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Daniel Mendes Pereira Ardisson-Araújo¹, Fernando Lucas de Melo¹, Miguel de Souza Andrade¹, William Sihler², Sonia Nair Bão¹, Bergmann Morais Ribeiro¹* and Marlinda Lobo de Souza²

Abstract

**Background:** Cassava (*Manihot esculenta*) is the basic source for dietary energy of 500 million people in the world. In Brazil, *Erinnyis ello ello* (Lepidoptera: Sphingidae) is a major pest of cassava crops and a bottleneck for its production. In the 1980s, a naturally occurring baculovirus was isolated from *E. ello* larva and successfully applied as a bio-pesticide in the field. Here, we described the structure, the complete genome sequence, and the phylogenetic relationships of the first sphingid-infecting betabaculovirus.

**Results:** The baculovirus isolated from the cassava hornworm cadavers is a betabaculovirus designated *Erinnyis ello* granulovirus (ErelGV). The 102,759 bp long genome has a G + C content of 38.7%. We found 130 putative ORFs coding for polypeptides of at least 50 amino acid residues. Only eight genes were found to be unique. ErelGV is closely related to ChocGV and PirahGV isolates. We did not find typical homologous regions and cathepsin and chitinase homologous genes are lacked. The presence of *he65* and *p43* homologous genes suggests horizontal gene transfer from Alphabaculovirus. Moreover, we found a nucleotide metabolism-related gene and two genes that could be acquired probably from Densovirus.

**Conclusions:** The ErelGV represents a new virus species from the genus Betabaculovirus and is the closest relative of ChocGV. It contains a dUTPase-like, a *he65*-like, *p43*-like genes, which are also found in several other alpha- and betabaculovirus genomes, and two Densovirus-related genes. Importantly, recombination events between insect viruses from unrelated families and genera might drive baculovirus genomic evolution.

**Keywords:** Biological control, Cassava hornworm, Baculovirus, Sphingidae, Horizontal gene transfer, Betabaculovirus evolution

Background

Cassava (*Manihot esculenta*) is the basic source for dietary energy of 500 million people in tropical and subtropical areas of Africa, Asia, and Latin America [1]. In Brazil, the hornworm *Erinnyis ello ello* (Lepidoptera: Sphingidae) is one of the most important pests [2] occurring throughout the year and greatly impacting cassava production [3,4]. This pest has been observed in 35 plant species, especially in the Euphorbiaceae family [5,6]. In large infestations, the cassava pest may reduce by 50% the roots yield. In the 1980s, a naturally occurring baculovirus was isolated from this pest and applied as a bio-pesticide in Brazil [5]. The biological control program has proven to be safe and economical [5,6]. However, genomic and structural information about this virus is lacking.

The *Baculoviridae* is a family of insect viruses with circular double-stranded genomic DNA [7-9] that have been successfully applied for the control of agricultural and forest pests [10]. So far, Alpha and Betabaculovirus are the most studied baculovirus genera; both infect Lepidoptera
The infection is initiated when larvae feed on foliage contaminated with orally infectious occlusion bodies (OBs) that release occlusion derived-virions (ODVs) in the midgut. Early after primary midgut epithelial cell infection, budded virions (BV) are produced and cause systemic infection. Infection symptoms include cuticle discoloration, movement loss, and incapability for feeding.

Few full-length betabaculovirus genome sequences are available compared to those from Alphabaculovirus and none of them was isolated from sphingid host. In this context, identification and sequencing of virus species from different lepidopteran families will provide a wider empirical database to help understand baculovirus evolution. Here, we presented the morphological characterization, the complete genome sequence, and the phylogenetic analyses of the natural cassava hornworm pesticide, the first completely sequenced betabaculovirus isolated from a sphingid host.

