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The complete genome of Rachiplusia nu nucleopolyhedrovirus (RanuNPV) and the identification of a baculoviral *CPD-photolyase* homolog

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ABSTRACT

We described a novel baculovirus isolated from the polyphagous insect pest *Rachiplusia nu*. The virus presented pyramidal-shaped occlusion bodies (OBs) with singly-embed nucleocapsids and a dose mortality response of 6.9×10^3 OBs/ml to third-instar larvae of *R. nu*. The virus genome is 128,587 bp long with a G + C content of 37.9% and 134 predicted ORFs. The virus is an alphabaculovirus closely related to Trichoplusia ni single nucleopolyhedrovirus, Chrysodeixis chalcites nucleopolyhedrovirus, and Chrysodeixis includens single nucleopolyhedrovirus and may constitute a new species. Surprisingly, we found co-evolution among the related viruses and their hosts at species level. Besides, auxiliary genes with homologs in other baculoviruses were found, *e.g.* a *CPD-photolyase*. The gene seemed to be result of a single event of horizontal transfer from lepidopterans to alphabaculovirus, followed by a transference from alpha to betabaculovirus. The predicted protein appears to be an active enzyme that ensures likely DNA protection from sunlight.

1. Introduction

Baculoviruses belong to a large family of rod-shaped insect-infecting enveloped viruses inside family *Baculoviridae*. The viruses harbor a supercoiled double-stranded DNA genome with size ranging from 80 to 180 kbp (Rohrmann, 2013). The family contains four genera that coevolved with their insect host at order level: members of *Alphabaculovirus* and *Betabaculovirus* are infectious to larvae of Lepidoptera (butterflies and moths), members of *Gammabaculovirus* are infectious to larvae of Hymenoptera (wasps with caterpillar-like behavior), and members of *Deltabaculovirus* are infectious to larvae of Diptera (mosquitoes). The virus infection process produces two viral phenotypes: (i) the occlusion-derived virus (ODV) and (ii) the budded virus (BV). These two morphologically different but genetically identical virions reflect their respective roles in insect-to-insect and cell-to-cell transmission, respectively. Moreover, as a hallmark of baculovirus, ODVs are occluded within a crystalline protein matrix, the occlusion body (OB). Based on OB morphology, baculoviruses are also classified into those that have a polyhedral-shaped OB called by nucleopolyhedrovirus (NPV) and those with a granular-shape OB called by granulovirus (GV) (Jehle et al., 2006b). This classification scheme is no longer used in baculovirus taxonomy, even though those two terms continue to be used in vernacular names of the viruses.

OB is a convergent adaptation in baculovirus and other insect viruses, including entomopoxviruses and cypovirus (Mitsuhashi et al., 2007; Axford et al., 2014). The OB protects the virion from environmental dissection and degradation and enables it to persist in a dormant form for a long period (Bergold, 2012). Nevertheless, the crystals are not able to protect baculovirus virions against ultraviolet (UV) radiation (Jeyarani et al., 2013), especially from the UV-B (290–320 nm). UV-B radiation may cause a drastic effect on the genomic viral DNA by means of forming two types of pyrimidine dimers, the more abundant cyclobutane-pyrimidine dimers (CPDs) and the less abundant (6–4)-photoproducts (6–4 PPs) (Sancar, 2004). Lesions might hamper polymerase activities through the DNA template, breaking down both transcription and replication processes, which may cause permanent mutation and virus attenuation (Weber, 2005; You et al., 2001; Choi and Pfeifer, 2005). In contrast, baculoviruses present in their genome an interesting

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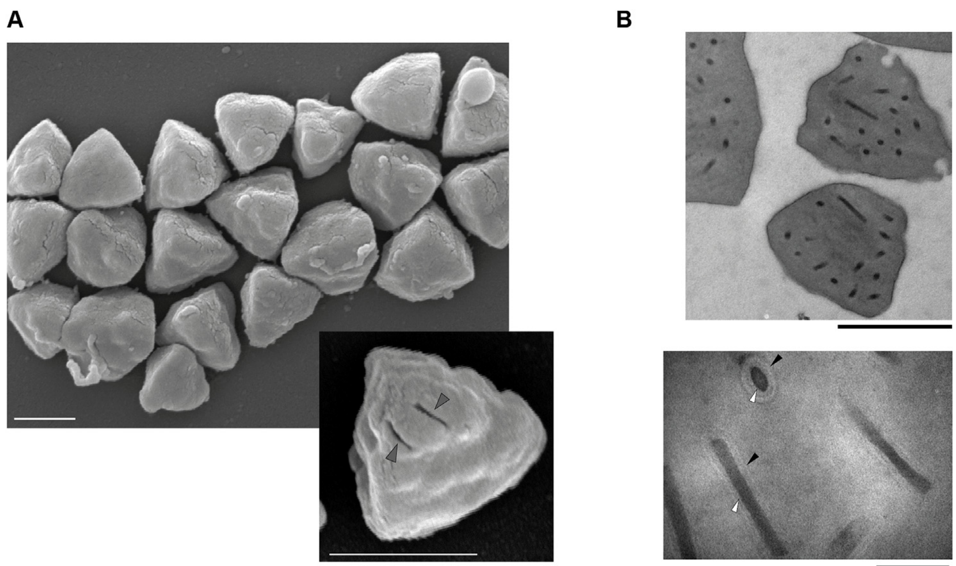

Fig. 1. Ultrastructure analyses of the RanuNPV occlusion bodies (OB). (A) Scanning electron micrography of OBs reveals pyramidal crystals with a predominant size of 1.2 μm. An inset reveals a reminiscent indentation of an immature OB that lost its ODV during preparation, which is pointed by the grey arrowhead. (B) Transmission electron micrography of sectioned OBs reveals several ODVs inside the proteinaceous polyhedrin occlusion. The ODVs present each a single rod-shaped nucleocapsid pointed by the white arrowhead and the envelopes by black. Scale bars = 1.0 μm.

