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Virulence and genetic characterization of six baculovirus strains isolated from different populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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ABSTRACT

Fall armyworm (FAW), *Spodoptera frugiperda* (Smith, 1797), is a polyphagous, voracious, and economically important agricultural pest. Biological control of FAW is a strategy that must be further explored. This study evaluated six baculovirus strains isolated from infected FAW larvae from Mexico, Argentina, Honduras, and the United States. Five alphabaculoviruses (SfNPV-An$_2$, SfNPV-Arg, SfNPV-Fx, SfNPV-Ho and SfNPV-Sin) and one betabaculovirus (SfGV-RV), were tested against FAW larvae, showing a wide diversity of virulence levels among strains when their estimated LC_{50}s were compared, being SfNPV-Arg, SfNPV-Ho and SfNPV-Fx more virulent than SfNPV-An$_2$, SfNPV-Sin and SfGV-RV. To determine any virulence difference *in vitro* studies of these isolates, Sf9 cell cultures were used. Interestingly, only ODVs from four of the test SfNPV strains showed infectivity on Sf9 cell cultures, and some differences in virulence were observed. Genomic restriction analyses and partial sequences of *lef-8, lef-9*, and *polh/granulin* genes showed little variability among alphabaculoviruses, both, among them and with previously reported sequences. However, sequences from SfGV-RV were closer to previously reported sequences from the SfGV-VG008 strain than the SfGV-Arg and SfGV-VG014 strains. The great difference in the *in vivo* virulence was not correlated with great similarity among the isolates. The characterization of these six baculoviruses isolates offers the basis for exploring their potential as biological control agents against *S. frugiperda*, as well the initial studies on their specific infection mechanisms, evolution, and ecology.

KEYWORDS: *Spodoptera frugiperda*, baculovirus, virulence, phylogeny, genetic comparison.

Introduction

Maize (*Zea mays* L. subsp. *mays*) (Poaceae) is one of the most important staple crops worldwide (Moya et al. 2018), and it is usually grown as an extensive monoculture system to satisfy human and animal consumption demand. At the same time, monoculture agricultural systems induce population increase of pests (Altieri et al. 2018). The fall armyworm (FWA), *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Smith, 1797), is a polyphagous, voracious, and economically important pest in North and South America (Cokola et al. 2021; Lee et al. 2020; Martínez et al. 2018), affecting a wide variety of plant species such as maize, sorghum, cotton and soybeans (Montezano et al. 2018), among many other crops. Management of FAW usually relies on the use of insecticides. However, resistance against chemical treatments has risen (Gutierrez et al. 2019; Yu 1991; Yu et al. 2003). Therefore, sustainable integrated pest management strategies must be explored, such as the use of biological control agents. Entomopathogens, parasitoids, and predators offer promising specific alternatives as they do not represent a risk to human health, the environment, or to other beneficial insects (Lacey et al. 2015; Melo et al. 2016; Tavares et al. 2010; Vega 2018; Wasim et al. 2009).

Baculoviruses belong to the family Baculoviridae (Harrison et al. 2018). These viruses are highly selective pathogens to insects from orders Lepidoptera, Hymenoptera and Diptera (Fuxa 2004; Kong et al. 2018). They are a diverse group of viruses with supercoiled, circular double-stranded DNA genomes, ranging in sizes from 80 to 180 kb and encoding between 90 and 180 genes. The two commonly found virion phenotypes in baculovirus are occlusion-derived virions (ODV) and budded virions (BV) (Blissard and Rohrmann 1990; Blissard and Theilmann 2018; Herniou et al. 2011; Jehle et al. 2006). Occlusion bodies (OBs) enclose the viral particles and allow viruses to survive in the environment. They are

composed of a crystalline matrix of protein, either polyhedrin in nucleopolyhedroviruses (NPV) and granulin in granuloviruses (GV) that occlude virions (Bilimoria 1991; Kelly et al. 2016). The Baculoviridae family is divided into 4 genera: *Alphabaculovirus* (Lepidoptera specific NPVs; group I and II), *Betabaculovirus* (Lepidoptera specific GVs), *Gammabaculovirus* (Hymenoptera specific NPVs), and *Deltabaculovirus* (Diptera specific NPVs) (Carstens and Ball 2009; Jehle et al. 2006).

