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Characterization and genomic analyses of a novel alphabaculovirus isolated from the black armyworm, *Spodoptera cosmioides* (Lepidoptera: Noctuidae)

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ABSTRACT

The black armyworm *Spodoptera cosmioides* is a pest of increasing importance in Cry1Ac-Bt toxin crops and non-Bt crops of soybean and cotton in Brazil. Here we characterized a baculovirus isolated from extracts of *S. cosmioides* that died with symptoms of nuclear polyhedrosis. The putative novel virus exhibited polyhedral occlusion bodies (OBs) with virions containing multiple rod-shaped nucleocapsids, characteristic of alphabaculoviruses. The virus isolate was named Spodoptera cosmioides nucleopolyhedrovirus isolate CNPSo-72 (SpcoNPV-CNPSo-72). SpcoNPV-CNPSo-72 was lethal to third-instar *S. cosmioides* caterpillars but not to *S. frugiperda* under the tested viral concentrations. Moreover, SpcoNPV-CNPSo-72 contained a circular 147,763 bp long genome and a G + C content of 44.8% with 151 annotated ORFs (10 unique for baculovirus) and five homologous regions (*hrs*). The 38 currently defined baculovirus core genes were found in the SpcoNPV-CNPSo-72 genome. After phylogenetic analysis, the novel virus was found to be closely related to other members of *Alphabaculovirus*, especially to the *Spodoptera*-infecting viruses, which included Spodoptera eridania nucleopolyhedrovirus isolate 251, Spodoptera litura nucleopolyhedrovirus isolate II, Spodoptera exigua multiple nucleopolyhedrovirus isolate US-1, Spodoptera eridania nucleopolyhedrovirus isolate CNPSo-165, and Spodoptera frugiperda multiple nucleopolyhedrovirus isolate 19. Surprisingly, the new baculoviral genome was found to code for a putative arginine-associated tRNA gene with a predicted intronic sequence of 105 nt. The gene was found inside the *bjdp* CDS. Overall, baculoviruses are pathogens that lethally infect insect larvae and their study allows a better understanding of large DNA virus evolution, which provides important insights for the development and improvement of biological control agents.

1. Introduction

The black armyworm *Spodoptera cosmioides* (Lepidoptera: Noctuidae) is a pest of expanding importance in both non-Bt and Cry1Ac-expressing soybean and cotton crops in Brazil (Bernardi et al., 2014; Araujo and Busoli, 2020). Sporadic infestations can cause yield loss due to defoliation and injuries to their pods (Sosa-Gómez et al., 2008). The main strategy to control the insect population is based on broad-spectrum chemical insecticides (Bernardi et al., 2014), which may lead to both selection of resistant pests and death of non-target organisms (*e.g.* natural enemies, pollinators, and soil arthropods). Moreover, chemical insecticides are important xenobiotics that accumulate in the environment and cause intoxication to human and other vertebrates (Gullan and Cranston, 2014). Therefore, methods based on the use of entomopathogens, including bacteria, fungi, and viruses are safer tools to complement the use of chemical pesticides in an agroecological and integrated pest management program (Sosa-Gómez, et al., 2017).

Baculoviruses are specific to the immature phase of insects and may be applied for pest population control in agriculture and forest (Clem and Passarelli, 2013; Sosa-Gómez et al., 2020). For instance, Anticarsia gemmatalis multiple nucleopolyhedrovirus (AgMNPV) has been applied since early 1980s for the biocontrol of the soybean caterpillar *Anticarsia gemmatalis* in Brazil and Latin America (Moscardi, 1999; Sosa-Gómez, 2017, 2008; Haase et al., 2015; Sosa-Gómez et al., 2020). Baculoviruses

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belong to *Baculoviridae*, a family of rod-shaped enveloped viruses with circular dsDNA genome whose size ranges from 80 to 180 kbp (Rohrmann, 2019). They are orally infectious to their hosts, which usually get infected from contaminated food or substrate. The viral infection begins when the insect feeds on substrates contaminated with occlusion bodies (OBs). The OBs dissolve and release the occlusion-derived virus (ODV) that fuses with the cell membrane of the midgut columnar epithelium. The nucleocapsid is carried to the nucleus, where the viral DNA replicates. Then, the infected cell produces the budded viruses (BVs), which mediate the secondary infections and spread the virus throw different host tissues like trachea, hemocytes, and fat body (Jehle et al., 2006; Slack and Arif, 2006; Ohkawa et al., 2010). Later, ODVs are occluded in OBs inside the cell nucleus and the diseased larvae exhibits a discolored and darkish tegument that can liquefy and release an enormous number of OBs in the environment (Blissard and Hohrmann, 1989; Federici, 1997; Sosa-Gómez et al., 2020).

The family *Baculoviridae* contains four genera, including members of *Alphabaculovirus* and *Betabaculovirus* that are infectious to larvae of Lepidoptera, members of *Gammabaculovirus* that are infectious to larvae of Hymenoptera, and members of *Deltabaculovirus* that are infectious to larvae of Diptera (Jehle et al., 2006; King et al., 2012; Javed et al., 2017). To date, 84 baculovirus species are recognized by the International Committee on Taxonomy of Viruses (ICTV), and only eight represent members isolated from *Spodoptera* hosts: six alphabaculoviruses and two betabaculoviruses. In Brazil, two *Spodoptera*-infecting baculoviruses have being isolated and had their genome sequenced, the Spodoptera frugiperda multiple nucleopolyhedrovirus isolate 19 (SfMNPV-19) (Wolff et al., 2008) and the Spodoptera eridania nucleopolyhedrovirus isolated CNPSo-165 (SperNPV-CNPSo-165) (Rodrigues et al., 2020). Here we characterized and sequenced the genome of a baculovirus isolated from field-collected *S. cosmioides* larvae with symptoms of nuclear polyhedrosis, which have been kept at the Virus Collection of the Brazilian Agricultural Research Corporation-Soybean (Portuguese acronym Embrapa-Soja, Empresa Brasileira de Pesquisa Agropecuária-Soja). The novel isolate was tentatively named Spodoptera cosmioides nucleopolyhedrovirus isolate CNPSo-72 (SpcoNPV-CNPSo-72) and may also represent a new species inside *Alphabaculovirus*.

