Journal of Biochemistry and Molecular Biology, Vol. 37, No. 2, March 2004, pp. 206-212

JOURNAL OF
Biochemistry and Molecular Biology

Identification, Characterization and Phylogenic Analysis of Conserved Genes within the *odvp-6e/odv-e56* Gene Region of *Choristoneura fumiferana* Granulovirus

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Received 14 April 2003, Accepted 7 July 2003

The genes that are located within the *odvp-6e/odv-e56* region of the *Choristoneura fumiferana* granulovirus (ChfuGV) were identified by sequencing the 11 kb *Bam*HI restriction fragment on the ChfuGV genome. The global GC content that was calculated from the data obtained from this genomic region was 34.96%. The open-reading frames (ORFs), located within the *odvp-6e/odv-e56* region, are presented and compared to the equivalent ORFs that are located at the same region in other GVs. This region is composed of 14 ORFs, including three ORFs that are unique to ChfuGV with no obvious homologues in other baculoviruses as well as eleven ORFs with homologues to granuloviral ORFs, such as granulin, CfORF2, *pk-1*, *ie-1*, *odv-e18*, *p49*, and *odvp-6e/odv-e56*. In this study, the conceptual products of seven major conserved ORFs (granulin, CfORF2, IE-1, ODV-E18, p49 and ODVP-6E/ ODV-E56) were used in order to construct phylogenetic trees. Our results show that granuloviruses can be grouped in 2 distinct groups as follows: Group I; *Choristoneura fumiferana* granulovirus (ChfuGV), *Cydia pomonella* granulovirus (CpGV), *Phthorimaea operculella* granulovirus (PhopGV), and *Adoxophyes orana* granulovirus (AoGV). Group II; *Xestia c-nigrum* granulovirus (XcGV), *Plutella xylostella* granulovirus (PxGV), and *Trichoplusia ni* granulovirus (TnGV). The ChfuGV conserved proteins are most closely related to those of CpGV, PhopGV, and AoGV. Comparative studies, performed on gene arrangements within this region of genomes, demonstrated that three GVs from group I maintain similar gene arrangements.

Keywords: Baculovirus, *Choristoneura fumiferana* granulovirus, Gene arrangement, Phylogeny

Introduction

At least eight families of insect viruses are known, but the viruses that are the most commonly used as viral bioinsecticides are viruses from the *Baculoviridae* family. Baculoviruses are a worldwide-distributed group of viruses that are specific to arthropods and are divided in two genera: the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs). Baculoviruses are characterized by the presence of a large protein matrix or occlusion body, which encase the viral particles. Baculovirus genome is composed of double-stranded circular DNA.

The high pathogenicity of GVs toward different insect pests of agricultural crops and forests make this group of viruses a very attractive candidate to be used as biological insecticides. Since 1950s, different GVs have been used as biological insecticides in different parts of the globe. In a method of classification based on tissue tropism, GVs are placed in three groups (Federici, 1997) as follows: Type I-GVs infecting the fat body (this group of GVs kill the insect host in a rather long process); Type II-GVs infecting most of the insect host tissues in a faster mode than Type I-GVs; Type III-Presently, the *Harrisina brillians* granulovirus (HbGV) is the only known

The nucleotide sequence data reported in this paper were submitted to the GenBank nucleotide sequence database and assigned the accession numbers AF58690, AF389081, AF406758, AF406760, and AY064489.

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member of this type that infects only the midgut cells.

Granuloviruses have large genomes (80-180 kbp) with the potential of encoding about 100 genes. Other viruses with big genome like poxvirus carry an extensive array of enzymes, which are essential for early gene transcription. However, granuloviruses, like other baculoviruses, carry no virion-associated proteins.

The genome of granuloviruses is composed almost entirely of unique DNA sequences, though several small repeated sequences, known as homologous regions (*hr*s), are known in their DNA. Open-reading frames are on either strand of the DNA. Most ORFs are separated by 2-200 bps. There are also some overlapping ORFs in the granulovirus genome, usually the termination codon TAA overlaps with the primary polyadenylation signal AATAAA. Some promoters are located within the neighboring ORFs. Frequently transcripts of one gene initiate within or through neighboring ORFs (Hayakawa *et al*., 1999; Hashimoto *et al.*, 2000; Luque *et al*., 2001).

