TESIS DOCTORAL

Genetic diversity in Chrysodeixis includens nucleopolyhedrovirus and factors involved in its generation

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Genetic diversity in Chrysodeixis includens nucleopolyhedrovirus and factors involved in its generation

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que la presente memoria de Tesis Doctoral titulada **"Genetic diversity in Chrysodeixis includens nucleopolyhedrovirus and factors involved in its generation"** elaborada por D. **Eduardo Aguirre Sánchez** ha sido realizada bajo nuestra dirección, y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

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RESUMEN

El control de las plagas de lepidópteros se está convirtiendo en un tema de urgencia debido a la creciente prevalencia de la resistencia a insecticidas, una reducción en el número de insecticidas sintéticos disponibles y una creciente demanda por parte del consumidor por productos libres de residuos. Los insecticidas basados en baculovirus son muy selectivos y no afectan a otros organismos ni producen residuos tóxicos. El estudio de la variabilidad genética y fenotípica de estos virus es una forma de seleccionar variantes genéticas que presenten propiedades insecticidas interesantes. El cribado de aislados naturales para identificar las características insecticidas de las variantes genotípicas es una técnica habitual en el estudio de los baculovirus. En la presente tesis se ha examinado la variabilidad genética de varios aislados de campo del nucleopoliedrovirus de *Chrysodeixis includens* (ChinNPV).

Partiendo de once larvas de *C. includens* muertas por virus recogidas de campos de soja en México, nueve aislados de ChinNPV mostraron diferencias en sus perfiles de restricción (REN). Esos aislados se utilizaron para crear una mezcla artificial, ChinNPV-Mex1. Se realizó un barrido de huéspedes para diferentes especies de lepidópteros plaga con esta mezcla, que resultó ser patogénico sólo para tres especies de la subfamilia Plusiinae (*C. includens*, *C. chalcites* y *T. ni*). La mezcla ChinNPV-Mex1 mostró una concentración letal al 50% (LC_{50}) de 6.5×10^4 cuerpos de inclusión (OBs)/mL en larvas de segundo estadio de *C. includens*. Este resultado resultó ser notablemente más bajo que el valor de LC_{50} obtenido para Crysogen®, el primer insecticida basado en ChinNPV, de 1.4×10^5 OBs/mL en larvas neonatas de *C. includens*.

La purificación en placa de las variantes genotípicas del ChinNPV-Mex1 se llevó a cabo en cultivo celular y resultó en 23 genotipos diferentes en función de su perfil REN de EcoRI y HindIII. La variante más prevalente fue ChinNPV-H, representando un 18% de todas las variantes aisladas. Se caracterizó de forma biológica los 23 genotipos mediante un ensayo de mortalidad a una sola concentración (10^4 OBs/mL), en el que la variante ChinNPV-R resultó ser la más

patogénica (39.5 ± 7.0% mortalidad) y cuyo resultado fue significativamente mayor que la mortalidad inducida por cualquier otra variante. Se seleccionaron cinco variantes genotípicas y se testó su tiempo medio de mortalidad y productividad para compararlos con el ChinNPV-Mex1. Estos genotipos no demostraron diferencias significativas en su tiempo medio de mortalidad, aunque la producción de OBs señaló a ChinNPV-F, K, R y V como las variantes más productivas, con valores que oscilaron entre 2.2×10^9 hasta 3.7×10^9 OBs/larva dependiendo del genotipo.

La segunda parte de la tesis se centró en la prevalencia de dos genotipos, ChinNPV-K y ChinNPV-E, en larvas de quinto estadio de *C. includens*. La frecuencia de los perfiles REN de ChinNPV-K en larvas individuales de *C. includens* infectadas con ChinNPV-Mex1 incrementó significativamente según disminuía la concentración de inóculo. A concentraciones elevadas de ChinNPV-Mex1, todas las larvas que murieron de infección viral mostraron el perfil REN de ChinNPV-K, mientras que, a concentraciones bajas de inóculo, el perfil REN predominante fue el de ChinNPV-E. A concentraciones intermedias de virus se recuperaron hasta 18 perfiles REN diferentes, pertenecientes a genotipos ya identificados previamente. Utilizando sólo OBs de ChinNPV-K como inóculo, la prevalencia del resto de variantes resultó la misma que para ChinNPV-K, con una clara correlación entre concentración de inóculo y la prevalencia de insectos muertos por virus que presentaban el perfil REN de ChinNPV-K. De nuevo, a concentración alta de ChinNPV-K como inóculo, su perfil REN fue el más prevalente, aunque se encontró mayor variabilidad que la observada cuando se utilizó ChinNPV-Mex1 como inóculo y además se encontraron nuevos perfiles REN. A baja concentración de ChinNPV-K se registró un incremento en la variabilidad de genotipos, con más variantes presentes y con variaciones en sus frecuencias. Sin embargo, cuando se utilizó ChinNPV-E como inóculo, los resultados contrastaron totalmente con los obtenidos para ChinNPV-K, sin ninguna correlación entre concentración y prevalencia de perfiles REN de ChinNPV-E. Todas las larvas que murieron de poliedrosis mostraron el perfil REN de ChinNPV-E a alta concentración excepto una, cuyo perfil REN no había sido registrado previamente. Por tanto, se

constató la correlación inversa existente entre concentración de virus y variabilidad genética, sugiriendo estos resultados que tal vez la variante ChinNPV-K estuviese teniendo un papel clave en la generación de la variabilidad.

Estos resultados me llevaron a comprobar la estabilidad genética de la progenie de los OBs provenientes de inocular larvas con una baja concentración de ChinNPV-K. Así, el inóculo obtenido tras inocular larvas con los genotipos obtenidos de esta baja concentración se utilizó para un siguiente pase. Aquellas larvas que murieron de poliedrosis mostraron el mismo perfil REN que la variante con la que habían sido inoculadas, sin evidencia de ChinNPV-K o de cualquier otra variante. Por tanto, las nuevas variantes generadas parecen ser estables genéticamente ya que actúan como genotipos puros.

Las mezclas de ChinNPV-K y -E a concentración elevada en las que ChinNPV-K estaba presente en una concentración similar o en proporciones mayores que ChinNPV-E, resultó en una mayor prevalencia del perfil REN de ChinNPV-K en la progenie viral de aquellos insectos que muertos por virus. También se recuperaron otros perfiles REN pertenecientes a otros genotipos. Sin embargo, cuando ChinNPV-E estaba presente en el inóculo en una proporción mayor que ChinNPV-K, sólo se observó el perfil REN de ChinNPV-E en los insectos muertos por virus. En la concentración baja de las mezclas, cuando ChinNPV-K tenía la misma proporción que ChinNPV-E en el inóculo, se observó mayor variabilidad. Sin embargo, cuando era ChinNPV-E el que se encontraba en una mayor proporción que ChinNPV-K, de nuevo la variabilidad de perfiles REN observados en los insectos muertos por virus descendía. Evidentemente, el inóculo con sólo ChinNPV-E resultó en que toda la progenie mostró este perfil REN.

Las muestras recogidas en pool de larvas inoculadas con ChinNPV-K a baja concentración mostró el perfil REN de ChinNPV-K. Tras utilizar este pool de OBs como inóculo para otra infección, la prevalencia del perfil REN de ChinNPV-K mostró la misma tendencia que hasta ahora, es decir, a concentración alta de inóculo, el perfil REN de ChinNPV-K fue el más observado, mientras que, a baja concentración de inóculo, de nuevo la prevalencia del perfil REN de ChinNPV-K disminuía en los insectos muertos por virus además de

aumentar el número de perfiles REN diferentes. Por tanto, la baja presencia de ChinNPV-K en larvas individuales es suficiente para convertirse en la variante mayoritaria cuando se recogen en pool y así observar la generación de variabilidad de manera dependiente de concentración cuando ese pool es usado como inóculo.

La infectividad de ChinNPV-K y -E en condiciones in vitro (indicada por la $TCID_{50}$) se estimó mediante un ensayo de dilución límite y resultó en una mayor producción de BVs en la hemolinfa de ChinNPV-K en larvas a las 48 horas de la infección. De modo similar, la cuantificación por qPCR de los BVs en la hemolinfa reveló una cantidad de 2,3 veces mayor de BVs en ChinNPV-K que en ChinNPV-E.

La prevalencia de ChinNPV-K en cultivo celular se examinó utilizando tanto BVs como ODVs en ensayos independientes. Los resultados obtenidos para ambos tipos de viriones mostraron que ningún clon de los obtenidos en cultivo celular presentó el perfil REN de ChinNPV-K. De nuevo, se encontró más variabilidad de la esperada para un solo genotipo en células infectadas con ChinNPV-K. En comparación, el mismo experimento llevado a cabo con ChinNPV-E resultó en que los clones recogidos mostraron únicamente el perfil REN de ChinNPV-E. Por tanto, parece que el genotipo ChinNPV-K es más inestable que el genotipo ChinNPV-E, cuyo comportamiento es el de un genotipo puro clásico.

Finalmente, la secuenciación de las variantes ChinNPV-K y -E desveló importantes diferencias entre ellas. Partiendo de un número similar de lecturas, el genoma de ChinNPV-E fue ensamblado en un único contig, mientras que el genoma de ChinNPV-K se ensambló en seis contigs diferentes y tuvo que ser cerrado mediante secuenciación Sanger. El perfil REN in silico de ChinNPV-E coincidió con el experimental, al contrario que el de ChinNPV-K, que mostró varias diferencias. La secuencia de ChinNPV-E mostró un bajo grado de variación localizado únicamente en el gen *hoar*, mientras que la secuencia de ChinNPV-K mostró mucha más variabilidad a lo largo de todo el genoma, con un total de 285 variaciones en la secuencia nucleotídica, muchas de las cuales fueron SNPs.

Estos resultados revelan la gran variabilidad presente en los aislados naturales de ChinNPV, confirma las diferencias existentes entre dos variantes genotípicas procedentes de esos aislados y, lo más importante, parecen identificar a la variante ChinNPV-K como generadora de variabilidad en esos aislados. En conclusión, hemos encontrado una gran variabilidad en aislados naturales de ChinNPV, con una sola variante que muestra un papel clave en la generación de esta diversidad de manera dependiente de la concentración.

SUMMARY

The control of lepidopteran pests is becoming ever more urgent due to growing prevalence of insecticide resistance, a reduction in the number of synthetic pesticides available for pest control and an increasing consumer demand for residue free produce. Baculovirus based insecticides are highly selective and do not harm other organisms or produce toxic residues. Studying the genetic and phenotypic variability of these viruses is a means to select genotypic variants with useful insecticidal properties. The screening of natural isolates to identify insecticidal characteristics of genetic variants is a common process in the study of baculoviruses. In the present thesis, the genetic variability of field-collected isolates of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) was examined.

From eleven virus-killed *C. includens* collected in soya fields in Mexico, nine ChinNPV isolates showed differences in their restriction endonuclease profiles (REN). These isolates were used to create an artificial mixture, named ChinNPV-Mex1. A host range test was performed with different lepidopteran pest species using the ChinNPV-Mex1 mixture, which proved to be pathogenic to only three species from the Plusiinae subfamily (*C. includens*, *C. chalcites* and *T. ni*). The ChinNPV-Mex1 mixture had a 50% lethal concentration (LC_{50}) of 6.5×10^4 OBs/mL in *C. includens* second instars. This result was notably lower than the first ChinNPV-based insecticide Crysogen® that had a reported LC_{50} of 1.4×10^5 OBs/mL in *C. includens* neonates.

Plaque purification of ChinNPV-Mex1 genotypic variants was performed in cell culture and resulted in 23 different genotypes that differed in their REN profiles with EcoRI and HindIII. The ChinNPV-H variant was the most prevalent variant, representing 18% of the plaque-purified variants. All 23 new genotypes were submitted to biological characterization through a single concentration (10^4 OBs/mL) mortality bioassay, in which ChinNPV-R was identified as the most pathogenic variant (39.5 ± 7.0% virus induced mortality) which was significantly higher than the mortality induced by any of the other variants. Five

variants were selected and tested for their speed-of-kill and productivity traits against ChinNPV-Mex1. Variants did not differ significantly in their mean time to death (MTD) values, whereas occlusion body (OB) production counts identified ChinNPV-F, -K, -R, and -V as the most productive variants with values ranging from 2.2×10^9 to 3.7×10^9 OBs/larva depending on variant.

The second part of the thesis focused on the prevalence of two selected genotypes, ChinNPV-K and ChinNPV-E in *C. includens* fifth instars. The frequency of the ChinNPV-K REN profile in individual *C. includens* larvae infected with ChinNPV-Mex1 increased significantly as inoculum concentration decreased. At high concentrations of ChinNPV-Mex1, all but one virus-killed larva showed the ChinNPV-K REN profile, whereas at low inoculum concentration, the predominant REN profile was that of ChinNPV-E. At the intermediate concentrations of inoculum, up to 18 different REN profiles belonging to previously identified genotypes were recovered from virus-killed larvae. When using ChinNPV-K OBs alone as inoculum, the prevalence of variants was the same as for the ChinNPV-Mex1 mixture, with a clear correlation between inoculum concentration and the prevalence of virus-killed insects showing the ChinNPV-K REN profile. Again, at a high concentration of ChinNPV-K inoculum, the ChinNPV-K REN profile was the most prevalent, although with more variability than the observed when ChinNPV-Mex1 was used as the inoculum. Novel REN profiles were also detected. A low concentration of ChinNPV-K inoculum resulted in an increase in genotype variability, with more variants present and variation in their frequencies. When ChinNPV-E OBs were used as inoculum the results contrasted with those of ChinNPV-K, with no correlation between inoculum concentration and the prevalence of ChinNPV-E REN profiles. All inoculated larvae that died of polyhedrosis disease showed the ChinNPV-E variant REN profile except one larva at the high concentration that showed a REN profile that did not match any of the previously described variants. Therefore, an inverse correlation between virus concentration and genetic variability was found and the results suggest that ChinNPV-K may have a key role in the generation of variability.

These results led me to assess the genetic stability of progeny OBs from larvae inoculated with a low concentration of ChinNPV-K. Larvae that died of polyhedrosis disease showed the same REN profile as the variant used as inoculum, with no evidence of the presence of the ChinNPV-K variant or any other variant. Hence, the new variants generated seem to be stable given that they act as pure genotypes.

Mixtures of ChinNPV-K and -E at high inoculum concentration in which ChinNPV-K was present at similar or higher proportions than ChinNPV-E resulted in a high prevalence of the ChinNPV-K REN profile in the virus-killed insects. Other REN profiles were also recovered. However, when ChinNPV-E was present in the inoculum at a higher proportion than ChinNPV-K, only the ChinNPV-E REN profile was observed in the OB progeny of virus-killed insects. At low inoculum concentration, when ChinNPV-K was at the same proportion than ChinNPV-E in the inoculum, more variability was observed, but when ChinNPV-E was present in a higher proportion than ChinNPV-K, the variability of REN profiles in virus-killed insects again decreased. Evidently, the inoculum comprising ChinNPV-E alone resulted in progeny with this REN profile and no other.

Pooled samples of larvae inoculated with ChinNPV-K at the lower concentration showed the ChinNPV-K REN profile. After using a pooled OB sample as inoculum, the prevalence of ChinNPV-K REN profile revealed the same trend, this is, at high inoculum concentration, the ChinNPV-K REN profile was the most frequently recovered but at the lower inoculum concentration, again, the prevalence of ChinNPV-K REN profile decreased in virus-killed insects while the number of different REN profiles increased. Therefore, the low presence of ChinNPV-K in individual larvae is enough to make it the majority variant in the pooled sample and observe the generation of variability in a concentration-dependent manner when using the pooled sample as inoculum.

The infectivity of ChinNPV-K and -E was assessed under in vitro conditions. The 50% tissue culture infectious dose ($TCID_{50}$) was estimated by end-point dilution assay and revealed a significantly higher production of budded virions (BV) in the hemolymph of ChinNPV-K infected larvae at 48 hours

post-infection. Similarly, qPCR quantification of BV in hemolymph samples revealed a 2.3-fold higher production of BV in ChinNPV-K infected insects compared to ChinNPV-E infected insects.

The prevalence of ChinNPV-K in cell culture was tested using BVs and ODVs in separate assays. Results for both type of virions showed that no REN profile of ChinNPV-K could be isolated from clones obtained from cell cultures. Again, more variability than expected for a single genotype was recovered from ChinNPV-K inoculated cells. In contrast, the same experiment carried out with ChinNPV-E resulted in clones that showed only the ChinNPV-E variant REN profile. Apparently, the ChinNPV-K genotype seems to be instable in comparison to ChinNPV-E, which acts as a classical pure genotype.

Finally, genome sequencing of the ChinNPV-K and -E variants revealed marked differences among them. Based on almost the same number of reads, the genome of ChinNPV-E was assembled in one unique contig whereas the ChinNPV-K genome had to be assembled in six different contigs and required Sanger sequencing to close the gaps. The in silico REN profile of ChinNPV-E matched the empirical REN profile, unlike ChinNPV-K which showed several differences. The ChinNPV-E sequence showed a small degree of variation located in the *hoar* gene, whereas the ChinNPV-K sequences showed variability along the entire genome, with a total of 285 variations in the nucleotide sequence, most of which were SNPs.

These results have revealed the great variability present within natural ChinNPV isolates, confirm the differences between two different genetic variants from these isolates and, more important, appear to identify the ChinNPV-K variant as a generator of variability in these isolates. In conclusion, a great variability has been revealed within ChinNPV natural isolates, with a single variant showing a key role in the generation of this diversity in a concentration-dependent manner.

CHAPTER I

Introduction

Contents

1. GENERAL INTRODUCTION AND SCOPE OF RESEARCH

The history of baculoviruses begins with their discovery in the ancient silk industry whose origins date from 5000 years ago in China (Rohrmann, 2019). Nevertheless, it was not until their activity as virulent and insect-specific pathogens was discovered and their potential as biological control agents was confirmed in field trials performed in the 1950s and 1960s (Ignoffo, 1973) that they were used as bioinsecticides. However, the rapid rise in the use of chemical insecticides and their remarkable insecticidal characteristics such as broad-spectrum toxicity and rapid speed of kill meant that baculoviruses were largely overlooked as pest control agents. Ironically, the same features that initially favored the adoption and widespread use of chemical insecticides resulted in overdependence on synthetic insecticides during the late 1970s and throughout the 1980s (Winstanley and Rovesti, 1993). Growing awareness of the hazards of chemical pesticides resulted in the urgent need to develop safe control products that could overcome the increasing problems of pest resistance and consumer demands for residue-free products (Nicolopoulou-Stamati et al., 2016). The inherent characteristics of baculoviruses as safe and specific biocontrol agents, like other microbial insecticides, have renewed interest in incorporating them into Integrated Pest Management (IPM) strategies in order to achieve a more sustainable pest control and products with little or no synthetic pesticide residues.

Using baculoviruses for the control of pestiferous insects has stimulated numerous studies on these viruses, particularly those focused on the biology, molecular processes and variability of these pathogens (Lapointe et al., 2012). In fact, the re-emergence of baculoviruses as potential biopesticides has spurred efforts to screen natural insect populations in order to discover new or more efficacious viral genotypes. These efforts highlight the great natural diversity that exists among the baculoviruses (Erlandson, 2009). The use of next generation sequencing (NGS) techniques has also improved the level of discrimination among variants, allowing the detection of high numbers of viral genotypes even within a single baculovirus isolate (Chateigner et al., 2015).

Given the high variability that baculoviruses display, the key question is how this variability is generated and maintained. Several studies have shed light on this topic, and have identified certain molecular mechanisms, in addition to point mutation, that are responsible for generating viral diversity (Van Oers and Vlak, 2007). Recombination, the exchange of genetic material between different virus strains and/or genotypes or even between the pathogen and the host, seems to be an important mechanism involved in the production of virus diversity (Barrera et al., 2015; Croizier et al., 1988; Inglis et al., 2020; Kamita et al., 2003). In multiple nucleopolyhedroviruses the genotypically diverse population of virions present in the occlusion bodies (OBs) ensures that this diversity is transmitted during insect-to-insect transmission of the pathogen (Clavijo et al., 2010), in the form of so-called collective infectious units (Sanjuán and Domingo-Calap, 2016).

This thesis examined the natural variability present in Chrysodeixis includens nucleopolyhedrovirus (ChinNPV). This included the study of the genetic variability present in a selection of natural ChinNPV isolates, in order to expand knowledge of this virus and to identify genetic variants that could be used as the active ingredient for a baculovirus-based bioinsecticide. The first question addressed was whether the variability of the isolates was similar to the variability found in other baculoviruses. The second part of the thesis focused on the maintenance of the variability of this virus. The mechanisms involved in generating the variability in ChinNPV was also studied and compared with those of other baculoviruses. As a spin-off from the main goals of the thesis, the genetic variants obtained from this virus may show useful insecticidal properties that could form the basis for a novel bioinsecticide for the control of *Chrysodeixis includens* in different parts of the world.

2. CHRYSODEIXIS INCLUDENS

2.1 Taxonomy and morphology

The soybean looper, *Chrysodeixis includens* (Walker, 1858) is an insect that belongs to the order Lepidoptera, the Noctuidae family (Latreille, 1809), Plusiinae subfamily (Boisduval, 1828) and the tribe Argyrogrammatini

(Eichlin and Cunningham, 1978; Lafontaine and Schmidt, 2010). Larvae of this insect are commonly known as semi-loopers (Barrionuevo and San Blas, 2016). Before 2003, this moth used to be assigned to the genus *Pseudoplusia* until a review of genital morphological characters of noctuids from Europe proposed that *Pseudoplusia* was a synonym of *Chrysodeixis* Hübner (1821) (Goater and Ronkay, 2003).

The life cycle of this moth with complete metamorphosis involves four development stages: egg, larva, pupa and adult (Fig.1). The eggs commonly present a color range from pale yellow to cream and are hemispherical in shape, with a slightly flattened top and base. Their height varies from 274 to 420 micrometers and the diameter from 490 to 580 micrometers (Barrionuevo and San Blas, 2016; Gomez Rolim et al., 2013) (Fig. 1A).

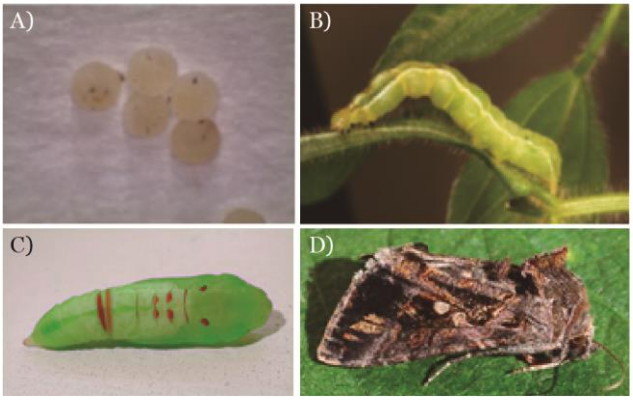

Figure 1. Morphology of *C. includens*: A) egg; B) larva; C) pupa; D) adult.

C. includens larvae develop through six instars followed by a pre-pupal stage. The larva presents three pairs of true thoracic legs and three pairs of prolegs (two in abdominal segments five and six and one in the anal segment) (Barrionuevo and San Blas, 2016; Xue and Hua, 2018). This allows them to move with a pronounced looping motion, characteristic of the Plusiinae larvae, due to the lack of abdominal prolegs compared to the normal

five present in most of the members of the Noctuidae family (Capinera, 2013) (Fig. 1B). Immature instars present a body length ranging from 2.65 ± 0.12 mm in first instars to 27.45 ± 2.74 mm in sixth instars, and typically present white stripes running down the dorsal and lateral portions of the body (Fig. 1B). The color pattern up to the third instar varies from hyaline to white when fed on artificial diet to light and dark green when fed on natural diet. From instar four to six, the color pattern is variable, more frequently from brightly green to yellowish green (Barrionuevo and San Blas, 2016).

Pupation usually occurs on the underside of leaves and this process normally takes from seven to nine days. The pupae have a fusiform-shape (Fig. 1C) and inhabit a silken cocoon produced by the larva during its final stage. Male pupae measure 18.6 ± 1.9 mm in length and 3.6 ± 0.1 mm in width. They are larger than female pupae that are 18.2 ± 0.1 mm long and 3.5 ± 0.1 mm wide. Diet consumed during the larval stage may influence pupal weight, which ranges from 180 to 270 mg approximately (Andrade et al., 2016; Canerday and Arant, 1967; Reid and Greene, 1973). The color is brightly green turning to light brown when the pupa is close to adult emergence (Barrionuevo and San Blas, 2016).

Figure 2. Adult soybean loopers, *Chrysodeixis includens* (Walker), with differing color patterns. Photographs by Lyle J. Buss, University of Florida.

Adults of *C. includens* have a wingspan of approximately 40mm. The forewing length is 15 to 17 mm, usually gold although some individuals present more of a bronze color. Forewings are darker in color than the hindwings, and

the first possess two oval silver spots, although in some individuals these are united. Two prominent crests are found on the thorax (Bretherton, 1983; Pinhey, 1975) (Fig. 2).

2.2 Geographical distribution and host plants

C. includens is mainly present in the occidental hemisphere, occurring from Canada to southern South America, although its presence has been reported in other countries like Sri Lanka, Japan and Australia (Alford and Hammond, 1982; Kogan, 1981) (Figure 3). It presents some of the characteristics of oogenesis-flight syndrome, which is associated with migratory insects, such as seasonal differences in levels of whole body lipids in adult moths (Mason et al., 1989).

Figure 3. Worlwide distribution of *C. includens*. Data source: (Cabi, 2015; "Falso Medidor de la Soya (*Chrysodeixis includens*), NaturaLista)

In the northern hemisphere, *C. includens* occurs in Canada (Nova Scotia, southern Quebec and southern Ontario), the USA (southern California, Arizona, New Mexico and southern Colorado) and Mexico. In most parts of the United States, *C. includens* is known to migrate, with annual emigrations of moths to the northern states (Palma et al., 2015). In the tropical Americas, it

can be found from Mexico to south America (Lafontaine and Schmidt, 2010; Pogue, 2005). In Brazil, where *C. includens* is one of the main pests in soybean crops, outbreaks of *C. includens* are detected frequently in west Bahia, Goiás, Mato Grosso, Mato Grosso do Sul, São Paulo, Paraná and Rio Grande do Sul, although it occurs in all states of the country (Moscardi et al., 2012; Wille et al., 2017).

C. includens has been named as "the soybean looper" as it constitutes a key pest in soybean crops in almost the whole American continent (Herzog, 1980; Meagher et al., 2013; Moscardi et al., 2012). Nevertheless, this moth can feed on numerous plants apart from soybean, which reflects its high level of polyphagy. A study carried out by Specht *et al.* (2015) identified the published host plant information in addition to recording new host plants in Brazil that had not been previously reported. Their results provided a list of 174 plants consumed by *C. includens*, belonging to 39 plant families, from which the most consumed by this moth were: Asteraceae (29), Solanaceae (21), Fabaceae (18), Lamiaceae (12), Brassicaceae (8), Poaceae (7), Amaranthaceae (6), Euphorbiaceae and Malvaceae (5). The authors contend that the great diversity of host plants and their availability in the Brazilian landscape guarantees the survival and persistence of *C. includens* populations even in the soybean off-season (Specht et al., 2015). Studies carried out in different plant hosts revealed that *C. includens* had similar biotic parameters, such as development time and survival rate, when fed on distinct hosts. (Andrade et al., 2016; Moonga and Davis, 2016; Specht et al., 2019). Indeed, sunflower was a more favorable host even than soybean (Andrade et al., 2016).

2.3 Biology and ecology

C. includens has been reported as a migratory species in the USA (Tingle and Mitchell, 1977) and up to four generations per year have been recorded in soybean fields (Burleigh, 1972), although this number can vary. Adults have a longevity of approximately 15-18 days (Canerday and Arant, 1967; Mitchell, 1967; Sosa-Gómez et al., 2014). Mating usually occurs at night, between 22.00 h and 4.00 h in the morning (Lingren et al., 1977) under the influence of sexual pheromones emitted by the females (Tumlinson et al.,

1972). After mating, females lay their eggs preferentially in the lower part of the plant and the abaxial surface of leaves (Hamadain and Pitre, 2002; Lacey and Kaya, 2007; Pereira et al., 2018; Zulin et al., 2018). This characteristic is similar to many lepidopteran species, given that eggs deposited on the abaxial surface are generally less exposed to extremes of heat and desiccation, as well to natural mortality agents, than eggs laid on the adaxial surface (Chapman, 2012a) (Fig. 4).

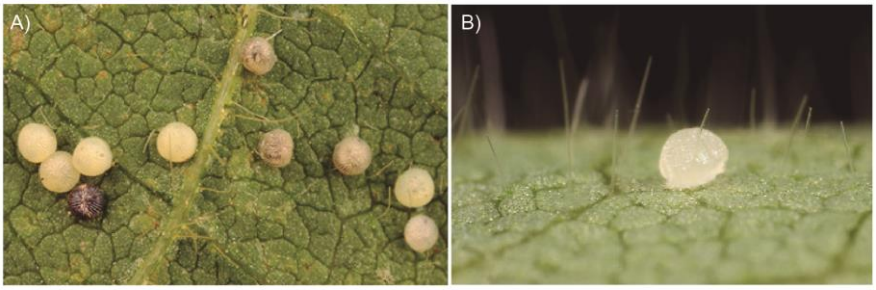

Figure 4. *C. includens* eggs on the abaxial surface of soybean leaves: A) group of eggs in different maduration stages and B) detail of single inmature egg.

The number of eggs that a female can oviposit in her lifetime and in favorable temperature conditions, is up to 700 eggs (Canerday and Arant, 1967; Jost and Pitre, 2002; Mitchell, 1967). Nevertheless, reports in the literature have shown variations in the reproductive potential of *C. includens* females from 500 to 1,300 eggs (Jensen et al., 1974). Emergence of the eggs take place after three to five days and the larvae develop through six instars (Mitchell, 1967; Strand, 1990). The larval period lasts from thirteen to twenty days, and the period from egg to adult varies from 24 to 34 days (Canerday and Arant, 1967; Mitchell, 1967; Reid and Greene, 1973) (Fig. 5), and an additional 6-8 days for the development of the pupae (Reid and Greene, 1973).

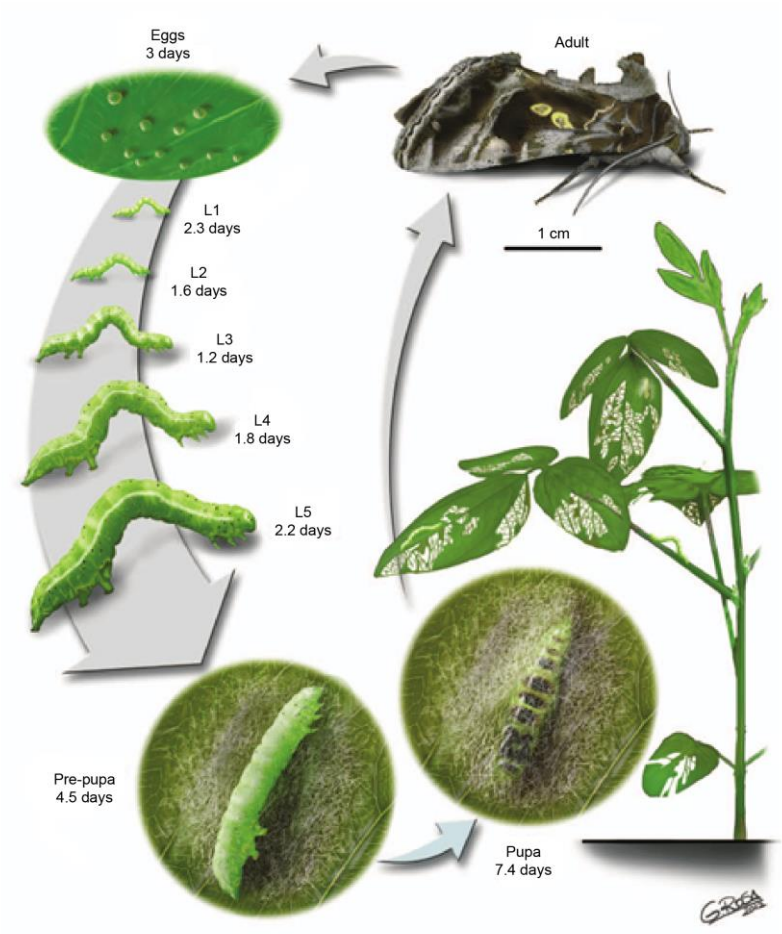

Figure 5. Development cycle of *Chrysodeixis includens*.

2.4 Damage and injury

In recent years, *C. includens* has become a major pest of soybean crops. United States and Brazil are the largest soybean producers of the world, followed by Argentina. Brazil is the second world soybean producer, accounting for approximately 30% of global production. Nevertheless, recent data shows that Brazil will overtake the United States as the first producer in the 2019/2020 season, (United States Department of Agriculture and Foreign

Agricultural Service, 2020) and it is in Brazil where the soybean looper causes significant damage to soybean crops (Bueno et al., 2017).

In the United States, *C. includens* was the third most damaging pest in the 2018 growing season, being responsible for 9.2% of all insect derived losses in soybean. However, in Delaware, Louisiana and South Carolina, this moth constituted the primary pest (Musser et al., 2018). In Brazil, in contrast, the importance of this pest has increased in the last decades. Until the 90s decade, *C. includens* was considered a secondary pest and was controlled mainly by entomopathogenic fungi and parasitoids (Bortolotto et al., 2015; Bueno et al., 2017). However, the overuse of non-selective insecticides, often associated with herbicides and fungicides, has increased the presence of *C. includens* in soybean, changing the status of the soybean looper to a primary pest widely distributed in the main soybean crops in Brazil (Bore´m and Almeida, 2011; Bortolotto et al., 2015; Moscardi et al., 2012)

C. includens attacks during the vegetative and reproductive phase (Carneiro et al., 2018). At small instars (from first to third), larvae tend to feed on parenchymatous tissues or highly digestible leaves because of their low fiber content whereas more developed larvae feed on older and more fibrous leaves including the veins (Kogan and Cope, 1974). During the first and second instars, larvae barely scrape the leaves and, when they reach the third instar, are able to pierce those, leaving the central and lateral veins untouched. This peculiar behavior is what distinguishes small *C. includens* larvae from other defoliating insect pests (Moscardi et al., 2012) (Fig.6). Indeed, in cotton crops *C. includens* prefers the lower one-half to two-thirds of the plant which hinders its early detection and timely insecticide control measures (Smith et al., 1994).

Total soybean leaf-consumption by *C. includens* larvae in the literature is highly variable, with values ranging from 64 to 200 cm^2 (De Freitas Bueno et al., 2011; Trichilo and Mack, 2015; Vázquez, 1988), which could be explained by the different leaf types and soybean varieties used in the experiments. For instance, an experiment carried out with “Bragg” soybean variety demonstrated that *C. includens* larvae could consume a mean of 82

cm² of leaf per larva, and the 97% of this quantity was consumed during the three last instars (Reid and Greene, 1973).

Figure 6. A) Damage caused by *C. includens* versus B) other defoliators. Photographs by A. de F. Bueno and A. C. Santos (Moscardi et al., 2012).

Despite the fact that soya is the crop in which *C. includens* causes more economical losses, this pest is recently gaining importance in other crops. For example, cotton crops are not greatly affected by this pest but it seems to act as a reservoir, facilitating the dispersion of insects from cotton to soybean (Schowalter, 2013). In this way, outbreaks tend to be of greater importance in agroecosystems where soybean and cotton crops coexist, in which this moth can be found in higher densities due to a higher rate of reproduction of adults fed on cotton (Burleigh, 1972).

Another crop that gains relevance as an alternative host for *C. includens*, is the sunflower (Andrade et al., 2016). When this plant is cultivated close to soybean residues, proliferation of *C. includens* occurs spontaneously, with larvae feeding heavily on sunflower seedlings. On developed plants larvae feed on tender leaves, bracts and the fleshy part of inflorescences (Leite et al., 2005).

More recently, reports have appeared of *C. includens* attacking pineapple plants in Brazil, in a crop that is often planted adjacent to final-stage soybean crops. Larvae feed on the floral remains of the pineapple plant, which led to holes in the fruit that reached the pulp. When compared to the damage

caused in other crops, such as soybean, in which tolerates up to 15-35% loss of leaves (Bortolotto et al., 2015), lesions in pineapple fruits are critical. This is because of the “aesthetic damage” that make these fruits unsuitable for commercialization (Detoni et al., 2018). As such, a polyphagous pest like *C. includens* should be considered to be a pest of "production systems” rather than a pest of particular agricultural crops as has been catalogued for other lepidopteran pests (Ventura et al., 2015).

2.5 Control methods

1.6.1. Natural biological control

C. includens is a common inhabitant of soybean crops and has been maintained in equilibrium by natural biological control agents such as the entomopathogenic fungi *Nomuraea rileyi, Pandora* sp. and *Zoophthora* sp. (Sosa-Gomez, 2005). Therefore, until the 1990s, *C. includens* was not considered as a threat to soybean crops. Unfortunately, the entrance of *Phakopsora pachyrhizi*, the causative agent of the Asian soybean rust, in Brazil during the 2000/2001 season, led to the use of fungicides rarely used on soybean crops in order to control this disease. The immediate consequence of those fungicides was to diminish epizootics caused by the beneficial fungi because the fungicides were not selective to *Phakopsora pachyrhizi*, therefore increasing *C. includens* populations (Sosa-Gómez et al., 2003). Vespidae and ants have also been described as key mortality factors in the regulation of *C. includens* populations (Pereira et al., 2018).

1.6.2. Chemical control

The use of chemical insecticides for the control of *C. includens* in soybean, cotton, common bean and sunflower crops in Brazil is still the main strategy used, reaching up to six chemical treatments per season in soybean crops (Bortolotto et al., 2015; Panizzi, 2013). Some authors are not convinced of the usefulness of chemical insecticides against *C. includens* due to its natural resistance to some chemicals and the fact that *C. includens* larvae tend to remain under the canopy of soybean plants, resulting in a reduced effect of chemical insecticide sprays (Bernardi et al., 2012; Dowd and Sparks, 1986).

These factors favor the development of resistance to chemical insecticides in *C. includens* (Stacke et al., 2019). The first case of field resistance to chemical insecticides in *C. includens* was reported for permethrin in 1987 in the U.S.A. (Leonard et al., 2015) and later for other insecticides. In Brazil, although no cases of resistance have been reported, there have been control failures of this pest when using pyrethroids (Sosa Gomez and Omoto, 2012).

1.6.3. Applied biological control

The objective of this control method is the use of natural enemies but in an augmentative or inoculative strategy. For this, the natural enemies are mass-produced and then applied to infested crops in order to reduce the prevalence of the pest. Several natural enemies of *C. includens* in soybean crops with promising features have been studied in order to develop them as a control strategy, although none has been incorporated into integrated pest management (IPM) programs (De Freitas Bueno et al., 2012). Baculoviruses and *Bacillus thuringiensis* (Bt) have a key role in the development of biological control methods. An outstanding example is the Anticarsia gemmatalis multiple nucleopolyhedrovirus (AgMNPV) used in the management of the velvetbean caterpillar, *Anticarsia gemmatalis*. This virus was applied in Brazil to over 2 million hectares during the 1997/1998 season, being considered as the greatest biological control program in the world at that time (Moscardi et al., 2011). For *C. includens*, its own nucleopolyhedrovirus (ChinNPV) has been studied in order to develop it as the basis for a bioinsecticide that may be used in the integrated management of this pest in soybean crops (Zanardo Botelho et al., 2019). In addition, Agbitech has developed the first baculovirus-based bioinsecticide using ChinNPV, called Chrysogen®, for the control of this pest. Transgenic soybean expressing a *Bacillus thuringiensis* (Bt) gene has been used for the control of *C. includens*, first in Brazil and then in other countries. Since 1994, when a soybean expressing the Bt Cry1Ab gene was first used (Parrott et al., 1994), other transgenic variants in soybean have been generated (Schünemann et al., 2014). Nevertheless, the extensive use of this strategy to control *C. includens* could lead to future resistance, which would reduce the value of this technology (Tabashnik et al., 2013).

3. BACULOVIRUS

3.1 Morphology and taxonomy

Insect viruses comprise RNA or DNA as their genetic material, and presently encompass 16 different virus families (Possee and King, 2014). From those families, only three (*Baculoviridae*, *Poxviridae* and *Reoviridae*) have their genetic material embedded in a proteinaceous matrix called the occlusion body (OB) (Ibarra and Del Rincón-Castro, 2008; Possee and King, 2014). This characteristic mode of transmission of virions within a protein matrix is found mainly in insects, and in the *Baculoviridae* family has led to the theory that these viruses evolved OBs in order to preserve them from degradation during diapause or the periods when the seasonal larval population is too low to sustain continued virus transmission (Miller, 1997). Nevertheless, both *Poxviridae* and *Reoviridae* include viruses that are infective to vertebrates (Hughes et al., 2010; Urbano and Urbano, 1994), which limits their value as bioinsecticides due to biosafety concerns. In contrast, the Baculoviridae family has never been reported to cause disease in any organism outside the phylum Arthropoda.

The earliest interest in baculoviruses was a result of the threat they posed to the silk industry in ancient China (Miller, 1997). More recently this interest has focused on the potential applications of these viruses, as agents for biological control of insect pests to vectors for expression of recombinant proteins in insect cells (Kost et al., 2005; Szewczyk et al., 2006). The viral occlusion within a protein matrix increases their resistance to chemical and physical decay and partially protects them from inactivation by ultraviolet (UV) light (Herniou et al., 2011). The high environmental stability provided by the proteinaceous structure of the occlusion body makes baculoviruses suitable candidates for the active ingredient of bioinsecticides.

Occlusion bodies in baculoviruses comprise a crystalline matrix which is composed mainly by a protein that is conserved in all baculovirus genomes, except for that of the dipteran viruses (Rohrmann, 2019). This major structure protein is named polyhedrin for the nucleopolyhedroviruses (NPV) and granulin for granuloviruses (GV), two closely related proteins despite

having different names, whereas Culex nigripalpus nucleopolyhedrovirus (CuniNPV) occlusion body protein is larger and not related in amino acid sequence to polyhedrin (Rohrmann, 2019). OBs have a dimension of 0.6 to 2 μM in diameter, far larger than oval-shaped granules which have a diameter of about 0.2 to 0.4 μM (Ackermann and Smirnoff, 1983). These OBs are surrounded by the calyx/polyhedron envelope (PE) which is thought to ensure OB stability (Rohrmann, 2019). Occlusion derived virions (ODVs) are embedded in the proteinaceous matrix. Several ODVs are occluded in each OB in NPVs. ODVs contain the nucleocapsids, containing the genetic material of the virus. Depending on the number of nucleocapsids enveloped in each ODV, baculoviruses are classified as single NPVs (in the case of one enveloped nucleocapsid per virion) or multiple NPVs (when more than one nucleocapsid is enveloped per virion), whereas GVs usually contain a single virion per OB with only one nucleocapsid in each virion (Funk et al., 1997) (Figure 7).

Another important structure in baculoviruses is a second type of virion, named budded virus (BV). In contrast to the occlusion bodies that are linked to baculovirus persistence in the environment and between-host transmission, BVs are involved in spreading the infection within the insect. BVs contain a single nucleocapsid with a single viral genome. This viral nucleocapsid contains the same genetic material as ODVs but differs in protein and lipid composition of the bilayer membrane of their envelope (Slack and Arif, 2007). A detailed comparison of ODV and BV structure will be explained in section 2.3.

