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A native variant of *Chrysodeixis chalcites* nucleopolyhedrovirus: The basis for a promising bioinsecticide for control of *C. chalcites* on Canary Islands' banana crops

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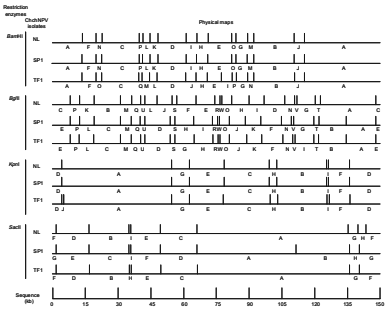
HIGHLIGHTS

- Five *C. chalcites* SNPV Canarian field isolates were identified.
- ChchSNPV-TF1 was the most prevalent and widespread variant.
- Physical maps of ChchSNPV isolates showed minimal differences at restriction sites.
- ChchSNPV-TF1 was the most pathogenic and fastest variant against *C. chalcites*.
- ChchSNPV-TF1 merits further evaluation as the basis for a biological insecticide.

GRAPHICAL ABSTRACT



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ABSTRACT

Chrysodeixis chalcites (Lepidoptera: Noctuidae) larvae cause up to 30% production loss in banana crops in the Canary Islands. Larvae of this species are susceptible to a nucleopolyhedrovirus (ChchNPV). This study aimed at evaluating the genetic diversity and bioinsecticidal activity of ChchNPV isolates collected from *C. chalcites* larvae in the Canary Islands. From a total 97 isolates collected in different banana greenhouses, restriction endonuclease analysis identified five genetic variants that differed slightly from ChchNPV isolates from Netherlands (ChchSNPV-NL) and Almería, Spain (ChchNPV-SP1). Physical maps revealed minimal differences at the genome level, mostly due to variation in the position/existence of restriction sites. ChchSNPV-TF1 was the most prevalent variant, representing 78% of isolates examined, and was isolated at all Canary Island sampling sites. This isolate was the most pathogenic isolate against *C. chalcites* second instars in terms of concentration-mortality metrics, compared to homologous variants or two heterologous viruses *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Anagrapha falcifera* multiple nucleopolyhedrovirus (AnfaMNPV). ChchSNPV-TF1 was also one of the fastest killing variants although no differences were observed in occlusion body production among the different variants in second instars. We conclude that ChchSNPV-TF1 merits further evaluation as the basis for a biological insecticide for control of *C. chalcites* in banana crops in the Canary Islands.

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1. Introduction

Chrysodeixis chalcites (Esper) (Lepidoptera: Noctuidae) is a major polyphagous pest in many countries (Shepard et al., 2009). In the Canary Islands, 100 km off the coast of Morocco, north-western Africa, *C. chalcites* populations have increased markedly during the past decade, possibly related to increased migration and range shifts in this and other noctuid pest species in response to global climate change (Sparks et al., 2007). Infestations of this pest frequently result in up to 30% losses in bananas grown under greenhouse conditions in the Canary Islands (Del Pino et al., 2011). Chemical-based control measures require multiple applications that can increase the risk of pest resistance, increase production costs, and can hamper the commercialization of produce that contain pesticide residues (Horowitz et al., 1998; Perera and Molina, 2007). Insect-infecting baculoviruses have been reported worldwide in over 600 host species, mainly from the order Lepidoptera (Jehle et al., 2006). Several of these viruses have been developed as the basis for effective biological insecticides against different crop and forest pests (Caballero et al., 2009; Moscardi, 1999).

Populations of *C. chalcites* can succumb to infection by a nucleopolyhedrovirus (ChchNPV; genus *Alphabaculovirus*). To date two ChchNPV isolates have been described; one from *C. chalcites* larvae on greenhouse-grown tomato and sweet pepper crops in the Netherlands that we will refer to as ChchSNPV-NL (van Oers et al., 2005, 2004), and the other from *C. chalcites* larvae on greenhouse-grown horticultural crops in El Ejido, Almería, Spain, named ChchNPV-SP1 (Murillo et al., 2000). ChchNPV infects members of an important group of Lepidoptera known as semiloopers (Noctuidae: Plusiinae) such as *Trichoplusia ni*, and has been considered as a potential candidate for biological control of semilooper pests. Other viruses with comparatively wide host ranges include *Autographa californica* NPV (AcMNPV), and its genotypic variant *Anagrapha falcifera* NPV (AnfaMNPV) (Harrison and Bonning, 1999), both of which can infect and kill *C. chalcites* larvae.

Considerable genetic heterogeneity is usually observed in natural baculovirus populations (Barrera et al., 2011; Erlandson, 2009; Graham et al., 2004; Murillo et al., 2006). This diversity has been demonstrated by the characterization of different geographical isolates of the same virus (Gordon et al., 2007; Williams et al., 2011) and also within single isolates, that can comprise a number of genotypic variants (Cory et al., 2005; Redman et al., 2010). Restriction endonuclease treatment of viral DNA provides profiles that are characteristic for each isolate or genotype (Erlandson et al., 2007; Harrison and Bonning, 1999). Closely related isolates do not usually show large phenotypic differences in terms of their insecticidal characteristics, although occasionally minimal changes in the genome of these variants may affect important biological traits such as pathogenicity, speed of kill, occlusion body (OB) yield, host range, OB size, and host liquefaction characteristics (Cory et al., 2005; Harrison et al., 2012; Rowley et al., 2011; Takatsuka et al., 2003). It has also been demonstrated that the geographical origin of both virus and host, can affect the characteristics of the dose–response curve and the survival period of infected hosts (Erlandson, 2009; Erlandson et al., 2007; Kouassi et al., 2009).

The aim of the present study was to select a nucleopolyhedrovirus isolate that could be evaluated for use in a control program targeted at *C. chalcites* in the Canary Islands. For this purpose, the natural diversity of ChchNPV isolates was evaluated by molecular and biological characterization of different isolates collected in the Canary Islands and compared with ChchNPV isolates from other geographical origins and with AcMNPV and AnfaMNPV isolates that can also infect *C. chalcites* larvae.

2. Material and methods

2.1. Insect source, rearing and viruses

C. chalcites larvae were obtained from a laboratory culture at the Universidad Pública de Navarra (Spain) established in 2007 using pupae received from the Instituto Canario de Investigaciones Agrarias, (ICIA), Tenerife, Spain, and refreshed periodically with pupae from the Canary Islands. Larvae were reared at 25 °C, 70 ± 5% humidity, and a photoperiod of 16:8 (light:dark), on a wheat germ, yeast and soybean meal based semisynthetic diet described by Greene et al. (1976). Adults were fed *ad libitum* with 30% w/v diluted honey.

During a period of high infestation of *C. chalcites* in banana crops in the Canary Islands, from October 2006 to November 2006, a total of 5479 larvae of *C. chalcites* were collected in greenhouses located at 11 different locations across the islands (Table 1). Collected larvae were individually reared on semisynthetic diet under laboratory conditions. A total of 1757 larvae died due to disease or undetermined causes (32.1%). Of these larvae, 97 died from lethal polyhedrosis disease which was confirmed by microscopic observation of OBs in giemsa-stained smears of infected cadavers (Table 1). These isolates were compared genetically and biologically with previously characterized isolates of ChchNPV from Almería (ChchNPV-SP1; Murillo et al., 2000) and The Netherlands (ChchSNPV-NL; van Oers et al., 2004), kindly provided by Dr. M.M. van Oers (University of Wageningen, The Netherlands). AcMNPV was kindly provided by Prof. R.D. Possee (CEH Oxford, UK) and AnfaMNPV by Dr. P. Támez-Guerra (Universidad Autónoma de Nuevo León, Mexico).

