

Bracovirus Gene Products Are Highly Divergent From Insect Proteins

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Recently, several polydnavirus (PDV) genomes have been completely sequenced. The dsDNA circles enclosed in virus particles and injected by wasps into caterpillars appear to mainly encode virulence factors potentially involved in altering host immunity and/or development, thereby allowing the survival of the parasitoid larvae within the host tissues. Parasitoid wasps generally inject virulence factors produced in the venom gland. As PDV genomes are inherited vertically by wasps through a proviral form, wasp virulence genes may have been transferred to this chromosomal form, leading to their incorporation into virus particles. Indeed, many gene products from *Cotesia congregata* bracovirus (CcBV), such as PTPs, I κ B-like, and cystatins, contain protein domains conserved in metazoans. Surprisingly however, CcBV virulence gene products are not more closely related to insect proteins than to human proteins. To determine whether the distance between CcBV and insect proteins is a specific feature of BV proteins or simply reflects a general high divergence of parasitoid wasp products, which might be due to parasitic lifestyle, we have analyzed the sequences of wasp genes obtained from a cDNA library. Wasp sequences having a high similarity with *Apis mellifera* genes involved in a variety of biological functions could be identified indicating that the high level of divergence observed for BV products is a hallmark of these viral proteins. We discuss how this divergence might be explained in the context of the current hypotheses on the origin and evolution of wasp-bracovirus associations. Arch. Insect Biochem. Physiol. 67:172–187, 2008. © 2008 Wiley-Liss, Inc.

KEYWORDS: polydnavirus; parasitism; braconidae; virulence factors; virus evolution

INTRODUCTION

In the past few years, a considerable amount of data has been obtained on the DNA contained in polydnavirus (PDV) particles injected into caterpillars by parasitoid wasps. The sequence of six PDV genomes has been achieved: *Cotesia congregata* BV (CcBV) (Espagne et al., 2004), *Microplitis demolitor* BV (MdBV) (Webb et al., 2006), *Campoplex sonorensis* Ichnovirus (CsIV) (Webb et al., 2006), *Tranosema rostrale* IV (TrIV), *Hyposoter fugitivus* IV (HfIV) (Tanaka et al., 2007) and *Glypta fumiferanae* IV (GfIV) (Lapointe et al., 2007). Furthermore, partial data are available in the public

databanks for other BVs such as *Cotesia plutellae* BV (CpBV) (Choi et al., 2005) and *Cotesia glomerata* BV (CgBV). Strikingly few PDV genes share significant similarities with genes from other viruses. The paucity of “virus-like” genes may be explained by the fact that the virus does not replicate in the host tissues (Wyder et al., 2003). Therefore, the genes involved in the production of virus particles are not required on the DNA circles and might reside permanently in the chromosomally integrated proviral form of the virus or elsewhere in the wasp genome. This was shown to be the case for at least one gene coding a structural protein of the CsIV particles (Deng et al., 2000). Albeit unsuccessful

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in determining the evolutionary link between PDVs and other known viruses, the sequencing of PDV genomes enabled the characterization of potential virulence factors produced in the host tissues, which are expressed from the DNA enclosed in the virus particles (Dupuy et al., 2006). Hopefully, the precise role of each product in the alterations of host physiology will be determined by in vivo approaches such as RNAi, which has already given promising results in caterpillars (Eleftherianos et al., 2006). Another interesting question concerns the origin of these virulence genes, i.e., how they have been acquired by PDVs. It was proposed that PDV virulence genes might originate from genes encoding venom products involved in parasitism success (Webb and Summers, 1990). Their integration into the chromosomal form of the virus might have allowed a less costly and more efficient production of the virulence products within the host tissues. Accordingly, the structure of the BV potential virulence factors strongly suggests they are of cellular origin; however, surprisingly, they do not show a clear phylogenetic link with insect genes (Provost et al., 2004; Espagne et al., 2005; Falabella et al., 2007; Friedman and Hughes, 2006). This divergence might be a feature of the virulence genes or of the parasitoid wasp genes. Indeed, an increased rate of molecular evolution in mitochondrial genes is a characteristic feature of Hymenoptera and the trend is more pronounced in parasitic lineages (Dowton and Austin, 1995; Castro et al., 2002). This divergence might be explained, in the context of the Nearly Neutral Theory, by speciation with founder events from populations of small effective size (Ohta, 1993) and indeed an extraordinary level of radiation has occurred within the parasitic Hymenoptera. Conversely, the adaptive theory of genetic evolution predicts that parasites are under greater selective pressure in general, compared with non-parasites. Both theories predict that nuclear genes might exhibit an elevated rate of divergence in the parasitic Hymenoptera, as found for mitochondrial genes.

In this report, we have characterized thoroughly the distances between CcBV potential virulence factors encoded by three CcBV gene families previ-

ously characterized (Provost et al., 2004; Espagne et al., 2005; Falabella et al., 2007) and related protein sequences. The sequencing of a wasp cDNA library enabled us to measure the distance between insect and wasp cellular gene products involved in different functions. We show that BV products are related but only very distantly to proteins from metazoans while in contrast *C. congregata* cellular proteins show a relatively close proximity to those of *Apis mellifera* and other insects. We discuss how the high divergence of CcBV gene products might be explained in the context of the different hypotheses regarding the origin and evolution of the virulence factors.

MATERIALS AND METHODS

Insects

Wasps (*C. congregata*; Hymenoptera, Braconidae, Microgastrinae) were reared under laboratory conditions on their natural host, the tobacco hornworm, *Manduca sexta* (Lepidoptera, Sphingidae, Sphinginae), which were maintained on an artificial diet at 27°C under a 16-h light/8-h dark photoperiod and 70 ± 5% relative humidity as previously described (Harwood et al., 1994).

cDNA Library Construction

Total RNA was extracted from *C. congregata* dissected ovaries according to the manufacturer's instructions (Total RNA isolation, Macherey-Nagel, France). A cDNA library was constructed using 500 ng of total RNA according to the manufacturer's user manual (Creator SMART cDNA library construction kit, BD Biosciences, France).

C. congregata cDNA Library Analysis

Sequencing reactions of 5,683 randomly selected clones were performed using dye terminator sequencing on an ABI 3700 (Applied Biosystems, France) at the National Sequencing Center (Genoscope Evry, France) thus generating 700-bp-length sequences of high quality. Sequences from *A.*

mellifera, *Tribolium castaneum*, *Anopheles gambiae*, *Aedes aegypti*, *Drosophila melanogaster* or *D. pseudoobscura* and *Homo sapiens* similar to *C. congregata* predicted proteins were retrieved using the BLASTP program (Altschul et al., 1997) at the NCBI (National Center for Biotechnology Information, Bethesda, MD). Proteins having an e-value below e^{-20} were selected as potential homologues. Alignments were performed using the ClustalX program (Thompson et al., 1997) on protein fragments of the same length. For CcBV virulence proteins, alignments were improved by visual assessment using MacCLADE (Maddison and Maddison, 2000).