The former classification of the *Baculoviridae* family comprised two genera, NPVs and GVs, as described in the 6th Report of the International Committee on Taxonomy of Viruses (ICTV) (Murphy et al., 1995). This classification remained unchanged until the year 2011, when the 9th ICTV report changed the taxonomy of the family following a proposal based on virus phylogeny (Jehle et al., 2006; King et al., 2011). Thus, the new classification enclosed a subdivision in four genera: 1) *Alphabaculovirus*, which are lepidopteran-specific NPVs with 47 species described, being the Autographa californica multiple nucleopolyhedrovirus (AcMNPV) the type species for this

viral genus. 2) *Betabaculovirus*, which groups the lepidopteran-specific GVs and comprises 26 species, with Cydia pomonella granulovirus (CpGV) as the type species; 3) *Gammabaculovirus*, which encompasses the hymenopteran-specific NPVs with two species as representatives of the genus and Neodipirion lecontei nucleopolyhedrovirus as the type species; and 4) *Deltabaculovirus*, containing the dipteran-specific NPVs with only one species, CuniNPV (King et al., 2011). The *Alphabaculovirus* genus can be subdivided into group I and II viruses defined by phylogenetic analysis of the *polh* gene (Zanotto et al., 1993). In all cases, viruses in the *Baculoviridae* family are named after the host from which they were first isolated, despite the confusion that this can generate with baculoviruses that infect multiple species of insects.

3.2 Infection cycle

Baculoviruses are adapted to initiate infection in the insect midgut, from which the infection spreads to other tissues throughout the insect. Consequently, baculoviruses have developed two types of virions (ODVs and BVs), each one with their specialized role in the infection cycle (Rohrmann, 2019). Consequently, it is important to understand the natural conditions present in the insect gastrointestinal tract under which baculoviruses have evolved to maximize their probability of infection (Rossignol, 1997).

Insects have their intestinal tract divided into three sections: fore-, mid- and hindgut (Chapman, 2012b). In Lepidoptera, the midgut is the major site where digestion of food and baculovirus infection takes place. Unlike the foregut and hindgut, the midgut lacks a cuticle layer, which is substituted with a tube-like structure composed by chitin fibers and glycoproteins called the peritrophic membrane (PM). This coating provides a barrier to gut cells from bacteria, viruses, fungi and physical damage from ingested plant material (Hegedus et al., 2009; Lehane, 1997; Pritchett et al., 1984; Terra, 2001).

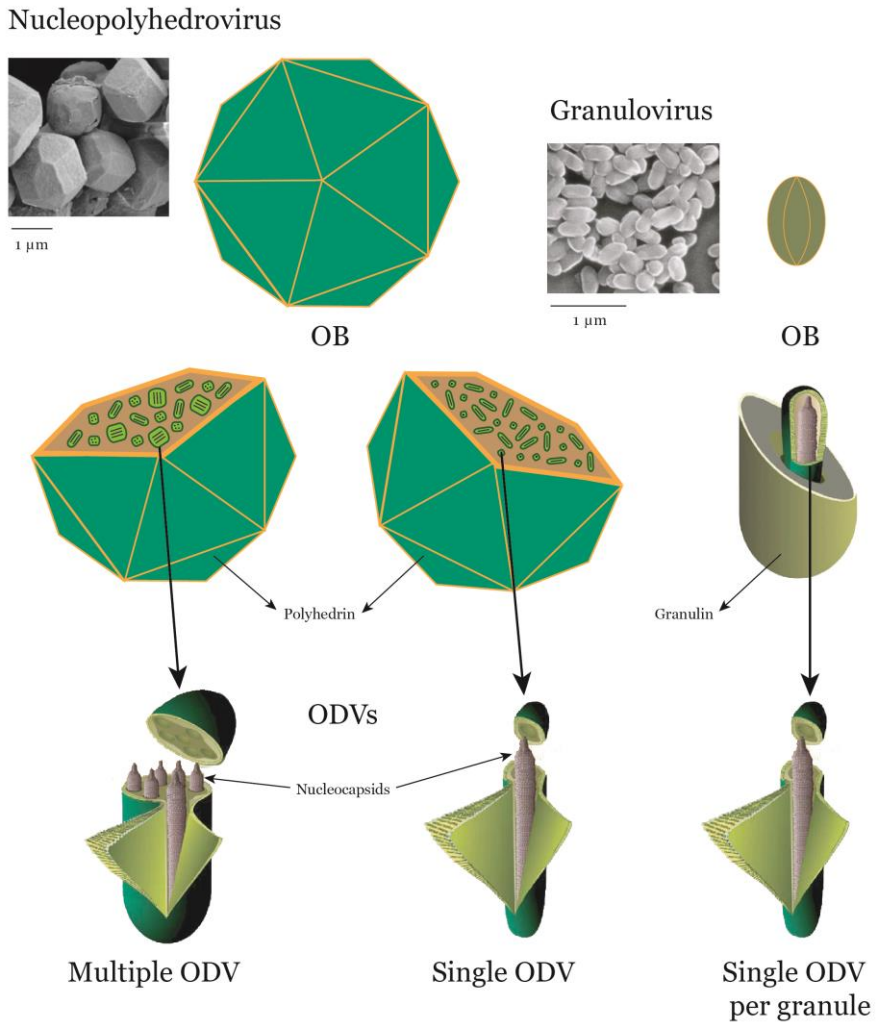

Figure 7. Structure of nucleopolyhedrovirus and granulovirus occlusion bodies. Multiple and single ODVs are depicted with their lipid bilayer membrane.

This is the first physical barrier that pathogen microorganisms, like baculoviruses, have to bypass in the insect midgut. They also face secreted antimicrobial compounds that act as immune proteins (He et al., 2011; Pauchet et al., 2008) (Figure 8). These antimicrobial peptides and proteases like lysozymes are important components of the insect immune system

(Lemaitre and Hoffmann, 2007). Toll-like receptors have also been described in the midgut of *Manduca sexta* (Ao et al., 2008) and are involved in recognition of structurally conserved molecules associated with pathogens (Zambon et al., 2005). Another key component of the immune response of the insect midgut is the natural microbiota, which can increase the host response against pathogens (Shao et al., 2017). Midgut cell sloughing is another mechanism of resistance against baculovirus infection (Hoover et al., 2000).

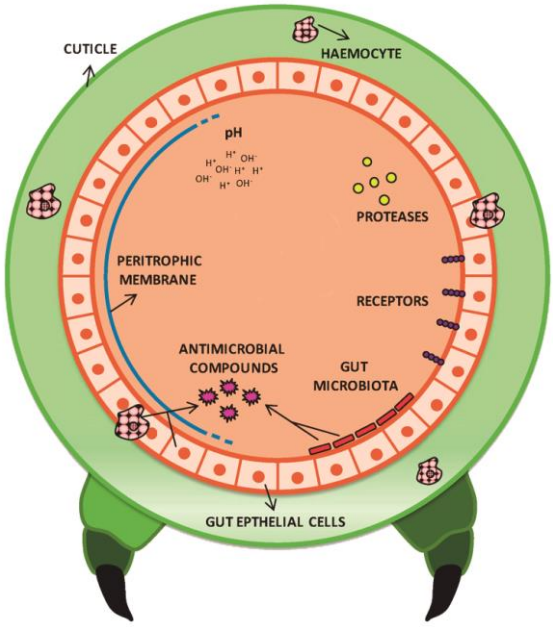

Figure 8. Schematic transversal section of the lepidopteran midgut tract.

Variation in pH is a notable characteristic of each section of the midgut, being neutral at the initial and final regions whereas at the central region pH varies from 10 to 12 in phytophagous Lepidoptera (Dow, 1984). This highly alkaline pH is unusual in nature and is a characteristic of the midgut of some insect species (Rohrmann, 2019), which is why baculovirus virions are occluded within highly stable alkali sensitive OBs. Therefore, the OB ensures environmental stability outside of the host body and simultaneously

accommodates the initiation of infection by dissolution of the protein matrix and the release of the ODVs in the insect midgut.

The baculovirus infection cycle is divided in two different phases: the oral infection known as primary infection and the spread of the viral infection throughout the organism known as secondary infection. The baculovirus infection cycle starts when a susceptible larva feeds on plant material contaminated with OBs (Figure 9). When the ingested OBs reach the insect midgut, the high pH present in the midgut dissolve the polyhedrin matrix, thus releasing the ODVs. Virion release is also facilitated by dismantling the polyhedron envelope by a combination of midgut proteinases (Rohrmann, 2019). The released virions must pass through the PM in order to access the epithelial cells. At this crucial step, baculoviruses may use ODV-E66 to degrade the chondroitin sulphate present at low levels in the PM due to its chondroitinase activity (Sugiura et al., 2011). Other baculoviruses use a set of viral proteinases called enhancins, which can be found either co-occluded in the OB matrix or on ODV surfaces (Gallo et al., 1991; Wang et al., 1994). These enzymes are metalloproteinases that specifically degrade the PM (Lepore et al., 1996; Wang and Granados, 1997).

Once the ODVs have passed the PM and can access the ectoperitrophic lumen, they are able to specifically bind to microvilli in the luminal side of columnar epithelial cells of the midgut (Adams and McClintock, 1991). The lipid bilayer of ODVs fuses with the midgut cell membrane, releasing the nucleocapsids into the cell (Horton and Burand, 1993; Mu et al., 2014; Summers, 1971) (Figure 10.1). This process seems to be mediated by specific binding of the ODV envelope to cell proteinase sensitive receptors (Horton and Burand, 1993). Successful oral infection depends on a group of viral proteins, called *per os* infectivity factors (PIFs), present on the ODV envelope (Boogaard et al., 2018; Peng et al., 2010; Wang et al., 2017). These proteins are essential for baculovirus oral infection but not for cell-to-cell spread of the virus. To date, up to ten PIF proteins have been identified (Boogaard et al., 2018; Fang et al., 2009; Javed et al., 2017; Kikhno et al.,

2002; Liu et al., 2016; Nie et al., 2012; Ohkawa et al., 2005; Pijlman et al., 2003; Sparks et al., 2011; Wang et al., 2019).

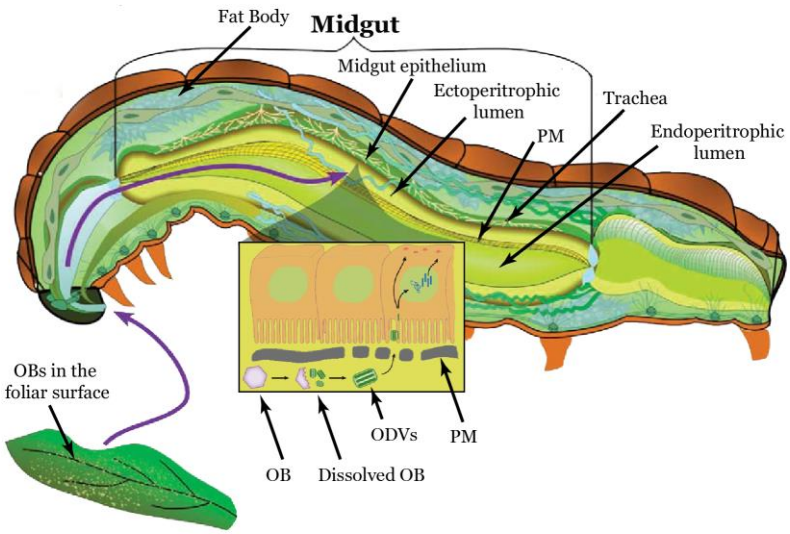

Figure 9. Schematic representation of the initial baculovirus infection process in insect hosts.

Nine of them form a complex that is stable in the unfavorable conditions of the midgut, suggesting a cooperation of these proteins in mediating ODV oral infection (Wang et al., 2019). Some studies suggest that ODV-E66, despite not being crucial for the oral infection, interacts with some components of the PIF complex (Dong et al., 2014; Peng et al., 2010). After ODV fusion with epithelial cells, nucleocapsids are released into the cytoplasm. Although microvilli typically contain a thick bundle of cross-linked actin filaments, it remains unknown whether nucleocapsids specifically interact with these pre-existing actin filaments (Gilbert, 2012). However, it is known that the P78/83 nucleocapsid protein promotes actin polymerization, which is crucial for capsid transport to the cell nucleus. (Goley et al., 2010, 2006; Ohkawa et al., 2010) (Figure 10.2). Viruses have multiple options to import their genetic material into the nucleus, including transport through the

Nuclear Pore Complex (NPC) of the whole nucleocapsid, the entry of the nucleic acid through the NPC leaving the empty capsid out of the nuclear membrane, capsid disassembly and subsequent release of generic material through the NPC and even nuclear entry bypassing the NPCs. It is thought that baculoviruses can use any of the mentioned paths to enter the nucleus although the nuclear import of baculovirus nucleocapsids is an understudied topic (Au et al., 2013). Viral gene expression and DNA replication commence in the nuclei of infected cells and structural proteins begin to be expressed. Newly formed nucleocapsids are assembled in the virogenic stroma (Figure 10.3) (Zhao et al., 2019) and are then transported to an electron-lucent region at the periphery of the nucleus, which has been named the "ring zone" (Figure 10.5).

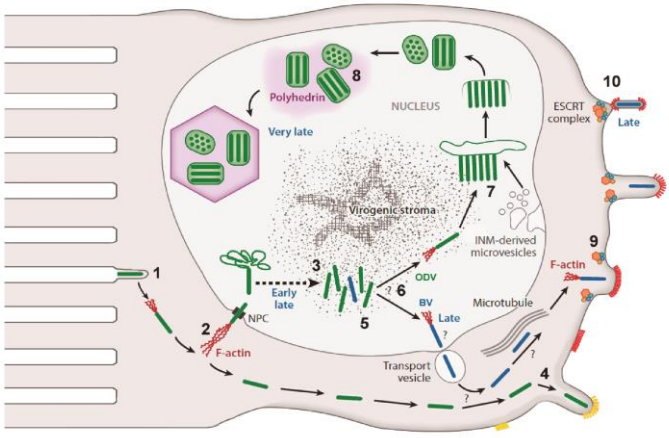

Figure 10. Baculovirus infection cycle whithin a midgut columnar epithelial cell. 1) Fusion between ODV and midgut columnar epithelial cell membranes. 2) ODV nucleocapsid transportation to the nucleus. 3) De novo synthesis of viral nucleocapsids. 4) Bypass of the nucleus-cycle by ODV nucleocapsids that bud directly to the haemocoele. 5) Localization of newly-formed nucleocapsids in the nuclear ring zone. 6) Formation of either ODV or BV. 7) Assembly of ODV nucleocapsids into nuclear membranes. 8) ODV occlusion in polyhedrin. 9) ESCRT recruitment of BV on budding sites. 10) Bud out of BVs acquiring midgut columnar epithelial cell plasmatic membrane.

For multiple nucleopolyhedroviruses, nucleocapsids may also circumvent the nucleus and transit directly through the midgut epithelial cell to the basal

membranes of those cells, where they bud into the hemocoel or tracheal cells (Granados and Lawler, 1981; Washburn et al., 1999) (Figure 10.4). Transport from the virogenic stroma to the ring zone is dependent on nuclear actin polymerization and nucleocapsid proteins VP80 (Ac104), P78/83 (Ac9), VP1054 (Ac54) and BV/ODV-C42 (Ac101) (Goley et al., 2006; Guan et al., 2016; Marek et al., 2011). The process that lead nucleocapsids to form either ODV or BV during assembly remains unknown (Figure 10.6), although it has been estimated that less than 16% of the synthetized viral genomic DNA is encapsulated in BV (Rosinski et al., 2002). The new synthetized nucleocapsids that are to become ODV in the ring zone come in contact with the inner nuclear membrane which envelopes the nucleocapsids and forms the known structure of ODVs. The assembly and occlusion of ODV in the nucleus requires a complex integration of events, including transference of ODV membrane proteins to the nucleus and formation of intranuclear membranes (INM), assembly and association of nucleocapsids with the latter and the wrapping or enclosing of nucleocapsids in membranes (Blissard and Theilmann, 2018) (Figure 10.7). At the last stage, the enveloped nucleocapsids that form the ODVs must associate with concentrated polyhedrin, which crystallizes around one or more ODVs to form the OB (Figure 10.8).

For those nucleocapsids that are to become BV, nucleocapsids are transported to the cytoplasm in vesicles surrounded by two membranes (formed by the inner and outer nuclear membrane) which are lost once they enter the cytoplasm, as free nucleocapsids are observed near the plasmatic membrane (Granados and Lawler, 1981; Williams and Faulkner, 1997). Once the nucleocapsids have been released from the vesicles, they are transported to the plasmatic membrane by actin polymerization (Ohkawa et al., 2010). Many viruses use the cellular ESCRT (endosomal sorting complexes required for transport) pathway for the budding and excision that releases the nucleocapsid from the plasma membrane (Chen and Lamb, 2008; Hurley, 2015; Votteler and Sundquist, 2013) (Figure 10.9). This pathway involves a series of protein complexes that mediate cargo recruitment, bud formation and excision of vesicles in the formation of multivesicular bodies in healthy cells. The

AcMNPV may also use this mechanism for budding at the plasma membrane, as a functional ESCRT pathway is required for BV production (Li and Blissard, 2012; Yue et al., 2017). The nucleocapsids then bud out of the cell acquiring an envelope and forming the BV (Figure 10.10). In this process, the major BV envelope glycoprotein GP64, dramatically influences the efficiency of budding but is not essential (Oomens and Blissard, 1999). Another viral protein, ME53 (AC140) has been shown to co-localize with GP64 at the plasma membrane (possibly at budding sites) and deletion of me53 gene results in a 1,000-fold reduction in infectious BV (De Jong et al., 2011, 2009). This whole process is called primary infection and once the midgut epithelial cells have been infected, the secondary infection starts to spread throughout the insect.

BVs are responsible for the secondary infection after budding from the basolateral membrane of midgut epithelial cells, bypassing the basal lamina in order to spread the infection to the other insect tissues. In this way, BVs spread the infection throughout the organism using the tracheal system which extends its branches through every tissue including the midgut epithelium (Engelhard et al., 1994; Kirkpatrick et al., 1994; Rahman and Gopinathan, 2004). Once BVs colonize the hemocoel, they can infect other insect cell types like hemocytes and fat body cells. Infection of hemocytes has a highly negative impact on the host response, as they stop working as insect immune cells and can spread the infection through the insect circulatory system (Slack and Arif, 2007). Hemocytes are the last barrier against pathogen infection, of which the most abundant in haemolymph are the granular cells and plasmatocytes, that have adhesion and phagocytosis activity against pathogens (Lavine and Strand, 2002). As the infection spreads through the tracheal system, it invades the fat body, epidermis and glial cells (Keddie et al., 1989; Knebel-Mörsdorf et al., 1996). As the infection progress, host cell metabolism and RNA synthesis increase while hormonal titers in the larva are also affected (Babu et al., 2009; Etebari et al., 2007; Granados and Williams, 1986). Infected cells throughout the insect accumulate OBs until their plasmatic membrane is disrupted and the OBs are released into the extracellular space. The external symptoms appear some days after infection,

including changes in the color of the integument, loss of appetite and a decrease in the activity of the larva. At the end of infection, the baculovirus induces enhanced locomotory activity and the larvae climb to the top of the plants (del-Angel et al., 2018; Hoover et al., 2011; Katsuma et al., 2012). Consequently, dead larvae are typically found hanging by their last abdominal pseudopods, and due to the process of disintegration and liquefaction of the tegument, OBs are released in the environment ready to be ingested and restart again the infection cycle. The viral enzymes responsible for this process are chitinases and cathepsins, among others (Hawtin et al., 1997).

3.3 Differences between BV and ODV

Despite carrying an identical genome, the two virion phenotypes production, structure, composition and function are quite different. As described previously, ODVs are formed in the cell nucleus by the enclosure of viral nucleocapsids in a lipid bilayer de novo synthetized. In contrast, nucleocapsids are transported to the cell membrane where they acquire their membrane to form the BVs. Regarding functionality, ODVs are occluded within the OBs and are directly involved in oral infection as they infect midgut epithelial cells. On the contrary, BVs are specialized in cell-to-cell infection and responsible for systemic infection. This results in differences in the efficiency of infection for different insect tissues. For instance, ODVs are approximately 10,000-fold more efficient than BVs at infection of midgut epithelial cells. However, BVs infect cell cultures up to 1,000-fold more efficiently than ODVs (Volkman et al., 1976; Volkman and Summers, 1977). As functionality is linked to virion structure, the following paragraphs highlight the main differences between these types of virions.

The main characteristic of the ODV envelope composition is that it contains at least 13 integral membrane proteins, of which nine are encoded by *pif* genes, as described in section 2.2 (Blissard and Theilmann, 2018). All the genes identified to date have the following properties in common: deletion results in the loss of per os infectivity but does not affect assembly of ODV virions, and homologs are present in all baculovirus genomes, that is, they form part of the core genes. In AcMNPV the nine proteins form a large high-

molecular weight complex in the ODV envelope (Peng et al., 2010; Wang et al., 2017). The importance of the PIF complex is that it exists and functions in the midgut environment at high pH and rich in proteases and that a stable complex is an essential condition for resistance to proteolytic degradation (Boogaard et al., 2017). The fact that *pif* genes involved in the complex are core genes suggests that the ODV entry mechanism is highly conserved in baculoviruses.

In contrast, the BV envelope is acquired from the cell plasma membrane during budding and contains a more limited number of virus-encoded proteins. Electron photomicrographs reveal the presence of spikes perpendicular to the viral envelope at both polar ends of the virions, correlated with the presence of fusion proteins, GP64 or F-protein (Qiushi Wang et al., 2015). While GP64 is the major envelope glycoprotein in the BV envelope of group I alphabaculoviruses, the major envelope glycoprotein in group II alphabaculoviruses and the remaining genera of baculoviruses is F protein (for fusion). Both proteins are functional analogues, mediating host cell binding and membrane fusion, but they differ in structure (Kadlec et al., 2008; Wang et al., 2015). Several lines of evidence support the concept that F protein represents an ancestral BV fusion protein, and that GP64 was more recently acquired by an ancestor of the group I alphabaculoviruses. Upon acquisition of GP64, the central role of F protein in entry was displaced (Lung et al., 2002, 2003; Pearson and Rohrmann, 2002; Wang et al., 2014). Interestingly, the F protein gene has been retained in viruses carrying GP64, which suggests a positive selection for another function(s) of F protein, while knockouts of the F protein performed in group II alphabaculoviruses, which carry no gp64 gene, are lethal (Wang et al., 2008; Westenberg and Vlak, 2008). In AcMNPV, the BV envelope contains at least six additional virus-encoded membrane proteins, present at lower levels. Although they may affect BV production levels, they are not essential for BV production or infectivity (Blissard and Theilmann, 2018).

There are also differences in the lipid composition of both virion types, but this aspect has been understudied and its implications in virion

function are unknown (Braunagel and Summers, 1994). Another structural difference between BV and ODV is the mechanism by which nucleocapsids are selectively tagged for nuclear egress (BV formation) or retention (ODV formation). Although this mechanism is still unclear, differences in the protein compositions of nucleocapsids isolated from BV and ODV suggest that one or more of the BV- or ODV-specific nucleocapsid proteins may regulate nuclear egress or retention (Braunagel et al., 2003; Hou et al., 2013; Wang et al., 2010). As substantial differences in the levels of ubiquitination of nucleocapsids from BV and ODV have been reported, with the level of ubiquitination in BV-derived nucleocapsids much higher than those from ODV, it was speculated that nucleocapsid ubiquitination may serve as a tag for nucleocapsid egress (Biswas et al., 2017). It is also possible that other post-translational modifications that have not yet been examined could be involved in regulating this process, and further studies in the structural analysis of nucleocapsids could yield important information in this area.

4. BACULOVIRUS GENOME

4.1 General description

The baculovirus genome is a supercoiled circular double strand of DNA. The size normally depends on the species, ranging from 81.7 Kb in Neodiprion lecontei nucleopolyhedrovirus to 178.7 Kb in Xestia c-nigrum granulovirus. This allows baculoviruses to encode from 89 to 183 genes, almost equally distributed on both strands (National Library of Medicine (US), n.d.) from which 38 have been identified as homologous or core genes (Rohrmann, 2019). As a convention, all baculovirus genomes are ordered in such way that *polyhedrin/granulin* is the first open reading frames (ORF) and the adenine of the ATG start codon of these genes is the first base in the genome. ORF numbers are assigned in a clockwise direction. Furthermore, intergenic regions are minimal, resulting in a highly compact genome (Van Oers and Vlak, 2007). Gene order is highly conserved in GVs, but not in NPVs. Gene order is more dissimilar in group II NPVs than in group I (Herniou et al., 2003). In fact, order does not respond to function or time of transcription

criteria (Ferrelli et al., 2012) and can be a measure of virus relatedness (Van Oers and Vlak, 2007). Only a cluster of four core genes is conserved in all baculoviruses sequenced to date. It is composed by *helicase*, *lef-5*, *ac96 (pif-4)* and *38K* (Herniou et al., 2003). These genes are involved in DNA replication, transcription, nucleocapsid assembly and oral infection (Fang et al., 2009; Guarino et al., 2002; McDougal and Guarino, 2000; Wu et al., 2006). However, the functional importance of the order of these genes within the cluster is still unknown. Distributed along the genome, most baculoviruses present regions of repeated sequences rich in AT and often-containing reiterated palindromic repeats, called homologous regions (*hrs*), which have been reported to function as enhancers of early gene expression and origins of DNA replication (Guarino et al., 1986; Lu et al., 1997; Rodems and Friesen, 1993; Theilmann and Stewart, 1992). Although these kinds of repeats are common in many baculoviruses, their number varies considerably among viral species and some of them lack the *hrs* sequences completely, as occurs for the nucleopolyhedroviruses isolated from *Chrysodeixis chalcites, Chrysodeixis includens* and *Trichoplusia ni* (Castro et al., 2015; Van Oers et al., 2005; Van Oers and Vlak, 2007; Willis et al., 2005). The ORFs do not contain introns in baculoviruses and always start with ATG instead of alternative start codons. In contrast, codon usage in baculoviruses is highly variable between genes within the same virus, and codon usage in homologous genes is not conserved among NPV species or in baculoviruses that share the same insect host (Levin and Whittome, 2000; Shi et al., 2016).

4.2 Gene expression regulation

Like other DNA viruses, baculovirus gene expression and DNA replication takes place in the nucleus of an infected cell and their genes are transcribed in a temporal sequence within a highly regulated process. Gene expression is developed in a stepwise-mode which ensures that all required proteins for promotion into the next phase of infection are available (Berretta et al., 2013), and this occurs in four stages: immediate-early, delayed-early, late and very late (Passarelli and Guarino, 2007; Rohrmann, 2019). The transcription of early genes does not require prior protein synthesis and is

performed by the host RNA polymerase II. This occurs before virus DNA replication, while late and very late gene transcription starts after viral DNA replication by a virus-encoded RNA polymerase (Berretta et al., 2013) (Figure 11).

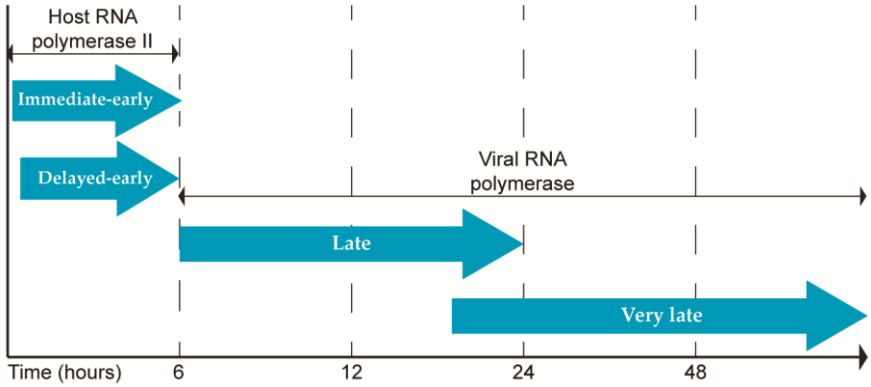

Figure 11. Schematic representation of the four phases of baculovirus gene expression. Figure by I. Beperet.

The main difference between immediate and delayed-early genes is that, while immediate-early genes do not require pre-existing viral factors for expression, the transcription of delayed-early genes requires activation by immediate-early genes, as demonstrated in transient expression assays (Berretta et al., 2013). Five different immediate early genes (*ieo, ie1, ie2, me53* and *p38*) have transcriptional regulatory functions (Ono et al., 2015; Rohrmann, 2019). Furthermore, they do not act independently in the regulatory network (Ono et al., 2015). Both kinds of early genes are transcribed between zero to six hours post infection (hpi) in cell culture, prior to DNA replication (Friesen, 1997; Rohrmann, 2019). Two major sequences have been identified and linked to early gene transcription: a TATA promoter and a CAGT (or CATT in some cases) transcription initiation consensus sequence (Rohrmann, 2019). The products of early genes are involved in DNA

replication, late gene expression and host-modification processes (Todd et al., 1996).

Transcription of late and very late genes in baculoviruses take place between 6 and 24 hpi for the late and from 18 to 72 hpi for the very late (Passarelli and Guarino, 2007). This process is developed by the viral RNA polymerase II, demonstrated by experiments carried out with α-amanitin, an inhibitor of RNA polymerase II. Late genes promoters contain a TAAG sequence motif that acts as a transcription initiator. This motif is less abundant in baculovirus genomes than expected by random occurrence, which suggest that this hypothetical random distribution is negatively selected by its activity as late promoter (Berretta et al., 2013). The integrity of this motif is strictly necessary for transcription and more than one functional TAAG is thought to exist upstream of the translational start codon (Morris and Miller, 1994; Thiem and Miller, 1989). Late transcriptional start sites always initiate at the second nucleotide of the TAAG late promoter element, but it remains unclear if the fact that transcripts begin with the sequence AAG acts as a signal for selective sorting or transport of mRNA (Rohrmann, 2019). Late transcripts usually encompass more than one ORF, which permits the existence of various transcripts with different 5' or 3' ends for each specific ORF. Coding regions for late genes are found on both DNA strands, which suggests that there may be opposite late transcripts sharing a complementary region (Berretta et al., 2013). Whether this plays any regulatory role remains unknown although this is probable, considering that baculovirus genes are susceptible to be silenced by double-stranded RNA (Flores-Jasso et al., 2004). What is certain is that these genes are transcribed at high levels and their products are mainly involved in viral assembly and occlusion (Lu and Miller, 1997).

4.3 Core genes and functions

To date, 38 conserved genes have been described as core genes in baculoviruses (Rohrmann, 2019). They are present in all baculoviruses independently of the taxonomic group they belong to. This number may vary over time, as careful analysis of sequenced baculovirus genomes may allow the identification of additional core genes, due to the fact that accumulation of

many mutations throughout divergent evolution may make it difficult to identify them (Rohrmann, 2019). Although there are homologous of these genes present in other DNA viruses, possessing the totality of the core genes seems to characterize a baculovirus (Van Oers and Vlak, 2007). This conservation has permitted phylogenetic comparison of baculoviruses, demonstrating that the core genes are highly suitable for phylogenetic studies (Herniou et al., 2003; Jehle et al., 2006; Wennmann et al., 2018). In 2006, the construction of a neighbor-joining tree of the amino acid alignment of the core genes sequenced to date allowed the establishment of the actual baculovirus classification (Jehle et al., 2006). The phylogenetic analysis of these core genes has also proved to be a useful tool for baculovirus species demarcation (Wennmann et al., 2018).

Core genes are involved in essential functions such as DNA replication (shaded in blue), gene transcription (in red), packaging and assembly (in yellow) and oral infectivity (in green) (Table 1). They may also be involved in essential interactions with the host (Van Oers and Vlak, 2007). AcMNPV is the type species for baculovirus and was the first baculovirus to be sequenced completely in 1994 (Ayres et al., 1994). Consequently, baculovirus ORFs are usually named after their homology to those of AcMNPV (Rohrmann, 2019). Accordingly, the information of the core genes described here is with reference to AcMNPV (Table 1).

Table 1. Baculovirus core genes function.

Gene	Gene in AcMNPV	Function
lef-2	Ac6	DNA primase accessory factor, required for transient DNA replication
lef-1	Ac14	DNA primase, required for transient DNA replication
pif-2	Ac22	Per os infectivity factor 2, required for oral infectivity
p47	Ac40	Subunit of the baculovirus polymerase, required for transient late gene transcription
lef-8	Ac50	Baculovirus RNA polymerase subunit, required for transient late gene expression

Gene	Gene in AcMNPV	Function
E3 ubiquitin ligase family	Ac53	Deletion of this gene causes defective virions
Vp1054	Ac54	Capsid protein required for nucleocapsid assembly
lef-9	Ac62	Baculovirus RNA polymerase subunit, required for transient late gene expression
DNA polymerase	Ac65	Deletion of this gene is lethal
formin related	Ac66	Involved in actin nucleation, myosin and components of a splicing complex, is required both for egress of virions from nuclei and also may be involved in the enucleation of polyhedra
pif-6	Ac68	Per os infectivity factor 6, required for oral infectivity and is suggested to be involved in polyhedron morphogenesis
vlf-1	Ac77	DNA integrase, required for the production of nucleocapsids
Integrin transmembrane domain	Ac78	Involved in the nuclear entry and egress of BV and also interacts with components of the ESCRT-III complex and may be involved in the egress of nucleocapsids at the nuclear membrane
gp41	Ac80	Tegument protein located between the virion envelope and capsid, involved in the nuclear entry and egress of BV and also interacts with components of the ESCRT-III complex and may be involved in the egress of nucleocapsids at the nuclear membrane
late expressed non-structural gene	Ac81	Deletion of this gene results in severe reduction in BV production
pif-8	Ac83	Per os infectivity factor 8, plays a major role in the ability of the virus to initiate midgut infection
VP39	Ac89	Involved in the transport of nucleocapsids destined to become BV to the cell membrane after their assembly in nuclei, it also appears to be required for proper DNA packaging and nucleocapsid assembly

Gene	Gene in AcMNPV	Function
lef-4	Ac90	RNA capping enzyme, essential for late transcription
p33	Ac92	FAD-linked sulfhydryl oxidase, involved in the protection of cells from oxidative stress caused by apoptosis
	Ac93	Formation of intranuclear microvesicles, may be involved in the nuclear entry and egress of BV, it also interacts with components of the ESCRT-III complex playing a role in the release of nucleocapsids at the nuclear membrane
p25	Ac94	Nuclear targeting signal localized in ODV and BV envelopes, may play a role in the shift from BV to ODV virions and also in virion occlusion, it also may be involved in nuclear entry and egress of BV
p143	Ac95	DNA helicase, required for transient DNA replication
pif-4	Ac96	Per os infectivity factor 4, required for oral infectivity, and may provide proteolytic resistance to the core structure on dissolution of polyhedral in the insect midgut
38k	Ac98	Required for nucleocapsid formation but not appear to be a structural component of ODV
lef-5	Ac99	RNA polymerase II initiation factor, required for transient late gene expression
p6.9	Ac100	DNA binding protein, essential for high levels of expression of very late genes
bv/odv-c42	Ac101	Capsid-associated protein of both BV and ODV, involved in actin polymerization
p45	Ac103	Involved in the envelopment of ODV and their incorporation into occlusion bodies, may be involved in the nuclear entry and egress of BV, also interacts with components of the ESCRT-III complex and may be involved in the release of nucleocapsids at the nuclear membrane
	Ac109	Deletion of this gene results in OBs lacking virions
pif-7	Ac110	Per os infectivity factor 7, required for oral infectivity
pif-3	Ac115	Per os infectivity factor 3, required for oral infectivity

Gene	Gene in AcMNPV	Function
pif-1	Ac119	Per os infectivity factor 1, required for oral infectivity
alkaline nuclease	Ac133	Involved in DNA recombination
p74	Ac138	The first infectivity factor described, in addition to pif-1 and pif-2 mediates specific binding of occlusion derived virus to midgut cells
p49	Ac142	Associated to ODV and BV virions, affecting nucleocapsid formation, also was reported to interact with components of ESCRT-III complex and therefore may be involved in the release of nucleocapsids at the nuclear membrane
odv-e18	Ac143	Translocation complex required for BV production
odv-ec27	Ac144	Associated to ODV and BV, involved in the production of nucleocapsids
pif-5	Ac148	Per os infectivity factor 5, required for oral infectivity

List of core genes described in baculoviruses. Genes involved in DNA replication are shaded in blue; Genes involved in transcription are shaded in red; Genes involved in packaging and assembly are shaded in yellow; Genes involved in oral infectivity are shaded in green.

4.4 Genera-defining genes

Just as core genes define baculoviruses, there are sets of genes that characterize particular groups of baculoviruses. These genera-defining genes serve to group some virus species.

Lepidopteran baculoviruses are divided into alphabaculoviruses and betabaculoviruses, that share another 30 genes in addition to the 38 core genes, of which nearly half need their function to be elucidated; the rest are listed in Table 2 (Van Oers and Vlak, 2007). From the enlisted genes in Table 2, the F protein is a curious example due to its presence in group I NPVs but in a truncated form, which serves to divide the lepidopteran NPVs in two groups. Lepidopteran NPV and GV genomes can also be distinguished by the presence of nine genes that are present in all NPVs and absent in all GVs: *orf1629*, *pkip*,

pp34/pep, *vp80/p87*, *ac19*, *ac34*, *ac55*, *ac59* and *ac108* (Van Oers and Vlak, 2007). The *orf1629* encodes a nucleocapsid-associated phosphoprotein (Vialard and Richardson, 1993) that interacts with a component of the nucleocapsid of BV and ODV (Braunagel et al., 2001). PKIP is a protein-kinase interacting protein and stimulates PK1, the virus associated protein kinase (Fan et al., 1998; McLachlin et al., 1998). *pp34/pep* encodes a phosphoprotein, which is a major component of the polyhedron envelope (Zuidema et al., 1989). The rest of the conserved ORFs in lepidopteran NPVs that are absent in GVs are of unknown function. GVs also have a characteristic set of genes, 22 of which are absent in sequenced NPVs. Among them, only two have their function described, *iap-5* which is an apoptosis inhibitor and *mp-nase*, a metalloprotease (Van Oers and Vlak, 2007).

Table 2. Conserved genes in lepidopteran NPVs and GVs. Adapted from Van Oers (2007).

Category	Gene name	ORF in AcMNPV	Function
Replication	dbp	ac25	ss-DNA binding protein
	lef-3	ac67	Single strand DNA binding; interaction with alk-exo and p143
	ie-1	ac147	Transactivator
	me-53	ac139	Zinc finger protein
	lef-11	ac37	Unclear
Transcription	pp31/39K	ac36	Phosphoprotein required for late gene expression
	lef-6	ac28	Not essential
	lef-10	ac53a	Late essential factor
Virus structure	F protein	ac23	BV envelope protein required for fusion
	fp25K	ac61	Intracellular trafficking of structural proteins
	odv-e66	ac46	Structural protein of ODV envelope
	pk1	ac10	Protein kinase
	polh	ac8	OB matrix protein
Host interaction	fgf	ac32	Attract cells to infection site
	ubiquitin	ac35	Prevents protein degradation

Hymenopteran NPVs characteristically lack an orthologue of the baculovirus F gene. These baculoviruses also lack homologs of the

transactivator *ie-1* and *lef-3* gene (Duffy et al., 2006). The only representative of dipteran NPVs, Culex nigripalpus nucleopolyhedrovirus (CuniNPV) lacks several genes present in lepidopteran NPVs, including *odv-e25*, *odv-e66* and *odv-e18* as well as a polyhedrin homolog and polyhedra morphogenesis-related genes (Afonso et al., 2001; Moser et al., 2001). Instead, this virus has a 90-kDa protein which has three times the size of NPV and GV OB proteins. Due to the little information available for this NPV, no firm conclusions can be extracted for their gene conservation and further studies will be required in order to better understand their properties and evolution.

4.5 Clade-specific genes

This section deals with those genes whose presence is associated with segregation among different lineages according to phylogenetic trees. These genes are thought to have been hypothetically acquired during evolution and the same selection process may have resulted in their loss (Herniou et al., 2003).

Alphabaculoviruses are phylogenetically divided into two groups by the presence of a functional F protein. This protein is truncated in group I NPVs. In this group of baculoviruses, the function of the F protein is performed by GP64 which is specific to this group (Monsma et al., 1996; Oomens and Blissard, 1999). This protein is not essential for infectivity but enhances viral pathogenicity (Lung et al., 2003).

There are other genes associated with more specific lineages. For instance, homologs of the *DNA photolyase* gene were thought to be present only in those NPV infecting insects of the subfamily Plusiinae (Van Oers et al., 2004; Willis et al., 2005; Xu et al., 2008). Nevertheless, a recent study in Rachiplusia nu NPV (RanuNPV), a virus related to the most common ancestor of Plusiinae viruses, showed a photolyase homolog gene with presence in other baculoviruses, including alphabaculoviruses and betabaculoviruses (Trentin et al., 2019). This may indicate that this gene is shared by narrow host-range viruses that infect Plusiinae insects, although it is not exclusive of these viruses.

Enhancin genes encode metalloproteinases located in the OBs that increase viral pathogenicity by disrupting the peritrophic membrane. They were mainly discovered in GVs but are also present in some NPVs. Indeed, phylogenetic analyses of all sequenced baculoviruses showed high levels of heterogeneity, suggesting that the enhancin genes of NPV and GV may have been acquired from independent sources (Slavicek, 2012).

Baculovirus repeated ORFs or *bro* genes are not present in all baculovirus genomes. These genes can be present as a single copy, like in AcMNPV, or up to eight copies per genome as in Mamestra configurata NPV-A (Li et al., 2002). Phylogenetic analyses of these genes showed that are distributed among invertebrate viruses but also in bacteriophages and bacterial transposons. Furthermore, their phylogenetic relationship suggests some degree of recombination between viral genomes that allowed the duplication and loss of genes but also their acquisition through horizontal gene transfer (Bideshi et al., 2003). Although their function is far from clear, they may play a role in transcription/replication processes by changing the chromatin structure of the host DNA (Zemskov et al., 2000).

Finally, there are also some examples of genes that have been acquired from an insect host by unrelated baculoviruses that share that common insect host and could be involved in viral adaptation to a particular ecological niche (Thézé et al., 2015). Genes like ecdysteroid UDP-glucosyltransferase (*egt*) and protein tyrosine phosphatase (*ptp*) are baculovirus accessory genes that appear to have a lepidopteran origin, acquired during coevolution with their hosts (Clem and Passarelli, 2013). In the same line, genes acquired from an insect host can be transmitted to other baculovirus coinfecting the same insect host, exemplified by the eukaryotic translation initiation factor 5 (*eif5*), whose orthologs have been found in two closely-related baculovirus, Choristoneura rosaceana NPV (ChroNPV) and Choristoneura occidentalis GV (ChocGV). This result indicates that the original gene was acquired from the insect host and subsequently one virus obtained it from the other when both viruses coinfected the same insect (Escasa et al., 2006; Thumbi et al., 2013).