Autographa californica multiple NPV (AcMNPV) *polyhedrin* (*polh*) gene was the first baculovirus gene sequenced (Hooft van Iddekinge et al. 1983; Rohrmann et al. 1981) and used for phylogenetic studies (Cowan et al. 1994; Zanotto et al. 1993). However, as genome sequences became available, some studies showed that different genes present different phylogenies (Harrison and Bonning 2004; Herniou et al. 2003), implying that to understand the evolution of baculoviruses, some genes were more valuable than others. This led to the definition of "core genes", and the selection of a set of conserved genes that together could offer robust phylogenetic results (Herniou et al. 2001; Herniou et al. 2003; Lange et al. 2004).

Another important aspect to consider in basic studies of baculoviruses as well as biological control agents is their potential large-scale, *in vitro* production, which has been possible since the establishment of the first cell line from pupal tissues of *Antheraea eucalypti* (Lepidoptera: Saturniidae) (Grace 1962), followed by more insect cell lines (Echalier 1971; Goblirsch et al. 2013; Grasela et al. 2012; Lynn and Hung 1986; Smagghe et al. 2009). These include the established IPLB-Sf21 (Sf21) pupal cell line of *S. frugiperda* (Vaughn et al. 1977) and the Sf9, a monoclonal cell line derived from Sf21 (Pasumarthy and Murhammer 1994).

Several baculoviruses with activity towards *S. frugiperda* have been isolated and studied throughout the American continent (Barrera et al. 2011; Barreto 2005; Berretta et al.

1998; Escribano et al. 1999; Fuxa 1987; García et al. 2020; Gómez et al. 2010; Ordóñez et al. 2020; Vieira et al. 2012; Yasem et al. 2009). The Food and Plant Biotechnology laboratory of the Life Sciences Division, Campus Irapuato-Salamanca of the University of Guanajuato, Mexico, has a collection of six isolates of SfNPVs, with some molecular variations and different biological activities towards *S. frugiperda* (Rangel-Núñez et al. 2014; Rios-Velasco et al. 2011; Ríos-Velasco et al. 2012). In this study we characterize those isolates with activity towards FAW larvae at the biological and phylogenetic level.

MATERIALS AND METHODS

Virus Strains

Five SfNPVs and one SfGV isolates were previously isolated from *S. frugiperda* or soil at different locations. SfNPV-An_2 was isolated in Coahuila, Mexico; SfNPV-Arg in Argentina; SfNPV-Fx in the United States; SfNPV-Ho in Honduras and SfNPV-Sin was isolated in Sinaloa, Mexico. The granulovirus SfGV-RV was isolated in Coahuila, Mexico. The six isolates were previously characterized using restriction endonuclease patterns. The isolates SfNPV-Arg, SfNPV-Fx and SfNPV-Ho were previously characterized by their virulence, establishing their lethal media concentration (LC_{50}) on *S. frugiperda* (Rangel-Nuñez et al. 2014; Ríos-Velasco et al. 2011; Ríos-Velasco et al 2012).

Virus propagation

Viral particles were amplified using larvae of *S. frugiperda*. Larvae were maintained in a semi-artificial diet containing 100 mL distilled water, 12.5 g bacteriological agar, 120 g corn flour, 50 g yeast, 5 g wheat germ, 25 g ground corn spike, 2.5 g sorbic acid, 5 g ascorbic acid, 3.125 g methylparaben, 8.75 g salt mixture, 62.5 g soybean, 3.125 mL formaldehyde

37%, 0.75 mg streptomycin, and 18.75 g Vanderzant vitamin mixture. They were maintained under insectarium conditions (60±10% relative humidity, 26±2°C and 16:8 h of light: dark photoperiod) in an environmental chamber (PERCIVAL) (Rangel Núñez et al. 2014). Insect infection was carried out by diet surface contamination, using 500 µl of $1x10^6$ OB/mL and 10 2nd instar FAW larvae per container. Larvae were placed individually within a grid on the diet and subsequently incubated for 6-7 days under the previously described conditions. The OBs from infected larvae were purified through sucrose density gradients by ultracentrifugation in a SW-32 rotor (Optia XPN-100, Beckman Coulter) at 24,000 rpm for 90 min. The concentrations of viral OBs were quantified with a hemacytometer and stored in aliquots of 500 µl of distilled water at 4°C until required.