Distances were measured based on the alignments using MEGA3 software (Kumar et al., 2004). Comparative protein sequence analyses were performed using a pairwise distance calculation method under the p-distance model with a pairwise deletion option, which takes into account insertions and deletions.

In Table 1, the CpBVPTP p-distance value average was calculated from the sequences homologous to CcBV PTPs: CpBVPTPA (AAZ04267), CpBVPTPD (AAV98017), CpBVPTPM (AAV98020), CpBVPTPR (AAV98009), CpBVPTPepsilon (AAZ04268), CpBVPTPdelta (AAZ04263), CpBVPTPB (AAV98015), CpBVPTPQ (AAV98018), CpBVPTPP (AAV98019), CpBVPTPL (AAV98021), CpBVPTPI (AAV98023). The CpBVIκB-like average was calculated from CpBVank1 (AAZ04265), CpBVank2 (AAZ04284), CpBVS2ank2 (AAZ04266), CpBVank3 (AAZ04279), CpBVank4 (AAZ04265). The MdBVIκB-like average was calculated from MdBVank1c (YP_239368) and MdBVH5 (YP_239384).

Phylogenetic Analyses

Protein sequences having similarities with CcBV predicted proteins were retrieved in the databanks using BLASTP and TBLASTN analyses. Alignments were performed using ClustalX (Thompson et al., 1997) and adjusted manually guided by the localization of the conserved motifs described using MacCLADE (Maddison and Maddison, 2000). Distance (neighbour-joining) and parsimony (heuris-

tic search) analyses were performed using the PAUP4 program using 1,000 replicates for bootstrap calculation (Swofford et al., 1996).

RESULTS

Protein Tyrosine Phosphatases (PTPs)

Like protein tyrosine kinases (PTK), protein tyrosine phosphatases (PTPs) are a key group of signal transduction enzymes, which control the levels of cellular protein phosphorylation (Andersen et al., 2001). CcBV genome contains a large gene family coding for PTPs (27 genes) and the mRNAs are expressed in tissues of the parasitized host *M. sexta* (Provost et al., 2004). CcBV PTPs are likely to interfere with several host signalling pathways. Sequences having significant homology with CcBV PTPs were retrieved by BLASTP analysis using each potential CcBV product. The highest scores were obtained with PTPs from BVs. Among cellular proteins, the highest scores were obtained with PTPs belonging to the MEG-2 type, which have been shown by mutations (in knock-out mice) to modulate murine development and platelet and lymphocyte activation through secretory vesicle function (Wang et al., 2005). However, in contrast to the MEG-2 PTPs where the PTP domain is associated with a conserved NH2 terminal lipid binding domain, BV PTPs consist of a PTP domain that is not associated with other domains (Provost et al., 2004).

Human and insect MEG-2 protein sequences together with CcBV and other homologous BV sequences were selected and aligned using ClustalX. After manual correction of the alignment using MacClade, a phylogenetic analysis was performed using the methods of distance and parsimony. The trees obtained are presented in Figure 1. The cluster of BV proteins showed much longer branches than the cluster of insect MEG-2 proteins indicating a high divergence of these sequences. Indeed the distances between CcBV protein sequences and that of the bee ranged from 0.644 to 0.815 (mean 0.720), values that are two-fold higher than the distance between PTPs from insects of different orders and *A. mellifera* (mean 0.316, Table 1). The

TABLE 1. Evolutionary Distance Estimations (p-Distance) of Viral and Cellular Protein Sequences*

Organisms	Protein tyrosine phosphatases			Organisms	IκB-like proteins (ankyrins)		
	Proteins name	p-distance values	Accession numbers		Proteins name	p-distance values	Accession numbers
<i>A. mellifera</i>	<i>AmMEG-2PTP</i>	0.000	(XP_394701)	<i>A. mellifera</i>	<i>AmlκB-like1</i>	0.000	(XP_001121009)
<i>T. castaneum</i>	<i>TcMEG-2PTP</i>	0.296	(XP_973901)		<i>AmlκB-like2</i>	0.281	(XP_394485)
<i>A. gambiae</i>	<i>AgMEG-2PTP</i>	0.334	(XP_322055)		<i>AmlκB-like3</i>	0.455	(XP_001121575)
<i>A. aegypti</i>	<i>AaMEG-2PTP</i>	0.308	(EAT43037)	<i>T. castaneum</i>	<i>TcIκB-like</i>	0.575	(XP_968785)
<i>D. melanogaster</i>	<i>DmMEG-2PTP</i>	0.325	(NP_001014728)	<i>A. gambiae</i>	<i>AgIκB-like</i>	0.537	(XP_317542)
<i>H. sapiens</i>	<i>HsMEG-2PTP</i>	0.521	(AAH71574)	<i>A. aegypti</i>	<i>AaIκB-like</i>	0.521	(EAT48251)
<i>C. congregata BV</i>	<i>CcBVPTPN</i>	0.687	(CAG17431)	<i>D. melanogaster</i>	<i>DmCactus</i>	0.562	(AAA85908)
	<i>CcBVPTPV</i>	0.677	(CAG17447)	<i>H. sapiens</i>	<i>HsIκBa</i>	0.567	(AAP36525)
	<i>CcBVPTPW</i>	0.711	(CAG17449)	<i>C. congregata BV</i>	<i>CcBVank1</i>	0.756	(CAG17432)
	<i>CcBVPTPU</i>	0.773	(CAG17444)		<i>CcBVank2</i>	0.773	(CAG17451)
	<i>CcBVPTPA</i>	0.696	(CAG17496)		<i>CcBVank3</i>	0.798	(CAG17445)
	<i>CcBVPTPH</i>	0.713	(CAG17404)		<i>CcBVank4</i>	0.750	(CAG17448)
	<i>CcBVPTPDec</i>	0.682	(CAG17389)		<i>CcBVank5</i>	0.657	(CAG17492)
	<i>CcBVPTPS</i>	0.703	(CAG17427)		<i>CcBVank6</i>	0.692	(CAG17493)
	<i>CcBVPTPM</i>	0.703	(CAG17386)		<i>CcBV26,5</i>	0.792	(CAG17495)
	<i>CcBVPTPO</i>	0.815	(CAG17405)	<i>C. plutellae BV average</i>	—	0.719	—
	<i>CcBVPTPE</i>	0.688	(CAG17426)	<i>M. demolitor BV average</i>	—	0.745	—
	<i>CcBVPTPX</i>	0.691	(CAG17455)	<i>Insects average</i>	—	0.489	—
	<i>CcBVPTPR</i>	0.644	(CAG17418)	<i>BVs average</i>	—	0.736	—
	<i>CcBVPTPepsilon</i>	0.789	(CAG17497)				
	<i>CcBVPTPdelta</i>	0.648	(CAG26748)				
	<i>CcBVPTPY</i>	0.685	(CAG17456)				
	<i>CcBVPTPB</i>	0.667	(CAG17379)				
	<i>CcBVPTPQ</i>	0.767	(CAG17388)				
	<i>CcBVPTPP</i>	0.759	(CAG17387)				
	<i>CcBVPTPL</i>	0.730	(CAG17385)				
	<i>CcBVPTPK</i>	0.740	(CAG17384)				
	<i>CcBVPTPI</i>	0.742	(CAG17380)				
	<i>CcBVPTPZ</i>	0.772	(CAG17457)				
	<i>CcBVPTPC</i>	0.763	(CAG17429)				
	<i>CcBVPTPalpha</i>	0.767	(CAG17458)				
<i>C. plutellae BV average</i>	—	0.700	—				
<i>G. indiensis BV</i>	<i>GiBVPTPB</i>	0.669	(AAZ30023)				
	<i>GiBVPTPQ</i>	0.759	(AAP37630)				
	<i>GiBVPTPP/Q</i>	0.724	(AAZ30024)				
	<i>GiBVPTPP</i>	0.728	(AAZ30025)				
	<i>GiBVPTPI</i>	0.725	(AAZ30027)				
<i>Insects average</i>	—	0.316	—				
<i>BVs average</i>	—	0.715	—				

