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# Deep sequencing of *Cotesia vestalis* bracovirus reveals the complexity of a polydnavirus genome

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ABSTRACT

Here we completed the whole genome sequence of *Cotesia vestalis* bracovirus (CvBV) by deep sequencing and compared the genome features of CvBV to those of other polydnaviruses (PDVs). The genome is 540,215 base pairs divided into 35 genomic segments that range from 2.6 to 39.2 kb. Comparison of CvBV with other PDVs shows that more segments are found, including new segments that have no corresponding segments in other phylogenetically related PDVs, which suggests that there might be still more segments not being sequenced in the present known PDVs. We identified eight gene families and five genes in CvBV, including new genes which were first found in PDVs. Strikingly, we identified a putative helicase protein displaying similarity to human Pif1 helicase, which has never been reported for other PDVs. This finding will bring new insights in research of these special viruses.



## Introduction

Parasitoid wasps are insects that develop as parasites of other arthropods as larvae but are free-living as adults. Some parasitoids have evolved an obligate association with viruses in the family Polydnaviridae, which can suppress the immune responses and disrupt the development of the host to create an environment favorable for the development of the parasitoid (Drezen et al., 2003). PDV genomes exist in two forms: the linear proviral form integrated into the genomic DNA of their wasp carrier and the circular episomal form assembled into nucleocapsids during replication in calyx cells (Fleming and Summers, 1991; Gruber et al., 1996). PDVs do not replicate in the wasp's host and the replication only occurs in the ovaries of the female wasps, so the transmission of PDVs relies on the survival of parasitoids and parasitoid survival depends on infection of the host by the virus (Webb and Strand, 2005). This mutualistic association between PDVs and wasps makes PDV a fascinating research model for viral evolution, replication and function.

PDV segments are closed circular DNA molecules ranging in size from 1.5 to over 50 kb and the segment number is ranging from 15 to over 105 (Desjardins et al., 2008; Espagne et al., 2004; Lapointe et al., 2007; Tanaka et al., 2007; Webb et al., 2006). Genomic segments are non-equimolar in abundance with some segments appearing to be present in hypermolar ratios relative to other segments (Beck et al., 2007). PDVs are categorized into two genera: the bracoviruses (BVs) and ichnoviruses (IVs), which are associated with braconid or ichneumonid wasps respectively (Webb, 1998). BVs and IVs differ from one another both morphologically and genetically, which indicates that their association with wasps arose independently (Kroemer and Webb, 2004; Turnbull and Webb, 2002). BVs typically enclose one or more barrel-shaped nucleocapsids per virion surrounded by a single envelope, whereas IVs typically contain one lenticular nucleocapsid per virion surrounded by two membranes (Webb, 1998). BVs are distributed among six subfamilies of Braconidae, (Cardiochilinae, Cheloninae, Khoikhoiinae, Mendesellinae, Microgastrinae and Miracinae). A phylogenetic analysis suggested that BVs carried by members of these wasp subfamilies might have a common origin (Whitfield, 1997). Characterization of genes encoding subunits of a viral RNA polymerase and structural components further indicated that BVs derived from an ancestral nudivirus (Bezier et al., 2009). The phylogenetic relationships among subfamilies in the Ichneumonidae are less clear. Analysis of virion structural components from IVs reveals that the set of structural genes is conserved among wasps associated with IVs and might originate from an ancestral virus (Volkoff et al., 2010). IVs generally have more but smaller segments than BVs (Kroemer and Webb, 2004). IV genomes generally encompass more than 20 DNA segments ranging in size from 2 to 28 kb with genome sizes estimated to range from 75 kb to greater than 250 kb (Webb, 1998). The newly completed genome of *Glypta fumiferanae* ichnoviruses (GfIV) has 105 genome segments with an aggregate genome size of 290 kb, displaying a degree of genome segmentation far greater than that reported for BVs or IVs (Lapointe et al., 2007). BV genomes usually have less than 30 DNA segments ranging in size from 2 to 50 kb with genome sizes estimated to range from 125 kb

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to greater than 567 kb (Desjardins et al., 2008; Espagne et al., 2004; Webb et al., 2006).

Unlike other viral genomes, the encapsidated form of PDV genomes is largely noncoding. The average coding density for *Microplitis demolitor* BV (MdBV) is only 17% with segments ranging from 2% to 43% (Webb et al., 2006), and they lack known viral structural proteins and DNA replication-associated enzymes (Deng et al., 2000). In addition, PDV genomes maintain genes and gene families that are unrelated to other viral genes (Kroemer and Webb, 2004). The variability of PDV gene families indicates that gene duplication is common in the evolution of these viruses (Webb, 1998). Each pair of duplicate genes was categorized as follows: (1) tandem gene duplication, (2) segmental duplication or (3) unresolved between the two hypotheses (Friedman and Hughes, 2006). Because PDVs do not replicate in parasitized hosts, gene duplication and non-equimolar segment ratios might be different strategies adopted by the virus to increase the copy number of essential genes and the levels of gene expression (Webb, 1998).