4.6 Species-specific genes

Individual baculoviruses contain a number of species-specific genes that have greatly influenced the diversification and evolution of these viruses (Ikeda et al., 2015). Most of baculovirus genomes sequenced to date include a set of species-specific genes, which may be related to the specific biological characteristics of the particular viral species, such as host range. For instance, in the Spodoptera frugiperda MNPV, up to 12 ORFs have been identified that are unique for this virus and do not present homologs in other sequenced baculoviruses (Harrison et al., 2008; Simón et al., 2011). In Diatraea saccharalis GV a G protein-coupled receptor (GPCR) was discovered that has not been found in other baculoviruses (Rohrmann, 2019), although it has orthologs in some entomopoxviruses. PARP protein (a poly ADP-ribose polymerase) was reported only in Anticarsia gemmatalis MNPV (de Castro Oliveira et al., 2006, 2008). Oxyplax ochracea nucleopolyhedrovirus (OxocNPV), which has recently been reported as a novel group I alphabaculovirus, presented three unique ORFs with no known homologs (Wang et al., 2018) Finally, in Chrysodeixis includens NPV (ChinNPV), two unique genes have been described, *psin5* and *psin8* although their function is not understood (Castro et al., 2015). Despite the hypothetical importance that unique genes may have in each virus species, the functional importance of these genes is not known in most of the cases.

Nevertheless, despite the conservation of all these genes, the total number of non-homologous genes present in baculovirus genomes seems to be enormous. For this reason, it is hypothesized that these variations have an important role in the specific patho-biological properties of each baculovirus species, providing the optimal genetic configuration for its particular ecological niche (van Oers and Vlak, 2007). It is reasonable to think that the large differences in host range, virulence and ecological fitness in baculoviruses could be explained, at least in part, by their collection of unique genes.

5. BACULOVIRUS GENOME DIVERSITY

Baculoviruses have a high level of diversity, as evidenced by the 76 species that comprise the four genera of the *Baculoviridae* family (ICTV, 2019).

5.1 Natural diversity of baculovirus species

The potential of baculoviruses as biological control agents for insect pests is due to their ability to initiate epizootics in lepidopteran pest populations. This interest in baculoviruses as potential biopesticides has led efforts to screen for more efficient isolates or strains (Erlandson, 2009). The objective of selection of individual or combined strains for this purpose has promoted the study of the diversity existing within this virus family. Variation has been reported in isolates from different geographical regions, between viruses recovered from different individuals within the same host population and even within those viral samples obtained from a single individual (Cooper et al., 2003; Crook et al., 1985; Gettig and McCarthy, 1982; Kislev and Edelman, 1982; Laitinen et al., 1996; Parnell et al., 2002; Shapiro et al., 1991; Vickers et al., 1991). Baculovirus variation in field isolates is usually analyzed at two levels: I) diversity that exists between different isolates and II) diversity at the genotype level.

1.6.4. Variation among different isolates

Great part of the information about genetic variation of baculoviruses has been obtained from virus species infecting economically important lepidopteran pests, but just a few examples illustrating high variability will be described here. In the Canary Islands, from 97 isolates collected in banana crops, five different genetic variants of Chrysodeixis chalcites NPV (ChchNPV) were obtained (Bernal et al., 2013b). Another study involving Helicoverpa armigera NPV (HearNPV) showed eight different strains in 20 isolates from 49 diseased larvae of *Helicoverpa armigera* (Figueiredo et al., 2009). More recently, seven field isolates of ChinNPV collected from different populations of *C. includens* larvae in south and central

Brazil showed that only two of the six isolates were identical in the number and mobility of restriction fragments (Alexandre et al., 2010).

In the case of GVs, their diversity is less well understood although several studies support evidence for variability among different isolates. Different studies have revealed the existence of diverse geographical isolates in several granulovirus species, such as CpGV and Tecia solanivora GV (TsGV) (Eberle et al., 2009; Espinel-Correal et al., 2010). Indeed, in the case of CpGV, in which the first case of resistance to baculovirus was reported, new field isolates have been screened in order to identify natural isolates capable of overcoming this resistance (Fan et al., 2020a). These few examples are sufficient to outline the high variability present in natural populations of baculoviruses and are a convenient introduction for the next level of genetic variability.

1.6.5. Variation in virus genotype

Baculovirus field isolates are usually found as a mixture of genotypic variants or genotypes (Harrison, 2009). In the studies mentioned above, the level of variation is determined on pooled or individual larvae in which the genotypic diversity present in the isolates is underrepresented. For isolation of the genotypes that comprise a natural isolate, in vivo or in vitro cloning of these has been the usual strategy, although the details of those techniques will be reviewed in the next section. The following studies have focused on single larval sampling strategies to isolate genotypic variants. The methodology of analyzing individual larvae instead of pooled samples is likely to increase the chances of quantifying the true genetic diversity present in a virus population (Figueiredo et al., 2009). There are many studies demonstrating that individual larvae from field populations may be infected by mixtures of genotypes. An extreme example is that of the Panolis flammea NPV, on which 24 different genotypes were isolated by in vivo cloning technique (Cory et al., 2005). Similarly, in Chrysodeixis chalcites NPV isolated form the Canary Islands, eight genetically distinct genotypes were cloned by plaque assay (Bernal et al., 2013a). More recently, ultra-deep sequencing of an isolate of AcMNPV has revealed an astonishing diversity for a unique isolate, with every

possible combination of variants present, albeit at different frequencies (Chateigner et al., 2015). A similar study surveyed in CpGV revealed a high SNP frequency in seven new isolates of that virus (Fan et al., 2020b). The outcome of this high degree of genetic heterogeneity is variation in the numerous phenotypic traits that modulate virus populations, and this is what is usually associated with genotypic variation in terms of pathogenicity, virus productivity and host range of baculoviruses (Erlandson, 2009).

5.2 Generation and maintaining of baculovirus diversity

The high genetic diversity in baculoviruses is the result of several mechanisms, in addition to the gradual accumulation of mutations over time. Baculovirus genomes contain regions considered as "hot spots" for variation, which include the homologous repeated regions (*hrs)* and the *bro* genes, both of which are target sites for intragenomic recombination. Regions with high variation containing *bro* genes are, for instance, found in two genotypes of Mamestra configurata NPV-A (Li et al., 2005). In Bombyx mori NPV strains, an active redistribution of *bro* genes and adjacent sequences due to intraspecific recombination has been postulated (López Ferber et al., 2001). In addition, an inversion of the order of a group of genes flanked by *hr* sequences in Cydia pomonella granulovirus (CpGV) is an example of intragenomic recombination involving *hr* sequences (Arends and Jehle, 2002).

Variation in *hr* sequences is thought to be correlated with viral replication. When baculoviruses are passaged in cell culture, genomic instability appears rapidly (Kool et al., 1991; Pijlman et al., 2001). In these conditions, the origin of replication *hr* and *non-hr* may be duplicated resulting in a strong replicative advantage over wild-type genomes (Kool et al., 1993; Lee and Krell, 1994, 1992). The *hr* and *non-hr* genes of a particular virus often show sequence similarities which indicates that the origin of replication of *hr* might have evolved by concatenation and internal rearrangements of a *non-hr* origin of replication (Jehle, 2002).

Homologous recombination is another mechanism by which baculoviruses exchange genetic information, and it appears to be a highly frequent event (Crouch and Passarelli, 2002; Hajos et al., 2000; Jehle et al.,

2003; Kamita et al., 2003). This usually takes place between virus variants or closely related viruses that infect a common host and can be as frequent as 7% (Summers et al., 1980). For instance, recombination between BmNPV and AcMNPV was reported to expand the host range of these viruses (Kondo and Maeda, 1991; Maeda et al., 1993). Although recombination in more distantly related baculoviruses is much less frequent, intraspecific and interspecific recombination of tortricid-specific GVs recombination has been reported within the granulin genes of CpGV and Cryptophlebia leucotreta granulovirus (CrleGV) (Jehle et al., 2003). Phylogenetical evidence of interspecific recombination is also found in different genome sequences of baculoviruses. For instance, a cluster of four genes of Xestia c-nigrum granulovirus (XecnGV) was found in the sequence of a particular isolate of Mamestra configurata NPV (MacoNPV), suggesting previous recombination in a common host (Li et al., 2002). The versatility of this genetic mechanism has been exploited as a technology to generate baculovirus expression vectors in which a parental viral DNA is recombined in insect cells with a transfer vector containing the foreign gene by homologous recombination (Chambers et al., 2018; King and Possee, 1992).

Transposable elements are frequent agents that favor recombination between the insect host genomes and baculoviruses (Blissard, 2002; Fraser, 1986). Most of the sequences derived from transposons that are present in baculovirus genomes originate from insertions of relatively small elements with high specificity for the sequence TTAA. Two examples of these elements are the short-inverted repeat elements in cell lines from *Trichoplusia ni* and *Spodoptera frugiperda*: piggyBac (formerly IFP2) and tagalong (formerly TFP3), respectively (Cary et al., 1989; Fraser et al., 1996, 1995). A copia-like retro-transposable element derived from the *T. ni* genome, called TED, is a frequent insertion in baculovirus genomes (Friesen and Nissen, 1990; Hajek and Friesen, 1998; Miller and Miller, 1982; Ozers and Friesen, 1996). This element is recognized by long terminal repeats and, characteristically, encodes enzymes for transcription and replication. A TED transposon was identified in

AcMNPV and was reported to show promoter activity in both directions that could be altering the expression of flanking genes (Friesen et al., 1986).

Apart from the molecular mechanisms that generate genetic variability in baculoviruses, other factors also have their impact in the variability of the pathogen, such as the host susceptibility to infection, food plants ingested by the host, host species-mediated selection and concentration of the virus (Baillie and Bouwer, 2013; Williams, 2018). The interaction between host-pathogen and its role in the variability of the baculoviruses is an important tool for unravelling the high diversity present in virus populations. Indeed, a study involving Lymantria dispar MNPV (LdMNPV) has demonstrated with a mathematical model that genetic drift within the host plays a strong role in pathogen variability (Kennedy and Dwyer, 2018). In addition, heterogeneity in susceptibility to infection for a determined host leads to polymorphisms in its own baculovirus, as reported for *Lymantria dispar* (Fleming-Davies et al., 2015). Similarly, a study on *Cydia pomonella* demonstrated the key role that host genotype has on the maintenance of virus genetic diversity (Graillot et al., 2019). The host plant on which the susceptible insects fed has been reported to influence the genotypic diversity of a baculovirus, with a clear interaction between the host food plant and the pathogenicity of different NPV genotypes (Hodgson et al., 2002). The host species can also influence the genetic diversity of baculoviruses which suggests a role for host selection of pathogen genotypes as a mechanism for maintaining diversity (Hitchman et al., 2007). Finally, an study involving Helicoverpa armigera NPV (HearNPV) demonstrated that the inoculum dose affected the transmission of different genetic variants, suggesting that those differences could influence the genetic structure of the virus in field populations with the consequent changes in pathogenicity, transmission and population dynamics (Baillie and Bouwer, 2013). To summarize, the multiple factors that directly affect the generation of genetic diversity in baculoviruses are becoming increasingly recognized. In addition, it is also important to recognize that the techniques we use to detect this diversity can influence the type of results obtained.

5.3 Detection of genotypic diversity in baculoviruses

Genotypic diversity in baculoviruses has been identified by separating genetic variants using in vitro plaque assay techniques (Knell and Summers, 1981; Lee and Miller, 1978; Lynn et al., 1993; Maeda et al., 1990; Smith and Summers, 1978). Cloning in vitro has demonstrated the high genetic variation that is present within field-collected baculovirus isolates, although it may not provide an accurate picture of the genotypes originally present in the wild type virus population. The cloning process involves selection of genetic variants that will grow in vitro with some evidence that it favors variants with sizeable deletions (Pijlman et al., 2001). However, in cases where in vitro cloning is not possible, in vivo cloning may be an alternative. This methodology can be applied by injecting larvae with a very low concentration of BVs (Muñoz et al., 1998). The objective is to achieve less than 10% mortality so that each dead larva is likely to have been infected by a single viral particle, containing a single genome. This technique may also favor those variants that are not infectious *per os*. For example, a study involving Spodoptera exigua NPV (SeMNPV) demonstrated the existence of two naturally occurring deletion mutants that were acting as parasitic genotypes in the virus population, which were detected using the in vivo technique (Muñoz et al., 1998; Serrano et al., 2013).

Apart from the method used to separate the genetic variants that conform a virus isolate, there are several methods by which the variants can be identified. This task began with the use of restriction endonuclease enzymes (REN), in the 1980s, used to characterize diversity within and between baculovirus isolates from the same and different host species (Williams, 2018). Indeed, the widespread use of restriction endonucleases for baculovirus characterization has demonstrated that genotypic variation within baculovirus populations is ubiquitous (Cory et al., 2005). This technique is still used nowadays due to its low cost and speed in visualizing the results. Nevertheless, the detection of polymorphisms by REN is limited to the enzyme recognition site, which reduces its ability to detect other types of variation. Another technique to identify genetic variants is denaturing gradient gel

electrophoresis (DGGE), which can be used to detect variations within a virus population, as demonstrated for HearNPV (Baillie and Bouwer, 2012). Using DGGE with HearNPV, these authors were able to detect up to 20 genetic variants in a single neonate larva. Despite the high efficiency of this technique, it is time-consuming. Nowadays, new techniques such as deep sequencing and metagenomics have established themselves as efficient methods for determining the diversity of genotypic variants present in natural virus populations (Baillie and Bouwer, 2012; Chateigner et al., 2015). Sequencing is a powerful tool by which the whole variability within a particular population can be assessed, although it is not suited to everyday screening of genetic variants due to the time that it requires and the high cost. A more recent technique, mass sequencing, provides an exact picture of the genotypic diversity within a viral population (Chateigner et al., 2015). Nevertheless, a major disadvantage of this method is the impossibility of isolating the genetic variants that comprise the sequenced virus, in order to evaluate the role of each variant in the phenotypic traits of the virus, or to isolate a particular genotype that could be used as the active ingredient for a bioinsecticide.

6. CHRYSODEIXIS INCLUDENS NUCLEOPOLYHEDROVIRUS

Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) is a group II alphabaculovirus that was isolated for the first time in the decade of 1970 and has been recently characterized morphologically and genetically (Alexandre et al., 2010; Alzogaray et al., 2018; Castro et al., 2015; Craveiro et al., 2013). Recently, a new viral species was discovered denominated ChinNPV#1 that differs from the currently existing ChinNPV species (Harrison et al., 2019). However, it has yet to be included in the ICTV Taxonomy Report (Harrison et al., 2018). All the information in this section refers only to the currently recognized species of ChinNPV.

At the moment of writing, 18 isolates of ChinNPV have been described, all originating from South America where the Brazilian isolates are the most abundant. According to the experiments carried to date, this virus has been proved to be infective only to its own host, *C. includens,* and a closely

related insect, *T. ni* (Morgado et al., 2020). As a relatively recently discovered baculovirus, little information about the genetic structure of this virus is available, although as occurs with other baculoviruses, it is suspected that ChinNPV isolates are composed of different genotypes (Alexandre et al., 2010; Castro et al., 2015; Sanches et al., 2019). As of 2020, 15 complete genome sequences of ChinNPV were available in the NCBI database (National Center for Biotechnology Information, USA), including the reference genome for this virus, PsinNPV-IE (GenBank accession number: KJ631622). All sequences show a nucleotide identity of at least 98% with the reference genome, PsinNPV-IE. The genome size of these sequences varies from 138,760 to 140,859 bp, containing from 140 to 142 ORFs. This virus is closely related to *C. chalcites* and T. ni NPVs, both members of the group II alphabaculoviruses (Thézé et al., 2018). The reference genome, PsinNPV-IE, is 139 Kb in size and encodes 140 ORFs, with a GC content of 39.3%. It has no *hrs* sequences, but shows two *bro* genes, as does TniNPV. It presents 134 homologs to ChchNPV and two unique ORFs (Castro et al., 2015). Three of the six isolates from Brazil and Guatemala sequenced in 2016 showed a third *bro* gene (*bro-c)* that was not present in the rest of their isolates or in the reference isolate (Craveiro et al., 2016). Due to the interest of using ChinNPV in IPM programs, several isolates have been tested against *C. includens* as potential active ingredients for a baculovirus-based insecticide, with promising results (Alexandre et al., 2010; Zanardo Botelho et al., 2019). Indeed, a ChinNPV-based bioinsecticide has been commercialized under the name of Crysogen® by AgBitech for the control of *C. includens* (Muraro et al., 2019). Furthermore, it has been demonstrated that *C. includens* larvae resistant to chemical insecticides are susceptible to the commercialized Crysogen® product (Godoy et al., 2019). Nonetheless, information on ChinNPV remains scant although these is growing interest in its development as the basis for potential bioinsecticide products for control of this important pest.

7. AIMS OF THE THESIS

This thesis has its origin in the need to evaluate the nucleopolyhedrovirus of Chrysodeixis includens (ChinNPV) and the increasing importance of this species as a pest of soybean in the Americas. Studying the natural variability could also provide potential variants for the future development of a ChinNPV-based insecticide. For these reasons, the thesis had two main aims:

1) To examine the variability present in natural isolates of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV), the genetic variants that conform the isolates and their contribution to the insecticidal characteristics of the isolates.
2) To determine the factors that promote variability in ChinNPV, with special attention to the mechanisms that generate variability and maintain variability in the viral population.

The specific aims of the thesis were:

1) To study the natural virus diversity present in larvae of *C. includens* that died of polyhedrosis after being collected from soybean fields in Mexico.
2) To examine the biological properties of an artificial mixture of the isolates collected, including the pathogenicity, speed-of-kill, OB production and host range of the virus and determine the genotypic composition of the artificial mixture.
3) To determine the biological activity of the genotypic variants of ChinNPV to understand their contribution to the pathogenicity of the virus population and select candidates for a ChinNPV-based insecticide.
4) To study the factors that contribute to the generation and maintenance of the genetic variability present in the ChinNPV population.
5) To investigate the role of the genetic variants present in the artificial mixture of the natural isolates in the generation and maintenance of ChinNPV diversity.

6) To obtain the genomic sequence of the genetic variants involved in the generation of the genetic diversity in order to better understand the molecular mechanisms that may be involved in propagating ChinNPV variability.

8. REFERENCES

Ackermann, H.-W., Smirnoff, W.A., 1983. A morphological investigation of 23 baculoviruses. J. Invertebr. Pathol. 41, 269–280.

Adams, J.R., McClintock, J.T., 1991. Chapter 6: Baculoviridae, nuclear polyhedrosis viruses Part 1. Nuclear polyhedrosis viruses of insects. En "Atlas Invertebr. Viruses" (J. R. Adams J. R. Bonami, eds.) CRC Press. Boca Raton. 87–180.

Afonso, C.L., Tulman, E.R., Lu, Z., Balinsky, C.A., Moser, B.A., Becnel, J.J., Rock, D.L., Kutish, G.F., 2001. Genome sequence of a baculovirus pathogenic for *Culex nigripalpus*. J. Virol. 75, 11157–11165. https://doi.org/10.1128/jvi.75.22.11157-11165.2001

Alexandre, T.M., Craveiro, S.R., Cunha, F., Fonseca, I.C.B., Castro, M.E.B., Moscardi, F., Ribeiro, Z.M.A., 2010. Evaluation of seven viral isolates as potential biocontrol agents against *Pseudoplusia includens* (Lepidoptera: Noctuidae) caterpillars. J. Invertebr. Pathol. 105, 98–104. https://doi.org/10.1016/j.jip.2010.05.015

Alford, A.R., Hammond, A., 1982. Plusiinae (Lepidoptera: Noctuidae) populations in Louisiana soybean ecosystems as determined with looplure-baited traps. J. Econ. Entomol. 75, 647–650.

Alzogaray, R.A., Arneodo, J.D., Taibo, C., Dami, L., Jakubowicz, V., 2018. First report of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) infecting *Chrysodeixis includens* (Lepidoptera: Noctuidae) in Argentina. Florida Entomol. 101, 515–516. https://doi.org/10.1653/024.101.0312

Andrade, K., Bueno, A. de F., da Silva, D.M., Stecca, C. dos S., Pasini, A., de Oliveira, M.C.N., 2016. Bioecological characteristics of *Chrysodeixis includens* (Lepidoptera: Noctuidae) fed on different hosts. Austral Entomol. 55, 449–454. https://doi.org/10.1111/aen.12208

Ao, J. qun, Ling, E., Yu, X.Q., 2008. A Toll receptor from *Manduca sexta* is in response to *Escherichia coli* infection. Mol. Immunol. 45, 543–552. https://doi.org/10.1016/j.molimm.2007.05.019

Arends, H.M., Jehle, J.A., 2002. Homologous recombination between the inverted terminal repeats of defective transposon TCp3.2 causes an inversion in the genome of Cydia pomonella granulovirus. J. Gen. Virol. 83, 1573–1578. https://doi.org/10.1099/0022-1317-83-7-1573

Au, S., Wu, W., Panté, N., 2013. Baculovirus nuclear import: Open, nuclear pore complex (NPC) sesame. Viruses. https://doi.org/10.3390/v5071885

Ayres, M.D., Howard, S.C., Kuzio, J., López-Ferber, M., Possee, R.D., 1994. The complete DNA sequence of Autographa californica nuclear polyhedrosis virus. Virology 202, 586–605.

Babu, K.R., Ramakrishna, S., Reddy, Y.H.K., Lakshmi, G., Naidu, N. V, Basha,

S.S., Bhaskar, M., 2009. Metabolic alterations and molecular mechanism in silkworm larvae during viral infection: A review. African J. Biotechnol. 8, 899–907.

Baillie, V.L., Bouwer, G., 2013. The effect of inoculum dose on the genetic diversity detected within Helicoverpa armigera nucleopolyhedrovirus populations. J. Gen. Virol. 94, 2524–2529. https://doi.org/10.1099/vir.0.052803-0

Baillie, V.L., Bouwer, G., 2012. High levels of genetic variation within Helicoverpa armigera nucleopolyhedrovirus populations in individual host insects. Arch. Virol. 157, 2281–2289. https://doi.org/10.1007/s00705-012-1416-6

Barrera, G.P., Belaich, M.N., Patarroyo, M.A., Villamizar, L.F., Ghiringhelli, P.D., 2015. Evidence of recent interspecies horizontal gene transfer regarding nucleopolyhedrovirus infection of *Spodoptera frugiperda*. BMC Genomics 16, 1008. https://doi.org/10.1186/s12864-015-2218-5

Barrionuevo, M.J., San Blas, G., 2016. Redescription of immature stages of the soybean looper (Lepidoptera: Noctuidae: Plusiinae). Can. Entomol. 148, 247–259. https://doi.org/10.4039/tce.2015.62

Bernal, A., Simón, O., Williams, T., Muñoz, D., Caballero, P., 2013a. A Chrysodeixis chalcites single-nucleocapsid nucleopolyhedrovirus population from the Canary Islands is genotypically structured to maximize survival. Appl. Environ. Microbiol. 79, 7709–7718. https://doi.org/10.1128/AEM.02409-13

Bernal, A., Williams, T., Hernández-Suárez, E., Carnero, A., Caballero, P., Simón, O., 2013b. A native variant of Chrysodeixis chalcites nucleopolyhedrovirus: The basis for a promising bioinsecticide for control of *C. chalcites* on Canary Islands' banana crops. Biol. Control 67, 101–110. https://doi.org/10.1016/j.biocontrol.2013.08.006

Bernardi, O., Malvestiti, G.S., Dourado, P.M., Oliveira, W.S., Martinelli, S., Berger, G.U., Head, G.P., Omoto, C., 2012. Assessment of the high-dose concept and level of control provided by MON 87701 × MON 89788 soybean against *Anticarsia gemmatalis* and *Pseudoplusia includens* (Lepidoptera: Noctuidae) in Brazil. Pest Manag. Sci. 68, 1083–1091. https://doi.org/10.1002/ps.3271

Berretta, M.F., Leticia, M., Salvador, R., Sciocco, A., Romanowski, V., 2013. Baculovirus gene expression, in: Current Issues in Molecular Virology - Viral Genetics and Biotechnological Applications. InTech. https://doi.org/10.5772/56955

Bideshi, D.K., Renault, S., Stasiak, K., Federici, B.A., Bigot, Y., 2003. Phylogenetic analysis and possible function of *bro*-like genes, a multigene family widespread among large double-stranded DNA viruses of invertebrates and bacteria. J. Gen. Virol. 84, 2531–2544. https://doi.org/10.1099/vir.0.19256-0

Biswas, S., Willis, L.G., Fang, M., Nie, Y., Theilmann, D.A., 2017. Autographa californica nucleopolyhedrovirus AC141 (Exon0), a potential E3 ubiquitin ligase, interacts with viral ubiquitin and AC66 to facilitate nucleocapsid egress. J. Virol. 92. https://doi.org/10.1128/jvi.01713-17

Blissard, G., 2002. Baculovirus diversity and molecular biology. Annu. Rev.

Entomol. 35, 127–155. https://doi.org/10.1146/annurev.ento.35.1.127
Blissard, G.W., Theilmann, D.A., 2018. Baculovirus entry and egress from insect cells. Annu. Rev. Virol. 5, 113–139. https://doi.org/10.1146/annurev-virology-092917-043356
Boogaard, B., Van Lent, J.W.M., Theilmann, D.A., Erlandson, M.A., van Oers, M.M., 2017. Baculoviruses require an intact ODV entry-complex to resist proteolytic degradation of per os infectivity factors by co-occluded proteases from the larval host. J. Gen. Virol. 98, 3101–3110. https://doi.org/10.1099/jgv.0.000974
Boogaard, B., van Oers, M.M., van Lent, J.W.M., 2018. An advanced view on baculovirus per os infectivity factors. Insects. https://doi.org/10.3390/insects9030084
Borém, A., Almeida, G.D. de, 2011. Plantas geneticamente modificadas : desafios e oportunidades para regio□es tropicais. Universidade Federal de Viçosa, Viçosa MG.
Bortolotto, O.C., Pomari-Fernandes, A., Bueno, R.C.O. de F., Bueno, A. de F., Cruz, Y.K.S. da, Sanzovo, A., Ferreira, R.B., 2015. The use of soybean integrated pest management in Brazil: a review. Agron. Sci. Biotechnol. 1, 25. https://doi.org/10.33158/asb.2015v1i1p25
Braunagel, S.C., Guidry, P.A., Rosas-Acosta, G., Engelking, L., Summers, M.D., 2001. Identification of BV/ODV-C42, an Autographa californica nucleopolyhedrovirus orf101-encoded structural protein detected in infected-cell complexes with ODV-EC27 and p78/83. J. Virol. 75, 12331–12338. https://doi.org/10.1128/jvi.75.24.12331-12338.2001
Braunagel, S.C., Russell, W.K., Rosas-Acosta, G., Russell, D.H., Summers, M.D., 2003. Determination of the protein composition of the occlusion-derived virus of Autographa californica nucleopolyhedrovirus. Proc. Natl. Acad. Sci. U. S. A. 100, 9797–9802. https://doi.org/10.1073/pnas.1733972100
Braunagel, S.C., Summers, M.D., 1994. Autographa californica nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: structural proteins, antigens, lipid and fatty acid profiles. Virology 202, 315–328. https://doi.org/https://doi.org/10.1006/viro.1994.1348
Bretherton, R.F., 1983. Noctuidae and Agaristidae, in: Heath, J., Maitland, E.A. (Eds.), The Moths and Butterflies of Great Britain and Ireland. Harley Books, Colchester, UK.
Bueno, R.C.O.F., Raetano, C.G., Dorneles Junior, J., Carvalho, F.K., 2017. Integrated management of soybean pests: The example of Brazil. Outlooks Pest Manag. 28, 149–153. https://doi.org/10.1564/v28_aug_02
Burleigh, J.G., 1972. Population dynamics and biotic controls of the soybean looper in Louisiana. Environ. Entomol. 1, 290–294. https://doi.org/10.1093/ee/1.3.290
Canerday, D.T., Arant, F.S., 1967. Biology of *Pseudoplusia includens* and notes on biology of *Trichoplusia ni*, *Rachiplusia ou*, and *Autographa biloba*. J. Econ. Entomol. 60, 870–871. https://doi.org/10.1093/jee/60.3.870
Capinera, J.L., 2013. Encyclopedia of entomology. Choice Rev. Online 42, 42-4375-42–4375. https://doi.org/10.5860/choice.42-4375

Carneiro, E., Silva, L.B., Silva, A.F. da, Lopes, G.N., Pavan, B.E., Rodrigues, R.H.F., Carvalhinho, D.T., Mielezrski, D.F., 2018. Lepidopteran pests associated with the soybean cultivars phenology. Biosci. J. 34. https://doi.org/10.14393/BJ-v34n1a2018-37172

Cary, L.C., Goebel, M., Corsaro, B.G., Wang, H.G., Rosen, E., Fraser, M.J., 1989. Transposon mutagenesis of baculoviruses: Analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. Virology 172, 156–169. https://doi.org/10.1016/0042-6822(89)90117-7

Castro, M., Grynberg, P., Báo, S.N., Ribeiro, Z., Craveiro, S.R., Melo, F.L., Inglis, P.W., Ribeiro, B.M., Togawa, R.C., 2015. The genome sequence of Pseudoplusia includens single nucleopolyhedrovirus and an analysis of p26 gene evolution in the baculoviruses. BMC Genomics 16, 127. https://doi.org/10.1186/s12864-015-1323-9

Chambers, A.C., Aksular, M., Graves, L.P., Irons, S.L., Possee, R.D., King, L.A., 2018. Overview of the baculovirus expression system. Curr. Protoc. Protein Sci. 91, 5.4.1-5.4.6. https://doi.org/10.1002/cpps.47

Chapman, R.F., 2012a. The insects: structure and function, The insects. Cambridge University Press, Cambridge. https://doi.org/10.1017/cbo9781139035460

Chapman, R.F., 2012b. Alimentary canal, digestion and absorption, in: The Insects. Cambridge University Press, pp. 38–68. https://doi.org/10.1017/cbo9780511818202.004

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, B.J., Lamb, R.A., 2008. Mechanisms for enveloped virus budding: Can some viruses do without an ESCRT? Virology. https://doi.org/10.1016/j.virol.2007.11.008

Clavijo, G., Williams, T., Muñoz, D., Caballero, P., Lopez-Ferber, M., 2010. Mixed genotype transmission bodies and virions contribute to the maintenance of diversity in an insect virus. Proc. R. Soc. London. Ser. B Biol. Sci. 277, 943–951. https://doi.org/10.1098/rspb.2009.1838

Clem, R.J., Passarelli, A.L., 2013. Baculoviruses: sophisticated pathogens of insects. PLoS Pathog. 9, e1003729. https://doi.org/10.1371/journal.ppat.1003729

Cooper, D., Cory, J.S., Myers, J.H., 2003. Hierarchical spatial structure of genetically variable nucleopolyhedroviruses infecting cyclic populations of western tent caterpillars. Mol. Ecol. 12, 881–890. https://doi.org/10.1046/j.1365-294X.2003.01785.x

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/http://dx.doi.org/10.1016/j.jip.2005.03.008

Craveiro, S.R., Melo, F.L., Ribeiro, Z.M.A., Ribeiro, B.M., Báo, S.N., Inglis, P.W., Castro, M.E.B., 2013. Pseudoplusia includens single nucleopolyhedrovirus: Genetic diversity, phylogeny and hypervariability

of the pif-2 gene. J. Invertebr. Pathol. 114, 258–267. https://doi.org/10.1016/j.jip.2013.08.005

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, 8–9. https://doi.org/10.1128/genomea.01192-16

Croizier, G., Croizier, L., Quiot, J.M., Lereclus, D., 1988. Recombination of Autographa californica and Rachiplusia ou nuclear polyhedrosis viruses in Galleria mellonella larvae. J. Gen. Virol. 69, 177–185. https://doi.org/10.1099/0022-1317-69-1-177

Crook, N.E., Spencer, R.A., Payne, C.C., Leisy, D.J., 1985. Variation in Cydia pomonella granulosis virus isolates and physical maps of the DNA from three variants. J. Gen. Virol. 66, 2423–2430. https://doi.org/10.1099/0022-1317-66-11-2423

Crouch, E.A., Passarelli, A.L., 2002. Genetic requirements for homologous recombination in Autographa californica nucleopolyhedrovirus. J. Virol. 76, 9323–9334. https://doi.org/10.1128/jvi.76.18.9323-9334.2002

de Castro Oliveira, J.V., De Melo, F.L., Romano, C.M., Iamarino, A., Rizzi, T.S., Yeda, F.P., Hársi, C.M., Wolff, J.L.C., De Andrade Zanotto, P.M., 2008. Structural and phylogenetic relationship of ORF 31 from the Anticarsia gemmatalis MNPV to poly (ADP-ribose) polymerases (PARP). Virus Genes 37, 177–184. https://doi.org/10.1007/s11262-008-0253-x

de Castro Oliveira, J.V., Wolff, J.L.C., Garcia-Maruniak, A., Ribeiro, B.M., de Castro, M.E.B., de Souza, M.L., Moscardi, F., Maruniak, J.E., de Andrade Zanotto, P.M., 2006. Genome of the most widely used viral biopesticide: Anticarsia gemmatalis multiple nucleopolyhedrovirus. J. Gen. Virol. 87, 3233–3250. https://doi.org/10.1099/vir.0.82161-0

De Freitas Bueno, A., Sosa-Gómez, D., Moscardi, B., Bueno, R.C., 2012. Inimigos naturais das pragas da soja. pp. 493–672.

De Freitas Bueno, R.C.O., De Freitas Bueno, A., Moscardi, F., Postali Parra, J.R., Hoffmann-Campo, C.B., 2011. Lepidopteran larva consumption of soybean foliage: Basis for developing multiple-species economic thresholds for pest management decisions. Pest Manag. Sci. 67, 170–174. https://doi.org/10.1002/ps.2047

De Jong, J., Arif, B.M., Theilmann, D.A., Krell, P.J., 2009. Autographa californica multiple nucleopolyhedrovirus me53 (ac140) is a nonessential gene required for efficient budded-virus production. J. Virol. 83, 7440–7448. https://doi.org/10.1128/jvi.02390-08

De Jong, J., Theilmann, D.A., Arif, B.M., Krell, P.J., 2011. Immediate-early protein ME53 forms foci and colocalizes with GP64 and the major capsid protein VP39 at the cell membranes of Autographa californica multiple nucleopolyhedrovirus-infected cells. J. Virol. 85, 9696–9707. https://doi.org/10.1128/jvi.00833-11

del-Angel, C., Lasa, R., Rodríguez-del-Bosque, L.A., Mercado, G., Beperet, I., Caballero, P., Williams, T., 2018. Anticarsia gemmatalis nucleopolyhedrovirus from soybean crops in Tamaulipas, Mexico: diversity and insecticidal characteristics of individual variants and their

co-occluded mixtures. Florida Entomol. 101, 404–410. https://doi.org/10.1653/024.101.0319

Detoni, A.M., Carvalho, S.L.C., Hoshino, A.T., Pastório, M.A., Schmidt, M.A.H., Menezes Junior, A.O., Androcioli, H.G., 2018. First report of *Chrysodeixis includens* (Walker, [1858]) (Lepidotera: Noctuidae) injurious to pineapple (*Ananas comosus L.*) (Bromeliaceae) in Brazil. Brazilian J. Biol. 78, 796–798. https://doi.org/10.1590/1519-6984.174384

Dong, Z.-Q., Zhang, J., Chen, X.-M., He, Q., Cao, M.-Y., Wang, L., Li, H.-Q., Xiao, W.-F., Pan, C.-X., Lu, C., Pan, M.-H., 2014. Bombyx mori nucleopolyhedrovirus ORF79 is a per os infectivity factor associated with the PIF complex. Virus Res. 184, 62–70. https://doi.org/https://doi.org/10.1016/j.virusres.2014.02.009

Dow, J.A., 1984. Extremely high pH in biological systems: a model for carbonate transport. Am. J. Physiol. 246. https://doi.org/10.1152/ajpregu.1984.246.4.r633

Dowd, P.F., Sparks, T.C., 1986. Characterization of a *trans*-permethrin hydrolyzing enzyme from the midgut of *Pseudoplusia includens* (Walker). Pestic. Biochem. Physiol. 25, 73–81. https://doi.org/10.1016/0048-3575(86)90033-7

Duffy, S.P., Young, A.M., Morin, B., Lucarotti, C.J., Koop, B.F., Levin, D.B., 2006. Sequence analysis and organization of the Neodiprion abietis nucleopolyhedrovirus genome. J. Virol. 80, 6952–6963. https://doi.org/10.1128/jvi.00187-06

Eberle, K.E., Sayed, S., Rezapanah, M., Shojai-Estabragh, S., Jehle, J.A., 2009. Diversity and evolution of the Cydia pomonella granulovirus. J. Gen. Virol. 90, 662–671. https://doi.org/10.1099/vir.0.006999-0

Eichlin, T.D., Cunningham, H.B., 1978. The Plusiinae (Lepidoptera: Noctuidae) of America north of Mexico, emphasizing genitalic and larval morphology. Tech. Bull. 1567 14–15.

Engelhard, E.K., Kam-Morgan, L.N.W., Washburn, J.O., Volkman, L.E., 1994. The insect tracheal system: A conduit for the systemic spread of Autographa californica M nuclear polyhedrosis virus. Proc. Natl. Acad. Sci. U. S. A. 91, 3224–3227. https://doi.org/10.1073/pnas.91.8.3224

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. https://doi.org/10.1007/s12250-009-3052-1

Escasa, S.R., Lauzon, H.A.M., Mathur, A.C., Krell, P.J., Arif, B.M., 2006. Sequence analysis of the Choristoneura occidentalis granulovirus genome. J. Gen. Virol. 87, 1917–1933. https://doi.org/10.1099/vir.0.81792-0

Espinel-Correal, C., Léry, X., Villamizar, L., Gómez, J., Zeddam, J.L., Cotes, A.M., López-Ferber, M., 2010. Genetic and biological analysis of colombian Phthorimaea operculella granulovirus isolated from *Tecia solanivora* (Lepidoptera: Gelechiidae). Appl. Environ. Microbiol. 76, 7617–7625. https://doi.org/10.1128/AEM.00999-10

Etebari, K., Matindoost, L., Mirhoseini, S.Z., Turnbull, M.W., 2007. The effect

of BmNPV infection on protein metabolism in silkworm (*Bombyx mori*) larva. Invertebr. Surviv. J. 4, 13–17.

Fan, J., Wennmann, J.T., Wang, D., Jehle, J.A., 2020a. Novel diversity and virulence patterns found in new isolates of Cydia pomonella granulovirus from China. Appl. Environ. Microbiol. 86. https://doi.org/10.1128/AEM.02000-19

Fan, J., Wennmann, J.T., Wang, D., Jehle, J.A., 2020b. Single nucleotide polymorphism (SNP) frequencies and distribution reveal complex genetic composition of seven novel natural isolates of Cydia pomonella granulovirus. Virology 541, 32–40. https://doi.org/10.1016/j.virol.2019.11.016

Fan, X., McLachlin, J.R., Weaver, R.F., 1998. Identification and characterization of a protein kinase-interacting protein encoded by the Autographa californica nuclear polyhedrosis virus. Virology 240, 175–182. https://doi.org/10.1006/viro.1997.8944

Fang, M, Nie, Y., Harris, S., Erlandson, M.A., Theilmann, D.A., 2009. Autographa californica multiple nucleopolyhedrovirus core gene ac96 encodes a per os infectivity factor (pif-4). J. Virol. 83, 12569–12578.