Organisms	Cystatins		
	Proteins name	p-distance values	Accession numbers
<i>A. mellifera</i>	<i>Amcyst</i>	0.000	(XP_392381)
<i>Nasonia vitripennis</i>	<i>Nvcyst</i>	0.478	(SCAFFOLD116)
<i>Locusta migratoria</i>	<i>Lmcyst</i>	0.696	(C0825360)
<i>Manduca sexta</i>	<i>Mscyst</i>	0.711	(BAE97580)
<i>D. melanogaster</i>	<i>Dmcyst_1</i>	0.761	(NP_572542)
	<i>Dmcyst_2</i>	0.770	(NP_476856)
	<i>Dmcyst_3</i>	0.770	(NP_731919)
	<i>Dmcyst_4</i>	0.782	(NP_650374)
<i>Homalodisca coagulata</i>	<i>Hccyst</i>	0.795	(C0641796)
<i>Gallus gallus</i>	<i>Ggcyst-type2</i>	0.745	(NP_990831)
<i>Coturnix japonica</i>	<i>Cjcyst-type2</i>	0.755	(P81061)
<i>Mus musculus</i>	<i>MmcysC-type2</i>	0.765	(AAA63298)
<i>Homo sapiens</i>	<i>HscysC-type2</i>	0.804	(AAP36501)
<i>C. congregata BV</i>	<i>CcBVcyst1</i>	0.723	(CAG17468)
	<i>CcBVcyst2</i>	0.723	(CAG17471)
	<i>CcBVcyst3</i>	0.703	(CAG17473)
<i>G. indiensis BV</i>	<i>GiBVcyst</i>	0.673	(ABK56999)
<i>Insects average</i>	—	0.720	—
<i>BVs average</i>	—	0.706	—

*See Materials and Methods and Results for details.

distances between the BV and *A. mellifera* protein sequences even exceeded the distance between the human and the bee protein sequences (0.521) (Table 1). Although some BV PTP sequences are very closely related, most probably because they result from recent duplications (such as PTPE and PTPX), most BV PTPs were very distant when compared to each other. For example, the distance between

CcBVPTPN and the other CcBVPTPs ranged from 0.625 (PTPV) to 0.774 (PTPI) (Table 2). This suggests that although the proteins are structurally very similar (single PTP domain), their sequences have diverged considerably since their acquisition by the virus genome. Although BV PTPs probably originate from insect genes, no phylogenetic link can be established because of the high divergence of BV PTPs.

TABLE 2. Evolutionary Distance Estimations (ρ -Distance) of *C. congregata* BV Protein Sequences of Protein Tyrosine Phosphatases, κ B-Like Proteins and Cystatins

p-distance values	<i>C. congregata</i> BV protein tyrosine phosphatases																								
	N	V	W	U	A	H	Drec	S	M	O	E	X	R	Epsilon	Delta	Y	B	Q	P	L	K	I	Z	C	Alpha
CcBVTPN	0.000																								
CcBVTPV	0.625																								
CcBVTPW	0.654	0.460																							
CcBVTPU	0.711	0.635	0.621																						
CcBVTPA	0.664	0.659	0.691	0.719																					
CcBVTPH	0.644	0.635	0.682	0.707	0.709																				
CcBVTPDrec	0.645	0.637	0.695	0.725	0.684	0.540																			
CcBVTPS	0.637	0.639	0.671	0.703	0.709	0.401	0.543																		
CcBVTPM	0.677	0.664	0.700	0.748	0.693	0.584	0.536	0.577																	
CcBVTP0	0.765	0.802	0.822	0.797	0.768	0.753	0.772	0.764	0.737																
CcBVTPe	0.667	0.655	0.705	0.726	0.702	0.617	0.643	0.621	0.649	0.776															
CcBVTPX	0.670	0.655	0.694	0.734	0.706	0.624	0.635	0.613	0.638	0.780	0.102														
CcBVTPR	0.702	0.675	0.699	0.748	0.685	0.708	0.694	0.705	0.712	0.821	0.699	0.706													
CcBVTPepsilon	0.767	0.750	0.759	0.761	0.828	0.790	0.808	0.777	0.811	0.832	0.778	0.787	0.696												
CcBVTPdelta	0.685	0.637	0.696	0.721	0.712	0.694	0.679	0.704	0.694	0.821	0.706	0.713	0.278	0.691											
CcBVTPY	0.658	0.689	0.668	0.722	0.702	0.726	0.700	0.723	0.718	0.811	0.736	0.736	0.640	0.749	0.664										
CcBVTPB	0.708	0.664	0.682	0.703	0.714	0.698	0.690	0.719	0.694	0.823	0.715	0.722	0.625	0.700	0.625	0.620									
CcBVTPQ	0.738	0.764	0.740	0.797	0.771	0.759	0.783	0.762	0.762	0.849	0.809	0.802	0.714	0.734	0.717	0.620	0.690								
CcBVTPP	0.753	0.773	0.790	0.803	0.762	0.767	0.771	0.774	0.774	0.844	0.809	0.813	0.743	0.811	0.739	0.621	0.692	0.534							
CcBVTPL	0.731	0.748	0.741	0.788	0.756	0.739	0.777	0.761	0.772	0.848	0.764	0.753	0.713	0.738	0.697	0.608	0.679	0.542	0.589						
CcBVTPK	0.748	0.766	0.758	0.791	0.754	0.776	0.760	0.768	0.760	0.845	0.815	0.807	0.721	0.734	0.697	0.648	0.702	0.582	0.570	0.479					
CcBVTPi	0.774	0.783	0.801	0.771	0.775	0.806	0.803	0.777	0.791	0.789	0.810	0.792	0.744	0.782	0.739	0.804	0.758	0.792	0.827	0.793	0.786				
CcBVTPZ	0.767	0.794	0.770	0.773	0.815	0.788	0.817	0.781	0.809	0.813	0.838	0.845	0.761	0.769	0.749	0.762	0.752	0.733	0.809	0.781	0.774	0.644			
CcBVTPC	0.764	0.773	0.780	0.780	0.786	0.789	0.815	0.782	0.792	0.858	0.841	0.834	0.749	0.836	0.748	0.799	0.773	0.789	0.814	0.808	0.775	0.717	0.652		
CcBVTPalpha	0.756	0.780	0.784	0.783	0.793	0.785	0.830	0.782	0.799	0.866	0.845	0.841	0.756	0.827	0.748	0.799	0.776	0.785	0.806	0.796	0.779	0.710	0.659	0.070	0.000