Only four granuloviral genomes have been completely sequenced. They are *Xestia c-nigrum* granulovirus (XcGV) (Hayakawa *et al*., 1999), *Plutella xylostella* granulovirus (PxGV) (Hashimoto *et al*., 2000), *Cydia pomonella* granulovirus (CpGV) (Luque *et al.*, 2001), and *Phthorimaea operculella* granulovirus (PhopGV) (GenBank/EMBL accession no. AF499596). Using the data currently available from the GV and NPV genomes, it is now possible to establish the relationships of the *Baculovirideae* family members. Baculoviruses may have a co-evolutive pathway with their insect host (Rohrmann, 1986), and this may contribute to the diversification of the different groups of baculoviruses.

Choristoneura fumiferana granulovirus (ChfuGV), which has been isolated from infected spruce budworm in several parts of eastern Canada, is considered a very interesting alternative to be used as a biological insecticide against spruce budworm larvae.

In this study, the *odvp-6e/odv-e56* region on the ChfuGV genome was characterized and compared to other GVs. The locations and transcriptional orientations of ORFs within this region in all GVs have been presented. We used the available sequences of conserved genes that were located in this area as well as the encoded gene products, to examine the phylogenetic relationship of ChfuGV to other GVs as well as NPVs.

Material and Methods

***In vivo* production and purification of virus and DNA Extraction** *Choristoneura fumiferana* fourth-instar larvae was infected by ChfuGV using a virus-contaminated artificial diet (Forté *et al*., 1999). The virus isolation from infected larvae was carried out as previously described (Bah *et al*., 1997).

DNA extraction, cloning, and sequencing ChfuGV genomic DNA was extracted from purified-enveloped nucleocapsids as previously described (Rashidan *et al*., 2002). An 11 kb *Bam*HI restriction fragment was identified and cloned into a plasmidic vector and sequenced as explained previously (Rashidan *et al*., 2002).

Computer analysis Sequence data assembly and analysis were performed with the Sequencher software version 4.0.5 (Gene Codes Corp., Ann Arbor, USA) and MacVector program version 4.5.0 (Eastman Kodak, Rochester, USA). The nucleotide sequence and its predicted amino acid sequence were compared to homologues in GenBank/EMBL and SWISSPROT by using BLAST (Altshul *et al*., 1990). CLUSTALX (1.81) (Thompson *et al.*, 1997) was used for the multiple amino acid sequence alignments. BOXSHADE version 3.21 was used for the similarity shading and scoring among the aligned sequences. The alignments were then used as the input to construct the phylogenetic tree with Branch and Bound search settings of PAUP 4.0b4a, employing the Neighbor-joining (NJ) method (Swofford, 2000). The reliability of the phylogenetic trees was evaluated by the bootstrap analysis with 1,000 bootstrap replicates.

Assignation of putative ORFs The information that was obtained from the sequencing of the 11 kb genomic fragment of ChfuGV was used to detect the homologue sequences in the GenBank database (whole database and restricted database containing only virus sequences) using the BLASTN (Altshul *et al*., 1990) and Fasta3 (Pearson and Lipman, 1988) programs. Alternatively, the putative ORFs were first translated from the ChfuGV nucleotide sequences. The amino acid sequences were compared with existing sequences in the GenBank database using the BLASTP program (Altshul *et al*., 1990). These data, and the one that was obtained from the gene order from other granulovirus genomes, were used to identify the ChfuGV ORFs.

Results and Discussion

An 11 kb *Bam*HI restriction fragment of ChfuGV was cloned and sequenced. The global GC content that was calculated from the data obtained from this genomic region was 34.96%. Among fourteen detected putative open-reading frames (ORFs), eleven ORFs were coded for proteins with known homologues in other GVs, while three of them were unique to ChfuGV (Table 1). The data, which was obtained from the sequencing of this fragment along with previous sequence data from the upstream region to ChfuGV granulin, helped us to obtain useful information about the gene content, transcription orientation (Fig. 1), and codon preference of ChfuGV. The comparative studies on ORFs that are present within this region, and their transcription orientations in ChfuGV and other GVs, proved that closely related GVs maintain almost the same gene arrangement and transcription orientation (Fig. 1).