Table 1
Dose-mortality response of third-instar larvae of *Rachiplusia nu* infected orally with RanuNPV-VPN54.

n[a]	Slope	LC_{50} (OB/ml)	Fiducial limits (95%)	X^2
270	1.018 ± 0.214	6,887.1	3,367.2–51,231.0	4.3271

[a] Number of insects tested.

strategy to overcome the UV impacts on DNA: several species genomes harbor CPD-Photolyase (CPD-Phr) homologs that repair CPD dimer at the damaged DNA (Gilbert et al., 2016). Large double-stranded DNA viruses exhibit genomic plasticity and may evolve by horizontal gene transfer (HGT). In this specific case of CPD-Phr, baculoviruses took advantage of an existing insect cell pathway and, through an unknown mechanism, incorporated it into their genome (Harrison et al., 2017).

The semilooper *Rachiplusia nu* (Guenée, 1852) (Lepidoptera: Noctuidae, Plusiinae) is a polyphagous leaf-feeder species widely distributed in South America (Young and Yearian, 1983). The main strategy for controlling the insect pest is based on chemical pesticides that may cause selection of resistant insects and pollute the environment. Therefore, biological control agents arise as an efficient approach to control insect pests in a green and safe fashion (Sun and Peng, 2007; Szewczyk et al., 2006), such as baculoviruses. Baculoviruses are specific to a narrow range of insects varying from one to dozens of hosts (Clem and Passarelli, 2013). This host specificity makes them widely used in the biological control of insect pests of forest and agricultural. For instance, in 1982 and 1983 a baculovirus-based biological control program was established using the Anticarsia gemmatalis multiple nucleopolyhedrovirus isolate 2D (AgMNPV-2D) to control a prevalent soybean (*Glycine max*) pest at that time, the velvetbean caterpillar *Anticarsia gemmatalis* (Moscardi, 1999; Sosa-Gómez, 2017). In this work, we characterized at biological and ultrastructural level a putative baculovirus found in larvae extracts of species *R. nu* with symptoms of baculovirus infection, the isolate VPN54. Moreover, we sequenced and described its complete genome. We found that Rachiplusia nu nucleopolyhedrovirus VPN54 (RanuNPV-VPN54) could be a representative of a novel species into genus *Alphabaculovirus* that nested close to the ancestor of three other previously characterized plusiinae-infecting virus species, including *Trichplusia ni single nucleopolyhedrvoirus*, *Chrysodeixis includens nucleopolyhedrovirus*, and *Chrysodeixis chalcites nucleopolyhedrovirus*. The related viruses presented strict genome architecture collinearity with no inversions. Moreover, we found several gene losses and acquisition along the genome, such as a homolog of the *late expression factor 12*, four *baculovirus repeat ORFs* (*bro-a, b, c,* and *d*), and a *bona fide* functional *CPD-photolyase class II* gene. We analyzed the evolution and structure of the CPD-Phr homolog in this work.

2. Materials and methods

2.1. Insects, virus purification, bioassay, and electron microscopy

RanuNPV was obtained from *R. nu* cadavers with characteristics of baculovirus infection death found in soybean crops in 1989. The place of collection was the city of Oliveiros (Santa Fe, Argentina). Cadavers were sent to EMBRAPA (Brazilian Agricultural Research Corporation) and kept frozen (catalog VPN54). The OBs in the extracts were used to infect field-collected caterpillars of species *R. nu* to confirm virus etiology and propagate the OBs. The insects were reared on soybean leaves and kept in an acclimatized room, at 26 ± 1 °C, 65 ± 10% RH, and a 12:12 (day:night) photoperiod. The cadavers were homogenized with the same volume of ddH2O (w/v), filtered on cotton gauze, and centrifuged at 5,000 × *g* for 10 min. The supernatant was discarded, the pellet suspended at the same volume of 0.5% SDS, and centrifuged at 5,000 × *g* for 10 min; the process was performed three more times. The pellet was suspended in 0.5 M NaCl, centrifuged at 5,000 × *g* for 10 min and suspended in 2 ml ddH2O. OBs were loaded onto a sucrose gradient (40–65%), centrifuged at 130,000 × *g* for 3 h. OBs were collected as a band and diluted five times with ddH2O. The suspension were collected by centrifugation at 7,000 × *g* for 10 min, diluted in ddH_2O (10^6 OBs/ml ddH_2O), and store at 4 °C (O'Reilly et al., 1992). For dose mortality response, eggs of *R. nu* caterpillars were obtained from soybean fields in Londrina (Paraná, Brazil) and hatched in laboratory. Third-instar caterpillars were fed on artificial diet (Greene et al., 1976). For bioassay, the diet was contaminated with six doses of RanuNPV-VPN54 OBs (3.12×10^2, 6.25×10^2, 1.25×10^3, 2.5×10^3, 5.0×10^3, and 1×10^4; an untreated group was established as control) and given in triplicate to 28–33 subjects of third instar larvae of *R. nu*. The insects were allowed to eat the contaminated food for 24 h. The infected insects were transferred to fresh diet and kept until death. Mortality was recorded by the number of dead insects for 12 days. The results were analyzed by Probit in PoloPlus version 1.0. Moreover, one hundred μl of the OB-containing suspension (10^5 OBs/ml of ddH2O) were used for Scanning electron microscopy and Transmission electron microscopy according to previously published protocols (Ardisson-Araújo et al., 2014).