p-distance values	<i>C. congregata</i> BV κ B-like proteins (ankyrins)		
	ank6	ank5rec	ank4
CcBV26.5	0.000		
CcBVank6	0.584		
CcBVank5	0.555	0.327	
CcBVank4	0.823	0.661	0.615
CcBVank1	0.720	0.573	0.550
CcBVank2	0.832	0.685	0.706
CcBVank3	0.766	0.669	0.642

<i>C. congregata</i> BV cystatins			
p-distance values	cyst1	cyst2	cyst3
CcBvcyst1	0.000		
CcBvcyst2	0.165		
CcBvcyst3	0.223	0.243	0.000

I κ B-Like Proteins (Ank Gene Products)

I κ B is a key regulator of the inflammatory response in vertebrates and its invertebrate homologue (Cactus) is involved in *Drosophila* development and innate immunity (Silverman and Maniatis, 2001). CcBV genome contains six genes encoding I κ B-like proteins (Espagne et al., 2004). Sequences of proteins having significant homology with CcBV I κ B-like proteins were retrieved by BLASTP analysis using each product. The highest scores were obtained with homologous proteins identified in CpBV and MdBV. Among cellular proteins, the highest scores were obtained with the I κ B-like proteins from *Drosophila* and other insects. As previously described, the structure of BV (and IV) I κ B-like proteins is very similar. They are made up almost entirely of an ankyrin domain comprising four repeats, which show similarity with repeats 3–6 of I κ B in *D. melanogaster* and in humans (Falabella et al., 2007; Thoetkiattikul et al., 2005; Kroemer and Webb, 2005). In *Drosophila*, the protein I κ B-homologue (Cactus) regulates multiple cellular responses activated by the nuclear import of a set of NF- κ B-like transcription factors, which control embryonic dorsoventral patterning (Bergmann et al., 1996) and antimicrobial responses (De Gregorio et al., 2001; Hoffmann, 2003). Some BV I κ B-like proteins likely play a role in the impairment of the host immune response by inhibiting the Toll/IMD pathways as suggested for two MdBV I κ B-like proteins that are capable of interacting with *Drosophila* NF- κ B/Rel proteins and blocking NF- κ B activation (Thoetkiattikul et al., 2005).

CcBV I κ B-like proteins, together with putative homologues from other BV and human and insect I κ B protein sequences, were selected and aligned using ClustalX and MacClade and a phylogenetic analysis was performed (Fig. 2). We then calculated the distances of the different protein sequences with three *A. mellifera* I κ B sequences retrieved from the databanks. In Table 1, the results are presented compared to one of these sequences; similar results were obtained with the other two (data not shown). The distances between CcBV I κ B-like protein sequences and the sequence of *A. mellifera*

ranged from 0.657 to 0.798 (mean 0.745), while the distance between I κ B-like proteins from insects and *A. mellifera* ranged from 0.281 to 0.575 (mean 0.489, Table 1). The distances between the BV I κ B-like proteins and *A. mellifera* sequences even exceeded the distance between human and bee sequences (0.567, Table 1). Unlike PTPs, no duplications seem to have occurred relatively recently in the BV I κ B-like gene family. The closest sequences are CcBVank6 and CcBVank5 (0.327, Table 2) and the duplication producing these copies most probably occurred before the separation of *Cotesia* species, since homologous sequences are found for both genes in the CpBV genome. Except for this pair of genes, the distance between CcBV protein sequences was high, ranging from 0.560 to 0.832 (Table 2), and generally exceeded the distance found between I κ B-like protein sequences of insects from different orders (0.521 to 0.575, Table 1).

Thus, like the PTPs, the I κ B-like proteins are characterized by a conservation of structure (repeats 3–6 of I κ B) but a high divergence of sequence. Due to the divergence of the I κ B-like proteins, it was not possible to demonstrate by phylogenetic analysis that the original gene copy has been acquired from the wasp genome.

Cystatin Proteins

Cystatins are cysteine protease inhibitors used as major immunoregulatory molecules by filarial nematodes (Maizels and Yazdanbakhsh, 2003). CcBV genome contains three genes on the same circle (circle 19) coding for type 2 cystatins. Type 2 cystatins are tight-binding reversible inhibitors of cysteine proteases belonging to the C1 family of peptidases such as cathepsins. As we reported previously, CcBV cystatins are likely to play a role in host caterpillar physiological deregulation by inhibiting host target proteases in the course of the host–parasite interaction (Espagne et al., 2005). Sequences having significant homology with BV cystatins were retrieved by BLASTP analysis using CcBV cystatin 1 sequence. The highest scores were obtained with a cystatin from *G. indiensis* BV, the