2. Material and methods

2.1. OB and DNA purification

Dead larvae of *S. cosmioides* with symptoms of nuclear polyhedrosis were collected in soybean fields in Paraná State, Brazil, in the 1980′s. After preliminary microscopical examination, polyhedral-shaped OBs were observed, and the sample was kept at the Virus Collection of the EMBRAPA-Soja and labeled isolate CNPSo-72. OBs and viral DNA were purified according to O'Reilly et al. (1992). Briefly, insect cadavers were homogenized with the same volume of ddH$_2$O (w/v), filtered through cotton gauze, and centrifuged at 7000 x g for 10 min. The supernatant was discarded, the pellet was resuspended in 0.5% SDS and centrifuged at 7000 x g for 10 min. This procedure was repeated twice. The final pellet was resuspended in 0.5 M NaCl, centrifuged once more, and resuspended with ddH$_2$O. The suspension was loaded onto a discontinuous sucrose gradient (40–80% sucrose in Phosphate Buffered Saline [PBS], 137 mM NaCl, 2.7 mM KCl, 10.0 mM Na$_2$HPO$_4$, 2.0 mM KH$_2$PO$_4$, pH 7.4) and centrifuged at 130,000 x g for 3 h. The collected band was diluted 1:1 in ddH$_2$O and the OBs were recovered by centrifuging at 7000 x g for 10 min. For DNA purification, 200 μl of the OB-containing suspension (10^{10} OBs/ml of ddH$_2$O) were heated for 20 min at 95 °C, placed on ice for 5 min, and treated with RQ1 RNase-Free DNase (Promega). The suspension was washed three times with SDS 0.5% and once with NaCl 0.5 M, centrifugated (7000 $\times$ g for 10 min) and resuspending with equal volumes. The last resulting pellet was suspended in ddH$_2$O. Purified OBs were dissolved in alkaline solution and the released

ODVs were treated with virus disruption buffer (10 mM Tris - HCl, pH 7.6; 10 mM EDTA, 0.25% SDS) and proteinase K (10 mg/ml in ddH2O) for nucleic acid release. The DNA was purified by sequential phenol, phenol-chloroform-IAA, chloroform extractions followed by an ethanol precipitation/washing step. Both quantity and quality of the purified DNA were determined by electrophoresis on a 0.8% agarose gel (Sambrook and Russell, 2001) and visualized, and photographed in AlphaImager® Mini (Alpha Innotech) (data not shown).

2.2. Transmission electron microscopy analysis

One hundred μl of purified OB-containing suspension (10^9 OBs/ml of ddH2O) were immersed for 2 h in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2). They were then post-fixed in 1% OsO4 in the same buffer for 1 h, dehydrated in acetone, and embedded in low viscosity Spurr's epoxy resin. Blocks were sectioned in a LKB ultratome III ultramicrotome equipped with a Diatome diamond knife and the sections were contrasted with 3% urany1 acetate and Reynold's lead citrate and examined in a JEOL JEM l00C transmission electron microscope (Ardisson-Araújo et al., 2014).

2.3. Insects and bioassay

The bioassay was carried out at EMBRAPA-Soja and the insects were obtained from a laboratory colony established in 2015 from individuals collected in the city of Londrina (Paraná State, Brazil). Early third-instar *S. cosmioides* larvae ($n = 42$ per concentration) were fed *ad libitum* with contaminated artificial diet (Greene et al., 1976) for 24 h (Lima et al., 2013). The diet contained six viral concentrations, including 0.6×10^3, 1.5×10^3, 3.75×10^3, 9.4×10^3, 23.4×10^3, and 58.6×10^3 OBs/ml. A group with no treatment ($n = 42$) was set up as control. For third-instar *S. frugiperda* assay, five virus concentrations ($n = 30$ insects per concentration) 1×10^6, 1×10^5, 1×10^4, 1×10^3 and 1×10^2 OBs/mL were used and an untreated group ($n = 40$) was set up as control. The insects were maintained at controlled temperature of 26±1.5 °C, with relative humidity 75±10% and photoperiod of 14:10 (L:D) (Rodrigues et al., 2020). The bioassay was carried out in triplicate and the mortality was recorded by scoring the number of dead insects at the end of 12 days. The results were analyzed by Probit in PoloPlus version 1.0.

2.4. Viral genome sequencing, assembly, and annotation

The viral DNA was sequenced by the 454 Genome Sequencer (GS) Titanium at Macrogen Company (South Korea). The resultant reads were trimmed and assembled using the *de novo* assembly method implemented in the software Geneious R10 (Kearse et al., 2012) with a pairwise minimal identity of 99.3%. For genome annotation, the open reading frames (ORFs) that started with a methionine codon (ATG) and encoded polypeptides of at least 50 amino acids were identified and annotated using the same software and the BLAST-X (Altschul et al., 1997). ORFs inside repeat regions were not considered to be annotated. The homologous regions (*hrs*) were annotated using the Tandem Repeat Finder implemented in Geneious (Benson, 1999), considering a minimum repeat length of 10 nt and a maximum mismatch of 20%. The conserved palindromes were aligned by MAFFT and the consensus sequence subject to DNA fold at 25 °C (Mathews, 2004) also implemented in Geneious. The genomic DNA sequence was submitted to GenBank under the accession number MK419955. Moreover, each ORF found in the genome sequence was submitted individually to BLASTX (Altschul et al., 1997) to find the identity to other baculoviruses. The ORFs with no BLAST matches were also submitted to HHpred and SMART to predict conserved domains (Schultz et al., 1998; Söding et al., 2005).
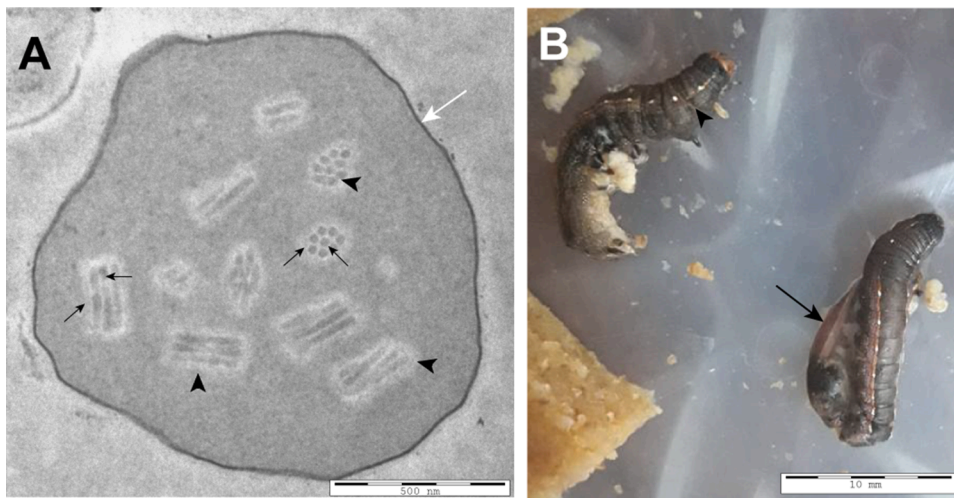
Fig. 1. Ultrastructural analysis of SpcoNPV-CNPSo-72 occlusion bodies (OBs) and the *post mortem* phenotype observed during viral etiology confirmation. (A) Representative transmission electron micrography of purified SpcoNPV-CNPSo-72 OBs. OBs were obtained from dead subjects of black armyworms, *S. cosmioides* that died symptoms of nuclear polyhedrosis (*e.g.* tree-top disease, flaccid/wilted larvae, ruptured cuticle, tegument discoloration) and presented a predominant polyhedral shape with numerous occlusion-derived viruses (ODVs) within. Each ODVs presented multiple rod-shaped nucleocapsids per envelope. In the micrography, black arrows point rod-shaped nucleocapsids sectioned transversally or longitudinally; black arrowheads point ODVs embed into the polyhedrin matrix; white arrow points the calyx deposited on the surface of a mature OB (Scall bar = 500 nm). (B) Two dead black armyworm subjects of species *S. cosmioides* depicting the *post mortem* phenotype after oral exposure to artificially contaminated diet with SpcoNPV-CNPSo-72 OBs. Black arrowhead shows cuticle discoloration and darkish pigmentation, and black arrow shows tegument disruption and liquefaction.