2.2. Occlusion body amplification, DNA extraction and restriction endonuclease analysis

Occlusion bodies (OBs) of the different isolates were amplified by a single passage in *C. chalcites* fourth instar larvae. Twenty-five larvae from the laboratory colony were starved overnight and then allowed to drink an OB suspension (10^6 OBs/ml) obtained from infected field-collected insects. Larvae that drank the suspension were individually reared on semisynthetic diet until death. As natural populations of Lepidoptera can harbour covert infections (Burden et al., 2003; Cabodevilla et al., 2011; Vilaplana et al., 2008), prior to amplification of field-collected isolates, 30 adult insects from the laboratory colony were subjected to qPCR analysis using primers targeted at the *DNApol* gene (forward primer 5′- taact ggagcggcaaagag-3′ and reverse primer 5′-cagatcacgcgagcaataaa-3′) (van Oers et al., 2005) to determine whether the laboratory colony harboured an inapparent nucleopolyhedrovirus infection. The fidelity of isolate amplification in colony insects was confirmed by restriction profile analysis following treatment with *Bgl*II.

OBs were extracted from dead larvae by homogenizing the cadavers in water and purified by filtration and differential centrifugation. OBs were then resuspended in double-distilled water and their concentration was determined by counting each sample three times using an improved Neubauer hemocytometer (Hawksley, Lancing, UK) under phase contrast microscopy at x400. Purified OBs were stored at 4 °C for up to 1 month prior to use in bioassays.

For DNA extraction, virions were released from OBs by mixing 100 μl of OB suspension containing 10^9 OBs/ml with 100 μl 0.5 M Na_2CO_3, 50 μl 10% (w/v) sodium dodecyl sulfate in a final volume of 500 μl and incubating for 10 min at 60 °C. Undissolved OBs and other debris were removed by low-speed centrifugation (3800*g*, 5 min). The supernatant containing the virions was treated with 25 μl proteinase K (20 mg/ml) for 1 h at 50 °C. Viral DNA was extracted twice with saturated phenol and once with chloroform

Table 1
Origin of the nucleopolyhedrovirus isolates obtained from *C. chalcites* larvae collected in greenhouses of the Canary Islands.

Location of greenhouses sampled		Coordinates		Isolates (n)	Canarian ChchSNPV isolates				
Island	Place (greenhouse)	Longitude (W)	Latitude (N)		TF1	TF2	TF3	TF4	TF5
Tenerife	Guargacho (Bueype)	16° 37′ 45″	28° 02' 41″	39	23	8	6	1	1
	Los Silos-Garachico	16° 48′ 15″	28° 22′ 11″	9	9	0	0	0	0
	El Fraile (San Lorenzo)	16° 40′ 46″	28° 00′ 15″	4	4	0	0	0	0
	Las Galletas (Valle Grande)	16° 39′ 07″	28° 01′ 47″	1	1	0	0	0	0
	Las Galletas (Laura)	16° 39′ 32″	28° 01′ 52″	4	4	0	0	0	0
	Puerto de la Cruz	16° 33′ 31″	28° 24′ 22″	7	7	0	0	0	0
	Las Galletas (R.F.)	16° 39′ 60″	28° 01′ 52″	3	3	0	0	0	0
	La Frontera (J.A).	18° 00′ 49″	27° 46′ 17″	5	5	0	0	0	0
La Palma	Los Llanos de Aridane	17° 55′ 53″	28° 37′ 27″	1	1	0	0	0	0
Gran Canaria	Vecindario	15° 25' 44″	28° 07' 52″	8	8	0	0	0	0
El Hierro	Frontera	18° 00′ 28″	27° 46′ 51″	16	16	0	0	0	0
Total				97	81	8	6	1	1

and isolated from the aqueous phase by alcohol precipitation. The pellet was resuspended in 50 to 100 μl of 0.1 × TE buffer (Tris–EDTA, pH 8) for 10 min at 60 °C. DNA concentration was estimated by reading the optical absorption at 260 nm. For restriction endonuclease analysis, 2 μg of viral DNA were mixed with 10 U of one of the following enzymes *Bam*HI, *Bgl*II, *Kpn*I and *Sac*II (Takara) and incubated for 4 to 12 h at 37 °C. These enzymes were selected among others as they allowed clear discrimination of ChchSNPV isolates. Reactions were stopped by addition of 4 μl of loading buffer (0.25% w/v bromophenol blue, 40% w/v sucrose). Electrophoresis was performed using horizontal 1% agarose gels in TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA, pH 8.0) at 20 V for 10 to 24 h. DNA fragments were stained with ethidium bromide and visualized on a UV transilluminator (Chemi-Doc, BioRad, California, USA).

2.3. Nucleocapsid packaging

To determine whether the ChchNPV isolates from the Canary Islands were single or multiple type nucleopolyhedroviruses, occlusion derived virions (ODVs) were released from samples of 5×10^8 OBs by exposure to alkaline buffer (0.1 M Na_2CO_3) for 30 min at 28 °C. Polyhedrin and other debris were removed by low-speed centrifugation (2500*g*, 2 min). The ODV-containing supernatant was banded by density equilibrium centrifugation (30,000*g*, 1 h) on 30–60% (w/v) continuous sucrose gradient. The banding pattern was visually inspected and photographed.

2.4. Construction of a ChchNPV genomic library

A partial *Bgl*II genomic library of the ChchNPV-TF1 isolate was constructed using the pSP70 plasmid (Promega) as receptor in conjunction with a DNA ligation kit (New England Biolabs). Genomic DNA was purified as described above, digested with *Bgl*II and ligated into the pSP70 plasmid at 16 °C overnight following manufacturer's instructions. The ligation reaction was dialyzed for 30 min against TE buffer and used to transform GeneHogs electrocompetent cells (Invitrogen), that were then selected on LB agar containing 100 μg/ml ampicillin.

To study the genomic variability among the field-collected isolates, the polymorphic restriction fragments of each isolate were cloned and subjected to terminal sequencing.

Colonies were amplified and DNA plasmids were purified and screened for the presence of inserts by *Bgl*II digestion and electrophoresis in 1% agarose gel. Inserts were authenticated by comparing their migration in agarose gels with the fragments from field collected variants ChchNPV-TF1, ChchNPV-TF2, ChchNPV-TF3, ChchNPV-TF4 and ChchNPV-TF5 generated by digestion with the same enzyme.

2.5. Physical mapping and sequencing

The construction of the physical map of ChchNPV-TF1 was obtained by ordering the restriction fragments on the viral genome according to the multiple digestions of cloned fragments. All cloned *Bgl*II fragments were digested with *Kpn*I, *Sac*II and *Bam*HI. The fragments resulting from multiple digestions were electrophoresed and the fragment sizes were then determined by analysis of the overlapping portions of cloned fragments. Mapping of the viral genome was confirmed by sequencing information obtained from the termini of cloned *Bgl*II fragments. Nucleotide sequences were determined in an ABI PRISM 377 automated DNA sequencer (Sistemas Genómicos S.A., Valencia, Spain), employing standard SP6 and T7 primers.

The physical maps of ChchNPV-TF2, ChchNPV-TF3, ChchNPV-TF4, and ChchNPV-TF5 were assembled by comparing their restriction profiles with that of ChchNPV-TF1, and using the sequencing information from the termini of each variant's polymorphic restriction fragments. The physical map previously constructed for the ChchNPV-TF1 variant was used for reference. The conventional practice of defining the restriction fragment which contains the *polyhedrin* gene as the start point for physical maps was followed (Vlak and Smith, 1982).