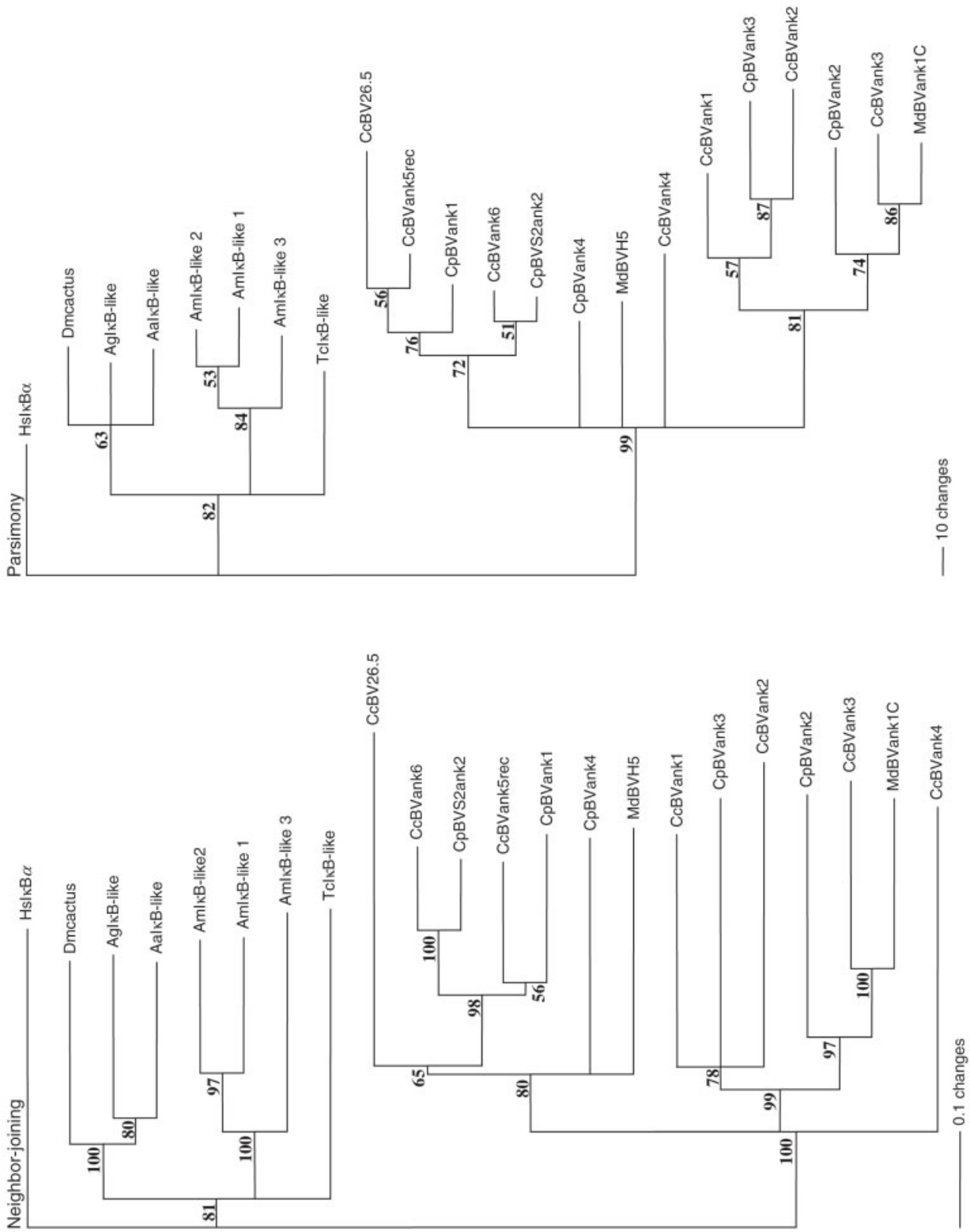


Fig. 2. Phylogenetic analysis of bracovirus IκB-like proteins. Neighbour-joining tree generated using PAUP4 from the alignment of the IκB-like sequences retrieved by BLASTP analysis using CcBV protein sequences as queries. The alignments were obtained using ClustalX and manual corrections. The *H. sapiens* sequence was used as outgroup.

unique related BV protein detected in the databanks. Among cellular proteins, the highest scores were those obtained with cystatins belonging to the type 2 cystatin family, such as the chicken egg cystatin, and two sequences from insects (*Locusta migratoria* and *Homalodisca coagulata*) retrieved from NCBI EST sequence databank (using TBLASTN analysis). CcBV and GiBV cystatins, human, avian, and insect cystatin sequences were selected and aligned using ClustalX and MacClade and a phylogenetic analysis was performed (Fig. 3).

The cellular cystatins appear to be under less stringent structural constraints than PTPs and IκB-like proteins. Indeed, whereas the PTPs comprise ten conserved domains and the IκB-like proteins four conserved ankyrin repeats, the conservation of cystatins is limited to a few conserved residues (data not shown). Unlike the PTPs and IκB-like gene families, it was not possible to find a group of homologous genes from different insect orders to compare to viral cystatins and most insect sequences retrieved belong to a different clade than the *A. mellifera* sequence (Table 1).

Cystatin sequences from CcBV are also distant from the *A. mellifera* sequence (0.703 to 0.723, mean 0.716, Table 1). However, interestingly, the three CcBV copies do not display the divergence of other virulence gene products but are highly similar (0.165 to 0.243, Table 2). They probably occurred from recent gene duplications, since the circle encoding the three cystatins is almost made up of three homologous segments: 2 of them (*cyst1* segment and *cyst2* segment) share 95.8% identity while the third one (*cyst3* segment) shares 63.8% with the *cyst1* segment. The fact that they have been detected only in closely related species such as *C. congregata* and *G. indiensis* and not in the completely sequenced *M. demolitor* genome suggests they might represent a recent acquisition of the BV genome. If such an interpretation proves true, cystatins might constitute good candidates to demonstrate that CcBV virulence genes originate from wasp genes. In the distance analysis, but not parsimony (Fig. 3), BV cystatins form a clade with insect and vertebrate type 2 cystatins. It would now be interesting to isolate cellular cystatins from hy-

menopteran parasitoid species. If these sequences were to group with BV sequences, this would strengthen the hypothesis of an acquisition of viral cystatins from the wasp genome.

***C. congregata* Cellular Proteins**

The analysis of the content of the *C. congregata* cDNA library revealed that most sequences share similarities with those of *A. mellifera*. We did not identify a sequence encoding either a cellular PTP or an IκB-like protein or a cystatin. We aligned 18 predicted amino acid sequences arbitrarily selected with those of other insect proteins (*A. aegypti*, *A. gambiae*, *D. melanogaster* or *D. pseudoobscura* and *T. castaneum*) for which the genomes are available in the databanks and with human sequences. From the alignment of the gene products, we measured the distance between insect or human sequences and those of *A. mellifera* (Table 3). The proteins analyzed displayed various levels of conservation, most probably reflecting different evolution rates of the genes. Strikingly, the similarity of *C. congregata* cellular gene product sequences with those of *A. mellifera* exceeded by far the level of similarity of CcBV sequences (PTPs, IκB-like proteins, and cystatins) with insect protein sequences (always below 60%). As an example of a gene displaying a relatively low conservation among the insect sequences retrieved, the *C. congregata* La Autoantigen product shared 55% identity and 72% similarity with the sequence of *A. mellifera* (Fig. 4, Table 3, Ssb gene). For a gene displaying a medium conservation, the phosphoglycerate mutase, *C. congregata* and *A. mellifera* proteins shared 77% identity and 88% similarity (Fig. 4, Table 3, GpmA gene). Finally, for the highly conserved gene coding for the developmental protein Mago Nashi, the *C. congregata* product showed 97% identity and 99% similarity with that of *A. mellifera* (Table 3, Mago gene).

Altogether, the *C. congregata* protein sequences always showed a close relation to the *A. mellifera* sequences (Table 3) indicating that the high level of divergence of CcBV PTPs and IκB-like proteins is a hallmark of BV proteins.