**Results and discussion**

**Virus characterization and genome features**

A naturally occurring baculovirus was isolated from dead cassava hornworm (E. ello ello) caterpillars in crops from the South of Brazil in 1986. As shown in Figure 1A, the larvae is usually found hanged in cassava apical leaves, which is a characteristic symptom of the baculovirus infections. Neither cuticle melanization nor post-mortem melting phenotypes were observed among the caterpillar cadavers, an attribute which probably facilitated virus collection and use for pesticide production as previously observed in another baculovirus (Anticarsia gemmatalis multiple nucleopolyhedrovirus - AgMNPV) [10]. Ultrastructural analyses revealed a granular OB with irregular form and size (Figure 1B) containing single rod-shaped nucleocapsid (Figure 1C). Both of these structural features, i.e. granular form and nucleocapsid shape, are typical of viruses from the genus Betabaculovirus [8,18] and thus, we named it Erinnyis ello granulovirus (ErelGV) isolate Br-S86 (Brazil/South/1986). Two other cassava hornworm-isolated granuloviruses were previously reported, one isolated in Colombia [19] and another from an undisclosed geographical source [20]. Restriction endonuclease profile analyses (Figure 1D) suggest that the Brazilian and the Colombian viruses (previously published in [19]) are either variants of the same species or are distinct species infecting the same host. However, the absence of sequence data from the latter prevents establishment of any phylogenetic relationship.

We sequenced the genome of ErelGV, the first completely sequenced sphingid host-isolated betabaculovirus (Genbank accession number KJ406702). The genome is 102,759 bp long with a G + C content of 38.7% (Table 1). We found 130 putative genes coding for polypeptides of at least 50 amino acid residues. Additional file 1: Table S1 summarizes the ErelGV genes and compares each predicted protein sequence with its orthologs in other baculoviruses. Eight of these were shown to be unique (ErelOrf-11, ErelOrf-15, ErelOrf-27, ErelOrf-53, ErelOrf-59, ErelOrf-70, ErelOrf-90, ErelOrf-102), and all of them are peptides with no significant similarity to any other sequence in GenBank. All 37 Baculoviridae core genes were found and no typical homologous regions (hrs)
were detected. However, we identified five putative homologous regions (hrs)/repeat regions lacking typical alpha-baculovirus hr palindromes. This feature is also found in both Choristoneura occidentalis granulovirus (ChocGV) and Pieris rapae granulovirus (PiraGV) genomes. As observed in ChocGV [21], ErelGV lacks both gp37 and exon0, which was previously predicted for being shared among all Alpha and Betabaculovirus [22].

**Phylogenetic analysis**

In order to better understand the evolutionary history of ErelGV and the genus Betabaculovirus, we carried out a maximum likelihood phylogenetic analysis using the 37 baculovirus core gene alignment from all baculovirus genome available. ErelGV clustered with ChocGV and both viruses share the same ancestor with PiraGV isolates (Figure 2). Since the Chinese and Korean PiraGV isolates are very similar to each other (99.5%), we have included only the Chinese isolate in our analyses. Using Mauve alignment [34], we found that ChocGV and PiraGV genomes have respectively 38.5% and 34.5% of global pairwise identity when compared to ErelGV genome. Additionally, our phylogenetic analyses did not find support for Betabaculovirus division in two clades (A and B), as described previously using neighbor joining clustering method [25,28]. Phylogenetic relationships in Baculoviridae, in particular in the genus Betabaculovirus, are difficult to discern due to the limited number of sequenced genomes available (Table 1). Therefore, we further evaluated ErelGV phylogenetic relationships using granulin, lef-8, and lef-9 partial gene dataset as previously carried out [20,35] (28 partial sequences), but including new sequences publicly available (seven sequences from completely sequenced baculovirus) totaling 35 granulovirus sequences. This analysis revealed that ErelGV isolate Br-S86 is closely related to another ErelGV (also called EeGV) from the Steinhaus collection [20] and that both are closer to Andraca bipunctata granulovirus (AnbiGV) (data not shown).

