Isolation and characterization of a baculovirus associated with the insect parasitoid wasp, *Cotesia marginiventris,* or its host, *Trichoplusia ni*

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Abstract

A multiple nucleopolyhedrovirus (MNPV) was isolated from *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) larvae that had been stung by the parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae). The wild type virus was plaque purified by infecting a *Heliothis subflexa* (BCIRL- HsAM1) cell line and isolating several clones. The mean estimated genomic size of this virus based on *Pst*I, *Bst*EII, *Sty*I, *Hin*dIII restriction profiles was estimated to be 106 ± 2.5 kbp (mean±SE). A clone designated as TnMNPV/CmBCL9 was used in bioassays against several lepidopteran pests and in comparative studies with the baculoviruses AcMNPV, AgMNPV, AfMNPV, PxMNPV and HzSNPV of *Autographa californica*, *Anticarsia gemmatalis*, *Anagrapha falcifera*, *Plutella xylostella*, and *Helicoverpa zea*, respectively. Infectivity studies showed that TnMNPV/CmBCL9 was highly infectious for *Heliothis subflexa* and *T. ni*, with an LC_{50} value 0.07 occlusion bodies/mm^2 in both species and also infectious for *H. zea* and *Heliothis virescens* with LC_{50} values of 0.22 and 0.27 occlusion bodies/mm^2, respectively. Restriction endonuclease analysis of the isolate and selected baculoviruses revealed profiles that were very similar to AfMNPV but different from the restriction endonuclease profiles of the other baculoviruses. Hybridization studies suggest that the TnMNPV/CmBCL9 was closely related to AfMNPV and AcMNPV-HPP. Further support for this comes from a phylogenetic analysis employing a split-graphs network, comparing the *polh, egt, and p10* genes from TnMNPV/CmBCL9 with those from other baculoviruses and suggests that this virus is closely related to the AcMNPV variants, AfMNPV and RoMNPV of *Rachiplusia ou*.

Keywords: TnMNPV/CmBCL9, *polh*, *egt*, *p10*, AfMNPV, *Anagrapha falcifera*, split-graphs network
Abbreviations: HzSNPV - Helicoverpa zea single nucleopolyhedroviruses, TnMNPV - Trichoplusia ni multiple nucleopolyhedroviruses, wt - wildtype
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Introduction

Baculoviruses are double stranded DNA viruses belonging to the family Baculoviridae that infect members of the phylum Arthropoda, mainly insects from the order Lepidoptera, but also other insect orders including Hymenoptera, Diptera, Coleoptera, Neuroptera, Thysanura and Trichoptera. They have also been reported to occur in the order Decapoda (shrimp) (Tanada and Kaya 1993; Possee 1993; Murphy et al. 1995). Baculoviruses have been successfully used worldwide to control Lepidopteran and Hymenopteran insect pests of agriculture and forestry importance (Granados and Federici 1986; Miller 1997; Moscardi 1999) and thus help reduce the need for chemical insecticides.

The Baculoviridae family is comprised of two genera, the *Nucleopolyhedrovirus* and the *Granulovirus* (Murphy et al. 1995). Members of these genera, such as the multiple and single nucleopolyhedroviruses (MNPV, SNPV), and granuloviruses, have a unique biphasic replicative cycle in which budded virus is produced early in the infection, and later, when viral particles are produced, they become occluded into proteinacious occlusion bodies formerly referred to as polyhedral inclusion bodies. The budded virus is responsible for the systemic spread of the virus within the host and is the entity used for infecting cell culture. The occlusion bodies are the main means by which the virus is disseminated in the environment between susceptible larvae. This is achieved through cell lysis of infected larvae resulting in contamination of the leaf surfaces and subsequent consumption of leaf tissue by healthy larvae.

There are many reports on the association of insect viruses with parasitoid wasps belonging to the families Braconidae and Ichneumonidae (Stoltz and Vinson 1977, 1979; Stoltz and Faulkner 1978; Vinson and Iwantsch 1980 A; Vinson and Iwantsch 1980 B; Fleming et al. 1983; Styer et al. 1987; Strand and Pech 1995; Doucet and Cusson 1996; Ferrarese et al. 2005). Such association may be as a contaminant on the parasitoid, or the virus may be internalized in the host tissues as is the case with the polydnaviruses that are the most studied (Kroemer and Webb 2004; Webb and Strand 2005).

The objectives of the present report were to establish the identity of the baculovirus isolated from parasitized *T. ni* larvae, to determine the relationship of this isolate to other well known baculoviruses, and to attempt to determine the possible origin of the newly isolated baculovirus.

Materials and Methods

History of the parasitoid

The braconid parasitoid, *Cotesia marginiventris* was originally obtained from the USDA, ARS, Stoneville, MS facility where it was reared on *Spodoptera exigua*. At the time, no indication of a possible baculovirus infection in the colony was reported. After receivership the parasitoid was then initially reared on *Spodoptera frugiperda* larvae obtained periodically from the USDA, ARS, Starkville, MS with no observable baculovirus symptoms reported either from that facility nor later at our laboratory. The parasitoid was then reared on *Trichoplusia ni* larvae available in-house from our insectary and there was no report of an observed baculovirus infection in the *T. ni* colony subsequent to exposure to the parasitoid.

Recovery and propagation of a baculovirus from *Cotesia marginiventris*

In the course of immunological studies employing *C. marginiventris*, it was found that several *T. ni* larvae that were stung by this parasitoid displayed typical baculovirus symptoms resulting in lysis of the larvae. Examination by light microscopy of the liquid contents from *T. ni* cadavers revealed the presence of occlusion bodies. *T. ni* larvae displaying typical baculovirus infection were consistently observed on other occasions following parasitization. Occlusion bodies from collected dead larvae were fed to 3rd instar *T. ni* by topical application to a wheat-soy diet (Bio-Serv, www.bio-serv.com) surface in order to amplify occlusion bodies as well as serve as a source of infectious hemolymph for inoculation of cell cultures.

Determination of possible latent viral infection in *T. ni larvae*

To investigate the possibility that individuals in the *T. ni* colony might harbor TnMNPV/CmBCL9 as a latent virus, 35 early 3rd instar *T. ni* larvae from the laboratory colony were stressed by incubating them at 37°C for six days to monitor for any pathogenic signs of an infection that would indicate a possible latent virus.

Viral source originating from the adult parasitoid interior

To investigate a possible viral source originating internally from the parasitoid, ten *C. marginiventris* from an exteriorly washed group of 40 insects resulting in *T. ni* infection were macerated in 2 ml Hanks' Balanced Salt Solution (HBSS) (Sigma, Co., www.sigmaaldrich.com), spun at 10,000 rpm in a tabletop centrifuge for 5 min to remove insect debris and then passed through a 0.22 µm filter. 30 µl undiluted samples of this filtrate were added to each of 15 wells of a 50-well tray each containing artificial diet and a 2nd instar *T. ni* larva. An equivalent number of larvae were used as controls. They were then incubated at 28°C to monitor for larval pathogenicity. Another 30 µl sample of undiluted filtrate was also used to inoculate three T-25 cm^2 flasks (5 ml) containing 1 x 10^5 cells/ml to determine possible budded virus presence in the parasitoid. Another flask containing the same TN-CL1 cell concentration was mock infected to act as a control.

Viral source originating from surface contact with a contaminated adult parasitoid

The question of whether or not the virus could have been transmitted through surface contact with an exteriorly contaminated parasitoid was also investigated. Forty adult parasitoids were collected and initially stored at −80°C. One ml of HBSS was added to the sample and then stored at 4°C for several days. The intention was to have the solution gently remove any potential parasitoid-surface virus so that it could be used as inoculum for both *in vitro* and *in vivo* assays. For the *in vitro* assay, 1 ml inoculum sterilized through a 0.22 µm filter was added to a T-25 cm^2 flask containing about 1 x 10^5 TN-CL1 cells/ml. The inoculum was removed after 2 h and replaced with 5 ml ExCell-401 (10% FBS) medium and incubated at 28°C. Another flask containing the same TN-CL1 cell concentration was mock infected to act as a control. To test whether the virus was present as occlusion bodies attached to the parasitoid body surface, 30 µl of surface-washed parasitoid solution was added to each of 15 wells of a 50-well tray each containing artificial diet and a 2nd instar *T. ni* larva. An equivalent number of larvae were used as controls. Trays were then incubated at 28°C to monitor for larval pathogenesis.

Plaque purification of wild type virus

Infectious hemolymph was collected from five 3rd instar *T. ni* larvae fed approximately 10^5 occlusion bodies and hemolymph collected on ice 48 h after exposure by snipping several prolegs. The infectious hemolymph was diluted at a ratio of 1:2 with ExCell 401 (SAFC Biosciences, www.sigmaaldrich.com/SAFC/Biosciences.html) and passed through a 0.45 µm millipore filter. A T-25 cm^2 flask of *Heliothis subflexa* cells (BCIRL-HS-AM1, McIntosh 1991) at 4 x 10^5 cells/ml in 5 ml of ExCell 401 containing 10% inactivated fetal bovine serum and antibiotics (McIntosh et al. 2005) were inoculated with 0.5ml of the filtered infectious hemolymph and incubated at 28°C for 5 days. Supernatant fluid was recovered by centrifugation at 1500 x g for 10 min and the cell pellet containing occlusion bodies were re-suspended in 5 ml of purified water and stored at −20°C. The supernatant from the infected BCIRL-HS-AM1 cell line was used to plaque purify the virus as previously described (McIntosh et al. 1997) and clones isolated. Selected clones and wild type virus were produced in 3 T-225 cm^2 flasks in BCIRL-HS-AM1 cells and the budded virus collected for DNA extraction (McIntosh et al. 2005).

Restriction enzyme analysis

Restriction endonuclease analyses were performed on the wild type virus as well as on selected clones that gave identical profiles. One of the clones, (TnMNPV/CmBCL9), was selected for a comparative study of its restriction endonuclease profile with those of several other baculoviruses and was used in all the remaining studies. The nucleopolyhedroviruses employed were: *Autographa californica* (AcMNPV) (McIntosh and Ignofo 1989), *Anagrapha falcifera* (AfMNPV), (McIntosh 1991), *Anticarsia gemmatalis* (AgMNPV), (Grasela and McIntosh 1998), *Plutella xylostella* (PxMNPV), (Kariuki et al. 2000) and the single nucleopolyhedrovirus from *Helicoverpa zea* (HzSNPV), (McIntosh et al. 2001). These baculoviruses were produced in cell culture as described and DNA extracted from budded virus as previously reported (McIntosh and Grasela 2006). The restriction enzymes used included *Hin*dIII, *Sty*I, *Vsp*I, *Bst*EII, *Xho*I and *Pst*I and digestion of the DNA was carried out according to the manufacturers instructions.

Hybridization studies

Hybridization studies were conducted with the 5 named baculoviruses as well as TnMNPV/CmBCL9 to determine the relationship if any, of the latter with the known baculoviruses. A previously described protocol (Kariuki and McIntosh 1999) using random primed DNA labeling of a *Vsp*I probe with digoxigenin-dUTP was followed for this comparative study.

Electron microscopy

Samples of TnMNPV/CmBCL9 occlusion bodies were prepared for transmission electron microscopy as previously described (Kariuki and McIntosh 1999) to determine whether the virus was a SNPV or MNPV, and were processed by the Electron Microscopy Core facility at the University of Missouri-Columbia.

In vivo infectivity studies of TnMNPV/CmBCL9 occlusion bodies

Occulsion bodies produced in BCIRL-HS-AM1 were used in infectivity studies against 24h old larvae from *T. ni*, *S. frugiperda*, *S. exigua*, *Helicoverpa zea*, *Heliothis virescens*, and *H. subflexa*. Both *T. ni* and *H. subflexa* larvae were obtained from the insectary at the Biological Control of Insects laboratory and the remaining larvae were obtained commercially (Bio-Serv, www.bio-serv.com). For each virus tested, the sample size consisted of three groups of 25 larvae per dosage replicated twice. Larvae were incubated at 28°C for 7 days, and mortalities were recorded daily for all insects and the LC_{50} values calculated employing PoloPlus v.1 (LeOra Software, leorasoftware.com).

