

# Characterization of *Chelonus inanitus* polydnavirus segments: sequences and analysis, excision site and demonstration of clustering

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Polydnaviruses (genera *Ichnovirus* and *Bracovirus*) have a segmented genome of circular double-stranded DNA molecules, replicate in the ovary of parasitic wasps and are essential for successful parasitism of the host. Here we show the first detailed analysis of various segments of a bracovirus, the *Chelonus inanitus* virus (CiV). Four segments were sequenced and two of them, CiV12 and CiV14, were found to be closely related while CiV14.5 and CiV16.8 were unrelated. CiV12, CiV14.5 and CiV16.8 are unique while CiV14 occurs also nested in another larger segment. All four segments are predicted to contain genes and predictions could be substantiated in most cases. Comparison with databases revealed no significant similarities at either the nucleotide or amino acid level. Inverted repeats with identities between 77% and 92% and lengths between 26 bp and 100 bp were found on all segments outside of predicted genes. Hybridization experiments indicate that CiV12 and CiV14 are both flanked by other virus segments, suggesting that proviral CiV segments are clustered in the genome of the wasp. The integration/excision site of CiV14 was analysed and compared to that of CiV12. On both termini of proviral CiV12 and CiV14 as well as in the excised circular molecule and the rejoined DNA a very similar repeat of 14 bp was found. A model to illustrate where the terminal repeats might recombine to yield the circular molecule is presented. Excision of CiV12 and CiV14 is restricted to the female and sets in at a very specific time-point in pupal–adult development.

## Introduction

Polydnaviruses are characterized by their unique genome structure consisting of multiple segments of double-stranded circular DNA and by their association with parasitic wasps of the families Ichneumonidae and Braconidae; accordingly they are classified as the genera *Ichnovirus* and *Bracovirus* (Stoltz *et al.*, 1995). Ichnoviruses and bracoviruses have distinct morphologies and appear to be evolutionarily unrelated (Whitfield, 1997). Polydnaviruses are essential for survival of the parasitoid larva and are involved in abrogation of the host's immune reaction (reviewed in Lavine & Beckage, 1995; Stettler *et al.*, 1998) and in various aspects of host

regulation (reviewed in Lawrence & Lanzrein, 1993). The genome of polydnaviruses is integrated in the wasp's genome and the viruses replicate from the integrated proviral DNA in nuclei of the calyx cells of the wasp ovary. The integration/excision sites have been analysed for two segments of the ichnovirus of *Campoletis sonorensis* (Fleming & Summers, 1991; Cui & Webb, 1997) and two bracovirus segments (Gruber *et al.*, 1996; Savary *et al.*, 1997) and they are all characterized by direct terminal repeats of variable length and similarity. The viruses are secreted from the calyx cells into the oviduct and injected into the host egg or larva along with the parasitoid egg (Stoltz *et al.*, 1995; Webb, 1998). In the parasitized host polydnaviruses do not replicate but expression of viral genes has been documented in several systems (reviewed in Webb, 1998). In the ichnovirus of *C. sonorensis* several segments have been sequenced and various gene families have been described and characterized (Cui & Webb, 1997; reviewed in Webb & Cui, 1998). In this ichnovirus and that of *Hyposoter fugitivus* complex homologies within and between segments have been

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observed and some small segments were shown to be nested in larger ones (Xu & Stoltz, 1993; Cui & Webb, 1997). Much less is known about bracovirus genome organization and gene families.

We are working with the bracovirus of the egg-larval parasitoid *Chelonus inanitus* (CiV) and have shown that its genome consists of at least 10 segments with sizes of between 7 and 31 kbp which appear to be singly encapsidated (Albrecht *et al.*, 1994). For a 12 kbp segment (CiV12) integration into the wasp's genome has been demonstrated as well as stage-specific excision and rejoining of flanking regions (Gruber *et al.*, 1996). Transcriptional activity has been analysed for three segments (CiV12, CiV14 and CiV16.8) and was seen to increase in the later phase of parasitization (Johner *et al.*, 1999). Here we show the complete sequences of four CiV segments (CiV12, CiV14, CiV14.5 and CiV16.8) and their analysis. We demonstrate that proviral CiV12 and CiV14 are flanked by other viral segments in the wasp's genome and that CiV14 is nested in a larger segment. We also show the sequence of the integration/excision site of CiV14 and compare it with that of CiV12 and that of a *Cotesia congregata* bracovirus segment. Furthermore, we demonstrate that excision of CiV14 and CiV12 sets in simultaneously at a particular stage of pupal-adult development.

## Methods

■ **Insects.** *C. inanitus* (Braconidae, Hymenoptera) is a solitary egg-larval parasitoid which was reared on its natural host *S. littoralis* (Noctuidae, Lepidoptera). Adult *S. littoralis* were kindly given to us by Novartis. Details about the biology and rearing of parasitoid and host are given in Grossniklaus-Bürgin *et al.* (1994).

■ **DNA isolation and sequencing.** Calyx DNA was collected and isolated as described in Gruber *et al.* (1996). Cloning and mapping of entire viral segments is described in Albrecht *et al.* (1994). Plasmid subcloning was done into pBluescript KS+ or pSP64 or pSP65 vectors. For sequencing of large subclones the GPS-1 Genome Priming System (New England Biolabs) was used according to the manufacturer's protocol. GPS-1 is a Tn7 transposon-based *in vitro* system which uses TnsABC transposase to insert a transprimer randomly into the DNA target. Plasmid DNA was prepared using Wizard Plus SV Minipreps (Promega). Sequencing reactions were performed using the Thermo Sequenase sequencing kit (Amersham) with IRD800-labelled primers. Automatic sequencing was carried out on a Gene ReadIR 4200 (Licor). Each sequence was determined at least three times and more determinations were done in case of ambiguities.