Fang, Minggang, Nie, Y., Theilmann, D.A., 2009. AcMNPV EXON0 (AC141) which is required for the efficient egress of budded virus nucleocapsids interacts with β-tubulin. Virology 385, 496–504. https://doi.org/http://dx.doi.org/10.1016/j.virol.2008.12.023

Ferrelli, M.L., Berretta, M., Belaich, M., Ghiringhelli, P., Sciocco-Cap, A., Romanowski, V., 2012. The baculoviral genome, in: Viral Genomes - Molecular Structure, Diversity, Gene Expression Mechanisms and Host-Virus Interactions. pp. 3–32. https://doi.org/10.5772/32209

Figueiredo, E., Muñoz, D., Murillo, R., Mexia, A., Caballero, P., 2009. Diversity of Iberian nucleopolyhedrovirus wild-type isolates infecting Helicoverpa armigera (Lepidoptera: Noctuidae). Biol. Control 50, 43–49. https://doi.org/10.1016/j.biocontrol.2009.02.005

Fleming-Davies, A.E., Dukic, V., Andreasen, V., Dwyer, G., 2015. Effects of host heterogeneity on pathogen diversity and evolution. Ecol. Lett. 18, 1252–1261. https://doi.org/10.1111/ele.12506

Flores-Jasso, C.F., Valdes, V.J., Sampieri, A., Valadez-Graham, V., Recillas-Targa, F., Vaca, L., 2004. Silencing structural and nonstructural genes in baculovirus by RNA interference. Virus Res. 102, 75–84. https://doi.org/10.1016/j.virusres.2004.01.018

Fraser, M.J., 1986. Transposon-mediated mutagenesis of baculoviruses: Transposon shuttling and implications for speciation, in: Annals of the Entomological Society of America. Oxford Academic, pp. 773–783. https://doi.org/10.1093/aesa/79.5.773

Fraser, M.J., Cary, L., Boonvisudhi, K., Wang, H.G.H., 1995. Assay for movement of lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. Virology 211, 397–407. https://doi.org/10.1006/viro.1995.1422

Fraser, M.J., Ciszczon, T., Elick, T., Bauser, C., 1996. Precise excision of TTAA-specific lepidopteran transposons piggyBac (IFP2) and tagalong (TFP3) from the baculovirus genome in cell lines from two species of

Lepidoptera. Insect Mol. Biol. 5, 141–151. https://doi.org/10.1111/j.1365-2583.1996.tb00048.x
Friesen, P.D., 1997. Regulation of baculovirus early gene expression. The baculoviruses 141–170. https://doi.org/10.1007/978-1-4899-1834-5_6
Friesen, P.D., Nissen, M.S., 1990. Gene organization and transcription of TED, a lepidopteran retrotransposon integrated within the baculovirus genome. Mol. Cell. Biol. 10, 3067–3077. https://doi.org/10.1128/mcb.10.6.3067
Friesen, P.D., Rice, W.C., Miller, D.W., Miller, L.K., 1986. Bidirectional transcription from a solo long terminal repeat of the retrotransposon TED: symmetrical RNA start sites. Mol. Cell. Biol. 6, 1599–1607. https://doi.org/10.1128/mcb.6.5.1599
Funk, C.J., Braunagel, S.C., Rohrmann, G.F., 1997. Baculovirus structure, in: The Baculoviruses. Springer US, pp. 7–32. https://doi.org/10.1007/978-1-4899-1834-5_2
Gallo, L.G., Corsaro, B.G., Hughes, P.R., Granados, R.R., 1991. In vivo enhancement of baculovirus infection by the viral enhancing factor of a granulosis virus of the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 58, 203–210. https://doi.org/10.1016/0022-2011(91)90064-W
Gettig, R.R., McCarthy, W.J., 1982. Genotypic variation among wild isolates of Heliothis spp nuclear polyhedrosis viruses from different geographical regions. Virology 117, 245–252. https://doi.org/10.1016/0042-6822(82)90523-2
Gilbert, L., 2012. Insect molecular biology and biochemistry, Insect molecular biology and biochemistry. Academic Press. https://doi.org/10.1016/C2009-0-62118-8
Goater, B., L. Ronkay, and M.F., 2003. Noctuidae Europaeae. Catocalinae & Plusiinae. Entomological Press.
Godoy, D.N., Führ, F.M., Stacke, R.F., Muraro, D.S., Marçon, P., Popham, H.J.R., Bernardi, O., 2019. No cross-resistance between ChinNPV and chemical insecticides in *Chrysodeixis includens* (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 164, 66—68. https://doi.org/10.1016/j.jip.2019.05.001
Goley, E.D., Ohkawa, T., Mancuso, J., Woodruff, J.B., D'Alessio, J.A., Cande, W.Z., Volkman, L.E., Welch, M.D., 2006. Dynamic nuclear actin assembly by Arp2/3 complex and a baculovirus WASP-like protein. Science. 314, 464–467. https://doi.org/10.1126/science.1133348
Goley, E.D., Rammohan, A., Znameroski, E.A., Firat-Karalar, E.N., Sept, D., Welch, M.D., 2010. An actin-filament-binding interface on the Arp2/3 complex is critical for nucleation and branch stability. Proc. Natl. Acad. Sci. U. S. A. 107, 8159–8164. https://doi.org/10.1073/pnas.0911668107
Gomez Rolim, A.A.S., Specht, A., Yano, S.A.C., Sosa-Gómez, D.R., De Jesus Andrade, C.G.T., 2013. Morphological and molecular characterization of the eggs of some noctuid species associated with soybean in Brazil. Ann. Entomol. Soc. Am. 106, 643–651. https://doi.org/10.1603/an13049
Graillot, B., Blachere-López, C., Besse, S., Siegwart, M., López-Ferber, M., 2019. Importance of the host phenotype on the preservation of the

genetic diversity in codling moth granulovirus. Viruses 11. https://doi.org/10.3390/v11070621
Granados, R.R., Lawler, K.A., 1981. In vivo pathway of Autographa californica baculovirus invasion and infection. Virology 108, 297–308. https://doi.org/http://dx.doi.org/10.1016/0042-6822(81)90438-4
Granados, R.R., Williams, K.A., 1986. In vivo infection and replication of baculoviruses., in: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses. CRC Press, Boca Raton, FL, pp. 89–108.
Guan, Z., Zhong, L., Li, C., Wu, W., Yuan, M., Yang, K., 2016. The Autographa californica multiple nucleopolyhedrovirus ac54 gene is crucial for localization of the major capsid protein VP39 at the site of nucleocapsid assembly. J. Virol. 90, 4115–4126. https://doi.org/10.1128/jvi.02885-15
Guarino, L.A., Dong, W., Jin, J., 2002. In vitro activity of the baculovirus late expression factor LEF-5. J. Virol. 76, 12663 LP – 12675. https://doi.org/10.1128/JVI.76.24.12663-12675.2002
Guarino, L.A., Gonzalez, M.A., Summers, M.D., 1986. Complete sequence and enhancer function of the homologous DNA regions of Autographa californica nuclear polyhedrosis virus. J. Virol. 60, 224–229.
Hajek, K.L., Friesen, P.D., 1998. Proteolytic processing and assembly of *gag* and *gag-pol* proteins of TED, a baculovirus-associated retrotransposon of the gypsy family. J. Virol. 72, 8718–8724. https://doi.org/10.1128/jvi.72.11.8718-8724.1998
Hajos, J.P., Pijnenburg, J., Usmany, M., Zuidema, D., Zavodszky, P., Vlak, J.M., 2000. High frequency recombination between homologous baculoviruses in cell culture. Arch. Virol. 145, 159–164. https://doi.org/10.1007/s007050050012
Hamadain, E.I., Pitre, H.N., 2002. Oviposition and larval behavior of soybean looper, *Pseudoplusia includens* (Lepidoptera: Noctuidae), on soybean with different row spacings and plant growth stages. J. Agric. Urban Entomol. 19, 29–44.
Harrison, R.L., 2009. Structural divergence among genomes of closely related baculoviruses and its implications for baculovirus evolution. J. Invertebr. Pathol. 101, 181–186. https://doi.org/http://dx.doi.org/10.1016/j.jip.2009.03.012
Harrison, R.L., Herniou, E.A., Jehle, J.A., Theilmann, D.A., Burand, J.P., Becnel, J.J., Krell, P.J., van Oers, M.M., Mowery, J.D., Bauchan, G.R., Consortium, I.R., 2018. ICTV Virus Taxonomy Profile: Baculoviridae. J. Gen. Virol. 99, 1185–1186. https://doi.org/https://doi.org/10.1099/jgv.0.001107
Harrison, R.L., Puttler, B., Popham, H.J.R., 2008. Genomic sequence analysis of a fast-killing isolate of Spodoptera frugiperda multiple nucleopolyhedrovirus. J. Gen. Virol. 89, 775–790. https://doi.org/10.1099/vir.0.83566-0
Harrison, R.L., Rowley, D.L., Popham, H.J.R., 2019. A novel alphabaculovirus from the soybean looper, *Chrysodeixis includens*, that produces tetrahedral occlusion bodies and encodes two copies of *he65*. Viruses 11. https://doi.org/10.3390/v11070579
Hawtin, R.E., Zarkowska, T., Arnold, K., Thomas, C.J., Gooday, G.W., King,

L.A., Kuzio, J.A., Possee, R.D., 1997. Liquefaction of Autographa californica nucleopolyhedrovirus-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. Virology 238, 243–253. https://doi.org/http://dx.doi.org/10.1006/viro.1997.8816

He, N., Zhang, S., Xu, Y., Fu, Q., Jia, L., Xiang, Z., 2011. Proteomic analysis of larval midgut from the silkworm (Bombyx mori). Comp. Funct. Genomics 2011, 1–13. https://doi.org/10.1155/2011/876064

Hegedus, D., Erlandson, M., Gillott, C., Toprak, U., 2009. New insights into peritrophic matrix synthesis, architecture and function. Annu. Rev. Entomol. 54, 285–302. https://doi.org/doi:10.1146/annurev.ento.54.110807.090559

Herniou, E., Arif, B., Becnel, J.J., Blissard, G., Bonning, B., Harrisson, R., Jehl, J.A., Theilmann, D., Vlak, J., 2011. Family baculoviridae.

Herniou, E.A., Olszewski, J.A., Cory, J.S., O'Reilly, D.R., 2003. The genome sequence and evolution of baculoviruses. Annu. Rev. Entomol. 48, 211–234. https://doi.org/10.1146/annurev.ento.48.091801.112756

Herzog, D.C., 1980. Sampling soybean looper on soybean. Springer, New York, NY, pp. 141–168. https://doi.org/10.1007/978-1-4612-9998-1_7

Hitchman, R.B., Hodgson, D.J., King, L.A., Hails, R.S., Cory, J.S., Possee, R.D., 2007. Host mediated selection of pathogen genotypes as a mechanism for the maintenance of baculovirus diversity in the field. J. Invertebr. Pathol. 94, 153–162. https://doi.org/10.1016/j.jip.2006.10.002

Hodgson, D.J., Vanbergen, A.J., Hartley, S.E., Hails, R.S., Cory, J.S., 2002. Differential selection of baculovirus genotypes mediated by different species of host food plant. Ecol. Lett. 5, 512–518. https://doi.org/10.1046/j.1461-0248.2002.00338.x

Hoover, K., Grove, M., Gardner, M., Hughes, D.P., McNeil, J., Slavicek, J., 2011. A gene for an extended phenotype. Science. 333, 1401. https://doi.org/10.1126/science.1209199

Hoover, K., Washburn, J.O., Volkman, L.E., 2000. Midgut-based resistance of *Heliothis virescens* to baculovirus infection mediated by phytochemicals in cotton. J. Insect Physiol. 46, 999–1007. https://doi.org/10.1016/S0022-1910(99)00211-5

Horton, H.M., Burand, J.P., 1993. Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. J. Virol. 67, 1860–1868.

Hou, D., Zhang, L., Deng, F., Fang, W., Wang, R., Liu, X., Guo, L., Rayner, S., Chen, X., Wang, H., Hu, Z., 2013. Comparative proteomics reveal fundamental structural and functional differences between the two progeny phenotypes of a baculovirus. J. Virol. 87, 829–839. https://doi.org/10.1128/jvi.02329-12

Hughes, A.L., Irausquin, S., Friedman, R., 2010. The evolutionary biology of poxviruses. Infect. Genet. Evol. https://doi.org/10.1016/j.meegid.2009.10.001

Hurley, J.H., 2015. ESCRTs are everywhere. EMBO J. 34, 2398–2407. https://doi.org/10.15252/embj.201592484

Ibarra, J.E., Del Rincón-Castro, M.C., 2008. Insect viruses diversity, biology, and use as bioinsecticides. Encycl. Life Support Syst. (EOLSS), Dev.

under auspices UNESCO, Eolss Publ. Oxford, UK 1–31.
Ignoffo, C.M., 1973. Effects of entomopathogens on vertebrates. Ann. N. Y. Acad. Sci. 217, 141–164. https://doi.org/10.1111/j.1749-6632.1973.tb32756.x
Ikeda, M., Hamajima, R., Kobayashi, M., 2015. Baculoviruses: diversity, evolution and manipulation of insects. Entomol. Sci. 18, 1–20. https://doi.org/10.1111/ens.12105
Inglis, P.W., Santos, L.A.V.M., Craveiro, S.R., Ribeiro, B.M., Castro, M.E.B., 2020. Mosaic genome evolution and phylogenetics of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) and virulence of seven new isolates from the Brazilian states of Minas Gerais and Mato Grosso. Arch. Virol. 166, 125–138. https://doi.org/10.1007/s00705-020-04858-2
International Committee on Taxonomy of Viruses [WWW Document], 2019.
Javed, M.A., Biswas, S., Willis, L.G., Harris, S., Pritchard, C., van Oers, M.M., Donly, B.C., Erlandson, M.A., Hegedus, D.D., Theilmann, D.A., 2017. Autographa californica multiple nucleopolyhedrovirus AC83 is a per os infectivity factor (PIF) protein required for occlusion-derived virus (ODV) and budded virus nucleocapsid assembly as well as assembly of the PIF complex in ODV envelopes. J. Virol. 91. https://doi.org/10.1128/jvi.02115-16
Jehle, J.A., 2002. The expansion of a hypervariable, non-hr ori-like region in the genome of Cryptophlebia leucotreta granulovirus provides in vivo evidence for the utilization of baculovirus non-hr oris during replication. J. Gen. Virol. 83, 2025–2034. https://doi.org/10.1099/0022-1317-83-8-2025
Jehle, J.A., Blissard, G.W., Bonning, B.C., Cory, J.S., Herniou, E.A., Rohrmann, G.F., Theilmann, D.A., Thiem, S.M., Vlak, J.M., 2006. On the classification and nomenclature of baculoviruses: A proposal for revision. Arch. Virol. 151, 1257–1266. https://doi.org/10.1007/s00705-006-0763-6
Jehle, J.A., Fritsch, E., Huber, J., Backhaus, H., 2003. Intra-specific and inter-specific recombination of tortricid-specific granuloviruses during co-infection in insect larvae. Arch. Virol. 148, 1317–1333. https://doi.org/10.1007/s00705-003-0104-y
Jensen, R.L., Newsom, L.D., Gibbens, J., 1974. The soybean looper: effects of adult nutrition on oviposition, mating frequency, and longevity. J. Econ. Entomol. 67, 467–470. https://doi.org/10.1093/jee/67.4.467
Jost, D.J., Pitre, H.N., 2002. Soybean looper and cabbage looper (Lepidoptera: Noctuidae) populations in cotton and soybean cropping systems in Mississippi. J. Entomol. Sci. 37, 227–235. https://doi.org/10.18474/0749-8004-37.3.227
Kadlec, J., Loureiro, S., Abrescia, N.G., Stuart, D.I., Jones, I.M., 2008. The postfusion structure of baculovirus gp64 supports a unified view of viral fusion machines. Nat. Struct. Mol. Biol. 15, 1024–1030. https://doi.org/nsmb.1484 [pii]10.1038/nsmb.1484 [doi]
Kamita, S.G., Maeda, S., Hammock, B.D., 2003. High-frequency homologous recombination between baculoviruses involves DNA replication. J. Virol. 77, 13053–13061. https://doi.org/10.1128/jvi.77.24.13053-13061.2003

Katsuma, S., Koyano, Y., Kang, W., Kokusho, R., Kamita, S.G., Shimada, T., 2012. The baculovirus uses a captured host phosphatase to induce enhanced locomotory activity in host caterpillars. PLoS Pathog. 8, e1002644.

Keddie, B.A., Aponte, G.W., Volkman, L.E., 1989. The pathway of infection of Autographa californica nuclear polyhedrosis virus in an insect host. Science. 243, 1728–1730. https://doi.org/10.1126/science.2648574

Kennedy, D.A., Dwyer, G., 2018. Effects of multiple sources of genetic drift on pathogen variation within hosts. PLoS Biol. 16, 1–17. https://doi.org/10.1371/journal.pbio.2004444

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.

King, A.M.Q., Lefkowitz, E., Adams, M.J., Carstens, E.B., 2011. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego (CA).

King, L.A., Possee, R.D., 1992. The baculovirus expression system. A laboratory guide. Chapman & Hall, London, United Kingdom.

Kirkpatrick, B.A., Washburn, J.O., Engelhard, E.K., Volkman, L.E., 1994. Primary infection of insect tracheae by Autographa californica M nuclear polyhedrosis virus. Virology 203, 184–186. https://doi.org/10.1006/viro.1994.1472

Kislev, N., Edelman, M., 1982. DNA restriction-pattern differences from geographic isolates of Spodoptera fittoralis nuclear polyhedrosis virus. Virology 119, 219–222. https://doi.org/10.1016/0042-6822(82)90081-2

Knebel-Mörsdorf, D., Flipsen, J.T.M., Roncarati, R., Jahnel, F., Kleefsman, A.W.F., Vlak, J.M., 1996. Baculovirus infection of *Spodoptera exigua* larvae : LacZ expression driven by promoters of early genes pe38 and me53 in larval tissue. J. Gen. Virol. 77, 815–824. https://doi.org/10.1099/0022-1317-77-5-815

Knell, J.D., Summers, M.D., 1981. Investigation of genetic heterogeneity in wild isolates of Spodoptera frugiperda nuclear polyhedrosis virus by restriction endonuclease analysis of plaque-purified variants. Virology 112, 190–197.

Kogan, M., 1981. Dynamics of insect adaptations to soybean: Impact of integrated pest managemenet. Environ. Entomol. 10, 363–371. https://doi.org/10.1093/ee/10.3.363

Kogan, M., Cope, D., 1974. Feeding and nutrition of insects associated with soybeans: food intake, utilization, and growth in the soybean looper, *Pseudoplusia includens*. Ann. Entomol. Soc. Am. 67, 66–72. https://doi.org/10.1093/aesa/67.1.66

Kondo, A., Maeda, S., 1991. Host range expansion by recombination of the baculoviruses Bombyx mori nuclear polyhedrosis-virus and Autographa californica nuclear polyhedrosis-virus. J. Virol. 65, 3625–3632.

Kool, M., Van Den Berg, P.M.M.M., Tramper, J., Goldbach, R.W., Vlak, J.M., 1993. Location of two putative origins of DNA replication of Autographa californica nuclear polyhedrosis virus. Virology 192, 94–101.

https://doi.org/10.1006/viro.1993.1011
Kool, M., Voncken, J.W., van Lier, F.L., Tramper, J., Vlak, J.M., 1991. Detection and analysis of Autographa californica nuclear polyhedrosis virus mutants with defective interfering properties. Virology 183, 739–46. https://doi.org/10.1016/0042-6822(91)91003-y
Kost, T.A., Condreay, J.P., Jarvis, D.L., 2005. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat. Biotechnol. 23, 567–575.
Lacey, L.A., Kaya, H.K., 2007. Field manual of techniques in invertebrate pathology: application and evaluation of pathogens for control of insects and other invertebrate pests. Springer, Dordrecht, The Netherlands.
Lafontaine, J.D., Schmidt, B.C., 2010. Annotated check list of the Noctuoidea (Insecta, Lepidoptera) of North America north of Mexico. Zookeys 40, 1–239. https://doi.org/10.3897/zookeys.40.414
Laitinen, A.M., Otvos, I.S., Levin, D.B., 1996. Genotypic variation among wild isolates of Douglas-fir tussock moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. J. Econ. Entomol. 89, 640–647. https://doi.org/10.1093/jee/89.3.640
Lapointe, R., Thumbi, D., J., C., 2012. Recent advances in our knowledge of baculovirus molecular biology and its relevance for the registration of baculovirus-based products for insect pest population control, in: Integrated Pest Management and Pest Control - Current and Future Tactics. InTech. https://doi.org/10.5772/31389
Lavine, M.D., Strand, M.R., 2002. Insect hemocytes and their role in immunity, in: Insect Biochemistry and Molecular Biology. Pergamon, pp. 1295–1309. https://doi.org/10.1016/S0965-1748(02)00092-9
Lee, H., Krell, P.J., 1994. Reiterated DNA fragments in defective genomes of Autographa californica nuclear polyhedrosis virus are competent for AcMNPV-dependent DNA replication. Virology 202, 418–429. https://doi.org/10.1006/viro.1994.1358
Lee, H.H., Miller, L.K., 1978. Isolation of genotypic variants of Autographa californica nuclear polyhedrosis virus. J. Virol. 27, 754–767.
Lee, H.Y., Krell, P.J., 1992. Generation and analysis of defective genomes of Autographa californica nuclear polyhedrosis virus. J. Virol. 66, 4339–4347. https://doi.org/10.1128/jvi.66.7.4339-4347.1992
Lehane, M.J., 1997. Peritrophic matrix structure and function. Annu. Rev. Entomol. 42, 525–550. https://doi.org/doi:10.1146/annurev.ento.42.1.525
Leite, R.M.V.M., Brighenti, A.M., Castro, C., 2005. Girassol no Brasil. Londrina, Brazil.
Lemaitre, B., Hoffmann, J., 2007. The host defense of *Drosophila melanogaster*. Annu. Rev. Immunol. 25, 697–743. https://doi.org/10.1146/annurev.immunol.25.022106.141615
Leonard, R.B., Boethel, D.J., Sparks, A.N., Layton, B.M., Mink, J.S., Pavloff, A.M., Burris, E., Graves, J.B., 2015. Variations in response of soybean looper (Lepidoptera: Noctuidae) to selected insecticides in Louisiana. J. Econ. Entomol. 83, 27–34. https://doi.org/10.1093/jee/83.1.27
Lepore, L.S., Roelvink, P.R., Granados, R.R., 1996. Enhancin, the granulosis

virus protein that facilitates nucleopolyhedrovirus (NPV) infections, is a metalloprotease. J. Invertebr. Pathol. 68, 131–140. https://doi.org/10.1006/jipa.1996.0070

Levin, D.B., Whittome, B., 2000. Codon usage in nucleopolyhedroviruses. J. Gen. Virol. 81, 2313–2325. https://doi.org/10.1099/0022-1317-81-9-2313

Li, L., Donly, C., Li, Q., Willis, L.G., Keddie, B.A., Erlandson, M.A., Theilmann, D.A., 2002. Identification and genomic analysis of a second species of nucleopolyhedrovirus isolated from *Mamestra configurata*. Virology 297, 226–244. https://doi.org/10.1006/viro.2002.1411

Li, L., Li, Q., Willis, L.G., Erlandson, M., Theilmann, D.A., Donly, C., 2005. Complete comparative genomic analysis of two field isolates of Mamestra configurata nucleopolyhedrovirus-A. J. Gen. Virol. 86, 91–105. https://doi.org/10.1099/vir.0.80488-0

Li, Z., Blissard, G.W., 2012. Cellular VPS4 is required for efficient entry and egress of budded virions of Autographa californica multiple nucleopolyhedrovirus. J. Virol. 86, 459–72. https://doi.org/10.1128/JVI.06049-11

Lingren, P.D., Greene, G.L., Davis, D.R., Baumhover, A.H., Henneberry, T.J., 1977. Nocturnal behavior of four lepidopteran pests that attack tobacco and other crops. Ann. Entomol. Soc. Am. 70, 161–167. https://doi.org/10.1093/aesa/70.2.161

Liu, J., Zhu, L., Zhang, S., Deng, Z., Huang, Z., Yuan, M., Wu, W., Yang, K., 2016. The Autographa californica multiple nucleopolyhedrovirus ac110 gene encodes a new per os infectivity factor. Virus Res. 221, 30–37. https://doi.org/10.1016/j.virusres.2016.05.017

López Ferber, M., Argaud, O., Croizier, L., Croizier, G., 2001. Diversity, distribution, and mobility of *bro* gene sequences in Bombyx mori nucleopolyhedrovirus. Virus Genes 22, 247–254. https://doi.org/10.1023/A:1011193603093

Lu, A., Miller, L., 1997. Regulation of baculovirus late and very late gene expression, in: Miller, L. (Ed.), The Baculoviruses. Springer US, pp. 193–216. https://doi.org/10.1007/978-1-4899-1834-5_8

Lu, M., Farrell, P.J., Johnson, R., Iatrou, K., 1997. A baculovirus (Bombyx mori nuclear polyhedrosis virus) repeat element functions as a powerful constitutive enhancer in transfected insect cells. J. Biol. Chem. 272, 30724–30728. https://doi.org/10.1074/jbc.272.49.30724

Lung, O., Westenberg, M., Vlak, J.M., Zuidema, D., Blissard, G.W., 2002. Pseudotyping Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV): F proteins from Group II NPVs are functionally analogous to AcMNPV GP64. J. Virol. 76, 5729–5736.

Lung, O.Y., Cruz-Alvarez, M., Blissard, G.W., 2003. Ac23, an envelope fusion protein homolog in the baculovirus Autographa californica multicapsid nucleopolyhedrovirus, is a viral pathogenicity factor. J. Virol. 77, 328–339. https://doi.org/10.1128/jvi.77.1.328-339.2003

Lynn, D.E., Shapiro, M., Dougherty, E.M., 1993. Selection and screening of clonal isolates of the Abington strain of gypsy moth nuclear polyhedrosis virus. J. Invertebr. Pathol. 62, 191–195.

https://doi.org/10.1006/jipa.1993.1095
Maeda, S., Kamita, S.G., Kondo, A., 1993. Host-range expansion of Autographa californica nuclear polyhedrosis virus (NPV) following recombination of a 0.6-Kilobase-pair DNA fragment originating from Bombyx mori NPV. J. Virol. 67, 6234–6238.
Maeda, S., Mukohara, Y., Kondo, A., 1990. Characteristically distinct isolates of the nuclear polyhedrosis virus from *Spodoptera litura*. J. Gen. Virol. 71, 2631–2639. https://doi.org/10.1099/0022-1317-71-11-2631
Marek, M., Merten, O.-W., Galibert, L., Vlak, J.M., van Oers, M.M., 2011. Baculovirus VP80 protein and the F-actin cytoskeleton interact and connect the viral replication factory with the nuclear periphery. J. Virol. 85, 5350–5362. https://doi.org/10.1128/jvi.00035-11
Mason, L.J., Johnson, S.J., Woodring, J., 1989. Seasonal and ontogenetic examination of the reproductive biology of *Pseudoplusia includens* (Lepidoptera: Noctuidae). Environ. Entomol. 18, 980–985. https://doi.org/10.1093/ee/18.6.980
McDougal, V. V, Guarino, L.A., 2000. The Autographa californica nuclear polyhedrosis virus *p143* gene encodes a DNA helicase. J. Virol. 74, 5273 LP – 5279. https://doi.org/10.1128/JVI.74.11.5273-5279.2000
McLachlin, J.R., Yang, S., Miller, L.K., 1998. A baculovirus mutant defective in PKIP, a protein which interacts with a virus-encoded protein kinase. Virology 246, 379–391. https://doi.org/10.1006/viro.1998.9210
Meagher, R., Goane, L., Murúa, M.G., Navarro, F., Barrionuevo, M.J., 2013. Life table studies of *Rachiplusia nu* (Guenée) and *Chrysodeixis* (= *Pseudoplusia*) *includens* (Walker) (Lepidoptera: Noctuidae) on artificial diet. Florida Entomol. 95, 944–951. https://doi.org/10.1653/024.095.0419
Miller, D.W., Miller, L.K., 1982. A virus mutant with an insertion of a copia-like transposable element. Nature 299, 562–564. https://doi.org/10.1038/299562a0
Miller, L.K., 1997. The baculoviruses. Springer Science & Business Media.
Mitchell, E.R., 1967. Life history of *Pseudoplusia includens* (Walker) (Lepidoptera : Noctuidae). J. Georg. Entomol. Soc. 2.
Monsma, S.A., Oomens, A.G., Blissard, G.W., 1996. The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. J. Virol. 70, 4607–4616.
Moonga, M.N., Davis, J.A., 2016. Partial life history of *Chrysodeixis includens* (Lepidoptera: Noctuidae) on summer hosts. J. Econ. Entomol. 109, 1713–1719. https://doi.org/10.1093/jee/tow156
Morgado, F. da S., Silva, L.A., Bernardes, L.M., Czepak, C., Strand, M.R., Ribeiro, B.M., 2020. *Trichoplusia ni* and *Chrysodeixis includens* larvae show different susceptibility to Chrysodeixis includens single nucleopolyhedrovirus per os infection. J. Pest Sci. (2004). https://doi.org/10.1007/s10340-020-01217-7
Morris, T.D., Miller, L.K., 1994. Mutational analysis of a baculovirus major late promoter. Gene 140, 147–153. https://doi.org/10.1016/0378-1119(94)90538-X
Moscardi, F., de Freitas Bueno, A., Sosa-Gómez, D.R., Roggia, S., Hoffmann-

Campo, C.B., Pomari, A.F., Corso, I.C., Yano, S.A.C., 2012. Artrópodes que atacam as folhas da soja, in: Hoffmann-Campo, C.B., Spalding, B.C.-F., Moscardi, F. (Eds.), Soja: Manejo Integrado de Insetos e Outros Artrópodes-Praga. Embrapa, Brasília, DF., pp. 209–334.

Moscardi, F., De Souza, M.L., De Castro, M.E.B., Lara Moscardi, M., Szewczyk, B., 2011. Baculovirus pesticides: Present state and future perspectives, in: Microbes and Microbial Technology: Agricultural and Environmental Applications. Springer New York, pp. 415–445. https://doi.org/10.1007/978-1-4419-7931-5_16

Moser, B.A., Becnel, J.J., White, S.E., Afonso, C., Kutish, G., Shanker, S., Almira, E., 2001. Morphological and molecular evidence that Culex nigripalpus baculovirus is an unusual member of the family Baculoviridae. J. Gen. Virol. 82, 283–297. https://doi.org/10.1099/0022-1317-82-2-283

Mu, J., van Lent, J.W.M., Smagghe, G., Wang, Y., Chen, X., Vlak, J.M., van Oers, M.M., 2014. Live imaging of baculovirus infection of midgut epithelium cells: a functional assay of per os infectivity factors (PIFs). J. Gen. Virol. https://doi.org/10.1099/vir.0.068262-0

Muñoz, D., Castillejo, J.I., Caballero, P., Munoz, 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.

Muraro, D.S., Giacomelli, T., Stacke, R.F., Godoy, D.N., Marçon, P., Popham, H.J.R., Bernardi, O., 2019. Baseline susceptibility of Brazilian populations of *Chrysodeixis includens* (Lepidoptera: Noctuidae) to C. includens nucleopolyhedrovirus and diagnostic concentration for resistance monitoring. J. Econ. Entomol. 112, 349—354. https://doi.org/10.1093/jee/toy361

Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A., Summers, M.D., 1995. Virus taxonomy: classification and nomenclature of viruses. Sixth report of the International Committee on Taxonomy of Viruses. Springer-Verlag, New York.

Musser, F.R., Catchot, A.L., Conley, S.P., Davis, J.A., DiFonzo, C., Greene, J., Lorenz, G.M., Owens, D., Reed, T., Reisig, D.D., Roberts, P., Royer, T.A., Seiter, N.J., Stewart, S.D., Taylor, S., Tilmon, K., Way, M.O., 2018. Soybean insect losses in the United States. Midsouth Entomol. 11, 1–23.

National Library of Medicine (US), N.C. for B.I., n.d. Complete genomes: Baculoviridae [WWW Document].

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., Hens, L., 2016. Chemical pesticides and human health: The urgent need for a new concept in agriculture. Front. Public Heal. 4, 1. https://doi.org/10.3389/fpubh.2016.00148

Nie, Y., Fang, M., Erlandson, M.A., Theilmann, D.A., 2012. Analysis of the Autographa californica multiple nucleopolyhedrovirus overlapping gene pair *lef3* and *ac68* reveals that AC68 is a per os infectivity factor and that LEF3 is critical, but not essential, for virus replication. J. Virol. 86, 3985–3994. https://doi.org/10.1128/jvi.06849-11

Ohkawa, T., Volkman, L.E., Welch, M.D., 2010. Actin-based motility drives baculovirus transit to the nucleus and cell surface. J. Cell Biol. 190, 187–195. https://doi.org/10.1083/jcb.201001162

Ohkawa, T., Washburn, J.O., Sitapara, R., Sid, E., Volkman, L.E., 2005. Specific binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus to midgut cells of *Heliothis virescens* larvae is mediated by products of pif genes ac119 and ac022 but not by ac115. J. Virol. 79, 15258–15264. https://doi.org/10.1128/jvi.79.24.15258-15264.2005

Ono, C., Sato, M., Taka, H., Asano, S., Matsuura, Y., Bando, H., 2015. Tightly regulated expression of Autographa californica multicapsid nucleopolyhedrovirus immediate early genes emerges from their interactions and possible collective behaviors. PLoS One 10, e0119580–e0119580. https://doi.org/10.1371/journal.pone.0119580

Oomens, A.G.P., Blissard, G.W., 1999. Requirement for GP64 to drive efficient budding of Autographa californica multicapsid nucleopolyhedrovirus. Virology 254, 297–314. https://doi.org/http://dx.doi.org/10.1006/viro.1998.9523

Ozers, M.S., Friesen, P.D., 1996. The env-like open reading frame of the baculovirus-integrated retrotransposon TED encodes a retrovirus-like envelope protein. Virology 226, 252–259. https://doi.org/10.1006/viro.1996.0653

Palma, J., Maebe, K., Guedes, J.V.C., Smagghe, G., 2015. Molecular variability and genetic structure of *Chrysodeixis includens* (Lepidoptera: Noctuidae), an important soybean defoliator in Brazil. PLoS One 10, 1–13. https://doi.org/10.1371/journal.pone.0121260

Panizzi, A.R., 2013. History and contemporary perspectives of the integrated pest management of soybean in Brazil. Neotrop. Entomol. https://doi.org/10.1007/s13744-013-0111-y

Parnell, M., Grzywacz, D., Jones, K.A., Brown, M., Oduor, G., Ong'aro, J., 2002. The strain variation and virulence of granulovirus of diamondback moth (*Plutella xylostella* Linnaeus, Lep., Yponomeutidae) isolated in Kenya. J. Invertebr. Pathol. https://doi.org/10.1016/S0022-2011(02)00001-0

Parrott, W.A., All, J.N., Adang, M.J., Bailey, M.A., Boerma, H.R., Stewart, C.N., 1994. Recovery and evaluation of soybean plants transgenic for a *Bacillus thuringiensis* var. *Kurstaki* insecticidal gene. Vitr. Cell. Dev. Biol. - Plant 30, 144–149. https://doi.org/10.1007/BF02632204

Passarelli, A., Guarino, L., 2007. Baculovirus late and very late gene regulation. Curr. Drug Targets 8, 1103–1115. https://doi.org/10.2174/138945007782151324

Pauchet, Y., Muck, A., Svatoš, A., Heckel, D.G., Preiss, S., 2008. Mapping the larval midgut lumen proteome of *Helicoverpa armigera*, a generalist herbivorous insect. J. Proteome Res. 7, 1629–1639. https://doi.org/10.1021/pr7006208

Pearson, M.N., Rohrmann, G.F., 2002. Transfer, incorporation, and substitution of envelope fusion proteins among members of the *Baculoviridae*, *Orthomyxoviridae*, and *Metaviridae* (insect retrovirus)

families. J. Virol. 76, 5301–5304. https://doi.org/10.1128/jvi.76.11.5301-5304.2002
Peng, K., van Oers, M.M., Hu, Z., van Lent, J.W.M., Vlak, J.M., 2010. Baculovirus per os infectivity factors form a complex on the surface of occlusion-derived virus. J. Virol. 84, 9497–9504. https://doi.org/10.1128/jvi.00812-10
Pereira, R.R., Neves, D.V.C., Campos, J.N., Santana, P.A., Hunt, T.E., Picanço, M.C., 2018. Natural biological control of *Chrysodeixis includens*. Bull. Entomol. Res. 108, 831–842. https://doi.org/10.1017/S000748531800007X
Pijlman, G.P., Pruijssers, A.J.P., Vlak, J.M., 2003. Identification of pif-2, a third conserved baculovirus gene required for per os infection of insects. J. Gen. Virol. 84, 2041–2049. https://doi.org/10.1099/vir.0.19133-0
Pijlman, G.P., Van Den Born, E., Martens, D.E., Vlak, J.M., 2001. Autographa californica baculoviruses with large genomic deletions are rapidly generated in infected insect cells. Virology 283, 132–138. https://doi.org/10.1006/viro.2001.0854
Pinhey, E.C.G., 1975. Moths of Southern Africa : descriptions and colour illustrations of 1183 species, 1st ed. ed. Tafelberg, Cape Town.
Pogue, M.G., 2005. The plusiinae (lepidoptera: noctuidae) of Great Smoky Mountains National Park, USA. Zootaxa 28, 1–75. https://doi.org/10.11646/zootaxa.2380.1.1
Possee, R.D., King, L.A., 2014. Insect viruses. eLS, Major Reference Works. https://doi.org/doi:10.1002/9780470015902.a0020712.pub2
Pritchett, D.W., Young, S.Y., Yearian, W.C., 1984. Some factors involved in the dissolution of Autographa californica nuclear polyhedrosis virus polyhedra by digestive fluids of *Trichoplusia ni* larvae. J. Invertebr. Pathol. 43, 160–168. https://doi.org/10.1016/0022-2011(84)90134-4
Rahman, M.M., Gopinathan, K.P., 2004. Systemic and in vitro infection process of Bombyx mori nucleopolyhedrovirus. Virus Res. 101, 109–118. https://doi.org/https://doi.org/10.1016/j.virusres.2003.12.027
Reid, J.C., Greene, G.L., 1973. The soybean looper: pupal weight, development time, and consumption of soybean foliage. Florida Entomol. 56, 203. https://doi.org/10.2307/3493125
Rodems, S.M., Friesen, P.D., 1993. The hr5 transcriptional enhancer stimulates early expression from the Autographa californica nuclear polyhedrosis virus genome but is not required for virus replication. J. Virol. 67, 5776–85.
Rohrmann, G., 2019. Baculovirus Molecular Biology 4th Edition, Bethesda (MD): National Center for Biotechnology Information (US).
Rosinski, M., Reid, S., Nielsen, L.K., 2002. Kinetics of baculovirus replication and release using real-time quantitative polymerase chain reaction. Biotechnol. Bioeng. 77, 476–480. https://doi.org/10.1002/bit.10126
Rossignol, P.A., 1997. Biology of the insect midgut. Parasitol. Today 13, 202. https://doi.org/10.1016/s0169-4758(97)84915-0
Sanches, M.M., Sihler, W., Silva, C.E.P., Guimarães, G.C., Benito, N.P., Sosa-Gómez, D.R., de Souza, M.L., 2019. Characterization of a Chrysodeixis includens nucleopolyhedrovirus isolate from Brazilian cerrado and

assessment of its co-infection with Anticarsia gemmatalis multiple nucleopolyhedrovirus. Brazilian Arch. Biol. Technol. 62, 1–17. https://doi.org/10.1590/1678-4324-2019180688

Sanjuán, R., Domingo-Calap, P., 2016. Mechanisms of viral mutation. Cell. Mol. Life Sci. 73, 4433–4448. https://doi.org/10.1007/s00018-016-2299-6

Schowalter, T.D., 2013. Insects and sustainability of ecosystem services, CRC Press. https://doi.org/10.1201/b14911

Schünemann, R., Knaak, N., Fiuza, L.M., 2014. Mode of action and specificity of *Bacillus thuringiensis* toxins in the control of caterpillars and stink bugs in soybean culture. ISRN Microbiol. 2014, 1–12. https://doi.org/10.1155/2014/135675

Serrano, A., Williams, T., Simón, O., López-Ferber, M., Caballero, P., Muñoz, D., 2013. Analagous population structures for two alphabaculoviruses highlight a functional role for deletion mutants. Appl. Environ. Microbiol. 79, 1118–1125. https://doi.org/10.1128/AEM.03021-12

Shao, Y., Chen, B., Sun, C., Ishida, K., Hertweck, C., Boland, W., 2017. Symbiont-derived antimicrobials contribute to the control of the lepidopteran gut microbiota. Cell Chem. Biol. 24, 66–75. https://doi.org/10.1016/j.chembiol.2016.11.015

Shapiro, D.I., Fuxa, J.R., Braymer, H.D., Pashley, D.P., 1991. DNA restriction polymorphism in wild isolates of Spodoptera frugiperda nuclear polyhedrosis virus. J. Invertebr. Pathol. 58, 96–105.

Shi, S.-L., Jiang, Y.-R., Yang, R.-S., Wang, Y., Qin, L., 2016. Codon usage in Alphabaculovirus and Betabaculovirus hosted by the same insect species is weak, selection dominated and exhibits no more similar patterns than expected. Infect. Genet. Evol. 44, 412–417. https://doi.org/https://doi.org/10.1016/j.meegid.2016.07.042

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

Slack, J., Arif, B.M., 2007. The baculoviruses occlusion-derived virus: Virion structure and function. Adv. Virus Res. 69, 99–165. https://doi.org/10.1016/s0065-3527(06)69003-9

Slavicek, J.M., 2012. Baculovirus enhancins and their role in viral pathogenicity, in: Adoga, M.M. (Ed.), Molecular Virology. Rijeka, Croatia. https://doi.org/10.5772/33285

Smith, G.E., Summers, M.D., 1978. Analysis of baculovirus genomes with restriction endonucleases. Virology 89, 517–527. https://doi.org/10.1016/0042-6822(78)90193-9

Smith, R.H., Freeman, B., Foshee, W., 1994. Soybean loopers: Late season foliage feeders on cotton. Alabama Ext.

Sosa-Gomez, D.R., 2005. Seletividade de agroquímicos para fungos entomopatogênicos. Embrapa Soja-Outras publicações científicas (ALICE).

Sosa-Gómez, D.R., Corrêa-Ferreira, B.S., Hoffmann-Campo, C.B., Corso, I.C.,

Oliveira, L.J., Moscardi, F., Panizzi, A.R., Bueno, A. de F., Hirose, E., Roggia, S., 2014. Manual de identificação de insetos e outros invertebrados da cultura da soja. Embrapa Soja-Documentos (INFOTECA-E).

Sosa-Gómez, D.R., Delpin, K.E., Moscardi, F., Nozaki, M. de H., 2003. The impact of fungicides on Nomuraea rileyi (Farlow) Samson epizootics and on populations of *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), on soybean. Neotrop. Entomol. 32, 287–291. https://doi.org/10.1590/S1519-566X2003000200014

Sosa Gomez, D.R., Omoto, C., 2012. Resistência a inseticidas e outros agentes de controle em antrópodes associados à cultura da soja, in: Soja - Manejo Integrado de Insetos e Outros Artrópodes-Praga. EMBRAPA.

Sparks, W.O., Harrison, R.L., Bonning, B.C., 2011. Autographa californica multiple nucleopolyhedrovirus ODV-E56 is a per os infectivity factor, but is not essential for binding and fusion of occlusion-derived virus to the host midgut. Virology 409, 69–76. https://doi.org/http://dx.doi.org/10.1016/j.virol.2010.09.027

Specht, A., de Paula-Moraes, S.V., Sosa-Gómez, D.R., 2015. Host plants of *Chrysodeixis includens* (Walker) (Lepidoptera, Noctuidae, Plusiinae). Rev. Bras. Entomol. 59, 343–345. https://doi.org/10.1016/j.rbe.2015.09.002

Specht, A., Sosa-Gómez, D.R., Roque-Specht, V.F., Valduga, E., Gonzatti, F., Schuh, S.M., Carneiro, E., 2019. Biotic potential and life tables of *Chrysodeixis includens* (Lepidoptera: Noctuidae), *Rachiplusia nu*, and *Trichoplusia ni* on soybean and forage turnip. J. Insect Sci. 19, 8. https://doi.org/10.1093/jisesa/iez072

Stacke, R.F., Giacomelli, T., Bronzatto, E.S., Halberstadt, S.A., Garlet, C.G., Muraro, D.S., Guedes, J.V.C., Bernardi, O., 2019. Susceptibility of brazilian populations of Chrysodeixis includens (Lepidoptera: Noctuidae) to selected insecticides. J. Econ. Entomol. 112, 1378–1387. https://doi.org/10.1093/jee/toz031

Strand, M.R., 1990. Characterization of larval development in *Pseudoplusia includens* (Lepidoptera: Noctuidae). Ann. Entomol. Soc. Am. 83, 538–544. https://doi.org/10.1093/aesa/83.3.538

Sugiura, N., Setoyama, Y., Chiba, M., Kimata, K., Watanabe, H., 2011. Baculovirus Envelope Protein ODV-E66 Is a Novel Chondroitinase with Distinct Substrate Specificity. J. Biol. Chem. 286, 29026–29034. https://doi.org/10.1074/jbc.M111.251157

Summers, M.D., 1971. Electron microscopic observations on granulosis virus entry, uncoating and replication processes during infection of the midgut cells of *Trichoplusia ni*. J. Ultrasructure Res. 35, 606–625. https://doi.org/10.1016/S0022-5320(71)80014-X

Summers, M.D., Smith, G.E., Knell, J.D., Burand, J.P., 1980. Physical maps of Autographa californica and Rachiplusia ou nuclear polyhedrosis virus recombinants. J. Virol. 34, 693–703. https://doi.org/10.1128/jvi.34.3.693-703.1980

Szewczyk, B., Hoyos-Carvajal, L., Paluszek, M., Skrzecz, I., Lobo de Souza, M., 2006. Baculoviruses: re-emerging biopesticides. Biotechnol. Adv. 24,

143–160. https://doi.org/S0734-9750(05)00119-9 [pii]10.1016/j.biotechadv.2005.09.001 [doi]
Tabashnik, B.E., Brévault, T., Carrière, Y., 2013. Insect resistance to Bt crops: Lessons from the first billion acres. Nat. Biotechnol. https://doi.org/10.1038/nbt.2597
Terra, W.R., 2001. The origin and functions of the insect peritrophic membrane and peritrophic gel. Arch. Insect Biochem. Physiol. 47, 47–61. https://doi.org/10.1002/arch.1036
Theilmann, D.A., Stewart, S., 1992. Tandemly repeated sequence at the 3′ end of the IE-2 gene of the baculovirus Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus is an enhancer element. Virology 187, 97–106. https://doi.org/10.1016/0042-6822(92)90298-4
Thézé, J., Lopez-Vaamonde, C., Cory, J.S., Herniou, E.A., 2018. Biodiversity, evolution and ecological specialization of baculoviruses: A treasure trove for future applied research. Viruses 10, 366. https://doi.org/10.3390/v10070366
Thézé, J., Takatsuka, J., Nakai, M., Arif, B., Herniou, E.A., 2015. Gene acquisition convergence between entomopoxviruses and baculoviruses. Viruses 7, 1960–1974. https://doi.org/10.3390/v7041960
Thiem, S.M., Miller, L.K., 1989. Identification, sequence, and transcriptional mapping of the major capsid protein gene of the baculovirus Autographa californica nuclear polyhedrosis virus. J. Virol. 63, 2008–2018.
Thumbi, D.K., Béliveau, C., Cusson, M., Lapointe, R., Lucarotti, C.J., 2013. Comparative genome sequence analysis of Choristoneura occidentalis Freeman and C. rosaceana Harris (Lepidoptera: Tortricidae) Alphabaculoviruses. PLoS One 8. https://doi.org/10.1371/journal.pone.0068968
Tingle, F.C., Mitchell, E.R., 1977. Seasonal populations of armyworms and loopers at Hastings, Florida. Florida Entomol. 60, 115. https://doi.org/10.2307/3494389
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, 2307–2317.
Trentin, L.B., Santos, E.R., Oliveira Junior, A.G., Sosa-Gómez, D.R., Ribeiro, B.M., Ardisson-Araújo, D.M.P., 2019. The complete genome of Rachiplusia nu nucleopolyhedrovirus (RanuNPV) and the identification of a baculoviral CPD-photolyase homolog. Virology 534, 64–71. https://doi.org/10.1016/j.virol.2019.05.019
Trichilo, P.J., Mack, T.P., 2015. Soybean leaf consumption by the soybean looper (Lepidoptera: Noctuidae) as a function of temperature, instar, and larval weight. J. Econ. Entomol. 82, 633–638. https://doi.org/10.1093/jee/82.2.633
Tumlinson, J.H., Mitchell, E.R., Browner, S.M., Lindquist, D.A., 1972. A Sex Pheromone for the Soybean Looper. Environ. Entomol. 1, 466–468. https://doi.org/10.1093/ee/1.4.466
United States Department of Agriculture, Foreign Agricultural Service, 2020. World Agrcultural Production [WWW Document].
Urbano, P., Urbano, F.G., 1994. The Reoviridae family. Comp. Immunol.