Determining the DNA sequences of the polyhedrin, *egt*, and *p10* genes

Polyhedrin protein sequences from *Autographa californica* MNPV (AcMNPV) (GenBank No. NC_001623), *Anticarsia gemmatalis* MNPV (AgMNPV) (GenBank No. NC_008520), *Bombyx mori* MNPV (BmMNPV) (GenBank No. L33180), *Spodoptera frugiperda* MNPV (SfMNPV) (Genbank No. AY250076), and *Orgyia pseudotsugata* MNPV (OpMNPV) (GenBank No. U75930) were used with the web-based software Block Marker and the algorithm MOTIF (Smith et al. 1990)

(http://blocks.fhcrc.org/blocks/ make_blocks.html) to find conserved blocks in the related five unaligned protein sequences. The blocks were then used with the algorithm CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primers) (Rose et al. 1998) (http://blocks.fhcrc.org/codehop.html) to generate the following pair of degenerate primers used to PCR amplify a predicted 651 bp fragment of the TnMNPV/CmBCL9 polyhedrin gene:

(1) TnMNPV/CmBCL9 (F) 5′-CCATCGGCCG AACCTACGTN TAYGAYAA
(2) TnMNPV/CmBCL9(R) 5′-GGGACA CCTCGA TCAGGATCTCYTCYTCYTC

A similar approach was also taken to determine theTnMNNPV/CmB *egt* sequence employing degenerate primers designed from the following protein sequences: AcMNPV, AgMNPV, *Helicoverpa armigera* MNPV (HaMNPV) (Genbank No. NC_003094), OpMNPV, *Rachiplusia ou* (RoMNPV) 4 (GenBank No. NC_004323), and SfMNPV. A predicted 1629 bp egt fragment was generated using the following primer pair:

(1) 5′-CGTGTTCCCTACCCCTGCTTW YWSNCAYCA
(2) 5′-TGCTGGTACTTGTGAGTGTGGTAGRMYTGRTCNCC

Likewise, a partial *p10* sequence was determined by employing the following degenerate primers based on the p10 amino acid sequences of AcMNPV, AgMNPV, BmMNPV, OpMNPV, and RoMNPV to generate a 204 bp p10 fragment:

(1) 5′-10 CCCAACATCCTG ACCCA GATHYTNGAN GC
(2) 5′-CGTCCCCGGTCAGC ATNSWYTGDAT

TnMNPV/CmBCL9 DNA (100 –200 ng/µl) was amplified using puReTaq Ready-To-Go PCR beads (Amersham Biosciences, www.apbiotech.com) under the following conditions: 95°C, 3 min (1X); 94°C, 45 s, 60°C, 1 min, 72°C, 2 min (40X); 72°C, 5 min; held 15°C. The reaction products were run on a 2% Metaphor gel containing 1 µg/µl ethidium bromide and visualized with a VersaDoc imaging system (Bio-Rad Laboratories, Inc., www.bio-rad.com). The expected amplicon product was gel extracted using QIAEX II gel extraction kit (Qiagen, Inc., www.qiagen.com). Purified amplicon products were then cloned into the pCR4-TOPO plasmid according to the protocol provided by the manufacturer (Invitrogen, Corp., www.invitrogen.com). To obtain a more reliable nucleotide sequence read of the putative TnMNPV/CmBCL9 *polyhedrin* gene, two clones, labeled TnMNPV/CmBCL1 and TnMNPV/CmBCL2, containing the amplicon from two separate PCR reactions were sequenced from the 5′- and the 3′-end of the amplicon insert using M13 Forward (–20) and M13 Reverse primers by the DNA Core facility at the University of Missouri. The four sequence reads were subsequently used to generate a consensus sequence of the TnMNPV/CmBCL9 polyhedrin gene employing the BioEdit Sequence Editor (Hall 1999).

Nucleotide sequence accession numbers

The *polh, egt, and p10* sequences described in this study have been deposited in GenBank; the accession numbers are EF418027 EF418026, and EF418025, respectively.

Analysis of sequence data

Multiple-sequence alignmenst of the nucleotide sequences were performed using T-Coffee (Notredame et al. 2000), which generates a library of the best global and local alignments based on the Sim algorithm from the Lalign package (Huang and Miller 1991). The CORE index was employed to evaluate the consistency between a multiple alignment and every pair of aligned residues contained in the library. To obtain a more accurate picture of the relationship between the newly isolated TnMNPV/CmBCL9 and other viruses in the Baculoviridae family, a network-based tool was employed for deciphering the evolutionary relationships in molecular sequence data. The method builds a network primarily constructed from distances determined from split-decomposition theory and can be implemented using the SplitsTree4 (v.4.6) program that generates a Split-graphs network (Huson 1998; Huson and Bryant 2006). A network-based approach has several advantages, one of which avoids the implicit assumption of a tree-like evolutionary process. This allows one the flexibility to determine if the data follow a tree-like evolutionary path or to identify some other underlying pattern the typical tree representation might not discern. The significance of the topology of the split-graphs was verified by bootstrap resampling (1000 replicates). The program allows one to choose among various distance matrixes to construct a graphical representation of the phylogenetic relationship.

Results and Discussion

Electron microscopy

Transmission electron microscopy of TnMNPV/CmBCL9 occlusion bodies are depicted in Figure 1. It shows the virus to be of the MNPV type because they contain many buddle-like structures embedded within a polyhedrin protein matrix each containing a multiple number of smaller virus particles.

Latent viral presence in the *T. ni* larvae

Of the 35 3rd instar larvae reared at 37°C, only five larvae died after six days of exposure. None of these larvae as well as the remaining insect showed any pathology typical of a baculovirus infection. This suggests that the source of the virus might come more from contamination rather than a possible latent virus.

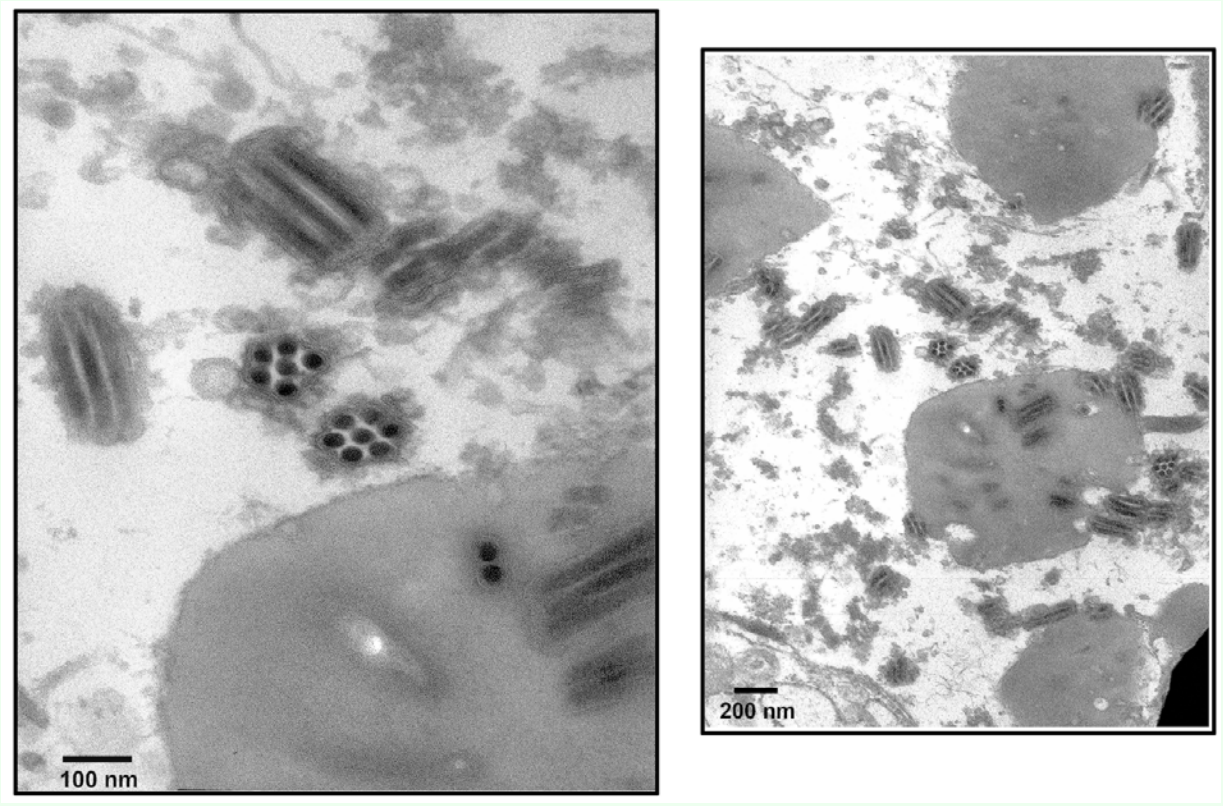

Figure 1. Transmission electron micrograph of the TnMNPV/CmBCL9 in BCIRL-HS-AM1 cells.

Viral source originating from the adult parasitoid interior

All 15 2nd instar larvae treated with a solution from macerated parasitoid showed no sign of pathogenicity after 7 days incubation. It is recognized that this test would only detect budded virus because the sample was passed through a 0.22 μm filter. It is also known that early instar larvae are not the most sensitive system for assaying for budded virus. There was no observed mortality in an equivalent number of control larvae. TN-CL1 cells inoculated with the filtered macerated parasitoid solution showed no signs of occlusion bodies formation after 7 days, other than general deterioration of cells probably from some component in the HBSS wash. Control cells were normal and almost confluent after 5 days.

Viral source originating from contact with parasitoid

TN-CL1 cells inoculated with the parasitoid-surface wash showed no sign of occlusion bodies formation after 7 days other than general deterioration of cells probably from some component in the HBSS wash. Control cells were normal and almost confluent after 5 days. When the supernatant collected from these cells was used to inoculate a fresh batch of TN-CL1 cells, no signs of viral infection were observed. Additionally, all 2nd instar larvae fed on diet treated with the parasitoid-surface wash also showed no pathogenesis nor did the untreated controls after 7 days. It is highly unlikely that budded virus would remain stable on the surface of an insect long enough to be somehow transmitted. Occlusion bodies attached to the parasitoid body that contaminate the diet surface would be the more plausible source of infection

Restriction endonuclease analysis and molecular weight of TnMNPV/CmBCL9

Two of the plaque purified clones (TnMNPV/CmBCL9 and TnMNPV/CmBCL14) and wt TnMNPV/CmB gave identical *Hin*dIII restriction patterns (data not shown). TnMNPV/CmBCL9 was employed in all other studies. A comparison of the *Hin*dIII restriction pattern between TnMNPV/CmBCL9 and four other baculoviruses revealed a number of differences both in the number as well as molecular size of the DNA fragments (Figure 2A). Six major bands ranging from 10.0 kb to 2.3 kb were found unique to TnMNPV/CmBCL9, whereas several *Hin*dIII restriction fragments present in AcMNPV-HPP (from 2.1 kb to 9.3 kb), PxMNPVCL3 (4.5 kb, 5.5 kb, 9.1 kb, 9.2 kb, 9.4 kb, 9.5 kb), AgMNPCL4-3A1 (from 6.0 kb to 9.3 kb) were absent in TnMNPV/CmBCL9. Most of the smaller *Hin*dIII bands (< 4.3 kb) of HzSNPV were absent in TnMNPV/CmBCL9 as well as in the other viruses examined. A comparison of the *Sty*I fragmentation profile showed a

