a* and *bro-c*, *ie-2* genes and ORF 137 (Fig. 3). For the Ag79-01, the total number of SNVs was more prominent in the *pe-38* gene (Fig. 3). Overall, the comparison of the total number of SNVs of both clones against the genome of reference AgMNPV-2D revealed that the AgL-16 has the greatest number of total SNVs. Among these SNVs, the genetic diversity in these clones was analyzed by comparison of the number of nonsynonymous mutations. Some genes showed higher diversity when the number of nonsynonymous mutations was compared. For the AgL-16, differences were mainly found in the *pe-38*, *ie-2*, and *bro-a/b* genes (Fig. 3). For the Ag79-01 clone, a high number of changes were found in the *pe-38* gene (Fig. 3). These results suggest that Ag79-01 genome is closer to AgMNPV-2D genome than to the AgL-16 clone. This finding was confirmed by the phylogenetic analysis of 20 AgMNPV isolates shown in Fig. 4. The genome synteny analysis revealed that all genomes were collinear, and large indels were located mainly near *pe-38*, some hrs and

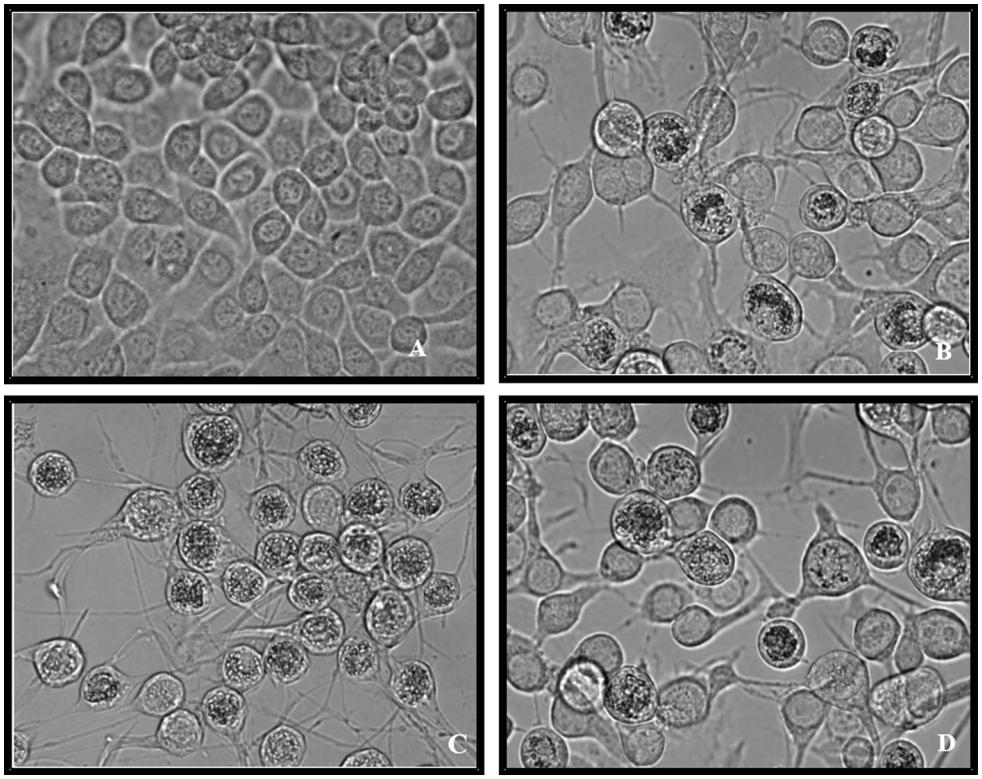

Fig. 1. Light microscopy images of Tn5B1-4 cells infected with AgMNPV-2D (B), Ag79-01 (C) e AgL-16 (D) at 48 h.p.i. Mock: non-infected cells (A).