2.5. Baculovirus phylogeny

The phylogenetic analysis of virus genome sequences inside family *Baculoviridae* was inferred by an amino acid MAFFT alignment (Katoh et al., 2002) predicted from all 38 baculoviral core, which were extracted from 99 genomes publicly available (Table S1). The concatenated protein sequences were used to construct a maximum likelihood tree by the Fast-Tree method with the Jones-Taylor-Thornton (JTT) model (Stamatakis et al., 2008) and a test for branch support with a Shimodaira-Hasegawa-like method (Anisimova et al., 2011).

2.6. Gene parity plot

A gene parity graph was constructed using the BLASTX (Altschul et al., 1997) results to identify homologs found between SpcoNPV-CNPSo-72 and other closely related *Spodoptera*-infecting alphabaculoviruses, including Spodoptera exigua multiple nucleopolyhedrovirus US1 (SeMNPV-US1), Spodoptera eridania nucleopolyhedrovirus isolate 251 (SperNPV-251), Spodoptera eridania multiple nucleopolyhedrovirus CNPSo-165 (SperMNPV-165), and Autographa californica multiple nucleopolyhedrovirus C6 (AcMNPV-C6).

2.7. Gene content comparison and new specie demarcation criteria

A Venn Diagram was constructed to identify the ORFs shared among SpcoNPV-CNPSo-72 and its closest relatives. To confirm that SpcoNPV-CNPSo72 may represent a new species of family *Baculoviridae*, the pairwise nucleotide distances was calculated using the Kimura 2-parameter (K2P) model of nucleotide substitution for conserved regions of *lef-8, lef-9,* and *polh* (Jehle et al., 2006) in MEGA7 (Kumar et al., 2016).

2.8. tRNA prediction

The genome of SpcoNPV-CNPSo-72 was submitted to the analyses by the ARAGON software (Laslett and Canback, 2004) that employs heuristic algorithms to predict tRNA secondary structure allowing for the presence of intronic regions. Moreover, the genomes of the closest relatives baculoviruses species of SpcoNPV-CNPSo-72 was downloaded and submitted to tRNA sequence prospection. To compare the tRNA-containing genomic region among the SpcoNPV and its close relatives, sequences containing the *bjdp* orthologs were extracted from SpcoNPV-CNPSo-72, SeMNPV-QD, SeMNPV-US-1, SfMNPV-19, SperNPV-CNPSo-165, SperNPV-251, SpexNPV-244.1, and SpltNPV-II and aligned by the MAFFT method (Katoh et al., 2002).

3. Results and discussion

3.1. Ultrastructural analysis of SpcoNPV-CNPSo-72 OBs and the viral etiology

Dead larvae of *S. cosmioides* with symptoms of nuclear polyhedrosis (*e.g.* tree-top disease, flaccid/wilted larvae, ruptured cuticle, tegument discoloration) were collected in Southern Brazil soybean fields in the 1980′s and assigned as SpcoNPV-CNPSo-72 at the EMBRAPA-Soja's virus collection. We carried out an ultrastructural analysis of purified OBs by TEM and found OBs with numerous ODVs inside the crystalline matrix and several rod-shaped nucleocapsids per envelope (Fig. 1A), typical of alphabaculoviruses. We assumed a predominant polyhedral shape and observed the calyx as an electron-dense structure surrounding the mature polyhedra (Fig. 1A, white arrow). The general structure observed was like those observed previously in other *Spodoptera*-infecting alphabaculoviruses OBs (Escasa et al., 2019; Rodrigues et al., 2020).

To confirm the infection etiology, we carried out an oral infection bioassay by exposing healthy third-instar subjects of species *S. cosmioides* to purified OBs spread on the top of an artificial diet. After seven days post-exposure, most of the larvae died with symptoms of baculovirus infection, including positive phototropism (data not shown), cuticle discoloration, easily ruptured tegument with darkish pigmentation, and *post morten* liquefaction (Fig. 1B). The melted content contained high yields of OBs (data not shown). This is consistent with the fact that viral *chitinase* and *cathepsin* are present in all described
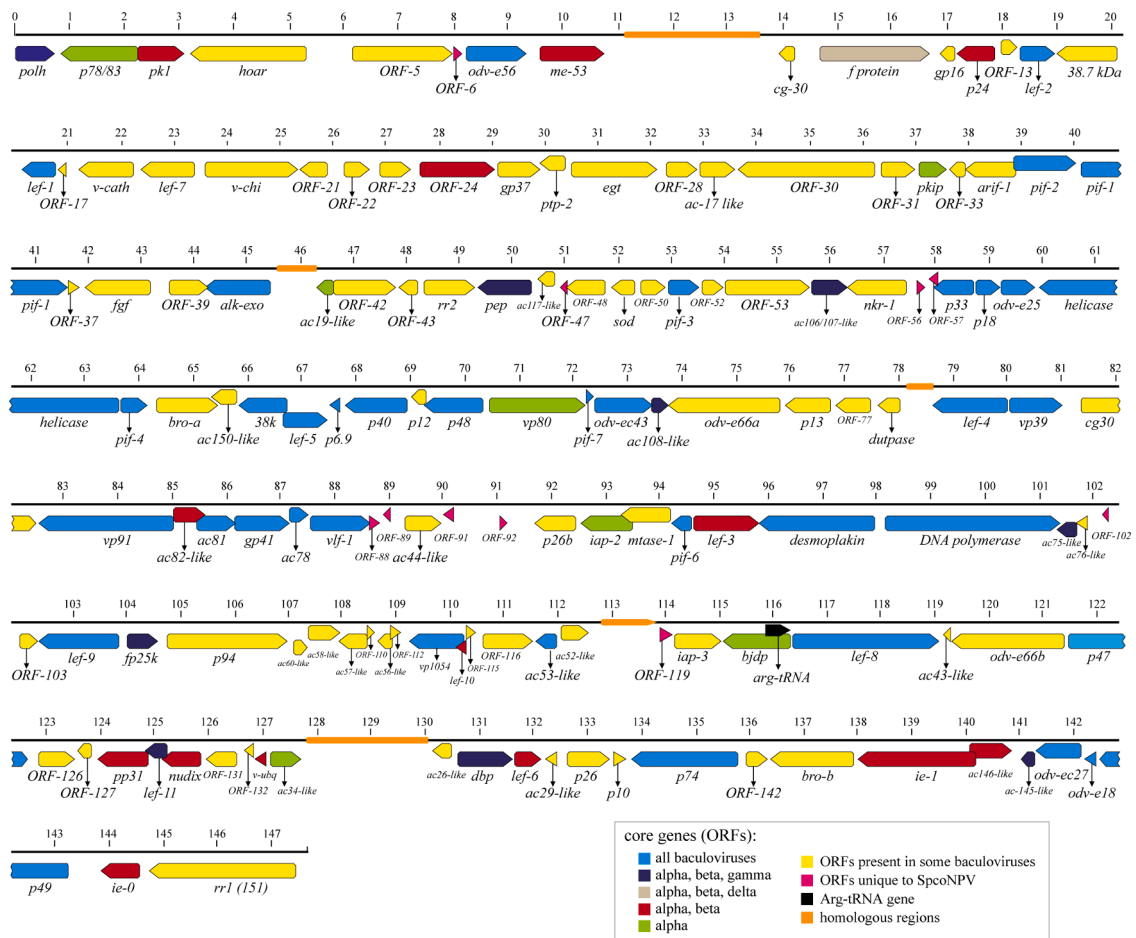
Table 1
Dose-mortality response of third instar larvae of *Spodoptera cosmioides* infected orally with SpcoNPV-CNPSo-72.

n*	Slope	LC$_{50}$ (OB. mL^{-1})	Fiducial Limits (95%)	LC$_{99}$ (OB. mL^{-1})
756	1.530 ± 0.194	2982.990	1237.763 – 6200.178	98,981.332

* number of insect tested.