2.6. Insect bioassays

The insecticidal activities of Canary Island variants were compared with those of ChchNPV-SP1, ChchSNPV-NL, AcMNPV and AnfaMNPV. Concentration-mortality metrics, mean time to death (MTD) and productivity (OBs/larva) were determined by *per os* bioassays performed using the droplet-feeding method (Hughes and Wood, 1981). For this, second-instar *C. chalcites* from the laboratory colony were starved for 8 to 12 h at 26 °C and then allowed to drink from an aqueous suspension containing 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue and OBs at one of five different concentrations for each variant. For AcMNPV and AnfaMNPV the OB concentrations used were 1×10^9, 1×10^7, 1×10^5, 1×10^3 and 1×10^1 OBs/ml, whereas for ChchNPV variants the OB concentrations were 1×10^5, 2×10^4, 4×10^3, 8×10^2 and 1.6×10^2 OBs/ml. These concentration ranges were previously determined to kill between 95% and 5% of the experimental insects. Larvae that ingested the suspension within 10 min were transferred to individual wells of a 25-well tissue culture plate with a cube of semisynthetic diet. Bioassays with 25 larvae per virus concentration and 25 larvae as negative controls were performed on three occasions. Larvae were reared at 25 °C and mortality was recorded every 12 h until the insects had either died or pupated. Virus induced mortality results were subjected to logit analysis using the POLO-PC program (LeOra Software, 1987).

Time mortality analysis, expressed as mean time to death (MTD), was performed only for insects infected by variants of ChchNPV. Groups of 25 second instars were allowed to drink OB suspensions during a 10 min period as described in the concentration-mortality bioassay. The OB concentration used for the time mortality analysis was 1×10^5 OBs/ml for all ChchNPV variants tested, except for ChchSNPV-TF1 which was inoculated at a concentration of 2×10^4 OBs/ml; these concentrations resulted in 92–98% mortality in all cases. The bioassay was performed three times. Larvae were individually reared at 25 °C and mortality was recorded every 8 h. Time mortality data were subjected to Weibull survival analysis using the Generalized Linear Interactive Modeling (GLIM) program (Crawley, 1993). Survival models, such as the Weibull, are preferred for analysis of time to death data as the variance in age at death tends to increase with the mean. The time mortality distribution of different isolates was analyzed graphically. Only individuals that died from polyhedrosis disease, confirmed by the microscopic observation of OBs, were included in these analyses.

The OB production of ChchNPV isolates was determined in *C. chalcites* second instars that were infected by treatment with the OB concentrations used in the time-mortality assay. Twenty-five larvae were inoculated for each treatment and the bioassay was performed three times. All the larvae that died of polyhedrosis disease (a minimum of 50 larvae per virus treatment) were collected and stored at −20 °C until used for OB counting. For this, each larva was homogenized in 100 µl distilled water, and the total yield of OBs per larva was estimated by counting each sample three times using a Neubauer hemocytometer. The average OB counts of each replicate were normalized by log-transformation and subjected to analysis of variance ANOVA using the SPSS v12 program (SPSS Inc., Chicago, IL).

3. Results

3.1. ChchNPV-TF1 was the most prevalent and widespread variant isolated from infected larvae

All of the 97 isolates collected from *C. chalcites* with typical signs of polyhedrosis disease could be classified as one of five different variants based on their restriction endonuclease profiles. The enzyme *Bgl*II proved particularly useful for differentiating among these isolates. All five variants had restriction profiles similar to those of the previously characterized ChchNPV-SP1 and ChchSNPV-NL isolates and therefore can be considered as geographically distinct variants of ChchNPV. The Canary Island variants were named ChchNPV-TF1, ChchNPV-TF2, ChchNPV-TF3, ChchNPV-TF4, and ChchNPV-TF5 (Fig. 1A). Quantitative PCR analysis revealed that the *C. chalcites* laboratory colony did not harbor a covert infection. The *Bgl*II restriction profiles of ChchNPV isolates recovered after *in vivo* multiplication were identical to those of the original material, indicating that no contamination had occurred during isolate amplification. The ChchNPV-TF1 isolate was selected as the reference variant because its *Bgl*II restriction profile was common to 78% of the isolates collected in field, indicating this to be the most prevalent variant. The prevalence of the remaining variants varied: 15% of isolates were classified as variant ChchNPV-TF2, 5% of isolates as variant ChchNPV-TF3, and two different single isolates were classified as variant ChchNPV-TF4 and variant ChchNPV-TF5. The ChchNPV-TF1 variant was present in all the greenhouses sampled, making it the most widespread variant in the Canary Islands, whereas the other four variants (ChchNPV-TF2 to ChchNPV-TF5) were only collected in Bueype greenhouse, Guargacho, Tenerife (Table 1).

The *Bgl*II profiles of the five variants from the Canary Islands were quite similar to one another. *Bgl*II treatment resulted in 23 visible fragments for ChchNPV-TF1, 25 fragments for ChchNPV-TF2, 24 fragments for ChchNPV-TF3, 23 fragments plus two submolar bands for ChchNPV-TF4 and 23 fragments plus one submolar band for the ChchNPV-TF5 variant (Table 2). Submolar bands were not visible in the restriction profiles of ChchNPV-TF1, ChchNPV-TF2 or ChchNPV-TF3, whereas submolar fragments were evident in ChchNPV-TF4 and ChchNPV-TF5 *Bgl*II profiles, suggesting the presence of a mixture of genotypes. It appears that ChchNPV-TF4 is likely to be a mixture of ChchNPV-TF1 and ChchNPV-TF2, whereas ChchNPV-TF5 is likely a mixture of ChchNPV-TF1 and ChchNPV-TF3 as polymorphic fragments of ChchNPV-TF2 and ChchNPV-TF3 appeared as submolar bands in these restriction profiles. All the isolates presented characteristic restriction fragments length polymorphisms. The characteristic restriction fragments were *Bgl*II-D for ChchNPV-TF1; *Bgl*II-L and *Bgl*II-N for ChchNPV-TF2; and *Bgl*II-M for ChchNPV-TF3. In contrast, the variants showing submolar fragments varied in *Bgl*II-D, *Bgl*II-M and *Bgl*II-O, in the case of ChchNPV-TF4, or *Bgl*II-D and *Bgl*II-M fragments in the case of ChchNPV-TF5 (Fig. 1A). The restriction profiles of the predominant variant ChchNPV-TF1 were compared with those of ChchSNPV-NL and ChchNPV-SP1 following treatment with *Bam*HI, *Bgl*II, *Kpn*I or *Sac*II (Fig. 1B). ChchNPV-SP1 and ChchNPV-TF1 appeared to be more similar to one another than to the ChchSNPV-NL variant. The genome size estimates derived from *Bgl*II fragments indicated that ChchNPV variants had genomes of 149.5 to 150.07 kb, similar in length to that of the ChchSNPV-NL genome, previously reported as 149.6 kb (van Oers et al., 2005) (Table 2).

3.2. ChchNPV variants from the Canary Islands were single nucleocapsid NPVs

The banding pattern observed following ODV centrifugation revealed that all the ChchNPV ODVs contained single nucleocapsids. They are, therefore, variants of a single type nucleopolyhedrovirus (SNPV) as indicated by the unique band visible in the sucrose gradient (Fig. 2).

3.3. Physical maps of ChchSNPV isolates showed minimal differences due to restriction site modifications

The similarity in variant genome sizes estimated from ChchSNPV-NL, ChchNPV-SP1 and ChchSNPV-TF1 restriction profiles suggested that differences in physical maps were likely due to modifications in restriction sites. To determine differences in physical maps among these variants an incomplete library of 15 of the 23 *Bgl*II fragments from ChchSNPV-TF1 was constructed (Table 2). Terminal sequence information from cloned *Bgl*II fragments was used to construct the physical map of ChchSNPV-TF1, using the ChchSNPV-NL genomic sequence as a reference (van Oers et al., 2005). The homologous ORFs identified in the library are shown in Table 3. All the *Bgl*II restriction fragments analyzed fell within ORFs that showed similarity to 30 genes from ChchSNPV-NL. This allowed adjacent restriction fragments to be mapped together and provided further confirmation of the position of most of these ORFs. Following this approach and using the ChchSNPV-NL reference sequence information the physical maps of the ChchSNPV-TF1 variant were constructed for each enzyme used (Fig. 3A).