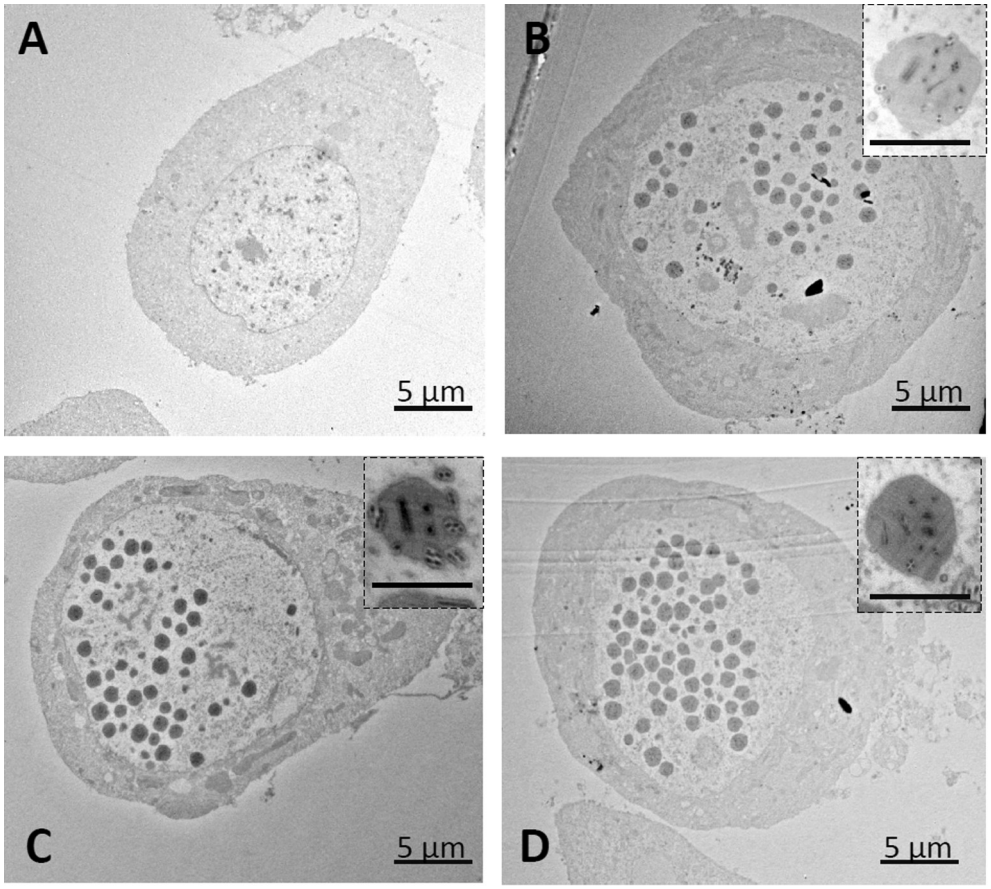

Fig. 2. Transmission electron microscopy of non-infected TN5B1-4 cells (A) and of infected TN5B1-4 cells (B-D). Cells showing polyhedra from AgMNPV-2D (B), AgMNPV-79-01 (C), and AgL-16 (D) 48 h.p.i. MOI: 10 and (bar = 5 µm). No ultrastructural differences were observed among infections.

