

Accepted Manuscript

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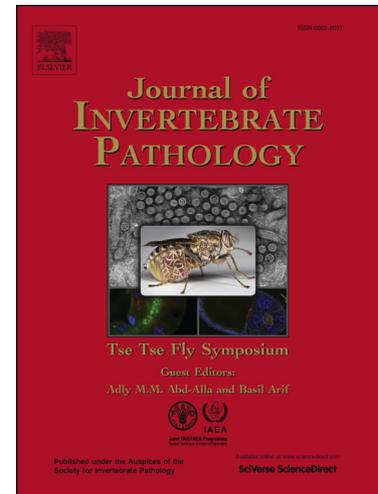
PII: S0022-2011(15)30008-2
DOI: <http://dx.doi.org/10.1016/j.jip.2015.08.009>
Reference: YJIPA 6725

To appear in: *Journal of Invertebrate Pathology*

Received Date: 13 July 2015
Revised Date: 7 August 2015
Accepted Date: 18 August 2015

Please cite this article as: Ferrelli, M.L., Taibo, C., Fichetti, P., Sciocco-Cap, A., Arneodo, J.D., Characterization of a new *Helicoverpa armigera nucleopolyhedrovirus* variant causing epizootic on a previously unreported host, *Helicoverpa gelotopoeon* (Lepidoptera: Noctuidae), *Journal of Invertebrate Pathology* (2015), doi: <http://dx.doi.org/10.1016/j.jip.2015.08.009>

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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 *COI* gene. Scanning electron microscope observations of purified OBs revealed their polyhedral morphology and an average diameter of $0.89 \pm 0.14 \mu\text{m}$. 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.

Keywords: *Helicoverpa gelotopoeon*; nucleopolyhedrovirus; electron microscopy; phylogenetic analysis.

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, *Helicoverpa 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 (Getting 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 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 *COI* 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.*, in press). 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% glutaraldehyde + 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 Na₂CO₃ 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 μ l (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 *COI* 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.*, in press) and the frequent occurrence of other heliothine moths. The sequence obtained (of which 761 bp

are available at GenBank under accession N° KP279738) shared 99.5% nucleotide identity with the sole *H. gelotopoeon* COI sequence published before (GenBank accession N° 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: $0.89 \pm 0.14 \mu\text{m}$, $n=30$) (Fig. 1A). Hemolymph of infected larvae, checked by light microscopy before TEM processing, had hypertrophied hemocytes at 2 days p. i., while polyhedra formation within nuclei became evident at 3 days p. i. (not shown). Ultrathin sections of these specimens showed virus proliferation in the nuclei of fat body cells. Numerous polyhedra containing embedded particles, as well as free, non-occluded virions were observed intranuclearly. Furthermore, it was determined that rod-shaped nucleocapsids were singly enveloped (Fig. 1B and 1C). According to its host and to the aforementioned microscopic features, the name HegeSNPV will be used for this new baculoviral isolate hereafter.

3.3 Genomic analysis

HegeSNPV *lef-8*, *lef-9* and *polh* partial sequences were deposited in Genbank with the accession numbers KP340515, KP340516 and KP340517, respectively. Phylogenetic analysis of concatenated *lef-8*, *lef-9* and *polh* partial sequences identified HegeSNPV as a variant of HearNPV belonging to the HzSNPV clade reported by Rowley *et al.* (2011) (Fig. 2). Indeed, when considering only these highly conserved genes, a pairwise Kimura-2-parameter nucleotide distance of 0.001 substitutions/site was demonstrated between HegeSNPV and isolate HzSNPV-F16 (accession N° AF334030), which genome has been completely sequenced (Chen *et al.*, 2002). This isolate was obtained from diseased *Helicoverpa zea* specimens

collected in USA (Ignoffo *et al.*, 1965). Phylogeny based on *hr3-orf62* (GenBank accession N° KP340518) also related HegeSNPV to the HzSNPV variants (see Figure S1, Supplementary Materials). As in other HzSNPV variants, the 59 aa protein encoded by *orf62* could be deduced from the nucleotide sequence. However, this protein is not exclusive to that clade and has been reported in HearSNPV-India and in some unassigned isolates, as well.