Fig. 2. Gene content of SpcoNPV-CNPSo-72. ORFs and predicted transcription direction are indicated as arrows. Gene names are indicated below arrows. Genome position is shown by a kb scale above the thin line. ORF shading is according to legend. Homologous regions are represented by brown bar above the ORFs. the arrows are filled with colors that represent genes previously reported in baculovirus genomes. Light blue: core genes in the genome of members of *Alphabaculovirus, Betabaculovirus, Gammabaculovirus*, and *Deltabaculovirus*; yellow: present in some baculovirus; pink: unique in SpcoNPV genome; dark-blue: core genes in *Alphabaculovirus, Betabaculovirus* and *Gammabaculovirus*; gray: core genes in *Alphabaculovirus, Betabaculovirus* and *Deltabaculovirus*; dark-red: core genes in *Alphabaculovirus*, and *Betabaculovirus*; light green: core genes in *Alphabaculovirus*; black: the putative tRNA gene.

Spodoptera-infecting alphabaculoviral genomes so far. The new baculovirus was able to melt, discolor and, consequently, liquefy the host tegument.

After confirming the susceptibility of *S. cosmioides* to this new putative virus, we carried out a dose-mortality response bioassay with purified OBs. SpcoNPV-CNPSo-72 was lethal to third-instar *S. cosmioides* larvae with a LC$_{50}$ of 2.9×10^3 OB/mL of artificial diet (Table 1). We also tested the ability of SpcoNPV-CNPSo-72 to kill larvae of *S. frugiperda*, the most important species inside the *Spodoptera*-complex in Brazil (Bernardi et al., 2014). The isolate was not able to orally infect *S. frugiperda* under the tested conditions, which is like that observed for SperNPV-CNPSo-165 (Rodrigues et al., 2020). This virus was able to kill third-instar caterpillars of two closely related species, *S. eridania* and *S. albula*, but not *S. frugiperda*, similarly to SpcoNPV-CNPSo-72. Surprisingly, in the case of SperNPV, larvae of *S. albula* were 400-fold more susceptible than *S. eridania* (Rodrigues et al., 2020). *Spodoptera*-infecting baculoviruses seem to vary their ability to kill more than one species. For instance, Spodoptera exigua MNPV did not cause mortality to larvae of neither *S. frugiperda* nor *S. littoralis* (Murillo et al., 2003). Moreover, both *S. exigua* and *S. littoralis* were found to be semi-permissive to the virus Spodoptera frugiperda MNPV (SfMNPV), whereas *S. exigua, S. littoralis*, and *S. frugiperda* were found to be permissive to Spodoptera littoralis NPV (Murillo et al., 2003; Simón et al., 2004). A remarkable bottleneck of using baculovirus relies on its specificity and often the restricted number of susceptible insects (Rohrmann, 2019; Sosa-Gómez et al.,

2020). Even insects inside same genus may exhibit different dose-mortality response to baculovirus isolates, as that observed for *S. frugiperda* (Cheng et al., 2005; Le Ru, et al., 2018; Rodrigues et al., 2020). Therefore, describing new baculovirus is an important contribution to build up a broad spectrum multispecies-based biopesticide and counteract the advance of the *Spodoptera*-complex over agriculture. No virus isolated from *S. cosmioides* had been sequenced completely and described so far. Certainly, it would be valuable to understand the host range of SpcoNPV-CNPSo-72 and the potential interaction among other species of baculovirus like SfMNPV, and SperNPV-CNPSo-165 to develop a product targeting the *Spodoptera*-complex. NPVs are transmitted as collective infectious units, which leads to genotypic diversity present in natural virus populations and to potential interspecific mixtures of viruses that can be applied to control complexes of lepidopteran pests (Williams et al., 2022).