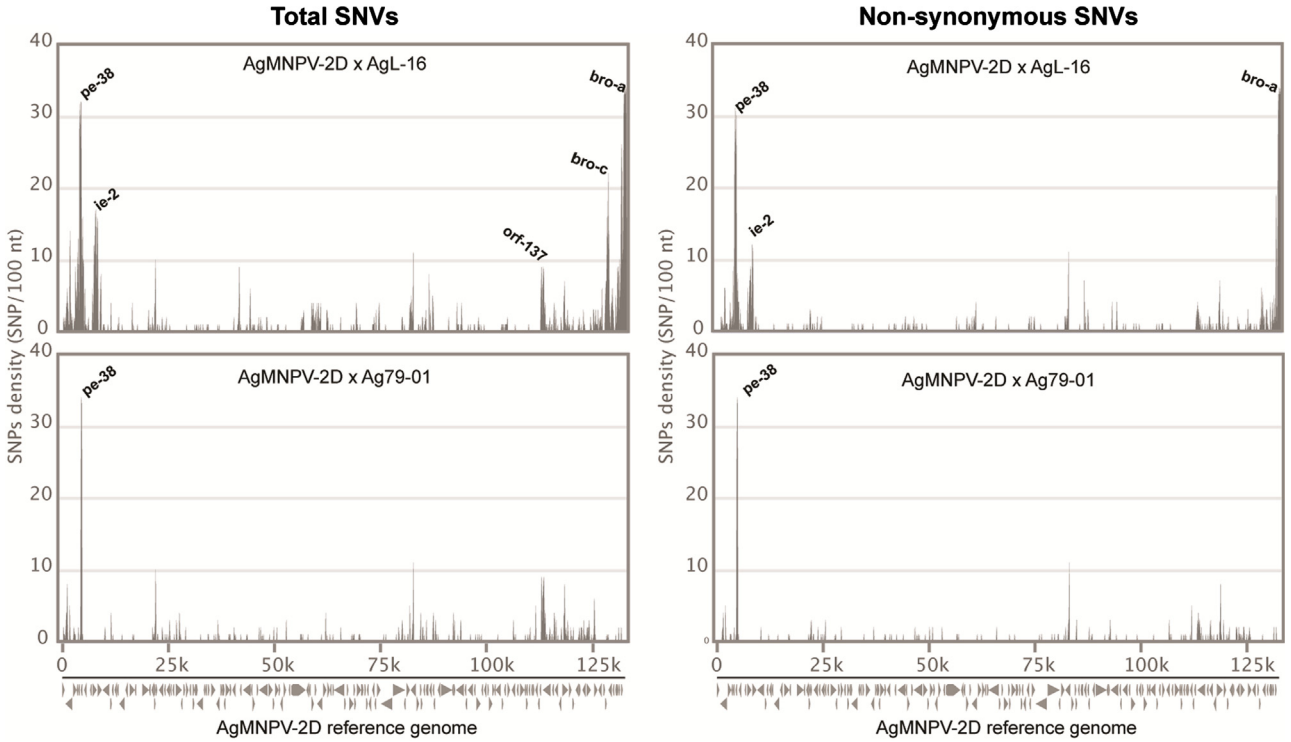

Fig. 3. A-D. Polymorphism analysis. To identify polymorphisms in coding and non-coding region the genome of both clones and AgMNPV-2D were compared and the single nucleotide variants (SNVs) density was plotted using R. The total number of SNVs was higher in AgL-16 compared to Ag79-01.

also *bro-a/bro-b* region (Fig. 4). The *hr* region number was also different among clones (AgMNPV-2D with nine, Ag79-01 with eight and AgL-16 with seven *hrs*).

4. Discussion

In the present work, AgMNPV clones derived from the field isolate AgMNPV-79 were selected by plaque assay and by end point dilution, their DNA were purified, and analyzed by REN. Bioassays against third-instar *A. gemmatalis* larvae showed different levels of virulence among the clones. The genome of two of these clones, with the lowest and highest LC_{50} values against *A. gemmatalis* larvae, were then sequenced. The REN analyses showed the presence of genotypic variants in the two groups of AgMNPV clones. AgMNPV genotypic variants have been previously detected by Johnson and Maruniak (1989) and Maruniak et al. (1999). A wild type preparation collected in a soybean field in 1979 in Brazil (AgMNPV-79) resulted in six different variants whereas the variant AgMNPV-2D was found in 40% of the plaques (Maruniak, 1989; Johnson and Maruniak, 1989). In addition, a viral commercial preparation that was applied consecutively in soybean fields in Brazil (AgMNPV-1985) resulted in eleven variants (Maruniak et al., 1999). The genomic variation of all the isolates was mapped showing that those from 1985 presented more heterogeneity with changes mapped in additional sites in comparison to the AgMNPV-79 variants.

Bioassays of the AgMNPV variants in groups A and B of this study revealed a very distinct pattern of virulence among them. A variant named Ag79-01 presented a very high virulence while the AgL-16 a very low, with the LC_{50} values of 73 OBs/ml and 1221 OBs/ml respectively. We observed that Ag79-01 was more pathogenic and faster to kill the larvae compared to the reference clone. In relation to the wild type isolate too, except for the LT_{50} in the highest OBs concentration. Simón et al. (2005) observed that the SfMNPV cloned genotypes with the lowest LC_{50} also kill the larvae faster. Although the wild type SfMNPV isolate have the LC_{50} values lower than these cloned genotypes, its speed of kill was slower compared to them. The authors suggested that these differences in virulence was related to deletion of certain genes involved in host regulation, as observed in other works (ÓReilly and Miller, 1991; Popham et al. 2001).

Ultrastrutural analysis of insect cells infected by both clones (Ag79-01 and AgL-16), as well as the AgMNPV-2D, showed that they induced all morphological changes characteristic of a baculovirus infection in a susceptible cell. No morphological alterations that could be associated to the disparity in virulence found for those variants were noticed.

Ribeiro et al. (1997) have also found differences in pathogenicity and occlusion body production in AgMNPV variants selected in *Diatraea saccharalis* larvae. Ten distinct plaque assay genotypes were obtained and compared. Bioassays have shown over one hundred-fold variation in LD_{50} values ranging from 1700 OBs to > 200,000 OBs/larva. The occlusion body production in infected larvae increased with the pathogenicity of the variant to the host, showing an average ten-fold reduction in *D. sacharalis* when compared to *A. gemmatalis* for the same variant (Ribeiro et al., 1997). An *in vitro* analysis of these variants showed no significant differences in viral replication in *Spodoptera frugiperda* (IPLB-SF-21) and *A. gemmatalis* (UFL-AG-286) cells (Maruniak, 1989).