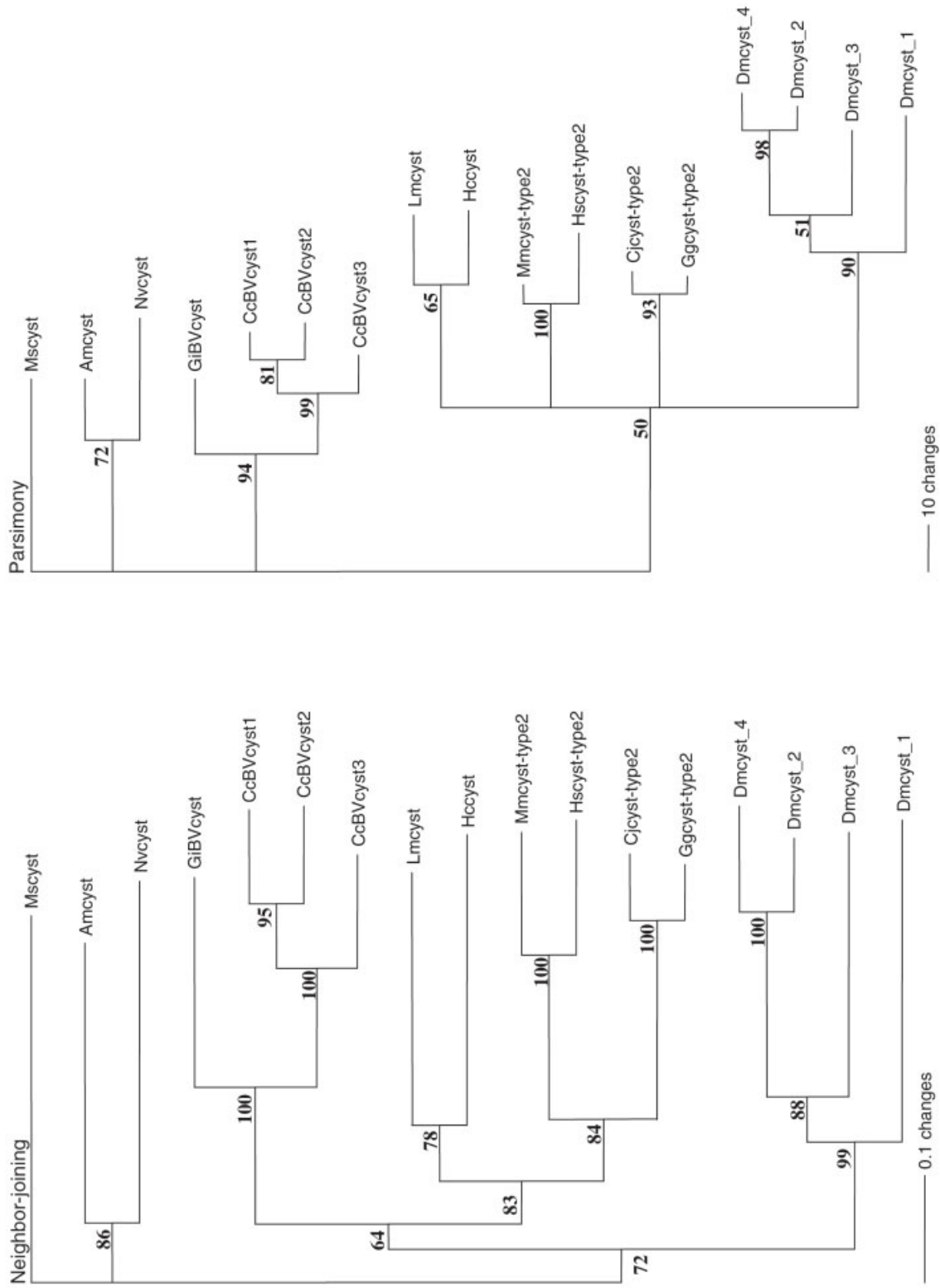


Fig. 3. Phylogenetic analysis of bracovirus cystatin proteins. Neighbour-joining tree generated using PAUP4 from the alignment of the cystatin sequences retrieved by BLASTP analysis using CcBV protein sequences as queries. The alignments were obtained using ClustalX and manual corrections. The *M. sexta* sequence was used as outgroup.

TABLE 3. Evolutionary Distance Estimations (p-Distance) of Cellular Protein Sequences from Insects and Human*

Proteins	<i>A. mellifera</i>	<i>C. congregata</i>	<i>T. castaneum</i>	<i>A. gambiae</i>	<i>A. aegypti</i>	<i>D. melano-</i> <i>gaster</i>	<i>D. pseudo-</i> <i>obscura</i>	<i>H. sapiens</i>	Overall average
Mago	XP_001120074 0.000	CAM34505 0.021	XP_967704 0.063	XP_318556 0.056	EAT36344 0.042	NP_476636 0.070	— —	NP_060518 0.091	0.066
Med31	XP_393244 0.000	CAM34512 0.055	XP_968682 0.155	XP_307924 0.182	EAT44514 0.191	NP_649483 0.236	— —	NP_057144 0.209	0.209
Mask	XP_393472 0.000	CAM34503 0.059	XP_974489 0.111	EAA08897 0.209	/ /	NP_788734 0.216	— —	NP_065741 0.359	0.250
Arf102F	XP_393787 0.000	CAM34509 0.060	XP_968567 0.153	XP_311973 0.080	AAW21993 0.160	NP_524631 0.067	— —	AAP36805 0.147	0.136
Cora	XP_392323 0.000	CAM34506 0.063	XP_973434 0.243	EAA00154 0.330	EAT40392 0.313	NP_523791 0.411	— —	/ /	0.288
Ari	XP_396912 0.000	CAM34511 0.075	XP_971560 0.109	XP_309801 0.184	EAT34761 0.177	NP_523399 0.211	— —	BAD92669 0.211	0.154
Hsp90	XP_623939 0.000	CAM34507 0.079	XP_967904 0.091	XP_308800 0.085	EAT36186 0.098	AAB46677 0.116	— —	ABC40730 0.128	0.101
Rps4	XP_623050 0.000	CAM34499 0.088	XP_969262 0.092	XM_308886 0.197	ABF18051 0.201	NP_729871 0.163	— —	AAH07308 0.247	0.192
Dlst	XP_392679 0.000	CAM34514 0.115	XP_971313 0.200	XP_309608 0.195	EAT45999 0.190	NP_650064 0.205	— —	CAG33008 0.260	0.197
Stathmin	XP_625029 0.000	CAM34500 0.161	XP_975021 0.360	XP_319067 0.416	EAT34958 0.429	NP_723158 0.419	— —	NP_110422 0.806	0.502
Gpx	XP_623095 0.000	CAM34513 0.168	XP_969937 0.327	XP_313166 0.461	AAQ02888 0.387	AAO41409 0.479	— —	NP_002076 0.464	0.435
Fis1	XP_623649 0.000	CAM34497 0.208	XP_972333 0.338	/ /	EAT39534 0.486	/ /	EAL29405 0.490	NP_057152 0.477	0.443
Hsp40	XP_394545 0.000	CAM34508 0.214	XP_967556 0.346	XP_319428 0.355	EAT41457 0.361	AAC23584 0.379	— —	AAH12115 0.399	0.359
RpL22e	XP_625009 0.000	CAM34502 0.217	XP_973501 0.283	XP_558423 0.311	EAT40512 0.277	NP_477134 0.294	— —	XP_001132707 0.403	0.332
GpmA	XP_625114 0.000	CAM34504 0.220	XP_971117 0.254	EAA01768 0.232	EAT42375 0.249	ABC66194 0.226	— —	CAG46544 0.409	0.263
Por	XP_623725 0.000	CAM34510 0.272	XP_967480 0.375	XP_318946 0.366	ABF18270 0.380	NP_476813 0.394	— —	P45880 0.444	0.357
GST	XP_624501 0.000	CAM34501 0.370	XP_967412 0.471	AAP13482 0.481	ABF18476 0.490	AAL48138 0.481	— —	CAD97673 0.583	0.488
Ssb	XP_395300 0.000	CAM34498 0.388	XP_972070 0.537	XP_319705 0.569	EAT45047 0.538	NP_477014 0.575	— —	NP_003133 0.602	0.549