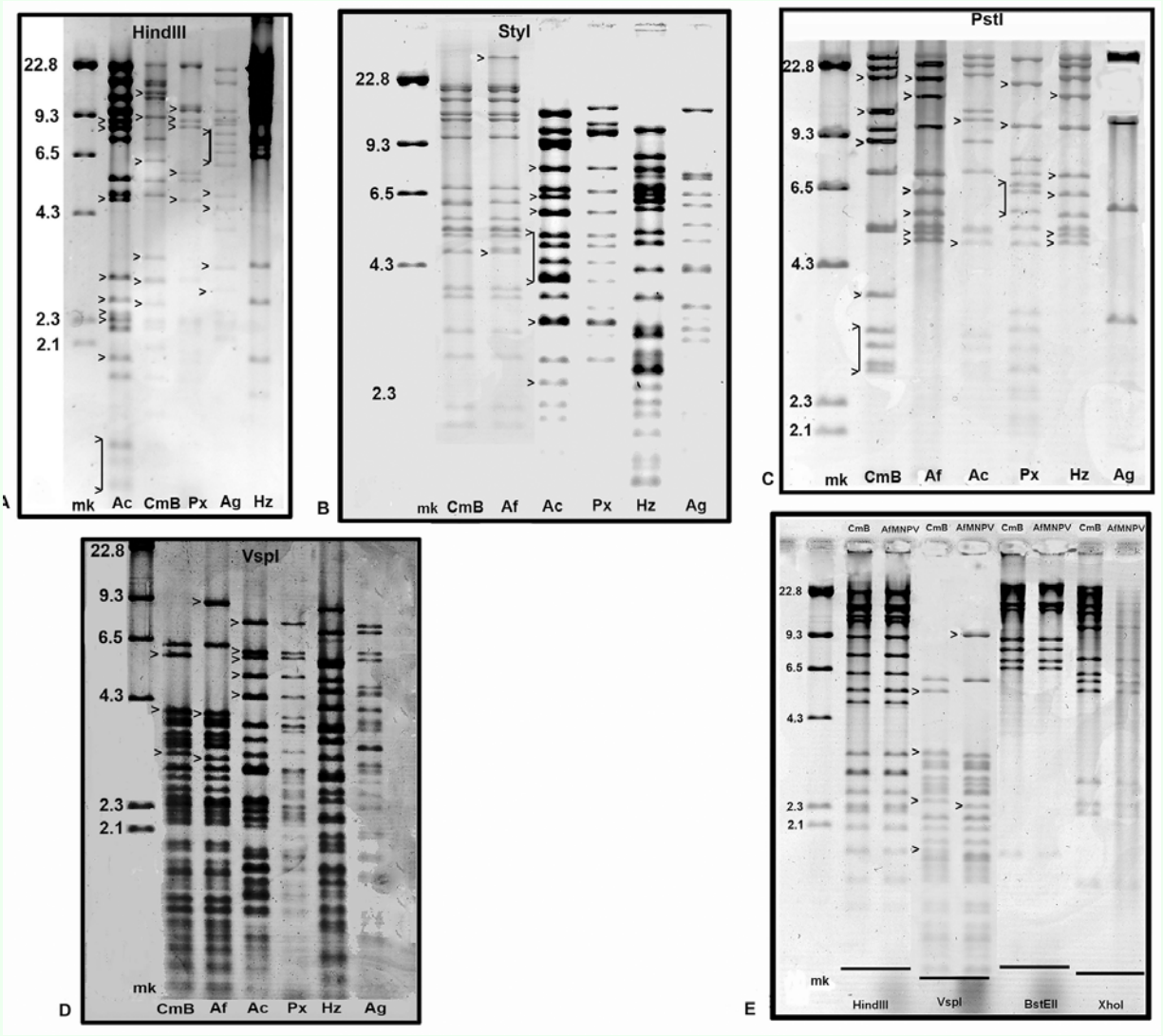

Figure 2. Comparison of five restriction profile patterns between TnMNPV/CmBCL9 and other baculoviruses. (A) *Hin*dIII restriction profile: (mk) DNA marker in kb; (Ac) AcMNPV-HPP; (CmB) TnMNPV/CmBCL9; (Px) PxMNPVCL3; (Ag) AgMNPV-CL4-3A1; and (Hz) HzSNPV. (B) *Sty*I restriction profile: (mk) DNA marker in kb; (CmB) TnMNPV/CmBCL9; (Af) AfMNPV; (Ac) AcMNPV-HPP; (Px) PxMNPVCL3; (Hz) HzSNPV; and (Ag) AgMNPV-CL4-3A1. (C) *Pst*I restriction profile: (mk) DNA marker in kb; (CmB)TnMNPV/CmBCL9; (Af) AfMNPV; (Ac) AcMNPV-HPP; (Px) PxMNPVCL3; (Hz) HzSNPV; and (Ag) AgMNPV-CL4-3A1; (D) *Vsp*I restriction profile: (mk) DNA marker in kb; (CmB) TnMNPV/CmBCL9; (Af) AfMNPV; (Ac) AcMNPV-HPP; (Px) PxMNPVCL3; (Hz) HzSNPV; and (Ag) AgMNPV-CL4-3A1: (E) Comparison of four different restriction profile patterns between TnMNPV/CmBCL9 and AfMNPV.

significant number of differences between TnMNPV/CmBCL9 and AcMNPV-HPP, PxMNPVCL3, AgMNPCL4-3A1, and HzSNPV (Figure 2B). In contrast, the overall *Sty*I restriction profile between TnMNPV/CmBCL9 and AfMNPV were indistinguishable except for the presence of two unique restriction fragments in AfMNPV. No further restriction pattern differences were detected on examination of these two viruses with the enzymes *Hin*dIII, *Bst*EII, and *Xho*I in terms of identical number and molecular size distribution of fragments (Figure 2C). However, the *Vsp*I restriction pattern revealed major differences between TnMNPV/CmBCL9 and AfMNPV especially in the higher molecular size region (> 6.5 kb) as well as three unique bands between the 4.3 – 6.5 kb region of AfMNPV (Figure 2D). A number of major band differences in the *Pst*I restriction profiles were also evident among TnMNPV/CmBCL9, AcMNPV-HPP, PxMNPVCL3, AgMNPV-CL1-3A1, and HzSNPV (Figure 2E). In particular, there were six unique *Pst*I fragments in AfMNPV that were absent in TnMNPV/CmBCL9, whereas eight unique bands were detected in TnMNPV/CmBCL9 that were absent in AfMNPV. In contrast, two unique *Pst*I fragments that were detected in AcMNPV were absent in TnMNPV/CmBCL9. The molecular weight of TnMNPV/CmBCL9 was estimated to be 106 ± 2.5 kbp

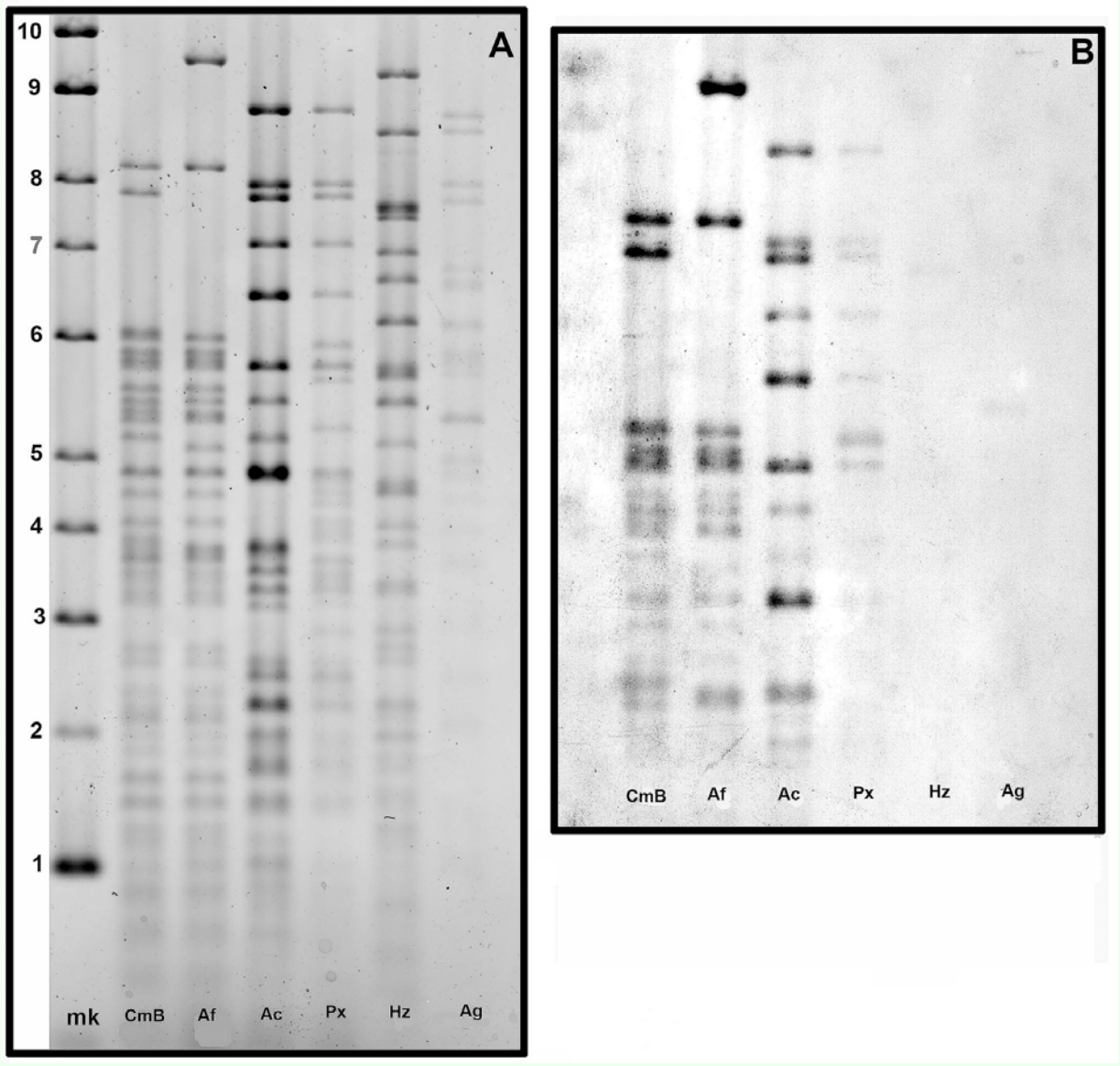

Figure 3. (A) Comparison of *Vsp*I restriction patterns between TnMNPV/CmBCL9 and five other baculoviruses. (mk) DNA marker in kb; (CmB) TnMNPV/CmBCL9; (Af) AfMNPV; (Ac) AcMNPV-HPP; (Px) PxMNPV-Cl3; (Hz) HzSNPV; and (Ag) AgMNPV-CL4-3A1. (B) Southern hybridization using a dig-labeled *Vsp*I probe from TnMNPV/CmBCL9. (CmB) TnMNPV/CmBCL9; (Af) AfMNPV; (Ac) AcMNPV-HPP; (Px) PxMNPVCL3; (Hz) HzSNPV; (Ag) and AgMNPV-CL4-3A1.

(mean ± SE) based on *Pst*I, *Bst*EII, *Sty*I, *Hin*dIII restriction profiles. This compares with an estimated mean genomic size for AfMNPV of 118.1 Kbp S.E. ± 6.9 in the host *T. ni* (Vail et al. 1993).

Hybridization studies

The *Vsp*I restriction profile showed some clearly distinct band differences specifically within the 6–10 kb range between TnMNPV/CmBCL9 and the other viruses. Hybridization analysis employing a *VspI* probe constructed from genomic TnMNPV/CmBCL9 revealed that the restriction pattern of the TnMNPV/CmB isolate appears to be more genetically similar to AcMNPV-HPP and AfMNPV than to PxMNPV, AgMNPV-CL1-3A1, or HzSNPV (Figure 3A, B). Federici and Hice (1997), also showed AfMNPV to be a genomic variant of AcMNPV based on Southern hybridization, the organization of the polyhedrin gene region, and nucleotide and deduced amino acid sequences of eight other viral genes in this region.

In vivo infectivity studies of TnMNPV/CmBCL9 occlusion bodies

Analysis of the hypothesis of equality and parallelism showed that changes in TnMNPV/CmBCL9 infectivity per unit change in dosage rate were significantly different

among the six species tested (P< 0.001) (Table 1). However, viral activity at a specific response level may still be similar between some comparisons. For example, the TnMNPV/CmB CL9 virus was equally effective against *H. zea* (LC_{50} = 0.22 occlusion bodies/mm^2) and *H. virescens* (LC_{50} = 0.26 occlusion bodies/mm^2). TnMNPV/CmBCL9 showed a 4.2 -12.2X lower infectivity against *S. exigua* larvae in comparison to reported LC_{50}s of *Plutella xylostella* MNPV (CL3) (0.70 occlusion bodies/ mm^2), AcMNPV (2.01 occlusion bodies/mm^2), and AfMNPVCL1 (1.67 occlusion bodies/mm^2) (Kariuki and McIntosh 1999). The LC_{50} of TnMNPV/CmBCL9 in *S. frugiperda* was 13.1 occlusion bodies/mm^2. Although they used 2nd-instar larvae incubated at 26°C and recorded an accumulative mortality after a 10-day period, Berretta et al. (1997) reported that *S. frugiperda* larvae infected with SfMNPV-AR and SfMNPV-ME isolates from Argentina had LC_{50}s (13.9 and 14.0 occlusion bodies/mm^2, respectively) similar to the TnMNPV/CmB CL9 LC_{50} reported here. Of the six species tested, the TnMNPV/CmBCL9 virus was most virulent against *H. subflexa* (LC_{50} = 0.07 occlusion bodies/mm^2) and *T. ni* (LC_{50} = 0.07 occlusion bodies/mm^2). Given that TnMNPV/CmB CL9 appears to be a variant of both AcMNPV and AfMNPV some further comparison can be made between TnMNPV/CmBCL9 and these two viruses. Although they used neonates instead of 24 h-old larvae, Hostetter and Puttler (1991) found AcMNPV and AfMNPV LC_{50} values to be somewhat higher for *H. virescens* (0.45 occlusion bodies/mm^2 and 0.35 occlusion bodies/mm^2, respectively) and *T. ni* (0.39 occlusion bodies/mm^2 and 0.15 occlusion bodies/mm^2, respectively) relative to TnMNPV/CmBCL9. Finally, a major difference can be seen in TnMNPV/CmB CL9 where this virus is more infectious in *H. zea* (LC_{50} = 0.22 occlusion bodies/mm^2) than AcMNPV (10.3 occlusion bodies/mm^2) as reported by Hostetter and Putler (1991).