Simón et al. (2005) also showed differences in pathogenicity and OB production in different *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV) variants derived form a Nicaraguan SfMNPV isolate. All single genotypes were significantly less pathogenic than the wild-type isolate. Three deletion genotypes were not able to infect larvae and the LC_{50} of other genotypes ranged from 2.45×10^5 to 1.53×10^5 OBs/ml compared to 5.47×10^4 OBs/ml of the wild-type isolate. On the other hand, further studies with this isolate revealed that two single genotypes were more virulent than the wild-type isolate (Simón et al., 2008). Moreover, the OB production of one deletion genotype were higher than the wild-type genotype. Improved OB production also occurred with two deletion genotypes mixed, resulting in increased likelihood of transmission. In addition, Barrera et al. (2013) showed that a SfMNPV isolate from Colombia presented six deletion genotypes more virulent than the wild-type isolate. However, the OB production was

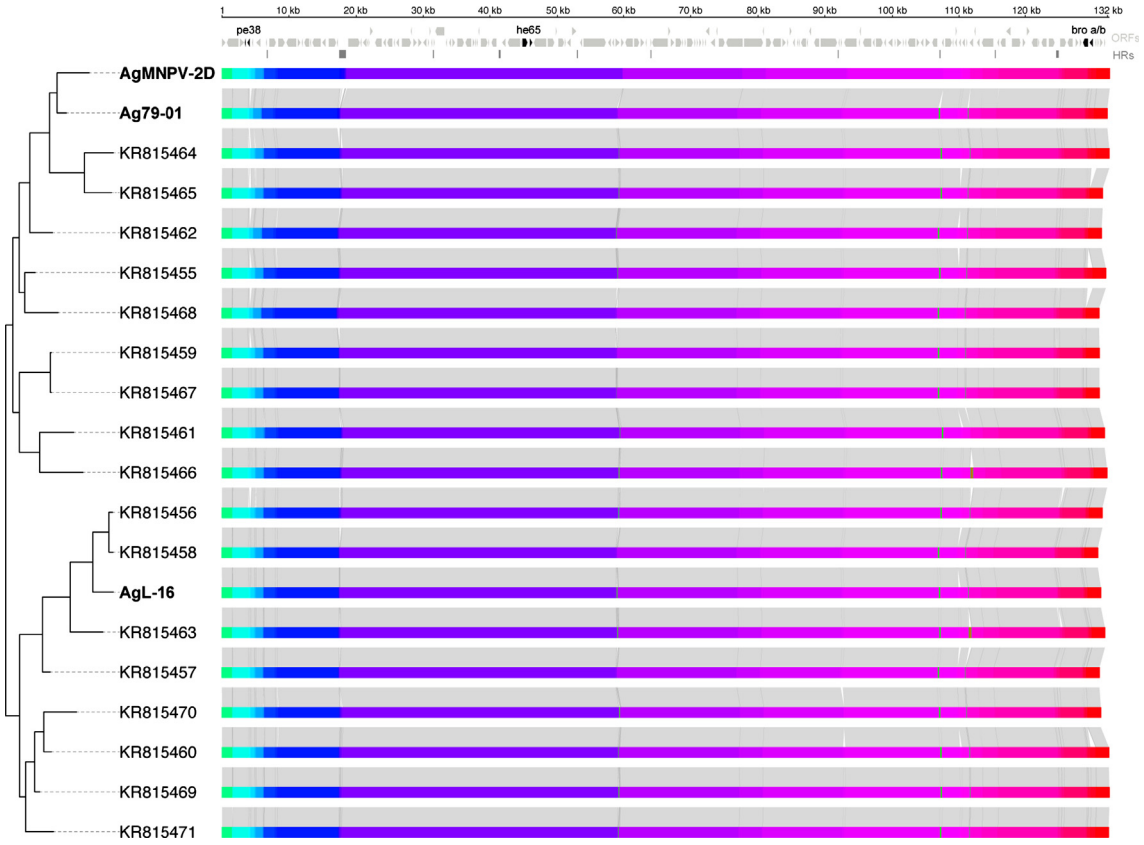

Fig 4. Phylogenetic relationship among AgMNPV isolates/clones and genome synteny analysis. AgMNPV-2D, Ag79-01 and AgL-16 are shown in bold. The phylogenetic tree was inferred based on the complete genome alignment of 20 genomes using FastTree algorithm. The data indicate the closest relationship of the Ag79-01 to the genome of reference AgMNPV-2D. The genome synteny analysis was performed with Mauve and plotted using genoPlotR package in R. The reference genome was AgMNPV-2D (DQ813662). Selected ORFs are shown in black. Gray shading indicates homologous regions among genomes, nucleotide syntenic blocks are represented by colored bars and the white spaces between blocks indicates missing regions. The genes with fusion/split pattern are shown in black bold (*pe-38*, *he65* and *bro-a/b*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