CfORF 1 a homologue to granulin Granulin, along with polyhedron, are found in the proteinous matrix of baculoviruses occlusion bodies. The ChfuGV granulin contains 747 nucleotides (nt) that encode a protein of 248 amino acids with a molecular mass equal to 29.299 kDa. The ChfuGV granulin

Table 1. Properties of ChfuGV ORFs detected in 11 kb restriction fragment and their related ORFs on other granulovirus genomes

ChfuGV			CpGV			PhopGV			PxGV			XcGV		
ORF	lenght (nt)	Dir	ORF	lenght (nt)	Dir	ORF	lenght (nt)	Dir	ORF	lenght (nt)	Dir	ORF	lenght (nt)	Dir
Cf1 *granulin*	747	>	Cp1 *granulin*	747	>	Po1 *granulin*	747	>	Px4 *granulin*	747	>	Xc1 *granulin*	747	>
Cf2	381	<	Cp2	525	<	Po2	522	<	Px5	396	<	Xc2	696	<
Cf3 *pk1*	834	>	Cp3 *pk1*	840	>	Po3 *pk1*	837	>	Px6 *pk1*	825	>	Xc3 *pk1*	909	>
Cf4 *orf594*	594	<	Cp4	567	<	Po4	573	<	Px7	1611	<	Xc4	975	<
Cf5	564	<	Cp5	243	>	Po5	276	>	Px8	528	<	Xc5	255	>
Cf6	255	>	Cp6	219	>	Po6 *ie1*	1281	<	Px9	282	>	Xc6	174	<
Cf7 *ie1*	1281	<	Cp7 *ie1*	1467	<	Po7	618	>	Px10 *ie1*	1182	<	Xc7	564	<
Cf8	684	>	Cp8	579	>	Po8	372	<	Px11	543	>	Xc8	261	>
Cf9	297	<	Cp9	306	<	Po9	1077	>	Px12	297	<	Xc9 *ie1*	1455	<
Cf10 *eIF-5*	1269	>	Cp10 *chitinase*	1785	<	Po10	171	>	Px13 *odv-e18*	276	<	Xc10	591	>
Cf11 *orf744*	744	>	Cp11 *cathepsin*	1002	>	Po11	240	>	Px14 *p49*	1341	<	Xc11	300	<
Cf12 *odv-e18*	267	<	Cp12	234	>	Po12 *odv-e18*	252	<	Px15	651	<	Xc12 *odv-e18*	252	<
Cf13 *p49*	1368	<	Cp13 *gp37*	756	<	Po13 *p49*	1281	<	Px16 *odv-e56*	1056	<	Xc13 *p49*	1362	<
Cf14 *odv-e56*	1062	<	Cp14 *odv-e18*	255	<	Po14	618	<				Xc14	690	<
			Cp15 *p49*	1374	<	Po15	960	<				Xc15 *odv-e56*	1062	<
			Cp16	591	<	Po16 *odv-e56*	1056	<						
			Cp17 *iap-3*	828	>									
			Cp18 *odv-e56*	1068	<									

Most homologous ORF: Cf2, Cp2, Po2, Px2, and Xc2; Cf5, Cp4, Po4, Px8, and Xc7; Cf6, Cp5, Po5, Px9, and Xc8; Cf8, Cp8, Po8, Px11, and Xc10; Cf9, Cp9, Po8, Px12, and Xc11

appears to be closely related to *Adoxophyes orana* granulovirus (AoGV, a Tortricidae-infecting GV). It is positioned in a cluster along with PhopGV (a Gelechiidae-infecting GV), CpGV (a Tortricidae-infecting GV), *Cryptophlebia leucotreta* granulovirus (ClGV, a Tortricidae-infecting GV), and HbGV (a Zygaenidae-infecting GV) apart from the cluster containing XcGV (a Noctuidae-infecting GV), PxGV (a Yponomeutidae-infecting GV), and *Trichoplusia ni* granulovirus (TnGV, a Noctuidae-infecting GV).

CfORF2 a homologue to CpORF2 A putative ORF of 381 nt is located immediately downstream of the granulin gene of ChfuGV. CfORF2 encodes 126 amino acids with an estimated molecular weight of 14.2 kDa. At the nucleotide level, the two putative TATA boxes and a baculovirus late promoter motif, ATAAG, precedes the CfORF2 start codon. The N-terminal region of CfORF2 demonstrates similarities with the N-terminus portion of the L11p protein family that is found in eukaryotic (*Guillardia theta*) and prokaryotic *(Thermotoga maritime)* cells. L11p proteins are ribosomal RNA-binding proteins and bind directly to ribosomal RNA. A phylogenetic tree of the CfORF2 product and the homologue ORFs in other GVs were constructed. The result placed ChfuGV, PhopGV, CpGV, and AoGV together in the same group.