***In vivo* Virulence of Baculovirus Isolates**

The LC_{50}s were estimated on FAW first instar larvae for the SfNPV-Sin, SfNPV-An_2 and SfGV-RV isolates and compared with those previously determined for isolates SfNPV-Arg, SfNPV-Fx and SfNPV-Ho (Rangel-Núñez et al. 2014). Bioassays were performed using 20 larvae per concentration, spreading ten larvae per petri dish containing the semi-artificial diet. Concentrations for NPV and GV isolates were quantified in a hemocytometer. The highest concentrations per bioassay were for SfNPV-Sin, $1.5x10^4$ OB/mm^2 of diet; for SfNPV-An_2, $3.2x10^2$ OB/mm^2, and for SfGV-RV, $3.1x10^4$ OB/mm^2. These concentrations were selected based on preliminary bioassays. For each isolate, six concentrations were calculated, using a dilution factor between 0.5-0.75. Larval mortality was documented after five days of incubation, and the results were analyzed using Probit analysis. A mean LC_{50} was estimated for each strain with the average of three replicates that satisfied the previously established statistical parameters (Ibarra and Federici 1987).

In vitro Virulence of Baculovirus Isolates

The possibility to observe some differences in the virulence of the baculovirus isolates on insect cell lines was tested. For this purpose, hemolymph of infected larvae with each isolate was inoculated to Sf9 (Thermofisher) cell cultures, supplemented with 5% fetal bovine serum. Due to negative results in all isolates, a less plausible alternative was tested, based on the inoculation of ODVs extracted from OBs. For each isolate, $2x10^8$ purified OBs were suspended in 300 μL of an alkaline buffer (0.1M Na_2CO_3, 0.1M NaCl, pH 10.8) and incubated at 50 rpm shaking for 10 min. Released virions were suspended in cell culture media Sf900 and sterilized by filtration (Lynn 2003). Filtrate was inoculated on cell line cultures and monitored for ten days using an inverted microscope (Zeiss Primo Vert). Supernatants from positive infections containing BV, were used to keep on the *in vitro* virus culture. Subsequent cell cultures started with $1X10^6$ cells in 25 cm^2 culture bottles and incubated for 24 h. These synchronized cell cultures were reinfected with a MOI of 10 and monitored for ten days to detect any virulence difference of each isolate, in terms of the number of cells showing OBs and the lysing period, when OBs were released.

Genetic Characterization

Restriction pattern analysis of isolates genomes and sequences of three core genes, were used to characterize each of the six baculovirus isolates, by comparing those sequences, both, among them, and with some previously reported.

171 **DNA Extraction**

172 The OBs from infected larvae were purified as described above. The purified OBs
173 were resuspended in a TE buffer solution (0.01 M Tris-HCl, 0.001 M EDTA, pH 7.6) and an
174 alkali solution (0.1 M Na_2CO_3, 0.1 M NaCl, pH 10.8) for 15 min in agitation. Virions were
175 purified through sucrose density gradients at 28,000 rpm for 40 min. Virions were then
176 suspended in buffer (10 mM Tris, 5 mM EDTA, 0.5% SDS) and incubated during 15 min at
177 60°C. Then, 100 µg of proteinase K (Invitrogen) was added and incubated again for 30 min
178 at 60°C. The mixture was washed with one volume of phenol:chloroform:isoamyl alcohol
179 (25:24:1), and centrifuged at 14,000 rpm for 10 min, then the aqueous phase was mixed with
180 one volume of cold isopropanol and centrifuged at 14,000 rpm for 10 min. The pellet was
181 washed with 70% ethanol, centrifuged again and the pellet was solubilized in sterile distilled
182 water (Del Rincón-Castro and Ibarra 1997). DNA was quantified in a Nanodrop (Thermo
183 Scientific) and its integrity corroborated by electrophoresis in 1% agarose gels.
184
185 **Genomic Restriction Pattern Analysis.** Once integrity of the genomic DNA from the NPVs
186 strains was corroborated, they were subjected to endonuclease digestion using *Bam*HI and
187 *Eco*RI restriction enzymes (Invitrogen), followed by electrophoresis in 0.8% agarose gels.
188 Digestion mixtures included 1 µg of genomic DNA, 1 µL restriction enzyme, 2 µL 10X
189 enzyme buffer, and water to complete 20 µL. Mixtures were incubated at 37°C for 2 h, and
190 restriction patterns were visualized in agarose gel electrophoresis carried out at 25V for 13
191 h. Patterns were recorded in a gel documentation system (Gel Doc™ EZ Imagen, Bio Rad)
192 and compared visually between each other. Differential bands between the isolates were
193 detected by comparing three replicates electrophoresed under different conditions and DNA
194 concentrations.