higher in the wild-type isolate than in the single genotypes. The OBs of all genotypes were orally infective, but the complete genotype OBs were approximately 4.4-fold more potent than the wild-type isolate. Interestingly, some variants did not liquefy infected larvae, as observed with the wild-type infected larvae, which was likely due to absence or lack of activity of *chitinase* and/or *cathepsin* genes (Barrera et al., 2013). The absence of *chitinase* and/or *cathepsin* genes activity was also observed in six genotypes of Nicaraguan isolate (Simón et al., 2011).

Genome analysis of two deletion genotypes of SfMNPV by Southern blot and PCR revealed they did not contain *pif-1* and *pif-2* genes (López-Ferber et al., 2003; Simón et al., 2004). These genotypes were not able to infect *S. frugiperda* larvae by ingestion, whereas both variants retained the ability to replicate in cell culture or by injection into larvae. The *pif-1* and *pif-2* genes are essential genes for peroral infectivity involved in the fusion between the virus envelope and the midgut cell microvilli (Kikhno et al., 2002). Although, another deletion genotype of SfMNPV that cannot infect larvae orally and contains all *pif* genes was observed. It was hypothesized that point mutations, which are not detected by restriction digestion analysis, were responsible for this phenotype (Simón et al., 2005) highlighting the importance of complete genome analysis of genotypes with distinct pathogenicity and virulence features.

In this work the genomes of the Ag79-01 and AgL-16 variants showed the presence of a single copy of the *pe-38* and *he65* genes which are divided into two ORFs in the genome of the AgMNPV-2D clone. It was noticed that one of the viral clones (AgL-16), with lower virulence, has many variations in the *ie-2* and *pe-38* genes, which are transcription regulatory genes responsible for the regulation of viral early gene expression during insect cell infection. The reduction of the virulence of the AgL-16 could be related to alterations on the *ie-2* and *pe-38* genes. The loss of virulence on deleted mutants for *ie-2* and *pe-38* genes has been also reported (Prikhod'ko et al., 1999; Milks et al., 2003). Recombinants of AcMNPV containing deletions of different functional regions within the *ie-2* gene exhibited delays in viral DNA synthesis, late gene expression, budded virus production, and occlusion body formation in SF-21cells but not inTN-5B1-4 cells. In TN-5B1-4 cells, the *ie-2* mutants produced more budded virus and fewer occlusion bodies but the infection proceeded without delay (Prikhod'ko et al., 1999). The virulence against the host insect was also affected. Determination of the dose-mortality response (LC_{50}) revealed the mutations in *ie-2* reduced the infectivity of AcMNPV OBs in both *S. frugiperda* and *T. ni* larvae, although the effect was more pronounced in *S. frugiperda*.

Comparison of a mutant with deletion on *pe-38* gene and wild-type (WT) viral replication in insect cell culture and virulence in *Heliothis virescens* larvae was reported by Milks et al. (2003). Compared to WT, the production of budded virus (BV) of the mutant was delayed by at least 3 h, and BV yields were reduced over 99%. Similarly, DNA synthesis levels were greatly reduced relative to those of WT, but onset of DNA replication was the same for both viruses. In bioassays, nearly sevenfold more of the mutant than WT virus was required. Furthermore, other genes showed alterations like the *odv-e56,* which have an essential role in the maturation and envelopment of the ODVs, and *bro-a* and *bro-b genes* which were found fused in only one single ORF. The polymorphism in the *hr* region was previous reported in AgMNPV (Maruniak et al., 1999, Brito et al., 2015). The absence of *hr1* was observed in eight wild-type isolates of AgMNPV from a total of 17

isolates analyzed by Brito et al. (2015). On the other hand, the Ag79-01 variant showed very few modifications in comparison to the AgMNPV-2D genome. Besides its high virulence, phylogenetic analysis of several AgMNPV genomes indicated a closest relationship of the Ag79-01 with the genome of reference. Further work using gene replacement with the mutated genes will be necessary to point out the DNA changes found in the genomes are responsible for the gain or loss of virulence towards *A. gemmatalis* larvae. In addition, selection analyses in AgMNPV genome population would help to inform if these genes potentially involved in virulence are subjected to positive selection.