CfORF3 a homologue to *pk-1* ORF number 3 on ChfuGV is a protein kinase homologue with the potential to encode a putative baculoviral protein kinase homologue with the predicted molecular mass of 32 kDa. Conceptual ChfuGV PK-1 protein, akin to its homologues in other baculoviruses, contains 11 conserved subdomains. These subdomains include motifs for serine/threonine protein kinases and ATP-binding. At

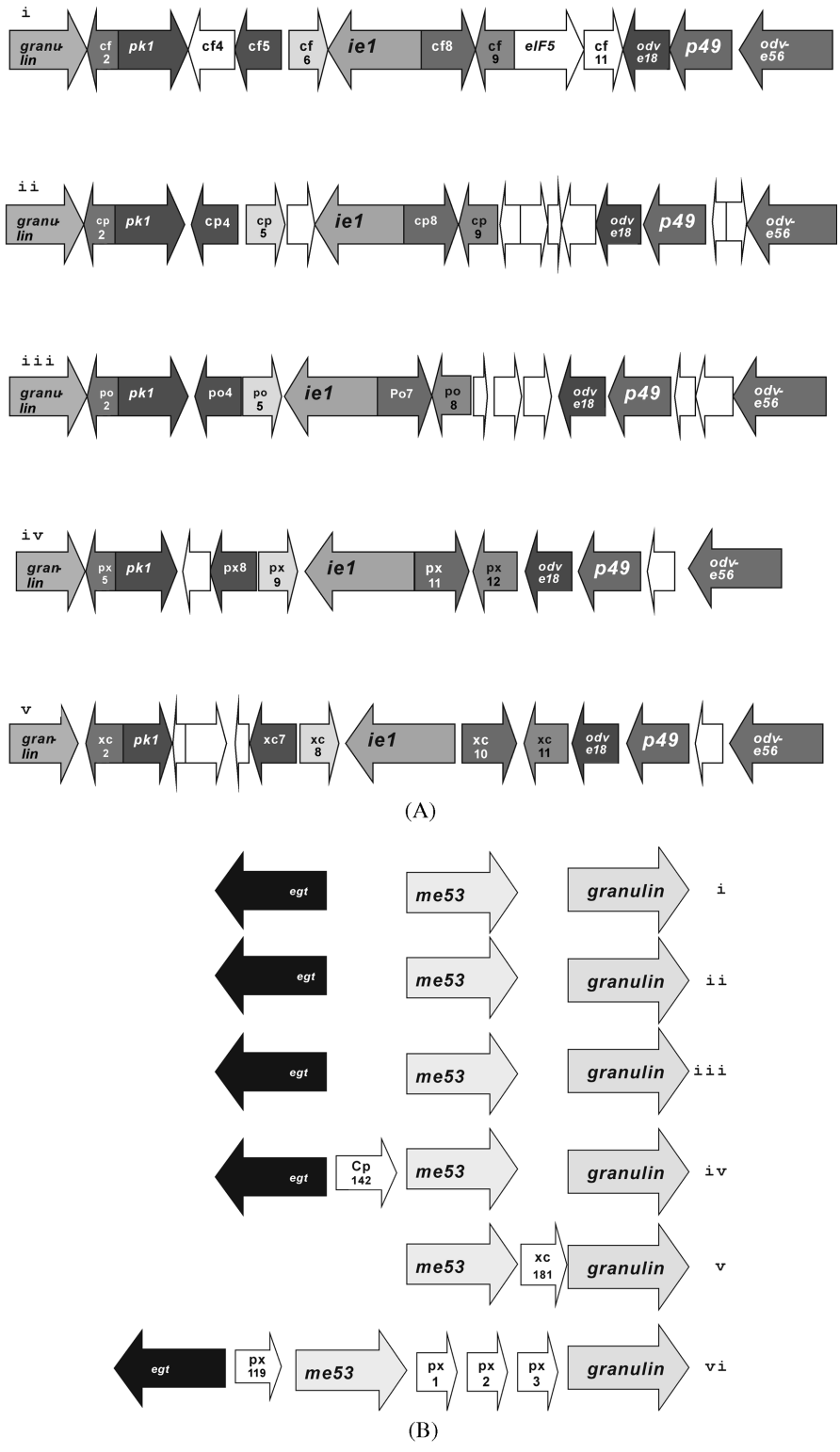

Fig. 1. Schematic illustration of the gene structure and orientation of (A) *odvp-6e/odv-e56*-containing region of various GVs (i-ChfuGV, ii-CpGV, iii-PhopGV, iv-PxGV, v-XcGV). (B) ORFs upstream to granulin (i- ChfuGV, ii-PhopGV, iii-AoGV, iv-CpGV, v- XcGV, vi- PxGV). The arrows represent the relative length and direction of the ORFs. White arrows indicate ORFs with no granuloviral homologue.