■ **Sequence analysis.** DNA sequence data were analysed using the GCG suite (Wisconsin Package version 10.1, Genetic Computer Group, Madison, WI, USA). Genes were predicted using Fgenesh 1.0 with *Drosophila* settings (Solovyev & Salamov, 1999; <http://genomic.sanger.ac.uk/gf/gf.html>). Sequence comparisons were made using BLAST 2 (Tatusova & Madden, 1999; <http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>) and dot plot sequence comparisons were generated with GCG with a sliding window of 20 nt and a stringency of 16 nt. Searches for motifs within predicted proteins were performed using the Prosite (Hofmann *et al.*, 1999; <http://www.expasy.ch/tools/scnpsit1.html>) and Blocks databases (Henikoff & Henikoff, 1994; <http://www.blocks.fhcr.org>). N-terminal signal and transmembrane

domains were screened using SignalP (Nielsen *et al.*, 1997; <http://www.cbs.dtu.dk/services/SignalP>) and Tmpred (Hofmann & Stoffel, 1993; <http://www.ch.embnet.org/software>) and localization of predicted proteins was analysed by pSORT (Nakai & Horton, 1999; <http://psort.nibb.ac.jp>). Potential N- and O-glycosylation sites were predicted by Prosite (Hofmann *et al.*, 1999) and NetOGlyc 2.0 (Hansen *et al.*, 1998; <http://www.cbs.dtu.dk/services/NetOGlyc>), respectively. Kyte and Doolittle hydrophobicity plots were generated using ProtScale (Expasy, <http://www.expasy.ch/cgi-bin/protscale.pl>).

■ **Analysis of the CiV14 integration/excision site and time-point of excision.** A male *C. inanitus* genomic library (Gruber *et al.*, 1996) was screened with the 1300 bp *Hind*III fragment of clone 2A6 from segment CiV14 (Figs 1 and 3) with methods described in Sambrook *et al.* (1989). DNA from male or female pupae of stages 2 to 4 (for designation of pupal stages see Albrecht *et al.*, 1994) was isolated as described in Gruber *et al.* (1996). PCR reactions were carried out in a volume of 50 µl with 100 ng of template DNA, 1 U of *Taq* polymerase, 0.2 µM primers (14LL/14RR or 14LR/14RL, see Fig. 3 and accession nos AJ278677 and AJ319653; 12LL/12RR or 12LR/12RL and accession nos Z58828 and Z58832) and 100 µM of each dNTP (Qiagen *Taq* PCR core kit) on a Mastercycler gradient (Eppendorf). The denaturation temperature was 95 °C, annealing was done at 55 °C (CiV14) or 60 °C (CiV12), each step lasting 1 min, and synthesis was at 72 °C for 2 min. Aliquots of 10 µl were taken from the reaction after 30 cycles and electrophoresed in MetaPhor agarose (FMC Bio Products) gels (35 g/l) in TAE buffer according to Sambrook *et al.* (1989). PCR clones were generated to investigate the frequency of the ATA and TAC sequence type variants of CiV14. The PCR product obtained with primers 14LL/14RR (circular DNA, see Fig. 3) and 10 ng of calyx DNA as template was polished with T4 polymerase and ligated into a *Sma*I-cut pBluescript vector. The PCR product obtained with primers 14LR/14RL (rejoined DNA, see Fig. 3) and 1 µg of DNA from adult female wasps as template was treated in the same way. PCR reactions of 30 cycles were carried out in a volume of 50 µl with 1 U *Taq* polymerase (Qiagen), 0.2 µM primers and 0.2 mM of each dNTP. The denaturation temperature was 95 °C, annealing was done at 55 °C, each step lasting 1 min, and synthesis was at 72 °C for 2 min.

■ **Southern blot.** DNA was separated on 8 g/l agarose gels in 0.5 × TAE (20 mM Tris, 10 mM sodium acetate, 0.5 mM EDTA, pH 7.8) by field inversion gel electrophoresis (FIGE) with program #1 on a PPI-200 programmable pulse inverter (MJ Research). Gels were blotted for 20 h by capillary transfer onto positively charged nylon membranes (Roche) according to Sambrook *et al.* (1989). The DNA was cross-linked to the membrane with a UV Stratalinker 2400 (Stratagene), 254 nm, 160 000 µJ/cm<sup>2</sup>. As probes fragments derived from wasp genomic clones flanking proviral CiV12 or CiV14, respectively, were used. For CiV12 flanking left (12FL) this was the 610 bp *Sal*I fragment of clone λA21 (Gruber *et al.*, 1996; accession no. AJ278674), and for flanking right (12FR) this was the 340 bp *Sal*I-*Spl*I fragment of λA21se461 (Gruber *et al.*, 1996; accession no. Z58831). For CiV14 flanking left (14FL) this was the 965 bp *Hind*III fragment of λ2B211 (see Fig. 3, accession no. AJ278673) and for flanking right (14FR) it was the 672 bp *Hind*III-*Sal*I fragment of λ1B231 (see Fig. 3, accession no. AJ278678). As segment-specific probes the PCR product obtained from clone 1G10 with primers U2779/L3394 (accession no. Z58828) was used for CiV12 (Fig. 1), the 1088 bp *Eco*RI fragment of 2A6 was used for CiV14 (Fig. 1), the 1600 bp *Eco*RI of 2B6 was used for CiV14.5 (Fig. 1) and the 1745 bp *Hind*III-*Eco*RI fragment of 2B1 was used for CiV16.8 (Fig. 1). Fragments were gel-purified twice and either digoxigenin (DIG)-labelled using DIG-High Prime (Roche) or [ $\alpha$ -<sup>32</sup>P]dCTP-labelled using Ready-To-Go DNA labelling beads (Amersham) according to the manufacturer's instructions.