**Betabaculovirus gene comparison**

We performed BLAST comparisons between ErelGV and all other full betabaculovirus genomes available in Genbank using the CGView Comparison Tool [36] and CIRCOS [37]. As shown in Figure 3, most of the ErelGV-encoded ORFs are conserved among all betabaculovirus, but protein similarity varies widely across the species. Some structural proteins, such as granulin and the per os infectivity factors (PIFs), were the most conserved genes. Conversely, F protein, the major Betabaculovirus envelope fusion protein (EFP, encoded by ErelOrf-28) and matrix metalloproteinase (MMP, a stromelysin-1-like protein, encoded by ErelOrf-39) were particularly variable despite of both being present in every betabaculovirus sequenced to date. The EFP is essential for cell-to-cell movement and systemic virus spread [7]. GP64 is the EFP found in Group I Alphabaculovirus and all orthologs are closely related to
each other (81% of protein sequence identity), whereas the F protein, found in both Alpha and Betabaculovirus [38], is very diverse (20 to 40% sequence identity). Interestingly, deletion of the gp64 or f protein genes is lethal for BV propagation in Autographa californiae multiple nucleopolyhedrovirus (AcMNPV) [39] and Helicoverpa armigera nucleopolyhedrovirus (HaNPV) [40,41], respectively. The deficiency can be rescued by efp homologs from many different viruses in the case of AcMNPV [42], but the opposite is not true; AcMNPV gp64 is not able to completely rescue an efp-deleted HaNPV. However, it is not clear why the F protein from Plutella xylostella granulovirus (PxyGV) is not able to rescue the infectivity of gp64-null AcMNPV [42] but that from AgseGV can. PxyGV causes systemic infection to the diamondback moth P. xylostella (Plutellidae) larvae [43] and AgseGV infects the cutworm A. segetum (Noctuidae) [44]. Thus, the betabaculovirus EFP variability might reflect the cell machinery adjustment at the insect family level considering that AcMNPV infects caterpillar from the same insect family of A. segetum. As ecologically highly variable gene, MMP, is a proteinase able to produce a distinct pattern of melanization in Bombyx mori larvae infected with the Xestia c-nigrum granulovirus (XecnGV) metalloproteinase-expressing Bombyx mori nucleopolyhedrovirus [45]. The enzyme is thought to enhance, replace, or act synergistically with proteins from virus or host playing an important role in the virus spread [46]. This variability is not unexpected since granulovirus genomes vary in content with respect to the presence or absence of the proteases cathepsin and enhancin genes and also the chitinase gene [12,45-47].

**Lack of cathepsin and chitinase genes**

ErelGV lacks cathepsin and chitinase genes, despite of their importance for promoting baculovirus horizontal transmission [48]. This feature can explain the integrity of caterpillar flesh and light color after death (Figure 1A). Other betabaculovirus genomes also lack both enzymes: complete deletion in ChocGV [21], Adoxophyes orana granulovirus (AdorGV) [23], Plthorimaea operculifera granulovirus (PhopGV) (unpublished), PxyGV [31] and Spodoptera litura granulovirus (SpliGV) [32]; Cryptophlebia leucotreta granulovirus (CrleGV) [26] chitinase has an interruption; and in Helicoverpa armigera granulovirus (HearGV) [29] only cathepsin is absent. Interestingly, most of these deletions seem to have occurred independently of each other within Betabaculovirus (data not shown), aside from ChocGV and ErelGV in which is strongly supported an ancestral lacking. Thus, it is reasonable to expect that AnbiGV, the closest relative to ErelGV, might also lack both cathepsin and chitinase. Taken together, these results reinforce the notion that both genes are most likely non-essential for the persistence of baculoviruses in the environment. Conversely, previous work from our research team has shown that introduction of cathepsin and chitinase from Choristoneura fumiferana defective...
nucleopolyhedrovirus into AgMNPV (which naturally lacks both genes) increases pathogenicity and occlusion body production relative to the wild type virus [49].

dUTPase-like gene

ErelOrf-5 codes for a nucleotide metabolism-related gene homologous to Orgyia pseudotsugata multiple nucleopolyhedrovirus (OpMNPV) Orf-31. The gene seems to be composed of a fusion between two distinct ORFs; the C-terminal portion is related to a baculovirus thymidylate kinase-like gene and the N-terminal portion is related to several dUTPase-like genes. The thymidylate kinase enzyme catalyzes a critical step in the biosynthesis of deoxythymidine triphosphate [50]. dUTPase catalyses...
dUTP dephosphorylation to generate dUMP [51]. High levels of dUTP can be deleterious for virus genomic DNA replication since dTTP can be substituted for dUTP during DNA synthesis [52]. A high dUTP/dTTP ratio promotes uracil incorporation into DNA. Uracils in DNA are then targeted by uracil DNA glycosylase and excised, leading to futile repair cycles and DNA breakage and or translesional DNA synthesis [53,54]. Nucleotide metabolism-related enzyme acquisition is common in baculoviruses [28] and could avoid this deleterious response by decreasing the dUTP/dTTP ratio, however how these genes alter the virus fitness is not clear [55].