The ChchSNPV-TF1 *Bgl*II physical map was used as reference for the construction of the physical maps of the remaining four ChchSNPV isolates from the Canary Islands, since this enzyme allowed clear discrimination between the different isolates (Fig. 3B). The different isolates collected in the Canary Islands were quite similar to one another. The unique *Bgl*II fragments of ChchSNPV-TF1, ChchSNPV-TF2 and ChchSNPV-TF3 isolates were cloned into pSP70 (Table 2) and terminal sequencing was carried out to determine the identity of these fragments (data not shown).

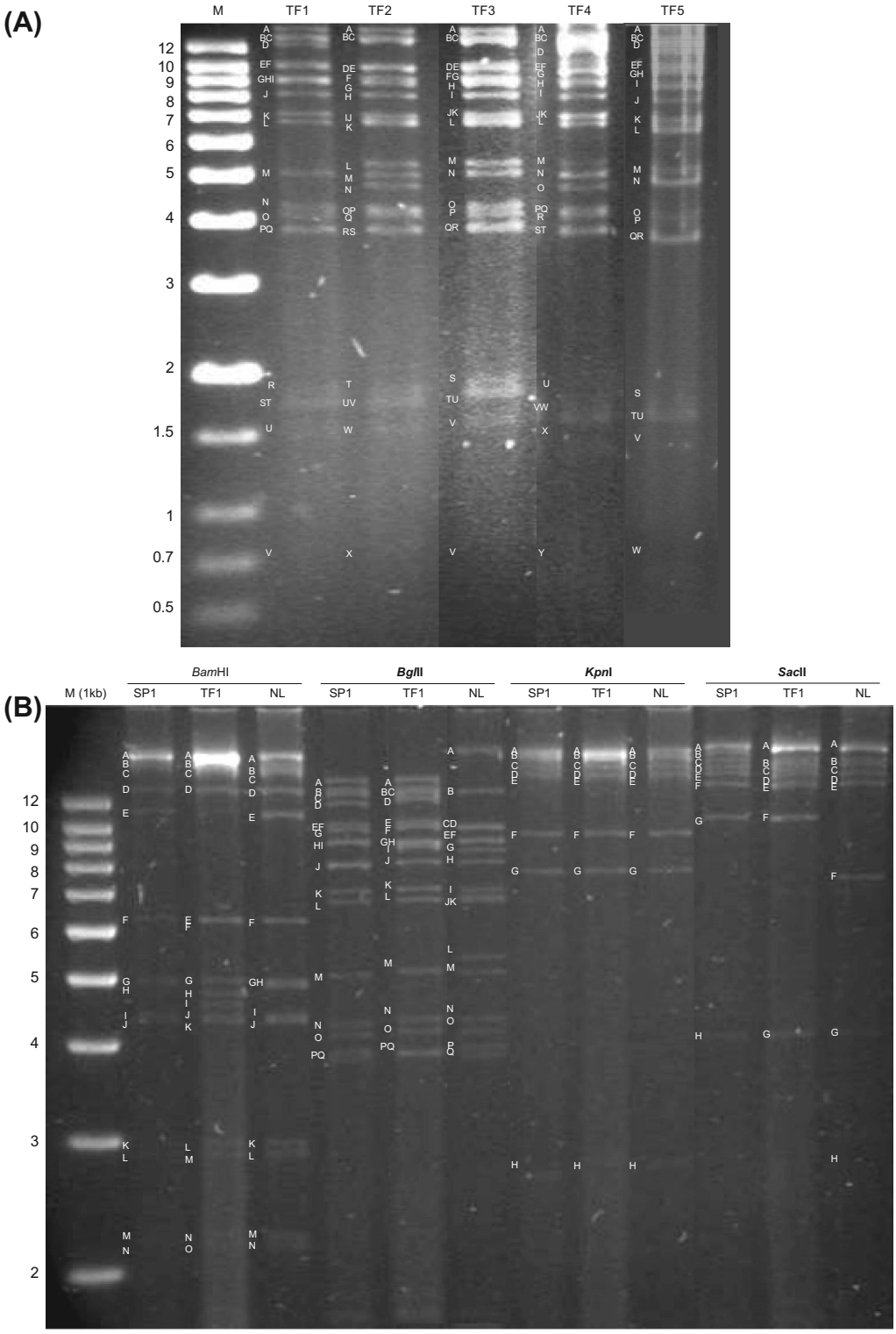

Fig. 1. (A) Restriction endonuclease profiles following digestion of ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5) DNAs with *Bgl*II. (B) ChchSNPV-TF1 (TF1) genomic DNA digested with *Bam*HI, *Bgl*II, *Kpn*I and *Sac*II compared with the restriction patterns of ChchSNPV-NL (NL) and ChchNPV-SP1 (SP1). The DNA 1 Kb Marker Ladder (Stratagene) was used as a molecular size marker (kbp) (M). Fragments were designated alphabetically giving the letter A to the largest fragment for each endonuclease digest.

The variability was located in two regions of the genome, between nucleotides 42,267 and 54,411, and nt 56,161 and 65,133 of the ChchSNPV-NL genome (van Oers et al., 2005), involving the *Bgl*II-L, -J and -F fragments, or *Bgl*II-D and -G of ChchSNPV-TF1 profile (Fig. 3B). ChchSNPV-TF4 and ChchSNPV-TF5 presented similar variability as the other strains, in that submolar restriction fragments suggested the presence of mixtures of variants that likely included ChchSNPV-TF2 and ChchSNPV-TF3.

Table 2
Molecular size (kb) of *Bgl*II restriction endonuclease fragments of the genomic DNAs of ChchNPV isolates; ChchSNPV-NL (NL) from Netherlands, ChchNPV-SP1 (SP1) from Almería, Spain, and ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5) from the Canary Islands, Spain. The DNA fragments are named alphabetically, starting with A for the largest fragment.

Genotypic variants							
Fragments	NL	SP1	TF1	TF2	TF3	TF4	TF5
A	27.70	14.40	14.41	14.41	14.41	14.41	14.41
B	12.78	13.28	13.28	13.29	13.29	13.28	13.28
C	9.83	12.78	12.78	12.77	12.77	12.78	12.78
D	9.76	12.11	12.11*	9.77	10.19*	12.11	12.11
E	9.05	9.93	9.93*	9.57*	9.77	9.93*	9.93*
F	8.97	9.79	9.79*	9.05	9.05	9.79	9.79
G	8.52	9.77	8.97*	8.72	8.95*	8.97	8.97
H	7.99	8.97	8.92*	7.99	8.72	8.92	8.92
I	7.01	8.92	8.71*	7.01	7.99	8.71	8.71
J	6.80	7.50	7.99	6.81*	7.01	7.99	7.99
K	6.69	7.02	7.02*	6.68*	6.81*	7.01	7.01
L	5.33	6.69	6.69	5.33*	6.68*	6.69	6.68
M	5.06	5.06	5.06*	5.06*	5.33*	(5.33)	(5.33)
N	4.31	4.31	4.29*	4.76*	5.06*	5.06	5.06
O	4.14	4.14	4.13	4.29	4.29	(4.76)	4.29
P	3.94	3.94	3.86*	4.20*	4.13	4.29	4.13
Q	3.83	3.83	3.83*	4.15	3.88*	4.13	3.88
R	1.86	1.86	1.86*	3.88*	3.83*	3.86	3.83
S	1.75	1.75	1.75*	3.83	1.86	3.83	1.86
T	1.74	1.74	1.74*	1.86	1.75	1.86	1.75
U	1.52	1.52	1.52*	1.75	1.74	1.75	1.74
V	0.77	0.77	0.77	1.74	1.52	1.74	1.52
W	0.15	0.15	0.15	1.52	0.77	1.52	0.77
X				0.77	0.15	0.77	0.15
Y				0.15		0.15	
Total size (kb)	149.60	149.67	149.68	149.50	150.07	149.67	149.67

These fragments were obtained after digestion of the ChchNPV genomic DNAs with *Bgl*II restriction enzyme.
()Submolar bands not included in isolates total size.
* Fragments cloned into pSP70 plasmid obtained by *Bgl*II digestion.