*See Materials and Methods and Results for details.

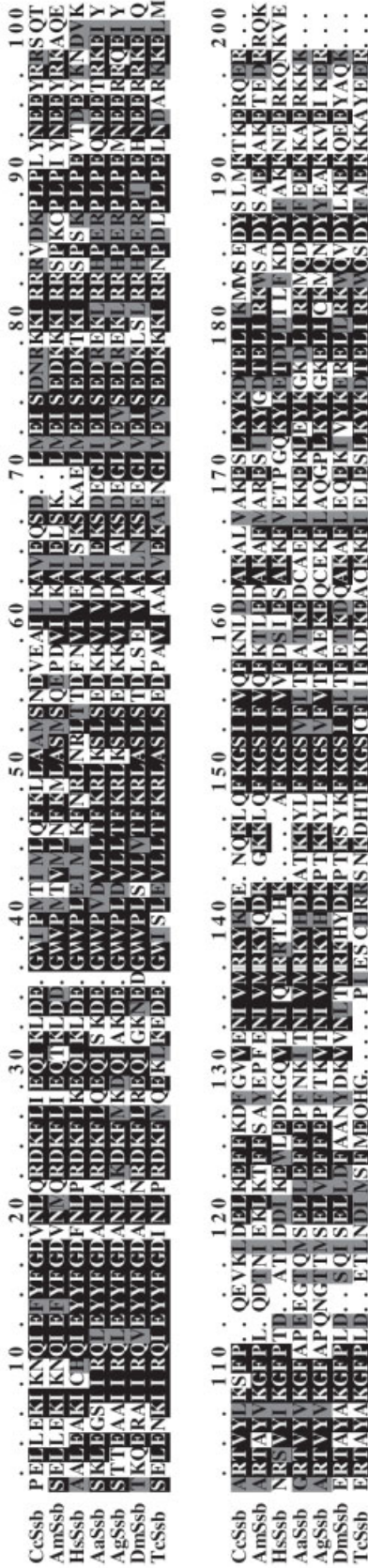
—, sequence not used in p-distance calculation; /, not available in databanks; Mago, mago nashi protein; Med31, mediator of RNA polymerase II transcription, subunit 31; Mask, ankyrin repeat domain protein 17; Arf102F, ADP ribosylation factor; Cora, coracle; Ari, ariadne; Hsp90, heat shock protein 90; Rps4, ribosomal protein S4; Dlst, dihydrolipoamide S-succinyltransferase; Gpx, phospholipid hydroperoxide glutathione peroxidase; Fis1, tetratricopeptide repeat domain protein 11; Hsp40, heat shock protein 40; RpL22e, ribosomal protein L22; GpmA, phosphoglycerate mutase; Por, Porin; GST, glutathione S-transferase; Ssb, La autoantigen.

DISCUSSION

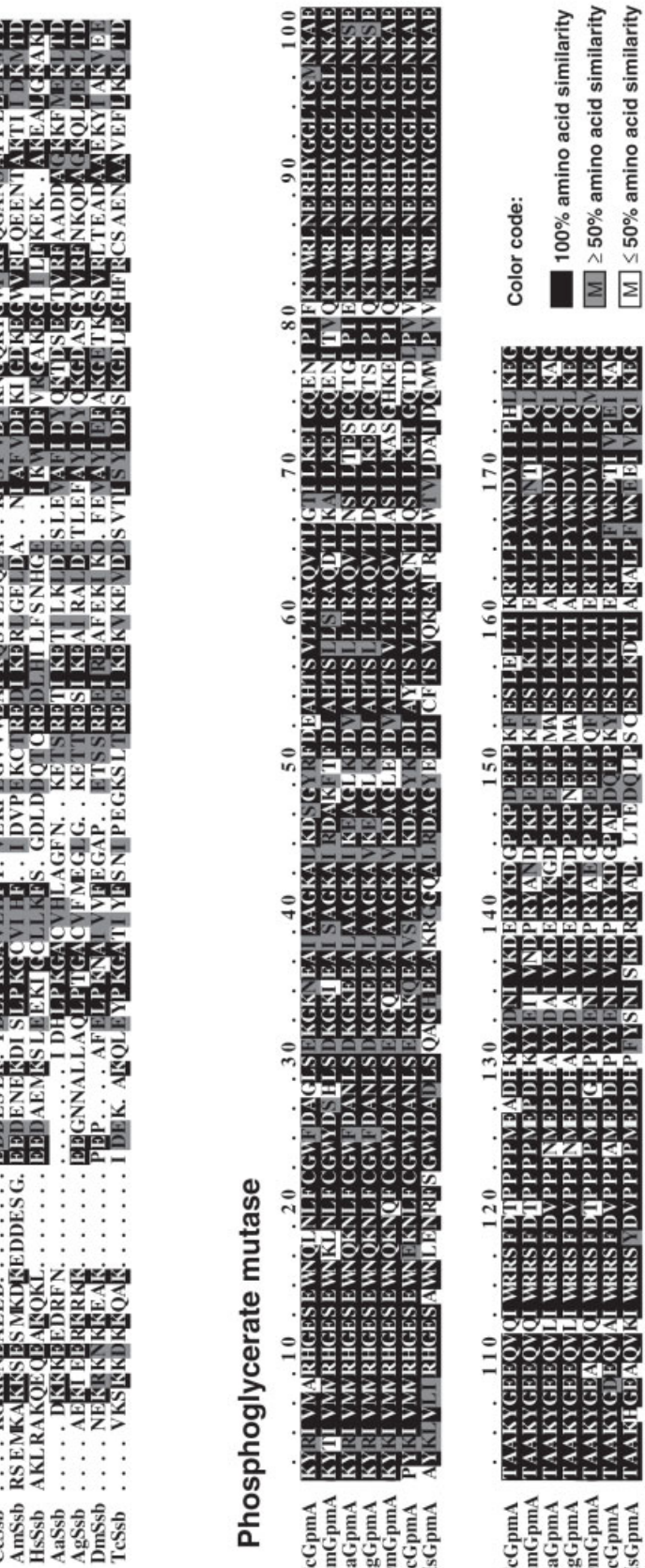
Since many BV virulence products are made up of a protein domain conserved in metazoans, we compared BV sequences to cellular proteins. Sur-

prisingly, they are not particularly close to insect proteins. The lack of a clear link between BV virulence and insect proteins might be due to the fact that BV sequences are evolving at a very fast rate. In support of this interpretation, we could show