Furthermore, we found in ErelGV genome a p43-like gene (ErelOrf-105) whose homologues were found only in baculovirus species from the genus Alphabaculovirus (Figure 4B) with conserved amino acid sequence and position in the genome [7]. Deletion of p43 in AcMNPV does not affect virus replication in cell culture and the reason for gene acquisition and preservation is not clear [57]. Two hypotheses can be raised for p43 introduction in ErelGV: (i) ErelGV acquired the p43-like gene from Group I Alphabaculovirus, specifically from AcMNPV-related viruses; or (ii) ErelGV acquired from Group II Alphabaculovirus, specifically from a baculovirus (e.g. Clanis bilineata nucleopolyhedrovirus - CbiNPV [58]) during co-infection of a sphingid host.

The he65-like and p43-like genes
The ErelGV genome contains homologues of the he65 and p43 genes. Homologues of he65 are harbored by several alphabaculoviruses, four betabaculoviruses (Agrotis segetum granulovirus (AgseGV), HearGV, Pseudaletia unipuncta granulovirus (PsunGV), and Xestia c-nigrum granulovirus (XecnGV)), and two betaentomopoxviruses (Anmacta moorei entomopoxvirus - AMV and Mythisma separate entomopoxvirus - MSV). This gene is a member of a distinct RNA ligase family related to the T4 RNA ligase gp63-like gene and is present in all the domains of life (Bacteria, Archaea, and Eukarya) [7,56]. The alignment of baculovirus and entomopoxvirus he65-like genes revealed large, independent, and recurrent deletions in the C-terminal region (data not shown), which contain five nucleotidyl transferase motifs [56]. The amino-terminal region was highly conserved although no previously characterized motifs were present. We performed a phylogenetic reconstruction based on this conserved domain. The he65 reconstruction revealed distinct horizontal gene transfer (HGT) events from Alphabaculovirus to Betabaculovirus and Betaentomopoxivirus (Figure 4A). Betabaculovirus likely endured two independent acquisitions from Group II Alphabaculovirus in distinct genomic regions: (i) a synapomorphic introduction for HearGV, PsunGV, and XecnGV (Figure 4A, orange rectangle); and (ii) an additional gain for AgseGV (Figure 4A, dark orange rectangle). Importantly, support for AgseGV branch is low. However, the genomic context of the gene is conserved among HearGV, PsunGV, and XecnGV but not in AgseGV (data not shown), reinforcing our hypothesis that two independent introductions occurred. Likewise, Betaentomopoxivirus homologues were probably acquired from Group II Alphabaculovirus (Figure 4, yellow rectangle). Remarkably, ErelGV is the first Betabaculovirus with a he65-like gene (ErelOrf-36 - Figure 4A, green rectangle) acquired from Group I Alphabaculovirus. It is not clear whether C-terminal deleted he65 remains functional in baculovirus. However the maintenance of the amino-terminal region indicates that this gene region is under positive selection pressure.

Acquisitions of Densovirus-related genes in Betabaculovirus
ErelOrf-57 and ErelOrf-100 are homologues to a non-structural Densovirus gene. Densovirus-related genes were previously described in two betabaculoviruses, ChocGV (ChocOrf-25) [21] and CrelGV (CrElOrf-9) [26], and one gammabaculovirus (baculovirus infective to hymenoptera), Neodiprion lecontei nucleopolyhedrovirus (NeleNPV - NeleOrf-81) [59]. The latter did not match the other two homologues (data not shown), suggesting these genes resulted from at least two HGT events between densoviruses and baculoviruses. Despite the limited number of Densovirus genomes available, we performed a phylogenetic analysis to help understand the origins of Betabaculovirus homologues. We found that the genes were dispersed over the phylogenetic tree, suggestive of multiple HGT events. As shown in Figure 5, Betabaculovirus homologues did not form a monophyletic cluster. To further substantiate our findings, we compared the likelihood of the observed tree to that estimated assuming a Betabaculovirus monophyletic clade (single-HGT event). Indeed, the likelihood ratio test rejected the monophyletic hypothesis favoring the multiple-HGT scenario, which was also supported by the distinct genomic context observed for the homologous betabaculovirus genes (data not shown). Moreover, both ErelOrf-57 and ErelOrf-100 form a well-supported clade, indicating that they probably represent a gene duplication event during ErelGV evolution.