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References

Ardisson-Araújo, D., Nunes Lima, R., Melo, F.L., Clem, R., Huang, N., Báo, S., Sosa-Gómez, D., Ribeiro, B.M., 2016. Genome sequence of Perigonia lusca single nucleopolyhedrovirus: insights into the evolution of a nucleotide metabolism enzyme in the family Baculoviridae. Sci Rep. 6, 24612. https://doi.org/10.1038/srep24612.

Barrera, G., Williams, T., Villamizar, L., Caballero, P., Simón, O., 2013. Deletion genotypes reduce occlusion body potency but increase occlusion body production in a colombian *Spodoptera frugiperda* Nucleopolyhedrovirus Population. PLoS One. 8 (10), e77271. https://doi.org/10.1371/journal.pone.0077271.

Bozzola, J.J., Russell, L.D., 1992. Electron Microscopy Principles and Techniques for Biologists. Jones and Bartlett Publishers, Boston, USA, pp. 542p.

Brito, A.F., Melo, F.L., Ardisson-Araújo, D.M.P., Sihler, W., Souza, M.L., Ribeiro, B.M., 2018. Genome-wide diversity in temporal and regional populations of the betabaculovirus *Erinnyis ello granulovirus* (ErelGV). BMC Genomics 19, 698. https://doi.org/10.1186/s12864-018-5070-6.

Brito, A.F., Braconi, C.T., Weidmann, M., Dilcher, M., Alves, J.M.P., Gruber, A., de Zanotto, P.M.A., 2015. The pangenome of the *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV). Genome Biol Evol. 8 (1), 94–108. https://doi.org/10.1093/gbe/evv231.

Caballero, P., Williams, T., 2008. Virus Entomopatógenos. In: Jacas, J.A., Urbaneja, A. (Eds.), Control Biológico de Plagas Agrícolas, Fascículo número 04. Phytoma, España, pp. 121–136.

Chateigner, A., Bézier, A., Labrousse, C., Jiolle, D., Barbe, V., Herniou, E.A., 2015. Ultra deep sequencing of a baculovirus population reveals widespread genomic variations. Viruses 7, 3625–3646. https://doi.org/10.3390/v7072788.

Chen, X., Zhang, W.J., Wong, J., Chun, G., Lu, A., McCutchen, B.F., Presnail, J.K., Herrmann, R., Dolan, M., Tingey, S., Hu, Z.H., Vlak, J.M., 2002. Comparative analysis of the complete genome sequences of *Helicoverpa zea* and *Helicoverpa armigera* singlenucleocapsid nucleopolyhedroviruses. J Gen Virol. 83, 673–684.

Cory, J.S., Green, B.M., Paul, R.K., Hunter-Fujita, F., 2005. Genotypic and phenotypic diversity of a baculovirus population within an individual insect host. J. Invertebr. Pathol. 89, 101–111. https://doi.org/10.1016/j.jip.2005.03.008.

Craveiro, S.R., Santos, L.A.V.M., Togawa, R.C., Inglis, P.W., Grynberg, P., Ribeiro, Z.M.A., Ribeiro, B.M., Castro, M.E.B., 2016. Complete genome sequences of six Chrysodeixis includens nucleopolyhedrovirus isolates from Brazil and Guatemala. Genome Announc. 4 (6), e01192–e1216. https://doi.org/10.1128/genomeA.01192-16.

Darling, A.C., Mau, B., Blattner, F.R., Perna, N.T., 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res. 14 (7), 1394–1403.

Erlandson, M., 2009. Genetic variation in field populations of baculoviruses: mechanisms for generating variation and its potential role in baculovirus epizootiology. Virol. Sin. 24, 458–469.

Ferreira, B.C., Melo, F.L., Souza, M.L., Moscardi, F., Báo, S., Ribeiro, B.M., 2014. High genetic stability of peroral infection factors from Anticarsia gemmatalis MNPV over 20 years of sampling. J Invertebr Pathol. 118. https://doi.org/10.1016/j.jip.2014.02.007.

Finney, D.J., 1971. Probit Analysis. Cambridge Univ. Press, London, New York, pp. 333p.

Finney, D.J., 1978. Statistical Method in Biological Assay, 3rd. ed. Charles Griffin & Company Ltd., London, pp. 508p.

Friesen, P.D., 1997. Regulation of baculovirus early gene expression. In: Miller, L.K. (Ed.), The Baculoviruses, pp. 141–170.

Granados, R.R., Guoxun, L., Derksen, A.C.G., McKenna, K.A., 1994. A new insect cell line from *Trichoplusia ni* (BTI-Tn-5B1-4) susceptible to *Trichoplusia ni* single nuclear polyhedrosis virus. J Invertebr Pathol. 64, 260–266.

Greene, G.L., Leppla, N.C., Dickerson, W.A., 1976. Velvetbean caterpillar: a rearing procedure and artificial medium. J Econ Entomol. 69, 487–488.