Analysis of the *polh*, *egt*, and p10 sequences

The distribution of BLAST hits showed that the TnMNPV/CmB CL9 polyhedrin nucleotide sequence had the best E values when aligned with RoMNPV (E = 0.0) and AfMNPV (E = 0.0). The TnMNPV/CmB CL9 *polyhedrin* sequence has a GC content of 46.7%, while the AfMNPV *polh* gene (Genbank no. AFU64896) was reported to have a GC content of 46.1%. The multiple-sequence alignment of the TnMNPV/CmB CL9 polyhedrin nucleotide and predicted protein sequences with seven viruses selected from the Genbank (BmMNPV [U75359], AfMNPV [AAB53357], OpMNPV [M14885], AcMNPV [KO1149], RoMNPV [DQ345451], SfMNPV [JO4333], AgMNPV [NC_008520]) is depicted in Figure 4A. If in the alignment only the partially determined TnMNPV/CmBCL9 sequence based on 651 nucleotides is considered, then the nucleotide composition between the polyhedrin sequence of TnMNPV/CmB CL9 and the other viruses (BmMNPV, AfMNPV, OpMNPV, AcMNPV, RoMNPV, SfMNPV, AgMNPV) consist of sequences having a 21.7 identity. If one makes a similar comparison using aligned amino acids sequences, then the residue composition between the TnMNPV/CmB CL9 *polh* gene and the other viruses consist of sequences having a 74.2% identity, and a 2.2%, 12.4% semi-conserved and conserved substitution level, respectively. Comparatively, the BLAST distribution is somewhat incomplete as there

Table 1. Probit analysis results of larval mortalities from six lepidopteran species infected with TnMNPV/CmBCL9.

				LC_{50} 95% C.I. (OB/mm^2)				
Species	**Regression line**	**Slope S.E.**	**LC_{50} (OB/mm^2)**	**L_1**	**L_2**	**χ^{2+}**	**df**	**heterogeneity**
S. frugiperda	Y = 1.96 + 0.76 X	0.092	13.1	7.96	26.34	1.52	1	0.152
S. exigua	Y = 0.15 + 1.27 X	0.086	8.48	6.87	10.80	0.26	1	0.26
H. zea	Y = –0.50 + 2.48 X	0.239	0.22	0.18	0.26	0.001	1	0.001
H. subflexa	Y = 2.16 + 1.65 X	0.128	0.07	0.06	0.08	1.83	2	0.913
H. virescens	Y = 1.14 + 1.68 X	0.134	0.26	0.31	0.67	0.67	1	0.67
T. ni	Y = –0.61 + 3.22 X	0.508	0.07	0.06	0.08	0.004	1	0.004

[+]Hypothesis of equality (equal slopes, equal intercepts): P2 = 1520.0; df = 10 ; P < 0.001
[+]Hypothesis of parallelism (equal slopes): P2 = 104.0; df = 5; P< 0.001

```
TnMNPV/CmBCL9  CCATCGGCCGAACCTACGTGTATGACAACAAATATTACAAAAACTTGGGTTCTGTTATTA  60
AfMNPV         CCATCGGGCGTACCTACGTGTACGACAACAAATATTACAAAAACTTGGGTTCTGTTATTA  60
BmMNPV         CCATCGGGCGTACTTACGTGTACGACAATAAATATTACAAAAACTTGGGCTGTCTTATCA  60
OpMNPV         CCATTGGTCGCACCTACGTGTACGACAACAAATACTACAAAAACTTGGGCTCCGTCATCA  60
AcMNPV         CCATCGGGCGTACCTACGTGTACGACAACAAGTACTACAAAAATTTAGGTGCCGTTATCA  60
RoMNPV         CCATCGGGCGTACCTACGTGTACGACAACAAATATTACAAAAACTTGGGTTCTGTTATTA  60
SfMNPV         CTTTGGGTCGCACCTACGTGTACGACAACAAGTTCTACAAAAATCTAGGTTCGGTCATCA  60
AgMNPV         CCATTGGTCGCACGTATGTGTATGATAACAAGTATTACAAAAACTTGGGTTCTGTGATTA  60
               *  * ** ** ** ** ***** ** ** ** *  ********  * **     * ** *

TnMNPV/CmBCL9  AAAACGCCAAGCGCAAGAAGCACCTAATCGAACATGAAGAAGAGGAGAAGCACTTGGATC 120
AfMNPVH        AAAACGCCAAGCGCAAGAAGCACCTAATCGAACATGAAGAAGAGGAGAAGCACTTGGATC 120
BmMNPV         AAAACGCCAAGCGCAAGAAGCACCTAGTCGAACATGAACAAGAGGAGAAGCAATGGGATC 120
OpMNPV         AAAACGCCAAGCGCAAGAAGCACCTTCTAGAACACGAAGAGGATGAAAAGCACCTGGACC 120
AcMNPV         AGAACGCTAAGCGCAAGAAGCACTTCGCCGAACATGAGATCGAAGAGGCTACCCTCGACC 120
RoMNPV         AAAACGCCAAGCGCAAGAAGCACCTAATCGAACATGAAGAAGAGGAGAAGCACTTGGATC 120
SfMNPV         AGAATGCCAAGCGCAAGGAGCACTTAGCTCTCCATGAAATCGAGGAGAGGACTTTGGACC 120
AgMNPV         AGAACGCCAAGCGCAAGAAGCACCTTCTTGAACATCAGGAGGAAGAAAAAAGCCTAGATG 120
               * ** ** ********* ***** *        **  *    ** **          **

TnMNPV/CmBCL9  CCTTAGACAATTACATGGTTGCCGAAGATCCTTTTCTAGGACCTGGCAAGAACCAAAAAC 180
AfMNPVH        CCTTAGACAATTACATGGTTGCCGAAGATCCTTTTCTAGGACCTGGCAAGAACCAAAAAC 180
BmMNPV         TTCTAGACAACTACATGGTTGCCGAAGATCCCTTTTTAGGACCGGGCAAAAACCAAAAAC 180
OpMNPV         CGCTAGACCACTACATGGTCGCCGAGGACCCCTTCTTGGGCCCCGGCAAAAACCAAAAAC 180
AcMNPV         CCCTAGACAACTACCTAGTGGCTGAGGATCCTTTCCTGGGACCCGGCAAGAACCAAAAAC 180
RoMNPV         CCTTAGACAATTACATGGTTGCCGAAGATCCTTTTCTAGGACCTGGCAAGAACCAAAAAC 180
SfMNPV         CTCTGGAAAGGTACGTTGTCGCCGAAGATCCCTTCCTGGGACCCGGCAAAAACCAAAAGC 180
AgMNPV         GGCTAGATCATTACATCGTGGCCGAAGACCCATTTTTAGGGCCCGGCAAAAACCAAAAAT 180
                  * **    *** * ** ** ** ** ** **  * ** ** ***** ********

TnMNPV/CmBCL9  TCACTCTTTTCAAAGAAATCCGTAATGTTAAACCCGATACCATGAAGTTGATCGTCAACT 240
AfMNPVH        TCACTCTTTTCAAAGAAATCCGTAATGTTAAACCCGATACCATGAAGTTGATCGTCAACT 240
BmMNPV         TTACCCTTTTTAAAGAAATTCGCAGTGTGAAACCCGATACCATGAAGTTAATCGTCAACT 240
OpMNPV         TGACCCTGTTTAAAGAAATTCGCAACGTCAAGCCCGACACGATGAAGCTCATCGTCAACT 240
AcMNPV         TCACTCTCTTCAAGGAAATCCGTAATGTTAAACCCGACACGATGAAGCTTGTCGTTGGAT 240
RoMNPV         TCACTCTTTTCAAAGAAATCCGTAATGTTAAACCCGATACCATGAAGTTGATCGTCAACT 240
SfMNPV         TGACTCTGTTCAAAGAGATCCGTATCGTCAAGCCCGACACCATGAAGCTGGTAGTAAACT 240
AgMNPV         TGACACTTTTTAAAGAAATCCGCAATGTAAAACCCGACACGATGAAGCTCATTGTTAACT 240
               * ** ** ** ** ** ** ** *  ** ** ***** ** ****** *  * **    *

TnMNPV/CmBCL9  GGAGCGGCAAAGAGTTTATGCGTGAAACTTGGACCCGTTTCGTTGAGGACAGTTTCCCCA 300
AfMNPVH        GGAGCGGCAAAGAGTTTATGCGTGAAACTTGGACCCGTTTCGTTGAGGACAGTTTCCCCA 300
BmMNPV         GGAGCGGCAAAGAGTTTTTGCGTGAAACTTGGACCCGTTTTGTTGAGGACAGCTTCCCCA 300
OpMNPV         GGAGCGGCAAGGAGTTTTTACGTGAAACCTGGACGCGCTTCGTGGAAGACAGCTTCCCCA 300
AcMNPV         GGAAAGGAAAAGAGTTCTACAGGGAAACTTGGACCCGCTTCATGGAAGACAGCTTCCCCA 300
RoMNPV         GGAGCGGCAAAGAGTTTATGCGTGAAACTTGGACCCGTTTCGTTGAGGACAGTTTCCCCA 300
SfMNPV         GGAGCGGCAAAGAGTTTCTCAGGGAAACTTGGACCCGTTTCATGGAAGACAGCTTCCCTA 300
AgMNPV         GGAGCGGTAAAGAGTTTCTGCGCGAAACTTGGACTCGTTTTGTTGAAGACAGCTTTCCCA 300
               ***  ** ** *****     * ***** ***** ** **  * ** ***** ** ** *

TnMNPV/CmBCL9  TTGTAAATGACCAAGAGGTGATGGACGTGTTCCTCGTCGTCAACCTCAGACCCACACGCC 360
AfMNPVH        TTGTAAATGACCAAGAGGTGATGGACGTGTTCCTCGTCGTCAACCTCAGACCCACACGCC 360
BmMNPV         TTGTAAACGACCAAGAGGTGATGGACGTGTACCTCGTCGCCAACCTCAAACCCACACGCC 360
OpMNPV         TCGTCAACGACCAAGAAGTAATGGACGTGTTTCTCGTCGTCAACATGCGTCCCACGCGCC 360
AcMNPV         TTGTTAACGACCAAGAAGTGATGGATGTTTTCCTTGTTGTCAACATGCGTCCCACTAGAC 360
RoMNPV         TTGTAAATGACCAAGAGGTGATGGACGTGTTCCTCGTCGTCAACCTCAGACCCACACGCC 360
SfMNPV         TCGTGAACGACCAAGAAATTATGGACGTGTTCCTAGTGATCAACATGAGACCCACTAGGC 360
AgMNPV         TTGTAAACGACCAAGAAGTGATGGATGTGTTTTTAGTCATTAACCTCCGCCCAACTCGCC 360
               * ** ** ********  * ***** ** *   * **    *** *    ** **  * *
```