Remarkably, a different situation was observed upon comparative analysis of HegeSNPV *orf5/5b* (GenBank accession N° KP340519). The topology of the resulting tree did not allow a clear classification of HegeSNPV in any of the groups of variants proposed by Rowley *et al.* (2011). Nevertheless, HegeSNPV *orf5/5b* clustered closer to the HearSNPV-China and -India groups than to the HzSNPV variants (see Figure S2, Supplementary Materials). In contrast to HzSNPV-F16 and almost all other HzSNPV isolates sequenced so far, HegeSNPV exhibited the 63-codon version of *orf5b*, which, in turn, is typical of the HearSNPV-China variants. This was also the case of isolate 566 (accession N° HQ246059) from *H. zea* (unknown geographic origin). The significance of the *orf5b*-encoded 53/63 aa proteins is not merely restricted to taxonomic approaches. They are homologues of AcMNPV *ac152*, and have been associated to higher virulence (Zhang *et al.*, 2005), which is a desirable characteristic for a biocontrol agent.

3.4. Bioassays

All larvae died when infected with initial doses of 54 and 18 OBs (40 dead out of 40 larvae tested and 38/38, respectively). Mortality decreased to 90.6% (29/32) and 75.0% (21/28) after inoculation with 6 and 2 OBs, respectively. A mortality of 3.3% (1/30) was recorded in uninfected controls, which developed normally until pupation. These data suggest that the median lethal dose for neonates was below 2 OBs, a value falling within the range observed for commercially available HearNPV isolates. The average time to death did not show significant differences among treatments: 4.0 ± 0.9 , 4.5 ± 0.8 , 4.3 ± 0.8 and 4.3 ± 0.7 days for doses of 54, 18, 6 and 2 OBs, respectively. Again, the results were in line with previous reports (Rowley *et al.*, 2011). Most diseased larvae exhibited a delay in molting or remained as 1st

instar larvae until death. Larval cadavers soon disintegrated releasing a whitish fluid, a common feature for several baculoviruses. The occurrence of OBs could be observed by optical microscopy in all symptomatic specimens, corroborating the etiology of the disease. These promising findings deserve additional experimental work for the exact calculation of both median lethal dose and median lethal time. Such information is essential before considering the development of a new baculovirus-based formulation.

The present study constitutes the first report of an entomopathogenic virus infecting *H. gelotopoeon*. In view of its potential as a bioinsecticide, the efficacy of HegeSNPV against this and other species in the heliothine pest complex is worthy of future research.

Acknowledgments

The authors are indebted to Eleonora Da Riva (Agidea S. A., Pergamino, Argentina) for providing virus-free insects to establish a laboratory colony and to Débora Moreyra and Roberto Igarza (IMyZA, CICVyA-INTA, Argentina) for insect rearing. Blanca García and Julián Diodati (Servicio de Microscopía, CICVyA-INTA) are gratefully acknowledged for their assistance in taking electron micrographs, and Matías Zapiola (IMyZA, CICVyA-INTA) for his help with electronic artwork. This work was funded by INTA Projects PNPV1135033 and PNBIO1131044.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at

Legends to Figures

Fig. 1. A. SEM micrograph of polyhedral OBs extracted from *H. gelotopoeon* larvae. B and C. Ultrathin sections showing polyhedra (black arrows) and nonoccluded virus particles (arrowheads) accumulating in the nuclei of infected fat body cells. White arrow indicates the nuclear membrane.

Fig. 2. Phylogeny of concatenated lef-8/lef-9/polh fragments. Minimum evolution (ME) phylograms inferred from the concatenated lef-8, lef-9, and polh alignments are shown with bootstrap values >50% for ME and maximum likelihood (ML) trees at each node where available (ME/ML). Apart from HegeSNPV, HzSNPV F16 (AF334030), HearSNPV G4 (AF271059), HearSNPV C1 (AF303045), HearNPV NNg1 (AP010907), HearNPV Australia (JN584482) and HzSNPV HS-18 (KJ004000), isolates used are representative of all isolates reported in Rowley et al. (2011). Accession numbers can be found in the supplementary table 1.