Grzywacz, D., 2017. Basic and Applied Research: Baculovirus. In: Lacey, L.A. (Ed.), Microbial Control of Insect and Mite Pests. Academic Press, Cambridge, pp. 27–46.

Guy, L., Kultima, J.R., Andersson, S.G.E., 2010. genoPlotR: comparative gene and genome visualization in R. Bioinformatics 26, 2334–2335.

Hill, T., Unckless, R.L., 2017. Baculovirus molecular evolution via gene turnover and recurrent positive selection of key genes. J Virol. 91, e01319–e1417. https://doi.org/10.1128/JVI.01319-17.

Hoffmann-Campo, C. B., Oliveira, E,B.,Moscardi, F., 1985. Criação massal da lagarta da soja (Anticarsia gemmatalis). Londrina, Embrapa-CNPSo, Documentos 10, 20 p.

Johnson, D.W., Maruniak, J.E., 1989. Physical map of Anticarsia gemmatalis nuclear polyhedrosis virus (AgMNPV-2) DNA. J Gen Virol. 70, 1877–1883.

Kikhno, I., Gutierrez, S., Croizier, L., Croizier, G., López-Ferber, M., 2002. Characterization of *pif*, a gene required for the per os infectivity of *Spodoptera littoralis* nucleopolyhedrovirus. J. Gen. Virol. 83, 3013–3022.

López-Ferber, M., Simon, O., Williams, T., Caballero, P., 2003. Defective or effective? Mutualistic interactions between virus genotypes. Proc. R. Soc. Lond. B. 270, 2249–2255.

Lu, A., Miller, L. K., 1997. Regulation of baculovirus late and very late gene expression, in: Miller, L. K. (Ed.)The Baculoviruses, pp.193–216.

Maruniak, J.E., 1986. Baculovirus structural proteins and protein and protein synthesis. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculovirus. CRC Press, Florida, pp. 129–146.

Maruniak, J.E., 1989. Molecular biology of *Anticarsia gemmatalis* baculovirus. Mem. Inst. Oswaldo Cruz. 84, 107–111.

Maruniak, J.E., Garcia-Maruniak, A., Souza, M.L., Zanotto, P.M.A., Moscardi, F., 1999. Physical maps and virulence of *Anticarsia gemmatalis nucleopolyhedrovirus* genomic variants. Arch Virol. 144, 1991–2006.

Miele, S.A.B., Garavaglia, M.J., Belaich, M.N., Ghiringhelli, P.D., 2011. Baculovirus: molecular insights on their diversity and conservation. Int J Evol Biol. 2011, 15.

Milks, M.L., Washburn, J.O., Willis, L.G., Volkman, L.E., Theilmann, D.A., 2003. Deletion of *pe38* attentuates AcMNPV genome replication, budded virus production and virulence in *Heliothis virescens*. Virol. 310, 224–234.

Morales, L., Moscardi, F., 1993. Comparação entre duas metodologias de bioensaios para vírus entomopatogênicos. An. Soc. Entomol. Brasil. 22, 535–540.

Morgado, F.S., Ardisson-Araújo, D., Ribeiro, B.M., 2017. Real-time expression analysis of selected anticarsia gemmatalis multiple nucleopolyhedrovirus gene promoters during infection of permissive semipermissive and nonpermissive cell lines. Viruses 9, 132. https://doi.org/10.3390/v9060132.

Moscardi, F., 1999. Assessment of the application of baculoviruses for control of Lepidoptera. Annu. Rev. Entomol. 44, 257–289.

Moscardi, F., Souza, M.L., Castro, M.E.B., Moscardi, M.L., Szewczyk, B., 2011. Baculovirus pesticides – present state and future perspectives, in microbes and microbial technology, springer science + business media. LLC 2011, 415–445. https://doi.org/10.1007/978-1-4419-7931-5_16.

Muñoz, D., Castillejo, J.I., Caballero, P., 1998. Naturally occurring deletion mutants are parasitic genotypes in a wild-type nucleopolyhedrovirus population of Spodoptera exigua. Appl. Environ. Microbiol. 64, 4372–4377.

Muñoz, D., Murillo, R., Krell, P.J., Vlak, J.M., Caballero, P., 1999. Four genotypic variants of a *Spodoptera exigua* nucleopolyhedrovirus (Se-SP2) are distinguishable by a hypervariable genomic region. Virus Res. 59, 61–74. https://doi.org/10.1016/S0168-702(98)00125-7.