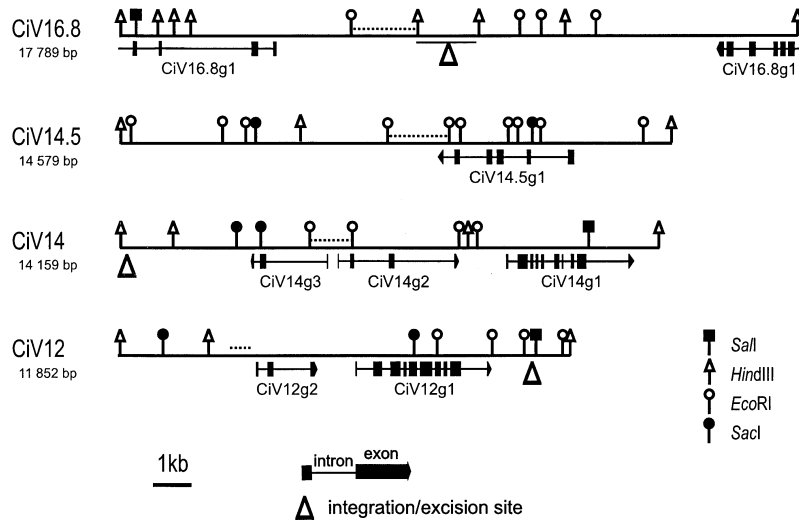


Fig. 1. Restriction maps of CiV12 (clone 1G10), CiV14 (clone 2A6), CiV14.5 (clone 2B6) and CiV16.8 (clone 2B1), predicted genes and integration/excision sites (CiV12 and CiV14) or region (CiV16.8). The circular molecules are presented in linear form. Dotted lines denote segment-specific probes. Positions of inverted repeats can be seen in the corresponding EMBL entries.

Blots were prehybridized for 5 h in  $5 \times$  SSC ( $1 \times$  SSC is  $0.15$  M NaCl,  $15$  mM sodium citrate, pH  $7.0$ ),  $20$  g/l blocking reagent (Roche),  $1$  g/l *N*-lauroylsarcosine,  $0.2$  g/l SDS,  $200$   $\mu$ g/ml of denatured calf thymus DNA and  $50\%$  formamide. Hybridization was in the same buffer at  $42^\circ\text{C}$  for  $48$  h with either  $15$  ng/ml of DIG-labelled probe or approximately  $10^5$  c.p.m. (Cerenkov counted) per ml of a  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ -labelled probe. High stringency washes were done in  $0.1 \times$  SSC,  $0.1\%$  SDS at  $65^\circ\text{C}$  twice for  $15$  min. Hybridization signals with DIG-labelled probes were detected by means of an alkaline phosphatase-conjugated anti-DIG antibody (Roche) and CDP-Star (Roche) and exposed for  $1$  h on a LAS-1000 Image Reader (Fuji). Hybridization signals of radiolabelled probes were detected by exposure to a PhosphorScreen (Molecular Dynamics) for  $1\text{--}7$  days depending on signal intensity and analysed on a PhosphorImager (Molecular Dynamics).

## Results

### Segment sequences and analyses

To characterize various segments of CiV and to investigate their relationship, four segments which had been previously cloned (Albrecht *et al.*, 1994) were sequenced and analysed (Fig. 1 and Table 1). The sequencing revealed that CiV12 (clone 1G10, accession no. Z58828) consists of  $11852$  bp,

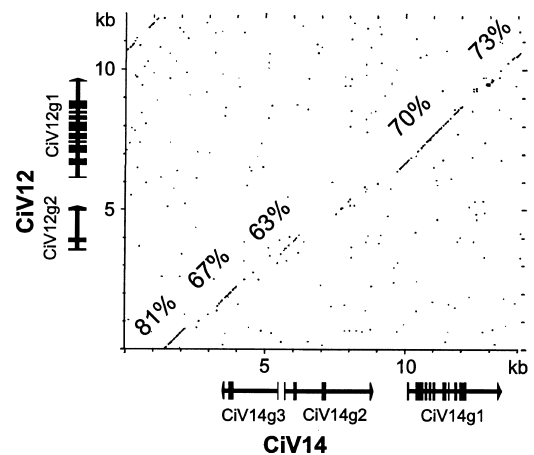


Fig. 2. Nucleotide sequence comparison between CiV12 and CiV14 by matrix dot plot. Percentages indicate degree of identity for a particular region. Predicted genes (Fig. 1) are also indicated on both axes.

CiV14 (clone 2A6, accession no. AJ278677) of  $14159$  bp, CiV14.5 (clone 2B6, accession no. AJ319654) of  $14579$  bp and CiV16.8 (clone 2B1, accession no. Z31378) of  $17789$  bp.

**Table 1.** Predicted proteins of CiV12, CiV14, CiV14.5 and CiV16.8 and their characteristics

Name	Location	Number of exons	Amino acids	$M_r \times 10^3$	Potential O-/N-glycosylation sites
CiV12p1	6199 → 9750	10	549	63.3	3/4
CiV12p2	3617 → 5118	3	80	8.9	0/2
CiV14p1	10113 → 13348	10	339	37.1	1/1
CiV14p2	5726 → 8801	4	96	10.9	0/2
CiV14p3	3446 ← 5477	3	89	10.2	1/0
CiV14.5p1	8436 ← 11996	6	225	25.8	3/2
CiV16.8p1	15704 ← 4052	10	447	50.7	3/6

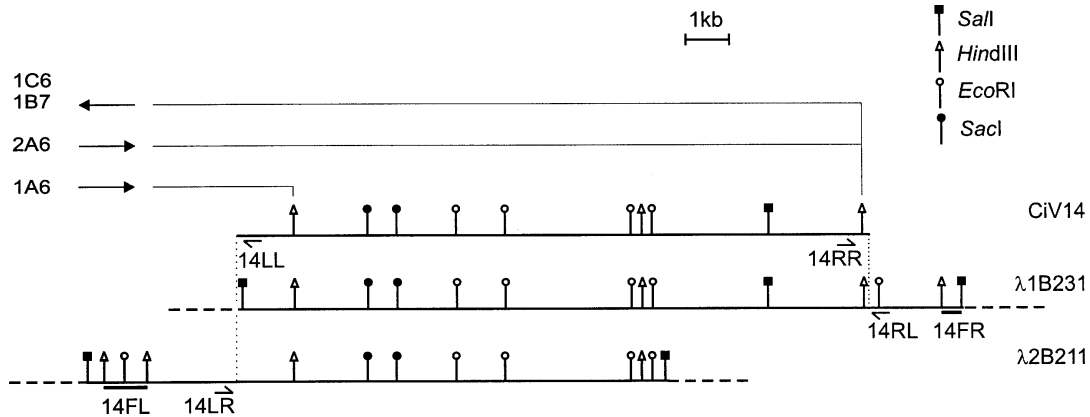


Fig. 3. Restriction map of CiV14 and genomic clones  $\lambda$ 1B231 and  $\lambda$ 2B211. In the CiV14 map the various clones as obtained by partial digestion of CiV DNA with *Hind*III or *Eco*RI and subsequent ligation with *Hind*III-cut pSP65 or *Eco*RI-cut pSP64 are given together with the orientation of the sP6 promoter (arrows). For details of cloning CiV14 see Albrecht *et al.* (1994) and of constructing the genomic male *C. inanitus* library see Gruber *et al.* (1996). Broken lines denote the  $\lambda$  arms. Bars denote probes used to analyse flanking regions (14FL and 14FR). Also primers 14LL, 14RR, 14LR and 14RL are indicated.