Microbiol. Infect. Dis. 17, 151–161. https://doi.org/10.1016/0147-9571(94)90040-X

Van Oers, M.M., Abma-Henkens, M.H.C., Herniou, E.A., de Groot, J.C.W., Peters, S., Vlak, J.M., 2005. Genome sequence of Chrysodeixis chalcites nucleopolyhedrovirus, a baculovirus with two DNA photolyase genes. J. Gen. Virol. 86, 2069–2080. https://doi.org/10.1099/vir.0.80964-0

Van Oers, M.M., Herniou, E.A., Usmany, M., Messelink, G.J., Vlak, J.M., 2004. Identification and characterization of a DNA photolyase-containing baculovirus from Chrysodeixis chalcites. Virology 330, 460–470. https://doi.org/10.1016/j.virol.2004.09.032

Van Oers, M.M., Vlak, J.M., 2007. Baculovirus genomics. Curr. Drug Targets 8, 1051–1068. https://doi.org/10.2174/138945007782151333

Vázquez, W., 1988. Biologia comparada de *Pseudoplusia includens* (Walker, 1857) (Lepidóptera: Noctuidae) em dietas naturais e artificiais e efeito de um vírus de poliedrose nuclear na sua mortalidade e no consumo da área foliar da soja. Universidade de São Paulo, Piracicaba.

Ventura, M.U., Roberto, S.R., Hoshino, A.T., Carvalho, M.G., Hata, F.T., Genta, W., 2015. First record of *Heliothis virescens* (Lepidoptera: Noctuidae) damaging table grape bunches. Florida Entomol. 98, 783–786. https://doi.org/10.1653/024.098.0259

Vialard, J.E., Richardson, C.D., 1993. The 1,629-nucleotide open reading frame located downstream of the Autographa californica nuclear polyhedrosis virus polyhedrin gene encodes a nucleocapsid-associated phosphoprotein. J. Virol. 67, 5859–5866. https://doi.org/10.1128/jvi.67.10.5859-5866.1993

Vickers, J.M., Cory, J.S., Entwistle, P.F., 1991. DNA characterization of eight geographic isolates of granulosis virus from the potato tuber moth (*Phthorimaea operculella*) (lepidoptera, gelechiidae). J. Invertebr. Pathol. 57, 334–342. https://doi.org/10.1016/0022-2011(91)90137-F

Volkman, L.E., Summers, M.D., 1977. Autographa californica nuclear polyhedrosis virus: Comparative infectivity of the occluded, alkali-liberated, and nonoccluded forms. J. Invertebr. Pathol. 30, 102–103. https://doi.org/https://doi.org/10.1016/0022-2011(77)90045-3

Volkman, L.E., Summers, M.D., Hsieh, C.H., 1976. Occluded and nonoccluded nuclear polyhedrosis virus grown in *Trichoplusia ni*: comparative neutralization comparative infectivity, and in vitro growth studies. J. Virol. 19, 820–832.

Votteler, J., Sundquist, W.I., 2013. Virus budding and the ESCRT pathway. Cell Host Microbe. https://doi.org/10.1016/j.chom.2013.08.012

Wang, J., Hou, D., Wang, Q., Kuang, W., Zhang, L., Li, J., Shen, S., Deng, F., Wang, H., Hu, Z., Wang, M., 2018. Genome analysis of a novel Group I alphabaculovirus obtained from *Oxyplax ochracea*. PLoS One 13, e0192279. https://doi.org/10.1371/journal.pone.0192279

Wang, M., Tan, Y., Yin, F., Deng, F., Vlak, J.M., Hu, Z., Wang, H., 2008. The F protein of Helicoverpa armigera single nucleopolyhedrovirus can be substituted functionally with its homologue from Spodoptera exigua multiple nucleopolyhedrovirus. J. Gen. Virol. 89, 791–798. https://doi.org/10.1099/vir.0.83466-0

Wang, M., Wang, J., Yin, F., Tan, Y., Deng, F., Chen, X., Jehle, J.A., Vlak, J.M., Hu, Z., Wang, H., 2014. Unraveling the entry mechanism of baculoviruses and its evolutionary implications. J. Virol. 88, 2301–2311. https://doi.org/10.1128/JVI.03204-13

Wang, P., Granados, R.R., 1997. An intestinal mucin is the target substrate for a baculovirus enhancin. Proc. Natl. Acad. Sci. U. S. A. 94, 6977–6982.

Wang, P., Hammer, D.A., Granados, R.R., 1994. Interaction of Trichoplusia ni granulosis virus-encoded enhancin with the midgut epithelium and peritrophic membrane of four lepidopteran insects. J. Gen. Virol. 75, 1961–1967. https://doi.org/10.1099/0022-1317-75-8-1961

Wang, Qiushi, Bosch, B.J., Vlak, J., Van Oers, M., Rottier, P., Lent, J., 2015. Budded baculovirus particle structure revisited. J. Invertebr. Pathol. 134. https://doi.org/10.1016/j.jip.2015.12.001

Wang, Q., Vlak, J.M., Rottier, P.J.M., Oers, M.M. van, Bosch, B.J., 2015. Structural and functional analysis of the baculovirus envelope fusion protein F. Wageningen University, Wageningen.

Wang, R., Deng, F., Hou, D., Zhao, Y., Guo, L., Wang, H., Hu, Z., 2010. Proteomics of the Autographa californica nucleopolyhedrovirus budded virions. J. Virol. 84, 7233–7242. https://doi.org/JVI.00040-10 [pii]10.1128/JVI.00040-10 [doi]

Wang, X., Liu, X., Makalliwa, G.A., Li, J., Wang, H., Hu, Z., Wang, M., 2017. Per os infectivity factors: a complicated and evolutionarily conserved entry machinery of baculovirus. Sci. China Life Sci. https://doi.org/10.1007/s11427-017-9127-1

Wang, X., Shang, Y., Chen, C., Liu, S., Chang, M., Zhang, N., Hu, H., Zhang, F., Zhang, T., Wang, Z., Liu, X., Lin, Z., Deng, F., Wang, H., Zou, Z., Vlak, J.M., Wang, M., Hu, Z., 2019. Baculovirus Per Os Infectivity Factor Complex: Components and Assembly. J. Virol. 93. https://doi.org/10.1128/JVI.02053-18

Washburn, J.O., Lyons, E.H., Haas-Stapleton, E.J., Volkman, L.E., 1999. Multiple nucleocapsid packaging of Autographa californica nucleopolyhedrovirus accelerates the onset of systemic infection in *Trichoplusia ni*. J. Virol. 73, 411–416.

Wennmann, J.T., Keilwagen, J., Jehle, J.A., 2018. Baculovirus Kimura two-parameter species demarcation criterion is confirmed by the distances of 38 core gene nucleotide sequences. J. Gen. Virol. 99, 1307–1320.

Westenberg, M., Vlak, J.M., 2008. GP64 of group I nucleopolyhedroviruses cannot readily rescue infectivity of group II F-null nucleopolyhedroviruses. J. Gen. Virol. 89, 424–431.

Wille, P.E., Pereira, B.A., Wille, C.L., Restelatto, S.S., Boff, M.I.C., Franco, C.R., 2017. Natural resistance of soybean cultivars to the soybean looper larva *Chrysodeixis includens* (Lepidoptera: Noctuidae). Pesqui. Agropecu. Bras. 52, 18–25. https://doi.org/10.1590/S0100-204X2017000100003

Williams, T., 2018. Viruses, in: Hajek, A.E., Shapiro-Ilan, D.I. (Eds.), Ecology of Invertebrate Diseases. John Wiley, Chichester, UK, pp. 213–285. https://doi.org/10.1002/9781119256106.ch7

Williams, G. V., Faulkner, P., 1997. Cytological changes and viral

morphogenesis during baculovirus infection, in: The Baculoviruses. Springer US, pp. 61–107. https://doi.org/10.1007/978-1-4899-1834-5_4
Willis, L.G., Siepp, R., Stewart, T.M., Erlandson, M.A., Theilmann, D.A., Theilmann, D.A., 2005. Sequence analysis of the complete genome of Trichoplusia ni single nucleopolyhedrovirus and the identification of a baculoviral photolyase gene. Virology 338, 209–226. https://doi.org/10.1016/j.virol.2005.04.041
Winstanley, D., Rovesti, L., 1993. Insect viruses as biocontrol agents, in: Jones D.G. (Ed.), Exploitation of Microorganisms. Springer Netherlands, pp. 105–136. https://doi.org/10.1007/978-94-011-1532-2_5
Wu, W., Lin, T., Pan, L., Yu, M., Li, Z., Pang, Y., Yang, K., 2006. Autographa californica multiple nucleopolyhedrovirus nucleocapsid assembly is interrupted upon deletion of the 38K gene. J. Virol. 80, 11475–11485. https://doi.org/10.1128/jvi.01155-06
Xu, F., Vlak, J.M., van Oers, M.M., 2008. Conservation of DNA photolyase genes in group II nucleopolyhedroviruses infecting plusiine insects. Virus Res. 136, 58–64. https://doi.org/10.1016/j.virusres.2008.04.017
Xue, S., Hua, B.Z., 2018. Morphology of egg and larva of the semi-looper Autographa nigrisigna (Walker) (Lepidoptera: Noctuidae: Plusiinae). Zool. Anz. https://doi.org/10.1016/j.jcz.2018.08.004
Yue, Q., Yu, Q., Yang, Q., Xu, Y., Guo, Y., Blissard, G.W., Li, Z., 2017. Distinct roles of cellular ESCRT-I and ESCRT-III proteins in efficient entry and egress of budded virions of Autographa californica multiple nucleopolyhedrovirus. J. Virol. 92. https://doi.org/10.1128/jvi.01636-17
Zambon, R.A., Nandakumar, M., Vakharia, V.N., Wu, L.P., 2005. The Toll pathway is important for an antiviral response in *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 102, 7257–7262. https://doi.org/10.1073/pnas.0409181102
Zanardo Botelho, A.B.R., da Silva, I.F., Ávila, C.J., 2019. Control of *Chrysodeixis includens* (Lepidoptera: Noctuidae) using Chin-IA (I-A) isolate as integrate component of management in soybean crops. Arq. Inst. Biol. (Sao. Paulo). 86. https://doi.org/10.1590/1808-1657000712018
Zanotto, P.M. de A., Kessing, B.D., Maruniak, J.E., 1993. Phylogenetic interrelationships among baculoviruses: evolutionary rates and host associations. J. Invertebr. Pathol. 62, 147–164. https://doi.org/http://dx.doi.org/10.1006/jipa.1993.1090
Zemskov, E.A., Kang, W., Maeda, S., 2000. Evidence for nucleic acid binding ability and nucleosome association of Bombyx mori Nucleopolyhedrovirus BRO proteins. J. Virol. 74, 6784–6789. https://doi.org/10.1128/jvi.74.15.6784-6789.2000
Zhao, S., He, G., Yang, Y., Liang, C., 2019. Nucleocapsid assembly of baculoviruses. Viruses 11. https://doi.org/10.3390/v11070595
Zuidema, D., Klinge-Roode, E.C., Van Lent, J.W.M., Vlak, J.M., 1989. Construction and analysis of an Autographa californica nuclear polyhedrosis virus mutant lacking the polyhedral envelope. Virology 173, 98–108. https://doi.org/10.1016/0042-6822(89)90225-0
Zulin, D., Ávila, C.J., Schlick-Souza, E.C., 2018. Population fluctuation and

vertical distribution of the soybean looper *Chrysodeixis includens* in soybean culture. Am. J. Plant Sci. 09, 1544–1556. https://doi.org/10.4236/ajps.2018.97113

CHAPTER II

Genetic variability of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) and the insecticidal characteristics of selected genotypic variants

ABSTRACT

Genetic variation in baculoviruses is recognized as a key factor, not only due to the influence of such variation on pathogen transmission and virulence traits, but also because genetic variants can form the basis for novel biological insecticides. In this study, we examined the genetic variability of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) present in field isolates obtained from virus-killed larvae. Different ChinNPV strains were identified by restriction endonuclease analysis, from which genetic variants were isolated by plaque assay. Biological characterization studies were based on pathogenicity, median time to death (MTD), and viral occlusion body (OB) production (OBs/larva). Nine different isolates were obtained from eleven virus-killed larvae collected from fields of soybean in Mexico. An equimolar mixture of these isolates, named ChinNPV-Mex1, showed good insecticidal properties and yielded 23 genetic variants by plaque assay, one of which (ChinNPV-R) caused the highest mortality in second instars of *C. includens*. Five of these variants were selected: ChinNPV-F, ChinNPV-J, ChinNPV-K, ChinNPV-R, and ChinNPV-V. No differences in median time to death were found between them, while ChinNPV-F, ChinNPV-K, ChinNPV-R and ChinNPV-V were more productive than ChinNPV-J and the original mixture of field isolates ChinNPV-Mex1. These results demonstrate the high variability present in natural populations of this virus and support the use of these new genetic variants as promising active substances for baculovirus-based bioinsecticides.

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1. INTRODUCTION

Genetic variability within baculoviruses is strongly related to host-pathogen interactions within each insect during viral infection. The genetic structure of baculovirus populations arises from the need to productively infect genetically diverse host insects and maximize the production of progeny viruses in the host to improve the likelihood of subsequent transmission (Cory and Myers, 2003; Hitchman et al., 2007). This variation in the genetic variants that conform baculovirus isolates is generated by multiple mechanisms, including point mutation, insertion and deletion, recombination and horizontal gene transfer. As a consequence, baculovirus isolates comprise mixtures of genetic variants. This allows variants to cooperate and complete among themselves, in interactions that modulate the survival of these viruses in nature (Ikeda et al., 2015; Williams, 2018).

Variation in genomic DNA is expressed phenotypically in differences in pathogenicity, speed of kill, the production of viral occlusion bodies (OBs), and even the mode of transmission of the pathogen to other susceptible hosts (horizontal transmission) or to the offspring (vertical transmission) (Cabodevilla et al., 2011; Cory et al., 2005; Harrison et al., 2008; Kamiya et al., 2004; Stiles and Himmerich, 1998). These traits are clearly important for virus transmission because they determine the likelihood of the host acquiring an infection (Williams, 2018).

Genotypic and phenotypic diversity have been widely reported for baculovirus isolates from naturally-infected insects. This has involved the characterization of different geographical isolates of the same virus species (Williams et al., 2011) and also the finding that individual isolates frequently comprise a mixture of genotypes (Cory et al., 2005; Redman et al., 2010; Rowley et al., 2011). In fact, field isolates of baculoviruses generally consist of a number of genotypic variants, which are easily distinguished by restriction endonuclease (REN) analysis (Harrison, 2009; Laviña et al., 2001). This high degree of genetic variability was first described for Autographa californica multiple nucleopolyhedrovirus (AcMNPV) (Lee and Miller, 1978) and then for

a variety of additional species, including Panolis flammea NPV (Weitzman et al., 1992), Spodoptera exigua MNPV (Muñoz et al., 1999), Spodoptera frugiperda MNPV (Simón et al., 2004) and Chrysodeixis chalcites NPV (Bernal et al., 2013a), among others.

Diversity within baculoviruses is also important in the development of these viruses as the active ingredients of biological insecticides. Artificial mixtures of selected genotypes have been developed for effective control of different insect pests. These mixtures had useful insecticidal characteristics such as high pathogenicity, virulence, and OB production (Caballero et al., 2009; P Caballero et al., 2013; Simón et al., 2016). In several cases, the mixtures had improved insecticidal properties over those of the component genotypes or the original field isolates from which they were obtained (Arrizubieta et al., 2015; Bernal et al., 2013a). Genetic diversity in the virus population can also be a valuable tool to avoid the development or overcome resistance in the pest population. For example, multiple resistance mechanisms have been identified in *Cydia pomonella* following extensive use of granulovirus-based insecticides (Gebhardt et al., 2014; Jehle et al., 2017). The use of different CpGV genotypes for pest control and insect resistance monitoring are important as individual CpGV variants may fail to control pest populations in certain orchards, which require the use genetically-distinct virus variants (Jehle et al., 2017).

The soybean looper, *Chrysodeixis includens* (Lepidoptera: Noctuidae), is an important agricultural pest that occurs from the northern United States to southern South America. The larvae feed on a range of crop plants, including soybeans, beans, cotton, sunflower, tomato, and potato (Eichlin and Cunningham, 1978). This pest is naturally infected by Chrysodeixis includens nucleopolyhedrovirus (ChinNPV), a single-nucleocapsid NPV that is classified as a group II alphabaculovirus (Alexandre et al., 2010; Thézé et al., 2018; Wennmann et al., 2018). This highly virulent virus could be a promising biological insecticide for control of *C. includens* (Alexandre et al., 2010), particularly given that resistance to chemical insecticides in this pest has become an issue of major concern in

some regions (Felland et al., 1990; Leonard et al., 2015; Mascarenhas and Boethel, 1997; Mink and Boethel, 1992). As a result, attention has shifted toward alternative control measures against *C. includens*, including the characterization of different ChinNPV isolates (Alexandre et al., 2010; Craveiro et al., 2016), with the aim of obtaining novel and genetically diverse variants that may be employed as the basis for an effective biological insecticide.

In this study, we aimed to evaluate the genetic variability of ChinNPV variants present in natural isolates of this virus and their contribution to virus biological activity. We also determined the biological properties of a selection of genotypes that could contribute to the insecticidal properties of the natural field isolates. We conclude that a selection of these variants could constitute the main ingredient of a biological insecticide targeted at this pest.

2. MATERIAL AND METHODS

2.1 Insects, cells, and viruses

A laboratory colony of *C. includens* was established from larvae collected in soya fields of Tamaulipas, Mexico and maintained under controlled conditions at 25 ± 1 °C, 75% relative humidity (RH) and 16 h light: 8 h dark photoperiod. Larvae were fed a wheatgerm-based semisynthetic diet (Greene et al., 1976). HighFive cells from *Trichoplusia ni* (ThermoFisher Scientific, Waltham, MA, USA) were maintained in TNM-FH medium (Gibco, Life technologies Ltd, Inchinnan, Renfrew, UK) with 10% fetal bovine serum (Gibco) at 28 °C.

Isolates of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) used in this study were obtained from individual *C. includens* larvae that died showing the typical signs of nucleopolyhedrovirus infection. Diseased larvae were collected in 2014 from soya fields in Tamaulipas in north-eastern Mexico, during studies on another soya pest, *Anticarsia gemmatalis* (Del-Angel et al., 2018). A total of 105 larvae of *C. includens* were collected in the experimental

field station of “Las Huastecas” located in Tamaulipas state, Mexico. Twenty-one of these larvae, representing 20% of the collected larvae, died of polyhedrosis. To purify viral OBs, each virus-killed larva was filtered through muslin and centrifuged with 0.1% SDS several times to eliminate insect debris. The resulting pellets were washed in distilled water and finally resuspended in milli-Q water. OB concentrations were determined using an improved hemocytometer (Hawksley Ltd., Lancing, UK) under phase-contrast microscopy. Purified OBs were stored at 4 °C until required.

2.2 Viral DNA extraction and restriction endonuclease analysis

Virions were released from OBs by incubating 100 µL of OB suspension (10^9 OBs/mL) with 100 µL of 0.5 M Na_2CO_3, 50 µL 10% SDS and 250 µL distilled water at 60 °C during 10 min. The suspension was centrifuged at 6000× g for 5 min and the supernatant containing the virions was transferred to a new 1.5 mL microcentrifuge tube and incubated for 45 min at 50 °C with 25 µL proteinase K (20 mg/mL). Viral DNA was then separated from proteins twice with an equal volume of phenol and once with an equal volume of chloroform and precipitated from the aqueous phase using 2.5 volumes of ice-cold absolute ethanol for 10 min at 12,000× g. Pelleted DNA was washed twice with 70% ice-cold ethanol and resuspended in 50 µL 0.1 × TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). Final DNA concentration was assessed using a Nanodrop One (ThermoFisher Scientific) spectrophotometer.

For restriction endonuclease (REN) analysis, 2 µg of viral DNA of each isolate were digested with BamHI, BglII, EcoRI, HindIII, and PstI (NEB Ltd., Hitchin, UK) for 4 h at 37 °C. The reactions were stopped by mixing with 4 µL of Gel Loading Dye buffer solution (6×, NEB Ltd., UK). Fragments were then separated by electrophoresis in 1% agarose gel immersed in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.0) running at 18 V for 15 h. DNA was stained with ethidium bromide and visualized on a UV transilluminator.

2.3 Construction of the ChinNPV-Mex1 mixture and its biological characterization

A sample of 10^8 OBs of each isolate was obtained from virus-killed field-collected larvae. These samples were used to prepare an equimolar mixture, in terms of OBs, of the nine isolates that we named the ChinNPV-Mex1 mixture.

Initially, to examine the host range of this virus, the ChinNPV-Mex1 mixture was used to inoculate eleven pestiferous lepidopteran species that we had in culture in the UPNA Insectary in Pamplona, Spain, namely: *Anticarsia gemmatalis*, *Chrysodeixis chalcites*, *Chrysodeixis includens*, *Helicoverpa armigera*, *Lobesia botrana*, *Mamestra brassicae*, *Spodoptera eridania*, *Spodoptera exigua*, *Spodoptera frugiperda*, *Spodoptera littoralis* and *Trichoplusia ni*. Two high concentrations of inoculum were chosen, 5×10^6 and 5×10^8 OBs/mL in order to ensure virus-induced mortality in the susceptible species. Inocula were administered to newly-molted second instars of each species by the droplet feeding method (Hughes and Wood, 1987). Groups of 24 larvae were inoculated with each concentration. Larvae that drank the OB suspensions within 10 min were individualized in 24-well plates with a piece of semi-synthetic diet. Control larvae drank a solution of sucrose and food dye containing no OBs. Mortality was recorded every 24 h until death or pupation. Results were classified as "non-permissive" (N.P.), or pathogenic in a range from 1+ to 4+, from less pathogenic to highly pathogenic, according to the prevalence of mortality. For susceptible species that experienced mortality in the previous test, 24 newly-molted fourth instars were allowed to drink a suspension of 10^8 OB/mL of the ChinNPV-Mex1 mixture of isolates. Larvae were then individualized in 24-well plates with semi-synthetic diet. Larvae that died of polyhedrosis were recovered. OBs were purified and DNA was extracted and submitted to REN analyses, as described in section 2.2, to check the identity of the virus that caused death.

For the biological characterization of the ChinNPV-Mex1 mixture, pathogenicity expressed as 50% lethal concentration (LC_{50}), was determined

by the droplet feeding method (Hughes and Wood, 1987). Newly-molted second instars of *C. includens* were allowed to drink OB suspensions containing 100 mg/mL sucrose and 0.05 mg/mL Fluorella Blue food dye and one of the following five OB concentrations: 1.2×10^3, 6.2×10^3, 3.1×10^4, 1.6×10^5, and 7.8×10^5 OBs/mL, which were expected to cause between 10% and 90% mortality according to results obtained in preliminary tests. Groups of 24 larvae were inoculated with each OB concentration. Those larvae that drank the viral suspension within 10 min were transferred individually to 24-well plates with a piece of semi-synthetic diet. Control larvae drank a solution of sucrose and food dye containing no OBs. Larvae were incubated at 25 ± 1 °C and 75% relative humidity. Virus mortality was recorded every 24 h until larvae had died or pupated. The experiment was performed on three occasions using different batches of insects. Concentration-mortality data were subjected to Probit analysis using the POLO-PC program (LeOra Software, 2003).

The median time to death (MTD) was determined in *C. includens* second instars that were allowed to consume an LC_{90} of ChinNPV-Mex1 OBs, previously determined as 2.3×10^6 OBs/mL. Groups of 24 larvae were inoculated by droplet feeding method, individualized on semi-synthetic diet as described in the LC_{50} bioassay, and were incubated at 25 ± 1 °C. Virus mortality was recorded at 8 h intervals until death or pupation. Control larvae were treated identically but did not consume OBs. The experiment was performed on three occasions using different batches of insects. Before performing a statistical analysis, the results were compared with the available survival time distributions, using the Akaike Information Criterion (AIC) in order to choose the most suitable distribution in R (2019). Data were subsequently analysed by Kaplan–Meier distribution using SPSS v25.0 (IBM Corporation, 2011).

OB production was determined in *C. includens* fifth instars inoculated with an LC_{99} of ChinNPV-Mex1 (10^8 OBs/mL) by the droplet feeding method. The experiment was performed three times using different batches of insects. Each dying larva was collected in a 1.5 mL microcentrifuge tube to prevent OB loss due to liquefaction of the larva and homogenized in a total volume of 1 mL

with distilled water. OBs from 20 randomly-selected larvae per treatment were counted in a Neubauer hemocytometer and each sample was counted three times. As results could not be normalized by transformation, they were subjected to Kruskal–Wallis test using SPSS v25.0 software (IBM Corporation, 2011).

2.4 Genotypic characterization and mortality response

For genotypic characterization, individual genotypes were isolated from the ChinNPV-Mex1 mixture by plaque purification (King and Possee, 1992). Briefly, fifth instar larvae of *C. includens* were inoculated with 10^8 OBs/mL of ChinNPV-Mex1 by the droplet feeding method. Hemolymph was extracted from 24 larvae by bleeding at 48 h post-infection (p.i.) and immediately frozen. The original sample of hemolymph was diluted in TNM-FH medium, filtered through a 0.45 µm filter and used for plaque purification. After 10 days p.i., wells containing clearly separated plaques were selected for plaque picking. Individual plaques were collected with a sterile Pasteur pipette and diluted in 300 µL of TNM-FH medium. Individual clones were injected intrahaemocelically in *C. includens* fifth instars that were then individualized in a plastic cup with semi-synthetic diet and incubated at 25 ± 1 °C and 75% relative humidity until death or pupation. Dead larvae showing signs of polyhedrosis disease were collected. REN analysis of the DNA (extracted as explained in Section 2.2) of the resultant OBs was performed with EcoRI and HindIII, as the combination of those enzymes allowed a clear discrimination of the different genotypes.

As a first approach, a test of pathogenicity involving one OB concentration close to the LC_{50} (10^4 OBs/mL) was performed for all the genotypic variants isolated by plaque purification, including the ChinNPV-Mex1 mixture as the reference treatment. Groups of 24 second instar larvae were orally inoculated with OBs of each variant following the droplet feeding method. Those larvae that drank the OB suspension within 10 min were transferred individually to 24-well plates with a piece of semi-synthetic diet. Control larvae drank a solution of sucrose and food dye containing no OBs.

Larvae were incubated at 25 ± 1 °C and 75% relative humidity. Mortality was recorded every 24 h. Analysis of the percentage of mortality was performed by Kruskal–Wallis test using SPSS v25.0 software.

2.5 Genotype selection and characterization of median time to death and OB production

According to the results obtained in the mortality responses to plaque-purified genotypes, the variants were divided into groups depending on the prevalence of mortality. Thus, one genotype was selected from the group of variants that caused low mortality (less than 15%), two from the group of medium mortality (15 to 30%) and two from the group that caused high mortality (31 to 40%). Median time to death and OB production studies were performed as described in section 2.3. Median time to death (MTD) was estimated using the log-rank test and OB production (OBs/larva) was subjected to Kruskal–Wallis test, as data could not be normalized by transformation.

3. RESULTS

3.1 Identification by REN of field ChinNPV isolates

DNAs of OBs from 11 virus-killed insects were analysed by REN to provide evidence for genetic variation. Analyses using EcoRI and HindIII revealed restriction fragment length polymorphisms among isolates, with a total of nine different restriction profiles (Figure 1a,b). The nine different isolates obtained were named Chin1 to Chin9. Two isolates, Chin5 and Chin6, were the genotypes with higher frequency that appeared twice. The REN profile of the ChinNPV-Mex1 mixture corresponded to that of the Chin9 isolate (Figure 1c).

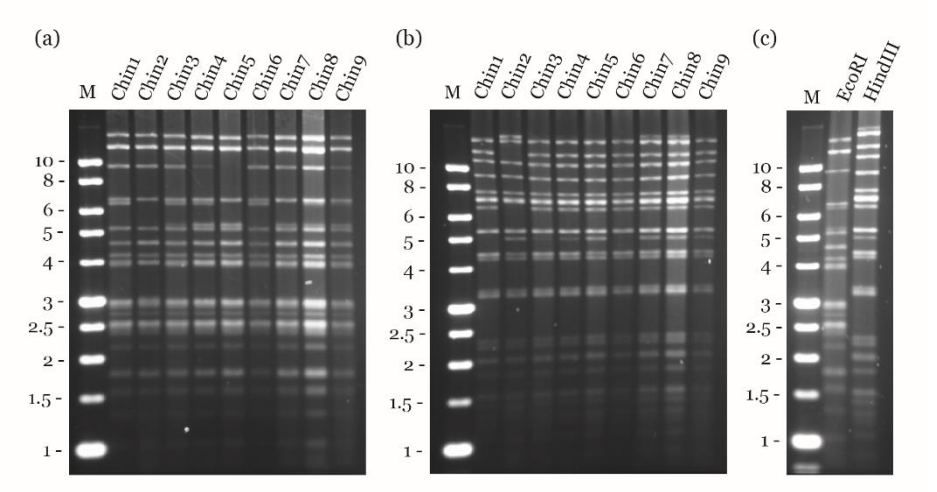

Figure 1. Restriction endonuclease profiles of the genomic DNA of nine different field isolates from virus-killed larvae (a) EcoRI, (b) HindIII and (c) ChinNPV-Mex1 mixture treated with both enzymes. M is the marker and the fragment size is shown in kilobases (Kb) on the left.

3.2 Biological and genotypic characterization of ChinNPV-Mex1

The host range study of the ChinNPV-Mex1 mixture showed that this virus was pathogenic to *C. includens*, *C. chalcites* and *T. ni*, all species from the Plusiinae subfamily. The other species tested were found to be non-permissive for this virus (Table 1). REN analysis of genomic DNA of OBs obtained from fourth instar larvae of *C. includens*, *C. chalcites*, and *T. ni* confirmed that ChinNPV was able to cause a productive infection in these insect species. The pathogenicity of the ChinNPV-Mex1 mixture, expressed as LC_{50}, was 6.5×10^4 OBs/mL. Median time to death, analysed with the log-normal model (selected as the best model for this analysis following a comparison of distribution-dependent AIC values), was estimated at a median value of 125 h post inoculation (hpi) in *C. includens* second instars. The OB production value was 2.2×10^9 OBs/larva in fifth instar larvae (Table 2). None of the control larvae died from polyhedrosis disease.

Table 1. Host range for ChinNPV in second instars of different lepidopteran species.

Species	5×10^6 OBs/mL	5×10^8 OBs/mL
Anticarsia gemmatalis	N.P.	N.P.
Chrysodeixis chalcites	++	+++
Chrysodeixis includens	++++	++++
Helicoverpa armigera	N.P.	N.P.
Lobesia botrana	N.P.	N.P.
Mamestra brassicae	N.P.	N.P.
Spodoptera eridania	N.P.	N.P.
Spodoptera exigua	N.P.	N.P.
Spodoptera frugiperda	N.P.	N.P.
Spodoptera littoralis	N.P.	N.P.
Trichoplusia ni	+	++++

N.P: Non-permissive. Percentage of mortality: + (1 to 25%), ++ (26 to 50%), +++ (51 to 75%), ++++ (76 to 100%).

Table 2. Results of Probit analysis used to estimate median lethal concentration (LC_{50}) and median time to death (MTD) values for ChinNPV-Mex1 in *C. includens* second instars and OB production values for ChinNPV-Mex1 in *C. includens* fifth instars.

Virus	LC_{50} * (OBs/mL)	95% Confidence Limits	
		Low	High
ChinNPV-Mex1	6.5×10^4	2.4×10^4	2.6×10^5
	MTD (h)	**95% Confidence limits**	
		Low	**High**
	125.0	121.8	128.2
	OB Production (OBs/larva)	**95% confident Limits**	
		Low	**High**
	2.2×10^9	1.2×10^9	3.1×10^9

* LC50 value estimated from a regression with slope (±SE) 0.826 ± 0.120 and intercept (±SE) −3.981 ± 0.559 (χ^2 goodness-of-fit test = 3.5641, d.f. = 3, heterogeneity = 1.1880).

Of the 185 virus-positive plaque picks, only 106 clones caused larval mortality by polyhedrosis following injection in *C. includens* fourth instars. Of the clones amplified in larvae, 23 different genotypic variants were obtained and were named ChinNPV-A to ChinNPV-W. Differences in their genomic restriction profiles were determined following digestion with EcoRI and HindIII. The 106

clones were then classified according to the prevalence of each of the 23 observed restriction profiles. The most prevalent genotype was ChinNPV-H with 19 clones obtained (representing 18% of the plaque-purified variants), whereas genotypic variants ChinNPV-J, T, U, V, and W were only observed in single clones (Figure 2).

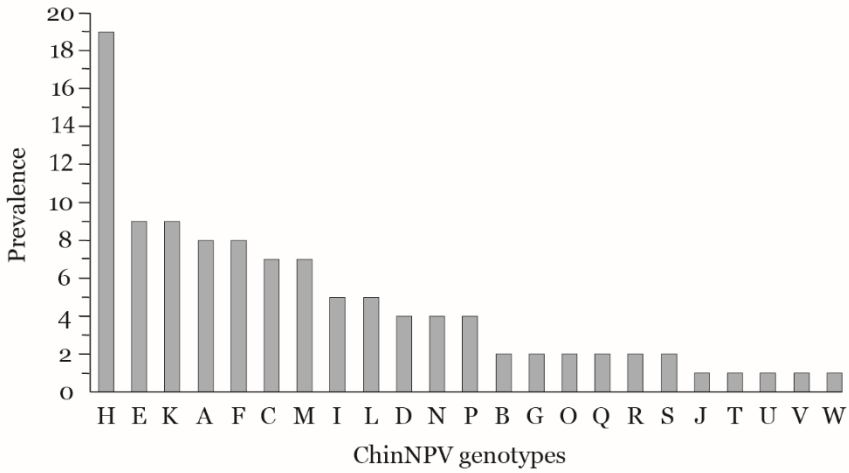

Figure 2. Prevalence of ChinNPV genotypes. Prevalence was calculated as the number of times each genotype was observed in 23 different restriction profiles from 106 clones isolated by plaque assay.

3.3 Biological characterization of ChinNPV genotypes

Insect bioassays, performed using a single concentration of inoculum, indicated that the most pathogenic genotype was ChinNPV-R, which resulted in 39.5 ± 7.0% mortality (Figure 3). In addition, statistical analysis revealed that ChinNPV-R was significantly different from the rest of the genotypes (Kruskal-Wallis $\chi^2 = 1170$, $p < 0.005$; Table A1).

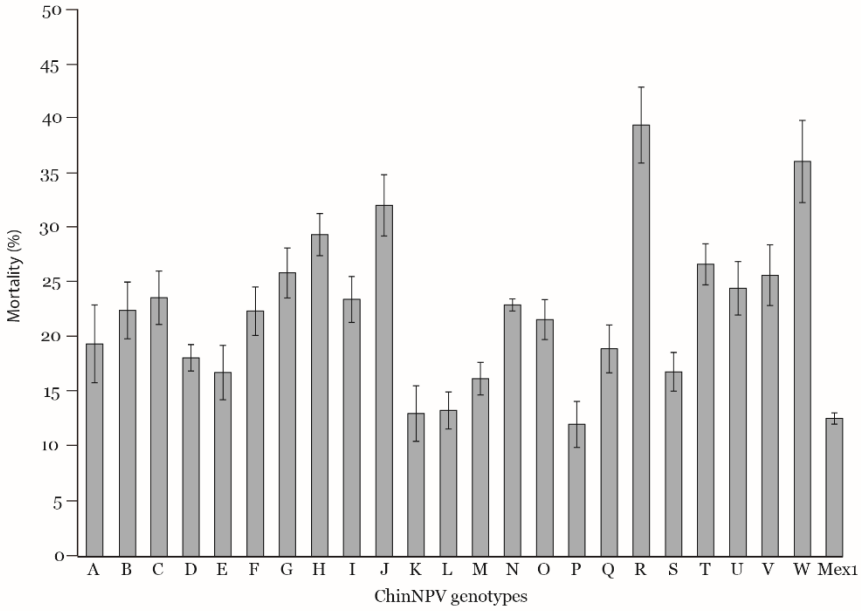

Figure 3. Percentage of mortality for each of the ChinNPV genotypic variants (A–W) and the ChinNPV-Mex1 mixture in *C. includens* second instars inoculated with 10^4 OBs/mL. Error bars indicate the standard error.

3.4 Virulence and OB production of the selected genotypes

The selected genotypes (ChinNPV-F, J, K, R, and V) did not differ significantly in MTD values and none of them differed significantly from the ChinNPV-Mex1 mixture (Log Rank $\chi^2 = 1.096$, df = 5, $p > 0.05$; Table 3). OB production estimated in *C. includens* fifth instars showed significant differences, with variants ChinNPV-F, K, R, and V as the most productive genotypes that produced between 2.2×10^9 and 3.7×10^9 OBs/larva depending on variant. This group of variants differed from genotype ChinNPV-J and ChinNPV-Mex1 that produced significantly fewer OBs/larva (Kruskal-Wallis $\chi^2 = 19.586$, $p < 0.001$, Figure 4).

Table 3. MTD values of genotypic variants estimated in *C. includens* second instars.

Variant	MTD (h)	95% Confidence Limits	
		Low	High
ChinNPV-F	125	120.3	129.7
ChinNPV-J	125	120.4	129.6
ChinNPV-K	119	115.5	122.5
ChinNPV-R	125	120.4	129.6
ChinNPV-V	119	115.7	122.3
ChinNPV-Mex1	125	121.8	128.2

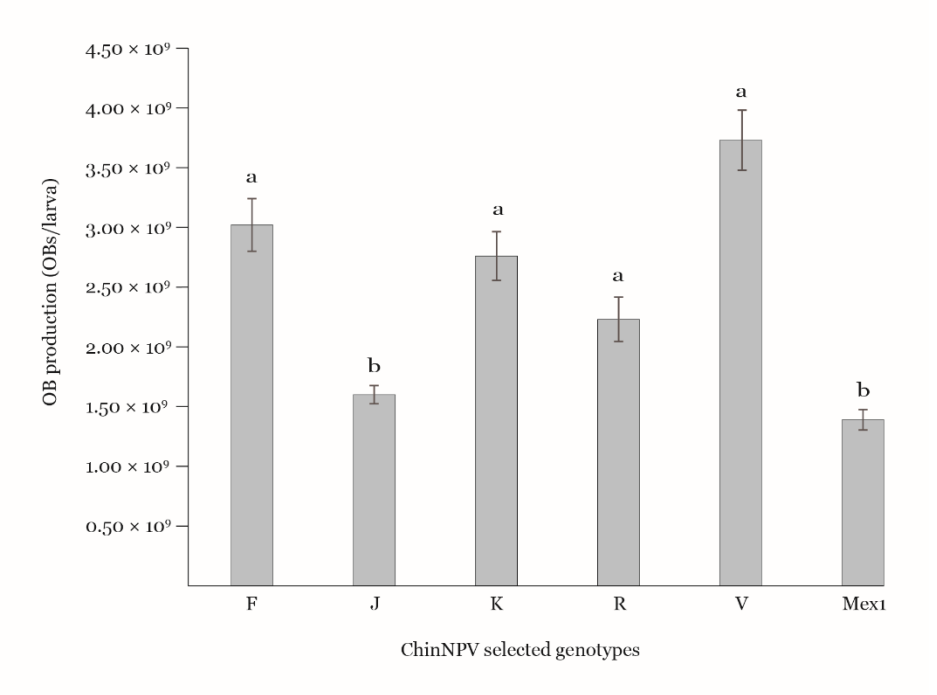

Figure 4. Mean OB production values of ChinNPV-Mex1 and selected genotypic variants in *C. includens* fifth instars. Error bars indicate the standard error. Columns headed by identical letters did not differ significantly (Kruskal-Wallis, $p > 0.05$).

4. DISCUSSION

The genetic variability within field isolates of ChinNPV and the insecticidal properties of their genotypes were examined with the objective of selecting variants with potential for bioinsecticide development. From the eleven corpses obtained from field-infected larvae, nine different REN profiles

were obtained, indicating a high variability in natural isolates of this virus under enzootic conditions, given that only 21 larvae died from lethal polyhedrosis out of 105 field-collected larvae (20% mortality). This may be an underestimate of the diversity present in the natural ChinNPV population due to the low number of samples and the omission of non-lethal NPV infection in the present study. The observed variability contrasts with results obtained in the closely-related species *C. chalcites*, a pest of banana crops in the Canary Islands, in which with the natural prevalence of virus-induced mortality of 2.3% resulted in just four different isolates out of 103 infected larvae analysed from a total of 4438 collected insects (Fuentes et al., 2017). In addition, geographically separated isolates usually show genetic differences, as reported by Alexandre et al. (2010), who identified five different isolates of ChinNPV from seven infected *C. includens* larvae obtained at different times and locations in Brazil. These results support the idea that isolates collected from distinct locations tend to differ genetically (Erlandson, 2009). In contrast, the ChinNPV used in this study had a behaviour and prevalence in field conditions that differed markedly from those described previously. Genetic variability has been proposed as an important tool through which baculoviruses can adapt to changing environmental conditions, and maintaining this genetic variability could even provide a means by which the viruses could adapt to alternative host species (Kolodny-Hirsch and Beek, 1997; Martignoni and Iwai, 1986; Weitzman et al., 1992). Nevertheless, the host range of ChinNPV tested in this study was limited to closely-related species in the Plusiinae subfamily, which reflects the high host specificity of most baculoviruses (Harrison and Hoover, 2012). Consequently, due to this narrow host range, genetic diversity in ChinNPV may not be especially relevant to infection of different host species because although *C. includens* populations naturally coexist with *T. ni* in the soya crop, the virus was not highly pathogenic to this alternative host (Table 1).