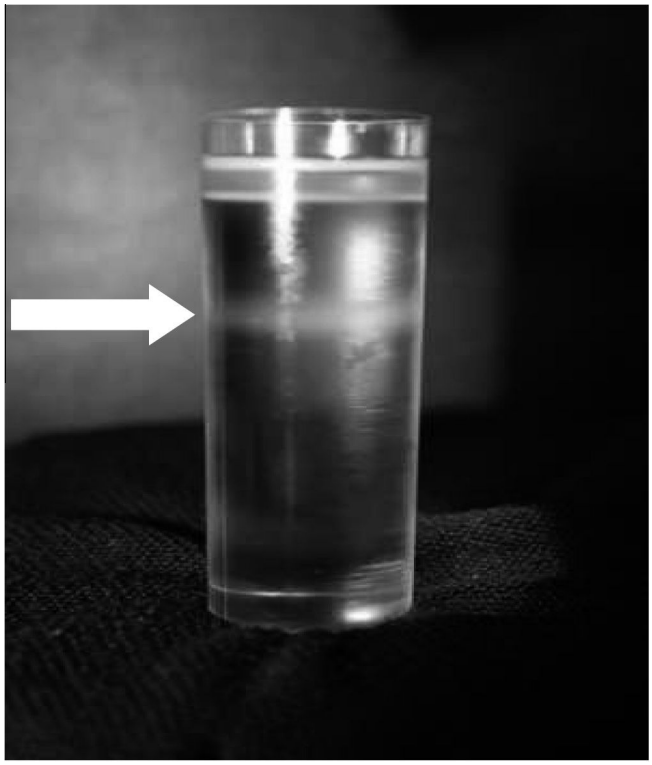

Fig. 2. ODV banding pattern of ChchSNPV-TF1 after continuous sucrose gradient separation. The arrow points out the unique band.

3.4. ChchSNPV-TF1 is the most pathogenic and one of the fastest killing variant

LC_{50} values of the ChchNPV variants ranged from 1.35×10^3 OBs/ml for the most pathogenic variant ChchSNPV-TF1, to 2.94×10^4 OBs/ml for the least pathogenic variant ChchSNPV-TF2 (Table 4). ChchSNPV-TF1 was significantly more pathogenic to insects from the Canary Islands' colony than any of the other ChchNPV variants tested being fifteen-fold more pathogenic in terms of concentration-mortality metrics than the ChchSNPV-NL variant and fourteen-fold more pathogenic than ChchNPV-SP1 or other isolates from the Canary Islands. The 95% fiducial limits of the relative potencies, representing the ratio of effective concentrations, overlapped broadly in ChchSNPV-NL, ChchNPV-SP1, ChchSNPV-TF2, ChchSNPV-TF3, ChchSNPV-TF4 and ChchSNPV-TF5, indicating no significant differences in pathogenicity among these variants. Both AcMNPV and AnfaMNPV, were markedly less pathogenic than any of the homologous viruses (Table 4). For this reason the heterologous viruses were not included in the speed of kill and OB productivity studies.

Analysis of mean time to death (MTD) values revealed significant differences among the different ChchNPV variants. ChchSNPV-NL, ChchSNPV-TF1 and ChchSNPV-TF3 were the fastest killing isolates for which the 95% confidence intervals of the MTD values broadly overlapped in each variant (Table 3). The majority of the insects infected with ChchNPV variants died between 106 and 120 h post-infection (Fig. 4A). Two mortality peaks were observed; one at ~115 h for ChchSNPV-NL, ChchNPV-SP1, ChchSNPV-TF1, ChchSNPV-TF2 and ChchSNPV-TF5 isolates and a later one at 120 h for insects infected with ChchSNPV-TF3 or ChchSNPV-TF4 (Fig. 4A).

Table 3
The position and orientation of the 33 open reading frames (ORFs) in the ChchSNPV-TF1 genome.

ORF N°	Gene family	Genomic fragment	Most homologous ORF	Size (nt)	Position in the ChchSNPV-NL genome	Dir*
1	*chch6*	*Bgl*II-P-E	*chch6*	503	7717–9165	>
2	*me-53*	*Bgl*II-P	*chch8*	41	11631–11755	<
3	*exon-0*	*Bgl*II-P	*chch10*	114	12038–12381	>
4	*p47*	*Bgl*II-M	*chch33*	51	31857–32011	<
5	*chch34*	*Bgl*II-M	*chch34*	158	32103–32557	>
6	*lef8*	*Bgl*II-M-Q	*chch37*	279	36221–37060	<
7	*bjdp*	*Bgl*II-Q	*chch38*	212	37084–37722	<
8	*chch41*	*Bgl*II-Q	*chch41*	142	40059–40485	>
9	*chch42*	*Bgl*II-Q-U	*chch42*	351	40505–41560	<
10	*lef-10*	*Bgl*II-U	*chch43*	12	41587–41623	<
11	*vp1054*	*Bgl*II-U-D	*chch45*	336	41932–42942	>
12	*DNA-polymerase*	*Bgl*II-D-S	*chch58*	292	53606–54487	<
13	*desmoplakin*	*Bgl*II-S-G	*chch59*	708	54487–56642	>
14	*lef3*	*Bgl*II-G	*chch60*	59	58159–58578	<
15	*gp37*	*Bgl*II-G	*chch67*	700	64428–65128	>
16	*DNA-photolyase I*	*Bgl*II-H	*chch68*	233	64329–65129	>
17	*chch75*	*Bgl*II-H-R	*chch74*	128	73255–73640	<
18	*vlf-1*	*Bgl*II-R	*chch76*	379	73715–74856	<
19	*gp41*	*Bgl*II-R	*chch78*	216	75272–75922	<
20	*chch88*	*Bgl*II-K	*chch88*	168	88902–88713	>
21	*p45*	*Bgl*II-K	*chch96*	59	94632–94453	<
22	*p87*	*Bgl*II-K-F	*chch97*	454	95233–96034	>
23	*chch106*	*Bgl*II-F-N	*chch106*	347	104455–105826	>
24	*chch107*	*Bgl*II-N	*chch107*	58	105651–105826	<
25	*chch108*	*Bgl*II-N	*chch109*	183	108518–109069	<
26	*chch109*	*Bgl*II-N	*chch110*	48	109174–109318	<
27	*chch110*	*Bgl*II-I	*chch111*	56	110097–110266	<
28	*chch111*	*Bgl*II-I	*chch112*	159	110420–110897	>
29	*chch119*	*Bgl*II-I	*chch119*	111	118015–118349	<
30	*dUTPase*	*Bgl*II-I	*chch120*	51	118504–118349	<
31	*calyx/pep*	*Bgl*II-I-T	*chch121*	331	118614–119609	>
32	*rr2*	*Bgl*II-T	*chch122*	266	119763–120563	<
33	*chch151*	*Bgl*II-E	*chch151*	266	148262–149062	<

* Direction of transcription in the same (>) or opposite (<) sense of the *polyhedrin* gene.

No significant differences were detected in total OB production among the different variants ($F_{(6,14)} = 2.204$; $P = 0.105$). The total yield values ranged from 8.40×10^6 to 1.03×10^7 OBs/larva for ChchNPV-SP1 and ChchSNPV-NL, respectively (Fig. 4B).

