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Short Communication

Characterization of a new *Helicoverpa armigera nucleopolyhedrovirus* variant causing epizootic on a previously unreported host, *Helicoverpa gelotopoeon* (Lepidoptera: Noctuidae)

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ABSTRACT

This paper reports the first biological and molecular characterization of a nucleopolyhedrovirus isolated from the soybean and cotton pest *Helicoverpa gelotopoeon*. Studies were performed following a virus outbreak in a rearing facility and in wild *H. gelotopoeon* populations in Córdoba, Argentina. Host identity was corroborated by partial sequencing of the *CO*I gene. Scanning electron microscope observations of purified OBs revealed their polyhedral morphology and an average diameter of 0.89 ± 0.14 μm. Ultrathin sections of infected larvae examined by transmission electron microscopy showed the intranuclear occurrence of polyhedra and virus particles in fat body cells. Nucleocapsids were singly enveloped. Phylogenetic analysis of *lef-8*, *lef-9*, *polh*, *orf5/5b* and *hr3-orf62* viral sequences identified this new NPV isolate (hereafter HegeSNPV) as a variant of *Helicoverpa armigera nucleopolyhedrovirus* (HearNPV). Furthermore, HegeSNPV was closely related to the so-called "HzSNPV Group" within HearNPV, although having particular characteristics.

1. Introduction

The neotropical noctuid *Helicoverpa gelotopoeon* (Dyar, 1921) is a widespread polyphagous pest in southern South-America. In Argentina, this moth has become an increasing problem in soybean crops during the last growing seasons (Cortés and Venier, 2013). In addition, it has been often found associated to various cultivated plants, such as cotton and chickpea, causing varying levels of economic damage (Cork and Lobos, 2003; Fichetti et al., 2009). To date, its control is commonly based on chemical insecticides. Transgenic Bt soybean and cotton lines are currently being used in the country, but information about their effect on the occurrence and dynamics of this pest is still scarce. Little is known about the natural enemies of *H. gelotopoeon*: it was shown to be susceptible to nematodes in the genus *Steinernema* under laboratory conditions (Caccia et al., 2014), and the occasional parasitization by the microhymenopteran *Campoletis grioti* has also been recorded (Murúa et al., 2009).

Several baculovirus strains have been found to infect other species in the Helicoverpa/Heliothis complex around the world (Rowley et al., 2011). *Heliothis virescens*, *Helicoverpa zea* and *Helicoverpa armigera* are all reported natural hosts of single-nucleocapsid (S) and multiple-nucleocapsid (M) nucleopolyhedroviruses (NPVs). Partial sequencing of the highly conserved genes *lef-8*, *lef-9* and *polh* allowed the classification of most SNPV isolates as variants of the same virus species, formerly named HzSNPV or HearSNPV depending on the insect in which they were initially identified. These have been recently merged into a single species, *H. armigera nucleopolyhedrovirus* (HearNPV), according to the last revision of the International Committee on Taxonomy of Viruses (Adams et al., 2015). Based on molecular analysis of additional loci, Rowley et al. (2011) revealed the existence of three main clades within HearNPV: HzSNPV variants, HearSNPV-China variants and HearSNPV-India variants. Other NPVs infecting heliothine include HearMNPV and AcMNPV (Gettig and McCarthy, 1982; Rowley et al., 2011).

By mid-2013, dramatic mortality was observed in a rearing facility of *H. gelotopoeon* in Córdoba city (Argentina). Most larvae died within a few days, exhibiting typical symptoms of baculovirus infection. Light microscopic examinations confirmed this

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preliminary diagnosis. Field specimens collected from chickpea crops in Córdoba province to establish a new laboratory insect population, revealed the high natural prevalence and incidence of the disease (unpublished data). In view of this, a study was undertaken in order to perform the biological and molecular characterization of the causal agent.

2. Materials and methods

2.1. Molecular confirmation of host species

Because no baculovirus infections had been recorded in *H. gelotopoeon* before, and due to the morphological similarity among heliothine species, partial sequencing of *CO*I gene was envisaged to corroborate the identity of the host. Total DNA from a laboratory diseased larva was extracted using CTAB. PCR was performed with primers H3Fw (5′-CGAGCAGAATTAGGTAAYCC-3′) and H3Rv (5′-GCTGATGTRAAATAAGCTCGAG-3′), designed for Heliothinae, with an expected product size of *ca.* 812 bp (Arneodo et al., 2015). The resulting amplicon was run in 1% w/v agarose gel. The product was purified with silica gel and directly sequenced in an ABI PRISM 3500 XL genetic analyzer (Applied Biosystems, USA) at Instituto de Biotecnología (INTA Castelar, Argentina).

2.2. Virus isolation and insect rearing

Occlusion bodies (OBs) were obtained from homogenates of symptomatic laboratory-reared *H. gelotopoeon* larvae from Córdoba. After filtration, centrifugation and treatment with 0.1% SDS, pellets were washed twice and resuspended in distilled sterile water. Purified OBs were quantified in a Neubauer chamber and used for subsequent electron microscopy analysis, genetic studies and bioassays. A virus-free colony of *H. gelotopoeon* needed to carry out the experiments originated from a laboratory rearing facility (AgIdea S. A., Pergamino, Argentina) and was further reared on artificial diet (Arneodo et al., 2010) at the Instituto de Microbiología y Zoología Agrícola (CICVyA-INTA, Hurlingham, Argentina). Third-instar *H. gelotopoeon* larvae were infected through diet (formaldehyde free)-surface contamination (Ignoffo et al., 1983) and conserved at −20 °C for future studies and applications.