To date, the genomes of four BVs [*Cotesia congregata* BV (CcBV), MdBV, *Glyptapanteles indiensis* BV (GiBV) and *Glyptapanteles flavicoxis* BV (GfBV)] and three IVs [*Campoletis sonorensis* IV (CsIV), GfIV and *Hyposoter fugitivus* IV (HfIV)] have been completed. Unfortunately, some atypical characteristics of PDVs make genome sequencing complicated. For instance, non-equimolar segmentation makes some low copy segments difficult to be sequenced and internal sequence homologies between segments make gap closing complicated. *Cotesia vestalis* (Haliday), a natural enemy of the diamondback moth, *Plutella xylostella* (Kurdjumov), was previously named *Cotesia plutellae* (Shaw, 2003). The *C. vestalis* BV (CvBV) genome has been partially sequenced using plasmid capture system (Choi et al., 2005). Here, we completed the sequencing of the whole CvBV genome using deep sequencing and compared the genome features of CvBV to those of other PDVs. By this method, we found some new genomic segments from CvBV displaying no similarity to those of other PDVs. On one of these new segments we found a putative helicase protein displaying similarity to human Pif1 helicase. This finding will bring new insights in research of these special viruses.

## Results and discussion

### *General features of the CvBV genome*

After deep sequencing, a total of 186,937 reads counting up to 70,720,255 bases were obtained, which yielded ca. 135× coverage of the genome. Assembly was performed using the GS de novo Assembler software (http://www.454.com/) and produced 269 contigs ranging from 500 base pairs (bp) to 15,370 bp, with a total size of 522,546 bp. For assembling the contigs, we used the genomic sequences of CcBV, CpBV, GiBV and GfBV as reference. Most of the CvBV genomic segments could be confirmed by matching the data to other BVs from *Cotesia* and *Glyptapanteles* species. For the rest of the contigs, their relationship was determined by multiplex PCR. After these contigs were assembled, we did multiplex PCR again to confirm their relationship by designing new primers from different sites. Our results yielded an estimated aggregate genome size for CvBV of 540,215 bp that was divided into 35 DNA segments ranging from 2.6 (Circle 33) to 39.2 kb (Circle 20) (GenBank accession no. HQ009524-HQ009558) (Fig. 1). As shown for other PDVs, the CvBV genome displays a strong A-T bias (65.68%) and a low coding


**Fig. 1.** Diagrammatic representation of sequence and annotation of the CvBV genome. The 35 non-redundant circular CvBV genome segments are represented as linear molecules to visualize segment size relationships within the genome. ORFs and members of gene families have been annotated by color and location to individual genome segments with color key to CvBV shown. Gray regions represent non-coding DNA. Scale bar is in bps.

**Table 1**
Features of the CvBV genes and gene families.

| Parameter | CvBV families | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PTP | BEN | Ank | EP | Ser-rich | RNase T2 | Lectin | Crp | CrV1 | Cyst | Duffy | Histone | DNA helicase |
| Number of related genes | 33 | 9 | 6 | 6 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 |
| Circle no.: no. of related genes | C4:5<br>C6:6<br>C9:2<br>C11:1<br>C12:9<br>C14:4<br>C19:2<br>C28:4 | C1:1<br>C8:1<br>C10:1<br>C13:1<br>C18:1<br>C21:1<br>C26:1<br>C27:2 | C2:1<br>C14:2<br>C17:2<br>C28:1 | C3:1<br>C5:1<br>C7:1<br>C11:1<br>C12:1<br>C32:1 | C13:1<br>C24:1 | C15:1<br>C16:1 | C23:1<br>C29:1 | C13:1<br>C24:1 | C29:1 | C22:1 | C5:1 | C11:1 | C35:1 |
| PDVs in which similar gene families are found | CcBV<br>MdBV<br>GiBV<br>GfBV<br>TnBV<br>GflV | CcBV<br>MdBV | CcBV<br>MdBV<br>GiBV<br>GfBV<br>TnBV<br>GflV<br>CsIV<br>HflV<br>TrIV | CcBV<br>CkBV | None | None | CrBV<br>CcBV | CcBV | CrBV | CcBV | CpBV | CcBV | None |

The features of each gene or gene family are detailed with the circle (C) localization of each gene and the number of related genes on each circle. Other PDVs containing such families are indicated. (PTP, protein tyrosine phosphatases; BEN, BEN domain-coding protein; Ank, ankyrin; EP, early expressed proteins; Ser-rich, Ser-rich proteins; Crp, cysteine-rich proteins; CrV1, CrV1-like protein; Cyst, cystatins; Duffy, Duffy-like protein).

density (27.0%), the values comparable to those reported for CcBV (66% A-T; less than 30% coding density) (Espagne et al., 2004). All DNA segments encoded at least one predicted open reading frame (ORF) >100 amino acids. Circles 30, 31, 32, 33 and 35, which all are smallest genomic segments, encoded only one ORF, while Circle 24, the second largest genomic segment, encoded 13 ORFs.

*General features of predicted ORFs*

In the CvBV genome, we identified 157 ORFs, which we numbered according to the genome segment on which they were found (Fig. 1). Unlike most viral genes, many CvBV genes contained introns (57.3%), which is close to that reported for CcBV (69%) but higher than those for CsIV (10%) and MdBV (14%). This large difference in the percentage of spliced genes is caused mainly by the identification criteria used for ORF prediction. Our previous studies indicated that when using Fgenesh for gene prediction, the *Brugia malayi* settings are more competent for PDV gene prediction, especially for the genes with introns (Chen et al., 2007). In order to improve PDV gene prediction, more experimental data about PDV gene transcription, processing and expression are needed (Gundersen-Rindal and Pedroni, 2006).