the nucleotide level, an early element (CAGT) and a late consensus (TAAG) motif were detected within the 5'-non translated region (NTR) of the ChfuGV *pk-1* gene. No obvious TATA element was observed in this region of the ChfuGV *pk-1*

gene. A phylogenetic analysis of the protein kinase proteins separated GVs from NPVs, and demonstrated that ChfuGV *pk-1* is most closely related to PhopGV and CpGV.

CfORF 7 a homologue to IE-1 IE1, an immediate-early baculoviral gene product, is a multifunctional transactivator with the capacity to regulate the expression from both viral and cellular gene promoters (Kovacs *et al.*, 1992). ORF potentially encodes a protein of 426 amino acids homologue to baculoviral IE1 with an estimated molecular weight of 50.33 kDa. At the nucleotide level, one TATA element, two putative CCAAT elements, a (A/C/T)CA(G/T)T motif (proposed consensus for arthropod transcriptional initiator elements), and two baculoviral late promoter motifs (TAAG) were detected within the 5' NTR of this gene. A domain, which is similar to the basic helix-loop-helix like (bHLH-like) domain in NPVs, was detected at the C-terminal region of IE-1 from ChfuGV (Rashidan *et al.*, 2002a). The phylogenetic estimation demonstrated an obvious separation between GVs and NPVs, and showed that ChfuGV IE-1 is most closely related to that of CpGV and PhopGV.

CfORF 12 a homologue to *odv-e18* ODV-E18 is a conserved baculoviral ODV-associated envelope protein. ORF coding for this protein were detected on the genome of several members of the *Baculoviridae* family. ODV-E18 has been characterized as a late protein in AcMNPV that is associated with both the ODV envelope and capsid (Braunagel *et al.*, 1996). The ORF number 12 on the ChfuGV genome potentially codes for a ODV-E18 homologue. The presumed ChfuGV ODV-E18 contains 88 amino acids with an estimated molecular weight of 9.28 kDa. Two baculoviral late promoter motifs (TAAG) were detected within the 5' NTR of the ChfuGV *odv-e18* gene at the positions −17 and −101 nt upstream of the start codon. A phylogenetic analysis of the baculoviral ODV-E18 protein separated GVs from NPVs and demonstrated that ChfuGV ODV-E18 is related the closest to those of PhopGV and CpGV (Fig. 2).

CfORF 13 a homologue to *p49* Three types of anti-apoptotic genes (*iap*, *p35* and *p49*) are known in baculoviruses. Also, p35 has the ability to suppress apoptosis that is induced by virus infection. A baculoviral protein (known as p49, a homologue to p35) is a novel apoptosis suppressor gene with the ability to inhibit insect and human effector caspases by inhibiting an initiator caspase (Pei *et al.*, 2002; Zoog *et al.*, 2002). The p49 homologue gene in ChfuGV contains 1368 nt that encode 455 amino acids with an estimated molecular weight of 53.720 kDa. The ChfuGV p49 gene contains a TATA element and two baculoviral late promoter motifs (TAAG) within the 5 NTR at the positions 56 nt and 17 nt, respectively, from the first ATG. The result of phylogenetic studies showed that the ChfuGV p49 protein, along with the CpGV and PhopGV p49 proteins, was placed in the same cluster.

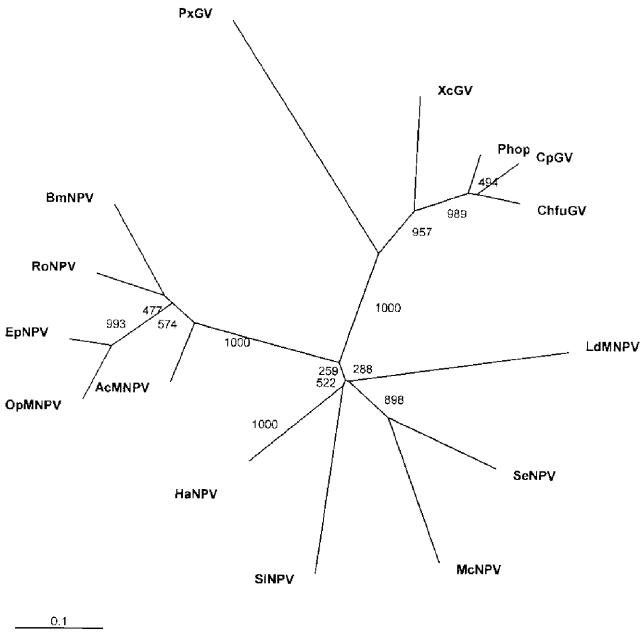

Fig. 2. Phylogenetic analysis of the protein product of baculoviral *odv-e18* gene located within the *odvp-6e/odv-e56* region of ChfuGV genome.