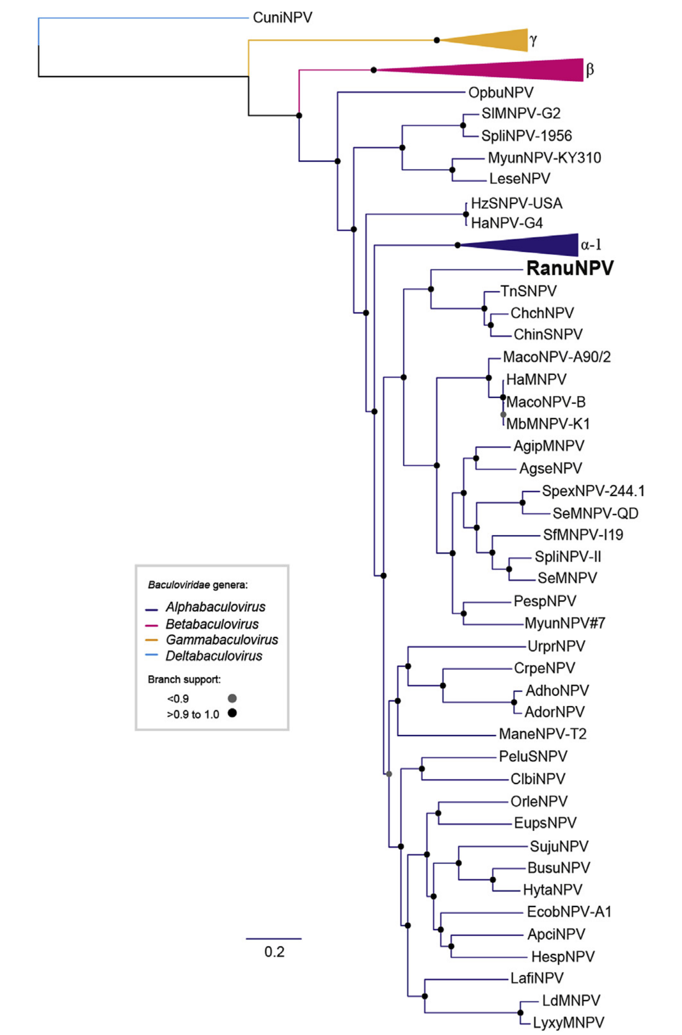

Fig. 2. RanuNPV is an alphabaculovirus. Maximum likelihood inference based on the concatenated nucleotide sequences of the 38 core genes from several selected baculovirus genomes (Table S1) using the FastTree method. The branch support was determined by a SH-like method (black and grey closed circles). Some branches were collapsed for clarity: alphabaculovirus group I, betabaculovirus (pink), gammabaculovirus (orange), and deltabaculovirus (CuniNPV, light blue). CuniNPV was used to root the tree. The genus *Alphabaculovirus* contained RanuNPV (boldface) that clustered together with TnNPV, ChchNPV, and ChinSNPV.

2.2. Viral genomic DNA extraction and amplification

For DNA purification, one hundred μl of the OB-containing suspension (10^6 OBs/ml ddH_2O) were heated for 20 min at 95 °C, placed on ice for 5 min, and treated with RQase RNase-Free DNase (Promega, Madison, WI, United States). The suspension was washed three times with SDS 0.5% and once with NaCl 0.5 M by centrifugation (7000 × *g* for 10 min), using equal volumes for each suspension. The resulting pellet was diluted in ddH2O and dissolved in alkaline solution for further DNA extraction (O'Reilly et al., 1992). The DNA pellet was dissolved in 10 μl of sterile ddH2O at 50 °C for 1 h and directly subjected to a rolling circle amplification (RCA) reaction using the phi29 DNA polymerase and a randon 3′-thiophosphate protected hexamer primer according to the manufacturer's protocols (New England Biolabs, Ipswich, MA, United States). The reaction was subjected to electrophoresis on a 0.8% agarose gel (w/v) (Sambrook and Russel, 2001), visualized, and photographed in AlphaImager® Mini (Alpha Innotech, San Leandro, CA, United States) (data not shown).

2.3. Sequencing and assembly of the viral genome and genome annotation

The sequencing of the viral DNA was performed with the 454 Genome Sequencer (GS) Titanium at the Macrogen (Seoul, South Korea). The reads were trimmed to remove regions of low quality sequencing. The *de novo* assembly method was used with no reference genome. Only one single contig was obtained by *de novo* assembly using an algorithm implemented in the Geneious 9.0 (Kearse et al., 2012). The open reading frames (ORFs) that started with a methionine codon (ATG) and encoded polypeptides of at least 50 amino acids were identified with Geneious 9.0 and annotated using BLAST-X (Altschul et al., 1997). The genomic DNA sequence was submitted to the GenBank with the accession number MK419956.

2.4. Analyses of gene content and genome comparison

The RanuNPV ORFs were compared with other baculoviruses by the BLASTX. We collected the identity of each gene with closely related baculoviruses, including Trichoplusia ni single nucleopolyhedrovirus (TnSNPV), Chrysodeixis chalcites nucleopolyhedrovirus (ChchNPV), and Chrysodeixis includens nucleopolyhedrovirus (ChinNPV) and also to Autographa californica multiple nucleopolyhedrovirus (AcMNPV), a virus in the type species baculovirus *Autographa californica nucleopolyhedrovirus*. The unique ORFs were submitted to the HMMER and the HHpred to search for conserved domains (Finn et al., 2011; Alva et al., 2016). We used the progressive Mauve algorithm implemented in the software Geneious R10 for genomic comparison and analysis. We reannotated the genomes of TnSNPV, ChchNPV, and ChinSNPV according to the same criteria used for the RanuNPV and constructed a Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) to represent the number of ORFs shared among RanuNPV and the closest relatives.