Another unique feature of the CvBV genome is the major gene families they encode. The amplification and diversification of genes might be a strategy for wasps to adapt the changing environment. In CvBV, thirteen gene families were identified (Table 1). With 33 members, protein tyrosine phosphatases (PTPs) were the largest family. The large member number reflects their important role in parasitism. Previous studies suggested that not all PTPs are functional and some PTPs function as a phagocytic inhibitor or apoptosis inducer, playing an important role in suppressing insect immune cells (Pruijssers and Strand, 2007; Suderman et al., 2008). PTPs are only found in BV genomes as well as the genome of GflV carried by the banchine ichneumonid *G. fumiferanae* (Lapointe et al., 2007). PTPs are mainly clustered on five segments: C12 (9 PTPs), C6 (6 PTPs), C4 (5 PTPs) C14 (4 PTPs) and C28 (4 PTPs). The second largest CcBV gene family is BEN domain-coding proteins, comprising 9 gene members. BEN domain-coding proteins are a new gene family found in BVs and their functions are still unknown. However, sequence analysis and contextual information suggest that it might act as a DNA-binding protein or an adaptor recruiting chromatin-modifying complexes (Abhiman et al., 2008). The CvBV genome was also found to enclose viral ankyrin (*ank*) genes, which are commonly shared by BVs and IVs. Six ankyrin genes located on 4 segments were found in CvBV. Our previous study characterized one of these ankyrins, CvBV-c17_4, indicating that this protein contains an ankyrin repeat domain with a high degree of similarity with IκB-like genes and it was confirmed to

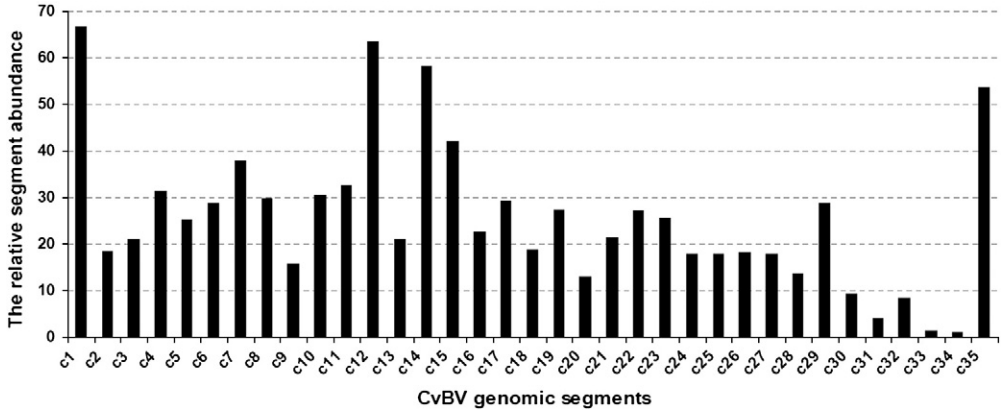


**Fig. 2.** The relative segment abundance of CvBV in calyx fluid. The abundance of the least abundant segment, CvBV-c34, was standardized to a level of 1. The abundance of the other segments was then expressed as an increase relative to the segment control. The relative abundance of each CvBV segment is the mean results for the relative abundance of all contigs the segment contains.