4. Discussion

The aim of this study was to select a native ChchNPV isolate that could be developed as the basis for a bioinsecticide-based control program against *C. chalcites* in the Canary Islands. The occurrence of distinct isolates from different geographical origins has been demonstrated for several other nucleopolyhedroviruses (Rowley et al., 2011; Takatsuka et al., 2003) and granuloviruses (Espinel-Correal et al., 2010; Léry et al., 1998). Given the inter- and intra-specific diversity in lepidopteran baculoviruses, the selection of isolates showing highly efficient insecticidal properties for biological control is an essential step in the development of a bioinsecticide product. Among the 97 field-collected ChchSNPV isolates, five genetic variants were identified by restriction endonuclease analysis, all of which were closely related to one another and showed clear similarities to the previously characterized ChchSNPV-NL from the Netherlands (van Oers et al., 2005) and ChchNPV-SP1 from Almería, Spain (Murillo et al., 2000). As *Bam*HI and *Kpn*I restriction profiles of ChchSNPV-NL and ChchNPV-SP1 were identical, as were those of ChchNPV-SP1 and ChchSNPV-TF1 with *Bgl*II, it is likely that these variants shared a similar sized genome. In addition, minimal differences were observed in the restriction profiles of the ChchSNPV variants from the Canary Islands. Terminal sequencing indicated that genetic differences among the variants were mainly due to point mutations and small deletions or insertions that modified restriction sites. These results find similarities with other studies that have reported that the genetic diversity of different isolates from distant geographical origins is often limited to minor differences reflected in the presence and distribution of restriction sites (Chen et al., 2002; Zhang et al., 2005). Differences were located mainly in two genomic regions, including *Bgl*II-L, -J and -F fragments of the ChchSNPV-NL genome (*Bgl*II-D and -G in ChchSNPV-TF1 profile) that include genes *vp1054*, *fp25k*, *lef-9*, *bro-a*, *DNA polymerase*, *desmoplakin*, *lef-3*, *iap-2*, *p26b*, *v-cathepsin*, *chitinase*, *pcna* or *gp37* and other ORFs of unknown function (van Oers et al., 2005).

ChchSNPV-TF1, ChchSNPV-TF2 and ChchSNPV-TF3 appeared to be composed of a dominant genotype or possibly a mixture of genotypes that were not revealed by restriction enzyme analysis, as previously observed for isolates of other nucleopolyhedroviruses (Muñoz et al., 1999; Simón et al., 2004). In contrast, ChchSNPV-TF4 and ChchSNPV-TF5 appeared to be a mixture of the dominant genotypes present in ChchSNPV-TF1 and ChchSNPV-TF2, and the predominant genotypes present in ChchSNPV-TF1 and ChchSNPV-TF3, respectively. Although ChchSNPV-TF1 was demonstrated to be a single nucleocapsid virus, multiple genomes are likely occluded within the same OB, favoring the presence of genotypic heterogeneity, as previously reported for other single nucleocapsid nucleopolyhedroviruses (Ogembo et al., 2007; Wang et al., 2003).

Differences in biological activity are common among virus isolates from distinct geographical regions (Alexandre et al., 2010; Rowley et al., 2011; Takatsuka et al., 2003), or even from the same regions (Barrera et al., 2011; Figueiredo et al., 2009; Milks, 1997), or among cloned variants derived from a single wild-type isolate (Cory et al., 2005; Ogembo et al., 2007). In addition, minor genetic differences can have significant effects on the phenotypic characteristics of these viruses (Maeda et al., 1993; Simón et al., 2012). For example, Simón et al. (2012) demonstrated that the lack of oral infectivity of SfMNPV-G genotype was due to minimal nucleotide

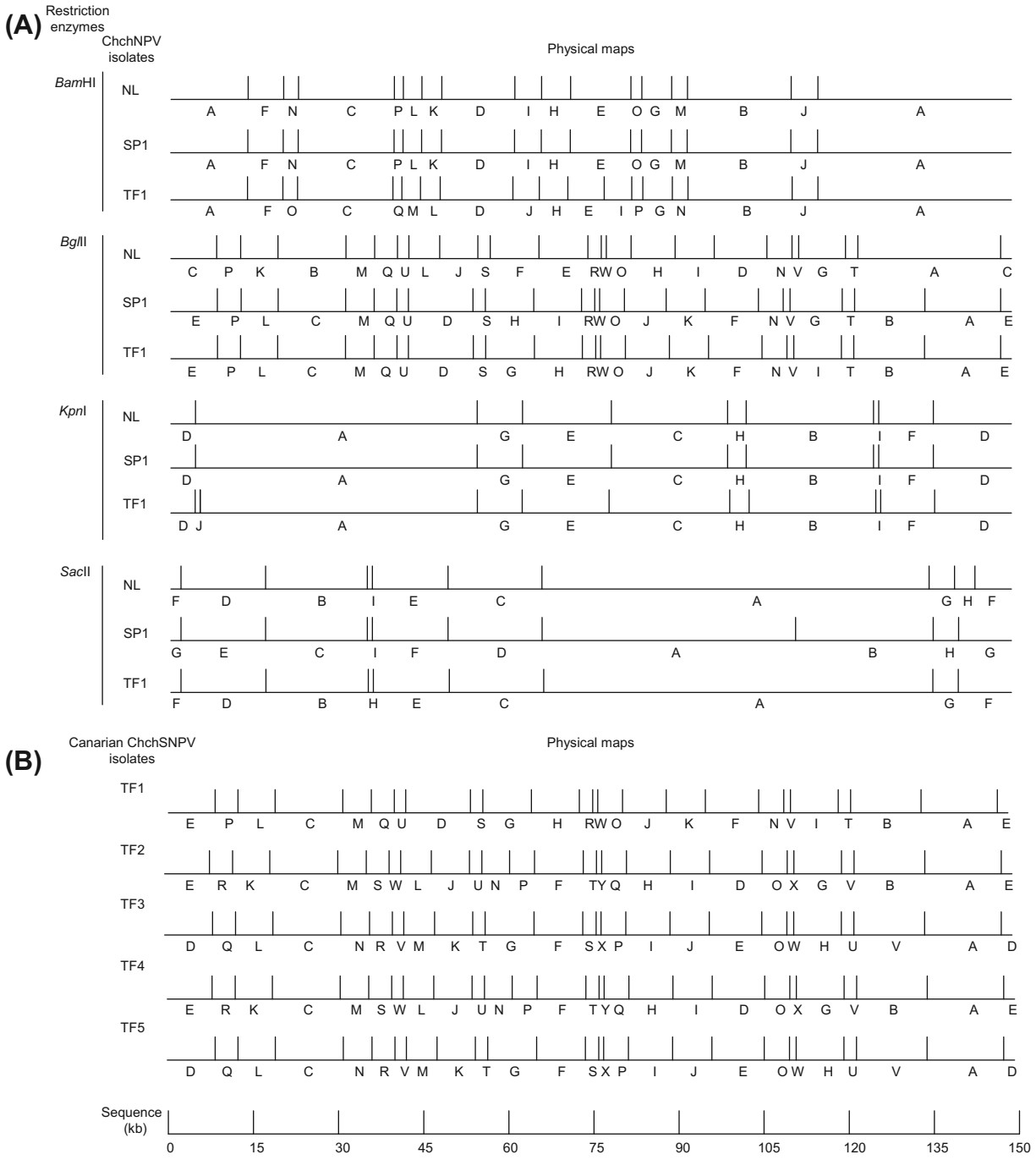

Fig. 3. (A) Physical maps of the ChchSNPV-NL (NL), ChchNPV-SP1 (SP1) and ChchSNPV-TF1 (TF1) genomes with *Bam*HI, *Bgl*II, *Kpn*I and *Sac*II. The genome size of ChchSNPV-TF1 was estimated to be 149.6 kbp. The first nucleotide in the different maps is the first nucleotide of the ChchSNPV-NL *Bgl*II-C fragment that carries the *polyhedrin* gene. The circular ChchNPV DNA is represented in linear form. Sequence (kb) representing restriction sites are indicating below the maps. (B) Physical maps of the genomes of the different isolates from the Canary Islands: ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5) with *Bgl*II enzyme. The genome size was estimated to be around 149 kbp.