Figure 4A. Multiple-sequence alignment of the TnMNPV/CmBCL9 *polh* nucleotide sequence with seven baculoviruses employing the T-Coffee package. [continued on next page]

```
TnMNPV/CmBCL9  CCAATAGGTGCTACAAGTTCCTCGCTCAACACGCTCTTAGATGGGACGAAGACTACGTGC 420
AfMNPVH        CCAATAGGTGCTACAAGTTCCTCGCTCAACACGCTCTTAGATGGGACGAAGACTACGTGC 420
BmMNPV         CCAACAGGTGCTACAAGTTCCTCGCTCAACACGCTCTTAGGTGGGAAGAAGACTACGTGC 420
OpMNPV         CCAACCGCTGCTACAAATTCTTGGCGCAACACGCTCTCAGGTGGGACTGCGACTACGTGC 420
AcMNPV         CCAACCGTTGTTACAAATTCCTGGCCCAACACGCTCTGCGTTGCGACCCCGACTATGTAC 420
RoMNPV         CCAATAGGTGCTACAAGTTCCTCGCTCAACACGCTCTTAGATGGGACGAAGACTACGTGC 420
SfMNPV         CCAACAGATGTTTCAGATTCCTGGCGCAACACGCTCTCCGTTGCGACCCTGACTACGTTC 420
AgMNPV         CTAACCGTTGCTACAAATTCCTGGCGCAACACGCTCTCCGTTGGGACTGCGATTACGTGC 420
               * **  * ** * **  *** * ** ***********    ** **    ** ** ** *

TnMNPV/CmBCL9  CCCACGAAGTAATCAGAATTGTGGAGCCATCTTACGTGGGTATGAACAACGAGTACAGAA 480
AfMNPVH        CCCACGAAGTAATCAGAATTGTGGAGCCATCTTACGTGGGTATGAACAACGAGTACAGAA 480
BmMNPV         CCCACGAAGTAATCAGAATTGTGGAGCCATCCTACGTGGGCATGAACAACGAATACAGAA 480
OpMNPV         CCCACGAGGTGATTAGGATTGTCGAGCCGTCGTACGTGGGCATGAACAACGAGTACCGCA 480
AcMNPV         CTCATGACGTGATTAGGATCGTCGAGCCTTCATGGGTGGGCAGCAACAACGAGTACCGCA 480
RoMNPV         CCCACGAAGTAATCAGAATTGTGGAGCCATCTTACGTGGGTATGAACAACGAGTACAGAA 480
SfMNPV         CTCACGAAGTCATTCGCATTGTGGAGCCCGTGTACGTAGGAAACAACAACGAATACCGCA 480
AgMNPV         CCCACGAGGTAATCCGCATTGTGGAGCCTTCCTACGTGGGCATGAACAACGAGTACAGAA 480
               * ** ** ** **  * ** ** *****    *  ** ** *  ******** *** * *

TnMNPV/CmBCL9  TTAGTCTGGCTAAGAAGGGCGGCGGCTGCCCAATCATGAACATTCACAGCGAATATACTA 540
AfMNPVH        TTAGTCTGGCTAAGAAGGGCGGCGGCTGCCCAATCATGAACATTCACAGCGAATATACTA 540
BmMNPV         TTAGTCTGGCTAAAAAGGGCGGCGGCTGCCCAATCATGAACATCCACAGCGAGTACACCA 540
OpMNPV         TCAGCCTGGCCAAAAAAGGCGGCGGCTGCCCTATCATGAACATTCACGCCGAATACACCA 540
AcMNPV         TCAGCCTGGCTAAGAAGGGCGGCGGCTGCCCAATAATGAACCTTCACTCTGAGTACACCA 540
RoMNPV         TTAGTCTGGCTAAGAAGGGCGGCGGCTGCCCAATCATGAACATTCACAGCGAATATACTA 540
SfMNPV         TCAGCCTGGCCAAGAAGGGCGGCGGCTGCCCTGTCATGAACCTTCACTCTGAGTACACGC 540
AgMNPV         TTAGCCTAGCCAAGAAAGGCGGTGGTTGCCCAATCATGAACATCCACAGCGAGTATACCA 540
               * ** ** ** ** ** ***** ** *****  * ****** * ***   ** ** **

TnMNPV/CmBCL9  ATTCGTTCGAGTCGTTTGTGAGCCGCGTCATATGGGAGAATTTCTACAAACCCATCGTTT 600
AfMNPVH        ATTCGTTCGAGTCGTTTGTGAGCCGCGTCATATGGGAGAATTTCTACAAACCCATCGTTT 600
BmMNPV         ACTCGTTCGAGTCGTTTGTGAACCGCGTCATATGGGAGAACTTCTACAAACCCATCGTTT 600
OpMNPV         ACTCGTTTGAATCGTTCGTAAACCGCGTCATCTGGGAGAACTTTTATAAGCCCATCGTGT 600
AcMNPV         ACTCGTTCGAACAGTTCATCGATCGTGTCATCTGGGAGAACTTCTACAAGCCCATCGTTT 600
RoMNPV         ATTCGTTCGAGTCGTTTGTGAGCCGCGTCATATGGGAGAATTTCTACAAACCCATCGTTT 600
SfMNPV         ACTCCTTCGAAGAGTTCATCAACCGTGTCATCTGGGAGAACTTCTACAAACCCATCGTCT 600
AgMNPV         ACTCGTTTGAGTCCTTTGTCAACCGCGTAATCTGGGAAAACTTTTACAAACCCATTGTGT 600
               * ** ** **    **  * **    ** ** ***** ** ** ** ** ***** ** *

TnMNPV/CmBCL9  ACATTGGCACAGACTCTGGCGAGGAAGAAGAGATCCTGATCGAGGTGTCCC 651
AfMNPVH        ACATTGGCACAGACTCTGGCGAAGAAGAGGAAATCCTAATTGAGGTTTCTC 651
BmMNPV         ACATCGGCACAGACTCTGCCGAAGAAGAGGAAATCCTAATTGAGGTTTCTC 651
OpMNPV         ACATTGGCACGGATTCGAGCGAGGAGGAGGAAATTCTCATCGAGGTGTCGC 651
AcMNPV         ACATCGGTACCGACTCTGCTGAAGAGGAGGAAATTCTCCTTGAAGTTTCCC 651
RoMNPV         ACATTGGCACAGACTCTGGCGAAGAAGAGGAAATCCTAATTGAGGTTTCTC 651
SfMNPV         ACGTAGGAACCGACTCTGGTGAGGAAGAGGAGATCCTCCTTGAACTGTCGC 651
AgMNPV         ACATTGGCACTGATTCGGGCGAAGAAGAAGAAATTCTTATTGAAGTCTCGC 651
               ** * ** ** ** **    ** ** ** ** ** **  * **  * ** *
```

Figure 4A. [continued] (*) indicate identical residues.

are no records of either the *egt* or the *p10* genes from AfMNPV in the GenBank database, and, as such, these comparisons are tentative. The TnMNPV/CmBCL9 *egt* has a GC content of 45.1% and the *p10* a GC content of 36.7%. For the partial aligned *egt* and *p10* nucleotide sequences, 33.0% and 4.8 identities, respectively, were found when TnMNPV/CmBCL9 was compared with six different viruses: BmMNPV, OpMNPV, AcMNPV, RoMNPV, SfMNPV, AgMNPV (Figure 5A, Figure 6A). For the partial *egt* and *p10* aligned protein sequences the semi-conserved substitution levels were 10.9% and 1.2%, respectively and conserved substitutions were 20.6% and 13.1%, respectively. Initial split-graph analysis of the *polh* nucleotide and translated protein sequences clearly placed TnMNPV/CmB CL9 into a group consisting of RoMNPV/AfMNPV/BmMNPV and this relationship can be explained for the most part as a split-graph network rather than a tree-like network (Figure 7). The

```
TnMNPV/CmBCL9  IGRTYVYDNKYYKNLGSVIKNAKRKKHLIEHEEEEKHLDPLDNYMVAEDPFLGPGKNQKL  60
AfMNPV         IGRTYVYDNKYYKNLGSVIKNAKRKKHLIEHEEEEKHLDPLDNYMVAEDPFLGPGKNQKL  60
BmMNPV         IGRTYVYDNKYYKNLGCLIKNAKRKKHLVEHEQEEKQWDLLDNYMVAEDPFLGPGKNQKL  60
OpMNPV         IGRTYVYDNKYYKNLGSVIKNAKRKKHLLEHEEDEKHLDPLDHYMVAEDPFLGPGKNQKL  60
AcMNPV         IGRTYVYDNKYYKNLGAVIKNAKRKKHFAEHEIEEATLDPLDNYLVAEDPFLGPGKNQKL  60
RoMNPV         IGRTYVYDNKYYKNLGSVIKNAKRKKHLIEHEEEEKHLDPLDNYMVAEDPFLGPGKNQKL  60
SfMNPV         LGRTYVYDNKFYKNLGSVIKNAKRKEHLALHEIEERTLDPLERYVVAEDPFLGPGKNQKL  60
AgMNPV         IGRTYVYDNKYYKNLGSVIKNAKRKKHLLEHQEEEKSLDGLDHYIVAEDPFLGPGKNQKL  60
               :*********:*****.:*******:*:  *: :*   * *:.*:***************

TnMNPV/CmBCL9  TLFKEIRNVKPDTMKLIVNWSGKEFMRETWTRFVEDSFPIVNDQEVMDVFLVVNLRPTRP 120
AfMNPV         TLFKEIRNVKPDTMKLIVNWSGKEFMRETWTRFVEDSFPIVNDQEVMDVFLVVNLRPTRP 120
BmMNPV         TLFKEIRSVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVYLVANLKPTRP 120
OpMNPV         TLFKEIRNVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVFLVVNMRPTRP 120
AcMNPV         TLFKEIRNVKPDTMKLVVGWKGKEFYRETWTRFMEDSFPIVNDQEVMDVFLVVNMRPTRP 120
RoMNPV         TLFKEIRNVKPDTMKLIVNWSGKEFMRETWTRFVEDSFPIVNDQEVMDVFLVVNLRPTRP 120
SfMNPV         TLFKEIRIVKPDTMKLVVNWSGKEFLRETWTRFMEDSFPIVNDQEIMDVFLVINMRPTRP 120
AgMNPV         TLFKEIRNVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVFLVINLRPTRP 120
               ******* ********:*.*.**** *******:***********:***:** *::****

TnMNPV/CmBCL9  NRCYKFLAQHALRWDEDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTN 180
AfMNPV         NRCYKFLAQHALRWDEDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTN 180
BmMNPV         NRCYKFLAQHALRWEEDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTN 180
OpMNPV         NRCYKFLAQHALRWDCDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHAEYTN 180
AcMNPV         NRCYKFLAQHALRCDPDYVPHDVIRIVEPSWVGSNNEYRISLAKKGGGCPIMNLHSEYTN 180
RoMNPV         NRCYKFLAQHALRWDEDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTN 180
SfMNPV         NRCFRFLAQHALRCDPDYVPHEVIRIVEPVYVGNNNEYRISLAKKGGGCPVMNLHSEYTH 180
AgMNPV         NRCYKFLAQHALRWDCDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTN 180
               ***::******** : *****:******* :** ****************:**:*:***:

TnMNPV/CmBCL9  SFESFVSRVIWENFYKPIVYIGTDSGEEEEILIEVS 216
AfMNPV         SFESFVSRVIWENFYKPIVYIGTDSGEEEEILIEVS 216
BmMNPV         SFESFVNRVIWENFYKPIVYIGTDSAEEEEILIEVS 216
OpMNPV         SFESFVNRVIWENFYKPIVYIGTDSSEEEEILIEVS 216
AcMNPV         SFEQFIDRVIWENFYKPIVYIGTDSAEEEEILLEVS 216
RoMNPV         SFESFVSRVIWENFYKPIVYIGTDSGEEEEILIEVS 216
SfMNPV         SFEEFINRVIWENFYKPIVYVGTDSGEEEEILLELS 216
AgMNPV         SFESFVNRVIWENFYKPIVYIGTDSGEEEEILIEVS 216
               ***.*:.*************:****.******:*:*
```

Figure 4B. The putative amino acid sequence of the TnMNPV/CmBCL9 *polh* gene: (*) indicate identical residues; (:) indicate semi-conserved residues; (.) designate conserved residues.

central portions of the *egt* (Figure 8) and *p10* (Figure 9) graphs also show box-like structures that indicate incompatible data suggesting a network-like rather than a simple evolutionary tree structure. The PHI test of recombination showed significant evidence for recombination in the *polh* aligned sequences ($p = 1.894 \times 10^{-5}$). In contrast, no evidence was found to indicate a recombination signal in either the *egt* ($p = 0.1052$) nor *p10* ($p = 0.1549$) sequences. The split graphs of the *egt* and *p10* DNA sequences are illustrated in Figure 8 and Figure 9, respectively. Parallel evolution, model heterogeneity, and sampling error along with recombination can result in misleading interpretation of phylogenetic histories. Baculoviruses are known for exhibiting recombination and their evolutionary histories may not be best represented by a bifurcating or multifurcating trees. We chose the use of a split-graph network as its premise is that it can represent a relationship among lineages without assuming a tree-like evolutionary process. Also a major difference between a network and a tree is that cycles are permitted in which paths start and end at the same node. In general, similar network patterns were generated between each of the DNA and protein aligned sequences of the three genes (Figures 7, 8, 9). Based on the *polh* and *egt* sequence alignments TnMNPV/CmBCL9 was grouped with RoMNPV, BmMNPV, AfMNPV, and AcMNPV.