**Table 2**
Similar segments of CvBV in other PDVs by BLASTN search.

| CvBV segment no. | Similar segments in other PDVs, query coverage and max identities | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CcBV | | | GiBV | | | GfBV | | |
| *Group 1* | | | | | | | | | |
| CvBV-c1 | Circle33 | 89% | 89% | Segment 6 | 46% | 87% | Segment 6 | 45% | 82% |
| | Circle9 | 88% | 89% | Segment 4 | 36% | 77% | Segment 4 | 34% | 75% |
| | Circle23 | 33% | 80% | Segment 7 | 14% | 81% | Segment 7 | 13% | 76% |
| | Circle25 | 31% | 82% | | | | | | |
| | Circle20 | 16% | 88% | | | | | | |
| | Circle6 | 12% | 77% | | | | | | |
| CvBV-c8 | Circle20 | 77% | 86% | Segment 7 | 43% | 84% | Segment 7 | 28% | 93% |
| | Circle6 | 35% | 81% | Segment 4 | 15% | 78% | Segment 4 | 15% | 77% |
| | Circle25 | 17% | 78% | Segment 6 | 16% | 77% | Segment 6 | 16% | 77% |
| | Circle23 | 15% | 78% | | | | | | |
| | Circle33 | 14% | 88% | | | | | | |
| | Circle9 | 14% | 88% | | | | | | |
| CvBV-c10 | Circle20 | 78% | 88% | Segment 7 | 43% | 84% | Segment 7 | 25% | 93% |
| | Circle6 | 33% | 81% | Segment 4 | 14% | 78% | Segment 4 | 13% | 77% |
| | Circle25 | 15% | 78% | Segment 6 | 14% | 77% | Segment 6 | 14% | 77% |
| | Circle23 | 14% | 78% | | | | | | |
| | Circle33 | 12% | 88% | | | | | | |
| | Circle9 | 12% | 88% | | | | | | |
| CvBV-c15 | Circle25 | 96% | 87% | Segment 4 | 66% | 80% | Segment 4 | 58% | 76% |
| | Circle23 | 59% | 92% | Segment 6 | 49% | 75% | Segment 6 | 51% | 75% |
| | Circle33 | 34% | 80% | Segment 7 | 23% | 81% | Segment 7 | 19% | 79% |
| | Circle9 | 34% | 80% | | | | | | |
| | Circle20 | 22% | 80% | | | | | | |
| | Circle6 | 20% | 81% | | | | | | |
| | Circle19 | 18% | 90% | | | | | | |
| | Circle30 | 14% | 79% | | | | | | |
| CvBV-c16 | Circle23 | 93% | 90% | Segment 6 | 56% | 97% | Segment 6 | 54% | 92% |
| | Circle25 | 60% | 89% | Segment 4 | 50% | 79% | Segment 4 | 50% | 73% |
| | Circle9 | 36% | 80% | Segment 7 | 18% | 84% | Segment 7 | 15% | 77% |
| | Circle33 | 34% | 80% | | | | | | |
| | Circle6 | 16% | 86% | | | | | | |
| | Circle20 | 16% | 80% | | | | | | |
| *Group 2* | | | | | | | | | |
| CvBV-c22 | Circle19 | 81% | 93% | Segment 1 | 21% | 89% | Segment 1 | 25% | 86% |
| | Circle30 | 39% | 79% | Segment 5 | 18% | 80% | Segment 3 | 20% | 89% |
| | Circle25 | 36% | 84% | Segment 3 | 17% | 89% | Segment 5 | 17% | 87% |
| | Circle13 | 14% | 93% | Segment 4 | 11% | 76% | Segment 4 | 10% | 73% |
| | Circle2 | 11% | 94% | | | | | | |
| CvBV-c23 | Circle30 | 60% | 92% | Segment 5 | 37% | 80% | Segment 5 | 36% | 89% |
| | Circle18 | 14% | 84% | Segment 1 | 19% | 78% | Segment 1 | 21% | 88% |
| | Circle13 | 9% | 80% | | | | | | |
| CvBV-c29 | Circle13 | 45% | 90% | Segment 5 | 21% | 77% | Segment 1 | 19% | 89% |
| | Circle18 | 18% | 90% | Segment 1 | 17% | 89% | Segment 5 | 18% | 78% |
| *Group 3* | | | | | | | | | |
| CvBV-c2 | Circle15 | 87% | 88% | Segment 15 | 54% | 87% | Segment 15 | 41% | 85% |
| | Circle35 | 29% | 82% | | | | Segment 12 | 21% | 81% |
| CvBV-c3 | Circle35 | 38% | 84% | Segment 15 | 31% | 80% | Segment 15 | 29% | 75% |
| | Circle15 | 20% | 86% | | | | Segment 12 | 19% | 79% |
| *Group 4* | | | | | | | | | |
| CvBV-c4 | Circle10 | 80% | 89% | Segment 23 | 63% | 82% | Segment 22 | 54% | 80% |
| | Circle17 | 42% | 91% | Segment 22 | 59% | 81% | Segment 23 | 51% | 82% |
| | | | | Segment 28 | 33% | 80% | Segment 28 | 29% | 80% |
| CvBV-c6 | Circle17 | 82% | 93% | Segment 22 | 65% | 85% | Segment 22 | 58% | 85% |
| | Circle10 | 49% | 89% | Segment 23 | 53% | 80% | Segment 23 | 45% | 80% |
| | | | | Segment 28 | 23% | 80% | Segment 28 | 19% | 79% |
| CvBV-c9 | Circle4 | 79% | 90% | Segment 28 | 26% | 81% | Segment 28 | 28% | 89% |
| | Circle10 | 19% | 91% | Segment 22 | 13% | 92% | Segment 22 | 12% | 90% |
| | Circle17 | 10% | 91% | Segment 23 | 13% | 93% | Segment 23 | 11% | 86% |
| *Group 5* | | | | | | | | | |
| CvBV-c20 | Circle32 | 24% | 83% | Segment 10 | 48% | 84% | Segment 10 | 40% | 78% |
| | | | | Segment 13 | 26% | 75% | Segment 13 | 24% | 85% |
| CvBV-c24 | Circle32 | 79% | 90% | Segment 13 | 33% | 81% | Segment 13 | 30% | 82% |
| | Circle18 | 14% | 90% | Segment 10 | 32% | 82% | Segment 10 | 29% | 90% |
| CvBV-c13 | Circle18 | 83% | 91% | Segment 13 | 14% | 81% | Segment 13 | 14% | 80% |
| | Circle32 | 20% | 89% | Segment 19&21 | 27% | 76% | Segment 19 | 25% | 74% |
| | | | | | | | Segment 17 | 8% | 79% |