sequence differences in the *sf*58 gene, which included 14 different nucleotides compared to the parental virus SfMNPV-B. As a result, the Gln-Leu-Val-Arg-Asn amino acid motif in SfMNPV-B (24-QLVSRN-29) was changed to a Ile-Lys-Glu-Thr-Gln sequence in SfMNPV-G (24-IKESTQ-29), which was associated with the complete loss of oral infectivity of SfMNPV-G OBs. In contrast, the host range of AcMNPV was modified following recombination of a 0.6 kb genomic fragment originating from *Bombyx mori* NPV (BmMNPV) (Maeda et al., 1993) genome in which a change in two adjacent nucleotides resulted in the modification of a single amino acid between BmMNPV (Asp) and AcMNPV (Ser) (Kamita and Maeda, 1997). For this reason, determining the relationship between differences at the genome level and virus phenotype will be clearly relevant to genotype selection in the development of virus-based insecticides.

The Canary Islands variants, although genetically very similar, differed significantly in OB pathogenicity. ChchSNPV-TF1 was the most pathogenic variant tested, whereas homologous viruses from the Canary Islands and from the other geographical regions, such as ChchNPV-SP1 and ChchSNPV-NL, were less pathogenic against

Table 4
LC_{50} values, relative potencies and MTD values of homologous ChchNPV variants, ChchSNPV-NL (NL) from Netherlands, ChchNPV-SP1 (SP1) from Almería, Spain, and ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5) from the Canary Islands, Spain and heterologous viruses *Autographa californica* (AcMNPV) and *Anagrapha falcifera* (AnfaMNPV) in *C. chalcites* second instar larvae.

Virus	LC_{50} (OBs/ml)	Relative Potency	Fiducial limits (95%)		MTD (h)	Fiducial limits (95%)	
			Low	High		Low	High
NL	2.03×10^4	1	–	–	126abc	123	127
SP1	1.89×10^4	1.11	0.47	2.58	127bc	125	129
TF1	1.35×10^3	15.47	7.37	32.48	123a	121	124
TF2	2.98×10^4	0.71	0.28	1.78	128bc	126	130
TF3	2.71×10^4	0.76	0.32	1.82	125ab	123	126
TF4	2.46×10^4	0.85	0.36	2.04	129c	127	130
TF5	2.41×10^4	0.86	0.36	2.03	127bc	125	129
AcMNPV	6.82×10^6	2.9×10^{-3}	–	–	–	–	–
AnfaMNPV	9.31×10^6	2.1×10^{-3}	–	–	–	–	–

Logit regressions were fitted in POLO Plus (LeOra Software, 1987). A test for non-parallelism was not significant for ChchNPV viruses ($\chi^2 = 4.52$; d.f = 6; $P > 0.05$). Relative potencies were calculated as the ratio of effective concentrations relative to the ChchSNPV-NL isolate. Mean time to death (MTD) values were estimated by Weibull survival analysis (Crawley, 1993).

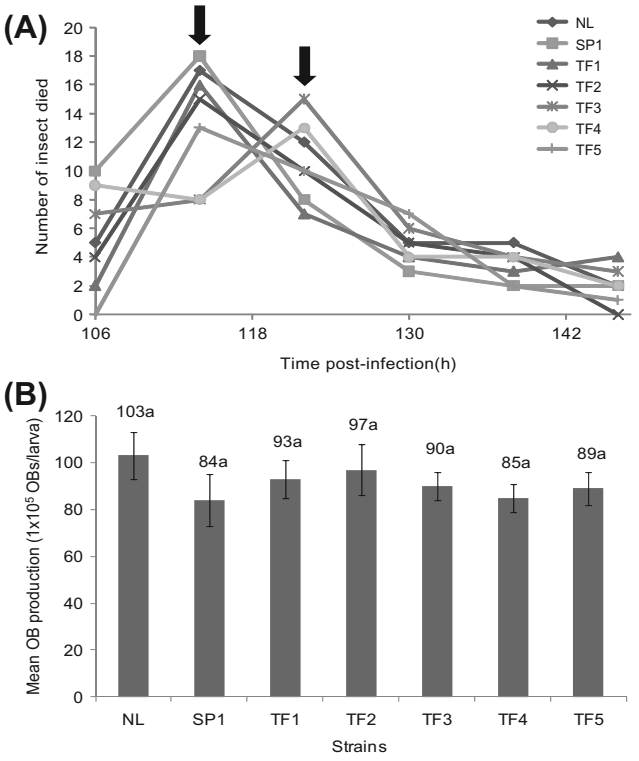

Fig. 4. (A) Mortality distribution over time of *C. chalcites* second instars killed by ChchSNPV-NL (NL), ChchNPV-SP1 (SP1), ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5) after inoculation with OB concentrations that resulted in ~90% mortality. (B) Mean OB production per insect ($\times 10^5$ OBs/larva) for *C. chalcites* larvae that died from ChchSNPV-NL (NL), ChchNPV-SP1 (SP1), ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5). Values above the bars indicate medians. Values followed by identical letters did not differ significantly for multiple post hoc comparisons (Tukey $P < 0.05$).

larvae from the Canary Islands laboratory colony. Studies on the genes present within the variable genomic region should help to determine their functional association with the biological activity of ChchSNPV. *C. chalcites* larvae from the Canary Islands were markedly more susceptible to the indigenous isolate ChchSNPV-TF1 compared to isolates from Netherlands and Almería or heterologous viruses. Indeed, homologous viruses are normally more pathogenic than viruses from other host species (Kouassi et al., 2009) and native isolates also tend to be more pathogenic to the local insect population than homologous isolates from elsewhere (Erlandson et al., 2007).

One of the factors that limits the effectiveness of baculoviruses as biological insecticides is that OBs are rapidly inactivated by solar ultraviolet (UV) radiation (Killick and Warden, 1991; Lasa et al., 2007). However, OB applications to greenhouse crops are not subjected to high levels of UV-inactivated due to the UV filtering properties of the greenhouse structure (Lasa et al., 2007). However, most of the banana crops in the Canary Islands are grown under plastic or nylon mesh greenhouses. As such, the persistence of ChchSNPV-TF1 OBs on banana crops requires study to determine the appropriate frequencies of OB applications for effective control of *C. chalcites* infestations. Currently, a low number of active substances are authorized for *C. chalcites* control in banana crops, with indoxicarb and *Bacillus thuringiensis var. kurstaki* being the most frequently used products. ChchSNPV-TF1-based products could be incorporated into integrated pest management programs, given the compatibility of these virus with biological and chemical control measures, thus reducing farmer dependence on synthetic insecticides and thereby reducing the likelihood of the development of insecticide resistance in the pest population.

In conclusion, the most prevalent and widespread strain of ChchSNPV in the Canary Islands, that we named ChchSNPV-TF1, should prove useful as the active ingredient in the development of a biological insecticide for *C. chalcites* control in banana crops on these islands. The high pathogenicity and rapid speed of kill of this virus is comparable to that of baculoviruses that are currently commercialized in effective bioinsecticide products targeted at other pests (Moscardi, 1999; Caballero et al., 2009).

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References

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

Barrera, G., Simón, O., Villamizar, L., Williams, T., Caballero, P., 2011. *Spodoptera frugiperda* multiple nucleopolyhedrovirus as a potential biological insecticide: genetic and phenotypic comparison of field isolates from Colombia. Biol. Control 58, 113–120.

Burden, J.P., Nixon, C.P., Hodgkinson, A.E., Possee, R.D., Sait, S.M., King, L.A., Haisl, R.S., 2003. Covert infections as a mechanism for long-term persistence of baculoviruses. Ecol. Lett. 6, 524–531.

