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Identification of nucleopolyhedrovirus that infect Nymphalid butterflies *Agraulis vanillae* and *Dione juno*

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ABSTRACT

Dione juno and *Agraulis vanillae* are very common butterflies in natural gardens in South America, and also bred worldwide. In addition, larvae of these butterflies are considered as pests in crops of *Passiflora spp.* For these reasons, it is important to identify and describe pathogens of these species, both for preservation purposes and for use in pest control. Baculoviridae is a family of insect viruses that predominantly infect species of Lepidoptera and are used as bioinsecticides. Larvae of *D. juno* and *A. vanillae* exhibiting symptoms of baculovirus infection were examined for the presence of baculoviruses by PCR and transmission electron microscopy. Degenerate primers were designed and used to amplify partial sequences from the baculovirus *p74*, *cathepsin*, and *chitinase* genes, along with previously designed primers for amplification of *lef-8*, *lef-9*, and *polh*. Sequence data from these six loci, along with ultrastructural observations on occlusion bodies isolated from the larvae, confirmed that the larvae were infected with nucleopolyhedroviruses from genus *Alphabaculovirus*. The NPVs from the two different larval hosts appear to be variants of the same, previously undescribed baculovirus species. Phylogenetic analysis of the sequence data placed these NPVs in *Alphabaculovirus* group I/clade 1b.

1. Introduction

The Baculoviridae family comprises a large number of viruses, which infect arthropods, mostly insects from the order Lepidoptera (Blissard et al., 2000; Kelly et al., 2007). The viral genome is double stranded circular DNA, with sizes ranging from 80 to 180 kbp and variable gene content (van Oers and Vlak, 2007; Jiang et al., 2008; Harrison, 2009). However, there are core genes conserved in all sequenced virus genomes such as *lef-8* and *lef-9* or those called *pifs* (per os infectivity factors) while including the *p74* gene (Belaich et al., 2006; Jehle et al., 2006a). Mature virions are assembled late in the infectious cycle and are included in an occlusion body (OB), constituted by a crystalline proteinaceous matrix mainly composed of polyhedrin (NPVs or nucleopolyhedroviruses) or granulin (GVs or granuloviruses), which after entering by *per os* route and disarm within the insect midgut releases the occlusion derived viruses (ODV) that naturally initiate the infection in the appropriate host (Ohkawa et al., 2005; Slack and Arif, 2007). Also, there is another phenotype called budded virus (BV) that is responsible for systemic infection (Kozlov et al., 1986; Rohrmann, 1992). Nowadays, the Baculoviridae is divided into four genera according to common biological and structural characteristics: *Alphabaculovirus*, that includes lepidopteran-specific baculoviruses, containing in turn a single (SNPV) or a multiple nucleocapsid (MNPV) into ODVs and Group I or Group II members accordingly with the presence of *gp64* gene; *Betabaculovirus*, which comprises the lepidopteran-specific genus granuloviruses; *Gammabaculovirus*, that comprise the hymenopteran-specific NeleNPV, NeseNPV and NeabNPV; and finally *Deltabaculovirus*, that includes to date only CuniNPV and possibly other not yet described dipteran-specific baculoviruses (Herniou et al., 2001, 2003; Jehle et al., 2006a).

Among the majority of baculoviral isolates made so far, many have demonstrated a great potential and are successfully used as bioinsecticides in pest management programs (Moscardi, 1999; Prater et al., 2006; Sun, 2007; Ashour et al., 2007; Lasa et al., 2008). On the other hand, baculoviruses actually have other applications such as those associated with protein expression systems or gene delivery in mammals (Hüser and Hofmann, 2003; Kim et al., 2008; Pan et al., 2009, 2010). In view of that, it remains important to apply simple methodologies that contribute to the discovery of new members non-previously described that potentially reveal new biological uses. In this way, reports constantly appear about the description of new isolates based on ultrastructural

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studies performed by electron microscopy or phylogenetic analysis based on partial sequence data (Wu and Wang, 2005; Cheng et al., 2005; Woo et al., 2006; Mukawa and Goto, 2006; AlaaEddeen, 2008). For the latter, the application of PCR assays based on baculovirus core genes emerges as a central tool that allows the amplification and proper isolation of virus genome fragments useful for subsequent viral classification by sequencing strategies or primer specificity (Jehle et al., 2006b; Murillo et al., 2006; Szewczyk et al., 2008; Zwart et al., 2008).

Dione juno (Stoll, 1782) (*Lepidoptera: Papilionoidea, Nymphalidae, Heliconiinae*), known as silverspot butterfly, silver-spotted flambeau or juno heliconian, and *Agraulis vanillae* (Boisduval and Le Conte, 1835) (*Lepidoptera: Papilionoidea, Nymphalidae, Heliconiinae*), known as gulf fritillary or passion butterfly, are insects with a wide geographical distribution in America ranging from Mexico to Argentina. Both lepidopterans are commonly bred in butterfly gardens worldwide because of their daily habits and beauty. It is also important to note that in their larval stages their natural diet is based on different plant species of the genus *Passiflora*, many of which have high commercial value in tropical and subtropical regions. For these reasons, the detection and characterization of natural pathogens affecting these butterflies is a relevant topic, either for the proper rearing of these insects in butterfly gardens; or conversely, to protect crops of *Passiflora sp*.

Taking into account previous data, in this study we designed degenerate primers based on the baculovirus *p74, cathepsin*, and *chitinase* genes and implemented them in PCR reactions with the aim to detect and classify baculovirus that infect Nymphalid

Table 1
Baculovirus complete genomes.