*(continued on next page)*

**Table 2** (*continued*)

| CvBV segment no. | Similar segments in other PDVs, query coverage and max identities | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CcBV | | | GiBV | | | GfBV | | |
| *Group 6* | | | | | | | | | |
| CvBV-c21 | Circle3 | 41% | 84% | Segment 14 | 46% | 82% | Segment 14 | 43% | 77% |
| | | | | Segment 11 | 45% | 76% | Segment 11 | 35% | 76% |
| CvBV-c27 | Circle3 | 75% | 87% | Segment 11 | 44% | 85% | Segment 11 | 46% | 92% |
| | | | | Segment 14 | 49% | 81% | Segment 14 | 43% | 82% |
| *Group 7* | | | | | | | | | |
| CvBV-c11 | Circle7 | 70% | 94% | Segment 24 | 61% | 89% | | | |
| CvBV-c14 | Circle26 | 73% | 91% | Segment 30 | 64% | 79% | | | |
| | Circle7 | 8% | 94% | Segment 24 | 61% | 77% | | | |
| CvBV-c33 | Circle7 | 74% | 88% | Segment 24 | 81% | 88% | | | |
| *Group 8* | | | | | | | | | |
| CvBV-c5 | Circle5 | 81% | 89% | Segment 8 | 37% | 80% | Segment 9 | 5% | 80% |
| CvBV-c32 | Circle8 | 41% | 74% | Segment 8 | 21% | 81% | Segment 9 | 23% | 79% |
| | Circle21 | 24% | 76% | | | | | | |
| | Circle5 | 15% | 79% | | | | | | |
| *No similarity* | | | | | | | | | |
| CvBV-c7 | Circle31 | 52% | 94% | Segment 5 | 17% | 78% | Segment 5 | 12% | 75% |
| | Circle2 | 50% | 94% | Segment 3 | 8% | 82% | Segment 3 | 9% | 84% |
| CvBV-c12 | Circle1 | 85% | 91% | Segment 25 | 58% | 82% | Segment 24 | 42% | 86% |
| | | | | Segment F | 58% | 80% | Segment 20 | 37% | 83% |
| | | | | Segment 20 | 43% | 85% | | | |
| CvBV-c17 | | | | Segment 19&21 | 64% | 81% | Segment 21 | 69% | 78% |
| CvBV-c18 | Circle12 | 93% | 92% | Segment 29 | 66% | 85% | Segment 29 | 59% | 89% |
| CvBV-c19 | | | | | | | | | |
| CvBV-c25 | Circle11 | 87% | 95% | | | | | | |
| CvBV-c26 | Circle6 | 56% | 88% | | | | | | |
| CvBV-c28 | Circle14 | 89% | 90% | Segment 26 | 85% | 80% | Segment 26 | 72% | 89% |
| CvBV-c30 | | | | | | | | | |
| CvBV-c31 | Circle4 | 10% | 85% | | | | | | |
| CvBV-c34 | Circle22 | 80% | 93% | | | | | | |
| | Circle36 | 52% | 86% | | | | | | |
| CvBV-c35 | | | | | | | | | |

be present within the nuclear region in hemocytes of the parasitized *P. xylostella* larvae after parasitism (Shi et al., 2008a). The fourth gene family encodes for six early-expressed proteins (EPs), among which five proteins are EP1-like protein and one is EP2-like protein. The EP genes encode secreted, glycosylated proteins that are expressed within 30 min in host and accumulate to comprise over 10% of total hemolymph proteins by 24 h post-parasitization (p.p.) (Harwood et al., 1994). The expression of EPs could induce significant reduction in total hemocyte numbers and suppress host immune response presumably by its hemolytic activity during parasitization (Kwon and Kim, 2008). Ser-rich proteins encode a serine-rich region and are mainly expressed in granulocytes (Shi et al., 2008b). RNase T2 is a widespread family of secreted RNases found in every organism examined thus far and some data showed that a fusion of the BEN domain to an RNase T2 domain in CpBV-S3 played an important role in inducing host immunosuppression (Park and Kim, 2010). In previous study, BEN and an RNase T2 domains were not identified but described as hypothetical proteins (Choi et al., 2005). We also found two lectins, two cysteine-rich proteins (Crp), one CrV1-like protein, one cystatin (Cyst) and one Duffy-like protein in CvBV. All these proteins were also found in other BVs, like CcBV and CrBV.

Like other PDVs, database searches failed to identify any genes in the CvBV genome with homology to genes that encodes structural proteins. However, on segment CvBV-c35 we identified an ORF which encode a protein displaying similarity to human Pif1 helicase with a high *E*-value of e-111, indicating that this protein might be involved in DNA replication. It was designated here as helicase-like protein. This ORF has a size of 4155 bp and is composed of four exons and three introns. The putative protein comprises 1384 amino acids with a predicted molecular mass of 158.5 kDa. Pif1 helicase and its paralogue Rrm3 helicase are involved in telomere DNA replication (Zhang et al., 2006). PDVs do not replicate in the wasp's host and the replication only occurs in the ovaries of the female wasps, so the function of this helicase-like protein is worthy for determination. In GfIV, researchers identified seven NTPase-like proteins displaying the pox-D5 domain of related NTPases found in several large dsDNA viruses, including the vaccinia virus D5 protein, although the levels of similarity of these GfIV gene products to viral proteins were not very high (Lapointe et al., 2007). The helicase-like protein in CvBV is located on segment CvBV-c35, which has no homologous sequences in other BVs. So there might be some similar genes in other PDVs not being uncovered.

### *The relative segment abundance of CvBV genome*

As with other PDVs, CvBV genomic segments vary in abundance. We assessed the relative abundance of these segments by the number of 454 reads assembled in each contig per kb. Segment C34 was the least abundant CvBV DNA and therefore was used as a calibrator that was standardized to a value of 1. In turn, segments C1 (14.2 kb), C12 (21.4 kb), C14 (13.7 kb), C35 (5.7 kb), C15 (10.9 kb) and C7 (17.5 kb) were 66.5-, 63.5-, 58.1-, 53.7-, 42.1- and 37.8-fold more abundant, respectively, than C34, whereas the other segments were 1.33- to 32.5-fold more abundant (Fig. 2). The six most abundant segments account for more than 35% of the viral DNAs in calyx fluid. The two largest segments, C20 (39.2 kb) and C24 (36.8 m kb), are only 13.0- and 18.0-fold more abundant, respectively. Non-equimolar segment ratios are one of the strategies adopted by the virus to increase the copy number of essential genes and the levels of gene expression in the absence of virus replication (Webb and Cui, 1998). Studies on MdBV revealed that high abundance segments primarily encode important virulence

factors, like PTPs and ankyrins. For CvBV, the most abundant segment CvBV-c1 encodes two conserved hypothetical proteins with unknown function, whereas the second and third most abundant segments, CvBV-c12 and -c14, encode 13 PTPs and 2 ankyrins. The fourth segment encodes the unique helicase-like gene we identified. No genes with known functions are found in the least abundant segments.