Specifically, the AfMNPV node in Figure7A might be interpreted as the ancestor of TnMNPV/CmBCL9. In the absence of a published AfMNPV *egt* gene sequence, analysis without AfMNPV still grouped TnMNPV/CmBCL9 with RoMNPV, AcMNPV, and BmMNPV. On the other hand, based on the *p10* gene network, the

```
TnMNPV/CMBCL9  CATCATATAGTGTACAAAGTGTATATTGAAGCTCTTGCCGAAAAATGTCACAACGTTACG  60
AGMNPV         CATCACCTGGTGTATCAAGCGTACGTGCAAGCTTTGGCAGACAAATGCCACAACGTGACC  60
ACMNPV         CACCATATAGTGTACAAAGTGTATATTGAAGCCCTTGCCGAAAAATGTCACAACGTTACG  60
OPMNPV         CACCACGTCGTGTACAGGGCGTACGTGCACGCGCTTGTGAAAAACTGCCACAACGTGACC  60
BMMNPV         CACCATATAGTCTACAAAGTGTATATTGAAGCCCTTGCCGAAAAATGTCACAACGTTACG  60
SFMNPV         CATCATAGCGTGTACAAAGTGTACATTCAAGCACTGGTCGAAAAAGGACACGAAGTCGTA  60
ROMNPV         CACCATATAGTGTACAAAGTGTATATTGAAGCTCTTGCCGAAAAATGTCACAACGTTACG  60
               ** **    ** **    * ***  *  * **  * *   * **  * *** * **

TnMNPV/CMBCL9  GTCGTCAAGCCCA--AACTGT-TTGAG--------TATTCAACCAAAACT----------  99
AGMNPV         GTGGTCAAGCCGC--AACTTT-TTGATTACGATGC--GGCAAACAAACAA---------- 105
ACMNPV         GTCGTCAAGCCCA--AACTGT-TTGCG--------TATTCAACTAAAACT----------  99
OPMNPV         GTGATCAAGCCAC--AGCTGC-TCGACTAC---GC-----CGTGCAGGAT----------  99
BMMNPV         GTCGTCAAGCCCA--AACTGT-TTGCG--------TATTCGACCAAAACT----------  99
SFMNPV         GTCGTCAAATCCACCAACAACGTAAACTACAAAGATATT-AACGACAATTACGCCGACGA 119
ROMNPV         GTCGTCAAGCCCA--AACTGT-TTGAG--------TATTCAACCAAAACT----------  99
               **  ****  *    * *    *

TnMNPV/CMBCL9  -TAT---------TGCGGTAATATCACGGAAATCAATGCCGACATGTCTGTCCAG--CAA 147
AGMNPV         -CGC---------TGCGGTCGTATCGAGCAGATCGACGCAGACATGTCTTCGCAG--CAA 153
ACMNPV         -TAT---------TGCGGTAATATCACGGAAATTAATGCCGACATGTCTGTTGAG--CAA 147
OPMNPV         -GAA---------TGCGGTCGTGTGGAGCAAATCGACGCCGACATGTCCGCGCAA--CAA 147
BMMNPV         -TAT---------TGCGGTAATATTACGGAAGTTAATTCCGACATGTCGGTCAAG--CAA 147
SFMNPV         CTATGAAGCGACACGCTACAACGTCACCGAAATTGATGC-GACGT-TATCGCAAGATTAT 177
ROMNPV         -TAT---------TGCGGTAATATCACGGAAATCAATGCCGACATGTCTGTCCAG--CAA 147
                           **       *    * * *  *    *** * *      *    *

TnMNPV/CMBCL9  TACAAAAAACTAGTGGCAAATTCGGCAATGTTTAGAAAGCGCGGAGTGGTGTCCGATACA 207
AGMNPV         TACAAAAAACTGGTGGCAAGTTCGGGCACGTTTCGCAAACGCGGCGTCGTGTCAGACGAA 213
ACMNPV         TACAAAAAACTAGTGGCGAATTCGGCAATGTTTAGAAAGCGCGGAGTGGTGTCCGATACA 207
OPMNPV         TACAAGAAGCTGGTGGCCAGTTCGGGCGTGTTCCGCAAGCGCGGCGTGGTGGCCGACGAG 207
BMMNPV         TACAAGAAACTAGTAACGAATTCGGCAATGTTTAGAAAGCGCGGAGTGGTGTCCGATACA 207
SFMNPV         TTCAAAAAACTCATGAAGAGGGCCCAAGTGTTTCGAAAGAGAGGTCTCGTCGCCGACAGC 237
ROMNPV         TACAAAAAACTAGTGGCAAATTCGGCAATGTTTAGAAAGCGCGGAGTGGTGTCCGATACA 207
               * *** ** **  *    *   *      ***  * **  * **  * **  * **

TnMNPV/CMBCL9  GACACGGTAACCGCCGCTAACTACCTGGGCTTGATTGAAATGTTCAAAGACCAGTTTGAC 267
AGMNPV         ACCACCGTCACGGCGGACAATTACATGGGGTTAGTGGAAATGTTTCGCGACCAGTTTGAC 273
ACMNPV         GACACGGTAACCGCCGCTAACTACCTAGGCTTGATTGAAATGTTCAAAGACCAGTTTGAC 267
OPMNPV         ACCACCGTCACCGCCGACAACTACATGGGCCTAATCGAAATGTTCAAGGACCAGTTTGAC 267
BMMNPV         GACACGGTAACCGCCGCCAACTACCTGGGCTTGATTGAAATGTTCAAAGACCAGTTTGAC 267
SFMNPV         TACTCTGTCACCGCAGATAACTACATGGGAATCGTGCGCATGATGAGTGATCAATTT-AA 296
ROMNPV         GACACGGTAACCGCCGCTAACTACCTGGGCTTGATTGAAATGTTCAAAGACCAGTTTGAC 267
                 * * ** ** ** *  ** *** * **  *  *    *** *    ** ** *** *

TnMNPV/CMBCL9  AATATTAA-CGTTCG-CAATTTC--ATTGCCAACAACCAGACGTTTGATTTGGTCGTCGT 323
AGMNPV         AACGTTCA-CGTGAA-AAATTTT--TTGGCCACAAACCGCACTTTTGACGTTGTAGTGGT 329
ACMNPV         AATATCAA-CGTGCG-CAATCTC--ATTGCCAACAACCAGACGTTTGATTTAGTCGTCGT 323
OPMNPV         AACGCCAA-TGTGCGTCGCTTTC---TCTCCACTAACCGCACATTTGACGCCGTGGTGGT 323
BMMNPV         AATATCAA-CGTGCG-CAATCTC--ATTGCCAACAACCAGACGTTTGATTTAGTTGTCGT 323
SFMNPV         ATTGCCTGCTGTGCAAAAATTTTTAAACAACAAAAAACAAAAATTTGATTTACTCATCAC 356
ROMNPV         AATATTAA-CGTTCG-CAATTTC--ATTGCCAACAACCAGACGTTTGATTTGGTCGTCGT 323
               *         **        * *        *   ** * *  *****     *  *

TnMNPV/CMBCL9  TGAAGCGTTTGCCGATTATGCGTTGGTGTTTGGCCATTTGT---ACGATCCGGCGCCCGT 380
AGMNPV         AGAGGCGTTTGCCGATTATGCGCTCGTATTTGGTCACCTGT---TTCGTCCAGCGCCCGT 386
ACMNPV         GGAAGCGTTTGCCGATTATGCGTTGGTGTTTGGTCACTTGT---ACGATCCGGCGCCCGT 380
OPMNPV         CGAGGCCTTTGCCGACTACGCGCTGGTGTTTGGGCACCTGT---TTCGCCCCGCGCCCGT 380
BMMNPV         GGAAGCGTTTGCCGATTATGCGTTGGTGTTTGGCCACCTGT---ACGATCCTGCGCCCGT 380
SFMNPV         CGAAGCGTTCATTGATTACACGTTGGTGTACTCGCATCTATTCAACGAT---ATACCCGT 413
ROMNPV         TGAAGCGTTTGCCGATTATGCGTTGGTGTTTGGCCATTTGT---ACGATCCGGCGCCCGT 380
                ** ** **     **    ** * *  *     **  * *             *****
```

Figure 5A. Multiple-sequence alignment of the TnMNPV/CmBCL9 *egt* nucleotide sequence with six baculoviruses employing the T-Coffee package. [continued on next page]

```
TnMNPV/CMBCL9  AATCCAAATCGCGCCTGGCTACGGTTTGGCAGAAAACTTTGA-TACGGTCGGCGCGGTTG 439
AGMNPV         GATCCAGATCGCGCCGGGCTACGGCCTGGCCGAAAACTTTGA-CGCCGTTGGCGCTGTGG 445
ACMNPV         AATTCAAATCGCGCCTGGCTACGGTTTGGCGGAAAACTTTGA-CACGGTCGGCGCCGTGG 439
OPMNPV         GATTCAAATTGCGCCGGGCTACGGCCTGGCCGAAAACTTTGAACGCCG-CCGCGCCGTGG 439
BMMNPV         AATCCAAATCGCGCCTGGCTACGGTTTGGCGGAAAACTTTGA-CACGGTAGGCGCCGTGG 439
SFMNPV         GATTCAAATCTCGTCGGGCTACGCTGTGGCGGAAAACTTTGA-AACTATGGGCGCGGTCG 472
ROMNPV         AATCCAAATCGCGCCTGGCTACGGTTTGGCAGAAAACTTTGA-TACGGTCGGCGCGGTTG 439
                ** ** **  ** * *******   **** ***********   *    ****  ** *

TnMNPV/CMBCL9  CGCGACACCCCGTCTATCATCCTAACATTTGGCGCAA---------CAATTTCGACGACA 490
AGMNPV         GACGCCACCCGATCCACTACCCCAACATTTGGCGCAGTAGCT-CGA-----TTGGCAACG 499
ACMNPV         CGCGGCACCCCGTCCACCATCCTAACATTTGGCGCAG---------CAATTTCGACGACA 490
OPMNPV         CGCGGCACCCGCTGCACTA-CCCAACATTTGGCGCAGCAGCTTTGA-----C--GCGGCG 491
BMMNPV         CGCGGCACCCCGTTCACCATCCTAACATTTGGCGCAA---------CAATTTCGACGACA 490
SFMNPV         GCAGACATCCCGTCTACTACCCCAACTTGTGGAGAGATAAATTTTACAATCTCAACGTCT 532
ROMNPV         CGCGACACCCCGTCTATCATCCTAACATTTGGCGCAA---------CAATTTCGACGACA 490
                    *   **    *  *  * *** * *** *                    *

TnMNPV/CMBCL9  CGGA---GGCAAACGTGATG-ACG--GAAATGCGTTTGTATAAAGAATTTAAAATTTTGG 544
AGMNPV         CGGA------CGGAGCGCTA-ATC--GAATGGCGTCTGTACAACGAATTTGAATTGTTGG 550
ACMNPV         CGGA---GGCAAACGTGATG-ACG--GAAATGCGTTTGTATAAAGAATTTAAAATTTTGG 544
OPMNPV         CGGC------GG-CGCGCTC-AGT--GAATGGCGCTTGCTGAACGAGTTCGAGCTGCTGG 541
BMMNPV         CGAA---GGCGAACTTGATG-ACG--GAAATGCGTTTGTATAAAGAATTTAAAATTTTGG 544
SFMNPV         GGGATCTGATCAACGAACTGTACGTCGAACTGAGGTTATACAATGAATTTTATAAATTGG 592
ROMNPV         CGGA---GGCAAACGTGATG-ACG--GAAATGCGTTTGTATAAAGAATTTAAAATTTTGG 544
                *                *  *    ***  * *  *    ** ** **  *     ***

TnMNPV/CMBCL9  CCAAACATGTCCAACGCGTTGCTCAAACAGCAGTTTGGACCCAATACACCGACAATTGAA 604
AGMNPV         CGCG-CCGTTCCGACGCGCTGCTCAAACTGCAATTTGGCCCCAACACGCCTACTATACGG 609
ACMNPV         CCAA-CATGTCCAACGCGTTGCTCAAACAACAGTTTGGACCCAACACACCGACAATTGAA 603
OPMNPV         CGCG-GCGGTCCGACGAACTGCTAAAACAACAATTCGGAAAAAGCACGCCCACCATCAGG 600
BMMNPV         CCAA-CATGTCCAACGCGTTGCTCAAACAGCAGTTTGGACCCGACACACCGACAATTGAA 603
SFMNPV         CCGATCAACAGAATCGT-TTGTTGAAAGAACAGTTTGGTCAAGACACGCCGACCATACAA 651
ROMNPV         CCAA-CATGTCCAACGCGTTGCTCAAACAGCAGTTTGGACCCAATACACCGACAATTGAA 603
               *    **       **   *    ***   ** ** **       ** ** ** **

TnMNPV/CMBCL9   AAACTACGCAAC 616
AGMNPV          CAATTGCGCAAC 621
ACMNPV          AAACTACGCAAC 615
OPMNPV          CAGCTGCGCGAC 612
BMMNPV          GAACTGCGCAAC 615
SFMNPV          GATCTACGCAAT 663
ROMNPV          AAACTACGCAAC 615
                 * *  *** *
```

Figure 5A. [continued] (*) indicate identical residues.

relation of TnMNPV/CmBCL9 with the other viruses was counter to what the *polh* and *egt* gene network showed by placing TnMNPV/CmBCL9 as a distantly unique virus (Figure 9). When all three gene sequences were concatenated using all the viruses except AfMNPV, TnMNPV/CmBCL9 was still grouped with RoMNPV and BmMNPV.