2.3. Electron microscopy

Purified OBs were processed for scanning electron microscopy (SEM) examination in a SEM JEOL JSM-T 100, based in a protocol developed by Torquato et al. (2006). Briefly, they were fixed for 2 h in an aqueous solution of 5% glutaraldhyde + 0.2% tannic acid, postfixed for 1 h in 1% osmium tetroxide and dehydrated in acetone series (50%, 80% and 90%). Some of the samples were metallized in order to get higher resolution images. For transmission electron microscopy (TEM), third-instar larvae were inoculated with approx. 1000 OBs by the droplet-feeding method (Hughes and Wood, 1981). Viral infection was checked by cutting prolegs and observing the hemolymph under light microscopy. Two infected larvae and a healthy control (fed with colorant + sucrose solution) were fixed in glutaraldehyde at 3 days post infection (p. i.), postfixed with osmium tetroxide, dehydrated and resin-embedded. Ultrathin sections were contrasted with uranyl acetate and lead citrate and observed in a TEM JEOL 1200 EX II.

2.4. PCR and sequencing

To obtain viral DNA, OBs were dissolved in 0.1 M Na_2CO_3 at room temperature for 15 min. After neutralization with 0.01 M Tris-HCl, virions were treated with proteinase K (0.5 mg/ml), 0.25% SDS and 10 mM EDTA for 3 h at 37 °C. DNA was phenol extracted and precipitated with 0.2 M NaCl and isopropanol. Primer pairs targeting viral sequences corresponding to *lef-8* (prL8-1/prL8-2), *lef-9* (HzSlef9-1/HzSlef9-2), *polh* (prPH-1/prPH-2), *orf5/5b* (HaC1-ORF5F/HaC1-ORF5R) and *hr3-orf62* (HaNNg1-hr3F1/HaNNg1-ORF62R1) were previously described (Lange et al., 2004; Rowley et al., 2011). PCR reactions for *lef-8* and *polh* consisted of an initial denaturation step of 95 °C for 5 min; 35 cycles of 95 °C for 40 sec, 50 °C for 15 sec, 72 °C for 50 sec and a final step of 72 °C for 5 min. Conditions for *lef-9*, *orf5/5b* and *hr3-orf62* were initial denaturation at 95 °C for 4 min; 35 cycles of 95 °C for 20 sec, 55 °C for 20 sec, 72 °C for 50 sec and a final step of 72 °C for 3 min. PCR products were visualized, purified and sequenced as described above.

2.5. Phylogeny

Phylogenetic relationships were inferred among concatenated partial *lef-8*, *lef-9* and *polh* sequences obtained in this study and those corresponding to previously described heliothine NPVs, available at GenBank. Analyses were conducted using MEGA ver. 6.0 (Tamura et al., 2013) with Minimum Evolution (ME) and Maximum Likelihood (ML) methods and 1000 bootstrap replicates. The more variable genomic regions *orf5/5b* and *hr3-orf62* were also compared to other reported HearNPV sequences. In the latter case, the presence/absence of the 5/5b protein homolog (encoded by *orf5/5b*) and of a 59 aa protein (encoded by *orf62*) was determined.

2.6. Bioassays

Initially healthy, laboratory-reared *H. gelotopoeon* neonate larvae were inoculated with different viral doses (2, 6, 18 and 54 OBs) by the droplet-feeding method (Hughes and Wood, 1981). The average liquid intake was previously determined as being 0.014 µl (unpublished data). Each treatment, including negative control, comprised 28–40 specimens. Larvae were further reared on artificial diet and checked three times a day until pupation. The mortality rate and the average time to death were calculated. Baculovirus infection in dead larvae was diagnosed by symptom assessment and dark-field microscopy.

3. Results and discussion

3.1. Host species

Analysis of the partial *CO*I gene sequence confirmed that the diseased larva belonged to the species *H. gelotopoeon*. Such molecular identification was especially meaningful given the recent detection of *H. armigera* in Argentina (Murúa et al., 2014; Arneodo et al., 2015) and the frequent occurrence of other heliothine moths. The sequence obtained (of which 761 bp are available at GenBank under accession no KP279738) shared 99.5% nucleotide identity with the sole *H. gelotopoeon CO*I sequence published before (GenBank accession no EU768938, Cho et al., 2008). The identity percentage was considerably lower when compared to *H. armigera* or *H. zea* (94.4% and 95.8% respectively). This was in agreement with previous microscopic examinations of the external genitalia of adults from the original laboratory population in Córdoba.

3.2. Electron microscopy

SEM observations of OBs revealed that they were polyhedral in shape, with variable diameters ranging from 0.6 to 1.2 µm (average: 0.89 ± 0.14 µm, $n = 30$) (Fig. 1A). Hemolymph of infected