2.5. Phylogeny of baculovirus and species demarcation criterion

Phylogenetic analysis was performed by the MAFFT method (Katoh et al., 2002) with the alignment of the baculovirus core genes from 97 publicly available baculovirus genomes (Table S1). A maximum likelihood tree was inferred using the Fast-tree method (Stamatakis et al., 2008) and a Shimodaira-Hasegawa-like test for branch support (Anisimova et al., 2011). To verify whether this virus corresponds to a new species, the nucleotide distances were estimated with the Kimura-2 parameter replacement model from partial sequences obtained from three conserved baculovirus genes, including *lef-8*, *lef-9,* and *polyhedrin* (Jehle et al., 2006a).

2.6. Analysis of congruence and virus co-evolution at species level

The virus phylogeny was inferred using the PhyML method (Guindon et al., 2010) based on the concatenated alignment of the 38 baculovirus core genes from nineteen species with the substitution model GTR + G (1.13) + I (0.20). For the host phylogeny, the inference was performed based on the mitochondrial *cytochrome oxidase I* (*coi*) gene with several noctuids from where the virus was isolated, using the PhyML method (Guindon et al., 2010) under the substitution model GTR + G (0.4) + I (0.4). A bombycoid host (*Bombyx mori*) and its virus isolate (Bombyx mori nucleopolyhedrovirus, BmNPV) was used as external group. The trees were presented as cladograms and the topologies were compared. For the branch statistical support, we performed Bootstrap analysis with 100 replicates.

2.7. In silico characterization of the RanuNPV phr reveals how this gene envolved in alphabaculovirus

A homolog of the insect photolyase protein (Phr) was identified in the RanuNPV genome (RanuNPV-ORF-68). The protein was

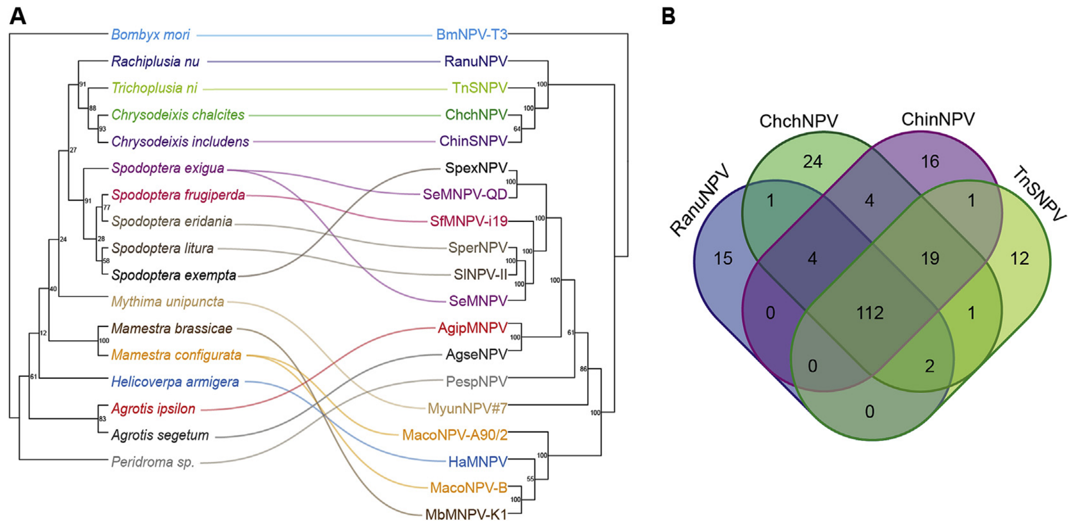

Fig. 3. Topology congruence plusiinaean hosts/alphabaculoviruses and RanuNPV gene content analyses. (A) Baculoviral tree topology of RanuNPV and other alphabaculoviruses against their respective hosts. We used the *cytochrome oxidase I* (*coi*) of mtDNA to infer lepidopteran phylogeny, especially for members of Noctuidae. Surprisingly, RanuNPV and its closest relatives, *i.e.* TnNPV, ChinSNPV, and ChchNpV presented congruence with host tree topology. (B) Venn diagram showing the number of genes shared among RanuNPV and its plusiinaean-infecting viruses.

characterized *in silico* in order to understand the conservation of the structure and putative functionality. Phylogenetic analyses based on the RanuNPV *phr* was performed using sequences retrieved from the BLASTX, with e-value less than 10^{-5}. The sequences were aligned by the MAFFT method (Katoh et al., 2002) and the alignment used for phylogenetic inference with the PhyML method (Guindon et al., 2010) under the substitution models LG + G (0.68). The model was predicted by the MEGA7 software (Kumar et al., 2016). To understand the evolution and acquisition of the *photolyase* gene, the genome context was evaluated in relation to the genome from closely related species. In addition, the conservation of secondary and tertiary structure were evaluated based on the homology model. The templates for three dimensional (3D) structure prediction of Phr were searched in Expasy SWISS-MODEL Server 59 (Schwede et al., 2003) using the predicted amino acid sequence as reference. The photolyase structure model was obtained from the crystallized functional enzyme model 5o8d. The model was validated by SAVES v. 5.0 (http://servicesn.mbi.ucla.edu/SAVES/) to obtain the Ramachandran plot. As a control, the same method was applied for ChchNPV phr1 and phr2.