195 **PCR Amplification of Core Genes**

196 Fragments of *lef-8, lef-9* and *polh/gran* genes were amplified from genomic DNA
197 extracted from the six baculovirus isolates by PCR, using degenerated primers (Table 1)
198 (Jehle et al. 2006; Lange et al. 2004). These primers included universal primer tails (M13
199 Rev, M13 Fw, and BGH Rev) to facilitate direct sequencing of amplicons. The specific
200 primers for gene *lef-8* should amplify a 702 bp fragment, while *lef-9* should amplify 295 bp,
201 and *polh* gene amplifies a 540 bp fragment. Amplification of *lef-8* and *lef-9* genes fragments
202 were performed by PCR touchdown (initial denaturalization at 95°C for 3 min; 15 cycles
203 decreasing the alignment temperature -1°C each cycle, 95°C for 30 s, 55°C for 30 s, 72°C
204 for 30 s; plus 20 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; and final extension at
205 72°C for 7 min). Gene *polh* was amplified by conventional PCR (initial denaturalization 95°C
206 for 4 min; 35 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min; with a final extension
207 at 72°C for 10 min). The PCR products were purified with the Pure Link PCR purification
208 Kit (Invitrogen), and the amplicons were sequenced on the Illumina platform at
209 MACROGEN Co. (Korea).

210

211 **Phylogenetic analysis.** Nucleotide sequences of *lef-8, lef-9* and *polh* were downloaded from
212 the NCBI GenBank from the following reported genomes: SfNPV-3AP2 (EF035042),
213 SfNPV-19 (EU258200), SfNPV-459 (MK503924), SfNPV-ColA (KF891883), SfNPV-
214 ArgM (MW162628), SfNPV-281 (MK503923), SfNPV-B (HM595733), SfGV-VG008
215 (KM371112), SfGV-Arg (MH170055), and SfGV-VG014 (KJ698693, KJ698695 and
216 KJ698691 for genes *lef-8, lef*-9 and *polh*, respectively). Nucleotide sequences were compared
217 to the partial sequences of the six FAW baculoviruses used in the present study.

SeqMan 5.0 software was used to assemble the sequences (DNASTAR Inc.). The nucleotide sequence alignment was performed in the Mega X program (Kumar et al. 2018) using the Muscle algorithm and fitting to the size of the fragments obtained from the sequencing, to be later concatenated in the Mesquite software (version 3.5.1). The phylogenetic analysis was completed in the Mega X software using the neighbor-joining method (Saitou and Nei 1987). The nucleotide substitution model applied was p-distance. Gaps were treated as missing data. Bootstrap analyses (using 1000 replications) were used to assess the confidence in the branching order.

RESULTS

Virulence characterization.