3.2. Features of the SpcoNPV-CNPSo-72 genome

We sequenced the genome of SpcoNPV-CNPSo-72 by the 454 Genome Sequencer (GS) FLX ™ Titanium method (Macrogen Inc., Korea). The sequencing produced 14,499 reads with a mean size of 589.8 ± 213.2 bp and a genome coverage of 50.4 X. We assembled *de novo* the reads into one single circular contig of 147,763 bp in size with a G + C nucleotide distribution content of 44.8%. The genome size and nucleotide distributions were within the same range as that reported for
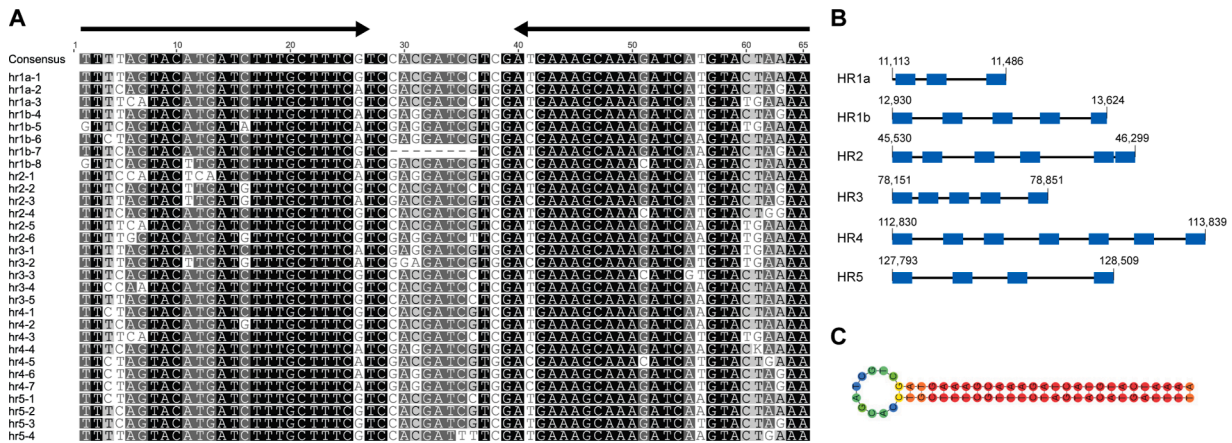
Fig. 3. SpcoNPV-CNPSo72 homologous region (*hr*) unit repeats. (A) A MAFFT alignment of the individual repeats found in the 5 SpcoNPV-CNPSo72 *hr*s are shown. The consensus sequence is located above the alignment. Black arrows point the complementary palindromes. Shading corresponds to the degree of nucleotide conservation: black, 100%, dark gray, >85%, light gray, > 60%. (B) Distribution of the hairpin-like palindromic structure along the 5 *hrs*, which is individually represented by blue rectangles. (C) Secondary structure prediction of conserved palindromic sequences spread along the 5 *hrs* based on DNA fold, revealing a hairpin-like structure.
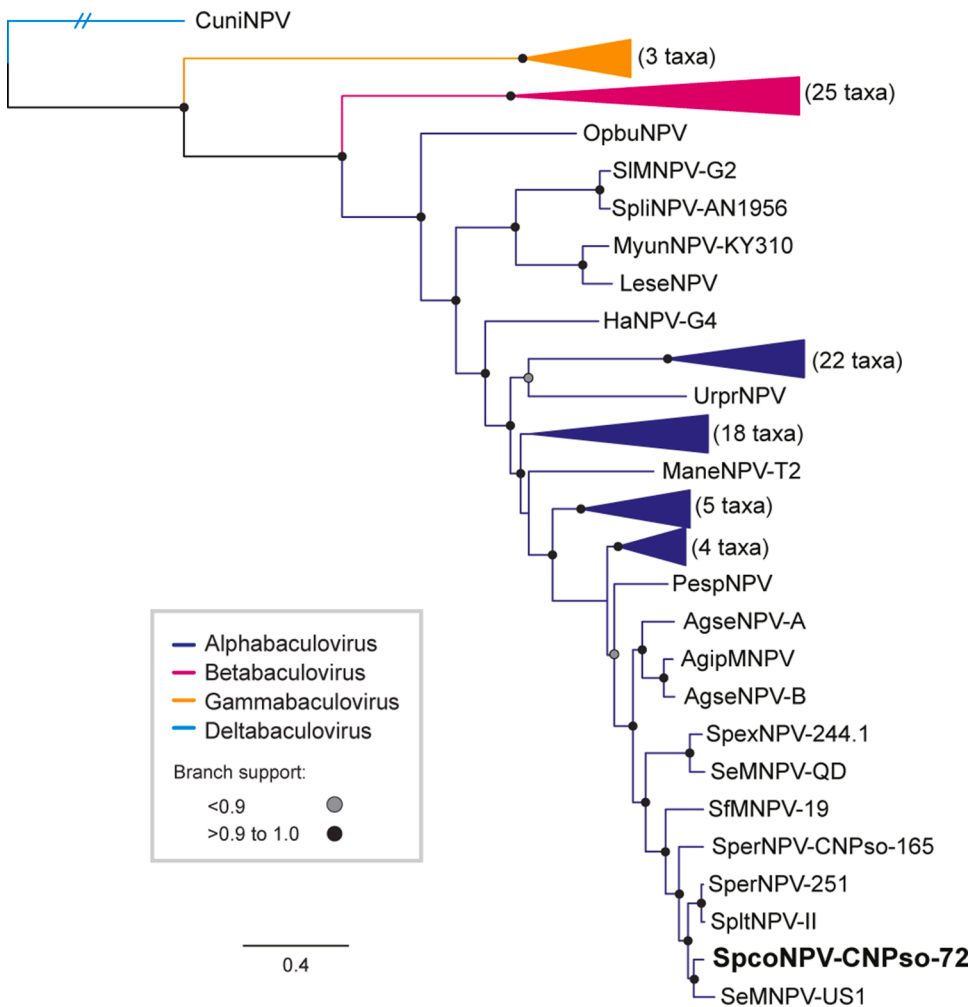

Fig. 4. Baculovirus phylogeny. The phylogenetic analysis shows that SpcoNPV-CNPso-72 is an *Alphabaculovirus* closely related to other *Spodoptera*-isolated viruses. The novel virus species shares a common ancestor with a branch containing SeMNPV-US1, SpltNPV-II, SperNPV-251, and SperNPV-CNPso-165, and SfMNPV-19. The maximum likelihood tree was inferred based on the concatenated nucleotide sequences of the 38 core genes from several selected baculovirus genomes using the Fast-Tree method. The branch support was determined by the SH-like method (black and gray closed circles). Some branches were collapsed: members of *Alphabaculovirus* group I (blue), other *Alphabaculovirus* (blue), *Betabaculovirus* (pink), and *Gammabaculovirus* (orange). CuniNPV, the unique representative of *Deltabaculovirus* (light blue), was used to root the tree.

other alphabaculoviruses (Table S2). We identified and annotated 151 ORFs encoding 50 or more amino acids, which covered about 86% of the genome. We also identified the 38 currently established baculovirus core genes and 26 genes shared between genomes of members of *Alphabaculovirus* and *Betabaculovirus* (Fig. 2) (Garavaglia et al., 2012).

We identify five homologous regions (*hrs*) in the SpcoNPV-CNPSo-72 sequence (Fig. 3). The SpcoNPV *hrs* were spread throughout the genome and contained 30 short repeats with a global pairwise identity of 87.3% (Fig. 3A). This is consistent with other described alphabaculoviral genomes (Harrison et al., 2019), especially those inside the
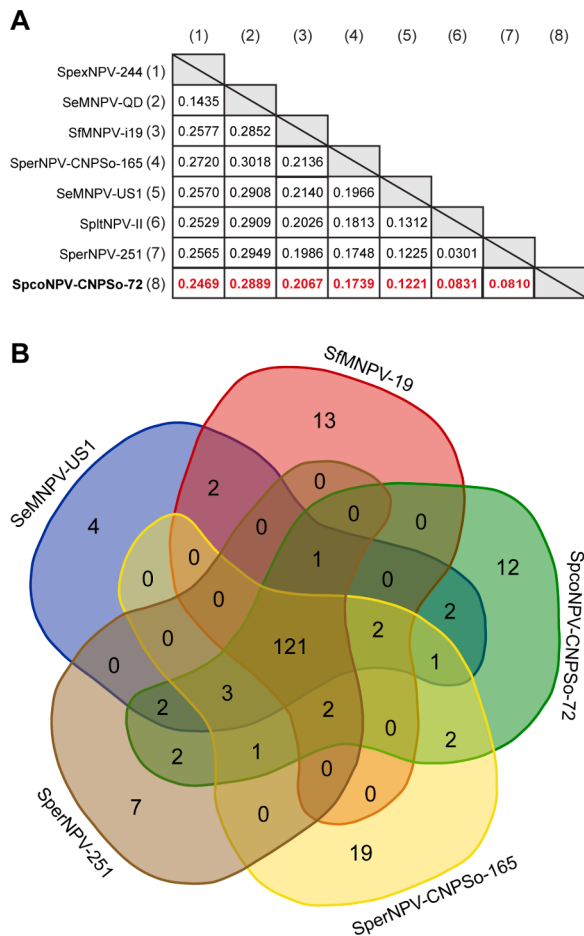
A

B

Fig. 5. Species demarcation criterion with the adjusted Kimura-2 parameter (aK2P) and gene content analysis of SpcoNPV-CNPSo-72 and other closely related baculovirus isolates. (A) aK2P based on the concatenated fragments of conserved partial *polh, lef-8, lef-9* genes of the SpcoNPV-CNPSo-72 and the closely related virus. The distances were calculated using MEGA (Kimura 2-parameter model) (Kumar et al., 2016), based on the species demarcation criteria. In red, we show SpcoNPV-CNPSo-72 values that fulfill the criterion to establish a new species (more than 0.072 substitution/site). (B) Venn diagram comparing the gene content among SpcoNPV-CNPSo-72 and its closest relatives (SeMNPV-US1, SperNPV-CNPSo-165, SfMNPV-19, and SperNPV-251). The gene content was compared by BLASTX to find homologs. 203 genes were found: 121 were shared among all four virus genomes, and 12 ORFs were found only in the SpcoNPV-CNPSo-72 genome, 10 of them are considered unique.