3. Results and discussion

3.1. Ultrastructure of RanuNPV occlusion bodies (OBs) and bioassay

We evaluated the RanuNPV purified OBs by transmission and scanning electron microscopy (TEM and SEM). By SEM, the OBs were found to be pyramidal (Fig. 1A). We found that the mean size of the pyramidal OBs was 1.4 ± 0.2 μm. The biggest size was 2.1 μm in length, whereas the smallest was 0.8 μm. We calculated that based on three representative fields by considering the pyramid as a planar figure, and measuring the highest length of 150 polyhedra. Immature OBs revealed rod-shaped indentations on the surfaces of OBs, which likely corresponded to ODV that were lost during isolation (inset's grey arrowhead, Fig. 1A). RanuNPV OB sections revealed several embedded ODVs with singly-enveloped nucleocapsids (Fig. 1B), in agreement to other plusiinaean-infecting alphabaculovirus. We analyzed several works where the authors had described OB shapes of alphabaculoviruses at ultrastructural levels (list of references and virus isolates in Table S3) and found no virus with that morphology found for RanuNPV OBs. The usual OB morphology of alphabaculovirus is polyhedral. The major protein responsible for OB formation is polyhedrin and RanuNPV polyhedrin presents one punctual mutation (A197N) in comparison to other closely related polyhedral OB-forming viruses (data not shown), *i.e.* ChchNPV and ChinNPV (Xu et al., 2010; Alexandre et al., 2010; Arneodo et al., 2018). An OB produced by the recombinant AcMNPV containing the SeMNPV polyhedrin had an altered morphology, being pyramidal with less virions occluded in comparison with the parental AcMNPV (Hu et al., 1999). Maybe, not only the polyhedrin sequence but also the cell machenary or other viral factor may alter OB shape and nucleocapside envelopement in OB-forming viruses (Silva et al., 2019).

To confirm the infection etiology found in *R. nu* subjects, we carried out a dose-mortality response in *R. nu* insects collected from soybean fields. We confirmed that the virus was lethal to third-instar subjects of *R. nu* with a LD50 of 6.9 × 10^3 OBs/ml. The infected caterpillars presented yellowish and liquefied tegument with melanotic pigments, as typical for other baculovirus infections (Rohrmann, 2013). The lethal dose obtained here is similar to that observed for several isolates of ChinNPV infecting third-instar *C. includens* larvae, which ranged from 2.5 × 10^3 to 9.3 × 10^3 OBs/ml (Alexandre et al., 2010) (see Table 1).

3.2. Properties of the RanuNPV genome sequence

We sequenced the genome of RanuNPV by the 454 Genome Sequencer (GS) FLX ™ Titanium method (Macrogen Inc., Korea). No virus isolated from *R. nu* had been sequenced completely and described so far. We assembled the reads from sequencing of RanuNPV DNA into a circular genome contig of 128,587 bp long. 8,305 reads with a mean size of 776.1 ± 210.7 nt were obtained and mapped, allowing for a coverage of 50.2 ± 12.4 X. The size of the genome and the G + C nucleotide distribution (37.9%) were within the range of genome sizes and nucleotide distributions that have been reported for other alphabaculoviruses (Table S1). Moreover, a total of 134 ORFs were annotated (Table S2).

3.3. Relationship of RanuNPV to other baculoviruses

RanuNPV belongs to genus *Alphabaculovirus* as a basal species of the clade formed by the plusiinaean-infecting alphabaculoviruses of species *Trichoplusia ni single nucleopolyhedrovirus*, *Chrysodeixis chalcites nucleopolyhedrovirus*, and *Chrysodeixis includens single nucleopolyhedrovirus* (Fig. 2). The phylogeny was based on the concatenated nucleotide sequence alignment of the 38 baculovirus core genes from several