***In vivo* Virulence of Baculovirus Strains.** Once bioassays of the evaluated baculovirus strains on FAW 1st instar larvae, Probit analysis estimated a wide variability of LC_{50}s. The SfNPV-Sin strain showed an LC_{50} of 1,619.24 OB/mm^2 of diet, while the SfNPV-An_2 isolate showed an LC_{50} of 21.24 OB/mm^2, and the SfGV-RV isolate showed an LC_{50} of 3,500 OB/mm^2 (Table 2). The virulence of the other three isolates included in the present study was previously estimated (Rangel Núñez et al. 2014), resulted in an LC_{50} of 1.15 OB/mm^2 for SfNPV-Arg, 3.42 OB/mm^2 for SfNPV-Fx, and 4.36 OB/mm^2 for SfNPV-Ho (Table 2). The isolate SfNPV-Sin turned out to be 1,408 times less virulent than the most virulent strain (SfNPV-Arg). Strains SfNPV-Fx, SfNPV-Ho, and SfNPV-Arg showed no statistical difference, but a highly significant difference was observed between these three strains when compared with the SfNPV-An2 and SfNPV-Sin, which showed very low virulence. Comparisons with the SfGV-RV strain has no meaning, as OBs from NPVs and GVs are not

242 comparable in size and virion content. All statistical requirements were fulfilled, as
243 previously described (Ibarra and Federici 1987).
244
245 ***In vitro*** **Virulence of Baculovirus Strains.** When Sf9 cell cultures were inoculated with
246 released ODVs from OBs of each isolate, only SfNPV-An_2, SfNPV-Arg, SfNPV-Fx, and
247 SfNPV-Ho were able of infect, replicate, and develop OBs within the cell. Isolate SfNPV-
248 Sin showed no infectivity, after several trials, and SfGV-RV was not expected to be
249 infectious, as it occurred, but still it was tested. The infected cells developed characteristic
250 cell changes from the baculovirus infection, such as nuclear hypertrophy, development of
251 virogenic stroma, presence of OBs in the nuclei, and cellular lysis. Additionally, the
252 supernatants from positive isolates were infective to subsequent cultures.
253 Synchronized cultures, inoculated with the same MOI, showed signs of infection at
254 day three post-infection (PI). On day 10 PI a significant number of infected cells was evident
255 in all isolates (Fig. 1). Almost all the cells infected with isolate SfNPV-An_2 showed OBs in
256 their nuclei, but no lysed cell were observed at this time, while cells infected with SfNPV-
257 Ho showed all the cell with OBs in their nuclei. All cells infected with SfNPV-Arg showed
258 OBs in their nuclei and some lysis, showing some released OBs. In contrast, cells infected
259 with SfNPV-Fx showed only some cells with OBs, because a great majority were already
260 lysed, and many free OBs were apparent (Fig. 1).
261
262 **Genetic characterization.**
263 **Genomic Restriction Pattern analysis.** Electrophoretic patterns obtained from the digestion
264 of the NPV strains genomes, using the *Bam*HI and *Eco*RI endonucleases are shown in figure
265 2. Differential bands are marked with an arrowhead. When the genomic DNA from each

isolate was digested with *Bam*HI (Fig. 2B), simple patters were obtained. Identical patterns were shown by isolates SfNPV-An2, SfNPV-Fx, and SfNPV-Ho, while isolates SfNPV-Arg and SfNPV-Sin showed identical patterns. Still, all patterns were highly similar. However, when the genomic DNAs from isolates were digested with *Eco*RI (Fig. 2A), more complex patterns were obtained. First, all patters showed at least one differential band when compared to each other. The highest similarity was observed between isolates SfNPV-Fx and SfNPV-Sin, while isolate SfNPV-An_2 showed the greatest difference when compared to the rest of the isolates.

Sequencing of *lef-8, lef-9,* and *polh* genes. The sequences of *lef-8*, *lef-9*, and *polh/gran* genes coming from the six strains were submitted to GenBank under the access numbers MK501795 to MK501800 (*lef-8*), MK507900 to MK507905 (*lef-9*) and MK558035 to MK558040 (*polh/gran*). Seven reference sequences of the *lef-8*, *lef-9* and *polh* genes from SfNPVs and 3 reference sequences of the *lef-8*, *lef-9* and *gran* genes from SfGV were downloaded from the NCBI GenBank for sequence comparison.

The sequencing of the six baculovirus resulted in amplicons of approximately 702, 295, and 540 bp from *lef-8*, *lef-9*, and *polh/gran*, respectively. After the sequence editing, the sequence alignments sizes were 613 pb for gene *lef-8*, 163 pb for *lef-9,* and 486 pb for *polh*.