*Segmental relationship among BVs*

To determine the relationship among BV genomic segments, homologous segments from other PDVs were retrieved by BLASTN analysis using CvBV genomic segments as query. A one to one correspondence between the genomic segments of CvBV, CcBV, GiBV and GfBV is proposed based on high similarities (Table 2). CvBV genomic segments have high identities with those of CcBV. For example, segment CvBV-c15 shares 96% query coverage with Circle 25 of CcBV, indicating that these two segments are closely related. The number of CcBV segments is 30, less than that of CvBV. Considering the high identities between CvBV and CcBV segments, it is suggested that there are still some CcBV segments not being sequenced. This one-to-one correspondence relationship could facilitate the genome sequence of other BVs. For some CvBV segments, more than one homologous segment from CcBV, GiBV and GfBV is retrieved, indicating that internal sequence homologies between segments are present in CvBV. For instance, six homologous segments from CcBV for CvBV-c1 were found and the query coverage ranges from 12% to 89%. So we categorized CvBV segments into 8 groups based on the homologous segments they shared. The segments in the same groups (Table 2) share some homologous sequences, which is also a strategy used by the virus to increase the copy number of essential genes and the levels of gene expression.

*Phylogenetic analyses*

To examine the relationships between BVs and IVs, we conducted two types of phylogenetic analyses: (i) a PTP phylogeny based on all known PTPs from five BVs (CvBV, CcBV, MdBV, GiBV and GfBV) and one IV (GflV); and (ii) an ankyrin phylogeny based on all known ankyrin proteins from five BVs (CvBV, CcBV, MdBV, GiBV and GfBV) and four IVs (GflV, CsIV, HflV and TrIV). The results from this analysis were close to those from Falabella et al. (2007) and Lapointe et al. (2007). Phylogenetic analysis of all PTPs revealed that CvBV PTPs

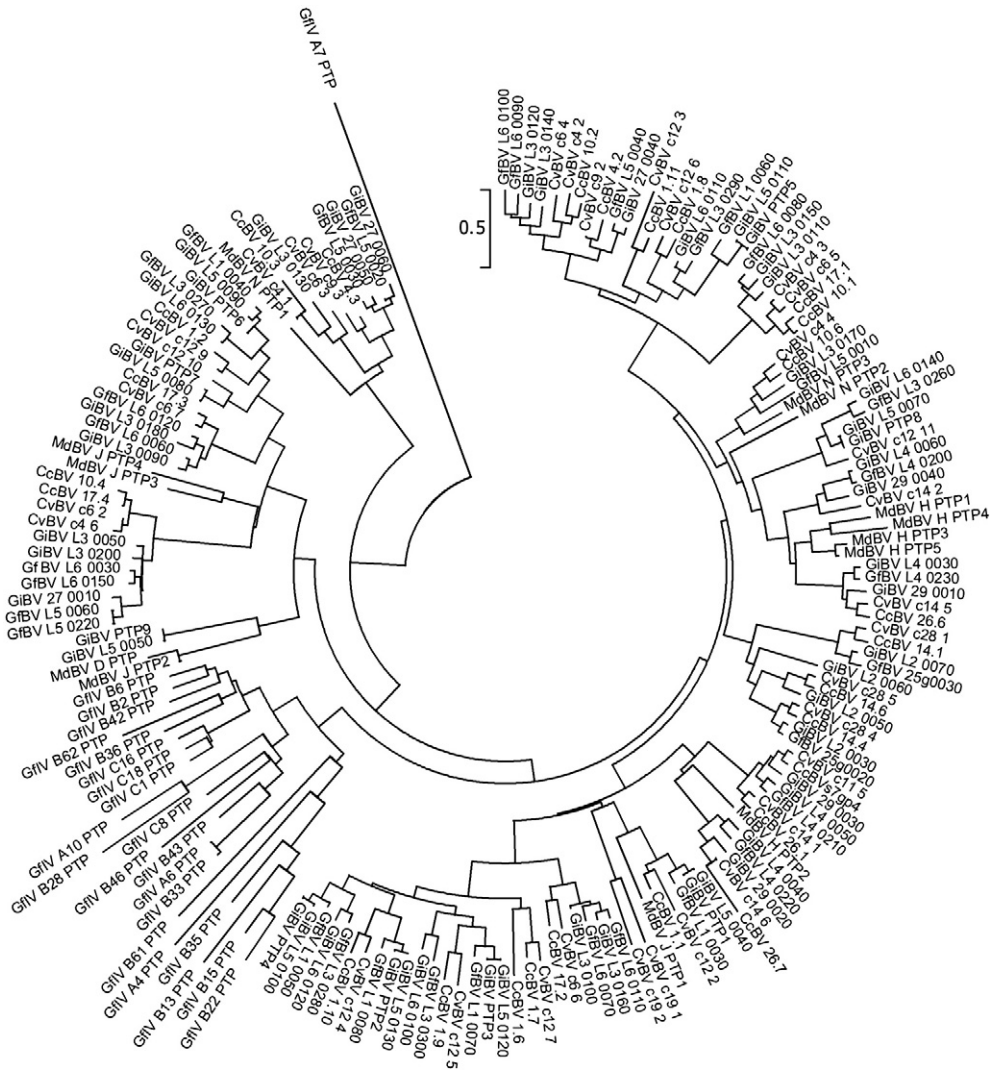


**Fig. 3.** Phylogenetic analysis of PTPs from the four BV and one IV genomes. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 53.47202211 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 289 positions in the final data set. Phylogenetic analyses were conducted in MEGA4.