Genus	Name	Abbreviation	Acc. number
Alphabaculovirus Group I	*Antheraea pernyi* NPV	AnpeNPV	NC_008035
	Antheraea pernyi NPV-L2	AnpeNPV-L2	EF207986
	Anticarsia gemmatalis MNPV-2D	AgMNPV-2D	NC_008520
	Autographa californica MNPV	AcMNPV	NC_001623
	Bombyx mandarina NPV	BomaNPV	NC_012672
	Bombyx mori NPV	BmNPV	NC_004323
	Choristoneura fumiferana DEF MNPV	CfDEFMNPV	NC_005137
	Choristoneura fumiferana MNPV	CfMNPV	NC_004778
	Epiphyas postvittana NPV	EppoNPV	NC_003083
	Hyphantria cunea NPV	HycuNPV	NC_007767
	Maruca vitrata MNPV	MaviMNPV	NC_008725
	Orgyia pseudotsugata MNPV	OpMNPV	NC_001875
	Plutella xylostella MNPV	PlxyMNPV	NC_008349
	Rachiplusia ou MNPV	RoMNPV	NC_004323
Alphabaculovirus Group II	*Adoxophyes honmai* NPV	AdhoNPV	NC_004690
	Adoxophyes orana NPV	AdorNPV	NC_011423
	Agrotis segetum NPV	AgseNPV	NC_007921
	Chrysodeixis chalcites NPV	ChChNPV	NC_007151
	Clanis bilineata NPV	ClbiNPV	NC_008293
	Euproctis pseudoconspersa NPV	EupsNPV	NC_012639
	Ecotropis obliqua NPV	EcobNPV	NC_008586
	Helicoverpa armigera MNPV	HearMNPV	NC_011615
	Helicoverpa armigera NPV-C1	HearNPV-C1	NC_003094
	Helicoverpa armigera NPV-G4	HearNPV-G4	NC_002654
	Helicoverpa armigera SNPV-NNg1	HearSNPV-NNg1	NC_011354
	Helicoverpa zea SNPV	HzSNPV	NC_003349
	Lymantria dispar MNPV	LdMNPV	NC_001973
	Lymantria xylina MNPV	LyxyMNPV	NC_013953
	Mamestra configurata NPV-90-2	MacoNPV-90-2	NC_003529
	Mamestra configurata NPV-B	MacoNPV-B	NC_004117
	Mamestra configurata NPV-90-4	MacoNPV-90-4	AF539999
	Orgyia leucostigma NPV	OrleNPV	NC_010276
	Spodoptera exigua MNPV	SeMNPV	NC_002169
	Spodoptera frugiperda MNPV	SfMNPV	NC_009011
	Spodoptera frugiperda MNPV-19	SfMNPV-19	EU258200
	Spodoptera litura NPV-II	SpliNPV-II	NC_011616
	Trichoplusia ni SNPV	TnSNPV	NC_007383
Betabaculovirus	*Adoxophyes orana* GV	AdorGV	NC_005038
	Agrotis segetum GV	AgseGV	NC_005839
	Choristoneura occidentalis GV	ChocGV	NC_008168
	Cryptophlebia leucotreta GV	CrleGV	NC_005068
	Cydia pomonella GV	CpGV	NC_002816
	Helicoverpa armígera GV	HearGV	NC_010240
	Phthorimea operculella GV	PhopGV	NC_004062
	Plutella xylostella GV	PlxyGV	NC_002593
	Pseudaletia unipuncta GV	PsunGV	EU678671
	Xestia c-nigrum GV	XnGV	NC_002331
Gammabaculovirus	*Neodiprion abietis* NPV	NeabNPV	NC_008252
	Neodiprion lecontei NPV	NeleNPV	NC_005906
	Neodiprion sertifer NPV	NeseNPV	NC_005905
Deltabaculovirus	*Culex nigripalpus* NPV	CuniNPV	NC_003084

This table contains all of baculoviruses used in experiments or bioinformatic studies, sorted by genus (and within them by alphabetical order). MNPV is the abbreviation of multicapsid nucleopolyhedrovirus; NPV is the abbreviation of nucleopolyhedrovirus; SNPV is the abbreviation of single nucleopolyhedrovirus; GV is the abbreviation of granulovirus. The accession numbers are from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and correspond to the sequences of complete genomes.

butterflies. This strategy was applied in combination with the methodology most widely recognized and used for such purposes based on *polyhedrin/granulin_lef-8_lef-9* genes (Lange et al., 2004; Jehle et al., 2006b). Thus, sequence analysis and ultrastructural observations on OBs were carried out to characterize a previously non-described *Alphabaculovirus* that infect *D. juno* and *A. vanillae* larvae.

2. Materials and methods

2.1. Virus stocks

Different species of baculoviruses were used throughout this work: AcMNPV (*Autographa californica* MNPV isolate C6; given by Robert D. Possee, Centre for Ecology and Hydrology, NERC, Oxford, United Kingdom), RoMNPV (*Rachiplusia ou* MNPV isolate R1; given by Bryony Bonning, Iowa State University, USA), RanuMNPV (*Rachiplusia nu* MNPV; isolated in our laboratory), AgMNPV-2D (*Anticarsia gemmatalis* MNPV isolate 2D; given by Bergmann Morais Ribeiro, University of Brasilia, Brasil), AgMNPV-SF (*Anticarsia gemmatalis* MNPV isolate SF; given by Juan Claus, Universidad Nacional del Litoral, Argentina), CfMNPV (*Choristoneura fumiferana* MNPV Ireland strain; given by Basil Arif, University of Guelph, Canada), LdMNPV (*Lymantria dispar* MNPV isolate CI 5–6; given by Jim Slavicek, USDA Forest Service, USA), SeMNPV (*Spodoptera exigua* MNPV isolate US1; given by Salvador Herrero, Universitat de Valencia, Spain), SfMNPV (*Spodoptera frugiperda* MNPV; isolated in our laboratory), XnGV (*Xestia c-nigrum* GV isolate alpha-4; given by Susumu Maeda, Institute of Physical and Chemical Research, Japan), CpGV (*Cydia pomonella*; isolate M1 given by David R. O'Reilly, Imperial College of Science, Technology and Medicine, UK) and EpapGV (*Epinotia aporema* GV; isolated in our laboratory).