Using the SfNPV-3AP2 isolate as a standard SfNPV reference strain, sequence alignment showed that the nucleotide homologies of *lef-8* ORFs of SfNPVs were above 98.7%. The homologies of the deduced amino acid sequences of all isolates were 100%. The aligned sequences of *lef-9* did not show changes in nucleotides or amino acids. In *polh,* differences were not present in the nucleotide or amino acid sequences.

289 For the studied betabaculovirus strain, the SfGV-VG008 isolate was used as a
290 standard SfGV reference. Sequence alignment showed that the nucleotide homologies of *lef-*
291 *8* ORFs of SfGVs were above 98.25%. The homologies of the deduced amino acid sequences
292 of all isolates were 99.03%. Sequence alignment showed that the nucleotide homologies of
293 *lef-9* ORFs of SfGVs were above 97.32%. The homologies of the deduced amino acid
294 sequences of all isolates were above 98.84%. Sequence alignment showed that the nucleotide
295 homologies of *gran* ORFs of SfGVs were above 98.27%. The homologies of the deduced
296 amino acid sequences of all isolates were above 98.84%.
297
298 **Phylogenetic analysis**
299 The phylogenetic analysis used a total of 499 positions of concatenated amino acid
300 sequences from Lef-8, Lef-9, and Polh proteins of the five nucleopolyhedroviruses (SfNPV-
301 Arg, SfNPV-Ho, SfNPV-Fx, SfNPV-An_2, and SfNPV-Sin) and the granulovirus SfGV-RV,
302 as well as several SfNPVs and SfGVs downloaded from the NCBI GenBank which were
303 used as references, for a total of 17 compared sequences. The optimal tree (Fig. 3) with the
304 sum of branch length was 0.67489023. The percentage of replicate trees in which the
305 associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the
306 branches. The tree is drawn to scale, with branch lengths in the same units as those of the
307 evolutionary distances used to infer the phylogenetic tree. The phylogenetic reconstruction
308 showed a great similarity between all the compared SfNPVs sequences. Specifically, an
309 identical identity between strain SfNPV-Arg and SfNPV-Sin, along with a reference strain
310 in the same clade is observed. Similar, bit in a different clade appears the SfNPV-Fx strain,
311 along two reference sequences. SfNPV-An2 and SfNPV-Ho share the same clade, whose
312 sequences were identical, as well as two reference sequences. Of course, SfGV-RV is in a

totally different clade, whose most similar reference strain was SfGV VG008. Other reference strains showed more differences. The sequences from the gammabaculovirus Neodiprion lecontei NPV was used as an outgroup.

DISCUSSION

This research was focused on the comparison of six baculovirus strains isolated from FAW larvae in four different countries. Virulence and some genetic features were the main characteristics to compare. Although virulence was highly variable, genetic features showed only limited differences between them.

Virulence of isolates SfNPV-Sin, SfNPV-An_2, and SfGV-RV was compared with other baculovirus isolates involved in the present study (Rangel-Núñez et al. 2014). Although the isolate SfNPV-An_2 showed the highest virulence among the three isolates estimated here, it was still 18 times less virulent than the SfNPV-Arg isolate. This great difference in virulence contrasts with other reports (Rowley et al. 2010) where SfNPV isolates showed more consistent levels of virulence. Trying to explain such a big difference in virulence, it is important to notice that those isolates showing the lowest virulence (SfNPV-Sin and SfNPV-An2) were originally isolated within the Mexican territory and tested on a Mexican population of FAW. This may indicate that Mexican NPV strains and Mexican FAW populations have co-evolved during a significant long period of time, developing natural resistance by Mexican FAW populations towards their NPVs. It would be interesting to test these isolates on Argentinian and/or Honduran FAW populations to prove this hypothesis. About the virulence of the SfGV-RV isolate, no comparison was done as NPV OBs not only are much bigger than GV OBs but also NPV OBs contain a great number of virions per OB, as compared with the single virion of a GV OB. However, if SfGV-RV is compared with

other SfGV strains, the former is about 100 times less virulent than the latter (Cuartas et al. 2014). The same hypothesis can be postulated to explain this phenomenon, based on some genetic differences.