In this study, information is presented on a new multiple nucleopolyhedrovirus variant, TnMNPV/CmB CL9 found in *T. ni* larvae following parasitization with the parasitoid *C. marginiventris*. Larval mortality studies showed that the virus is highly infectious for 24 h-old *T. ni* and *H. subflexa* larvae and infectious for both 24 h-old *H. zea* and *H. virescens* larvae. Restriction DNA and hybridization profiles indicated that TnMNPV/CmB CL9 is genetically similar to AfMNPV, but appears to be a new isolate of the multiple nucleopolyhedrovirus type. The partially determined *polh, egt, and p10* nucleotide sequences in a split-graph analysis further demonstrated a close relationship to AfMNPV, BMNPV, and RoMNPV, previously determined genetic variants of AcMNPV. Various possible sources of the virus that were examined in this study included (1) surface contamination of parasitoid, (2) virus sequestered by the parasitoid, and (3) a latent virus present in the *T. ni* colony. The latter

```
TnMNPV/CmBCL9  HHIVYKVYIEALAEKCHNVTVVKPKL----------FEY--STKTYCGNITEINADMSVQ  48
AcMNPV         HHIVYKVYIEALAEKCHNVTVVKPKL----------FAY--STKTYCGNITEINADMSVE  48
AgMNPV         HHLVNQVYVQALADKCHNVTVVKPQL----------FDYDAANKQRCGRIEQIDADMSSQ  50
RoMNPV         HHIVYKVYIEALAEKCHNVTVVKPKL----------FEY--STKTYCGNITEINADMSVQ  48
BmMNPV         HHIVYKVYIEALAEKCHNVTVVKPKL----------FAY--STKTYCGNITEVNSDMSVK  48
OpMNPV         HHVVYRAYVHALVKNCHNVTVIKPQL----------LDY--AVQDECGRVEQIDADMSAQ  48
SfMNPV         HHSVYKVYIQALVEKGHEVVVVKSTNNVNYKDINDNYAD--DYEATRYNVTEIDATLSQD  58
               ** * :.*:.**..: *:*.*:*.                    : .: :::: :* .

TnMNPV/CmBCL9  QYKKLVANSAMFRKRGVVSDTDTVTAANYLGLIEMFKDQFDNINVRNFIANN-QTFDLVV 107
AcMNPV         QYKKLVANSAMFRKRGVVSDTDTVTAANYLGLIEMFKDQFDNINVRNLIANN-QTFDLVV 107
AgMNPV         QYKKLVASSGTFRKRGVVSDETTVTADNYMGLVEMFRDQFDNVHVKNFLATN-RTFDVVV 109
RoMNPV         QYKKLVANSAMFRKRGVVSDTDTVTAANYLGLIEMFKDQFDNINVRNFIANN-QTFDLVV 107
BmMNPV         QYKKLVTNSAMFRKRGVVSDTDTVTAANYLGLIEMFKDQFDNINVRNLIANN-QTFDLVV 107
OpMNPV         QYKKLVASSGVFRKRGVVADETTVTADNYMGLIEMFKDQFDNANVRRFLSTN-RTFDAVV 107
SfMNPV         YFKKLMKRAQVFRKRGLVADSYSVTADNYMGIVRMMSDQFKLPAVQKFLNNKKQKFDLLI 118
                :***:  :  *****:*:*  :*** **:*::.*: ***.   *:.:: .: :.** ::

TnMNPV/CmBCL9  VEAFADYALVFGHLYDPAPVIQIAPGYGLAENFDTVGAVARHPVYHPNIWRNNFDDTEAN 167
AcMNPV         VEAFADYALVFGHLYDPAPVIQIAPGYGLAENFDTVGAVARHPVHHPNIWRSNFDDTEAN 167
AgMNPV         VEAFADYALVFGHLFRPAPVIQIAPGYGLAENFDAVGAVGRHPIHYPNIWRSSSIGNADG 169
RoMNPV         VEAFADYALVFGHLYDPAPVIQIAPGYGLAENFDTVGAVARHPVYHPNIWRNNFDDTEAN 167
BmMNPV         VEAFADYALVFGHLYDPAPVIQIAPGYGLAENFDTVGAVARHPVHHPNIWRNNFDDTKAN 167
OpMNPV         VEAFADYALVFGHLFRPAPVIQIAPGYGLAENFERRRAVARHPLHYPTFGAAAL-TRRGG 166
SfMNPV         TEAFIDYTLVYSHLFNDIPVIQISSGYAVAENFETMGANTKRLCCRIPVYRTY------- 171
               .*** **:**:.**:   *****:.**.:****:   *  :: .

TnMNPV/CmBCL9  VMTEMRLYKEFKILAKH 184
AcMNPV         VMTEMRLYKEFKILANM 184
AgMNPV         ALIEWRLYNEFELLA-- 184
RoMNPV         VMTEMRLYKEFKILA-N 183
BmMNPV         LMTEMRLYKEFKILA-N 183
OpMNPV         ALSEWRLLNEFELLARR 183
SfMNPV         ----------------- 171
```

Figure 5B. The putative amino acid sequence of the TnMNPV/CmBCL9 *polh* gene: (*) indicate identical residues; (:) indicate semi-conserved residues; (.) designate conserved residues.

possibility seems unlikely since no infection of larvae was observed in the stock colony of *T. ni* and attempts to activate latency were unsuccessful. The actual origin of the virus at this time remains unknown.

References

Berretta M, Rios ML, Sciocco de Cap A. 1997. Characterization of a nuclear polyhedrosis virus of *Spodoptera frugiperda* from Argentina. *Journal of Invertebrate Pathology* 71: 280-282.

Bruen TC, Herve P, Bryant D. 2006. A simple and robust statistical test for detecting the presence of recombination. *Genetics* 172: 2665-2681.

Doucet D, Cusson M. 1996. Role of the calyx fluid in alterations of immunity in *Choristoneura fumiferana* larvae parasitized by *Tranosema rostrale*. *Comparative Biochemistry and Physiology* 114A: 31-317.

Federici BA, Hice RH. 1997. Organization and molecular characteriization of genes in the polyhedrin region of the *Anagrapha falcifera* multinucleocapsid NPV. *Archives of Virology* 142: 333-348.

Ferrarese R, Brivio M, Congiu T, Falabella P, Grimaldi A, Mastore M, Perletti G, Pennacchio F, Sciacca L, Tettamanti G, Valvassori R, de Eguileor M. 2005. Early suppression of immune response in *Heliothis virescens* larvae by the endophagous parasitoid *Toxoneuron nigriceps*. *Invertebr Surv J* 2: 60-68.

Fleming JGW, Blissard GW, Summers MD. 1983. Expression of *Campoletis sonorensis* virus in the parasitoid host. *Journal of Virology* 48: 74-78.

Grasela JJ, McIntosh AH. 1998. *In vitro* and *in vivo* host range of *Anticarsia gemmatalis* multiple nuclear polyhedrosis virus. *In Vitro Cellular and Developmental Biology* 34: 79-83.

Granados RR, Federici BA. 1986. *The Biology of Baculoviruses* 1 & 2. CRC Press.

Hall T. 1999. BioEdit: a user-friendly biological sequence alignment and analysis program for Windows 95/98/NT. *Nucleic Acid Sym Ser* 41: 95-98.

```
TnMNPV/CmBCL9  CC-AACATCCTGACCCAGATACTAGAGG-CGTA-------CAACCA-TTTAATTTGAGAG  50
BMMNPV         CCTAACGTTTTGACACGAATTTTAGACGCCATTGCGGAAACTAACA-CAAAGGTTGACAG  59
ACMNPV         CCTAACGTTTTGACGCAAATTTTAGACGCCGTTACGGAAACTAACA-CAAAGGTTGACAG  59
AGMNPV         CCTAGCATTTTGACGCAAATTTTGGAGGCCGTAAGGGCTGTTGATAGCAAAG—TAACCG   58
ROMNPV         CCTAACGTTTTAACGCAAATTTTAGACGCCGTTACGGAAACCAACA-CAAAGGTTGACAG  59
OPMNPV         CCCAGCATTTTGACGCAAATCCTAGACGCCGTAAGGGCCGTTGACAGC-AAGG-TCACTG  58
SFMNPV         AATAAGTTTATTCCTCAAATTATA-ATTATATTAAACATTATGAG-TCAAAATATTTTAC  58
                  *   *  *  * *  **  *  *     *                 *   *

TnMNPV/CmBCL9  -TCGTAAAACGGCTCATAACAATA----CGTGT-CG--GTTGACAATTGTTTCCA----T  98
BMMNPV         -TGTTCAAA----------CTCAG-----TTAAACGGGCTGGAAGAATCGTTCCAGCCTT 103
ACMNPV         -TGTTCAAA----------CTCAG-----TTAAACGGGCTGGAAGAATCATTCCAGCTTT 103
AGMNPV         CTTTGCAAA----------CACAG----GTTGA-CCAGTTGGTAGAGGATTCCAAGACAC 103
ROMNPV         -TGTTCAAA----------CTCAG-----TTGAACGGACTAGAGGAGTCATTCCAGCTTT 103
OPMNPV         CGCTGCAAA----------CCCAA----GTTGA-CCAGCTGGTCGAGGATTCCAAAACCC 103
SFMNPV         --TTTTAATCCGTTCCGACATCAAAGACGTCGA-CGCCAAAGTTGACGCTTTGCAAAGTT 115
                     **                          *      *   *    *   *

TnMNPV/CmBCL9  ---TGCACGCTTTGCACCACACAATCCATGTCCATCAACAGCAAATAATTGTACGCGACG 155
BMMNPV         ---TGGACGGTTTGC-CCGCTCAAT------------------------TGAC--CGATT 133
ACMNPV         ---TGGACGGTTTGC-CCGCTCAAT------------------------TGAC--CGATC 133
AGMNPV         ---TTGAAGCCCTCA-CCGATCAAC------------------------TCGG--CGAAC 133
ROMNPV         ---TGGACGGTTTGC-CCGCTCAAT------------------------TGAC--CGATC 133
OPMNPV         ---TAGAAGCGCTCA-CCGATCAAC------------------------TCGG--TGAGC 133
SFMNPV         CCGTGGATGATGTAA-AGGCTAACC-----TCCC-CGACACCACAGAATTATCCAACAAA 168
                     * *   *         *                            *      *

TnMNPV/CmBCL9  TCCAACAGGTGTGGGT-------TTAACATTGCAGTTACTTTTATATAATCAATTTTG-C 207
BMMNPV         T----------------------TAACACTAAAATCTCAGAAATTCAATCCATATTGAC 170
ACMNPV         T----------------------TAACACTAAGATCTCAGAAATTCAATCCATATTGAC 170
AGMNPV         T----------------------CGACAATAAAGTTAGCGACATCCAATCAATGTTGAG 170
ROMNPV         T----------------------TAACACTAAAGTTTCAGAAATTCAATCTATATTGAC 170
OPMNPV         T----------------------TGACAATAAAGTCAGCGATATTCAATCAATGCTGAG 170
SFMNPV         TTAGAC-GCTCAAGCTACTACTTTGGACACTATCGTATCT-CAAGTCAA-CAACATTC-- 223
                                    *** *    *       *   ** * *   *

TnMNPV/CmBCL9  CAGC-ACGT-----TTCATTATACTGT--C---AACAGAGTTTTTATAATAA---TGAGG 253
BMMNPV         CGGTGACACTG---CTCCGGATCCTCCAG----ACT-----------CC----------- 201
ACMNPV         CGGCGACATTG---TTCCGGATCTTCCAG----ACTC--ACTA-AAGCCTA-AGCTGAAA 219
AGMNPV         CCTTGATGACT---TGCCTGAACCACCAGC---ACCCGAACCG-CAGCCTGAACCTGAAC 223
ROMNPV         CGGCGACATCG---TTCCGGATCTTCCAA----ACTC--ACTC-AAGCCTA-ATCTGAAA 219
OPMNPV         TGTCGAGGAGGAGCTGCCCGAGCCGCCAGC---ACCAGC-------GCCCGAGCCTGAGC 220
SFMNPV         --TCGAAGTTTTGAACCCTGAAATTCCAGATCTACCAGT-------GCCC-AACTTG--- 270
                    *          *   *            *

TnMNPV/CmBCL9  ATACGCTTT-TTTATAATTTATGAGATTTA-------TGTTTTTGCAAACGGGCAGTTGT 305
BMMNPV         ------------------------------------------------------------ 201
ACMNPV         A--CCCAAGCTTTTGA-ACTCG---ATTC-AGACGCTCGTCGTGGTAAAC--GCAGTTCC 270
AGMNPV         C--GG-AACC-TGAGG-TCCCGGAAATTCCCAACACCCGGCG------AA--GCCG---C 267
ROMNPV         A--GCCAAGCGTTTGA-ACTCG---ATTC-AGACACTCGTCGTGGTAAAC--GCAGTTCC 270
OPMNPV         T--CCC-----CGAGA-TCCCCGACGTTCCCGGCCTTCGCC-----------GCAGTCGC 261
SFMNPV         -------------------CGTAAAAAGACAAC--TACCACTACTA-----CCACTAAA 303

TnMNPV/CmBCL9  A-GCGAT-- 311
BMMNPV         ------TAA 204
ACMNPV         AA---GTAA 276
AGMNPV         AA---GTAA 273
ROMNPV         AA---GTAA 276
OPMNPV         AAGCAGTAA 270
SFMNPV         AAG---TAA 309
```