CfORF 14 a homologue to *odvp-6e/odv-e56* ORF 14 on the ChfuGV genome contains 1062 nt potentially-encoding 353 amino acids with an estimated molecular mass of 38.5 kDa. This ORF is a homologue to the baculoviral ODVP-6E/ODV-E56 proteins. The baculoviral ODVP-6E/ODV-E56 protein is a highly conserved ODV envelope-associated protein. At the nucleotide level, the TATA box and CCAAT element were centered, respectively, at −134 nt and −150 nt from the putative start triplet. A late promoter motif (GTAAG) was located at −14 bp upstream of the first ATG. The gene contained a slight variant of the polyadenylation signal, AATAAT, at 3' NTR at the position +10 nt downstream of the termination signal. ChfuGV ODVP-6E/ODV-E56 contained two potential membrane-spanning regions at the C-terminal and many putative *N*- and *O*- glycosylation, *N*-myristoylation, and phosphorylation sites (Rashidan *et al.*, 2002b). The phylogenetic estimation demonstrated that ChfuGV ODVP-6E/ODV-E56 is related the closest to those of CpGV and PhopGV.

Evolutionary relatedness of granuloviruses It is crucial to establish the relationship among the different members of the Baculoviridae family in order to extend our knowledge of the biology and evolution history of this virus family. With the aim of establishing such a relationship between ChfuGV and other GVs and NPVs, we used the following two different approaches: (*i)* phylogenetic study on several conserved genes in GVs and NPVs, (*ii)* gene arrangement within a region that contains two genes that code for the major highly-conserved ODV-associated proteins (granulin and ODVP-6E/ODV-E56).

A true comprehension of the evolutionary history of these conserved structural proteins will help us to achieve a more accurate understanding of their defined role in the virus infection cycle. To date, one of the major sources that was used as information to build phylogenetic trees was primarily based on the sequence of the occlusion body matrix proteins, granulin and polyhedrin. The validity of this approach was first questioned by Herniou and his associates (2001). Alternatively, a more plausible approach was to apply more than one conserved baculoviral protein in these studies. An approach that is similar to this was previously employed by Mitchell and his associates (2000), which combined data from different genes for phylogenetic studies on the Noctuoidea family (Insecta: Lepidoptera).

Using this approach, several phylogenetic trees were constructed. Each agreed on the separation of the GVs and NPVs and the division of NPVs into groups I and II, as postulated by other researches (Zanotto *et al.*, 1993; Bulach *et al.*, 1999), supported with high bootstrap values (Fig. 2).

Here we demonstrate that GVs can be grouped into the following 2 distinct groups: Group I (ChfuGV, CpGV, PhopGV and AoGV) and Group II (XcGV, PxGV, LoGV and TnGV). The ChfuGV-conserved proteins are most closely related to those of CpGV, PhopGV, and AoGV.

ChfuGV (along with CpGV, PhopGV, and AoGV) shares a high degree of gene order preservation within the studied genome region (Fig. 1). GVs and NPVs demonstrated different patterns in their gene order in the polyhedrin/granulin, *odvp-6e/odv-e56*, and granulin/polyhedrin genes regions (data not shown).

An extensive search is now underway to determine the complete genome sequences of more GVs. Baculoviruses are rapidly being dissected by sophisticated tools in attempt to illuminate the evolutionary pathway of this family of viruses. Hence, the results in this article can offer useful information for future studies on ChfuGV molecular biology. These results can also be used in other baculoviral phylogenetic studies in which the major goal would be the achievement of a better understanding about those biological and ecological characteristics that divide this group of viruses.

Acknowledgments The authors would like to thank Dr. Anne Bruneau (Department of Biology, University of Montreal) for helping us in the phylogenetic studies. We also thank Dr. David Morse (Department of Biology, University of Montreal) for his scientific discussions. This research was funded by the Ministère des Ressources Naturelles du Québec, Grant number 0316-240S to CG.

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