2.2. Cell culture and viral DNA isolation

Sf9 (Invitrogen) and UFL-Ag-286 cells (Sieburth and Maruniak, 1988) were grown at 27 °C in GRACE's Medium (Invitrogen) containing 10% foetal bovine serum (FBS; Bioser, Buenos Aires, Argentina) and 1× Antibiotic–Antimycotic (Invitrogen, 100×). Sixty-percent confluent monolayers of Sf9 cells were infected with RanuMNPV, AcMNPV or RoMNPV, and the same was done with AgMNPV-2D or AgMNPV-SF in UFL-Ag-286 cells (0.1 multiplicity of infection in all assays). After incubating during 7 days at 27 °C, budded viruses (BV) were collected from supernatants, centrifuged for cell debris clarification at 5000 rpm during 10 min in a microfuge, ultracentrifuged for concentration at 24000 rpm during 1.5 h in 25% sucrose cushion (SW28, Beckmann XL-70) and then resuspended in lysis buffer (10 mM Tris–HCl, 10 mM EDTA, 0.25% SDS). Completed a 37 °C overnight treatment with proteinase K (20 mg/ml; Sigma), virus DNA was purified by standard phenol/chloroform extraction and ethanol precipitation (Sambrook et al., 1989). The DNA isolation was tested by electrophoresis in 0.6% agarose gel, ethidium bromide-stained and quantified by OD_{260} spectroscopy (SmartSpec 3000 Bio-Rad).

2.3. Larval rearing and viral DNA isolation

Larvae of *Cydia pomonella*, *Epinotia aporema* and *Spodoptera frugiperda* were obtained from colonies established at IMyZA-INTA and maintained on an artificial diet at 25 °C, 14:10 h light darkness cycle and 60% relative humidity (Greene et al., 1976). To the aim of virus genome isolation, larvae were infected by diet contamination with 4000 OBs/mm^2 using CpGV, EpapGV and SfMNPV, respectively. The OBs of CpGV and EpapGV were counted in a 0.02 mm deep Petroff-Hauser chamber under dark field illumination; the

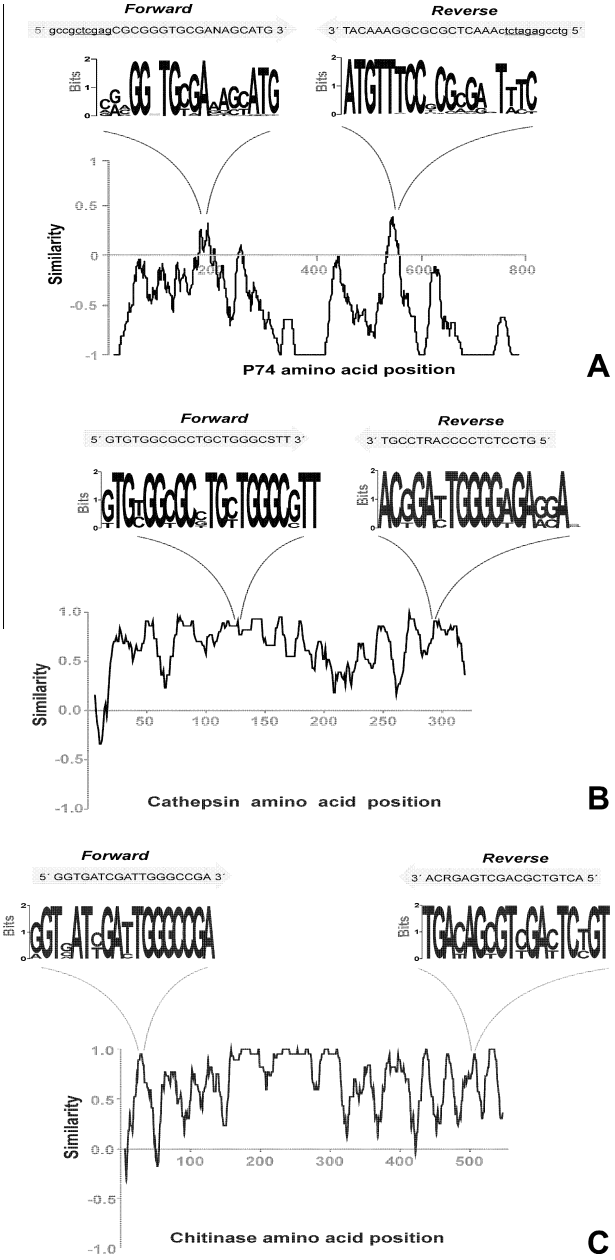

Fig. 1. Primer design. (A) Line plot shows the similarity pattern of all reported P74 proteins from baculoviruses that infect Lepidoptera. (B and C) Line plots show the similarity pattern of Cathepsin and Chitinase proteins from AcMNPV, RoMNPV, PlxyMNPV, BmNPV, OpMNPV, CfDEFMNPV and EppoNPV, respectively. In all cases, the amino acid position corresponds to the relative position in the multiple alignments. Above the two identified more conserved regions, sequence logos and universal primer sequences are also showed.