On the other hand, the *in vitro* virulence of the six strains tested on Sf9 cell cultures showed some differences, too, but not at the same level than those found in the *in vivo* bioassays. First, there is an unsolved question about why hemolymph from infected larvae failed to infect Sf9 cell cultures, while ODVs were able to infect them. It is important to notice that, when infection of Sf9 cells was finally achieved with ODVs, such infections occurred in a very limited number of cells, showing low infectivity, similar to results shown in some pioneering reports (Volkman et al. 1976; Volkman and Summers 1977). Subsequent cultures used supernatants containing BVs from the established cultures. Still, only four out of six isolates were able to replicate *in vitro* as SfNPV-Sin was unable to grow, which also showed very little virulence on larvae. Also, the SfGV-RV strain did not grow in cultured cells, although this result was expected, as very few cases of stale GV *in vitro* cultures have been achieved (Ma et al. 2019).

Differences were observed mostly on the length of the infection process. Three replicates of synchronous cultures, with the same cell concentration and the same inoculum consistently showed that infection with isolate SfNPV-An2 was slower than infections with isolates SfNPV-Arg, and SfNPV-Fx. To some extent, these results may explain those obtained in the *in vivo* bioassays.

In reference to the genetic characterization of the isolates, both, the genomic restriction analysis, and the sequence comparison of the three selected genes, they showed a high degree of similarity between the isolates. Minor differences were observed in terms of additional or missing bands in the restriction patterns of the NPVs tested. The great difference

361 in the *in vivo* virulence between isolates SfNPV-Sin and SfNPV-Arg contrasted with the
362 almost identical restriction patterns (only one differential band). In fact, these results refuted
363 our suspicion that SfNPV-Sin was not actually an SfNPV. However, it is.

364 Other reports have reported restriction patterns of SfNPVs isolates from other
365 American countries such as Colombia, Nicaragua, and some Caribbean islands, additional to
366 strains with the same geographical origin as ours (Argentina, Honduras, Mexico and, United
367 States) (Barrera et al. 2011; Berretta et al. 1998; Escribano et al. 1999; Rangel-Núñez et al.
368 2014; Ríos-Velasco et al. 2012), showing more difference than those found in this report.
369 Interestingly, some of those differences were correlated to the virulence level of those strains,
370 opposite to our results.

371 On the other hand, sequencing of specific baculovirus genes have been widely used
372 to establish phylogenetic relationships between different species and strains, using highly
373 conserved genes (Kaur et al. 2014; Gani et al. 2017). Sequences of *lef-8, lef-9,* and
374 *polh/granulin* genes have been recommended to be used as a minimum information to detect
375 phylogenetic relationships between strains (Jehle et al. 2006). The size of the sequences
376 obtained from the six isolates used in this report agree with other reports (Jehle et al. 2006;
377 Lange et al. 2004); however, minimal variations were detected in the *lef8* and *lef9* genes, and
378 no variation in the *polh* gene, among the SfNPVs studied here and when compared with
379 reported sequences. Interestingly, identical sequences were detected when SfNPV-Arg and
380 SfNPV-Sin were compared. Again, as observed in the genomic restriction analysis, a highly
381 virulent strain shows identical sequences with the low virulent strain SfNPV-Sin, both from
382 totally different origin. The same result was observed between the SfNPV-AN_2 and the
383 SfNPV-Ho strains. These results are not surprising as a previous report shows little variability
384 on the *lef8* and *polh* genes, when 40 SfNPV isolates from the USDA-ARS collection were

compared (Rowley et al. 2010). Therefore, sequence variation on those genes cannot be used to identify the geographical origin nor the virulence of each strain. Genomic sequencing may clarify some of these questions. This study agrees with previously reported results by other authors regarding the high specificity exhibited by SfNPVs (Popham et al. 2021), which creates interest for deeper studies on virus-host interaction and their specificity mechanisms. The genotypic and phenotypic variability found between the studied SfNPVs isolates, mostly those related to the evident difference in virulence among isolates, emphasize the need for sequencing their genomes to compare virulence variability at the genomic level. All this information would make it possible to determine in the future which of these American isolates of SfNPV are the most suitable to be developed for biological control programs against the FAW.