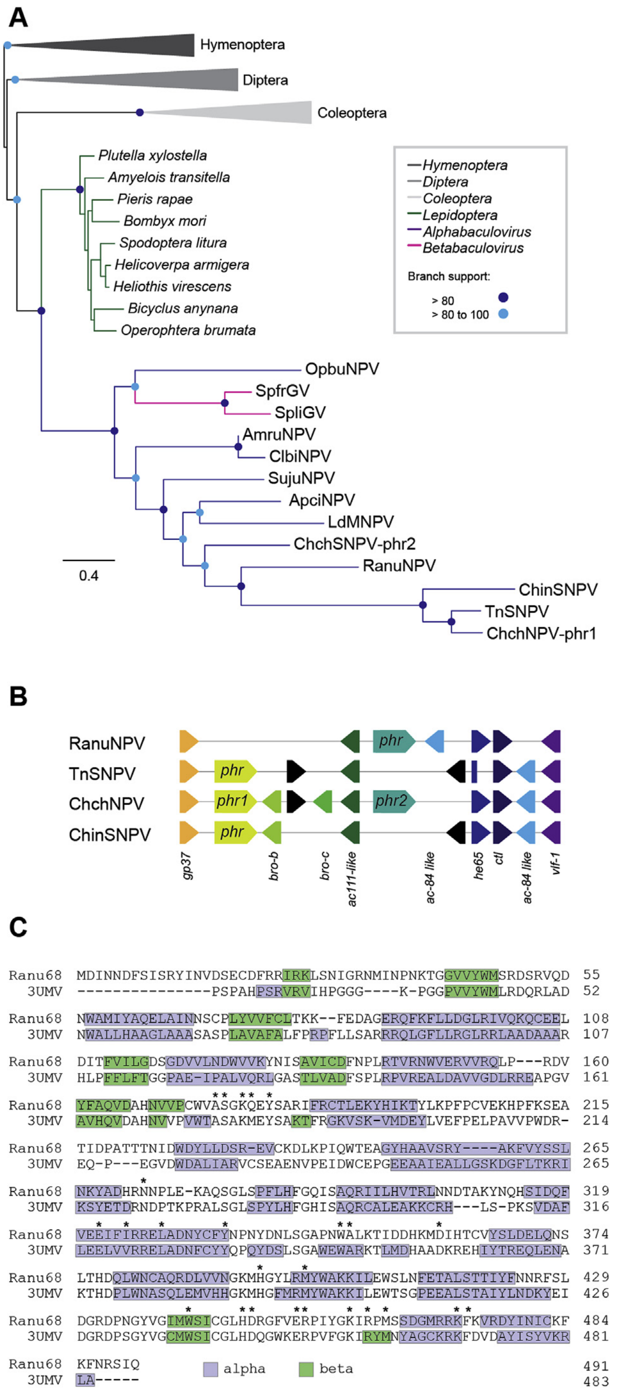

Fig. 4. Photolyase evolution in RanuNPV-related alphabaculovirus and alignment with a homolog Phr crystal. (A) Phylogeny of Phr homologs based on the predicted amino acid sequences by the PhyML method under LG + G model. We hypothesized that a single event of horizontal transfer of the *phr* gene from lepidopterans to alphabaculovirus (blue branches) and then to betabaculovirus (pink braches). (B) The genomic contexts of the photolyase in the RanuNPV and related species, including TnSNPV, ChchNPV, and ChinNPV. The arrowhead depicts gene direction in the genome. Arrowheads with similar colors depict orthology, and the black ones independent acquisitions. (C) Individual alignments of the RanuNPV *phr* gene with the crystal structure of the 3UMV protein identifying the secondary structures. Conserved β-sheets and α-helices are boxed in green and purple, respectively.

baculovirus genomes publicly available. The pairwise nucleotide identity of the RanuNPV core genes with all completely sequenced alphabaculovirus genome is shown in Table S1. The mean identity of RanuNPV and the closely related species were about 59.4%, which depicts a very divergent lineage inside the genus. Indeed, the branch length separating RanuNPV from the other virus species is in a range similar to that observed for other recognized baculovirus species, pointing RanuNPV as a representative of a putative novel virus species.

3.4. RanuNPV may represent a novel species inside Alphabaculovirus

There are only 45 species recognized by the International Committee on Taxonomy of Virus (ICTV) within genus *Alphabaculovirus*. From those, only four had been described in Brazil, *Anticarsia gemmatalis multiple nucleopolyhedrovirus*, *Chysodeixes includes nucleopolyhedrovirus*, *Lonomia obliqua nucleopolyhedrovirus*, and *Perigonia lusca nucleopolyhedrovirus*. ChinNPV is the unique isolate described in Brazil from *Chysodeixes includens*, one of the most important soybean pests (Craveiro et al., 2015). A specific demarcation criterion was established for baculoviruses (Jehle et al., 2006a), which is based on the comparative analyses of the conserved nucleotide region from three genes, *polyhedrin*, *lef-8*, and *lef-9* by using the Kimura-2-parameters model (K2P).

A virus is considered to be a member of a novel species when the number of substitutions is higher than 0.05 per site. We suggest that RanuNPV is a member of a new species, which we are tentatively naming as *Rachiplusia nu nucleopolyhedrovirus* (Table S2). In two previous works, a *R. nu*-infecting virus was isolated and described (Rodríguez et al., 2012; Young and Yearian, 1983). In Young and Yearian (1983), a nucleopolyhedrovirus (NPV) was isolated from larvae collected from soybean in Argentina in 1980. The virus was lethal to larvae of *R. ou* and presented OBs similar to that observed for RanuNPV in the present work, regarding shape and number of nucleocapsids per envelope. In Rodríguez et al., 2012, the virus was found to have OBs with multiply-enveloped ODVs and was called Rachiplusia nu multiple nucleopolyhedrovirus (RanuMNPV). The partial sequences of the genes *p74*, *polyhedrin*, *v-cathepsin*, *v-chitinase*, *lef-8*, and *lef-9* genes revealed that this virus is a closely related variant of AcMNPV found in a different host range. *Autographa californica*, *R. ou*, and *R. nu* belongs to subfamily Plusiinae.

3.5. Coevolution analysis at species level

We evaluated whether there would be congruence between the baculoviral tree topology of RanuNPV, TnSNPV, ChchNPV, and ChinSNPV against their respective hosts, including *R. nu*, *T. ni*, *C. chalcites*, and *C. includens*. RanuNPV and its related viruses were isolated from hosts that belong to family Noctuidae and subfamily Plusiinae. Surprisingly, we found strict congruence between the trees, as observed in Fig. 3A. We constructed the trees based on the mtDNA *cytochrome oxidase subunit i* (*COI*) for hosts, which is widely used to infer phylogenies of lepidopterans (Taft and Cognato, 2017; Kirichenko et al., 2018) and concatenated core genes for baculoviruses. We selected the non-plusiinaean bombycoid host *B. mori* and the alphabaculovirus isolated from this species as external groups to the trees. Baculoviruses evolved with their host at insect order level generating alpha, beta, gamma, and deltabaculovirus (Herniou et al., 2004). Coevolution, a reciprocal evolution in interacting species driven by natural selection, is a powerful determinant for the biology and genetics of infection, pointing to the historical association between pathogens and their hosts (Woolhouse et al., 2002). The selection pressure exerted on baculoviruses makes them subject to coevolution (Herniou et al., 2004). Nevertheless, different phylogeny-trait correlation tests show unequivocal significant associations between the taxonomy of insect hosts at superfamily, family, and subfamily and the BV species phylogeny (Thèzè et al., 2018). In contrast, virus and host coevolution at species level is not clear.