The high biological activity of the ChinNPV-Mex1 mixture means that this mixture could be a promising active ingredient for a baculovirus-based insecticide, as the biological activity of this mixture was similar to that of other

viruses that are already registered as insecticidal products (Caballero et al., 2007, 2013(a), 2013(b). The LC_{50} value of 6.5×10^4 OBs/mL and a MTD of 125 h in *C. includens* second instars suggest that this mixture may be significantly more active than the first registered ChinNPV-based product, Chrysogen, from Brazil, which has an LC_{50} of 1.4×10^5 OBs/mL in *C. includens* neonates (Muraro et al., 2019).

The number of genotypic variants obtained from the ChinNPV-Mex1 mixture was similar to the variability found in other NPVs like Panolis flammea NPV and Helicoverpa zea NPV (Corsaro and Fraser, 1987; Hodgson et al., 2001). Although this level of variability has been reported for other viruses, the context changes when the comparison shifts to a more related virus, such as C. chalcites NPV, in which eight genotypic variants were isolated from a single wild-type isolate using the same methodology as used here (Bernal et al., 2013b). For the ChinNPV, 23 genotypic variants were identified from a mixture of nine different wild-type isolates collected from the same area of soya fields at a single time. It is notable however, that this variability was present in a small sample (11 larvae) collected from the same crop at a single moment in time. Although we do not know how the genotypic variants were originally distributed across the eleven infected insects (as the ChinNPV-Mex1 mixture was used as inoculum for the plaque purification), this is still an elevated number of genotypic variants. The fact that *C. includens* larvae infected with ChinNPV and *A. gemmatalis* larvae infected with AgMNPV were collected at the same crop field and time, and that both viruses show high genetic variation (Del-Angel et al., 2018), indicates that genetically-complex natural NPV populations can coexist in field crop conditions. Indeed, the particular conditions of the crop or field may constitute a source of viral diversity. For instance, the soya crop itself may influence the genetic composition of baculovirus field isolates, as already described elsewhere (Hodgson et al., 2002) .

For initial characterization of the phenotypic characteristics of variants, a single concentration bioassay was performed, in which the ChinNPV-R variant was the most pathogenic genotype in terms of induced

mortality. This was not one of the most prevalent of the plaque purified variants, a finding which supports the idea that in vitro cloning may not accurately reflect the genotypic variation in wild type NPV isolates and may favour genotypes better adapted to cellular culture conditions (Cory et al., 2005). Other techniques for studying the genetic structure of NPVs have been developed (Baillie and Bouwer, 2013; Chateigner et al., 2015), but only in vitro cloning allows the isolation and subsequent characterization of the individual genetic variants. The correlation between genotype prevalence and pathogenicity is however unpredictable. For example, the most prevalent genotypic variant may not be the most pathogenic (Bernal et al., 2013b; Del-Angel et al., 2018; Simón et al., 2004), whereas other studies report the contrary (Arrizubieta et al., 2015; Barrera et al., 2013; Muñoz et al., 1998). Interestingly, all genotypes induced higher or similar mortalities in experimental insects as the ChinNPV-Mex1 mixture, indicating that genotypic interactions within the artificial mixture may diminish its overall pathogenicity. However, although key to understanding the genetic structure of viral populations, the outcomes of such interactions are usually complex and difficult to predict (Williams, 2018). Variation in the content of occlusion derived virions within OBs was not evaluated and may have contributed to the observed differences in the pathogenicity of ChinNPV genotypic variants. However, we considered the OB as the infection unit of interest as, under natural conditions, the likelihood of establishing a lethal infection is dependent on the number of OBs that insects consume when feeding on contaminated foliage.

All ChinNPV genotypes were classified into one of three groups (high, medium, and low mortality), according to their capacity to infect and kill larvae. Five genotypes were selected ChinNPV-F, J, K, R, and V, as a representative sample of all the genotypes present in the natural virus population. Speed-of-kill studies demonstrated no marked differences among the five selected variants, none of which was faster-killing than the ChinNPV-Mex1 mixture. In contrast, OB production of ChinNPV-F, K, R, and V variants was higher than the ChinNPV-Mex1 mixture. Taking into account all these

results, the genotypes ChinNPV-F, J, R, and V could be candidates for a new bioinsecticide for control of *C. includens*. ChinNPV-J induced high mortality rate but had low OB production, ChinNPV-F and V induced moderate mortality but were both highly productive, and ChinNPV-R had suitable insecticidal characteristics across all the properties that we studied. Nevertheless, due to the fact that ChinNPV-Mex1 showed lower mortality and OB production than most of the individual genotypes, it seems that genotype interactions may result in a reduction in the biological activity of the ChinNPV population. Therefore, the unpredictable nature of virus interactions makes it necessary to test the possible combinations of these genotypes for the design of the final active ingredient of a potential bioinsecticide.

Our results indicate that there is high genotypic variability within the natural population of ChinNPV isolates from north-eastern Mexico. The study of the biological properties of those variants did not explain the reason for the persistence of the high diversity, as the individual genotypes had similar or more active insecticidal characteristics than a mixed-isolate preparation (ChinNPV-Mex1). Most NPV infections involve mixtures of genotypes and the analysis of individual clonal genotypes is unlikely to provide ecologically useful information. In addition, the genotypic heterogeneity seen in natural viral populations facilitates virus survival during stochastic changes in environmental conditions (Williams, 2018). Although studies are required on how variant interactions may affect phenotypic traits, insecticidal characteristics of the ChinNPV-F, J, R, and V variants appear promising for the development of the active ingredient for a new baculovirus-based insecticide to control *C. includens*.

5. CONCLUSIONS

The study of the genetic variability in ChinNPV samples collected in a soya field revealed high natural diversity. Nine different isolates were obtained from eleven virus-killed larvae and 23 genetic variants were obtained by plaque purification. Among the variants, ChinNPV-F, J, R, and V were

identified as having useful characteristics for the development of a ChinNPV-based insecticide for control of *C. includens*, in terms of pathogenicity, speed-of-kill, and OB production/larva. The results of these studies did not explain the persistence of high diversity in the pathogen population, which may reflect the outcome of variant interactions within infected insects.

6. REFERENCES

Alexandre, T.M., Craveiro, S.R., Cunha, F., Fonseca, I.C.B., Castro, M.E.B., Moscardi, F., Ribeiro, Z.M.A., 2010. Evaluation of seven viral isolates as potential biocontrol agents against *Pseudoplusia includens* (Lepidoptera: Noctuidae) caterpillars. J. Invertebr. Pathol. 105, 98–104. https://doi.org/10.1016/j.jip.2010.05.015

Arrizubieta, M., Simón, O., Williams, T., Caballero, P., 2015. A novel binary mixture of Helicoverpa armigera single nucleopolyhedrovirus (HearSNPV) genotypic variants provides improved insecticidal characteristics for control of the cotton bollworm. Appl. Environ. Microbiol. 81, 3984–3993. https://doi.org/10.1128/AEM.00339-15

Baillie, V.L., Bouwer, G., 2013. The effect of inoculum dose on the genetic diversity detected within Helicoverpa armigera nucleopolyhedrovirus populations. J. Gen. Virol. 94, 2524–2529. https://doi.org/10.1099/vir.0.052803-0

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, e77271. https://doi.org/10.1371/journal.pone.0077271

Bernal, A., Simón, O., Williams, T., Muñoz, D., Caballero, P., 2013a. A Chrysodeixis chalcites single-nucleocapsid nucleopolyhedrovirus population from the Canary Islands is genotypically structured to maximize survival. Appl. Environ. Microbiol. 79, 7709–7718. https://doi.org/10.1128/AEM.02409-13

Bernal, A., Simón, O., Williams, T., Muñoz, D., Caballero, P., 2013b. A

Chrysodeixis chalcites single nucleopolyhedrovirus population from the Canary Islands is genotypically structured to maximize survival. Appl. Environ. Microbiol. 79, 7709–7718. https://doi.org/10.1128/aem.02409-13

Caballero, P., Bernal, A., Simón, O., Carnero, A., Hernández-Suárez, E., Williams, T., 2013(a). Nuevos genotipos del NPV simple de Chrysodeixis chalcites (ChchSNPV): procedimiento para su producción y uso como agente de control biológico.

Caballero, P., Bernal, A., Simón, O., Carnero, A., Hernández-Suárez, E., Williams, T.G., 2013(b). Nuevos genotipos del nucleopoliedrovirus simple de Chrysodeixis chalcites (ChchSNPV), procedimiento para su producción y uso como agente de control biológico.

Caballero, P., Murillo, R., Muñoz, D., Williams, T., 2009. El nucleopoliedrovirus de Spodoptera exigua (Lepidoptera: Noctuidae) como bioplaguicida: análisis de avances recientes en España. Rev. Colomb. Entomol. 35, 105–115.

Caballero, P., Willliams, T., Muñoz, D., Murillo, R., Lasa, R., 2007. Nuevos genotipos del nucleopoliedrovirus de Spodoptera exigua y uso de los mismos en el control de las plagas producidas por este insecto. WO2007122278A2.

Cabodevilla, O., Ibanez, I., Simon, O., Murillo, R., Caballero, P., Williams, T., 2011. Occlusion body pathogenicity, virulence and productivity traits vary with transmission strategy in a nucleopolyhedrovirus. Biol. Control 56, 184–192. https://doi.org/10.1016/j.biocontrol.2010.10.007

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

Core R Team, 2019. A Language and Environment for Statistical Computing. R Found. Stat. Comput. 2, https://www.R--project.org.

Corsaro, B.G., Fraser, M.J., 1987. Characterization of genotypic and phenotypic variation in plaque-purified strains of HzSNPV elkar isolate. Intervirology 28, 185–198. https://doi.org/10.1159/000150016

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/http://dx.doi.org/10.1016/j.jip.2005.03.008

Cory, J.S., Myers, J.H., 2003. The ecology and evolution of insect baculoviruses. Annu. Rev. Ecol. Evol. Syst. 34, 239–272. https://doi.org/10.1146/annurev.ecolsys.34.011802.132402

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, 8–9. https://doi.org/10.1128/genomea.01192-16

del-Angel, C., Lasa, R., Rodríguez-del-Bosque, L.A., Mercado, G., Beperet, I., Caballero, P., Williams, T., 2018. Anticarsia gemmatalis nucleopolyhedrovirus from soybean crops in Tamaulipas, Mexico: diversity and insecticidal characteristics of individual variants and their co-occluded mixtures. Florida Entomol. 101, 404–410. https://doi.org/10.1653/024.101.0319

Eichlin, T.D., Cunningham, H.B., 1978. The Plusiinae (Lepidoptera: Noctuidae) of America north of Mexico, emphasizing genitalic and larval morphology. Tech. Bull. 1567 14–15.

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. https://doi.org/10.1007/s12250-009-3052-1

Felland, C.M., Pitre, H.N., Luttrell, R.G., Hamer, J.L., 1990. Resistance to Pyrethroid Insecticides in Soybean Looper (Lepidoptera: Noctuidae) in Mississippi. J. Econ. Entomol. 83, 35–40. https://doi.org/10.1093/jee/83.1.35

Fuentes, E.G., Hernández-Suárez, E., Simón, O., Williams, T., Caballero, P., 2017. Chrysodeixis chalcites nucleopolyhedrovirus (ChchNPV): Natural occurrence and efficacy as a biological insecticide on young banana plants in greenhouse

and open-field conditions on the Canary Islands. PLoS One 12, e0181384. https://doi.org/10.1371/journal.pone.0181384

Gebhardt, M.M., Eberle, K.E., Radtke, P., Jehle, J.A., 2014. Baculovirus resistance in codling moth is virus isolate-dependent and the consequence of a mutation in viral gene *pe38*. Proc. Natl. Acad. Sci. 111, 15711–15716. https://doi.org/10.1073/pnas.1411089111

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

Harrison, R., Hoover, K., 2012. Baculoviruses and other occluded insect viruses, in: Insect Pathology. Elsevier Inc., pp. 73–131. https://doi.org/10.1016/B978-0-12-384984-7.00004-X

Harrison, R.L., 2009. Structural divergence among genomes of closely related baculoviruses and its implications for baculovirus evolution. J. Invertebr. Pathol. 101, 181–186. https://doi.org/http://dx.doi.org/10.1016/j.jip.2009.03.012

Harrison, R.L., Puttler, B., Popham, H.J.R., 2008. Genomic sequence analysis of a fast-killing isolate of Spodoptera frugiperda multiple nucleopolyhedrovirus. J. Gen. Virol. 89, 775–790. https://doi.org/10.1099/vir.0.83566-0

Hitchman, R.B., Hodgson, D.J., King, L.A., Hails, R.S., Cory, J.S., Possee, R.D., 2007. Host mediated selection of pathogen genotypes as a mechanism for the maintenance of baculovirus diversity in the field. J. Invertebr. Pathol. 94, 153–162. https://doi.org/10.1016/j.jip.2006.10.002

Hodgson, D.J., Vanbergen, A.J., Hartley, S.E., Hails, R.S., Cory, J.S., 2002. Differential selection of baculovirus genotypes mediated by different species of host food plant. Ecol. Lett. 5, 512–518. https://doi.org/10.1046/j.1461-0248.2002.00338.x

Hodgson, D.J., Vanbergen, A.J., Watt, A.D., Hails, R.S., Cory, J.S., 2001. Phenotypic variation between naturally co-existing genotypes of a Lepidopteran baculovirus. Evol. Ecol. Res. 3, 687–701.

Hughes, P.R., Wood, H.A., 1987. In vivo and in vitro bioassay methods for baculoviruses, in: Granados, R.R., Federici, B.A. (Eds.), The Biology of

Baculoviruses. CRC Press, Boca Raton, FL., pp. 1–30.

IBM Corporation, 2011. IBM SPSS Statistics for Windows.

Ikeda, M., Hamajima, R., Kobayashi, M., 2015. Baculoviruses: diversity, evolution and manipulation of insects. Entomol. Sci. 18, 1–20. https://doi.org/10.1111/ens.12105

Jehle, J.A., Schulze-Bopp, S., Undorf-Spahn, K., Fritsch, E., 2017. Evidence for a second type of resistance against Cydia pomonella granulovirus in field populations of codling moths. Appl. Environ. Microbiol. 83. https://doi.org/10.1128/AEM.02330-16

Kamiya, K., Zhu, J., Murata, M., Laviña-Caoili, B.A., Ikeda, M., Kobayashi, M., Kawamura, S., 2004. Cloning and comparative characterization of three distinct nucleopolyhedroviruses isolated from the common cutworm, Spodoptera litura (Lepidoptera: Noctuidae) in Japan. Biol. Control 31, 38–48. https://doi.org/10.1016/j.biocontrol.2004.04.003

King, L.A., Possee, R.D., 1992. The baculovirus expression system. A laboratory guide. Chapman & Hall, London, United Kingdom.

Kolodny-Hirsch, D.M., Beek, N.A.M. Van, 1997. Selection of a morphological variant of Autographa californica nuclear polyhedrosis virus with increased virulence following serial passage in *Plutella xylostella*. J. Invertebr. Pathol. 69, 205–211. https://doi.org/https://doi.org/10.1006/jipa.1997.4659

Laviña, B.A., Padua, L.E., Wu, F.Q., Shirata, N., Ikeda, M., Kobayashi, M., 2001. Biological characterization of a nucleopolyhedrovirus of Spodoptera litura (Lepidoptera: Noctuidae) isolated from the Philippines. Biol. Control 20, 39–47. https://doi.org/10.1006/bcon.2000.0877

Lee, H.H., Miller, L.K., 1978. Isolation of genotypic variants of Autographa californica nuclear polyhedrosis virus. J. Virol. 27, 754–767.

Leonard, R.B., Boethel, D.J., Sparks, A.N., Layton, B.M., Mink, J.S., Pavloff, A.M., Burris, E., Graves, J.B., 2015. Variations in response of soybean looper (Lepidoptera: Noctuidae) to selected insecticides in Louisiana. J. Econ. Entomol. 83, 27–34. https://doi.org/10.1093/jee/83.1.27

LeOra Software, 2003. Polo Plus.

Martignoni, M.E., Iwai, P.J., 1986. Propagation of multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata* in larvae of *Trichoplusia ni*. J. Invertebr. Pathol. 47, 32–41. https://doi.org/10.1016/0022-2011(86)90160-6

Mascarenhas, R.N., Boethel, D.J., 1997. Responses of Field-Collected Strains of Soybean Looper (Lepidoptera: Noctuidae) to Selected Insecticides Using an Artificial Diet Overlay Bioassay. J. Econ. Entomol. 90, 1117–1124. https://doi.org/10.1093/jee/90.5.1117

Mink, J.S., Boethel, D.J., 1992. Development of a Diagnostic Technique for Monitoring Permethrin Resistance in Soybean Looper (Lepidoptera: Noctuidae) Larvae. J. Econ. Entomol. 85, 1056–1062. https://doi.org/10.1093/jee/85.4.1056

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 Env. 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.

Muraro, D.S., Giacomelli, T., Stacke, R.F., Godoy, D.N., Marçon, P., Popham, H.J.R., Bernardi, O., 2019. Baseline susceptibility of Brazilian populations of *Chrysodeixis includens* (Lepidoptera: Noctuidae) to C. includens nucleopolyhedrovirus and diagnostic concentration for resistance monitoring. J. Econ. Entomol. 112, 349—354. https://doi.org/10.1093/jee/toy361

Redman, E.M., Wilson, K., Grzywacz, D., Cory, J.S., 2010. High levels of genetic diversity in Spodoptera exempta NPV from Tanzania. J. Invertebr. Pathol. 105, 190–193. https://doi.org/10.1016/j.jip.2010.06.008

Rowley, D.L., Popham, H.J.R., Harrison, R.L., 2011. Genetic variation and virulence of nucleopolyhedroviruses isolated worldwide from the heliothine pests Helicoverpa armigera, Helicoverpa zea, and Heliothis virescens. J.

Invertebr. Pathol. 107, 112–126. https://doi.org/10.1016/j.jip.2011.03.007

Simón, O., Caballero, P., Williams, T.G., Arrizubieta, M., 2016. Nuevos genotipos del nucleopoliedrovirus simple de Helicoverpa armigera (HearSNPV), procedimiento para su producción y uso como agente de control biológico.

Simón, O., Williams, T., López-Ferber, M., Caballero, P., 2004. Genetic structure of a Spodoptera frugiperda nucleopolyhedrovirus population: High prevalence of deletion genotypes. Appl. Environ. Microbiol. 70, 5579–5588. https://doi.org/10.1128/AEM.70.9.5579-5588.2004

Stiles, B., Himmerich, S., 1998. Autographa californicaNPV Isolates: Restriction Endonuclease Analysis and Comparative Biological Activity. J. Invertebr. Pathol. 72, 174–177. https://doi.org/10.1006/jipa.1998.4758

Thézé, J., Lopez-Vaamonde, C., Cory, J.S., Herniou, E.A., 2018. Biodiversity, evolution and ecological specialization of baculoviruses: A treasure trove for future applied research. Viruses 10, 366. https://doi.org/10.3390/v10070366

Weitzman, M.D., Possee, R.D., King, L.A., 1992. Characterization of two variants of Panolis flammea multiple nucleocapsid nuclear polyhedrosis virus. J. Gen. Virol. 73, 1881–1886. https://doi.org/10.1099/0022-1317-73-7-1881

Wennmann, J.T., Keilwagen, J., Jehle, J.A., 2018. Baculovirus Kimura two-parameter species demarcation criterion is confirmed by the distances of 38 core gene nucleotide sequences. J. Gen. Virol. 99, 1307–1320.

Williams, H.L., Monge-Monge, K.S., Otvos, I.S., Reardon, R., Ragenovich, I., 2011. Genotypic variation among Douglas-fir tussock moth nucleopolyhedrovirus (OpNPV) isolates in the western United States. J. Invertebr. Pathol. 108, 13–21. https://doi.org/10.1016/j.jip.2011.06.004

Williams, T., 2018. Viruses, in: Hajek, A.E., Shapiro-Ilan, D.I. (Eds.), Ecology of Invertebrate Diseases. John Wiley, Chichester, UK, pp. 213–285. https://doi.org/10.1002/9781119256106.ch7

CHAPTER III

Generation of variability in Chrysodeixis includens nucleopolyhedrovirus (ChinNPV): the role of a single variant

ABSTRACT

The mechanisms generating variability in viruses are diverse. Variability allows baculoviruses to evolve with their host and with changes in their environment. We examined the role of one genetic variant of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) and its contribution to the variability of the virus under laboratory conditions. A mixture of natural isolates (ChinNPV-Mex1) contained two genetic variants that dominated over other variants in individual larvae that consumed high (ChinNPV-K) and low (ChinNPV-E) concentrations of inoculum. Studies on the ChinNPV-K variant indicated that it was capable of generating novel variation in a concentration-dependent manner. In cell culture, cells inoculated with high and low concentrations of ChinNPV-K virions showed the same response to the ChinNPV-K genotype. Interestingly, the ChinNPV-K variant could not be recovered from plaques derived from low concentration inocula originating from budded virions or occlusion derived virions of ChinNPV-K. Genome sequencing revealed marked differences between ChinNPV-K and ChinNPV-E, with high variation in the ChinNPV-K genome, mostly due to single nucleotide polymorphisms. We conclude that ChinNPV-K may be an unstable genetic variant that is responsible for generating much of the detected variability in the natural ChinNPV isolates used in this study.

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1. INTRODUCTION

Baculoviruses are double-stranded DNA viruses, with a large circular genome ranging from 80 to 180 Kb (Harrison et al., 2018). Due to the negative correlation between mutation rate and genome length, baculoviruses are supposed to be less prone to mutation than most other DNA viruses (Sanjuán and Domingo-Calap, 2016). Nevertheless, natural isolates of baculoviruses are known to comprise mixtures of genotypes (Erlandson, 2009), which suggests that phenomena such as recombination, mutation, and transposition take place frequently during baculovirus replication (Gebhardt et al., 2014; Gilbert et al., 2014; Kamita et al., 2003; Li et al., 2002; López-Ferber et al., 2001). In addition, SNPs (single nucleotide polymorphisms) are generated during the replication of viral DNA, which could reasonably be linked to a lack of proof reading activity of the viral polymerase, although polymerases from Bombyx mori NPV (BmNPV) and Autographa californica MNPV (AcMNPV), both present 3' to 5' exonuclease activity, which implies a high degree of fidelity during the DNA replication cycle (Hang and Guarino, 1999; McDougal and Guarino, 1999; Mikhailov et al., 1986).

The presence of high diversity within baculoviruses is particularly evident when sequencing these viruses. Two recent examples, a study carried out on Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) (Masson et al., 2020) and other on Autographa californica multiple nucleopolyhedrovirus (AcMNPV) (Loiseau et al., 2020), revealed the high variability present in natural baculovirus isolates and the different mechanisms involved in its generation. Factors such as food plants or host genetic heterogeneity can also directly influence the genetic variability present in baculovirus isolates (Cory, 2010; Williams, 2018). A previous study in *Helicoverpa armigera* identified the role of the inoculum dose on the genetic variability present in an isolate of Helicoverpa armigera NPV (HearNPV) (Baillie and Bouwer, 2013). In that study, the authors examined the positive effect of inoculum dose in the variability observed when *Helicoverpa armigera* larvae were fed with 5% or 95% lethal doses (LD_5 or LD_{95}) of HearNPV. The variability observed was similar in insects that consumed either of the two

doses, which was unexpected given that the authors hypothesized that the genetic diversity detected in the lower dose would be lower than the genetic diversity of the samples used for inoculation (Baillie and Bower, 2013).

High genetic diversity is present in isolates of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV, previously known as Pseudoplusia includens single nucleopolyhedrovirus) collected in soybean fields in Mexico (Aguirre et al., 2019). Nine genetically distinct isolates were obtained from eleven virus-killed larvae. In vitro cloning from an equimolar mixture of the nine isolates, named ChinNPV-Mex1, revealed the presence of 23 genetic variants by restriction endonuclease analysis (Aguirre et al., 2019). Here we examined the effect of host heterogeneity and inoculum concentration on the genetic variability of this artificial mixture. We also reveal the key role of a single variant, named ChinNPV-K, in generating variability in a concentration-dependent manner. This variation was mainly present as SNPs throughout the genome. The molecular mechanisms that generate this variability are unclear, although several possibilities are discussed.

2. MATERIALS AND METHODS

2.1 Insects, cells, and viruses

A laboratory colony of *C. includens* was established from larvae collected in soybean fields of Tamaulipas, Mexico and maintained under controlled conditions at 25 ± 1 °C, 75% relative humidity (RH) and 16 h light: 8 h dark photoperiod. Larvae were reared on a wheatgerm-based semi-synthetic diet (Greene et al., 1976). This population was tested by qPCR following the method described by Virto et al. (2014) and declared as ChinNPV-free (data not shown). HighFive cells from *Trichoplusia ni* (ThermoFisher Scientific, Waltham, MA, USA) were maintained in TNM-FH medium (Gibco, Life Technologies Ltd, Renfrew, UK) with 10% fetal bovine serum (Gibco) at 28 °C.

ChinNPV-Mex1 was prepared from an equimolar mixture of OBs from nine ChinNPV field isolates described previously (Aguirre et al., 2019).

Individual genotypes used in this study were isolated from ChinNPV-Mex1 by plaque purification in HighFive cells, multiplied in *C. includens* fifth instars and identified as distinct variants using the restriction endonucleases EcoRI and HindIII (Aguirre et al., 2019). To purify viral OBs, each virus-killed larva was filtered through muslin and centrifuged with 0.1% SDS twice at 2.300×*g* to eliminate insect debris. The resulting pellets were washed in distilled water and resuspended in milli-Q water. OB concentrations were determined by counting in an improved hemocytometer (Hawksley Ltd., Lancing, UK) under phase-contrast microscopy. Purified OBs were stored at 4 °C until required.

2.2 Viral DNA extraction and restriction endonuclease analysis (REN)

Occlusion derived virions (ODVs) were released from OBs by incubating 20 µL of OB suspension (~10^{10} OBs/mL) with 50 µL of 0.5 M Na_2CO_3 and 180 µL of distilled water at 60 °C during 10 min. The suspension was centrifuged at 6000×*g* for 5 min and the supernatant containing the virions was incubated for 1 h at 65 °C with 15 µL proteinase K (20 mg/mL), 25 µL of 10% SDS and 25 µL of 0.5 M EDTA. Viral DNA was then separated from proteins by addition of 150 µL of MPC reagent (Masterpure™ Kit, Lucigen Corp., WI, USA) and centrifuged for 10 min at 10.000× *g*. DNA was precipitated using 2.5 volumes of ice-cold absolute ethanol for 10 min at 12.000× *g*. Pelleted DNA was washed twice with 70% ice-cold ethanol and resuspended in 50 µL 0.1 × TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). The final DNA concentration was estimated using a Nanodrop One (ThermoFisher Scientific, Waltham, MA, USA) spectrophotometer.

For restriction endonuclease (REN) analysis, 2 µg of viral DNA of each ChinNPV genotype were digested with EcoRI and HindIII (FastDigest™, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 1 h at 37 °C. The reactions were loaded into an 1% agarose gel and fragments were then separated by electrophoresis in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.0) running at 18 V for 15 h. DNA was stained with GelRed® (Biotium Inc.,

Fremont, CA, USA) and visualized on a UV transilluminator (G-Box Syngene, Synoptics Ltd, Cambridge, UK).

2.3 Effects of host heterogeneity and inoculum concentration on genetic diversity following inoculation of larvae with ChinNPV-Mex1

To examine the effects of host heterogeneity and inoculum concentration on variant diversity, individual fifth instar *C. includens* larvae were inoculated with one of four different OB concentrations: 1×10^4, 5×10^5, 5×10^6 and 1×10^8 OBs/ml using the droplet feeding method (Hughes and Wood, 1987). This range of OB concentrations was expected to result in between 20% and 100% mortality, based on the results of previous assays. Larvae were inoculated with each OB concentration and each larva was considered as a replicate insect. A total of 447 larvae were inoculated for the 1×10^4 OBs/ml concentration, 72 larvae for the 5×10^5 OBs/ml concentration, 150 larvae for the 5×10^6 OBs/ml concentration and 100 larvae for the 10^8 OBs/ml concentration. Differences in the numbers of inoculated larvae for each concentration reflected the need of achieve a minimum of 60-100 virus-killed individuals for each concentration. Larvae were reared individually on semi-synthetic diet and incubated in 16 h light: 8 h dark photoperiod at 25 ± 1 °C and 75% relative humidity. Virus induced mortality was recorded every 24 h until larvae had died or pupated. Control larvae (20 individuals) consumed the same inoculum but without OBs. All virus-killed larvae were recovered individually, and the OBs purified as described in section 2.1. REN analysis was performed on the DNA extracted from the recovered OBs using EcoRI and HindIII. The most abundant genetic variant present in each insect was identified by comparing each REN profile with the REN profiles of the genotypes previously isolated from the ChinNPV-Mex1 mixture (Aguirre et al., 2019). New REN profiles that did not correspond to the genetic variants identified in the previous study were classified together in the same group as “unidentified” variants. The prevalence of each dominant genotype was expressed as the percentage of larvae that produced OBs with a particular REN profile. The effect of OB concentration on

the frequency of ChinNPV-K REN profiles was examined using Pearson's χ^2 test in SPSS v25.0 software (IBM SPSS Statistics for Windows v.25.0, IBM Corp., Armonk, NY).

2.4 Genetic diversity present following inoculation with variants ChinNPV-K and ChinNPV-E

The ChinNPV-E and -K variants were selected for study because their characteristic REN profiles were the most prevalent in insects inoculated with low and high concentrations of OBs, respectively, in the previous experiment (section 2.3). A similar experiment to that of section 2.3 was performed using OBs comprising ChinNPV-E and -K variants alone. To simplify the experiment, we used only two OB concentrations (5×10^4 and 1×10^8 OBs/ml), estimated to result in approximately 20% and 99% mortality, respectively. A total of 145-163 larvae were inoculated with the 1×10^8 OBs/ml concentration, and 169-368 larvae for the 5×10^4 OBs/ml concentration, in order to achieve a total of 144-162 virus-killed larvae for the 1×10^8 OBs/ml and 57-80 virus-killed larvae for the 5×10^4 OBs/ml concentration. A group of 20 control larvae were treated identically but did not consume OBs. The results were analyzed as described in section 2.3. The frequencies of ChinNPV-K or -E virus killed larvae were compared by Fisher's exact test with SPSS v25.0 (IBM SPSS Statistics for Windows v.25.0, IBM Corp., Armonk, NY).

2.5 Genetic stability of progeny OBs obtained following infection with ChinNPV-K OBs at low concentration

Due to the high variability of genotypes obtained in the ChinNPV-K low concentration treatment in the previous experiment (section 2.4), we selected four OB samples from the previous experiment that showed the dominant REN profile of genotypic variants ChinNPV-H, -L, -N and -P. Each of these variants had been recovered from the 5×10^4 OBs/ml concentration treatment in section 2.4. Each OB sample was used as inoculum for another assay involving high inoculum concentration to determine whether the ChinNPV-K would become dominant when high concentration was used (suggesting its presence as a minor

component in the ChinNPV-H, -L, -N and -P dominant samples obtained in section 2.4). For this, a total of 20 *C. includens* fifth instars were allowed to consume 1 × 10^8 OBs/ml of each sample, which was expected to cause a 90-100% mortality. Inoculated larvae were reared individually in plastic cups until death or pupation. A group of 20 control larvae were treated identically but did not consume OBs. The results were analyzed as described in section 2.3 considering each larva as an individual replicate.

2.6 Prevalence of ChinNPV-K and ChinNPV-E REN profiles in larvae inoculated with mixtures of variants K and E

In order to confirm the suspected concentration-diversity effect associated with the ChinNPV-K genotype, an experiment was designed to assess whether the ChinNPV-K genotype had to be present at a minimum concentration in the inoculum to generate variability and if so, to determine the threshold concentration at which the ability of ChinNPV-K to generate that variability was lost. For this, we prepared different mixtures of ChinNPV-K OBs and ChinNPV-E OBs in a range of proportions maintaining a final concentration of 1 × 10^8 OBs/ml, expected to result in >90% mortality (Table 1). A total of 20 fifth instar larvae were inoculated, reared individually and those that died of polyhedrosis were individually subjected to OB extraction and REN analysis.

Table 1. Concentrations of ChinNPV-K and ChinNPV-E in inoculum mixtures with a total concentration of 10^8 OBs/ml.

Treatment	ChinNPV-K	ChinNPV-E
Control	0	0
1	10^8	0
2	10^8	10^2
3	10^8	10^5
4	9 × 10^7	10^7
5	5 × 10^7	5 × 10^7
6	10^7	9 × 10^7
7	10^5	10^8
8	10^2	10^8
9	0	10^8

Additional tests were performed using variants K and E at a lower concentration of 5 × 10^4 OBs/ml (Table 2), expected to result in 20-30%

mortality. In this case only three treatments were performed due to the low mortality that this concentration produces and the high number of insects that have to be inoculated to achieve a sufficient number of samples for analysis. For this, 150-200 larvae were inoculated in each treatment, reared individually and those that died of polyhedrosis were subjected to OB extraction and REN analysis.

Table 2. Concentrations of ChinNPV-K and ChinNPV-E in inoculum mixtures with a total concentration of 5×10^4 OBs/ml.

Treatment	ChinNPV-K	ChinNPV-E
Control	0	0
1	2.5×10^4	2.5×10^4
2	5×10^3	4.5×10^4
3	0	5×10^4

2.7 Prevalence of ChinNPV-K REN profile in pooled samples

In all the previous experiments REN profiles were obtained from individual virus-killed larvae. The following experiment was performed to determine whether the ChinNPV-K REN profile could be detected when progeny OBs from virus-killed larvae inoculated with a low concentration of ChinNPV-K OBs were collected in a pooled sample. For this, 20 *C. includens* fifth instars were allowed to consume 5×10^4 OBs/ml of ChinNPV-K pure variant using the droplet feeding method and were reared individually in plastic cups with diet. Larvae that died of polyhedrosis disease were collected, pooled and purified to produce a first passage inoculum. The purified inoculum was quantified and subjected to REN profile analysis.

We also examined whether the concentration-diversity effect was maintained after using the pooled OB sample as inoculum. For this, groups of 150 - 200 *C. includens* fifth instars were inoculated with the lower concentration (5×10^4 OBs/ml) and 15-20 larvae were inoculated with the higher concentration (1×10^8 OBs/ml) of the pooled OB inoculum, which was expected to cause between 20 and 99% mortality, respectively. Inoculated larvae were reared individually in plastic cups. Virus-killed larvae were recovered individually and each dead larva was processed and analyzed as

described in section 2.3. A total of approximately 100 larvae were analyzed for the low concentration experiment and 20 for the high concentration. Each larva was considered as a replicate.

2.8 ChinNPV-K and ChinNPV-E infectivity in cell culture

The *in vitro* infectivity of the budded virus of ChinNPV-K and ChinNPV-E was determined by end point dilution assay. Briefly, 20 fifth instars of *C. includens* were inoculated with 10^8 OBs/mL of each genotype by the droplet feeding method. Hemolymph was extracted from larvae by bleeding at 48 h post-infection (h.p.i.), pooled within each variant and immediately frozen. The sample of hemolymph of each genotype was diluted 1:10 in TNM-FH medium (10^{-1}), filtered through a 0.45 µm filter and serially diluted 1:10 in TNM-FH medium (10^{-2} to 10^{-6}). For each variant, a 10 µL volume of each dilution was used to inoculate wells containing 10^4 HighFive cells in a 96 well plate. A total of 24 wells were inoculated with each dilution and the experiment was performed three times (replicates). Plates were sealed with masking tape, incubated at 28 °C for 7 days and were then examined for signs of virus infection. Results were analyzed by the Spearman-Kärber method (Lynn, 1992) in order to determine the 50% tissue culture infectious dose ($TCID_{50}$) of each variant. The resulting values were compared by Welch's t-test for unequal variances using SPSS v25.0 (IBM SPSS Statistics for Windows v.25.0, IBM Corp., Armonk, NY).

To compare BV production *in vivo*, the quantity of budded virus of each genotype present in pooled hemolymph samples was determined by qPCR using specific primers for ChinNPV in a CFX96 thermal cycler (Bio-Rad, Hercules, CA, USA). For this, specific primers targeted at the ChinNPV *dnaPol* gene were used in a reaction prepared in 96-well optical plates and containing a mixture of 3 µl of SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.2 µl of each specific primer (10 µM), 5.6 µl of distilled water and 1 µl of template DNA, reaching a final volume of 10 µl. Genomic DNA of ChinNPV-K and -E pure variants was used as an internal standard for each qPCR assay. The amplification reaction protocol was: denaturation step at 95 °C for 2.3 minutes,

44 amplification cycles at 95 °C for 15 s and 60 °C for 30 s. The last step, a melting curve analysis was performed with a dissociation stage of 60 °C for 15 s and 95 °C for 5 s, to confirm the presence of the target amplicon by the visualization of a single peak. Hemolymph samples of each variant were quantified in independent qPCR reactions. Posterior data analysis was performed using Bio-Rad CFX Manager software (Bio-Rad Laboratories, Hercules, CA, USA).

2.9 ChinNPV-K progeny in cell culture

To assess whether the behavior and productivity of the ChinNPV-K variant at high and low concentrations in cell culture was similar to that of the experiments performed *in vivo*, a series of experiments were performed *in vitro*. First, cells were inoculated with BVs at a high concentration. For this, 2×10^6 HighFive cells were deposited in 25 cm^2 flasks. After 1 hour, cells attached to flask surface were inoculated with hemolymph collected from fifth instar larvae infected with ChinNPV-K or -E pure variants at a multiplicity of infection (MOI) of 10. At 10 days after infection, cells were recovered and centrifuged at 3000× *g* for 10 min. Pelleted cells were subjected to DNA extraction following the MasterPure DNA Extraction Kit protocol (Epicentre Biotechnologies, Madison, Wisconsin, USA). Extracted DNA was analyzed by REN. This experiment was performed in triplicate.

Second, as it was not possible to analyze REN profiles directly from cell cultures inoculated with a low concentration of BVs due to the low production of virus progeny, plaque purification was performed and the recovered plaques were individually amplified in *C. includens* fifth instars. Samples of hemolymph from larvae infected with ChinNPV-K and -E pure variants were diluted in TNM-FH medium and then filtered through a 0.45 µm filter. HighFive cells in 6-well tissue culture plates were inoculated with a range of dilutions (10^{-5} – 10^{-7}) of hemolymph samples. After 10 days p.i., wells containing clearly separated plaques (i.e., wells with fewer than ten plaques) were selected for plaque picking and the individual plaques were collected with a sterile Pasteur pipette and diluted in 300 µL of TNM-FH medium. Individual plaque picks were then

injected intrahemocelically in *C. includens* fifth instars that were placed individually in a plastic cup with semi-synthetic diet and incubated at 25 ± 1 °C and 75% relative humidity until death or pupation. Virus-killed larvae were collected individually and the OBs were extracted, purified as described in section 2.1 and subjected to REN analysis (section 2.2). The prevalence of the recovered genotypes was expressed as the percentage of each variant's REN profiles from the total of virus-killed insects.

Third, an additional plaque purification procedure was performed using ODVs instead of BVs in order to examine the possible differences between the two types of virions. Briefly, to release ODVs from the protein matrix, a 20 µL volume of ChinNPV-K or -E pure OB suspension (10^{10} OBs/mL) was incubated with 50 µL of 0.1 M Na_2CO_3 and 180 µL of distilled water at 28 °C during 30 min. This was performed three times. The suspension was centrifuged at 6000× *g* for 5 min and the supernatant containing ODVs was diluted in TNM-FH medium and filtered through a 0.45 µm filter for use in plaque purification. The remaining steps of plaque purification and analysis were identical to those described in the study on BVs in hemolymph.

2.10 ChinNPV-K and ChinNPV-E genome sequencing

In order to determine whether differences in the ChinNPV-K and -E genome sequences could account for the biological differences observed, genomic DNA from ChinNPV-K and ChinNPV-E variants was extracted from purified OBs and its integrity was checked by gel electrophoresis. DNA samples were shipped to StabVida laboratories (Stab Vida Lda, Caparica, Portugal) for library construction and sequencing using an Illumina Miseq with 300 bp paired-end sequencing reads. Genome gap closing was performed using the Sanger technique after PCR amplification of the non-overlapping regions using specific primers. Next, *de novo* assembly was carried out using CLC Genomics Workbench 10.1.1 (www.qiagenbioinformatics.com/). Raw sequence data were quality filtered by removing low quality or ambiguous reads that were <Q30, according to Illumina quality scores. Reads shorter than 50 bp were discarded. Reads were *de novo* assembled using a stringent criterion of overlap of at least

98 bp of the read and 98% identity. After contig creation, reads were mapped back to the contigs for assembly correction. The consensus sequences were digested in silico with BamHI, EcoRI, HindIII and PstI (Vincze et al., 2003) to compare the REN profile patterns of the consensus sequence with those of the empirical observations. The raw sequences of each genotype were mapped against its consensus sequence to assess the intrinsic variability of both genomes using the variant detector tool of CLC (Chateigner et al., 2015). Genome annotation for ChinNPV-K and ChinNPV-E consensus sequences was performed using SnapGene® (GSL Biotech; available at snapgene.com).

3. RESULTS

3.1 Effects of host heterogeneity and inoculum concentration on genetic diversity following inoculation of larvae with ChinNPV-Mex1

Virus-induced insect mortality varied significantly with inoculum concentration from a minimum of 14% to a maximum of 100% at the lowest and highest concentrations, respectively. The frequency of the ChinNPV-K variant REN profile increased significantly with inoculum concentration ($\chi^2 = 174.0$, df = 3, $p < 0.001$). The high inoculum concentration (10^8 OBs/mL), resulted in 100% mortality and 99 out of 100 recovered larvae showed the ChinNPV-K variant REN profile, whereas the single remaining insect had the dominant profile of the ChinNPV-E variant (Figure 1A). The intermediate concentrations of 5×10^6 and 5×10^5 OBs/mL resulted in 76% and 40% mortality, respectively. These concentrations also resulted in intermediate prevalences of 32% and 28%, respectively, of the ChinNPV-K REN profile in OBs from virus-killed insects. At a concentration of 5×10^6 OBs/mL, ChinNPV-I was the most prevalent variant followed by twelve other variants (Figure 1B). At 5×10^5 OBs/ml, ChinNPV-C was the most prevalent of the minority variants (Figure 1C). The lowest inoculum concentration, 10^4 OBs/mL, resulted in 14% mortality, but none of the virus-killed larvae produced a ChinNPV-K REN profile, whereas

the ChinNPV-E variant was the most prevalent REN profile, followed by eleven other variants (Figure 1D).