OBs of SfMNPV were counted in a 0.1 mm deep Neubauer chamber under dark field illumination. After a 5-day incubation period, dead insects were collected. At the same time, dead larvae of *A. vanillae* and *D. juno* were collected with apparent symptoms of baculoviral infection in a butterfly garden where these insects were reared (Escobar, Buenos Aires province, Argentina). All artificially and naturally infected larvae were macerated and homogenized in distilled water, and filtered through cheesecloth. Granules or polyhedra were purified by two cycles of centrifugation in continuous 30–60% (w/w) sucrose gradient at 100,000*g* for 1 h, at 4 °C. The bands containing OBs were removed, pooled, diluted 1:3 with distilled water and spun down at 8000*g* for 1 h, at 4 °C. The OBs

were resuspended in distilled water, and virions were released by hydrolysis of granules or polyhedra in alkaline buffer (0.1 M Na_2CO_3, 0.01 M NaCl, 0.1 M EDTA; pH 10.5) at 37 °C, 30 min. Later a clarification by centrifugation for 10 min at 14,000 rpm, SDS were added up to 0.5% of final concentration, initially incubated at 65 °C for 10 min and then incubated overnight at 37 °C with proteinase K (20 mg/ml). Finally, the DNA was purified in the same way as described for genome isolation derived from BVs.

2.4. Primer design and PCR reactions

Fragments of *p74, cathepsin and chitinase* genes were amplified by PCR with Taq DNA polymerase (Invitrogen) using the following primers: *p74* with *Fw_p74* 5′gccgctcgagCGCGGGTGCGANAGCATG3′ (non-*p74* sequences are indicated in lowercase and the XhoI site is underlined) and *Rev_p74* 5′caggctctagaGAAACTCGCGCGGAAACAT3′ (non-*p74* sequences are indicated in lowercase and the XbaI site is underlined); *cathepsin* with p_cat_{398} 5′GTGTGGCGCCTGCTGGG CSTT3′ and p_cat_{896} 5′GTCCTCTCCCCARTCCGT3′; *chitinase* with p_quit_{68} 5′GGTGATCGATTGGGCCGA3′ and p_quit_{1513} 5′ACRGAGTC GACGCTGTCA3′. All primers were designed from amino acid multiple sequence alignments made using the *Clustal X* program (Thompson et al., 1994, 1997). Similarity plots were calculated using the *Clustal X* consensus symbols (∗, and .) as the input sequence, in an overlapping windows-based strategy. The sum of assigned values for each residue in each window (11 amino acids) was divided by the window width and allotted to the central position to generate the plots (P.D. Ghiringhelli, unpublished), assigning arbitrary values of +1 for identical (∗), +0.5 and +0.25 for different degree of residue conservation (: and ., PAM 350 based; Schwartz and Dayhoff, 1979) and −1 for non-identical residues. After identification of high similarity candidate regions, the corresponding nucleic acid sequences were recovered, piled up and nucleotide *Sequence logos* were made using weblogo platform (Crooks et al., 2004). The PCR

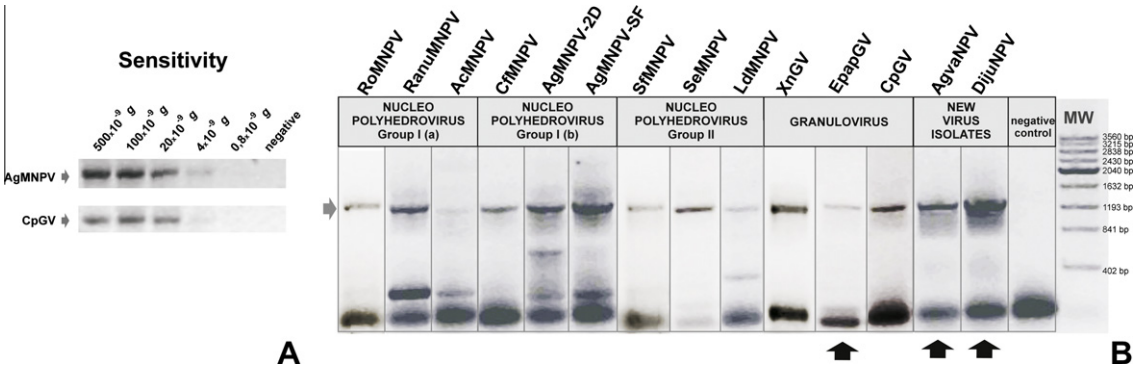

Fig. 2. *p74* PCR assay. (A) Method sensitivity measured using serial dilutions of DNAs from one *Alphabaculovirus* (AgMNPV) and one *Betabaculovirus* (CpGV). (B) Amplicons of *p74* gene from different baculovirus templates resolved by electrophoresis in 1% agarose gel. The identity of virus template is indicated at the top of each lane and the reactions are clustered by genus and subgenus. A gray arrow indicates the expected amplification fragments, and black arrows at the bottom of three lanes indicate amplicons non-described previously. MW: molecular weight marker 400 bp (PB-L; Argentina).