3.6. RanuNPV ORF content

The RanuNPV genome contains all the 38 core genes identified to date in every baculovirus genome, including the recently described gene *ac110* (*pif-7*, the RanuNPV-ORF-93) (Javed et al., 2017). The genome also presents the 26 ORFs identified by Garavaglia et al. (2012) as present in genomes of alpha- and betabaculoviruses (Table S2). We also found four copies of the *baculovirus repeated ORF* (*bro*), and homologs of auxiliary genes such as the *inhibitor of apoptosis 2* (*iap-2*, the RanuNPV-ORF-61) and *iap-3* (RanuNPV-ORF-39), *chitinase* (RanuNPV-ORF-65), *cathepsin* (RanuNPV-ORF-64), and *he65* (RanuNPV-ORF-70). Among the hypothetical ORFs annotated, nine were found to be unique (RanuNPV-ORF-5, RanuNPV-ORF-6, RanuNPV-ORF-9, RanuNPV-ORF-20, RanuNPV-ORF-33, RanuNPV-ORF-40, RanuNPV-ORF-112, RanuNPV-ORF-115, and RanuNPV-ORF-125), *i.e.* not found in any other baculovirus genomes so far (Table S3). Only one hypothetical gene (RanuNPV-ORF-5) did not show any hit with any other gene from Genbank and no domains were found using either the HHpred or Smart. We also performed an ORF content comparison among RanuNPV and its closest relatives (*i.e.* TnNPV, ChchNPV, and ChinSNPV) and we plotted the result in a Venn Diagram (Fig. 3B). A total of 211 different genes were found considering the four species genomes. For this comparison, we reannotated the four genomes under the same criterion and found 32 new genes not annotated before, including three in the TnNPV, 16 in the ChchNPV, and 13 in the ChinSNPV. Only 112 genes were shared among the four species. Fifteen ORFs were found only in the RanuNPV genome: nine unique in baculovirus, three *baculovirus repeat ORFs* (*bro-a*, *bro-c*, and *bro-d*) with no ortholog in the related species, one *late expression factor 12* (*lef-12*, the RanuNPV-ORF-34), and two other hypothetical proteins (RanuNPV-ORF-63, RanuNPV-ORF-128). The RanuNPV-ORF-63 did not present hit using BLASTX; however, we did found hit with the alphabaculovirus Agrotis segetum nucleopolyedrovirus (nt identity of 25.7%, e-value 2.2E-11) by using the HMMER algorithm. The RanuNPV-ORF-128 was found to be related to Spodoptera frugiperda multiple nucleopolyhedrovirus (nt identity of 38%, e-value 3.00E-12). Two ORFs were shared solely by RanuNPV and both TnSNPV and ChchNPV (RanuNPV-ORF-31 and RanuNPV-ORF-35 [ac43-like]) and one single ORF was shared between RanuNPV and ChchNPV, a *CPD-phr* homolog (RanuNPV-ORF-68).

3.7. The evolution of photolyase genes in plusiinaean-infecting alphabaculovirus

The RanuNPV genome presents a homolog of a *CPD-phr* gene (RanuNPV-ORF68). As a virus related to the most recent common ancestor of ChchNPV, ChinNPV, and TnSNPV, the ortholog of a *CPD-phr* found in the genome of RanuNPV, allowed for a wide comprehension of *CPD-phr* in *Baculoviridae*. To investigate the evolutionary history of this gene, we performed a BLASTX search in the NCBI non-redundant database to find homologs. The retrieved sequences were aligned by the MAFFT and used to infer the phylogeny by the PhyML. We found that the *CPD-phr* gene is present in several baculoviruses, including members of *Alphabaculovirus* and *Betabaculovirus* (Fig. 4A). The baculovirus clade nested as a monophyletic group sharing a unique ancestor with lepidopterans (Biernat et al., 2012). This finding was previously observed (Harrison el al., 2017; van Oers et al., 2004; Willis et al., 2005). Some alphabaculoviruses that infect also members of subfamily Plusiinae do not contain *phr* genes, including AcMNPV, Thysanoplusia orichalcea nucleopolyhedrovirus (ThorNPV), and Rachiplusia ou multiple nucleopolyhedrovirus (RoMNPV) (Van Oers and Vlak, 2007). The gene topology in Fig. 4A depicts a single event of HGT from insects to baculovirus, specifically to alphabaculoviruses. This ability to acquire genes from insects is a remarkable feature in baculoviruses, which include genes related to nucleotide metabolism (Ardisson-Araújo et al., 2016), nucleases (Ardisson-Araújo et al., 2018), innate immune response (Ardisson-Araújo et al., 2015), and apoptosis control (Harrison et al., 2016). In the specific case of *phr*, the gene was acquired by alphabaculovirus and transferred to betabaculovirus. Viruses of only two *Spodoptera*-infecting betabaculovirus species harbor a *phr* homolog. ChchNPV is the unique virus harboring two copies of the *phr*, called by *phr1* and *phr2*. van Oers et al. (2005) described the gene as a product of duplication rather than independent horizontal gene transfer. In a wide perspective, the enzymes are divided into two classes based on the differences in the amino acid sequences of the organisms that harbor the gene (Kanai et al., 1997). Interestingly, the RanuNPV added a small piece to this puzzle that clarifies the evolution of *phr* in baculovirus. We took advantage of the genome *loci* for the related species to understand the gene evolution (Fig. 4B). RanuNPV is a virus closely related to the ancestor of the plusiinae-infecting alphabaculoviruses and presents a *phr2* homolog but not a *phr1*. Two main hypothetical pictures might be draw from this evolutionary scenario. In the first, *phr2* is present in the most recent common ancestor (m.r.c.a.) of RanuNPV-related species. In the primary speciation event, the RanuNPV lineage maintained the *phr2* and the lineage that originate the m.r.c.a. of TnSNPV, ChchNPV, and ChinNPV underwent a duplication event of *phr2* that led to *phr1* followed by two independent losses, one by the TnSNPV lineage and the other one by the ChinNPV lineage. In the second scenario, the most recent common ancestor (m.r.c.a) of TnSNPV, ChchNPV, and ChinNPV acquired the *phr1*. After, the ChchNPV lineage underwent an independent duplication that generated *phr2* and transferred the gene to the RanuNPV lineage. Besides being less parsimonious than the second picture, the first hypothesis is reinforced by two important facts. First, the *phr1* homologs seemed to have lost the CPD-repairing activity whereas the *phr2* maintained this activity (Van Oers et al., 2008). Second, the hypothetical independent loss of *phr2* undergone by TnSNPV lineage is reinforced by the presence of a reminiscent non-coding fragment of *he65* (Fig. 4B).