La autoantigen



Phosphoglycerate mutase



Color code:
 ■ 100% amino acid similarity
 ■ ≥ 50% amino acid similarity
 □ ≤ 50% amino acid similarity

Fig. 4. Alignment of proteins displaying a relatively low (La autoantigen) or intermediate conservation (Phosphoglycerate mutase) from *C. congregata* (Cc), *A. mellifera* (Am), *T. castaneum* (Tc), *A. gambiae* (Ag), *A. aegypti* (Aa), *D. melanogaster* (Dm), and *H. sapiens* (Hs).

that BV virulence PTPs and I κ B-like proteins are more distant among themselves than homologous proteins from different insect orders. A faster rate of evolution has been shown to act on parasitic Hymenoptera compared to Diptera (Castro et al., 2002). This faster evolution might be explained by founder effects during the extraordinary level of radiation that occurred within the parasitic Hymenoptera, or by specific features in the physiology of the host endoparasitoid relationship in the hymenopteran compared to the dipteran (Castro et al., 2002; Downton and Austin, 1995). In particular, dipteran endoparasitoids larvae breathe outside the host while hymenopteran endoparasitoids live totally engulfed in their host. The faster rate of evolution has been shown to act on mitochondrial genes and if the same phenomenon occurs for chromosomal genes, it might explain in part the divergence observed in BV genes. To determine whether the BV gene divergence was due to a general divergence of *C. congregata* nuclear sequences from those of *A. mellifera* or was a particular feature of CcBV virulence genes, we have analyzed a *C. congregata* cDNA library and identified the homologues of 18 insect genes. The *C. congregata* genes obtained are closely related to those of *A. mellifera*, indicating that the high divergence observed for I κ B-like and PTPs sequences is not a general trend of the parasitoid wasp genes but a specific feature of BV genes. The divergence of BV proteins is most probably a major indication of the dynamic of forces that shape the evolution of polydnviruses and has to be explained in the context of the current hypotheses on the origin and evolution of wasp-bracovirus associations.

The source of virulence genes might be a first clue towards understanding why they have diverged. The wasps harboring BVs form a monophyletic group (Downton and Austin, 1998). All BVs are thus thought to originate from a virus originally integrated in the ancestor wasp of this group (Whitfield, 2002). Viruses are known to pick up cellular genes that are beneficial for their life in infected hosts (Herniou et al., 2003). It is thus likely that some BV virulence genes might have been present in the genome of this ancestor virus.

In agreement with this hypothesis, I κ B-like and PTP sequences are present in almost all the sequenced BVs, except in the partially sequenced *Chelonius inanitus* BV associated with a wasp from the Cheloninae (Annaheim and Lanzrein, 2007). The I κ B-like and PTP genes may have been lost in this particular lineage, where the wasps do not have to face the host immune challenge since they oviposit within the host's eggs.

As an alternative hypothesis, BV virulence genes might have been acquired from the wasp genome after the integration of the ancestor virus. Residing originally in a non-viral region of the wasp genome, they may have been transferred to the proviral form at different times during the radiation of the microgastrid complex, leading to their incorporation into virus particles. In the case of cystatin genes, so far detected only in closely related species (Espagne et al., 2005), the CcBV cystatins lack the intron present in the cellular cystatins and it was thus suggested that the virus acquired the gene via integration of a cDNA into the provirus (Espagne et al., 2005).

Whether BV virulence genes were picked up by the ancestor virus, most probably infectious for Hymenoptera and/or Lepidoptera, or were acquired since the integration in the wasp genome, these virulence genes have in theory an insect origin. The divergence between BV and insect genes may have occurred before or after the integration of the ancestor virus or both.


Genes present in "free viruses" are known to undergo rapid evolution resulting in divergence of sequences. For example, it results from this phenomenon that a virus of vertebrates (ASFV) uses an I κ B-like protein highly divergent from the swine cognate sequence to inhibit the host inflammatory response (Revilla et al., 1998). Part of the divergence observed between BV and insect genes might be explained by a similar phenomenon where the free virus evolved rapidly before integration into the wasp genome. Divergence of BV sequences may also have occurred since the integration in the wasp genome in response to selection pressures imposed by the host-parasite relationship. Interestingly, a recent "adaptive radiation" model (Francino, 2005)

based on experimental work performed on *E. coli* (Kugelberg et al., 2006) proposes that the evolution of a new function may start with the amplification of an existing gene with some level of preadaptation to that function. This model might apply to BVs since a prominent feature of their genomes is their abundance in gene families (Dupuy et al., 2006). Different copies have most likely been produced by duplications of segments of the viral genome, which in the most recent cases are still detectable (Friedman and Hughes, 2006).

The new function might be to interfere with a lepidopteran pathway involved in host defence. The preadapted protein might correspond to a gene product originating from the wasp and having a limited affinity with a lepidopteran molecule involved in defence. Or, in the case of host shift, the preadapted protein might be a BV product already fitted to a lepidopteran target (from the former host) but having to interact with the homologous molecule of another lepidopteran species (the new host). According to the adaptive radiation model, new copies are produced by successive rounds of duplications, to compensate for, by dosage effect, the low affinity of the preadapted protein. Due to the fact that several copies are available, the selection pressure acting on each copy is relaxed and the effect of different mutations can be assessed, until a protein with increased affinity to the new target is produced. Once such a goal is achieved, the other copies become pseudogenes and are eventually lost. Such a process of mutation scanning results in rapid divergence of gene copies and may account for the high divergence of I κ B-like and PTP BV genes as well as for the presence of pseudogenes in the CcBV gene families. An important implication of this model is that the products of the duplicated copies of CcBV do not necessarily have different functions. The fact that different copies are present in the BV genome indicates rather that the process of adaptation to a new target is still ongoing. Comparison of homologous genes in different species will allow for the measurement of the selection pressures operating on BV genes (Dupas et al., 2003) and for the determination of whether

the model of adaptive radiation may offer a general framework to understand the evolution of BV.

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