O'Reilly, D.R., Miller, L.K., 1991. Improvement of a baculovirus pesticide by deletion of the *egt* gene. Bio/Technology 9, 1086–1089.

O'Reilly, D.R., Miller, L.K., Luckow, V.A., 1992. Baculovirus Expression Vectors: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

Oliveira, J.V.C., Wolff, J.L.C., Garcia-Maruniak, A., Ribeiro, B.M., Castro, M.E.B., Souza, M.L., Moscardi, F., Maruniak, J.E., Zanotto, P.M.A., 2006. Genome of the most widely used viral biopesticide: *Anticarsia gemmatalis* multiple nucleopolyhedrovirus. J Gen Virol. 87, 3233–3250.

Payton, M.E., Greenstone, M.H., Schenker, N., 2003. Overlapping confidence intervals or standard error intervals: What do they mean in terms of statistical significance? J Insect Sci. 3, 34–39.

Popham, H.J., Bischoff, D.S., Slavicek, J.M., 2001. Both *Lymantria dispar* nucleopolyhedrovirus enhancin genes contribute to viral potency. J Virol. 75, 8639–8648.

Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2–approximately maximum-likelihood trees for large alignments. PLoS One. 10 (5(3)), e9490.

Prikhod'ko, E.A., Lu, A., Wilson, J.A., Miller, L.K., 1999. In vivo and in vitro analysis of baculovirus *ie-2* mutants. J Virol. 73 (3), 2460–2468.

Development Core Team, R., 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria,. http://www.R-project.org.

Ribeiro, H.C.T., Pavan, O.H.O., Muotri, Y.A.R., 1997. Comparative susceptibility of two different hosts to genotypic variants of the Anticarsia gemmatalis nuclear polyhedrosis virus. Entomol. Exp. Appl. 83, 233–237.

Rohrmann, G.F., 2013. Baculovirus Molecular Biology, 3rd Ed. National Center for Biotechnology Information, Bethesda.

Simón, O., Williams, T., Lopez-Ferber, M., Caballero, P., 2004. Genetic structure of a spodoptera frugiperda nucleopolyhedrovirus population: high prevalence of deletion genotypes. Appl. Environ. Microbiol. 70, 5579–5588.

Simón, O., Williams, T., López-Ferber, M., Caballero, P., 2005. Functional importance of deletion mutant genotypes in an insect nucleopolyhedrovirus population. Appl. Environ. Microbiol. 71, 4254–4262.

Simón, O., Williams, T., Caballero, P., López-Ferber, M., 2006. Dynamics of deletion genotypes in an experimental insect virus population. Proc. R. Soc. B. 273, 783–790. https://doi.org/10.1098/rspb.2005.3394.

Simón, O., Williams, T., López-Ferber, M., Taulemesse, J.M., Caballero, P., 2008.

Population genetic structure determines speed of kill and occlusion body production in Spodoptera frugiperda multiple nucleopolyhedrovirus. Biol. Control. 44, 321–330.
Simón, O., Palma, L., Beperet, I., Muñoz, D., López-Ferber, M., Caballero, P., Williams, T., 2011. Sequence comparison between three geographically distinct Spodoptera frugiperda multiple nucleopolyhedrovirus isolates: Detecting positively selected genes. J. Invertebr. Pathol. 107, 33–42. https://doi.org/10.1016/j.jip.2011.01.002.
Sosa-Gómez, D.R., 2017. Microbial control of soybean pest insects and mites. In: Lacey, L.A. (Ed.), Microbial control of insect and mite pests: from theory to practice. Elsevier, Amsterdam, pp. 199–208.
Theilmann, D.A., Blissard, G.W., Bonning, B., Jehle, J., O'Reilly, D.R., Rohrmann, G.F., Thiem, S., Vlak, J.M., 2005. Family Baculoviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy: Eight Report of the International Committee on Taxonomy of Viruses, pp. 177–185.
Todd, J.W., Passarelli, A.L., Miller, L.K., 1995. Eighteen baculovirus genes, including lef-11, p35,39K, and p47, support late gene expression. J. Virol. 69, 968–974.
Todd, J.W., Passarelli, A.L., Lu, A., Miller, L.K., 1996. Factors regulating baculovirus late and very late gene expression in transient-expression assays. J. Virol. 70 (4), 2307–2317.
van Oers, M.M., Pijlman, G.P., Vlak, J.M., 2015. Thirty years of baculovirus-insect cell protein expression: from dark horse to mainstream technology. J Gen Virol. 96 (1), 6–23. https://doi.org/10.1099/vir.0.067108-0.
Vaughn, J.L., Goodwin, R.H., Tompkins, G.J., McCawley, P., 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Vitro. 13, 213–217.