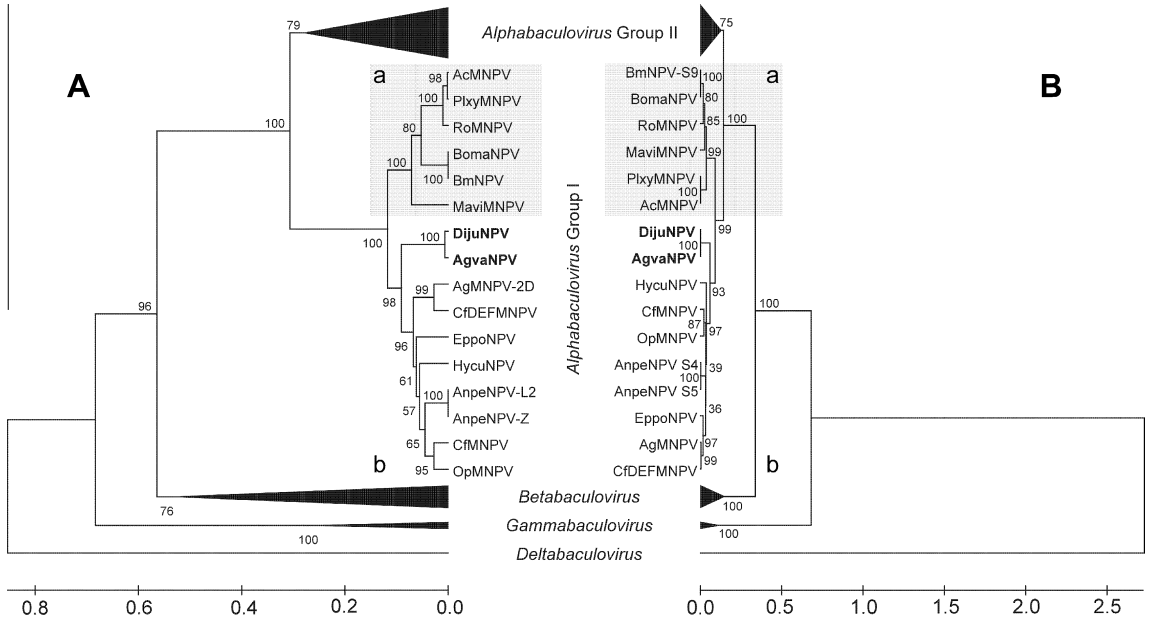

Fig. 3. DijuNPV and AgvaNPV classification. Translated sequences of PCR amplicons from 53 different baculoviruses belonging to the 4 genera proposed, together with the experimental obtained sequences from *Agraulis vanillae* and *Dione juno* infected larvae (identified as AgvaNPV and DijuNPV, respectively), were phylogenetically analyzed according to what described in Materials and Methods. In the cladograms *Alphabaculovirus* Group II, *Beta-*, *Gamma-* and *Deltabaculovirus* are shown in condensed form. (A) Cladogram derived from an internal *p74* fragment. (B) Cladogram derived from a Polyhedrin/Granulin_Lef-8_Lef-9 concatemer.

templates (10–20 ng for each reaction tube) were viral DNAs of AcMNPV, RanuMNPV, RoMNPV, AgMNPV-2D, AgMNPV-SF, CfMNPV, LdMNPV, SeMNPV, SfMNPV, CpGV, EpapGV, XnGV and viral DNAs from infected *D. juno* and *A. vanillae*. The *p74* PCR cycle was 94 °C for 2 min (1 cycle); 92 °C for 10 s, 48 °C for 12 s, 72 °C for 1 min 30 s (20 cycles); 92 °C for 10 s, 60 °C for 12 s, 72 °C for 1 min 30 s (20 cycles); 72 °C for 10 min (1 cycle). The *p74* PCR method sensitivity was estimated in units of viral genomes using AgMNPV and CpGV as reaction templates and standard equivalences between DNA mass and number of molecules [(*DNA mass* × *Avogadro's Number*)/(*genome Molecular Weight*)]. The *cathepsin* PCR profile was 94 °C for 2 min (1 cycle); 92 °C for 15 s, 40 °C for 20 s, 72 °C for 40 s (5 cycles); 92 °C for 10 s, 48 °C for 15 s, 72 °C for 40 s (30 cycles); 72 °C for 6 min (1 cycle). The *chitinase* PCR profile was 94 °C for 2 min (1 cycle); 92 °C for 15 s, 48 °C for 20 s, 72 °C for 1 min 30 s (35 cycles); 72 °C for 6 min (1 cycle). All amplicons were individually cloned in pZErO-2 (Invitrogen) previously digested with EcoRV or XhoI/XbaI (Fermentas) using T4-DNA ligase and *Top 10 Escherichia coli* (Invitrogen), by standard protocols (Sambrook et al., 1989). Finally, plasmids were purified (GeneJet™ Plasmid Miniprep kit, Fermentas) and the inserts were completely sequenced using universal *Sp6* and *T7* primers (Macrogen services, Korea).

2.5. Partial sequencing of polyhedrin, lef-8 and lef-9 genes

Fragments of *polyhedrin*, *lef-8* and *lef-9* genes of viral DNAs isolated from infected *D. juno* and *A. vanillae* larvae were amplified by PCR with Taq DNA polymerase using primers prPH-1/prPH-2, prL8-1/prL8-2, prL9-1/prL9-2 and reaction conditions previously described (Lange et al., 2004; Jehle et al., 2006b). The amplicons were purified from agarose gel after electrophoresis resolution (HiYield™ Gel/PCR DNA MiniKit, BioAmerica) and completely sequenced using *M13 forward* and *reverse* primers (Macrogen services, Korea).