3.8. Ranu68 is a bona fide functional CPD-Photolyase

Since most of the *phrs* described so far have the same basic architecture, we took advantage of a previously established crystal structure to solve by homology modeling the predicted RanuNPV *phr*. Therefore, in order to determine whether the predicted amino acid sequence of RanuNPV-ORF68 potentially encodes a functional Phr, we performed an alignment against homologs with solved crystal structures and built a 3D model (Fig. S1A). The identity between the viral sequences and its homolog was 44% (PDB ID: 5o8d). The model was checked by the SAVES v5.0 and we found that 91.3% of the amino acid residues were in most favored regions and only three residues were in disallowed regions, which represents 0.7% of the residues (Fig. S1A). Essentially, the RanuNPV Phr model represents a globular protein that is composed by two defined domains, an N-terminal α/β-domain (residues 1–200) and a C-terminal α-helical domain (residues 227–486) (Fig. S1A). A long inter-domain loop (residues 201–226) links the two major domains. Most of the secondary structures observed for the Phr model is predicted for the Ranu068, as also observed for the both fuctional ChchNPV CPD-Phr2 (Fig. S1B) and Phr1 (Fig. S1C). Importantly, for ChchNPV CPD-Phr2 92.1% of the amino acid residues were in most favored regions and only four residues were in disallowed regions, which represents 0.9% of the residues. In contrast, phr1 presented 86.8% of the amino acid residues were in most favored regions and four residues were in disallowed regions, which represents 0.9% of the residues. In a previous work characterizing the activity of baculoviral Phrs, Van Oers et al. (2008) identified several directly conserved amino acids among the active baculoviral enzymes and other characterized enzymes. Interestingly, *phr1* of ChchNPV does not encode a functional CPD-disrupting enzyme, besides sharing a similar ancestor to *phr2*, the CPD-disrupting functional enzyme. Twenty-six amino acid residues were conserved among ChchNPV Phr2 and other functionally tested Phr homologs and not conserved in the non-functional counterpart Phr1 (Fig. 4C). Of those, Ranu068 conserved 25 residues with only one mutation at position 325. Conversely, all the analyzed homologs, including Ranu068 presented a hydrophobic amino acid residue at that changed position (valine or isoleucine). Therefore, based on the tertiary, secondary and, primary conservation of the Phr, we believe that Ranu068 is likely a *bona fide* active CPD-Phr. Active CPD-photolyases can revert CPD lesion by means of a catalytic cofactor, the FAD that assimilates light energy and reverts the dimer (Deisenhofer, 2000; Eker et al., 2009). Photoreactivation is a pathway used to revert the pyrimidine dimers to their monomeric form (Sancar, 2004). An enzyme activity remains to be carried out in order to elucidate the functional activity of Ranu068.

4. Conclusion

In this work, we have characterized at the ultrastructural and genomic level a baculovirus isolated from the plusiinaean soybean pest *R. nu*. The virus presented the peculiar feature of having a pyramidal-shaped OB with several singly-enveloped ODVs within and a lethal dose of 6.9 OBs/ml to the third-instar field-derived caterpillars of species *R. nu*. The virus could represent a novel species in genus *Alphabaculovirus* and be correlated with members that infect other insects in subfamily Plusiinae. It has 134 ORFs and nine were shown to be unique in baculovirus genomes. In addition, we identified a hypothetical *phr* gene with a predicted role for the repair of CPD lesions in DNA. In particular, it was found that the *phr* gene underwent a single event of horizontal transfer from lepidopteran to alphabaculovirus and then to betabaculovirus. The RanuNPV genome provides a key baculovirus genome to clarify the evolution of the *phr* in alphabaculovirus, since it is related to the m.r.c.an of the plusiinaean-isolated viruses. Overall, alphabaculovirus genome sequencing is of importance to the field as few genomes are publicly accessible. RanuNPV is a widely distributed polyphagous pest in South America that causes great damage to several crops of economic importance. Certainly, both discovery and description of novel baculoviruses may lead to the development of greener and safer pesticides in order to counteract and effectively control crop damage-causing insect populations. Moreover, that allows us to understand the evolution of baculovirus in a wider perspective.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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