Spodoptera-infecting closely related viruses (Escasa et al., 2019). Most of the *hrs* genomic positions were conserved in other related viral genomes like the SperNPV-251 and SeMNPV-US1 genomes. The 30 repeats were spread in different numbers throughout the *hrs* and interrupted by non-repeat sequences (Fig. 3B). The consensus sequence extracted from the 30 palindromic repeats aligned presented a DNA fold similar to a hairpin structure (Fig. 3C). *Hrs* are highly variable and closely related within a genome, albeit they may show limited nucleotide identity and size between different viruses (Rorhmann, 2019). They seem to be regulatory sequences with implication as transcriptional enhancers and origins for virus DNA replication in several baculoviruses (Guarino and Summers, 1986; Leisy and Rohrmann, 1993; Pearson et al., 1993; Pearson and Rohrmann, 1995).

3.3. Baculovirus phylogeny

To understand the evolutionary relationship of SpcoNPV-CNPSo-72 to other baculoviruses, we carried out a phylogenetic analysis inferred

from the concatenated amino acid alignments of the 38 baculoviral core genes from 99 baculoviral genomes (Table S1). The tree exhibited a topology with a single branch containing all alphabaculoviruses (Fig. 4, blue branches). SpcoNPV-CNPSo-72 clustered in a highly supported clade formed by the *Spodoptera*-infecting viruses SpexNPV-244.1, SeMNPV-QD, SfMNPV-19, SperNPV-CNPso-165, SeMNPV-US1, SperNPV-251, and SpltNPV-II. The SpcoNPV-CNPSo-72 isolate was found to be closely related to the most recent common ancestor (m.r.c. a.) of SeMNPV-US1 (Fig. 4). The branch length separating SpcoNPV-CNPSo-72 from the other completely sequenced alphabaculoviruses is in a range that is comparable to the branch lengths separating viruses that are members in other recognized species (Trentin et al., 2019; Rodrigues et al., 2020). SpcoNPV-CNPSo-72 is related to other group II alphabaculoviruses, which are characterized by having a F protein homolog as the major BV envelope fusion protein (Rohrmann 2019). The clade containing SpcoNPV and the other closely related *Spodoptera*-infecting viruses nested together with other viruses which have been isolated from noctuid hosts. Interestingly, alphabaculovirus group I remained monophyletic whereas group II was found to be polyphyletic, as previously observed in other phylogenetic inferences (Santos et al., 2018; Trentin et al., 2019; Rodrigues et al., 2020; Harrison et al., 2017).

3.4. Species demarcation criterion

We investigated whether SpcoNPV-CNPSo-72 was potentially a representative member of new species inside genus *Alphabaculovirus*. We found that SpcoNPV-CNPSo-72 fulfilled the criterion to establish a novel baculovirus species. A virus isolate may represent a new species if the number of substitutions per site is higher than 0.050 (Jehle et al., 2006) using the Kimura-2-parameter (K2P) substitution model in selected regions of three viral genes, *lef-8, lef-9* and *polh*. SpcoNPV-CNPSo-72 showed an K2P-based pairwise distance of 0.0810 (Fig. 5A).

3.5. Gene content analysis

We annotated 151 putative ORFs encoding polypeptides of at least 50 amino acid residues in the genome of SpcoNPV-CNPSo-72 and identified among them the 38 baculoviral core genes. The genome also contained the 26 ORFs shared between alphabaculoviruses and betabaculoviruses (Table S3) (Garavaglia et al., 2012). Regarding auxiliary genes, we found two copies of the *baculovirus repeated ORF* (*bro*) (SpcoNPV-ORF-63 and SpcoNPV-ORF-143). Homologs of *bro* gene were previously reported in the genome of members of *Alphabaculovirus, Betabaculovirus*, and *Deltabaculovirus* but not in members of *Gammabaculovirus* (Rohrmann, 2019). The number of copies may vary in different baculoviral genomes with common events of gene duplication and/or loss (Harrison and Lynn, 2007; Zhou et al., 2012). The function of the *bro* genes remains unclear, and it was reported that the protein product may exhibit nucleic acid and nucleosome association capabilities, single-stranded DNA (ssDNA) binding motif (Zemskov et al., 2000) or even being related to the virion structure (Wang et al., 2010), and some genes seem to be highly required for viral replication (Kang et al., 1999).