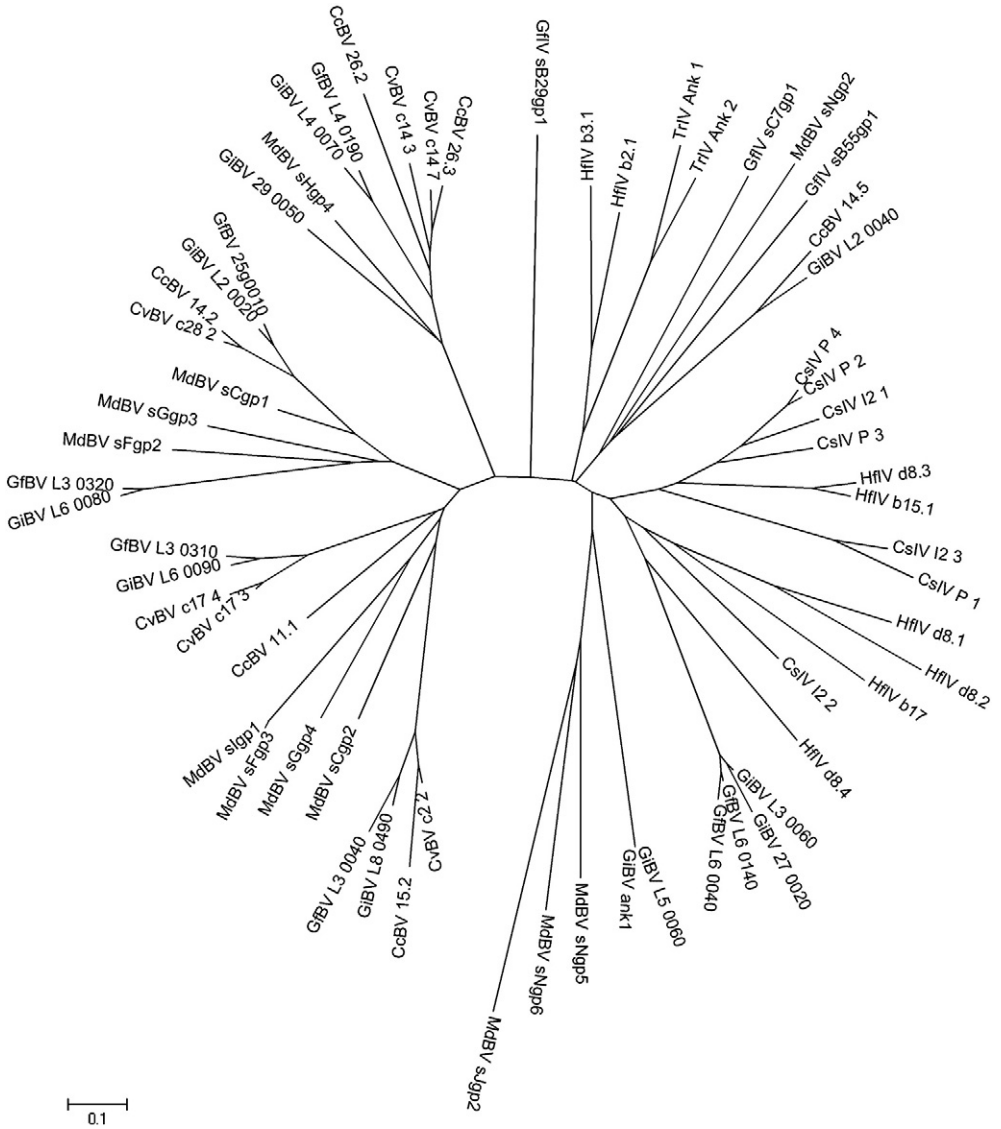


**Fig. 4.** Phylogenetic analysis of ankyrins from the four BV and four IV genomes. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 17.80265794 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 67 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

dispersed into all lineages except for the GfIV lineage (Fig. 3). This result proposes that the two wasp groups acquired the viruses independently. PTPs from corresponding segments of CvBV and CcBV always clustered into the same group, further indicating that these two BVs are closely related. Four pairs of gene duplicates were identified in CvBV PTPs. CvBV-c4_2, _3 and _6 were gene duplicates of CvBV-c6_4, _5 and _2 respectively. They favored the hypothesis of segmental gene duplication because these PTPs cluster on two segments. The pair of CvBV-c19_1 and _2 favored the hypothesis of tandem gene duplication since both genes cluster on the same segment. Phylogenetic analysis of the ankyrins showed that all ankyrins from BVs did not form a lineage separated from the IV ankyrins (Fig. 4). Only tandem gene duplication occurred in CvBV ankyrins and the duplicate pairs were CvBV-c17_3 and _4, CvBV-c14_3 and _7. Gene duplication is very common in PDVs and is also a strategy used by PDVs to increase gene copies (Friedman and Hughes, 2006). Both segmental and tandem gene duplication occurred in PTPs, while only tandem gene duplication occurred in CvBV ankyrins.