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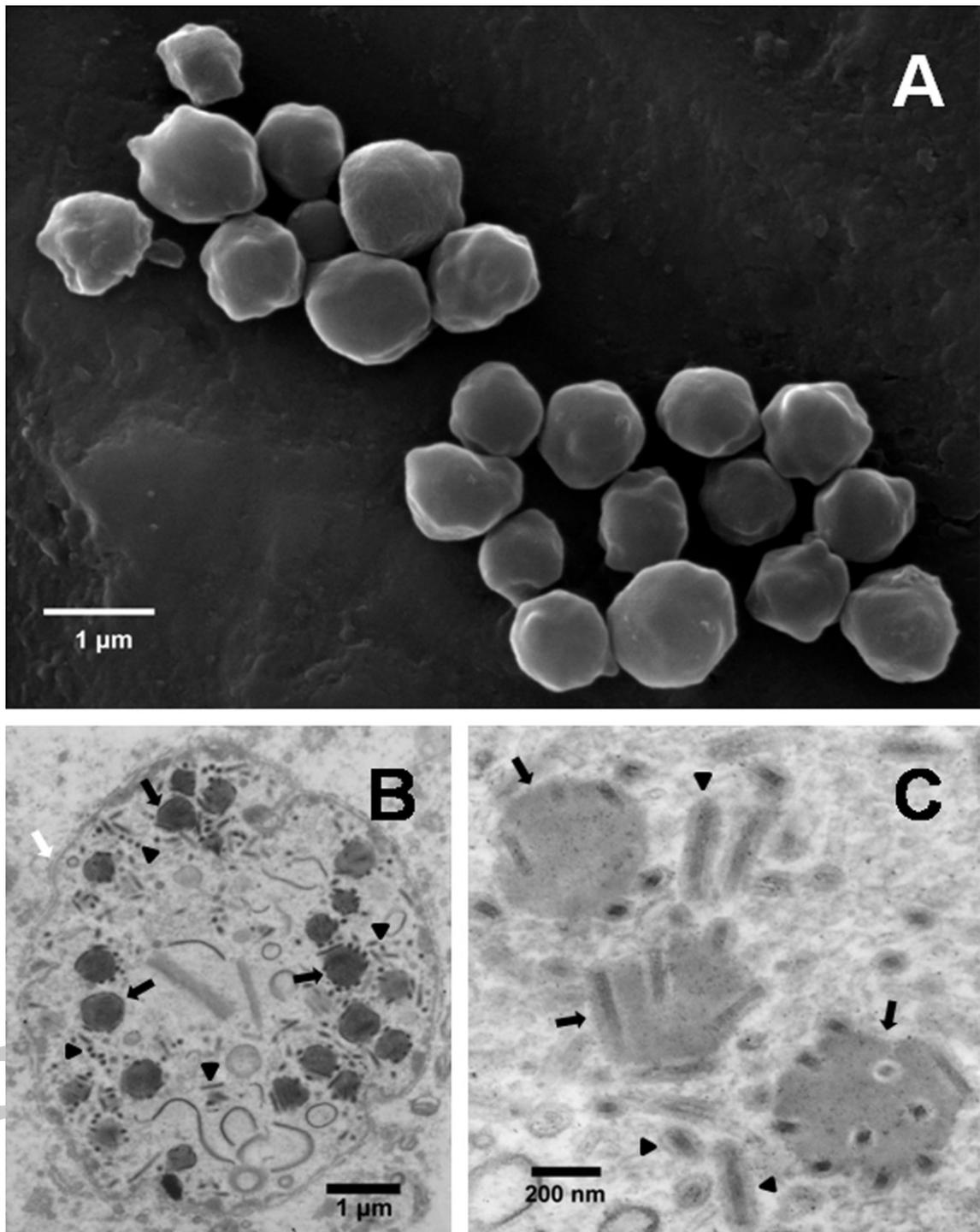
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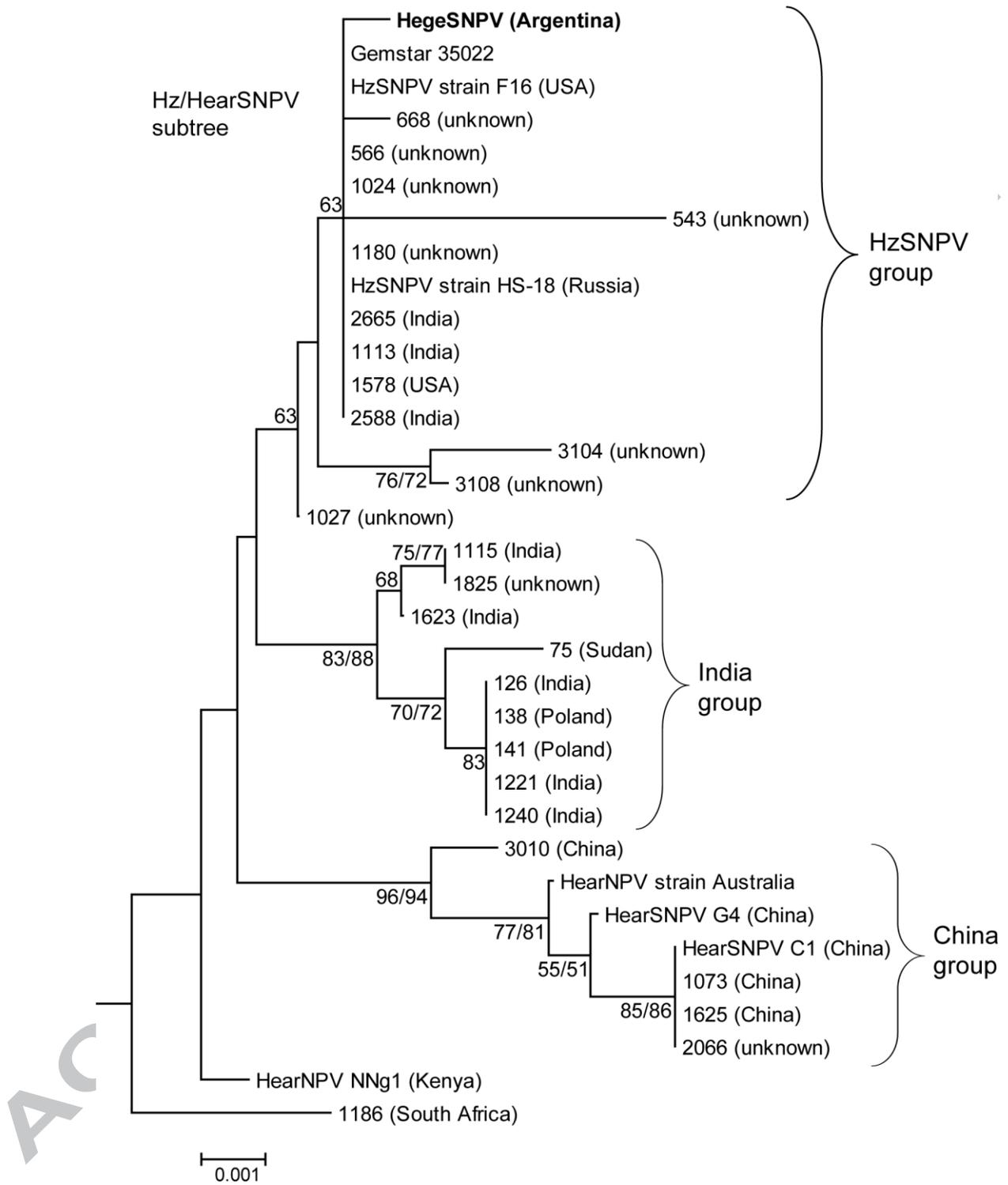
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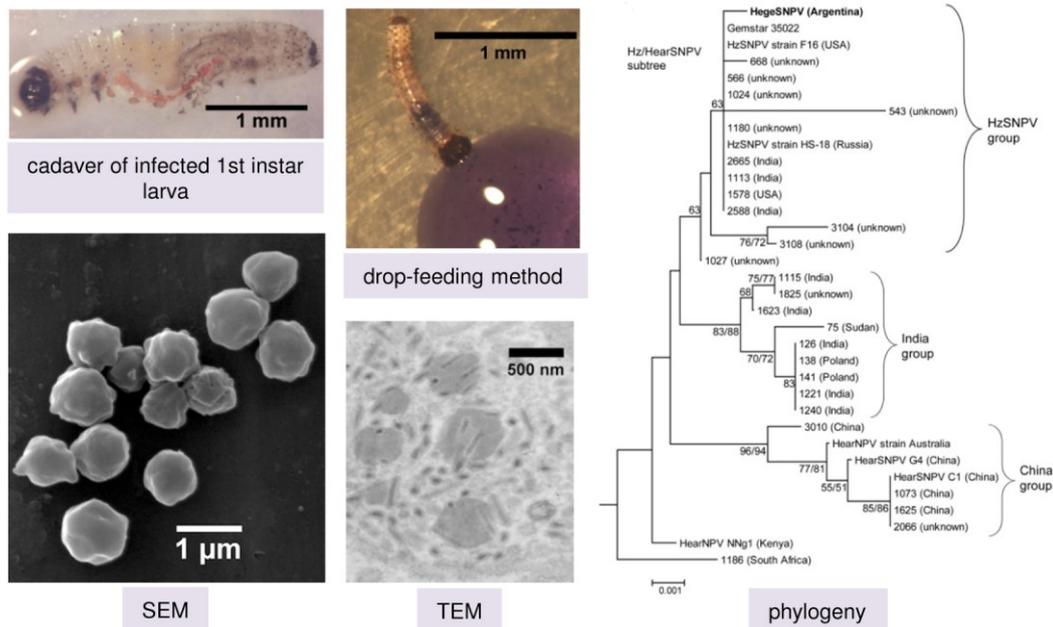
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Graphical abstract

Highlights

- This is the first report of a baculovirus infecting the bollworm *Helicoverpa gelotopoeon*, a serious pest of soybean, cotton and chickpea in South-America.
- A comprehensive research including bioassays, electron microscopy, sequencing and phylogenetic analyses was performed to characterize the virus.
- The virus was shown to be related to, but not identical, with the so-called HzSNPV variants of HearNPV.