SpcoNPV-CNPSo-72 encodes homologs of auxiliary genes such as the *inhibitor of apoptosis* (*iap*) 2 (SpcoNPV-ORF-94) and *iap-3* (SpcoNPV-ORF-120), *chitinase* (SpcoNPV-ORF-18), *cathepsin* (SpcoNPV-ORF-20). The *iap* proteins contain a zinc-binding motif called BIR (Baculovirus IAP Repeat) and a zinc-binding motif called RING domain (Clem, et al., 2001). Apoptosis is an important defense against virus infection. Several insect DNA viruses, including the baculoviruses, entomopoxviruses, iridoviruses, nudiviruses, and asfarviruses encode *iap* genes to inhibit the apoptosis caused by viral infection and thereby expedite virus multiplication and dissemination (Byers et al., 2016). The *chitinase* and *cathepsin* homologous are coded by several baculovirus genomes, and all viruses inside the SpcoNPV-CNPSo-72-containing branch harbor these genes in their genomes. Baculovirus *chitinase* seems to be closely related to a proteobacteria homologous gene (Hughes and Friedman, 2003).
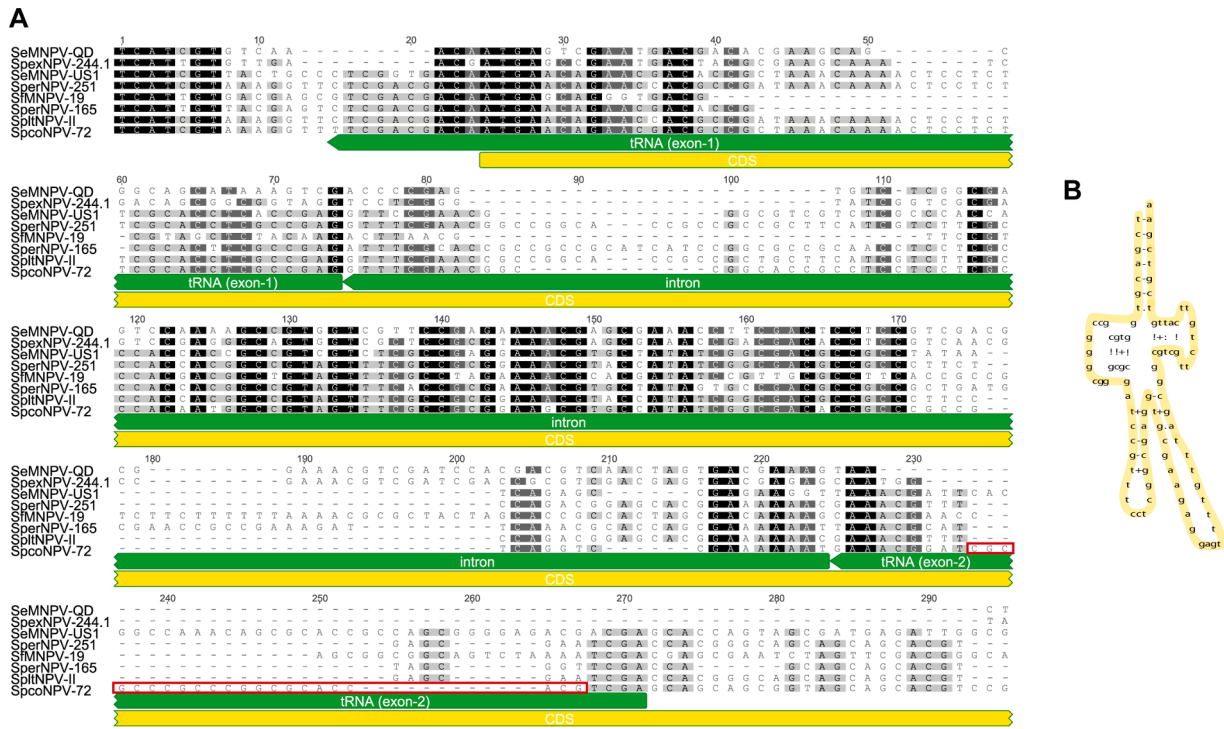
A

B

Fig. 6. SpcoNPV-CNPSo-72 tRNA gene for the arginine (Arg) codon found inside the *bjdp* CDS. (A) The nucleotide sequences of SpcoNPV *bjdp* orthologs were aligned: the predicted *bjdp* CDS is indicated as yellow bars and tRNA sequence in green bars, specifying tRNA-exon 1, tRNA-intron, and tRNA-exon 2 regions. A red box highlights the short SpcoNPV autapomorphic sequence found inside the predicted tRNA-exon 2. (B) Secondary structure of SpcoNPV tRNA-Arg-gene with a classical cloverleaf structure, highlighting interacting bases, 5′, and 3′ ends.

Among the hypothetical ORFs annotated, 10 ORFs were found to be unique (SpcoNPV-ORF-6, −47, −56, −57, −88, −89, −91, −92, −102, −119), *i.e.* not found in any other baculovirus genome described so far. Moreover, SpcoNPV-ORF-6, ORF-56, and ORF-119 contained trans-membrane domains with no predicted signal peptide.

We performed a gene content comparison among SpcoNPV-CNPo-72 and its phylogenetically closest viruses (SeMNPV-US1, SfMNPV-19, SperNPV-251, and SperNPV-CNPSo-165) and the results were plotted in a Venn Diagram (Fig. 5B). A total of 203 different genes were found considering all the five genomes analyzed (Table S4). For this, we reannotated all ORFs of the five isolates using the same criterion of annotation, not considering genes inside *hrs*. Out of 203 genes, only 121 genes were shared among the five genomes. 12 ORFs were present only in the SpcoNPV-CNPSo-72 genome: 10 found to be autapomorphic acquisition in baculovirus and two with homologs in genomes other than the four compared. We also found five ORFs shared between SpcoNPV-CNPSo-72 and SperNPV-251: 4 ORFs coding for hypothetical proteins and 1 ORF was related to a *bro* gene. Two ORFs were shared between SpcoNPV-CNPSo-72 and SeMNPV-US1, the *ac44* gene and a copy of *cg30* gene and two ORFs shared between SpcoNPV-CNPSo-72 and SperNPV-CNPSo-165, a hypothetical protein and a putative homolog of the *NAD-glutamate desidrogenase* gene.

Importantly, the systematic description of novel species at biological and molecular levels allows for a deeper understanding of both the evolution of baculoviruses, as well as their potential relationship to their hosts. SpcoNPV and SperNPV are viruses that do not infect *S. frugiperda*; therefore, understanding the genomic difference between these viruses and SfMNPV would allow the identification of potential host-specific factors. Taking that, we found 13 genes, which code for hypothetical proteins present only in the genome of SfMNPV-19 in comparison with both the SpcoNPV-CNPSo-72 and SperNPV-CNPSo-165 genomes. Some of these genes may play a role in host specificity and could be used to formulate recombinants to likely expand host range. Baculoviruses usually have narrow insecticidal spectrum with not clearly described

mechanism to controle host-range. For instance, a cell culture-isolated recombinant AcMNPV mixture with a SeMNPV fragment insertion had a broader host range than the parental viruses and was found to being able to infect not only the permissive hosts of its parental viruses but also a nonpermissive host (*Spodoptera litura*) (Wu et al., 2016).