CiV14 had been designated CiV15 in an earlier publication by Albrecht *et al.* (1994). The average GC content of all four segments was between 32% and 33%, with 38–41% in the coding regions and 30–32% in the non-coding regions. For prediction of coding exons, various programs were used, namely gscan 1.0 (Burge & Karlin, 1997), genie (Kulp *et al.*, 1996), grail 1.3 (Xu & Uberbacher, 1997), GeneBuilder (<http://www.itba.mi.chr.it/webgene>) and Fgenesh (Solovyev & Salamov, 1999) and the results were compared with two cloned cDNAs of CiV14, namely CiV14g1 and CiV14g2 (A. Johner, personal communication). Fgenesh 1.0 using *Drosophila* settings gave the best predictions and was thus applied to predict genes on the sequenced CiV segments. Two genes were predicted for CiV12, three genes for CiV14 (CiV14g3 however with human settings only) and one gene each for CiV14.5 and CiV16.8 and all appear to contain introns (Fig. 1 and Table 1). The percentage of nucleotides predicted to be coding is 16% for CiV12, 11% for CiV14, 8% for CiV16.8 and 5% for CiV14.5. The structure of the genes varies, CiV12g1 and CiV14g1 having many short introns and the others having few but long introns (Fig. 1). The location, size and characteristics of the corresponding hypothetical proteins are given in Table 1. It shows that predicted proteins have a length between 80 and 549 amino acids with an  $M_r$  between 8.9 and 63.3. Some potential *N*- and/or *O*-glycosylation sites are predicted for all seven proteins. For none of the predicted proteins was a signal peptide detected and hydrophobicity plots did not reveal any particular pattern. Comparison of the nucleotide sequences with EMBL databases or of the hypothetical proteins with swiss-PROT databases using BLAST (Altschul *et al.*, 1997) did not reveal any significant similarities. Analysis of the predicted proteins with GeneQuiz (Hoersch *et al.*, 2000) did not reveal any functional annotations. Search for motifs using Prosite and Blocks databases suggested for the CiV12g1 hypothetical protein functionally contradictory char-

acteristics such as a calmodulin-binding motif, two transmembrane helices, a transposase domain and a leucine zipper. For CiV16.8p1 two nuclear localization signals were found (PSORT).

When the nucleotide sequences of the four segments were compared using BLAST, high similarities over large stretches were observed between CiV12 and CiV14, including the regions where genes are predicted (Fig. 2). For the hypothetical proteins of CiV12g1 and CiV14g1 38% of the amino acids are identical in a stretch of 448 amino acids and for the hypothetical proteins of CiV12g2 and CiV14g2 35% of the amino acids are identical over a stretch of 42 amino acids. Besides these high similarities between CiV12 and CiV14 a stretch of 252 bp with 82% identity was found between CiV14.5 and CiV16.8 and a stretch of 86 bp with 90% identity between CiV14 and CiV16.8. A search for repeats with the use of RUMMAGE (Taudien *et al.*, 2000) revealed the presence of inverted repeats on all four segments with identities between 77% and 92%, lengths of 26 bp to 100 bp and AT contents between 33% and 73%. Thus, the length of the various repeats, their distances and their AT content are variable and their sequences share no obvious similarity.

#### Integration/excision site

We then analysed the integration/excision region of CiV14 and compared it to that of CiV12, which we had studied earlier (Gruber *et al.*, 1996). We screened a male genomic *C. inanitus* library (Gruber *et al.*, 1996) with a DIG-labelled probe of CiV14 (a 1300 bp *Hind*III fragment of clone 2A6) and obtained clones  $\lambda$ 1B231 and  $\lambda$ 2B211, which are shown together with the entire CiV14 segment in Fig. 3. It shows that  $\lambda$ 1B231 contains a collinear copy of CiV14 and flanking regions on the right side and  $\lambda$ 2B211 contains part of a collinear copy of CiV14 and flanking regions on the left side. Both flanking regions and the rejoined DNA were sequenced (accession nos AJ278675,