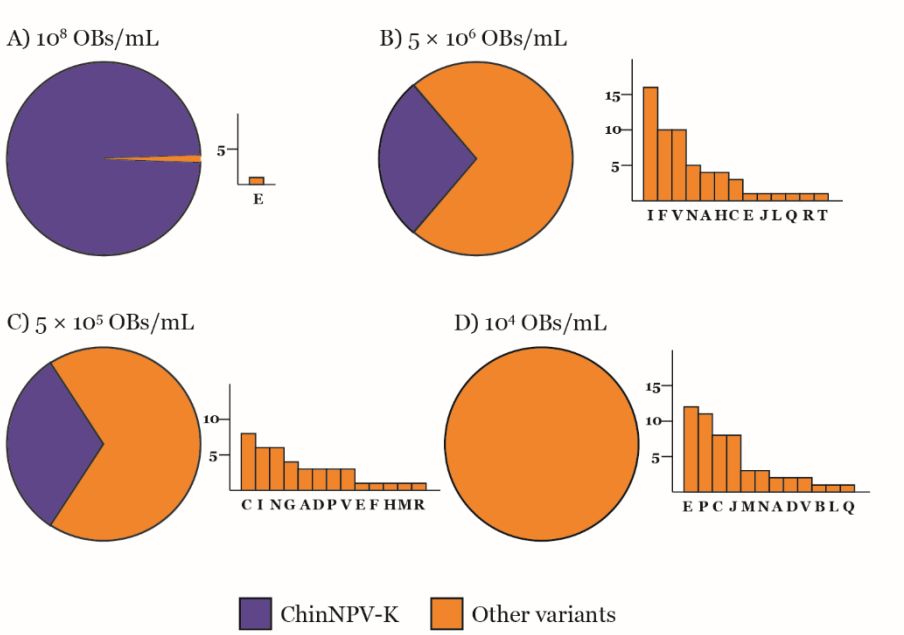

Figure 1. Prevalence of ChinNPV variants by dominant REN profile in *C. includens* fifth instar larvae inoculated with ChinNPV-Mex1 at concentrations of A) 10^8, B) 5×10^6, C) 5×10^5, D) 10^4 OBs/mL. Circular charts represent the proportions of dominant REN profiles similar or different to that of ChinNPV-K OBs recovered from virus-killed larvae (indicated as ChinNPV-K and Other variants, respectively). A bar chart is depicted next to each circular chart specifying which REN profiles were observed among the other variants.

3.2 Genetic diversity present following inoculation with variants ChinNPV-K and ChinNPV-E

Inoculation of ChinNPV-K and ChinNPV-E pure variant OBs in *C. includens* fifth instars resulted in marked differences in the frequency of variants in virus-killed insects. Inoculum concentration significantly affected the prevalence of larvae showing the ChinNPV-K REN profile ($\chi^2 = 41.84$, d.f. = 1, $p<0.001$). The higher inoculum concentration of 10^8 OBs/ml ChinNPV-K resulted in 99% mortality, but only 57% of larvae (90 out of 157 virus-killed insects) inoculated with the ChinNPV-K inoculum showed the

ChinNPV-K variant REN profile (Figure 2A). The remaining 43% of insects showed one of nine different variants, the most prevalent of which was a previously unknown variant, present in 26% (41 out of 157 larvae) of virus-killed insects. The unknown variant (shown as "?" in Fig. 2) did not match any of the variants identified previously (Aguirre et al., 2019). The 5 × 10^4 OBs/mL concentration of ChinNPV-K inoculum resulted in 22% mortality and the prevalence of ChinNPV-K REN profiles in virus-killed insects was 13% (10 out of 79 insects). The remaining 87% of insects showed REN profiles of thirteen different variants, the most prevalent of which was a group of unknown variants present in 16% (13 out of 79 larvae) of virus-killed insects comprising REN profiles that did not match any of the variants identified previously (Aguirre et al., 2019).

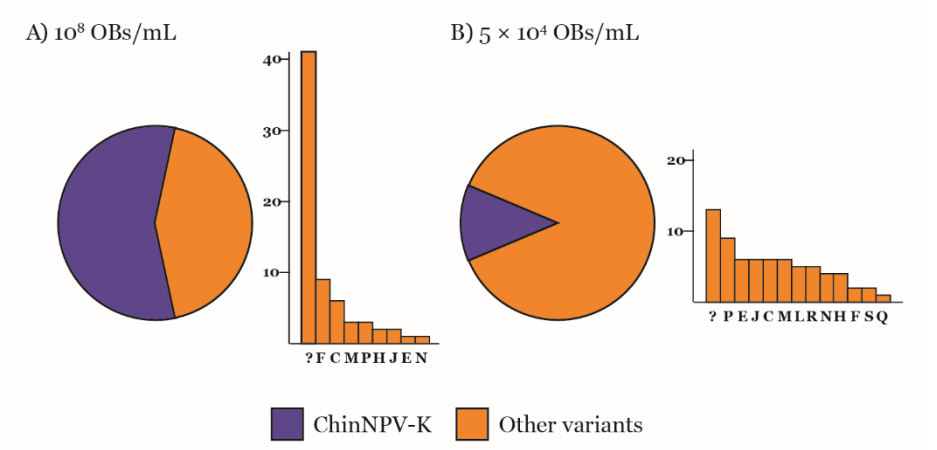

Figure 2. Prevalence of ChinNPV-K REN profiles in OB samples from virus-killed *C. includens* fifth instar larvae. Circular charts represent the proportions of REN profiles of ChinNPV-K and other variants recovered from virus-killed larvae that had been inoculated with A) 10^8 OBs/mL or B) 5 x 10^4 OBs/mL. A bar chart is depicted next to each circular chart to indicate which REN profiles were recovered among the other variants including a variant that could not be identified by their REN profiles (shown as "?").

In contrast, inoculum concentration did not significantly affect the prevalence of larvae showing the ChinNPV-E REN profile (χ^2 = 1.62, d.f. = 1, p=0.579). When a high concentration of ChinNPV-E inoculum was used, 99% of larvae died of polyhedrosis disease and 97% (135 out of 139 larvae) of virus-

killed insects showed the ChinNPV-E REN profile. A single unknown variant profile recovered from ChinNPV-E inoculated insects did not match any of the known variants. Larvae inoculated with the low concentration of ChinNPV-E inoculum experienced 24% mortality and the ChinNPV-E variant REN profile was the only profile observed in these insects. None of the control larvae died of polyhedrosis disease in any treatment.

3.3 Genetic stability of progeny OBs obtained following infection with ChinNPV-K OBs at low concentration

Following inoculation of larvae with a low concentration (5×10^4 OBs/ml) of ChinNPV-K variant OBs in the previous experiment (section 3.2), we selected four samples of the resulting variants showing the REN profiles of ChinNPV-H, -L, -N and -P. These samples were then used to inoculate larvae at high OB concentration (10^8 OBs/ml) to see whether it was possible to recover the original ChinNPV-K REN profile. Analysis of OBs from virus-killed larvae revealed that all four samples tested conserved their original REN profile (i.e. ChinNPV-H, -L, -N and -P) and no evidence of the ChinNPV-K variant or any other variant was detected in any of the virus-killed insects. This suggests that for the ChinNPV-K variant to dominate in the OB progeny it must be present at a minimum concentration in the OB inoculum administered to larvae under high concentration conditions.

3.4 Prevalence of ChinNPV-K and ChinNPV-E REN profiles in larvae inoculated with mixtures of variants K and E

The mixtures comprising similar or higher proportions of ChinNPV-K than ChinNPV-E revealed that ChinNPV-K REN profiles were observed in the OB progeny from the majority of insects that were inoculated with a high OB concentration. This was so even in the treatment in which the inoculum comprised both variants in equal proportions (5×10^7 OBs/ml of ChinNPV-K + 5×10^7 OBs/ml of ChinNPV-E) (Figure 3). Other variant REN profiles (variants C, E, F, J, M, N, P and one new variant, not classified previously) appeared in mixtures in which ChinNPV-K was present in the inoculum in a similar or

higher proportion than ChinNPV-E. When ChinNPV-E was present in the inoculum at a higher concentration than ChinNPV-K, only the ChinNPV-E REN profile was observed in OBs from virus killed insects, except for one case (variant ChinNPV-C) recovered in the mixture in which ChinNPV-E OBs were nine-fold more abundant than ChinNPV-K OBs (1×10^7 K + 9×10^7 E OBs/mL, not shown in Figure 3).

For the lower concentration, 5×10^4 OBs/ml, in both inoculum mixtures that contained the ChinNPV-K genotype (2.5×10^4 K + 2.5×10^4 E and 5×10^3 K + 4.5×10^4 E OBs/mL) the K variant REN profile appeared only once in each treatment. In the equimolar mixture (2.5×10^4 K + 2.5×10^4 E OBs/mL), 34% (13 out of 38 insects) of the recovered REN profiles showed the ChinNPV-E REN profile and up to 10 different REN profiles were detected in virus-killed insects (Figure 4A). In the inoculum in which the concentration of ChinNPV-K OBs was ten-fold higher than that of the ChinNPV-E OBs (5×10^3 K + 4.5×10^4 E OBs/mL), the ChinNPV-E REN profile was observed in 72% of virus-killed insects, with only four different variant REN profiles among the other REN profiles observed. In contrast, the diversity of different variant profiles decreased with the increasing concentration of ChinNPV-K OBs in the inoculum, with only five variants observed (apart from ChinNPV-K), most of which were of ChinNPV-E (Figure 4B). In the inoculum comprising ChinNPV-E OBs alone, no other variant profiles were recovered (not shown in Figure 4). These results indicate again that ChinNPV-E behaved as a pure genotype and was not involved in the spontaneous generation of ChinNPV diversity.

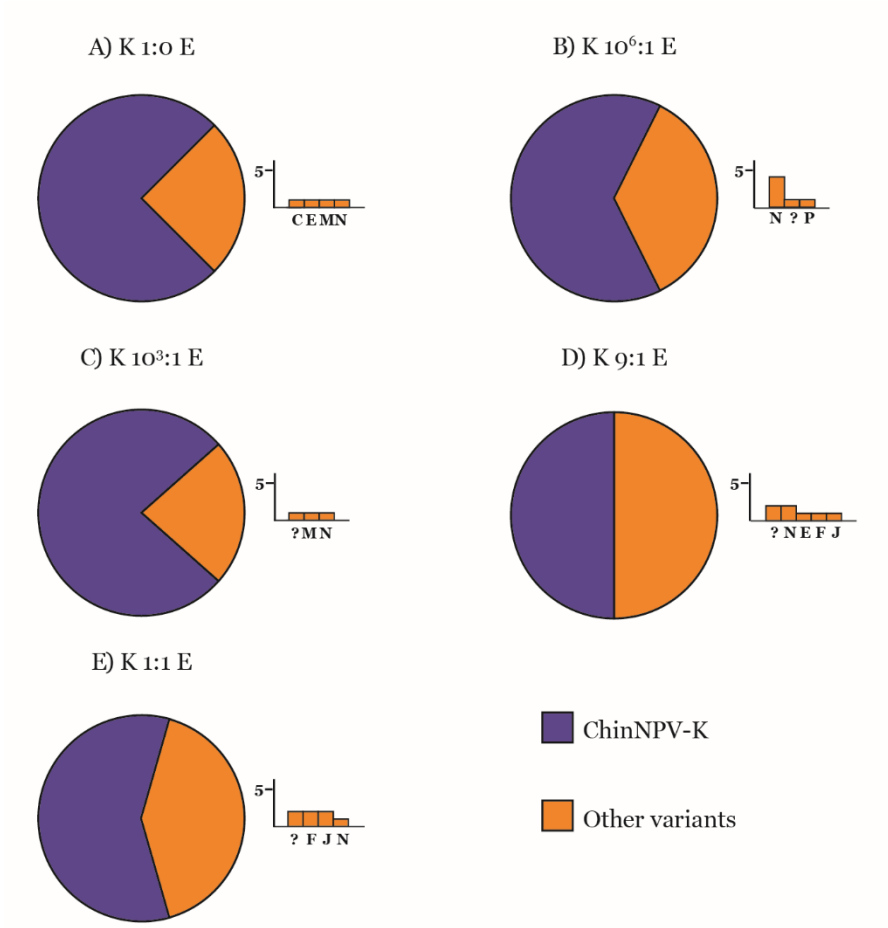

Figure 3. Prevalence of REN profiles in *C. includens* fifth instar larvae that died following inoculation of mixtures of variant K OBs and variant E OBs (total concentration 10^8 OBs/mL). Inocula were A) 1×10^8 K, B) 10^8 K + 10^2 E, C) 10^8 K + 10^5 E, D) 9×10^7 K + 10^7 E, E) 5×10^7 K + 5×10^7 E OBs/mL, shown as ratios of K:E in the figure. Circular charts represent the proportions of REN profiles of ChinNPV-K and other variants recovered from dead larvae. A bar chart depicted next to each circular chart indicates which REN profiles were recovered among the other variants and their frequency. Only those concentrations in which ChinNPV-K appears are represented; the inocula comprising 1×10^7 K + 9×10^7 E, 10^5 K + 10^8 E, 10^2 K + 10^8 E and 10^8 E OBs/ml, in which only the ChinNPV-E REN profile was recovered, are not depicted. Novel REN proflies were classified as unknown (shown as "?" in bar charts).

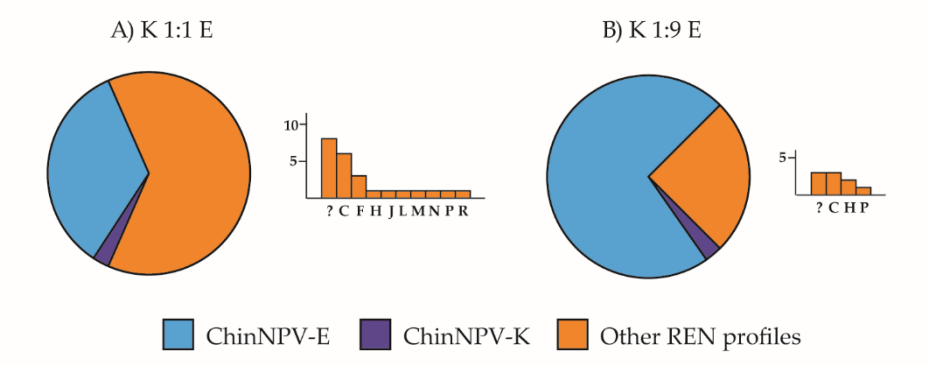

Figure 4. Prevalence of ChinNPV-K and ChinNPV-E REN profiles in *C. includens* fifth instar larvae that died following inoculation with variant mixtures (K + E) at a total concentration of 5×10^4 OBs/ml. Inocula were A) 2.5×10^4 K + 2.5×10^4 E, B) 5×10^3 K + 4.5×10^4 E OBs/mL, shown as ratios of K:E in the figure. Circular charts represent the proportions of REN profiles of ChinNPV-E, ChinNPV-K and other variants recovered from dead larvae. A bar chart depicted next to each circular chart indicates which REN profiles were recovered among the other variants and their frequency. Novel REN proflies were classified as unknown (shown as "?" in bar charts).

3.5 Prevalence of ChinNPV-K REN profile in pooled samples

The OB samples collected following inoculation of larvae with ChinNPV-K at the lower concentration (5×10^4 OBs/ml) were pooled and used to inoculate new batches of larvae at two inoculum concentrations to determine whether the ChinNPV-K variant could be recovered by a single passage of pooled OB samples. The REN profile of the pooled OB sample was identical to that of the ChinNPV-K variant profile. When pooled OBs were used to inoculate larvae at the higher concentration (1×10^8 OBs/ml), 9 out of 12 virus-killed larvae (75%) presented the ChinNPV-K variant profile, whereas the remaining 3 larvae (25%) presented two different variant profiles (Figure 5A). When pooled OBs were used to inoculate larvae at the lower concentration (5×10^4 OBs/ml), the ChinNPV-K variant was again the most prevalent profile present in 14 out of 32 larvae (44%) of the total recovered genotypes. The remaining 18 insects (56%) presented one of six different variant profiles in different frequencies (Figure 5B).

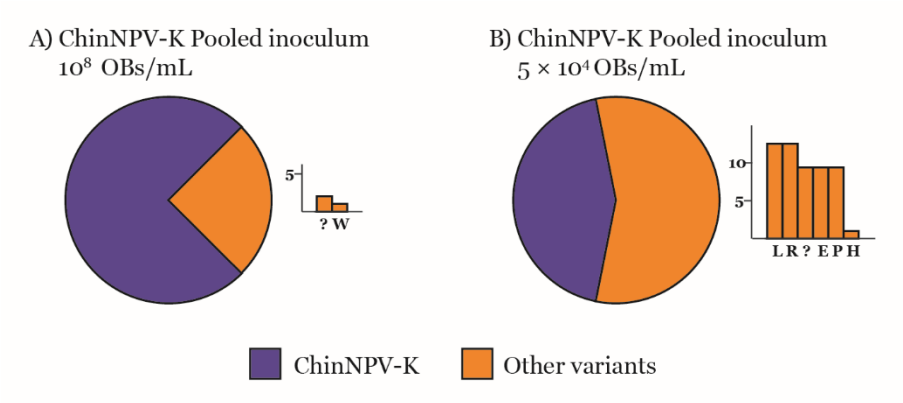

Figure 5. Prevalence of REN profiles in *C. includens* fifth instar larvae that were inoculated with A) 10^8 OBs/mL or B) 5 x 10^4 OBs/mL of a pooled OB inoculum derived from insects that had been originally inoculated with ChinNPV-K variant OBs. Circular charts represent the proportion of REN profiles of ChinNPV-K and other variants recovered from dead larvae. A bar chart is depicted next to each circular chart specifying which REN profiles were recovered among the other variant profiles and their frequency. Novel REN proflies were classified as unknown (shown as "?" in bar charts).

3.6 ChinNPV-K and ChinNPV-E infectivity in cell culture

$TCID_{50}$ values estimated by end-point dilution assay were $1.3 \times 10^6 \pm 2.3 \times 10^5$ pfu/ml for ChinNPV-K and $2.8 \times 10^5 \pm 1.9 \times 10^4$ pfu/ml for ChinNPV-E indicating a significantly higher production of BV in the hemolymph of ChinNPV-K infected larvae at 48 hours post-infection (Welch's t = 19.88, d.f. = 1, 2.03, p = 0.046). Budded virus quantification by qPCR indicated the presence of 1.95×10^8 genome copies/ml of hemolymph for ChinNPV-K and 8.5×10^7 copies/ml for ChinNPV-E, i.e. a 2.3-fold higher production of BV in ChinNPV-K infected insects.

3.7 ChinNPV-K progeny in cell culture

The infection of HighFive cells with a high concentration of ChinNPV-K BVs resulted in a DNA profile that matched that of the ChinNPV-K variant in each of the three replicates performed. For the lower concentration, a total of 48 plaque picks that originated from ChinNPV-K BVs in hemolymph were injected in *C. includens* fifth instars and virus-killed larvae that produced OBs were analyzed by REN. Ten different REN profiles were identified in virus-killed

insects but none of the 48 clones presented the characteristic ChinNPV-K variant REN profile (Figure 6). The most prevalent REN profile in cell culture was that of ChinNPV-P that was present in 18 out of 48 (38%) virus-killed larvae. In contrast, plaque picks derived from ChinNPV-E BV in hemolymph, produced virus-killed larvae that showed the ChinNPV-E variant profile alone; no other REN profiles were observed. Thus, ChinNPV-E again behaved as a pure genetic variant and did not spontaneously generate variant diversity.

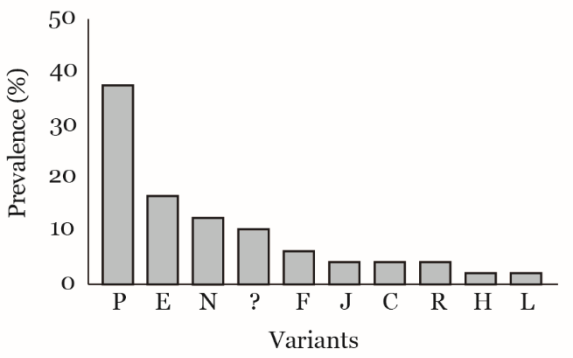

Figure 6. Prevalence of REN profiles from virus-killed larvae infected by injection of plaque picks derived from BVs in hemolymph samples obtained following inoculation of *C. includens* fifth instars with ChinNPV-K pure variant OBs. New genotypes whose REN profiles did not match any of the previous studied variants were included in the unknown group (shown as "?"). Total sample size = 48 plaque picks.

The previous plaque purification procedure was repeated using ChinNPV-K ODVs instead of BVs, with similar results. Seven different REN profiles from 23 analyzed plaque picks were observed in virus-killed insects but none of these corresponded to the ChinNPV-K variant profile (Figure 7). The genetic variants obtained from the plaque picks using ODVs as inoculum differed from those obtained using BV in hemolymph as inoculum, although variant P was the dominant variant profile in both cases. This result suggests that the ChinNPV-K variant is not a mixture of genetic variants but is capable of generating variant diversity de novo.

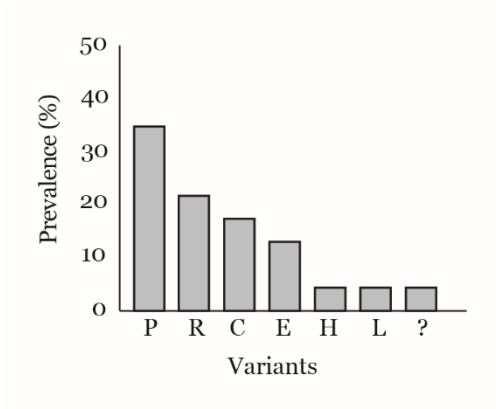

Figure 7. Prevalence of REN profiles from virus-killed *C. includens* fifth instars infected by injection of plaque picks derived from ChinNPV-K pure variant ODVs. Those new genotypes whose REN profiles did not match any of the previous genotypes were included in the unknown group (shown as "?"). Total sample size = 23 plaque picks.

3.8 ChinNPV-K and ChinNPV-E genome sequencing

Raw sequence data of both variants gave an entire coverage of 6291 for ChinNPV-E and 6180 for ChinNPV-K. Reads obtained by Illumina technology for ChinNPV-E DNA were assembled in a unique contig, whereas the sequence of ChinNPV-K was assembled in six different contigs and subsequently closed by Sanger sequencing. For both sequences, the Phred quality score was >30. ChinNPV-K and ChinNPV-E consensus sequences were digested *in silico*. The ChinNPV-E *in silico* REN profile with EcoRI and HindIII both exactly matched the empirical profiles, whereas the ChinNPV-K in silico REN profile did not match either the EcoRI or the HindIII empirical profiles, although it did match those of the other restriction enzymes tested such as PstI and BamHI. After mapping the raw reads of both ChinNPV-E and ChinNPV-K against its own consensus sequence, ChinNPV-E showed a small degree of variation located in the *hoar* gene, which did not include any restriction site. However, the ChinNPV-K sequences showed variability along the entire genome (Figure 8). Indeed, 285 variations were detected in ChinNPV-K, most of which were SNPs. Four of the SNPs were located in EcoRI restriction sites and one in a HindIII restriction site, which may explain the fact that the in silico and the experimental REN profile did not match. Genome annotation using the

Pseudoplusia includens SNPV-IE genome as reference resulted in 139 ORFs for both genomes (two fewer than the PsinSNPV reference genome), of which three ORFs had no correspondence with any of the sequenced ChinNPV genomes available in the NCBI GenBank. Differences between ChinNPV-K and -E genomes were mainly SNPs, which did not affect the length of any of the detected ORFs, with one marked exception in the *hoar* gene, which was 114 bp longer in ChinNPV-E compared to the PsinSNPV reference genome.

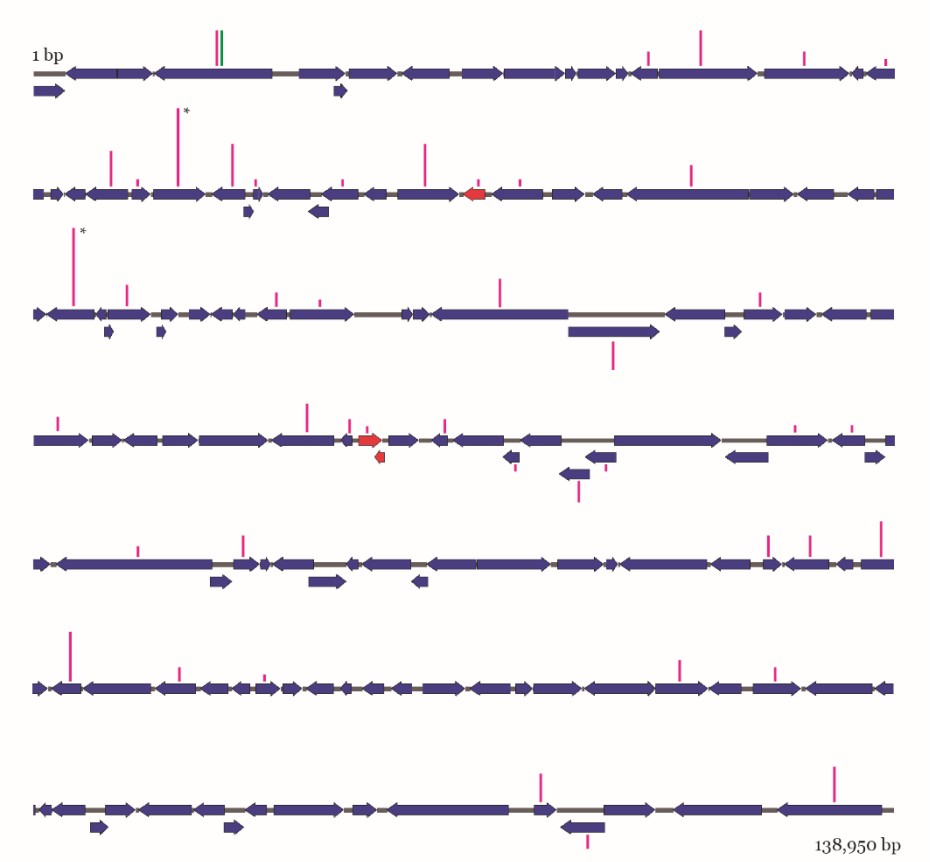

Figure 8. Schematic representation of ChinNPV-K genome ORFs. Vertical bars represent the number of cumulative SNPs in each ORF in pink for ChinNPV-K and green for ChinNPV-E, resulting in different heights according to the number of SNPs. Bars labelled with asterisks indicate the highest number of SNPs detected, which was 22 SNPs per ORF. Only the ChinNPV-K genome is depicted due to the high similarity between the ChinNPV-K and ChinNPV-E ORF length and distribution. The three ORFs in red represent ORFs that are not present in the ChinNPV genomes sequenced previously.

4. DISCUSSION

The generation of variability in ChinNPV and the probable mechanisms involved in its generation were examined. In a previous study, we detected high ChinNPV diversity in larvae collected from soybean fields, despite an intermediate prevalence (~20%) of natural virus-induced mortality (Aguirre et al., 2019). Notably, the number of genotypic variants present in individual virus-killed larvae increased as the concentration of inoculum decreased.

This result finds support from previous studies on Helicoverpa armigera nucleopolyhedrovirus (HearNPV) (Baillie and Bouwer, 2013) who also observed high variability of HearNPV in insects inoculated with low doses of OBs. Nevertheless, there are important differences with the procedures of Baillie and Bouwer (2013) in that the HearNPV study was performed using two isolates, whereas we used a mixture of nine different field isolates of ChinNPV, i.e., our initial inoculum was likely to comprise a greater diversity of variants than the inoculum used by Baillie and Bouwer (2013).

Baillie and Bouwer (2013) argued that genetic events like mutation and recombination are expected to occur at similar rates under both high and low dose conditions, although a lower production of OBs in the insects that consumed high dose inoculum (due to the fast speed-of-kill of high inoculum concentrations) may reduce the genetic diversity detected. The relationship between the speed-of-kill of the virus and the concentration or dose of OBs consumed has been recognized often in alphabaculovirus pathosystems (Cory, 2010; Williams, 2018).

Recombination events take place when the same cell is infected by at least two different genotypes and this may be exacerbated at high multiplicities of infection (M.O.I.) (Bull et al., 2001), which would result in higher variability when large quantities of virus are used as inoculum. However, we observed the contrary, namely greater diversity at low inoculum concentrations. This may be explained by a founder effect, in which a few founder genomes undergo changes in the early stages of infection which are then transmitted and amplified in other cells via the BV progeny and become the dominant variant in the majority of OB

progeny collected from virus-killed insects. This effect relies on a combination of (i) the paucity of founder virus genomes in low concentration inocula and (ii) instability in the replication of these genomes early in infection. Notably, of all the variants that we identified, instability was only observed in the ChinNPV-K variant; all other variants were stable during passage in *C. includens*.

The variability found in the low concentration inoculum treatments is consistent with the diversity present in the field-collected isolates in which 11 larvae that died from natural ChinNPV infection yielded 9 different REN profiles (Aguirre et al., 2019). However, the REN profiles obtained from individual field-collected larvae (Aguirre et al., 2019) and insects inoculated in the laboratory did not match, suggesting that the variability generated in ChinNPV populations at low inoculum concentrations does not follow a fixed pattern. In the studies carried out with different inoculum concentrations, we observed that, in addition to the increased variability with the decrease of inoculum concentration, the prevalence of the genetic variants also varied. At high concentrations of OB inoculum, the ChinNPV-K genotype dominated all other variants in progeny OBs and at the same time, the ChinNPV-E variant increased in prevalence at the intermediate inoculum concentrations (5×10^6 and 5×10^5 OBs/ml), and was the most prevalent variant at the lowest inoculum concentration. In contrast, Baillie and Bouwer (2013) did not observe any consistent pattern in the distribution of genetic variants across virus doses in HearNPV. It has been hypothesized that, when one genetic variant is particularly virulent, it can dominate over the other variants during the spread of an epizootic of infection (Gelernter and Federici, 1990). This is usually observed in wild-type baculoviruses where the most abundant variant is often the one with the highest fitness in a given environment at a given moment (Bernal et al., 2013; Ferreira et al., 2019). Nevertheless, according to the experiments carried out previously (Aguirre et al., 2019) the ChinNPV-K genotype was not the most pathogenic variant i.e., the variant that produced the highest prevalence of mortality in inoculated insects. Indeed, the ChinNPV-K was grouped among the least pathogenic variants, and resulted in mortality similar to that of the ChinNPV-Mex1 mixture (Aguirre et al., 2019).

In the present study we examined the effect of inoculum concentration upon two selected genotypes, ChinNPV-K and ChinNPV-E, because of their observed behavior in *C. includens* fifth instars after inoculation with ChinNPV-Mex1. In larvae inoculated with ChinNPV-E OBs, the progeny OBs from virus-killed larvae consistently presented the ChinNPV-E REN profile, except for a single larva in the higher dose. However, when larvae were inoculated with ChinNPV-K OBs, several different variant REN profiles were obtained, suggesting a role of ChinNPV-K in the generation of viral diversity. Although more than half of the ChinNPV-K inoculated insects died showing the K variant profile, nine different REN profiles (of which one was not previously known and was classified as a new variant) were detected among the virus-killed insects. The new REN profile recovered was five-fold more prevalent than the next most common variant, ChinNPV-F (Figure 2A). However, in insects inoculated with a lower concentration of ChinNPV-K OBs, the prevalence of ChinNPV-K decreased markedly in the OBs collected from virus-killed insects and the fraction of other variants increased conspicuously (Figure 2B). The appearance of so many variants and the differences in the presence of ChinNPV-K in virus-killed insects from high and low inoculum concentrations suggested that ChinNPV-K was not a structured mixture of genotypes. Indeed, considering that both genotypes (ChinNPV-K and ChinNPV-E) were obtained through plaque purification, it is highly unlikely that all the genotypes produced in ChinNPV-K killed insects were collected along with the ChinNPV-K variant when this genotype was isolated by plaque-purification.

Following this hypothesis, we examined the genetic stability of the OB progeny obtained from ChinNPV-K-inoculated insects that died at the lower inoculum concentration in which a diversity of variant profiles were observed in OBs from virus-killed larvae. We wondered whether ChinNPV-K was present in these OBs but at a level that was undetectable in by REN analysis. If so, according to our previous results, a high prevalence of ChinNPV-K REN profile could be expected if larvae were inoculated with a high concentration of these OBs. However, the ChinNPV-K REN profile was not recovered in any of the larvae that were inoculated. We then hypothesized that a minimum

concentration of ChinNPV-K was required in the inoculum to recover ChinNPV-K dominance in larvae inoculated with a high concentration of OBs.

To assess whether the dose-dependent generation of variant diversity by ChinNPV-K was associated with total OB dose (all variants) or whether a minimum concentration of ChinNPV-K was required in the inoculum, we designed mixtures in which the proportions of the variants ChinNPV-K and ChinNPV-E varied, but the total concentration of OB inoculum remained constant. Following analysis of OBs from insects that died at the high inoculum concentration, we observed that ChinNPV-K was the most prevalent variant recovered in all inoculum mixtures involving >50% of variant K OBs (Figure 3). In addition, very few variants other than ChinNPV-K and ChinNPV-E were detected. As the prevalence of ChinNPV-K decreased and the prevalence of ChinNPV-E increased in the inocula, only ChinNPV-E was detected in the virus-killed insects, which indicated that a minimum quantity of ChinNPV-K was required for the K variant and other genotypes to appear in REN profiles. This suggests that ChinNPV-K contributed to genetic variability but only when this variant was present at a high proportion in the inoculum. A similar result was obtained in the variant mixtures administered at low inoculum concentration. An equimolar mixture of ChinNPV-K and ChinNPV-E inoculum resulted in a high diversity of variants in the progeny OBs that decreased markedly when the prevalence of ChinNPV-K in the inoculum OBs was nine-fold lower than that of ChinNPV-E. Again, the inoculum comprising only ChinNPV-E did not result in the appearance of any other variants in progeny OBs. In conclusion, a minimum quantity of ChinNPV-K was required in the inoculum in order to observe the dose-dependent generation of variability. At high concentrations, a minimum of 50% of ChinNPV-K OBs is needed to observe the dominance of the ChinNPV-K variant in REN profiles, whereas the generation of diversity at low concentrations is observed even when only 10% of ChinNPV-K OBs is present. This finding confirmed that the ChinNPV-K variant differs from the other ChinNPV variants, and functions as a generator of variability in a concentration-dependent manner.

All the previous experiments involved the analysis of OB progeny from individual larvae. Analysis of the pooled OB sample obtained from ChinNPV-K inoculum at the lower concentration (5×10^4 OBs/mL), unexpectedly resulted in the ChinNPV-K variant profile. Based on the previous experiments in which only 13% of virus-killed larvae produced the K variant REN profile (section 3.2), we expected that the pooled inoculum would show a profile that differed from that of ChinNPV-K. This new result suggested that ChinNPV-K was present as a minority variant in virus-killed larvae (the source of the pooled OBs) at a prevalence too low to be detected by the REN technique. However, by pooling OBs from multiple host insects, the accumulation of low levels of ChinNPV-K in the presence of multiple other variants in the individual larvae may have converted ChinNPV-K into the majority genetic variant of the pooled OBs and, thus, the only REN profile detectable.

Larvae inoculated with the high concentration of pooled OBs mostly showed the presence of the ChinNPV-K variant profile, with a low prevalence of other variants. At the low concentration, the prevalence of ChinNPV-K decreased and the diversity of REN profiles increased again. As the REN analysis indicated that the ChinNPV-K variant was the majority variant in the pooled OB inoculum the unusual behavior of this variant in generating diversity at low inoculum concentrations was confirmed. As we did not know the complete variant composition of the pooled OBs, due to limitations of the qPCR technique in the presence of high variation, we were unable to identify the founder variants or detect events such as recombination between the variants during the early stages of the infection process that may have affected the composition of the viral progeny recovered from virus-killed insects.

Given these results from in vivo experiments, we asked whether ChinNPV-K would show the same characteristics when infecting cells in vitro. For this, we first compared the in vitro infectivity of ChinNPV-K and ChinNPV-E and detected minimal differences. Cells inoculated with a high inoculum concentration produced the ChinNPV-K variant, indicating the same behavior as observed in the in vivo experiments. Plaque purification procedures performed at low concentration were designed so that cells were likely to be

infected by a single BV or ODV, both of which contain a single genome (as ChinNPV is a single nucleocapsid NPV) (Craveiro et al., 2013) However, we found it impossible to isolate a clone of ChinNPV-K using BVs or ODVs. Unexpectedly, instead of ChinNPV-K clones, up to ten different REN variant profiles were detected in BV-infected cells and up to seven variants in cells infected using ODVs. The number and identity of variants also varied when individual plaque picks were injected into larvae. This result points strongly to ChinNPV-K being a genetically unstable variant. Moreover, recombination is unlikely to have generated the observed variants as it normally involves at least two variants infecting the same cell, which is unlikely in cell culture conditions involving low concentrations of BV or ODV inocula. Furthermore, the phenomenon of superinfection exclusion has been described in baculoviruses (Beperet et al., 2014; Salem et al., 2012; Xu et al., 2013) and has recently been proposed as a mechanism of protection against coinfection of cells by parental viruses and their progeny that may harbor deleterious mutations (Zhang et al., 2017). Superinfection exclusion is employed by many viruses to avoid coinfection of cells by similar viruses, which could mean that recombination may rarely be the cause for the diversity observed in our study. It appears more likely that variants were generated during replication in cell culture principally by point mutation. Recent studies in different baculoviruses using deep sequencing seem to indicate that point mutations are a frequent source of diversity in this virus family (Alletti et al., 2017; Chateigner et al., 2015; Masson et al., 2020). Therefore, genetic variability within baculovirus strains is probably far higher than that observed with techniques such as REN profiles or the DGGE (denaturing gradient gel electrophoresis) analysis used by Baillie and Bouwer (2012). Furthermore, superinfection exclusion could also be involved in the establishment of a founder effect. At low concentrations, the few founder variants that successfully overcome the bottlenecks at the initial stages of infection (midgut cell sloughing, apoptosis, dissemination of BVs, etc.) will be protected from the replication of other variants by the superinfection exclusion mechanism and are more likely to be the progenitor genomes of viral progeny.

To unravel the molecular processes that might be involved in the ability of ChinNPV-K to generate variant diversity, genome sequencing was performed. The ChinNPV-E variant genome was included in this study as a reference genome that did not generate de novo diversity. The first notable difference between the two genomes was that, despite a similar coverage of approximately 6200, ChinNPV-E was assembled in a single contig, whereas the ChinNPV-K genome sequencing required the assembly of six contigs. To better understand this, we mapped the raw reads of each genome against its own consensus sequence. As suspected, the ChinNPV-E reads showed little variability compared to the consensus sequence (with only 9 SNPs detected) and that variability was exclusively located in the *hoar* gene, which is known as a primary source of variability among baculovirus genotypes (Bideshi et al., 2003; Erlandson, 2009; Le et al., 1997; Rohrmann, 2019). In contrast, the ChinNPV-K reads had up to 285 variations in comparison to the consensus sequence that were distributed throughout the genome. This suggested that the ChinNPV-K genome is highly unstable compared to that of ChinNPV-E. Furthermore, the presence of these SNPs at restriction sites is likely to be the origin of some of the variability observed. When one of these SNPs is fixed, the REN profile changes and, therefore, the resulting progeny OBs are considered as a new genetic variant. In addition, initial comparison of the ChinNPV-E and -K genomes did not reveal evidence for large deletions or insertions observed in variants from some other baculovirus populations (Muñoz et al., 1998; Redman et al., 2010; Simón et al., 2005), although a subset of ChinNPV isolates was recently identified comprising chimeric genomes that appear to survive in natural populations of this virus (Inglis et al., 2020). The pattern of variation in the ChinNPV-K genome did not match the regions of phylogenetic incongruence (PIRs) that have been described in Brazilian isolates of ChinNPV by Inglis et al. (2020), in which variability was grouped across six defined zones of the genome, meaning that mechanisms other than horizontal gene transfer are responsible for the variability observed in ChinNPV-K. The replication instability of ChinNPV-K also complicated potential further studies, such as the quantification of ChinNPV-K present in individual larvae by qPCR. Specifically,

the unique primer targets for ChinNPV-K are likely to be compromised by SNPs or involve the *hoar* region that comprises numerous repeat sequences that invalidate its use in qPCR amplification. Consequently, despite its evident limitations, the use of restriction endonucleases was one of the few informative techniques available to us for variant characterization in the present study.

In this study we examined characteristics of OBs collected from insects inoculated with different concentrations of ChinNPV-K and ChinNPV-E OBs. Genome sequencing revealed that the ChinNPV-K variant harbors a high level of intrinsic genetic variability that could be readily detected by the REN technique. The unstable ChinNPV-K variant was capable of generating a range of genotypes that appeared on multiple occasions and at different prevalences in insects inoculated with low concentrations of ChinNPV-K OBs. This was likely due to a combination of a paucity of founder genomes in insects that consumed low inoculum concentrations and replication instability in the founder genomes that gave rise to the majority of progeny OBs. If the variability in ChinNPV-K was generated by point mutation, then small changes at certain points on the genome might result in phenotypic differences in biological activity (Cory et al., 2005). Most SNP type modifications would not have biological relevance unless they produced a significant change in an amino acid at the active site of a particular protein or a key cellular receptor, for example. The ChinNPV-K variant was able to generate high levels of diversity in the ChinNPV population which may have implications for virus fitness, host-virus interactions and the transmission of diversity via the progeny OBs from insects infected by this unusual variant.

5. REFERENCES

Aguirre, E., Beperet, I., Williams, T., Caballero, P., 2019. Genetic variability of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) and the insecticidal characteristics of selected genotypic variants. Viruses 11, 581. https://doi.org/10.3390/v11070581

Alletti, G.G., Sauer, A.J., Weihrauch, B., Fritsch, E., Undorf-Spahn, K., Wennmann, J.T., Jehle, J.A., 2017. Using next generation sequencing to identify and quantify the genetic composition of resistance-breaking commercial isolates of Cydia pomonella granulovirus. Viruses 9, 250.

https://doi.org/10.3390/v9090250

Baillie, V.L., Bouwer, G., 2013. The effect of inoculum dose on the genetic diversity detected within Helicoverpa armigera nucleopolyhedrovirus populations. J. Gen. Virol. 94, 2524–2529. https://doi.org/10.1099/vir.0.052803-0

Baillie, V.L., Bouwer, G., 2012. High levels of genetic variation within Helicoverpa armigera nucleopolyhedrovirus populations in individual host insects. Arch. Virol. 157, 2281–2289. https://doi.org/10.1007/s00705-012-1416-6

Beperet, I., Irons, S.L., Simón, O., King, L. a, Williams, T., Possee, R.D., López-Ferber, M., Caballero, P., 2014. Superinfection exclusion in alphabaculovirus infections is concomitant with actin reorganization. J. Virol. 88, 3548–56. https://doi.org/10.1128/JVI.02974-13

Bernal, A., Simón, O., Williams, T., Muñoz, D., Caballero, P., 2013. A Chrysodeixis chalcites single-nucleocapsid nucleopolyhedrovirus population from the Canary Islands is genotypically structured to maximize survival. Appl. Environ. Microbiol. 79, 7709–7718. https://doi.org/10.1128/AEM.02409-13

Bideshi, D.K., Renault, S., Stasiak, K., Federici, B.A., Bigot, Y., 2003. Phylogenetic analysis and possible function of *bro*-like genes, a multigene family widespread among large double-stranded DNA viruses of invertebrates and bacteria. J. Gen. Virol. 84, 2531–2544. https://doi.org/10.1099/vir.0.19256-0

Bull, J.C., Godfray, H.C.J., O'reilly, D.R., 2001. Persistence of an occlusion-negative recombinant nucleopolyhedrovirus in *Trichoplusia ni* indicates high multiplicity of cellular infection. Appl. Environ. Microbiol. 67, 5204–5209. https://doi.org/10.1128/AEM.67.11.5204-5209.2001

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

Cory, J., 2010. The ecology of baculoviruses. Insect Virol. 405–421.