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Declarations

Conflict of interests The authors declare no conflicts of interest.

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640 **Figure Captions**

641

642 **Fig. 1** Genomic restriction analysis of the five SfNPV isolates. A) Genomes digested with
643 *Eco*RI; B) Genomes digested with *Bam*HI. An2: SfNPV-An_2, Ar: SfNPV-Arg: Fx: SfNPV-
644 Fx, Ho: SfNPV-Ho, and Sin: SfNPV-Sin. MWM: Molecular weight marker. Differential
645 bands are pointed by an arrowhead

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647 **Fig. 2** Synchronized cultures of four SfNPV isolates cultured on Sf9 cells, after 10 days post
648 infection. A) SfNPV-An_2; B) SfNPV-Ho; C) SfNPV-Arg; D) SfNPV-Fx

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650 **Fig. 3** Phylogenetic tree constructed from concatenated nucleotide sequences of *lef-8*, *lef-9*,
651 and *polh* genes from five SfNPV strain and one SfGV strain, using the neighbor-joining
652 analysis method. Bootstrap resampling was done 1000 times, and resulting bootstrap values
653 are shown on the corresponding branches. Eleven sequences were added from the NCBI
654 GenBank for comparison. The NeleNPV virus was used as outgroup. Scale bar indicates the
655 number of substitutions per site

656

Table 1 Primers used for the amplification of the genes *lef-8*, *lef-9*, and *polh/gran* of six baculovirus isolates with activity towards *S. frugiperda.*

Primer	**Sequence** [a, b]	**Universal primer**	**bp**[c]	**Source**
lef-8 forward	CAGGAAACAGCTATGACCAYGGHGARATGAC	M13 Rev	702	(Lange et al. 2004)
lef-8 reverse	GTAAAACGACGGCCAGAYRTASGGRTCYTCSGC	M13 Fw		
lef-9 forward	CAGGAAACAGCTATGACAARAAYGGITAYGCBG	M13 Rev	295	(Lange et al. 2004)
lef-9 reverse	GTAAAACGACGGCCAGTTGTCDCCRTCRCARTC	M13 Fw		
Polh/gran forward	TAGAAGGCACAGTCGAGGNRCNGARGAYCCNTT	BGH Rev	540	(Jehle et al. 2006)
polh /gran reverse	CAGGAAACAGCTATGACCDGGNGCRAAYTCYTT	M13 Rev		

[a] *Underlined nucleotides* indicate standard sequencing M13 forward, M13 reverse, T7 and BGHrev primers (this part of the primer allows for the direct sequencing of polymerase chain reaction products); degenerate baculovirus primers are not underlined.

[c] Expected size of the amplification product.

673 **Table 2** Probit analysis parameters obtained from the bioassays of baculovirus strains
674 included in this work, on FAW 1st instar larvae, as compared to those previously reported
675 by Rangel-Núñez et al. (2014)

Viral isolates	**n**	**Slope (±SE)**	**LC_{50} (OB/mm^2) (Fiducial limits)**	**X^2**	**Ref.**
SfNPV-Sin	360	1.36.(±0.13)	1,619.24 (1,123.51-2,334.01)	2.23	This work
SfNPV-An_2	360	0.96 (±0.10)	13.7 (21.24-83.32)	1.46	This work
SfGV-RV	360	1.31 (±0.21)	3,500 (2,200.10-5,700.43)	1.30	This work
SfNPV-Arg	360	1.72 (±0.19)	1.15 (0.65-2.14)	4.5	R-N (2014)
SfNPV-Fx	360	1.45 (±0.08)	3.42 (1.85-6.37)	4.8	R-N (2014)
SfNPV-Ho	360	2.06 (±0.14)	4.36 (2.39-7.95)	1.4	R-N (2014)

676 n: Number of evaluated larvae; X^2: chi-square value; R-N (2014): Rangel-Núñez et al. 2014.
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