2.6. Sequence analysis

Sequence analyses were performed using available genome sequences of baculoviruses (Table 1). Multiple alignments were obtained with *ClustalX* program, using *Gap opening* 10 and *Gap extension* 0.1 as parameters for pairwise alignments, and *Gap opening* 10 and *Gap extension* 0.05 for multiple alignments. Dayhoff distance corrected UPGMA distance analyses (gamma shape parameter: 2.25; gaps/missing data: complete deletion, respectively) and phylogenetic trees (1000 bootstrap replicates) were inferred from the amino acid sequence alignments by using MEGA, version 4.0 (Nei and Kumar, 2000; Kumar et al., 2004). The pairwise comparisons of amino acid sequences were performed using *ClustalW* with the default parameters. Distance matrices from aligned nucleotide sequences were determined by using the Pairwise Distance calculation of the same software applying the Kimura 2-parameter (K-2-P) model (Kumar et al., 2004).

2.7. Morphological studies

D. juno and *A. vanillae* purified occlusion bodies, were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3)–0.25 M sucrose for 3 h, postfixed in 1% osmium tetroxide for 1 h, dehydrated through an ethanolpropylene oxide series, and embedded in Epon-Araldite resin. Ten ultrathin sections (~70 nm thick), stained in 2% uranyl acetate (20 min.) and lead citrate (3 min.), were examined with a JEM-1200 EX II electron microscope. The relative diameters of polyhedra of some samples were determined by measurement of the cross sections in electron micrographs.

Table 2
Baculovirus Kimura 2-parameter nucleotide distances.

p74 fragment	Concatemer of *polH/lef-8/lef-9* fragments															
	AnpeNPV-Z	AnpeNPV-L2	OpMNPV	CfMNPV	DijuNPV	AgvaNPV	HycuNPV	EppoNPV	CfDEFMNPV	AgMNPV-2D	MaviNPV	BmNPV	BomaNPV	RoMNPV	PlxyMNPV	AcMNPV
AnpeNPV-Z		**0.001**	0.198	0.228	0.254	0.255	0.258	0.259	0.215	0.226	0.372	0.379	0.388	0.380	0.421	0.423
AnpeNPV-L2	**0.001**		0.197	0.229	0.255	0.256	0.259	0.260	0.216	0.227	0.373	0.380	0.389	0.382	0.423	0.424
OpMNPV	0.197	0.196		0.157	*0.183*	*0.184*	0.186	0.269	0.220	0.235	0.386	0.372	0.378	0.383	0.380	0.384
CfMNPV	0.218	0.218	0.181		*0.219*	*0.220*	0.204	0.265	0.234	0.240	0.363	0.358	0.366	0.351	0.374	0.372
DijuNPV	0.253	0.251	*0.181*	*0.248*		**0.007**	0.288	0.347	0.290	0.311	0.308	0.300	0.303	0.290	0.319	0.315
AgvaNPV	0.248	0.248	*0.180*	*0.245*	**0.002**		0.288	0.339	0.283	0.304	0.309	0.301	0.304	0.291	0.322	0.317
HycuNPV	0.241	0.240	0.239	0.237	0.250	0.251		0.260	0.214	0.226	0.361	0.372	0.374	0.356	0.390	0.391
EppoNPV	0.321	0.319	0.315	0.303	0.286	0.288	0.292		0.208	0.222	0.327	0.340	0.347	0.333	0.391	0.390
CfDEFMNPV	0.235	0.234	0.257	0.273	0.256	0.257	0.286	0.291		0.041	0.339	0.336	0.346	0.340	0.374	0.376
AgMNPV-2D	0.265	0.264	0.284	0.289	0.266	0.267	0.293	0.300	0.096		0.331	0.342	0.349	0.343	0.382	0.385
MaviNPV	0.439	0.441	0.411	0.424	0.425	0.420	0.435	0.407	0.375	0.389		0.112	0.113	0.127	0.164	0.162
BmNPV	0.359	0.361	0.349	0.360	0.361	0.356	0.380	0.366	0.316	0.343	0.180		**0.008**	0.062	0.113	0.110
BomaNPV	0.361	0.363	0.351	0.361	0.363	0.358	0.382	0.365	0.317	0.345	0.178	**0.001**		0.059	0.112	0.109
RoMNPV	0.369	0.368	0.340	0.365	0.379	0.376	0.372	0.364	0.303	0.331	0.171	0.091	0.092		0.110	0.104
PlxyMNPV	0.346	0.347	0.328	0.354	0.371	0.366	0.359	0.359	0.300	0.336	0.157	0.056	0.056	0.045		**0.008**
AcMNPV	0.346	0.344	0.326	0.354	0.369	0.366	0.357	0.359	0.299	0.335	0.160	0.058	0.057	0.044	**0.004**	
	Group Ib										Group Ia					

Bold values show isolates that could be considered as the same species. Italicized values indicate the AgvaNPV/DijuNPV more related Group Ib species.

3. Results

3.1. Detection of baculovirus in D. juno and A. vanillae larvae

To the aim of identifying if affected larva (*D. juno* and *A. vanillae*) bred in a butterfly garden in Argentina (Escobar, Buenos Aires province) were infected with baculoviruses, a PCR method based on different genes was applied. According to this, degenerate primers for *p74*, *cathepsin* and *chitinase loci* were designed using available sequence data (Fig. 1).

First, *p74* PCR assay was optimized testing different viral genomes as templates and a double temperature profile; a first permissive stage with 20 cycles at 48 °C of annealing, and a second stringent profile with another 20 cycles at 60 °C were tested. Serial dilutions of two viral genomes (one of *Alphabaculovirus* and other of *Betabaculovirus*) were used as PCR templates to test the method sensitivity (Fig. 2A). Similar values of target molecules (about 10^6) were detected.