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/http://dx.doi.org/10.1016/j.jip.2005.03.008

Craveiro, S.R., Melo, F.L., Ribeiro, Z.M.A., Ribeiro, B.M., Báo, S.N., Inglis, P.W., Castro, M.E.B., 2013. Pseudoplusia includens single nucleopolyhedrovirus: Genetic diversity, phylogeny and hypervariability of the pif-2 gene. J. Invertebr. Pathol. 114, 258–267. https://doi.org/10.1016/j.jip.2013.08.005

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. https://doi.org/10.1007/s12250-009-3052-1

Ferreira, B.C., Melo, F.L., Silva, A.M.R., Sanches, M.M., Moscardi, F., Ribeiro, B.M., Souza, M.L., 2019. Biological and molecular characterization of two Anticarsia gemmatalis multiple nucleopolyhedrovirus clones exhibiting contrasting virulence. J. Invertebr. Pathol. 164, 23–31. https://doi.org/10.1016/j.jip.2019.03.008

Gebhardt, M.M., Eberle, K.E., Radtke, P., Jehle, J.A., 2014. Baculovirus resistance in codling moth is virus isolate-dependent and the consequence of a mutation

in viral gene *pe38*. Proc. Natl. Acad. Sci. 111, 15711–15716. https://doi.org/10.1073/pnas.1411089111

Gelernter, W.D., Federici, B.A., 1990. Virus epizootics in californian populations of *Spodoptera exigua*: dominance of a single viral genotype. Biochem. Syst. Ecol. 18, 461–466. https://doi.org/https://doi.org/10.1016/0305-1978(90)90093-U

Gilbert, C., Chateigner, A., Ernenwein, L., Barbe, V., Bézier, A., Herniou, E.A., Cordaux, R., 2014. Population genomics supports baculoviruses as vectors of horizontal transfer of insect transposons. Nat. Commun. 5, 3348. https://doi.org/10.1038/ncomms4348

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

Hang, X., Guarino, L.A., 1999. Purification of Autographa californica nucleopolyhedrovirus DNA polymerase from infected insect cells. J. Gen. Virol. 80, 2519–2526. https://doi.org/10.1099/0022-1317-80-9-2519

Harrison, R.L., Herniou, E.A., Jehle, J.A., Theilmann, D.A., Burand, J.P., Becnel, J.J., Krell, P.J., van Oers, M.M., Mowery, J.D., Bauchan, G.R., Consortium, I.R., 2018. ICTV Virus Taxonomy Profile: Baculoviridae. J. Gen. Virol. 99, 1185–1186. https://doi.org/https://doi.org/10.1099/jgv.0.001107

Hughes, P.R., Wood, H.A., 1987. In vivo and in vitro bioassay methods for baculoviruses, in: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses. CRC Press, Boca Raton, FL., pp. 1–30.

IBM Corporation, 2017. IBM SPSS Statistics for Windows.

Inglis, P.W., Santos, L.A.V.M., Craveiro, S.R., Ribeiro, B.M., Castro, M.E.B., 2020. Mosaic genome evolution and phylogenetics of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) and virulence of seven new isolates from the Brazilian states of Minas Gerais and Mato Grosso. Arch. Virol. 166, 125–138. https://doi.org/10.1007/s00705-020-04858-2

Kamita, S.G., Maeda, S., Hammock, B.D., 2003. High-frequency homologous recombination between baculoviruses involves DNA replication. J. Virol. 77, 13053–13061. https://doi.org/10.1128/jvi.77.24.13053-13061.2003

Le, T.H., Wu, T., Robertson, A., Bulach, D., Cowan, P., Goodge, K., Tribe, D., 1997. Genetically variable triplet repeats in a RING-finger ORF of Helicoverpa species baculoviruses. Virus Res. 49, 67–77. https://doi.org/10.1016/S0168-1702(97)01454-8

Li, L., Donly, C., Li, Q., Willis, L.G., Keddie, B.A., Erlandson, M.A., Theilmann, D.A., 2002. Identification and genomic analysis of a second species of nucleopolyhedrovirus isolated from *Mamestra configurata*. Virology 297, 226–244. https://doi.org/10.1006/viro.2002.1411

Loiseau, V., Herniou, E.A., Moreau, Y., Lévêque, N., Meignin, C., Daeffler, L., Federici, B., Cordaux, R., Gilbert, C., 2020. Wide spectrum and high frequency of genomic structural variation, including transposable elements, in large double-stranded DNA viruses. Virus Evol. 6. https://doi.org/10.1093/ve/vez060

López Ferber, M., Argaud, O., Croizier, L., Croizier, G., 2001. Diversity, distribution, and mobility of *bro* gene sequences in Bombyx mori nucleopolyhedrovirus. Virus Genes 22, 247–254. https://doi.org/10.1023/A:1011193603093

Lynn, D.E., 1992. Improved efficiency in determining the titer of the Autographa californica baculovirus nonoccluded virus. Biotechniques 13, 282–285.

Masson, T., Fabre, M.L., Pidre, M.L., Niz, J.M., Berretta, M.F., Romanowski, V., Ferrelli, M.L., 2020. Genomic diversity in a population of Spodoptera frugiperda nucleopolyhedrovirus. bioRxiv 2020.10.27.358317. https://doi.org/10.1101/2020.10.27.358317

McDougal, V. V., Guarino, L.A., 1999. Autographa californica nuclear polyhedrosis virus DNA polymerase: Measurements of processivity and strand displacement. J. Virol. 73, 4908–4918. https://doi.org/10.1128/jvi.73.6.4908-4918.1999

Mikhailov, V.S., Marlyev, K.A., Ataeva, J.O., Kullyev, P.K., Atrazhev, A.M., 1986. Characterization of 3'→5' exonuclease associated with DNA polymerase of silkworm nuclear polyhedrosis virus. Nucleic Acids Res. 14, 3841–3857. https://doi.org/10.1093/nar/14.9.3841

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 Env. Microbiol 64, 4372–4377.

Redman, E.M., Wilson, K., Grzywacz, D., Cory, J.S., 2010. High levels of genetic diversity in Spodoptera exempta NPV from Tanzania. J. Invertebr. Pathol. 105, 190–193. https://doi.org/10.1016/j.jip.2010.06.008

Rohrmann, G.F., 2019. Baculovirus molecular biology 4th edition.

Salem, T., Cheng, X.-H., Cheng, X.-W., 2012. AcMNPV enhances infection by ThorNPV in Sf21 cells and SeMNPV in Hi5 cells. Arch. Virol. 157, 1875–1885. https://doi.org/10.1007/s00705-012-1347-2

Sanjuán, R., Domingo-Calap, P., 2016. Mechanisms of viral mutation. Cell. Mol. Life Sci. 73, 4433–4448. https://doi.org/10.1007/s00018-016-2299-6

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. https://doi.org/10.1128/AEM.71.8.4254-4262.2005

Vincze, T., Posfai, J., Roberts, R.J., 2003. NEBcutter: A program to cleave DNA with restriction enzymes. Nucleic Acids Res. 31, 3688–3691. https://doi.org/10.1093/nar/gkg526

Virto, C., Navarro, D., Tellez, M.M., Herrero, S., Williams, T., Murillo, R., Caballero, P., 2014. Natural populations of *Spodoptera exigua* are infected by multiple viruses that are transmitted to their offspring. J. Invertebr. Pathol. 122c, 22–27. https://doi.org/10.1016/j.jip.2014.07.007

Williams, T., 2018. Viruses, in: Hajek, A.E., Shapiro-Ilan, D.I. (Eds.), Ecology of Invertebrate Diseases. John Wiley, Chichester, UK, pp. 213–285. https://doi.org/10.1002/9781119256106.ch7

Xu, X., Chen, Y., Zhao, Y., Liu, X., Dong, B., Jones, I.M., Chen, H., 2013. Baculovirus superinfection: a probable restriction factor on the surface display of proteins for library screening. PLoS One 8, e54631. https://doi.org/10.1371/journal.pone.0054631 [doi]PONE-D-12-28041 [pii]

Zhang, X.-F., Sun, R., Guo, Q., Zhang, S., Meulia, T., Halfmann, R., Li, D., Qu, F., 2017. A self-perpetuating repressive state of a viral replication protein blocks superinfection by the same virus. PLOS Pathog. 13, e1006253.

https://doi.org/10.1371/journal.ppat.1006253

CHAPTER IV

General discussion

Genetic variability in baculoviruses has been deeply discussed because of its clear implications in the development of biological insecticides (Guo et al., 2006). This variability will have marked consequences for the probability of transmission of the virus (Cory and Myers, 2003; Hitchman et al., 2007). Specifically, genetic variability allows virus adaptation to the constant changes in their environment and may permit viruses to adapt to variation in the host or in alternative host species (Kolodny-Hirsch and Beek, 1997; Martignoni and Iwai, 1986; Weitzman et al., 1992). During replication, baculoviruses undergo changes in their genome that can alter key biological traits such as occlusion body (OB) pathogenicity, speed-of-kill and the number of OBs produced in each infected host for subsequent transmission. These traits are invariably used to select the isolate or variants that comprise the active ingredient for a bioinsecticide (Ibarra and Del Rincón, 2001). In fact, the potential of baculoviruses as bioinsecticides is gaining recognition and their use is currently increasing worldwide (Lacey et al., 2015).

Chrysodeixis includens, the soybean looper, is a harmful pest of soybean crops, especially in Brazil, where the control of this pest has become an important issue in the production of soybean. For this reason the variability of ChinNPV was evaluated following the collection of isolates from a soybean crop in Tamaulipas, Mexico, the results of which are described in **Chapter II**. The genetic variability of the collected field isolates of ChinNPV and the insecticidal characteristics of five selected genotypes were determined. Initially, high diversity was identified in field-collected larvae, with nine different ChinNPV restriction endonuclease (REN) profiles out of eleven virus-killed larvae. This result differed from those obtained in other closely-related insect species and its homologous virus, where a lower number of isolates were recovered from a higher number of host larvae (Fuentes et al., 2017). The low

prevalence of the virus and high variability present in the same soybean field initially suggested that this variability could be a virus adaptation to the presence of an alternate host species, but host range testing revealed that this idea was probably not accurate. The Mexican ChinNPV only infected closely-related species in the Plusiinae subfamily, with higher efficiency in its homologous host. The difference in the oral infectivity found between Plusiinae-insects, i.e. between *C. includens* and *Trichoplusia ni*, may be related to problems in the establishment of the primary infection in the heterologous host (Morgado et al., 2020). Resistance to peroral infection that differed from the insect's susceptibility to intrahemocelic injection was previously reported in the Autographa californica multiple nucleopolyhedrovirus (AcMNPV) - *Spodoptera frugiperda* pathosystem (Haas-Stapleton et al., 2005). Whether the mechanisms involved in the ChinNPV - *T. ni* system are similar to those described for AcMNPV - *S. frugiperda* remain unclear and may be examined in future studies.

Biological characterization of the ChinNPV-Mex1 mixture revealed high OB pathogenicity with an LC_{50} value of 6.5×10^4 OBs/mL in *C. includens* second instars. To date, several natural isolates of ChinNPV have been subjected to concentration-mortality bioassays, although none of them have been shown to be more pathogenic than ChinNPV-Mex1 (Harrison et al., 2019; Muraro et al., 2019). All other studied isolates were collected in South America, compared to ChinNPV-Mex1 as the first ChinNPV isolates from North America (Mexico). Despite the promising LC_{50} value, it is important to bear in mind that is common for natural isolates to be better adapted to local populations of lepidopteran pests (Barrera et al., 2015; Escribano et al., 1999), which would explain the lower LC_{50} value of ChinNPV-Mex1 when tested against the pest population from which it was isolated (Barrera et al., 2013; García-Banderas et al., 2020).

High variability was present in the natural isolates of ChinNPV, which comprised up to 23 different genetic variants (based on REN profile characteristics), an elevated number for a nucleopolyhedrovirus collected at one site at one moment in time. Usually, the procedure of segregating

genotypes is performed using a single natural isolate, in order to reveal its genotypic composition. Nevertheless, in this thesis the different isolates found in the same soybean field in Mexico were mixed first, to sample all the variability present in those isolates and, second, to allow all variants to compete among themselves during replication to produce a mixture with favorable insecticidal traits. Although the number of genotypic variants isolated from ChinNPV-Mex1 was broadly similar to the numbers from other nucleopolyhedrovirus (Corsaro and Fraser, 1987; Hodgson et al., 2001), the ChinNPV-Mex1 mixture clearly differed in variability compared to related viruses such as Chrysodeixis chalcites nucleopolyhedrovirus (ChchNPV) and Anticarsia gemmatalis multiple nucleopolyhedrovirus (AgMNPV). For example, the study carried out by Bernal et al. (2013) revealed the presence of eight genotypic variants in one natural isolate of ChchNPV from the Canary Islands. Similarly, a study conducted on AgMNPV in Mexico resulted in five different genotypic variants isolated from a pooled sample of 30 field isolates (Del-Angel et al., 2018).

The high genotypic variability present in ChinNPV-Mex1 was a promising start for the identification of variants with traits that could be selected for the basis of an insecticide against *C. includens*. A single-concentration bioassay performed on all the plaque-isolated genetic variants revealed the ChinNPV-R as the most pathogenic variant, even more so than the ChinNPV-Mex1 artificial mixture. According to the OB pathogenicity criterion, five genetic variants were selected and subjected to speed-of-kill and OB productivity assays. As no significant differences were detected in median time to death (MTD), and OB productivity was similar among variants but higher than observed in the ChinNPV-Mex1 mixture (all tested variants except ChinNPV-J), it appears that ChinNPV-R could be selected as a candidate component of a bioinsecticide. In spite of this, the possible interactions among variants were not examined in this thesis although mixtures of genotypes can have better insecticidal characteristics than individual genotypic variants (Arrizubieta et al., 2015; Bernal et al., 2013). This is an interesting issue, as baculovirus genotypic variants often present lower OB pathogenicity than the

original isolate from which they originated (Lei et al., 2020; Redman et al., 2016). In the case of ChinNPV, the variants purified from natural isolates may compete for host resources or perhaps the different combinations of variants within natural isolates result in the mortality differences observed. Regardless, it would be of interest to further investigate this unusual characteristic of these ChinNPV genotypes.

The high variability observed in ChinNPV-Mex1 stimulated a study on the source of this variability and the possible mechanisms involved in its generation. **Chapter III** described several experiments on the prevalence of virus variants in the homologous host, *C. includens*, and described sequencing studies on two genetic variants with remarkable behavior in insects treated with different concentrations of OBs. Inoculation of larvae with one of four concentrations of ChinNPV-Mex1 OBs revealed that variability in the virus progeny depended on the inoculum concentration. Baillie and Bouwer (2013) performed a similar study on Helicoverpa armigera NPV and reached a similar conclusion, in that at low inoculum concentration, an increase in genetic variability was detected. Indeed, an increase in variability and changes in the frequencies of the variants were detected in ChinNPV inoculated insects. Several hypothesis concerning the generation of genotype variability were discussed. Firstly, recombination was recognized as a likely source of variability in the ChinNPV natural isolates. Nevertheless, recombination between different genotypes usually requires high multiplicity of infection (M.O.I) (Bull et al., 2001) due to the need of at least two different variants co-infecting the same cell. This event is evidently more frequent when a high concentration of inoculum is used, which implies a greater probability of different variants infecting the host for subsequent recombination. In any case, the results with ChinNPV contradict this assertion as higher variability was observed when insects were inoculated with lower concentrations of ChinNPV-Mex1 OBs.

In addition to the effect of low inoculum concentration on variant diversity, the prevalence of the different variants was also affected by the inoculum concentration. For instance, ChinNPV-K was the dominant variant

at high inoculum concentration, whereas ChinNPV-E increased in prevalence as the inoculum concentration decreased. This was clearly in contrast to the findings of Baillie and Bouwer (2013) on HearNPV. The fact that ChinNPV-K was the dominant variant might be explained by a specific highly competitive phenotype, as observed in other wild-type baculoviruses (Bernal et al., 2013a; Ferreira et al., 2019). However, when the biological properties of ChinNPV-K were examined in **Chapter II** (Aguirre et al., 2019), it became apparent that this variant was neither the most virulent nor the most pathogenic of the ChinNPV variants. Therefore, to investigate the effect of inoculum concentration two variants were selected: ChinNPV-K and ChinNPV-E. Experiments revealed that ChinNPV-K was the source of variability in this virus, whereas ChinNPV-E did not generate almost any variation in the same experimental conditions. These results, were notable in two aspects; the first one was that the inoculum concentration effect was inherent to ChinNPV-K and the second one was that using ChinNPV-K as inoculum resulted in a new variant that had not been recovered before. It was also clear that ChinNPV-K was not a structured mixture of genotypes as could be suspected from the previous results obtained. Indeed, it is important to highlight that both variants, ChinNPV-K and ChinNPV-E were recovered from a plaque assay, ruling out the possibility that ChinNPV-K was in fact a complex mixture of multiple variants.

The genetic stability of the variants obtained at the lower concentration of ChinNPV-K was then examined. Surprisingly, none of the variants tested produced any other variant apart from the original. This is consistent with each variant being a pure genotype. Furthermore, the role of ChinNPV-K was then investigated in the presence of another variant. It was also studied whether it was necessary for ChinNPV-K to be present at some minimum threshold concentration for variability to appear in the virus progeny. Those experiments indicated that ChinNPV-K had to be present at least in an equimolar concentration in the mixture to obtain a high diversity of variants in the progeny OBs when using a high inoculum concentration, and

that this variability disappeared when the concentration of ChinNPV-K was below that limit.

Nevertheless, the previous results were obtained from individual larvae, so analysis of a pooled sample was performed using material from dead larvae inoculated with ChinNPV-K at low concentration. The pooled sample presented the ChinNPV-K REN profile, suggesting that ChinNPV-K was also present in the individual larvae but its REN profile was not detectable due to the limits of the REN technique. This may be the reason for the lack of detection of the ChinNPV-K REN profile in individual larvae while it was evident in the pooled sample. Larvae inoculated with the pooled OB sample at high and low concentration showed similar results as the previous experiments carried out with individual larvae. A possible founder effect was then considered which could explain why high variability was observed when a low concentration of ChinNPV-K OBs was used, although this theory could not be confirmed experimentally due to the limitations of the qPCR technique in the presence of high variation. The sequencing of all genetic variants was considered in order to design specific qPCR DNA probes and thus, distinguish specifically each genetic variant in a mixture, thereby allowing the calculation of the proportions of each variant in a more accurate way. Nevertheless, sequencing of all variants and probe design would have had a high cost, so that variants had to be distinguished using the REN method. This was clearly not the best method for this task but was fast and had a considerable degree of resolution for the study.

So far, experimental data focused on of the variability generated by ChinNPV-K in vivo, but it was uncertain whether this variant would behave the same way in vitro conditions. To answer this, the infectivity of ChinNPV-K and ChinNPV-E was compared in cell culture, although only a small difference was detected that could hardly explain the differences observed among those genotypes in vivo. Using plaque purification of budded virions (BVs) and occlusion-derived virions (ODVs), different REN profiles were obtained, none of which were ChinNPV-K. This result clearly pointed to ChinNPV-K being a genetically unstable variant and generator of variability rather than a source of

recombination events, given that this scenario normally involves a minimum of two variants infecting the same cell, which would be highly improbable given the low concentrations of virus used for plaque purification. In addition, the superinfection exclusion phenomenon described for many viruses (including baculoviruses) supports the theory of genetic instability of ChinNPV-K, because this event is used by many viruses to avoid coinfection of cells by very similar viruses that could represent a competitor for the infected cell resources (Beperet et al., 2014; Salem et al., 2012; Xu et al., 2013; Zhang et al., 2017). Indeed, the variability observed at low concentrations as a consequence of the few founder variants that successfully infect the midgut cells are also protected by the superinfection exclusion mechanisms. This means that not only the founder effect would favor the variability but the superinfection exclusion would assure that those variants would not have to compete for the same cell.

The last step in understanding ChinNPV-K variability involved complete genome sequencing. The ChinNPV-E genome was also sequenced as a stable reference variant. As expected, little variation was detected in ChinNPV-E reads and variability was mainly located in the *hoar* gene. In contrast, a high number of variations were identified in ChinNPV-K that were distributed along the entire genome. It was clear then that in comparison to ChinNPV-E, the genome of ChinNPV-K appears to be markedly more unstable and presents much most variability in the form of single nucleotide polymorphisms (SNPs). Due to the REN technique used in this study, an SNP affecting an EcoRI/HindIII restriction site would change the REN profile and, therefore, would be considered a new genotype. So, the key issue here is the definition of a genotypic variant, this is, if only a few changes (SNPs) in an entire genome are sufficient to create new genotype or whether sequence variation must be considered as a part of the whole genome. In virology, the term quasispecies refers to complexes composed by large numbers of closely related variant genomes linked through mutation and subjected to competition and selection (Domingo and Perales, 2019; Lauring and Andino, 2010). Quasispecies theory is usually applied to RNA viruses that have high

mutation rates (Sanjuán and Domingo-Calap, 2016). However, advances in sequencing techniques have also revealed high variability present in baculovirus isolates (Alletti et al., 2017; Chateigner et al., 2015; Masson et al., 2020). This could be the situation of the ChinNPV-K genotype. Nevertheless, recent studies about the genetic variability in this virus have demonstrated the existence of chimeric genomes in natural populations (Inglis et al., 2020). This could explain the variability observed in the ChinNPV-K genome, although the regions of phylogenetic incongruence (PIRs) described by Inglis et al., (2021) do not fit the variability observed in the ChinNPV-K genome, or its hypothesized origin via horizontal gene transfer as the main cause of diversity. As a result, I postulate that the replication process is the source of the variability in ChinNPV-K, which implies the difficulty of tracing those punctual mutations by qPCR in further studies due to the high number of SNPs that would have to be traced.

In this thesis I have demonstrated the high genetic variability present in field-collected ChinNPV samples, both at the level of individual larvae and at the level of the virus population. This thesis reveals the importance of the inoculum concentration on the genetic diversity observed in virus killed insects and especially the role of a particular genetic variant, ChinNPV-K, in the generation of that variability. This information is of interest not only for the development of specific bioinsecticides for the Soybean looper, *Chrysodeixis includens*, but also for the understanding of genetic variability in baculoviruses.

REFERENCES

Aguirre, E., Beperet, I., Williams, T., Caballero, P., 2019. Genetic variability of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) and the insecticidal characteristics of selected genotypic variants. Viruses 11, 581. https://doi.org/10.3390/v11070581

Alletti, G.G., Sauer, A.J., Weihrauch, B., Fritsch, E., Undorf-Spahn, K., Wennmann, J.T., Jehle, J.A., 2017. Using next generation sequencing to identify and quantify the genetic composition of resistance-breaking commercial isolates of Cydia pomonella granulovirus. Viruses 9, 250.

https://doi.org/10.3390/v9090250

Arrizubieta, M., Simón, O., Williams, T., Caballero, P., 2015. A novel binary mixture of Helicoverpa armigera single nucleopolyhedrovirus (HearSNPV) genotypic variants provides improved insecticidal characteristics for control of the cotton bollworm. Appl. Environ. Microbiol. 81, 3984–3993. https://doi.org/10.1128/AEM.00339-15

Baillie, V.L., Bouwer, G., 2013. The effect of inoculum dose on the genetic diversity detected within Helicoverpa armigera nucleopolyhedrovirus populations. J. Gen. Virol. 94, 2524–2529. https://doi.org/10.1099/vir.0.052803-0

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, e77271. https://doi.org/10.1371/journal.pone.0077271

Barrera, G.P., Belaich, M.N., Patarroyo, M.A., Villamizar, L.F., Ghiringhelli, P.D., 2015. Evidence of recent interspecies horizontal gene transfer regarding nucleopolyhedrovirus infection of *Spodoptera frugiperda*. BMC Genomics 16, 1008. https://doi.org/10.1186/s12864-015-2218-5

Beperet, I., Irons, S.L., Simón, O., King, L. a, Williams, T., Possee, R.D., López-Ferber, M., Caballero, P., 2014. Superinfection exclusion in alphabaculovirus infections is concomitant with actin reorganization. J. Virol. 88, 3548–56. https://doi.org/10.1128/JVI.02974-13

Bernal, A., Simón, O., Williams, T., Muñoz, D., Caballero, P., 2013a. A Chrysodeixis chalcites single-nucleocapsid nucleopolyhedrovirus population from the Canary Islands is genotypically structured to maximize survival. Appl. Environ. Microbiol. 79, 7709–7718. https://doi.org/10.1128/AEM.02409-13

Bull, J.C., Godfray, H.C.J., O'reilly, D.R., 2001. Persistence of an occlusion-negative recombinant nucleopolyhedrovirus in *Trichoplusia ni* indicates high multiplicity of cellular infection. Appl. Environ. Microbiol. 67, 5204–5209. https://doi.org/10.1128/AEM.67.11.5204-5209.2001

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

Corsaro, B.G., Fraser, M.J., 1987. Characterization of genotypic and phenotypic variation in plaque-purified strains of HzSNPV elkar isolate. Intervirology 28, 185–198. https://doi.org/10.1159/000150016

Cory, J.S., Myers, J.H., 2003. The ecology and evolution of insect baculoviruses. Annu. Rev. Ecol. Evol. Syst. 34, 239–272. https://doi.org/10.1146/annurev.ecolsys.34.011802.132402

Del-Angel, C., Lasa, R., Rodríguez-del-Bosque, L.A., Mercado, G., Beperet, I., Caballero, P., Williams, T., 2018. Anticarsia gemmatalis nucleopolyhedrovirus from soybean crops in Tamaulipas, Mexico: diversity and insecticidal characteristics of individual variants and their co-occluded mixtures. Florida Entomol. 101, 404–410. https://doi.org/10.1653/024.101.0319

Domingo, E., Perales, C., 2019. Viral quasispecies. PLoS Genet. 15, e1008271. https://doi.org/10.1371/journal.pgen.1008271

Escribano, A., Williams, T., Goulson, D., Cave, R.D., Chapman, J.W., Caballero, P., 1999. Selection of a nucleopolyhedrovirus for control of Spodoptera frugiperda (Lepidoptera: Noctuidae): structural, genetic, and biological comparison of four isolates from the Americas. J. Econ. Entomol. 92, 1079–1085. https://doi.org/10.1093/jee/92.5.1079

Ferreira, B.C., Melo, F.L., Silva, A.M.R., Sanches, M.M., Moscardi, F., Ribeiro, B.M., Souza, M.L., 2019. Biological and molecular characterization of two Anticarsia gemmatalis multiple nucleopolyhedrovirus clones exhibiting contrasting virulence. J. Invertebr. Pathol. 164, 23–31. https://doi.org/10.1016/j.jip.2019.03.008

Fuentes, E.G., Hernández-Suárez, E., Simón, O., Williams, T., Caballero, P., 2017. Chrysodeixis chalcites nucleopolyhedrovirus (ChchNPV): natural occurrence and efficacy as a biological insecticide on young banana plants in greenhouse

and open-field conditions on the Canary Islands. PLoS One 12, 1–19. https://doi.org/10.1371/journal.pone.0181384

García-Banderas, D., Tamayo-Mejía, F., Pineda, S., de la Rosa, J.I.F., Lasa, R., Chavarrieta-Yáñez, J.M., Gervasio-Rosas, E., Zamora-Avilés, N., Morales, S.I., Ramos-Ortiz, S., Valle, J., Martínez-Castillo, A.M., 2020. Biological characterization of two Spodoptera frugiperda nucleopolyhedrovirus isolates from Mexico and evaluation of one isolate in a small-scale field trial. Biol. Control 149, 104316. https://doi.org/10.1016/j.biocontrol.2020.104316

Guo, Z.J., Ge, J.Q., Wang, D., Shao, Y.M., Tang, Q.Y., Zhang, C.X., 2006. Biological comparison of two genotypes of Helicoverpa armigera single nucleocapsid nucleopolyhedrovirus. BioControl 51, 809–820. https://doi.org/10.1007/s10526-006-9008-6

Haas-Stapleton, E.J., Washburn, J.O., Volkman, L.E., 2005. Spodoptera frugiperda resistance to oral infection by Autographa californica multiple nucleopolyhedrovirus linked to aberrant occlusion-derived virus binding in the midgut. J. Gen. Virol. 86, 1349–1355. https://doi.org/10.1099/vir.0.80845-0

Harrison, R.L., Rowley, D.L., Popham, H.J.R., 2019. A novel alphabaculovirus from the soybean looper, *Chrysodeixis includens*, that produces tetrahedral occlusion bodies and encodes two copies of *he65*. Viruses 11. https://doi.org/10.3390/v11070579

Hitchman, R.B., Hodgson, D.J., King, L.A., Hails, R.S., Cory, J.S., Possee, R.D., 2007. Host mediated selection of pathogen genotypes as a mechanism for the maintenance of baculovirus diversity in the field. J. Invertebr. Pathol. 94, 153–162. https://doi.org/10.1016/j.jip.2006.10.002

Hodgson, D.J., Vanbergen, A.J., Watt, A.D., Hails, R.S., Cory, J.S., 2001. Phenotypic variation between naturally co-existing genotypes of a Lepidopteran baculovirus. Evol. Ecol. Res. 3, 687–701.

Ibarra, J.E., Del Rincón, M.C., 2001. Capacidad insecticida de los baculovirus, Los baculovirus y sus aplicaciones como bioinsecticidas en el control biológico de plagas. Phytoma, Valencia, Spain.

Inglis, P.W., Santos, L.A.V.M., Craveiro, S.R., Ribeiro, B.M., Castro, M.E.B., 2020. Mosaic genome evolution and phylogenetics of Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) and virulence of seven new isolates from the Brazilian states of Minas Gerais and Mato Grosso. Arch. Virol. 166, 125–138. https://doi.org/10.1007/s00705-020-04858-2

Kolodny-Hirsch, D.M., Beek, N.A.M. Van, 1997. Selection of a morphological variant of Autographa californica nuclear polyhedrosis virus with increased virulence following serial passage in *Plutella xylostella*. J. Invertebr. Pathol. 69, 205–211. https://doi.org/https://doi.org/10.1006/jipa.1997.4659

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: Back to the future. J. Invertebr. Pathol. 132, 1–41. https://doi.org/10.1016/j.jip.2015.07.009

Lauring, A.S., Andino, R., 2010. Quasispecies theory and the behavior of RNA viruses. PLoS Pathog. https://doi.org/10.1371/journal.ppat.1001005

Lei, C., Yang, J., Wang, J., Hu, J., Sun, X., 2020. Molecular and biological characterization of Spodoptera frugiperda multiple nucleopolyhedrovirus field isolate and genotypes from China. Insects 11, 1–11. https://doi.org/10.3390/insects11110777

Martignoni, M.E., Iwai, P.J., 1986. Propagation of multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata* in larvae of *Trichoplusia ni*. J. Invertebr. Pathol. 47, 32–41. https://doi.org/10.1016/0022-2011(86)90160-6

Masson, T., Fabre, M.L., Pidre, M.L., Niz, J.M., Berretta, M.F., Romanowski, V., Ferrelli, M.L., 2020. Genomic diversity in a population of Spodoptera frugiperda nucleopolyhedrovirus. bioRxiv 2020.10.27.358317. https://doi.org/10.1101/2020.10.27.358317

Morgado, F. da S., Silva, L.A., Bernardes, L.M., Czepak, C., Strand, M.R., Ribeiro, B.M., 2020. *Trichoplusia ni* and *Chrysodeixis includens* larvae show different susceptibility to Chrysodeixis includens single nucleopolyhedrovirus per os infection. J. Pest Sci. (2004). https://doi.org/10.1007/s10340-020-01217-7

Muraro, D.S., Giacomelli, T., Stacke, R.F., Godoy, D.N., Marçon, P., Popham, H.J.R., Bernardi, O., 2019. Baseline susceptibility of Brazilian populations of *Chrysodeixis includens* (Lepidoptera: Noctuidae) to C. includens nucleopolyhedrovirus and diagnostic concentration for resistance monitoring. J. Econ. Entomol. 112, 349—354. https://doi.org/10.1093/jee/toy361

Redman, E.M., Wilson, K., Cory, J.S., 2016. Trade-offs and mixed infections in an obligate-killing insect pathogen. J. Anim. Ecol. 85, 1200–1209. https://doi.org/10.1111/1365-2656.12547

Salem, T.Z., Cheng, X.H., Cheng, X.W., 2012. AcMNPV enhances infection by ThorNPV in Sf21 cells and SeMNPV in Hi5 cells. Arch. Virol. 157, 1875–1885. https://doi.org/10.1007/s00705-012-1347-2

Sanjuán, R., Domingo-Calap, P., 2016. Mechanisms of viral mutation. Cell. Mol. Life Sci. 73, 4433–4448. https://doi.org/10.1007/s00018-016-2299-6

Weitzman, M.D., Possee, R.D., King, L.A., 1992. Characterization of two variants of Panolis flammea multiple nucleocapsid nuclear polyhedrosis virus. J. Gen. Virol. 73, 1881–1886. https://doi.org/10.1099/0022-1317-73-7-1881

Xu, X., Chen, Y., Zhao, Y., Liu, X., Dong, B., Jones, I.M., Chen, H., 2013. Baculovirus superinfection: a probable restriction factor on the surface display of proteins for library screening. PLoS One 8, e54631. https://doi.org/10.1371/journal.pone.0054631 [doi]PONE-D-12-28041 [pii]

Zhang, X.-F., Sun, R., Guo, Q., Zhang, S., Meulia, T., Halfmann, R., Li, D., Qu, F., 2017. A self-perpetuating repressive state of a viral replication protein blocks superinfection by the same virus. PLOS Pathog. 13, e1006253. https://doi.org/10.1371/journal.ppat.1006253

CONCLUSIONES

1. Se ha encontrado una gran variabilidad en aislados naturales de ChinNPV recogidos en campos de soja en Tamaulipas (México). De once larvas que murieron por infección natural de ChinNPV, nueve presentaron perfiles REN diferentes y fueron considerados como aislados diferentes.

2. La mezcla de los nueve aislados diferentes, llamada ChinNPV-Mex1, se construyó como modelo para estudiar las propiedades genéticas y biológicas del virus. Esta mezcla ha demostrado ser patogénica tan solo en tres especies de plúsidos (*C. includens*, *C. chalcites* y *T. ni*). El estudio de su actividad biológica reveló que este virus tiene un gran potencial como ingrediente activo de un insecticida biológico para el control de *C. includens*.

3. Se encontraron al menos 23 variantes genéticas (desde ChinNPV-A hasta ChinNPV-W) formando parte de la mezcla ChinNPV-Mex1, siendo ChinNPV-H la más prevalente. Sin embargo, la variante más patogénica resultó ser ChinNPV-R para larvas de segundo estadio de *C. includens*. Se realizó una selección de las variantes identificadas y se agruparon según su alta (ChinNPV-R, -J), media (ChinNPV-V, -F, -E) o baja (ChinNPV-K) patogenicidad.

4. En cinco de esas variantes (ChinNPV-F, -J, -K, -R and -V) también se estudiaron otras características insecticidas como son el tiempo medio de mortalidad y la producción de OBs/larva. No se encontraron diferencias significativas entre ellas en cuanto al tiempo medio de mortalidad, mientras que en producción de OBs/larva, ChinNPV-F, -K, -R, y -V fueron los más productivos. Por tanto, las variantes ChinNPV-F, -J, -R, y -V se identificaron como variantes con características útiles para desarrollar un insecticida biológico basado en ChinNPV para el control de *C. includens*.

5. Cuando se infectaron larvas de quinto estadio con una concentración alta de ChinNPV-Mex1, se encontró que la variante ChinNPV-K era la más frecuente en la progenie viral. Sin embargo, cuando las larvas eran inoculadas con una baja concentración de ChinNPV-Mex1, la progenie viral estaba compuesta por una gran diversidad de variantes genotípicas entre las cuales destacaba ChinNPV-E como la más prevalente.

6. La progenie viral resultante de inocular larvas con ChinNPV-E mostró el perfil de restricción de ChinNPV-E en el 97-100% de las larvas analizadas. Por el contrario, en la progenie viral obtenida cuando las larvas se inoculaban con la variante ChinNPV-K, se generaba gran diversidad de perfiles de restricción de forma dependiente de la concentración. De esta manera, la diversidad encontrada es inversamente proporcional a la concentración de inóculo utilizada.

7. El uso de altas concentraciones de ChinNPV-K como inóculo en experimentos llevados a cabo en condiciones tanto in vivo como in vitro resultó en la obtención del perfil de restricción de ChinNPV-K en la mayoría de las muestras analizadas.

8. Por el contrario, en los experimentos en los que se utilizaron bajas concentraciones de ChinNPV-K como inóculo en condiciones tanto in vivo como in vitro, se recuperaron diferentes genotipos a diferentes frecuencias, obteniendo ChinNPV-K sólo en unas pocas muestras en condiciones in vivo. Por tanto, la variante ChinNPV-K utilizada a bajas concentraciones es capaz de generar diversidad, pero de manera impredecible.

9. Se encontró una elevada variabilidad intragenómica por todo el genoma de ChinNPV-K en comparación con ChinNPV-E, en gran parte debida a polimorfismos de un solo nucleótido.

10. Este trabajo confirma la alta variabilidad presente en los aislados naturales de ChinNPV aquí estudiados y demuestra el papel clave de una variante genética, ChinNPV-K, en la generación de esta diversidad de manera dependiente de la concentración.

CONCLUSIONS

1. High natural diversity has been revealed in ChinNPV samples collected in a soya field in Tamaulipas (Mexico). Eleven larvae that died from natural ChinNPV infection yielded nine different REN profiles that were considered as different field isolates.

2. A mixture of the nine different ChinNPV isolates named ChinNPV-Mex1 was constructed as a model to study the biological and genetic properties of the virus. The ChinNPV-Mex1 is pathogenic only to three species from the Plusiinae subfamily (*C. includens*, *C. chalcites* and *T. ni*). The study of the biological activity of the mixture revealed that this virus has a great potential as an active ingredient in a biological insecticide for the control of pests caused by *C. includens*.

3. At least 23 genetic variants (from ChinNPV-A to ChinNPV-W) were comprised within the ChinNPV-Mex1 mixture being the ChinNPV-H the most prevalent. Nevertheless, ChinNPV-R resulted to be the most pathogenic variant in *C. includens* second instars. A selection of the identified variants was grouped according to their high (ChinNPV-R, -J), intermediate (ChinNPV-V, -F, -E) or low (ChinNPV-K) pathogenicity.

4. In five of the variants (ChinNPV-F, -J, -K, -R and -V), other insecticidal properties were also studied, such as the speed-of-kill and the production of OBs/larvae. No differences were observed in terms of speed-of-kill among them, whereas ChinNPV-F, -K, -R, and -V showed the highest OB production per larvae. Therefore, ChinNPV-F, -J, -R, and -V were identified as having useful characteristics for the development of a ChinNPV-based insecticide for control of *C. includens*, in terms of pathogenicity, speed-of-kill, and OB production/larva.

5. When fifth instar larvae of *C. includens* were inoculated with a high concentration of ChinNPV-Mex1, the ChinNPV-K variant was the most

frequent variant in the viral progeny. On the contrary, when the larvae were inoculated with a low concentration of ChinNPV-Mex1, the viral progeny consisted of a great diversity of variants of which ChinNPV-E was the most prevalent.

6. The viral progeny of the larvae that died from polyhedrosis after inoculation with the ChinNPV-E variant showed the ChinNPV-E REN profile in 97-100% of the analyzed larvae. In contrast, in the viral progeny of the larvae inoculated with the ChinNPV-K variant, a diversity of REN profiles was generated in a concentration-dependent manner. In this way, the diversity found is inversely proportional to the inoculum concentration used.

7. The use of high ChinNPV-K concentrations as inocula in experiments performed under in vivo/in vitro conditions resulted in the recovery of ChinNPV-K REN profile in most of the samples analysed.

8. On the other hand, experiments carried out using ChinNPV-K at low inoculum concentration under in vivo/in vitro conditions resulted in the recovery of different genotypes at different frequencies, recovering ChinNPV-K only in a few samples of in vivo conditions. That is to say, ChinNPV-K used at low concentration shows its capability of generating diversity but in an unpredictable form.

9. High intra-genomic variability was present throughout the genome in ChinNPV-K in comparison to ChinNPV-E, mostly due to single nucleotide polymorphisms.

10. The present work confirms the high variability present in the natural isolates of the ChinNPV studied and reveals the specific role of a single variant, named ChinNPV-K, in the generation of that diversity in a concentration-dependent manner.

LIST OF PUBLICATIONS

Aguirre, E.; Simón, O. ; Williams, T. ; Caballero, P. 2015. Efecto de la quitinasa del alphabaculovirus de Chrysodeixis chalcites (ChchSNPV) sobre la actividad insecticida de varios alphabaculovirus. [Conference presentation]. SEEA 2015 Conference, Valencia, Spain.

Aguirre, E.; Simón, O. ; Williams, T. ; Caballero, P. 2016. Effect of the Chrysodeixis chalcites single nucleopolyhedrovirus (ChchNPV) chitinase upon the insecticidal activity of several other alphabaculoviruses. Oral communication. 49th Annual Meeting of the Society for Invertebrate Pathology. International Congress on Invertebrate Pathology and Microbial Control. Tours, France.

Aguirre, E.; Caballero, J. ; Wandeler, H. ; Muñoz, D.; Murillo, R.; Caballero, P. 2016. Insecticidal activity of Phthorimaea operculella granulovirus isolates from Southern Europe on Tuta absoluta. Poster communication. 49th Annual Meeting of the Society for Invertebrate Pathology. International Congress on Invertebrate Pathology and Microbial Control. Tours, France.

Aguirre, E.; Beperet, I. ; Williams, T. ; Caballero, P. 2017. Importancia funcional de las variantes genotípicas de aislados del alphabaculovirus de Chrysodeixis includens (ChinSNPV) en la patogenicidad del virus. Oral communication. X Congreso Nacional de Entomología Aplicada. Logroño, Spain.

Aguirre, E.; Beperet, I. ; Williams, T. ; Caballero, P. 2019. Genetic Variability of Chrysodeixis Includens Nucleopolyhedrovirus (ChinNPV) and the Insecticidal Characteristics of Selected Genotypic Variants. Viruses 2019, 11, 581.

Aguirre, E.; Beperet, I.; Williams, T.; López-Ferber, M.; Caballero, P. 2019. Genetic variability in Chrysodeixis includens nucleopolyhedrovirus. Oral communication. International Congress on Invertebrate Pathology and Microbial Control & 52nd Annual Meeting of the Society for Invertebrate Pathology & 17th Meeting of the IOBC-WPRS Working Group “Microbial and Nematode Control of Invertebrate Pests”. Valencia, Spain.

Aguirre, E.; Beperet, I. ; Williams, T. ; Caballero, P. 2021. Generation of variability in Chrysodeixis includens nucleopolyhedrovirus (ChinNPV): the role of a single variant. Viruses. Submitted.

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