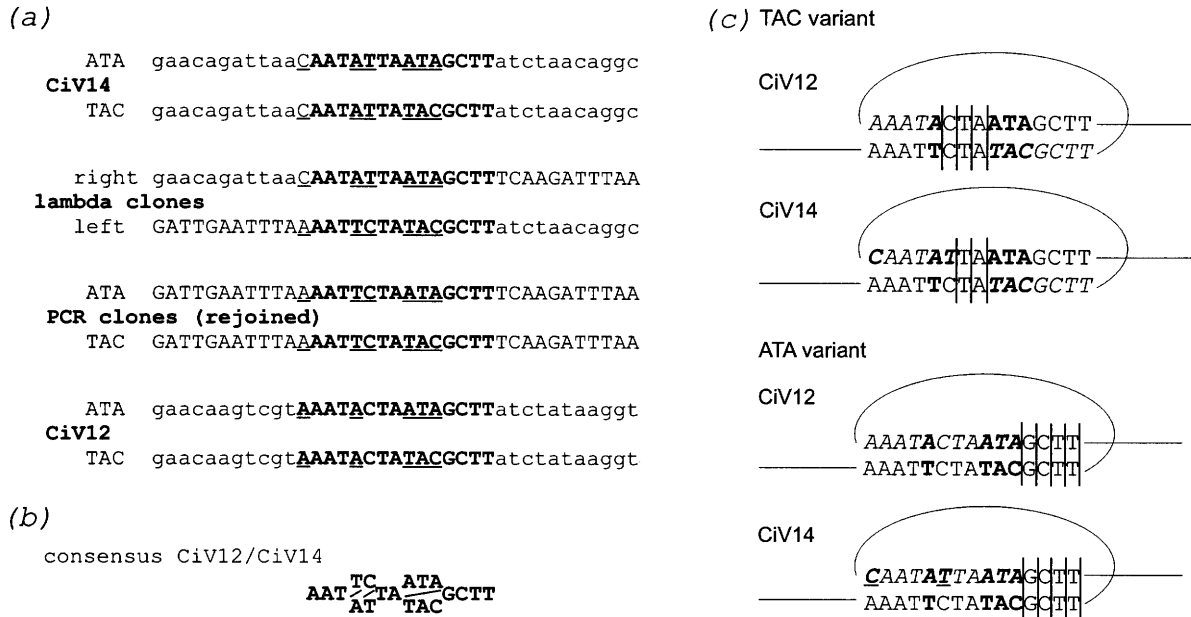


Fig. 4. (a) Sequence analyses of the junction region in various CiV14 clones, right and left junctions of CiV14 in  $\lambda$  clones ( $\lambda$ 1B231 and  $\lambda$ 2B211, Fig. 3) and in PCR clones of rejoined DNA after excision of CiV14 and comparison with the junction region in CiV12 (from Gruber *et al.*, 1996). Lower-case letters designate virus DNA, roman capital letters designate flanking sequences and bold capital letters designate direct repeats. Underlined letters denote divergent nucleotides. The triplets ATA and TAC categorize the sequence variants found in the CiV14 clones (seven ATA, three TAC) and in the PCR clones of the rejoined DNA (four ATA, five TAC). (b) Consensus between CiV12 and CiV14 in the repeat region. (c) Model to illustrate the hypothetical juxtaposition of left and right direct repeats and possible sites of recombination (bars) to yield either the TAC or ATA variant of CiV12 and CiV14. Letters in italics designate nucleotides which will end up in the excised circular CiV12 or CiV14.

AJ278676, AJ319653) and a small portion of the sequences is given in Fig. 4 along with the corresponding regions of CiV12. Fig. 4(a) shows the presence of an imperfect consensus sequence of 14 nucleotides in CiV14, in the terminus of the right and left  $\lambda$  clones and in the rejoined DNA. As observed earlier for CiV12 (Gruber *et al.*, 1996), two sequence variants (ATA and TAC) were found in CiV14 and rejoined DNA. For CiV14 the ATA variant was seen in seven clones and the TAC variant in three clones whereas for rejoined DNA the ATA variant was found in four clones and the TAC variant in five clones. The CiV12 repeat with its two variants is shown on the bottom of Fig. 4(a) and the consensus sequence with the repeat of CiV14 in Fig. 4(b). The repeats flanking proviral CiV12 and CiV14 are very similar over 14 nucleotides. At position 4 in CiV12 and CiV14, all virus clones and the right junction had an A while the left junction and the rejoined DNA had a T. At position 5 CiV12 always had a C whereas CiV14 had a T in all virus clones and the right junction and a C in the left junction and rejoined DNA. At positions 8–10 the two sequence types ATA or TAC were found in CiV12 and CiV14 and rejoined DNA corresponding to the right and left junction respectively. Fig. 4(c) shows a hypothetical model for the excision of the ATA and TAC variants of CiV12 and CiV14. According to this model the wasp DNA would form a loop in such a way as to juxtapose the terminal repeats for recombination. For formation of the TAC type of CiV12, recombination would

take place between positions 5, 6, 7 or 8, resulting in a rejoined site of the ATA type.

In the EPI segment of the bracovirus of *Cotesia congregata* direct repeats were also observed and the sequences were found to contain a potential binding site for a recombinase of the Hin family, namely the *hixC* half site which constitutes the DNA binding motif of the Hin recombinase of *Salmonella typhimurium* (Savary *et al.*, 1997). We thus compared the repeats and adjoining sequences of the CiV12 and CiV14 junction region with that of the *C. congregata* EPI segment (Fig. 5). The sequence comparison reveals that there is very little similarity between the EPI repeats and those of CiV12 and CiV14. A weak similarity is seen to the potential Hin recombinase binding site in CiV12 and to a lesser extent in CiV14. In the former, the G at position 3 and in the latter, the C at position 8 would prevent Hin binding *in vitro* according to Feng *et al.* (1994). On the bottom of Fig. 5 we also show the sequence of the putative junction region of CiV16.8. Repeated screening of the male genomic library with CiV16.8-specific probes was without success but the integration region could be identified with a PCR approach. We designed four pairs of primers distributed over the entire CiV16.8 segment and carried out PCR with the Roche Expand long template PCR system. As template we used either male or female adult *C. inanitus* DNA. With the DNA of females all four primer pairs gave a product of the expected length while with DNA of

<b>Hin recombinase binding site (<i>hixC</i> half-site)</b>	<b>TTA-TCAAAAA-CCT</b>
<b>direct repeat (consensus CiV12/CiV14)</b>	<b>AA<u>T</u><sup>AC</sup><u>TT</u>TA<sup>ATA</sup><u>TAC</u>GCTT</b>
<i>Cotesia congregata</i> EP1 5' DRJ 3' DRJ	CAA-TCAAAAAAGCTATAAGAAA TTATTCAAAAA-GCTATAGAAGT
<i>Chelonus inanitus</i> CiV12 left (5) right (3)	AA <u>G</u> -TCGTAAATAC <u>TA</u> ATAGCTT TAA-TCTGAAATTCATACGCTT
<i>Chelonus inanitus</i> CiV14 left (5) right (3)	GAA-TTTAAATTCATACGCTT AGA-TTAA <u>CA</u> ATATTAATAGCTT

*Chelonus inanitus* CiV16.8 putative circle junction

AAA-TAACAATACTTATAGCTT

Fig. 5. Comparison of the junction region between *Cotesia congregata* EP1 (Savary *et al.*, 1997) and CiV12, CiV14 and hypothetical site in CiV16.8. Potential recombinase binding sites along with direct repeats are shown. Underlined letters designate identity with the potential recombinase binding site and bold letters designate identity with the repeat. Boxed letters indicate nucleotides which would prevent *Hin* binding *in vitro* according to Feng *et al.* (1994). Shaded letters designate sequence variants (ATA or TAC).