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. Colom. Entomol. 35, 105–115.

Cabodevilla, O., Ibáñez, I., Simón, 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.

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

Cory, J.S., Green, B.M., Paul, R.K., Hunter-Fujita, F., 2005. Genotypic and phenotypic diversity of a baculovirus population within an individual insect host. J. Invertebr. Pathol. 89, 101–111.

Crawley, M.J., 1993. GLIM for Ecologists. Blackwell Science, Oxford, UK.

Del Pino, M., Carnero, A., Cabello, T., Hernández-Suárez, E., 2011. La lagarta o bicho camello, *Chrysodeixis chalcites* (Esper, 1789), una plaga emergente en los cultivos de platanera de Canarias. Phytoma 225, 21–24.

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

Erlandson, M.A., Newhouse, S., Moore, K., Janmaat, A., Myers, J., Theilmann, D., 2007. Characterization of baculovirus isolates from *Trichoplusia ni* populations from vegetable greenhouses. Biol. Control 41, 256–263.

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:Noctuidae). Appl. Environ. Microbiol. 76, 7617–7625.

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.

Gordon, J., Chaeychomsri, S., Kamiya, K., Ishikawa, H., Katou, Y., Ikeda, M., Kobayashi, M., 2007. Cloning and comparative characterization of nucleopolyhedroviruses isolated from African Bollworm, *Helicoverpa armigera* (Lepidoptera:Noctuidae) in different geographic regions. J. Insect Biotech. Sericol. 76, 39–49.

Graham, R.I., Tyne, W.I., Possee, R.D., Hails, R.S., 2004. Genetically variable nucleopolyhedroviruses isolated from spatially separate populations of the winter moth *Operophtera brumata* (Lepidoptera: Geometridae) in Orkney. J. Invertebr. Pathol. 87, 29–38.

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.L., Bonning, B.C., 1999. The nucleopolyhedroviruses of *Rachoplusia ou* and *Anagrapha falcifera* are isolates of the same virus. J. Gen. Virol. 80, 2793–2798.

Harrison, R.L., Popham, H.J.R., Breitenbach, J.E., Rowley, D.L., 2012. Genetic variation and virulence of *Autographa californica* multiple nucleopolyhedrovirus and *Trichoplusia ni* single nucleopolyhedrovirus isolates. J. Invertebr. Pathol. 110, 33–47.

Horowitz, A.R., Weintraub, P.G., Ishaaya, I., 1998. Status of pesticide resistance in arthropod pests in Israel. Phytoparasitica 26, 231–240.

Hughes, P.R., Wood, H.A., 1981. A synchronous per oral technique for the bioassay of insect viruses. J. Invertebr. Pathol. 37, 154–159.

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.

Kamita, S.G., Maeda, S., 1997. Sequencing of the putative DNA helicase-encoding gene of the *Bombyx mori* nuclear polyhedrosis virus and fine-mapping of a region involved in host range expansion. Gene 190, 173–179.

Killick, H.J., Warden, S.J., 1991. Ultraviolet penetration of pine trees and insect virus survival. Entomophaga 36, 87–94.

Kouassi, L.N., Tsuda, K., Goto Mukawua, S., Sakamaki, Y., Nakamura, M., 2009. Biological activity and identification of nucleopolyhedroviruses isolated from *Mythimna separata* and *Spodoptera litura* in Japan. BioControl 54, 537–548.

Lasa, R., Ruiz-Portero, C., Alcázr, M.D., Belda, J.E., Caballero, P., Williams, T., 2007. Efficacy of optical brightener formulations of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) as a biological insecticide in greenhouses in southern Spain. Biol. Control 40, 89–96.

Léry, X., Abol-Ela, S., Giannotti, J., 1998. Genetic heterogeneity of *Phthorimaea operculella* granulovirus: restriction analysis of wild-type isolates and clones obtained *in vitro*. Acta Virol. 42, 13–21.

Maeda, S., Kamita, S.G., Konda, 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.

Milks, M.L., 1997. Comparative biology and susceptibility of cabbage looper (Lepidoptera: Noctuidae) lines to a nuclear polyhedrosis virus. Environ. Entomol. 26, 839–848.

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

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.

Murillo, R., Lipa, J.J., Muñoz, D., Amate, J., Barranco, P., Cabello, T., Caballero, P., 2000. Caracterización bioquímica de un nucleopoliedrovirus de *Chrysodeixis chalcites* autóctono de España. Bol. San. Veg. Plagas 26, 637–644.

Murillo, R., Elvira, S., Muñoz, D., Williams, T., Caballero, P., 2006. Genetic and phenotypic variability in *Spodoptera exigua* nucleopolyhedrovirus isolates from greenhouse soils in southern Spain. Biol. Control 38, 157–165.

Ogembo, J.G., Chaeychomsri, S., Kamiya, K., Ishikawa, H., Katou, Y., Ikeda, M., Kobayashi, M., 2007. Cloning and comparative characterization of nucleopolyhedroviruses isolated from African bollworm, *Helicoverpa armigera*, (Lepidoptera: Noctuidae) in different geographical regions. J. Insect Biotech. Sericol. 76, 39–49.

Perera, S., Molina, M.J., 2007. Plagas y enfermedades en el cultivo ecológico de la platanera. In: Nogueroles, C., Líbano, J. (Eds.), El cultivo ecológico de la platanera en Canarias. Gabinete de Proyectos Agroecológicos S.L, Tenerife, pp. 77–118.

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.

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.

Shepard, B.M., Hammig, M.D., Carner, G.L., Ooi, P.A.G., Smith, J.P., 2009. Implementing integrated pest management in developing and developed countries. In: Peshin, R., Dhawan, A.K. (Eds.), Integrated Pest Management: Dissemination and Impact. Springer, Netherlands, pp. 275–305.

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.

Simón, O., Palma, L., Williams, T., López-Ferber, M., Caballero, P., 2012. Analysis of a naturally-occurring deletion mutant of *Spodoptera frugiperda* multiple nucleopolyhedrovirus reveals *sf58* as a new *per os* infectivity factor of lepidopteran-infecting baculoviruses. J. Invertebr. Pathol. 109, 117–126.

LeOra Software, 1987. POLO-PC. A User's Guide to Probit or Logit Analysis. LeOra Software, Berkeley, CA.

Sparks, T.H., Dennis, R.L.H., Croxton, P.J., Cade, M., 2007. Increased migration of Lepidoptera linked to climate change. Eur. J. Entomol. 104, 139–143.

Takatsuka, J., Okuno, S., Nakai, M., Kunimi, Y., 2003. Genetic and biological comparisions of ten geographic isolates of a nucleopolyhedrovirus that infects *Spodoptera litura* (Lepidoptera: Noctuidae). Biol. Control 26, 32–39.

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.

van Oers, M.M., Abma-Henkens, M.H., Herniou, E.A., de Groot, J.C., 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.

Vilaplana, L., Redman, E.M., Wilson, K., Cory, J.S., 2008. Density-related variation in vertical transmission of a virus in the African armyworm. Oecologia 155, 237–242.

Vlak, J.M., Smith, G.E., 1982. Orientation of the genome of *Autographa californica* nuclear polyhedrosis virus: a proposal. J. Virol. 41, 1118–1121.

Wang, H., Deng, F., Pijlman, G.P., Chen, X., Sun, X., Vlak, J.M., Hu, Z., 2003. Cloning of biologically active genomes from a *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus isolates by using a bacterial artificial chromosome. Virus Res. 97, 57–63.

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 United States. J. Invertebr. Pathol. 108, 13–21.

Zhang, C.X., Ma, X.C., Guo, Z.J., 2005. Comparison of the complete genome sequences between C1 and G4 isolates of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus. Virology 333, 190–199.