Because the family Baculoviridae presents high variability in sequence between the known members, the use of viruses representing all phylogenetic lineages of baculoviruses that infect Lepidoptera as PCR templates was decided. So that, nine members of alphabaculoviruses including three from Group Ia MNPV viruses (AcMNPV, RoMNPV, RanuMNPV), three from Group Ib MNPV viruses (AgMNPV-SF, AgMNPV-2D, CfMNPV), and three members of Group II MNPVs (SeMNPV, LdMNPV, SfMNPV), and on the other hand three members of betabaculoviruses (CpGV, XcGV, EpapGV) were selected (Jehle et al., 2006a; Oliveira et al., 2006). Note that the proposed prototypes in the main Lepidopteran clades (AcMNPV, AgMNPV, LdMNPV and CpGV) were included and in all cases amplified an expected fragment size of about 1200 bp. But most importantly, this assay showed a positive result when DNAs isolated from dead larvae of *D. juno* and *A. vanillae* were used as templates, suggesting the presence of baculovirus infection (Fig. 2B).

3.2. D. juno and A. vanillae baculovirus classification

To reach the successful outcome of the *p74* PCR reaction, the amplicon identity was established by cloning in *Escherichia coli* and sequencing. In these, the sequences obtained from clones of the different viruses were in agreement with the information reported in GenBank (Table 1). On the other hand, the sequence corresponding to the fragment amplified from EpapGV genome (previously non-reported, GenBank DQ345450.1) showed high similarity with *p74* ORFs. In this way, the PCR assay allowed the properly detection of either evaluated virus from the different selected lineages. However, the most relevant result were the *p74* internal sequences obtained from the apparently infected *D. juno*

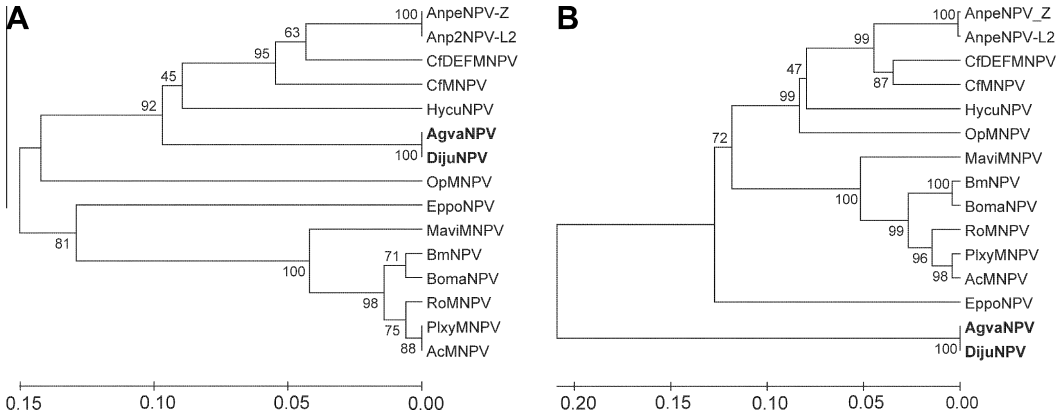

Fig. 4. Classification based on *cathepsin and chitinase* genes. Translated sequences of *cathepsin* (V-CATH, A) and *chitinase* (B) internal fragments (theoretical PCR amplicons) from 13 different baculoviruses belonging to *Alphabaculovirus* genus, together with the experimental obtained sequences from *Agraulis vanillae* infected larvae (identified as AgvaNPV) and *Dione juno* infected larvae (identified as DijuNPV), were phylogenetically analyzed.

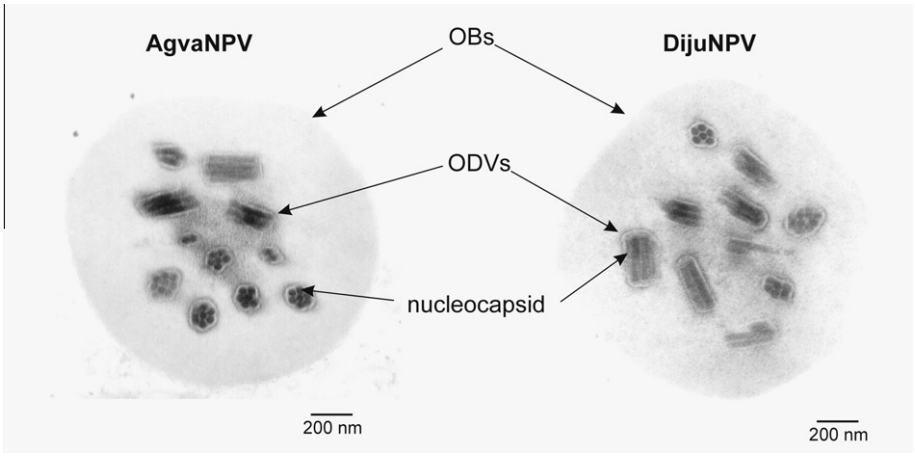

Fig. 5. Morphological studies. Electron microscopy on polyhedra sections of about 70 nm thick purified from infected *Agraulis vanillae* and *Dione juno* larvae were done. The resulting images from the *Agraulis vanillae* sample (identified as AgvaNPV) and from the *Dione juno* sample (identified as DijuNPV) show typical paracrystalline protein matrix (polyhedra) containing envelopes including nucleocapsids with bacilliform shape.

and *A. vanillae* larvae, in which the amino acid sequence were 99% identical to each other (DijuNPV *p74*: GU443971; AgvaNPV *p74*: GU443972). Thus, these results have shown that these natural samples contained non-previously identified baculoviruses, which were initially called DijuNPV and AgvaNPV based on the subsequent bioinformatic analysis realized using all known *p74* sequences along with new data obtained in this work (Fig. 3A).