## Conclusions

PDVs are a special group of DNA viruses because of their segmented double-stranded DNA genomes and the obligately mutualistic relationship with their hymenopteran hosts. Genome sequence of PDVs would bring insights into our understanding about viral origin, evolution and the replication mechanism used by virus. But the PDV genomes are very difficult to be fully sequenced because the genome is segmented and internal sequence homologies appear between segments. The number of genomic segments is hard to estimate. Different segments of similar size make it difficult to determine whether a given band is a high abundance segment or a mixture of segments (Beck et al., 2007). For example, 13 segments were determined by field inversion gel analysis of the GiBV viral genome, and the viral genome size was estimated to be approximately 250 kb (Chen and Gundersen-Rindal, 2003), but after a whole genome shotgun sequencing, 29 segments were discovered and the genome size was renewed to be 517 kb (Desjardins et al., 2008). The genome of CvBV has been partially sequenced using plasmid capture

system and 24 segments with an aggregated genome size of 351 kb were determined, but the segments with the size over 30 kb are not be covered due to the restriction of this system (Choi et al., 2009; Choi et al., 2005). So we conducted a deep sequencing approach to complete this genome. The complete genome sequence of CvBV presents some new features for PDVs. The close relationship between BVs will facilitate the characterization of other BV genomes. Strikingly, we identified a putative helicase protein displaying similarity to human Pif1 helicase, which has never been reported for other PDVs. This finding will bring new insights in research of these special viruses.

## Materials and methods

### *Insect rearing*

*C. vestalis* and its host *P. xylostella* were maintained as previously described (Chen et al., 2008). Briefly, individual *P. xylostella* larva was exposed to a single *C. vestalis* female within a 10 mm × 80 mm test tube to ensure 100% parasitization. After a single oviposition was observed, each parasitized larva was removed from the test tube and reared on cabbage. Once emerged, the wasp was fed with 20% (vol./vol.) honey water. Both *C. vestalis* and its host were maintained at 25 ± 1 °C, 65% relative humidity under 14 h light: 10 h dark.

### *Virus purification and viral DNA isolation*

PDV virions and viral DNA were collected from 400 to 500 2-day-old female wasps as previously described by Webb and Cui (1998), and Beck et al. (2007). Briefly, each pair of ovaries was dissected in a 10 μl drop of cold PBS and punctured individually with forceps, causing the calyx fluid to diffuse into the PBS drop. The calyx fluid was filtered through a 0.45 μm filter to remove eggs and cellular debris. The filter-purified viral fluids were first incubated with DNase I to eliminate the contamination of genomic DNA from wasp. After removing the DNase I, the fluids were incubated in an equal volume of extraction buffer (4% sarcosyl, 1% SDS, 50 mM EDTA, 10 mM Tris, 0.2 M NaCl, 20 μg RNase $ml^{-1}$, 50 μg proteinase K $ml^{-1}$) at 50 °C for 2 h, followed by two extractions with ultrapure buffer-saturated phenol (Invitrogen). DNA was precipitated in 2 volumes of cold ethanol and centrifuged at 20,000*g* for 30 min. The viral DNA pellet was resuspended in TE buffer and stored at 4 °C.

### *Genome sequencing*

The genome of CvBV was sequenced using massively parallel pyrosequencing technology (454 GS FLX) (Margulies et al., 2005). Assembly was performed using the GS de novo Assembler software (http://www.454.com/) and produced 269 contigs ranging from 500 bp to 15,370 bp, with a total size of 522,546 bp. Relationship of the contigs was firstly determined by comparing the contigs with genome of CpBV using blastn, and then validated by PCR. The relationship of remaining contigs was determined by multiplex PCR (Tettelin et al., 1999). Primers of both ends of each contig were pooled into different groups, and PCR amplication was performed using each two groups as primers. PCR products were separated on 1% agarose gel and the brightest DNA bands were extracted for sequencing. The sequences of each PCR product were aligned with contig sequences to determine the relationship between contigs. Once the relationship was determined, gaps were then filled in by sequencing the PCR products using ABI 3730xl capillary sequencers. Finally, the sequences were assembled using Phred, Phrap and Consed software packages (http://www.genome.washington.edu), and low quality regions of the genome were resequenced. Each circular segment was confirmed by multiplex PCR and sequencing.

### *Annotation*

Putative open reading frames (ORFs) were predicted using FGENESV (http://linux1.softberry.com/berry.phtml) and GENSCAN (http://genes.mit.edu/GENSCAN.html). Database searches were performed using NCBI's BLASTN, BLASTX, and BLASTP (www.ncbi.nlm.nih.gov/BLAST/). Functional annotation of ORFs was performed through searching against GenBank's non-redundant (nr) protein database using BLASTP (Altschul et al., 1997). Regulatory motifs were identified using the PFAM database (www.sanger.ac.uk/Software/Pfam).

### *Sequence analyses*

Pyrosequencing produces a huge number of reads such that the more abundant a given sequence is, the more are the reads one obtains. So here the relative abundance of CvBV segments is approximately represented by the number of 454 reads assembled in each contig per kb. The relative abundance of each CvBV segment is the mean results for the relative abundance of all contigs the segment contains. The abundance of the least abundant segment, C34, was standardized to a level of 1. The abundance of the other segments was then expressed as an increase relative to the segment C34 control.

Homologous segments from other PDVs were retrieved by BLASTN analysis using CvBV genomic segments as query. The homologous segments were selected and the query coverage and max identities were recorded.

### *Phylogenetic analyses*

For the phylogenetic analysis of ank and PTP genes, all of the sequences were aligned using Clustal X version 1.81 (Benner et al., 1994). Portions of the sequences that did not correspond among gene copies were then pruned. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) in MEGA4 (Tamura et al., 2007). Bootstraping with 1000 replicates was conducted to evaluate the tree branches (Felsenstein, 1985). The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992) and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option).

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