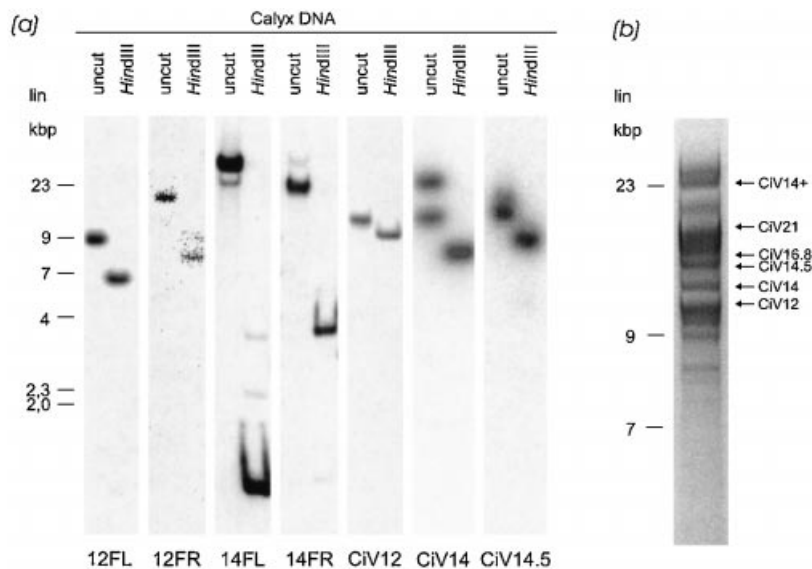
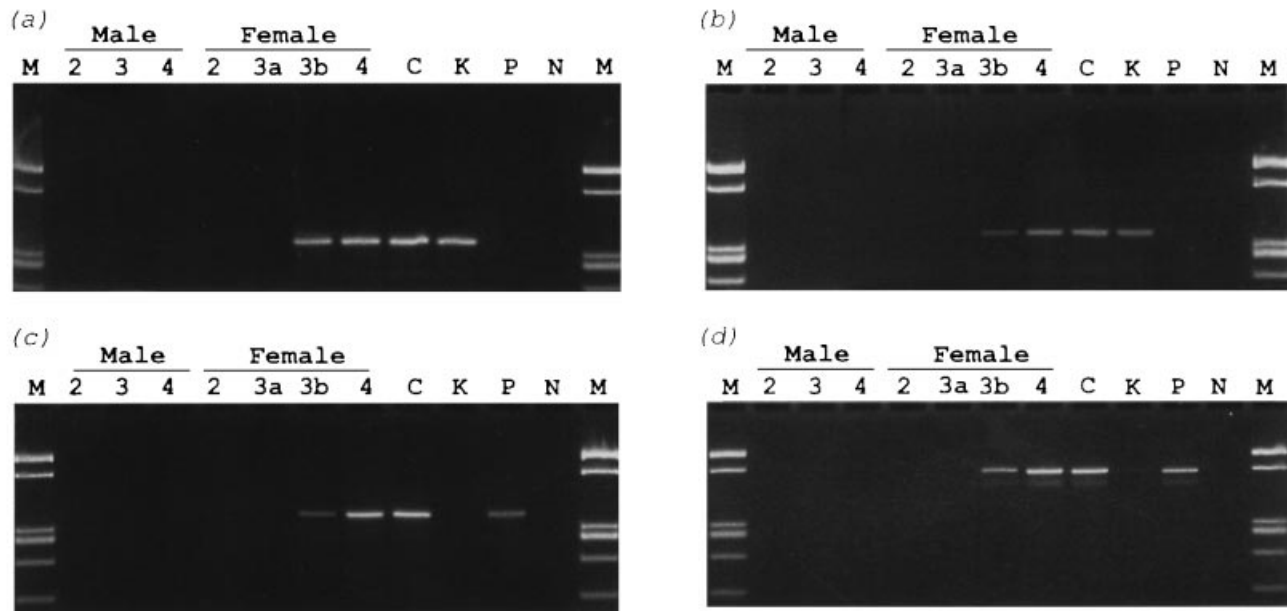


Fig. 6. (a) Analysis of flanking regions and viral segments by Southern blot. Undigested and *Hind*III-digested calyx DNA (1–3 µg) was separated by FIGE. The probes used to analyse left and right flanking regions of CiV14 (14FL, 14FR) are indicated in Fig. 3. The probe used to analyse the left flanking region of CiV12 (12FL) was clone λA21s610 (see Gruber *et al.*, 1996 and accession no. AJ278674) and the one to analyse the right flanking region (12FR) was a 340 bp *Sall*–*Sspl* fragment of λA21se461 (accession no. Z58831). The segment-specific probes for CiV12, CiV14 and CiV14.5 were as indicated in Fig. 1. As relaxed circular markers are not available linear λ digested with *Hind*III was used. (b) Ethidium bromide-stained FIGE gel of undigested calyx DNA showing the region above 7 kbp and the position of segments CiV12, CiV14, CiV14.5, CiV16.8 and CiV21 as deduced from hybridizations.

males one pair of primers gave no product, indicating that this region contained the integration/excision site. With an additional pair of primers within this region it was possible to locate the integration/excision site between position 7711 and 9566 in a *Hind*III fragment as indicated in Fig. 1. The search for the consensus of the repeat elements of CiV12 and CiV14 and for the *Hin* recombinase binding site in this part of CiV16.8 gave the sequence shown on the bottom of Fig. 5 (position

8337–8358, reverse complementary). Twelve nucleotides are identical with the 14 nucleotides of the repeat of the consensus between CiV12 and CiV14. At positions 10 and 15 in CiV16.8 a T instead of an A is seen. At positions 12–13 sequence variants between flanking regions, excised circle and rejoined DNA were observed in CiV12 and CiV14 as described above (positions 4–5 in Fig. 4) whereby the excised circle always had an A at position 12 which is also the case in CiV16.8. With