Conclusion
ErelGV is a new betabaculovirus species closely related to ChocGV and PiraGV isolates. Its genome encodes 130 ORFs, eight of which are unique. We found evidence suggesting horizontal gene transfers from Alphabaculovirus and Densovirus to Betabaculovirus. The he65-like gene was independently acquired tree times from Alphabaculovirus. We found a dUTPase-like gene homologous to OpMNPV Orf-31 and two Densovirus-related genes. The contribution of these genes to baculovirus fitness is not clear and is
being experimentally tested in our lab. Importantly, recombination events between insect viruses from unrelated families and genera might drive baculovirus genomic evolution.

Nucleotide sequence accession number

The ErelGV genome sequence was submitted to GenBank under accession number KJ406702.

Availability of supporting data

The complete ErelGV genome sequence has been submitted to GenBank (accession number KJ406702). All supporting data is included as additional files.

Figure 4 Phylogeny of he65 and p43 reveals horizontal gene transfer in ErelGV from Alphabaculovirus. (A) The maximum likelihood (ML) tree was inferred using the conserved amino-terminal region alignment of he65-like gene for 36 baculoviruses and two entomopoxviruses. Circles indicate the presence (blue) or absence (red) of the carboxy-terminal region. The postulated horizontal gene transfer (HGT) events are highlighted for Betabaculovirus (light and dark orange), Betaentomopoxvirus (yellow), and ErelGV (green). (B) ML-Phylogenetic reconstruction for p43-like gene found in ErelGV genome. The trees are midpoint rooted for purposes of clarity.

Figure 5 Densovirus-related genes in betabaculovirus and phylogenetic relationship. ML tree was inferred using the alignment of ErelOrf-57 and ErelOrf-100 from ErelGV with non-structural protein (NS) from Bombyx mori densovirus 2 and 3 (BmDENSV-2 / Genbank YP_007714627.1 and BmDENSV-3/Genbank YP_007714627), NS3 from Diatraea saccharalis densovirus (DisaDENSV/Genbank NP_046812.1), Mythimna loreyi densovirus (MyloDENSV/Genbank NP_958098.1), Helicoverpa armigera densovirus (HaDENSV/Genbank NP_958098.1), Helicoverpa armigera densovirus (HaDENSV/Genbank AFK91821), Galleria mellonella densovirus (GameDENSV/Genbank NP_899649.1), Junonia coenia densovirus (JucoDENSV/Genbank NP_899649.1), and Pseudoplusia includens densovirus (PsinDENSV/Genbank YP_007003822.1), Orf-25 from ChocGV, and Orf-9 from CrleGV. The tree is midpoint rooted for purposes of clarity only. We hypothesized gene duplication for both ErelGV genes (boldface).
Methods purification

Virus cadavers of the hornworm *E. ello ello* with baculovirus infection symptoms were collected in cassava crops in the South of Brazil (Itajai, Santa Catarina) in 1986. They were kindly provided by Dr. Renato Arcano Pegoraro (EPAGRI). The cadavers were kept in the freezer and later used for OBs purification. Insect cadavers were homogenized with ddH₂O (w/v), filtered through three layers of gauze, and centrifuged at 7,000 x g for 10 min. The pellet was resuspended in 0.5% (w/v) SDS and again centrifuged at 7,000 x g for 10 min. The dilution and centrifugation steps were repeated four times, and the final pellet was washed in 0.5 M NaCl. The pellet was resuspended in ddH₂O, loaded onto a continuous 40-65% sucrose gradient, and centrifuged at 104,000 x g for 40 min at 4°C. The OB band was collected, diluted 4-fold in ddH₂O, and centrifuged at 7,000 x g for 15 min at 4°C.