3.6. Description of a putative tRNA-coding gene in the genome of SpcoNPV-CNPSo-72

Prompted by the unexpected finding in the genome of the large dsDNA tailed giant Tupanvirus, which possesses the most complete translational apparatus of the known virosphere (Abrahão et al., 2018), we prospected for tRNA-coding sequences in the SpcoNPV-CNPSo-72 genome using ARAGORN (Laslett and Canback, 2004). Surprisingly, we found that the SpcoNPV genome codes for a unique putative tRNA gene for the arginine (Arg) codon. The tRNA-Arg-gene was found to be located inside the CDS of the *bjdp* or *DNA J domain protein* gene. Homologs of *bjdp* can be found in all alphabaculoviral genomes (Rohrmann, 2019). Moreover, the SpcoNPV tRNA-Arg-gene was predicted to contain a short intronic sequence of 105 nt between tRNA-exon 1 and tRNA-exon 2 (Fig. 6A). Introns are quite common inside tRNA genes, which can be removed from the transcribed pre-tRNA by a self-splicing mechanism of maturation (Fujishima and Kanai, 2014). We carried out a multiple alignment of *bjdp* ortholog gene to all SpcoNPV closely related viruses (Fig. 6A) and found that SpcoNPV gene contained a short autapomorphic sequence inside the predicted tRNA-exon 2 (Fig. 6A, red rectangle). The region containing the tRNA-exon 1 in the alignment exhibited a global nucleotide pairwise identity of 56.6%, whereas the region containing the tRNA-exon 2 exhibited only 27.5%. We did not find tRNA-Arg-gene orthologs in the *bjdp* sequences obtained from the other closely related SpcoNPV viruses, including SeMNPV-QD, SpexNPV-244.1, SeMNPV-US1, SperNPV-251, SfMNPV-19, SperNPV-CNPSo-165, and SpltNPV-II. The tRNA secondary structure of the predicted mature form of the SpcoNPV tRNA is shown in Fig. 6B
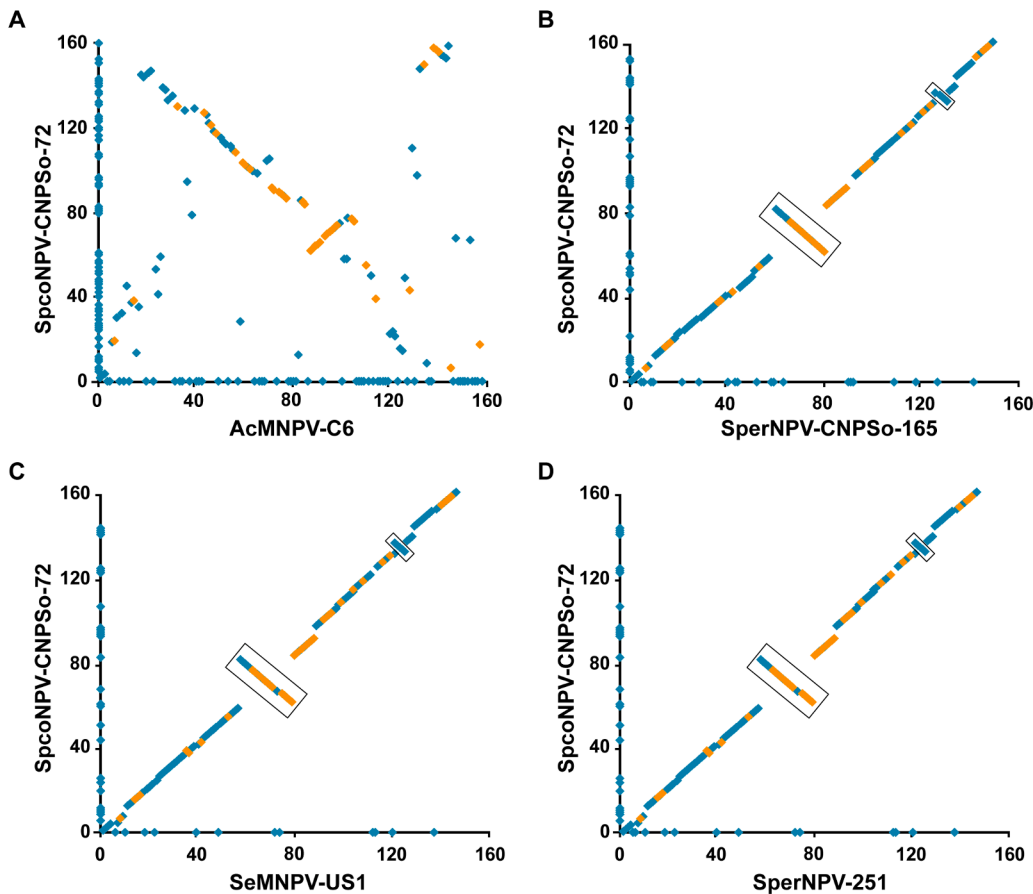
Fig. 7. Gene parity plots of the SpcoNPV-CNPSo72 genome. Plots show the gene content and respective position in the SpcoNPV-VPN72 genome with those from the (A) AcMNPV-C6, (B) SperNPV-CNPSo-165, (C) SeMNPV-US1, and (D) SperNPV-251 genomes. Each blue diamond represents a homologous gene, and the golden diamonds represent one of the 38 core genes. The autapomorphic inversion of the SpcoNPV-CNPSo-72 genome can be seen inside de black rectangle, in comparison to the other Spodoptera-infecting baculoviruses.

with a classical cloverleaf-like structure. tRNA sequences have not been described and annotated in baculovirus genomes so far and the fitness gain of coding for a tRNA is not clear in baculovirus as well as the origin and the reason to be lacked by other closely related genomes. Viruses depend on their hosts translation machinery for the synthesis of their own proteins, and it is expected that there is a selective pressure for the virus to adjust the codon usage in order to corresponding host organism to enable the efficient translation of viral proteins (Walsh and Mohr, 2011; Albers and Czech, 2016). However, genes related to replication, transcription and/or translation, mainly tRNA genes, are occasionally identified in viruses (Delesalle et al., 2016). tRNA genes have been observed in some virus families of double-stranded DNA (Albers and Czech, 2016; Morgado and Vicente, 2019).

3.7. SpcoNPV-CNPSo72 comparison to other related species

We have compared by gene-parity plot analysis the genome of SpcoNPV-CNPSo-72 with the genome of SperNPV-CNPSo-165, SeMNPV-US1, SperNPV-251, AcMNPV-C6 (Fig. 6A to D). We found a very distinct distribution of genes along the genome of SpcoNPV-CNPSo72 and AcMNPV-C6 (Fig. 7A) with a large inversion observed. In contrast, the gene order among SpcoNPV-CPSo72 and the closely related viruses genomes revealed high collinearity (Fig. 7B, 7C, and 7D), and we found two inversions in the genome. We have colored the core genes using orange dots and non-core genes using blue dots that together depict the location of the genes along the genome. Regarding the inversions, the first one was flanked by the *p33* (SpcoNPV-ORF-58) and *dut* (SpcoNPV-ORF-78) homolog genes, which were also flanked by two repeat regions, a direct repeat, and the *hr3*. The second inversion is smaller than the first one and occurred between SpcoNPV-ORF-127 and ORF-131 genes, which are also flanked by two direct repeats. The presence of inversions

in parts of the viral genomes might be a force that drives baculovirus genome evolution (Aragão-Silva et al., 2016; Wilkinson and Weller, 2003). Inversions were reported among closely related species, although there is a discrete level of collinearity. Moreover, inversions usually occur flanked by homologous regions (Kuzio et al., 1999).

4. Conclusion

We have found in the extract of dead *S. cosmioides* larvae a novel and distinct alphabaculovirus named Spodoptera cosmioides nucleopolyhedrovirus isolate CNPSo-72 (SpcoNPV-CNPSo-72). The novel virus exhibited OBs with polyhedral shape and several ODVs with multiple nucleocapsids within. The virus was confirmed to be lethal to *S. cosmioides* but not to *S. frugiperda*. The SpcoNPV-CNPSo-72 complete genome was assembled and found to encode 151 putative ORFs with 10 found to be unique in baculovirus, five *hrs* and a single tRNA-Arg-coding sequence predicted inside the *bjdp* gene. SpcoNPV-CNPSo-72 fulfilled the criterium to establish a novel specie inside genus *Alphabaculovirus*. After phylogenetic analysis, SpcoNPV-CNPSo-72 nested with SfMNPV, SperNPV, SeMNPV, and SpltNPV in a highly supported branch. Those viruses contained 231 ORFs, in which 121 were shared among them all. The description of the SpcoNPV gene content and comparison with other *Spodoptera*-infecting virus could bring light to genes related to virus host range among the *Spodoptera* complex. Overall, the discovery, characterization, and description of new baculovirus species can assist in a better understanding the evolution of baculoviruses and improve biopesticides to minimize the intensive use of chemical insecticides.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary materials

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