**Fig. 7.** Comparison of the appearance of excised circular CiV12 (a, c) and CiV14 (b, d) DNA and rejoined flanking sequences in pupal–adult development. DNA (100 ng) of male and female pupae of stages 2–4 (for designation of stages see Albrecht *et al.*, 1994) was amplified by PCR. For detection of the excised circular CiV12 and CiV14, primers 12LL/12RR (a, expected product length 265 bp) or 14LL/14RR (b, expected product length 264 bp) respectively were used. For detection of the rejoined flanking sequences of CiV12 and CiV14, primers 12LR/12RL (c, expected product length 267 bp) or 14LR/14RL (d, expected product length 386 bp) respectively were used. M, marker, pSP65 digested with *Hpa*II; C, calyx DNA (20 ng); K, cloned segment CiV12, namely 2 pg of clone 1G10 (a, c) or cloned CiV14, namely 2 pg of clone 2A6 (b, d); P, cloned PCR products of rejoined DNA as obtained with primers 12LR/12RL (a, c) or primers 14LR/14RL (b, d); N, no DNA.

respect to the ATA and TAC sequence variants we do not know whether a TAC version also exists for CiV16.8 as we have not sequenced other clones.

### Flanking sequences and time-point of excision of CiV12 and CiV14

To investigate whether proviral CiV12 and CiV14 are flanked by other proviral segments in the wasp's genome and whether CiV12, CiV14 and CiV14.5 are nested in other segments Southern blots were made. Undigested and *Hind*III-digested (1–3 µg) calyx DNA was separated by FIGE and hybridized with probes of left and right flanking regions of CiV12 and CiV14 or segment-specific probes. The bands seen with uncut calyx DNA and probes of flanking regions (12FL, 12FR, 14FL, 14FR) show that both CiV12 and CiV14 are flanked by other viral segments on both sides (Fig. 6a). Analysis of 15 µg of male genomic DNA digested with *Hind*III with the same probes gave a weak band at the same position as with 1 µg of calyx DNA in the case of 14FL and 14FR and only very weak and smeary signals in the case of 12FL and 12FR (data not shown). This indicates that the hybridization signals seen with calyx DNA cannot be attributed to contamination with genomic DNA. With the segment-specific probes (CiV12, CiV14, CiV14.5) one band was seen in the case of CiV12 and CiV14.5 and two bands in the case of CiV14; the bands of *Hind*III-digested segments were of the expected size

(Fig. 6a). To test whether the entire CiV14 is nested or only parts of it, three additional probes located at different positions in CiV14 were also used; they all gave the same two bands (data not shown). This indicates that CiV14 is nested in a larger segment while CiV12 and CiV14.5 are not. The ethidium bromide-stained gel of undigested calyx DNA (the region above 7 kbp) and the position of cloned segments as deduced from hybridizations is shown in Fig. 6(b) together with a linear marker. It shows that some of the identified segments are seen as distinct bands.

We then investigated whether CiV14 is excised at the same developmental stage as CiV12 and analysed more precisely the time-point when excised circular virus molecules first appear. For this purpose DNA from male and female pupae from stages 2 to 4 of pupal–adult development (for definition of stages see Albrecht *et al.*, 1994) was used as template. Primers specific for the excised CiV12 (Fig. 7a) and excised CiV14 (Fig. 7b) or for the rejoined CiV12 (Fig. 7c) and rejoined CiV14 (Fig. 7d) were used. The data show that excised CiV12 and CiV14 and their corresponding rejoined DNA both appear at stage 3b and are absent in males.

## Discussion

### Segment similarities, nesting and clustering

This is the first detailed analysis of segments and their relatedness and genetic information for a bracovirus. Se-

quencing of four CiV segments (Fig. 1) revealed that two of them, namely CiV12 and CiV14, have regions of high similarity (Fig. 2), suggesting the possibility of segment duplication. CiV14.5 and CiV16.8 appear to be unrelated. This contrasts to the situation in the ichnovirus of *C. sonorensis*, where a high amount of cross-hybridization between segments and a 540 bp repeat common to most segments was observed (Theilmann & Summers, 1987). Hybridization with segment-specific probes indicated that CiV12, CiV14.5 and CiV16.8 are unique (Fig. 6a; Albrecht *et al.*, 1994), whereas CiV14 occurs also nested in another larger segment (Fig. 6a). Unique as well as nested segments have also been observed in the ichnoviruses of *Hyposoter fugitivus* (Xu & Stoltz, 1991, 1993) and *C. sonorensis* (Cui & Webb, 1997; Webb & Cui, 1998). In these analyses both superhelical and relaxed circular molecules of the segments were observed. When gels are stained with ethidium bromide after separation of the DNA the covalently closed and nicked circular relaxed forms comigrate (Fig. 6; Albrecht *et al.*, 1994); in the presence of ethidium bromide the covalently closed relaxed circular form can be forced into the superhelical form. When calyx DNA was separated in gels containing ethidium bromide and was then probed with a CiV12-specific probe, nicked relaxed circular and superhelical molecules (covalently closed circles) could be seen as separate bands (data not shown). Based on these observations and the Kleinschmidt spreads (Albrecht *et al.*, 1994) we assume that *in vivo* CiV DNA occurs as covalently closed relaxed circles.

Hybridization of calyx DNA with probes specific for the flanking regions of CiV12 and CiV14 indicated that both are flanked by other viral segments (Fig. 6a), suggesting that proviral CiV segments are integrated in tandem arrays in the wasp's genome. We cannot show physical linkage to non-viral wasp genomic sequences so far as we have not yet analysed segments at the edge of the proviral cluster. Estimation of the approximate size of the flanking segments by comparison with the size of identified segments and contour length data (Albrecht *et al.*, 1994) suggests that CiV12 is flanked by a ca. 9 kbp and 20 kbp segment and CiV14 by a ca. 25 kbp and 31 kbp segment. In the bracovirus of *C. congregata* the EP1 circle is flanked on one side by another viral circle and on the other side by wasp DNA (Savary *et al.*, 1997). In contrast, segments B and W of the ichnovirus of *C. sonorensis* have been shown to be flanked by wasp DNA on both sides (Fleming & Summers, 1991; Cui & Webb, 1997).