Electron microscopy

For scanning electron microscopy (SEM), 100 μl of the OB-containing solution (10⁹ OBs/ml) were incubated with 300 μl of acetone at 25°C for 1 hour. The solution was loaded in a metallic stub, dried overnight at 37°C, coated with gold in a Sputter Coater (Balzers) for 3 min, and observed in a scanning electron microscope Jeol JSM 840 A at 10 kV. For transmission electron microscopy (TEM) pellets of purified granules were fixed in Karnovsky fixative (2.5% glutaraldehyde, 2% paraformaldehyde, in 0.1 M, cacodylate buffer, pH 7.2) for 2 h, post-fixed in 1% osmium tetroxide in the same buffer for 1 h and then stained *en bloc* with 0.5% aqueous uranyl acetate, dehydrated in acetone, and embedded in Spurr’s low viscosity embedding medium. The ultrathin sections were contrasted with 2% uranyl acetate and observed in a ZEISS TEM 109 at 80 kV.

Genomic DNA restriction analyses

Purified granules (10⁹ OBs/ml) were dissolved in an alkaline solution and used to extract DNA according to O’Reilly et al. [60]. The quantity and quality of the isolated DNA were determined by electrophoresis on 0.8% agarose (data not shown). The viral DNA (1–2 μg) was individually cleaved with the restriction enzymes HindIII, EcoRI, and BamHI (Promega) according to manufacturer’s instructions. The DNA fragments generated were analyzed by 0.8% agarose gel electrophoresis [61], visualized, and photographed in Alphalmager™ Mini (Alpha InnoTech).

Genome sequencing, assembly, and annotation

ErelGV genomic DNA was sequenced with the 454 Genome Sequencer (GS) FLX™ Standard (Roche) at the Centro de Genômica de Alto Desempenho do Distrito Federal (Brasília, Brazil). The genome was assembled *de novo* using Geneious 6.0 [62] and confirmed using restriction enzyme digestion profile. The annotation was performed using Geneious 6.0 to identify the open reading frames (ORFs) that started with a methionine codon (ATG) encoding at least 50 amino acids and blastp [63] to identify homologues.

Phylogeny, genome, and gene comparisons

For *Baculoviridae* phylogenetic analysis, a MAFFT alignment [64] was carried out with concatenated amino acid sequences predicted for 37 baculovirus core genes. A maximum likelihood tree was inferred using PhyML with 100 repetitions of a non parametric bootstrap [65], implemented in Geneious, with LG + I + G + F model selected by Prottest 2.4 [66]. Moreover, a genomic comparison was performed using the protein dataset of all the complete *Betabaculovirus* genomes available in Genbank. The dataset was compared using CGView Comparison Tool [36] and the results were plotted using CIRCOS [37]. We also compared ChocGV and PiraGV genomes with ErelGV genome using Mauve alignment [34]. The horizontal gene transfer (HGTs) events were investigated comparing the maximum likelihood phylogenetic tree inferred using the RAxML method [67] and a MAFT alignment of homologues for *he65*-like and *p43*-like, and *Densovirus*-related genes with 100 repetitions of a non parametric bootstrap for branch support.

Additional file

Additional file 1: Table S1. Characteristics of the *Ennnys ello granulovirus* (ErelGV) genome: analysis and homology search. Predicted ORFs are compared with homologous genes in three related genomes.

Competing interests

The authors declare that they have no competing interests.

Author’s contributions

Conceived and designed the experiments: DMPAA, FLM, BMR, MLS; Contributed reagents/materials/analysis tools: BMR, DMPAA, FLM, MSA, SNB, MLS; Wrote the paper: DMPAA, FLM, BMR, MLS. All authors read and approved the final manuscript.

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Author details

1 Cell Biology Department, Laboratory of Baculovirus, University of Brasilia, 70910-900 Brasilia, DF, Brazil. 2 Embrapa Genetic Resources and Biotechnology, Biological Station Park, 70770-917 Brasilia, DF, Brazil.

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