To confirm and improve the lineage baculovirus assignation, PCR reactions (data not shown) and amplicon sequencing of *polyhedrin*, *lef-8* and *lef-9* genes (AgvaNPV *polyhedrin*: HQ010116; AgvaNPV *lef-8*: HQ010114; AgvaNPV *lef-9*; DijuNPV *polyhedrin*: HQ010113; DijuNPV *lef-8*: HQ010111; DijuNPV *lef-9*: HQ010112) were carried out using the virus DNAs from DijuNPV and AgvaNPV as templates. The cladograms derived from a Polyhedrin/Granulin_Lef-8_Lef-9 concatemer and P74 fragment showed the same species distribution and similar support bootstrap values (Fig. 3B).

Considering this information, the closest phylogenetic relationship was with nucleopolyhedroviruses that infect lepidopteran (*Alphabaculovirus*), and in particular with those of Group Ib. The K-2-P distance between both of *polyhedrin_lef-8_lef-9* concatemer and *p74* nucleotide sequence of DijuNPV and AgvaNPV (Table 2), revealed that DijuNPV and AgvaNPV are more related to OpMNPV and CfMNPV than the others. In addition, both K-2-P distances between DijuNPV and AgvaNPV are smaller than 0.015, suggesting that both isolates belong to the same baculovirus species.

On the other hand, the sequence corresponding to EpapGV amplicon clustered with the granulovirus members (data not shown) coincided with previous reports (Parola et al., 2002; Manzán et al., 2002; Sciocco-Cap et al., 2001).

3.3. Cathepsin and chitinase sequence analysis

Subsequently, in order to provide more sequence information for the new baculovirus species additional PCR assays targeted to regions of *cathepsin* and *chitinase* genes were performed using two pairs of primers previously designed (Fig. 1B and C). Both selected *loci* are considered as baculovirus genes encoding factors associated with auxiliary function (Hawtin et al., 1997; Jehle et al., 2006b). Except the reported case of AgMNPV (Oliveira et al., 2006), the rest of Group I alphabaculoviruses have orthologous sequences of *cathepsin* and *chitinase* genes. PCR assays with the new primers and the viral DNAs isolated from infected *D. juno* and *A. vanillae* larvae as reaction templates were performed and amplicons of predicted size were obtained (data not shown). Thus, after cloning and sequencing (AgvaNPV *cathepsin*: GU443969; AgvaNPV *chitinase*: GU443970; DijuNPV *cathepsin*: HQ010117; DijuNPV *chitinase*: HQ010118), phylogenetic analysis including the PCR theoretical equivalent sequences from other alphabaculoviruses of Group I was performed, confirming the relationship with other members of this clade (Fig. 4). In addition, the presence of both auxiliary genes in the new isolated baculovirus was confirmed.

3.4. Morphological studies

Finally, morphological studies by electron microscopy of purified OBs obtained from infected *D. juno* and *A. vanillae* larvae were performed (Fig. 5). The observed occlusion bodies were polyhedral in shape, with a diameter mean size of 1.56 (±0.055) μm. In general, each polyhedron contained more than ten virion bundles, with a multiple and variable number (2–11) of nucleocapsids surrounded by a common lipid bilayer. This result is in agreement with the virus description for the multiple nucleopolyhedroviruses (MNPVs) of Lepidoptera (Jehle et al., 2006a).

4. Discussion

Baculoviruses are well-studied insect pathogens because they have multiple applications in several fields of science, such as a tool for pest biocontrol, as a platform to heterologous protein expression, or as a delivery system to gene therapy and vaccination (Hüser et al., 2006; Sun, 2007; Ashour et al., 2007; Lasa et al., 2008; Kim et al., 2008; Pan et al., 2009, 2010; Hitchman et al., 2009). In view of that, the discovery and characterization of new baculoviruses will be welcome and very useful. The tests that assist in the stated purpose are mainly based on the amplification of fragments from several viral genes such as *polyhedrin*/*granulin*, *DNA polymerase*, *lef-8* and/or *lef-9* (Bulach et al., 1999; Christian et al., 2001; Graham et al., 2004; Lange et al., 2004; Murillo et al., 2006; AlaaEddeen, 2008). In particular, in this study the *p74, cathepsin, chitinase, lef-8, lef-9 and polyhedrin loci* were used as PCR target to detect and classify a new virus isolated from dead larvae of Nymphalid butterflies.

The only available sequence from baculovirus that infect *Agraulis sp.* derives from the extensive study realized by Jehle's group (Jehle et al., 2006b). The comparison between the sequences described in this work and the previous report shows an identity of 99%, suggesting that the isolated virus described here might be evolutionarily closely related. On the other hand, in view of the low variability manifested in sequence data between the viruses isolated from *D. juno* and *A. vanillae* and the concurrent natural infection of those butterfly larvae colonies, both pathogens seem to be the same baculovirus species.

Taking into account all the results presented in this report, the name AgvaNPV (*A. vanillae* multiple nucleopolyhedrovirus) and the inclusion within the Group Ib into the genus *Alphabaculovirus* is proposed for this new baculovirus partially characterized that infect and kill *A. vanillae* and *D. juno* larvae.

To our knowledge, this is the first report of a polyhedrosis virus of Group Ib that infects two different genera of butterflies.

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