### Sequence analyses and gene prediction

Comparisons of CiV nucleotide sequences with sequences in databases did not reveal any significant similarities. Analysis for repeats showed that inverted repeats with identities between 77% and 92%, lengths of 26–100 bp and AT contents between 33% and 73% were found on all four sequenced segments outside of the predicted genes. Palindromic structures of comparable sizes have been reported to act as origins of replication in baculoviruses (Pearson *et al.*, 1992; Ahrens *et al.*,

1995; Kool *et al.*, 1995) and it is thus conceivable that certain AT-rich palindromic structures in CiV might serve the same function. A large palindromic structure was also found in the EP1 circle of the *C. congregata* bracovirus (Savary *et al.*, 1997).

All four sequenced segments are predicted to contain genes, namely one on CiV14.5 and CiV16.8, two on CiV12 and three on CiV14 (Fig. 1 and Table 1). The predicted proteins consist of 80–549 amino acids and have some potential O- and/or N-glycosylation sites. With the exception of CiV14g3, experimental information has been obtained to substantiate the existence of these genes. For CiV14g1, CiV14g2 and CiV12g2 the cDNA has been cloned (A. Johner, D. Kojic and B. Lanzrein, unpublished). For CiV12g1, CiV14.5g1 and CiV16.8g1 primers have been designed and quantitative reverse transcription PCR analyses have shown that they are all transcribed in a stage-specific manner (S. Zumbach, D. Kojic and B. Lanzrein, unpublished). The accuracy of the predictions was as follows. The percentage of nucleotides correctly predicted as coding was 68% for CiV14g1, 56% for CiV14g2 and 100% for CiV12g2. The percentage of false positives at the nucleotide level was 7% for CiV14g1, 67% for CiV14g2 and 0% for CiV12g2.

Comparison of the predicted proteins with databases did not reveal any significant similarities to known proteins. Nor did we find motifs such as cystein-rich motifs observed in some genes of the ichnovirus of *C. sonorensis* (Dib-Hajj *et al.*, 1993; Cui & Webb, 1996) and the bracovirus of *M. demolitor* (Strand *et al.*, 1997; Trudeau *et al.*, 2000). Thus, the identified and predicted genes and proteins from the four CiV segments appear to be unrelated to proteins reported for other bracoviruses (Harwood *et al.*, 1994; Asgari *et al.*, 1996; Yamanaka *et al.*, 1996; Strand *et al.*, 1997; Varricchio *et al.*, 1999; Trudeau *et al.*, 2000) or ichnoviruses (Cui & Webb, 1996; Cui *et al.*, 1997; Deng & Webb, 1999; Béliveau *et al.*, 2000). All these bracoviruses and ichnoviruses are from larval parasitoids and many of the identified proteins have been shown to be involved in abrogation of the immune response of the host. In the egg–larval parasitoid *C. inanitus* abrogation of the host's immune system appears to be different from that of larval parasitoids (Stettler *et al.*, 1998) and thus the predicted CiV proteins found here may be involved in other aspects of host regulation.

### Integration/excision site and time-point of excision

Analysis of the integration/excision site of CiV14 and comparison to that of CiV12 (Gruber *et al.*, 1996) revealed great similarities. On both termini of proviral CiV12 and CiV14 as well as in the excised circular molecule and the rejoined DNA a very similar repeat of 14 bp was found (Fig. 4). For both CiV12 and CiV14 two sequence variants (ATA, TAC) were seen in the excised segment and the rejoined DNA and a model illustrating where the terminal repeats might recombine to yield the two variants of CiV12 and CiV14 is

presented in Fig. 4(c). For the EP1 bracovirus segment of *C. congregata* rejoining of DNA after excision of the viral DNA was also observed and in this case one base pair is lost during the excision process, possibly at the position where DNA strand exchange occurs (Savary *et al.*, 1997). The direct repeats of the EP1 segment have a length of 24 bp (5') and 22 bp (3') and contain a DNA motif that resembles a *Hin* recombinase recognition site (Savary *et al.*, 1997). Searches for this motif in the CiV12 and CiV14 repeats revealed weak similarity (Fig. 5) and the existence of nucleotides which would prevent *Hin* binding *in vitro* according to Feng *et al.* (1994). The existence of direct repeats on the termini of proviral segments appears to be a general feature of polydnaviruses, but the size of the repeats is very variable. For segment W of the *C. sonorensis* ichnovirus 1185 bp repeats with 100% identity were found (Cui & Webb, 1997) and for segment B 59 bp repeats with 83% identity (Fleming & Summers, 1991). Up to now rejoined DNA after excision of viral DNA has not been documented in ichnoviruses. It is not known whether this indicates a difference in the excision process between ichnoviruses and bracoviruses and has to do with the fact that the ichnoviral segments analysed to date are flanked by wasp DNA (Fleming & Summers, 1991; Cui & Webb, 1997) while the bracoviral segments appear to be clustered.

Excision of viral DNA appears to be restricted to females and sets in at a very precise time-point of pupal–adult development for both CiV12 and CiV14 (Fig. 7; Gruber *et al.*, 1996). The appearance of excised viral DNA was also seen to be stage-dependent in the ichnovirus of *C. sonorensis* (Norton & Vinson, 1983; Webb & Summers, 1992) and the bracovirus of *C. congregata* (Savary *et al.*, 1999). This bracovirus was also excised in diploid, but not haploid males (Savary *et al.*, 1999). The absence of excised viral molecules in the *C. inanitus* males (Fig. 7; Gruber *et al.*, 1996) might thus indicate that our *C. inanitus* colony contains no diploid males or that diploid males do not occur in this species. Extrachromosomal viral DNA was found also in males of the braconid *Cotesia melanoscela* (Stoltz *et al.*, 1986) and the ichneumonids *C. sonorensis* (Fleming & Summers, 1986; Cui & Webb, 1997) and *Hyposoter fugitivus* (Xu & Stoltz, 1991), but it is not known whether this has to do with the existence of diploid males also in these species.

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