Figure 6A. Multiple-sequence alignment of the TnMNPV/CmBCL9 *p10* nucleotide sequence with five baculoviruses employing the T-Coffee package. (*) indicate identical residues.

```
TnMNPV/CmBCL9   PHYYKNSVDSIMKRAGKIDYIKVTAMLNPHLLDVAYNYL-----LLMDMDCVVQSVQWKQ 55
BMMNPV          PNVLTRILDAIAETNTKVDSVQTQLN----GLEESFQPLDGLPAQLTDFNTKISEIQ-SI 55
AcMNPV          PNVLTQILDAVTETNTKVDSVQTQLN----GLEESFQLLDGLPAQLTDLNTKISEIQ-SI 55
AgMNPV          PSILTQILEAVRAVDSKVTALQTQVD----QLVEDSKTLEALTDQLGELDNKVSDIQ-SM 55
RoMNPV          PNVLTQILDAVTETNTKVDSVQTQLN----GLEESFQLLDGLPAQLTDLNTKVSEIQ-SI 55
OpMNPV          PSILTQILDAVRAVDSKVTALQTQVD----QLVEDSKTLEALTDQLGELDNKVSDIQ-SM 55
SfMNPV          QNILLLIRSDIKDVDAKVDALQSSVDDVKANLPDTTELSNKLDAQATTLDTIVSQVN-NI 59
                         . :     *:  ::          *    :    ::         :..:: .

TnMNPV/CmBCL9   LSTDTYCYEPFYDSQIKWL-------------YASSIWVRML 84
BMMNPV          LTGDTAPDPP--DS---------------------------- 67
AcMNPV          LTGDIVPDLP--DSLKPKLK--TQ-AFELDSDARRGKRSS-K 91
AgMNPV          LSL---DDLP--EPPAPEPQPEPEPEPEVP-EIPNTRRSR-K 90
RoMNPV          LTGDIVPDLP--NSLKPNLK--SQ-AFELDSDTRRGKRSS-K 91
OpMNPV          LSV--EEELP--EPPAPAPEPE---LPEIP-DVPGLRRSRKQ 89
SfMNPV          LEV-LNPEIP--DLPVPNLR--KKT-TTTTTTK--------K 87
                *        *  :
```

Figure 6B. The putative amino acid sequence of the TnMNPV/CmBCL9 *polh* gene: (*) indicate identical residues; (:) indicate semi-conserved residues; (.) designate conserved residues.

Hostetter DL, Puttler B. 1991. A new broad host spectrum nuclear polyhedrosis virus isolated from a celery looper: *Anagrapha falcifera* (Kirby) (Lepidoptera: Noctuidae). *Journal of Environmental Entomology* 20(5): 1480-1488.

Huang X, Miller W. 1991. A time-efficient, linear-space local similarity algorithm. *Advances in Applied Mathematics* 12: 337-357.

Huson DH. 1998. SplitsTree: A program for analyzing and visualizing evolutionary data. *Bioinformatics* 14: 68-73.

Huson DH, Bryant D. 2006. Application of Phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* 23(2): 254-267.

Kariuki CW, McIntosh AH, Goodman CL. 2000. *In vitro* host range studies with a new baculovirus isolate from the diamondback moth *Plutella xylostella* (L.) (Plutellidae: Lepidoptera). *In Vitro Cellular and Developmental Biology* 36: 271-276.

Kariuki CW, McIntosh AH. 1999. Infectivity studies of a new baculovirus isolate for the control of the diamondback moth (Plutellidae: Lepidoptera). *Journal of Economic Entomology* 92(5): 1093-98.

Kroemer JA, Webb BA. 2004. Polydnavirus genes and genomes: emerging gene families and new insights into polydnavirus replication. *Annual Review of Entomology* 49: 431-456.

LeOra Software 1997. POLO-PC Probit and Logit Analysis. *User's manual*. 28pp. Berkeley, California.

McIntosh AH, Ignoffo CM. 1989. Replication of *Autographa californica* nuclear polyhedrosis virus in five lepidopteran cell lines. *Journal of Invertebrate Pathology* 54: 97-102.

McIntosh AH. 1991. *In vitro* infectivity of a clonal isolate of *Syngrapha falcifera* (celery looper) multiple nuclear polyhedrosis virus. *Journal of Invertebrate Pathology* 17: 649-650.

McIntosh AH, Goodman L, Grasela JJ. 1997. Virus production and plaque-forming ability of *Helicoverpa zea* (Lepidoptera: Noctuidae) clonal cell lines. *Applied Entomology and Zoology* 32(4): 657-658.

McIntosh AH, Grasela JJ, Goodman CL. 2001. Growth of a clonal cell line of *Helicoverpa zea* (Lepidoptera: Noctuidae) in suspension culture and replication of its homologous baculovirus HzSNPV. *Applied Entomology and Zoology* 36(3): 349-352.

McIntosh AH, Goodman CL, Grasela JJ. 2006. A simplified and rapid method for extraction of DNA from Baculovirus occlusion bodies. *Bioprocessing Journal (July/August, Tech Rev)* 5(2): 59-61.

McIntosh AH, Grasela JJ, Popham HJR. 2005. AcMNPV in permissive, semipermissive, and nonpermissive cell lines from Arthropoda. *In Vitro Cellular and Developmental Biology* 41: 298-304.

Miller LK. 1997. *The Baculoviruses*. Plenum Press.

Moscardi F. 1990. Assessment of the application of baculoviruses for control of lepidoptera. *Annual Review of Entomology* 14: 257-289.

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD. 1995. Virus taxonomy. Classification and nomenclature of viruses. Sixth report of the International Committee on Taxonomy of Viruses. Springer-Verlag.

Notredame C, Higgins D, Heringa J. 2000. T-Coffee: A novel method for multiple sequence alignments. *Journal of Molecular Biology* 302: 205-217.

Possee RD. In: Kim L, editor. 1993. Viral approaches for insect control. *Advanced Engineered Pesticides* 99-112. CRC Press.

Rose T, Schultz ER, Henikoff JG, Pietrokovski S, McCallum CM, Henikoff S. 1998. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly-related sequences. *Nucleic Acid Research* 26(7): 1628-1635.

Smith HO, Annau TM, Chandrasegaran S. 1990. Finding sequence motifs in groups of functionally related proteins. *Proceedings of the National Academy of Science USA* 87: 826-830.

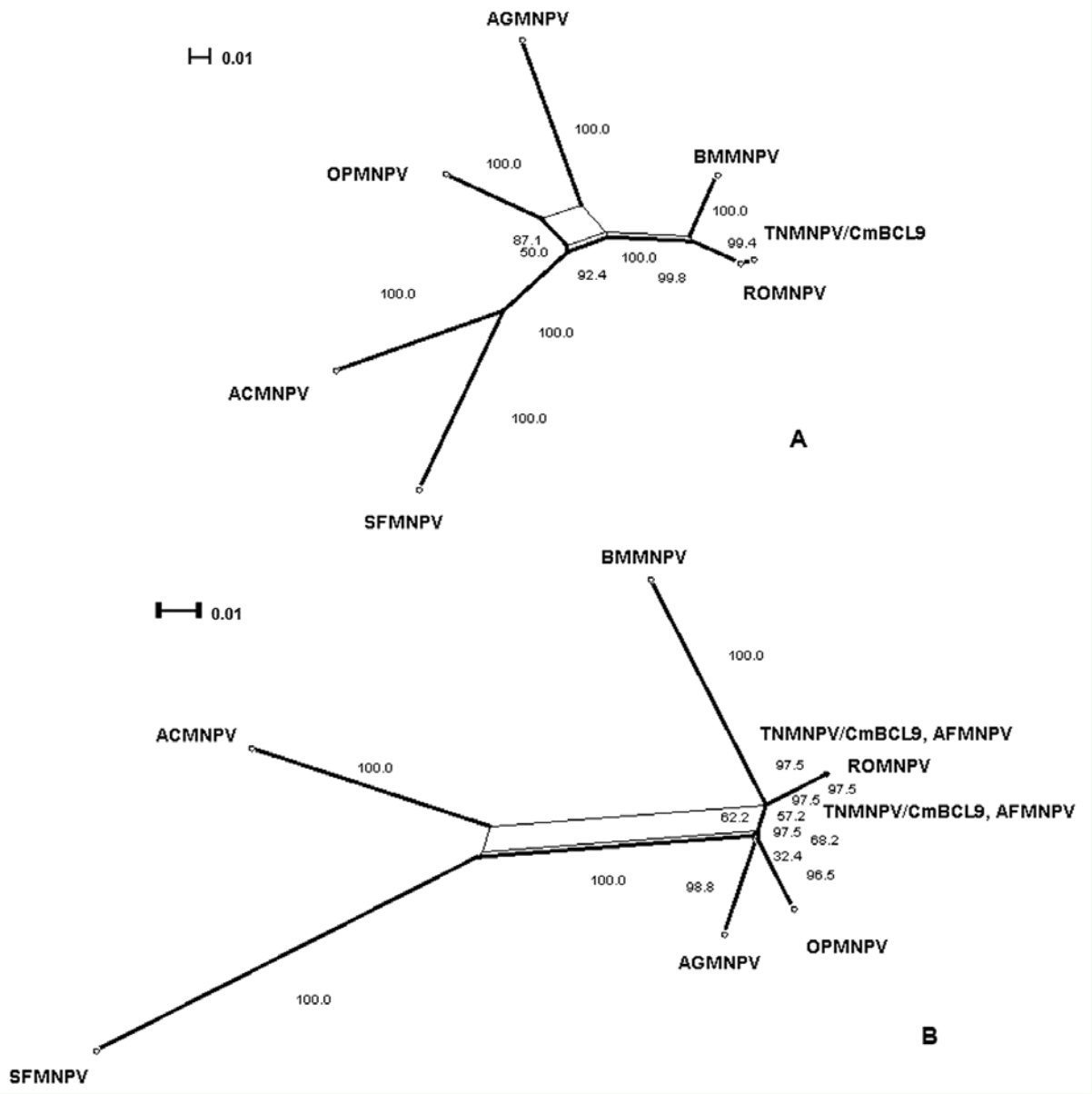

Figure 7. The split-graph of the *polh*, *egt*, and *p10* genes shows that the phylogenetic relationship among the viruses is more appropriately depicted by a non-tree like data set (box-like structures) rather than the typical bifurcating tree. The Hamming distances (d_h), which are computed by giving equal weight to the number of nucleotide differences as well as insertions-deletions between each sequence pair between taxa are accurately represented by the split-graph based on a fit index of 91.7. The number besides each branch is the percent of computed graphs in which a split corresponding to the branch occurred by bootstrap re-sampling.

Stoltz DB, Vinson SB. 1977. Baculovirus-like particles in the reproductive tracts of female parasitoid wasps. II: The genus *Apantales*. *Canadian Journal of Microbiology* 23: 28-37.

Stoltz DB, Vinson SB. 1979. Viruses and parasitism in insects. *Advances in Virus Research* 24: 125-171.

Stoltz DB, Faulkner G. 1978. Apparent replication of an unusual virus-like particle in both a parasitoid wasp and its host. *Canadian Journal of Microbiology* 24: 1509-1514.

Strand MR, Pech LL. 1995. Immunological compatibility in parasitoid-host relationships. *Annual Review of Entomology* 40: 31-56.

Styer EL, Hamm JJ, Nordlund DA. 1987. A new virus associated with the parasitoid *Cotesia marginiventris* (Hymenoptera : Braconidae): Replication in Noctuid host larvae. *Journal of Invertebrate Pathology* 50: 302-309.

Tanada Y, Kaya HK. 1993. *Insect Pathology*. Academic Press.

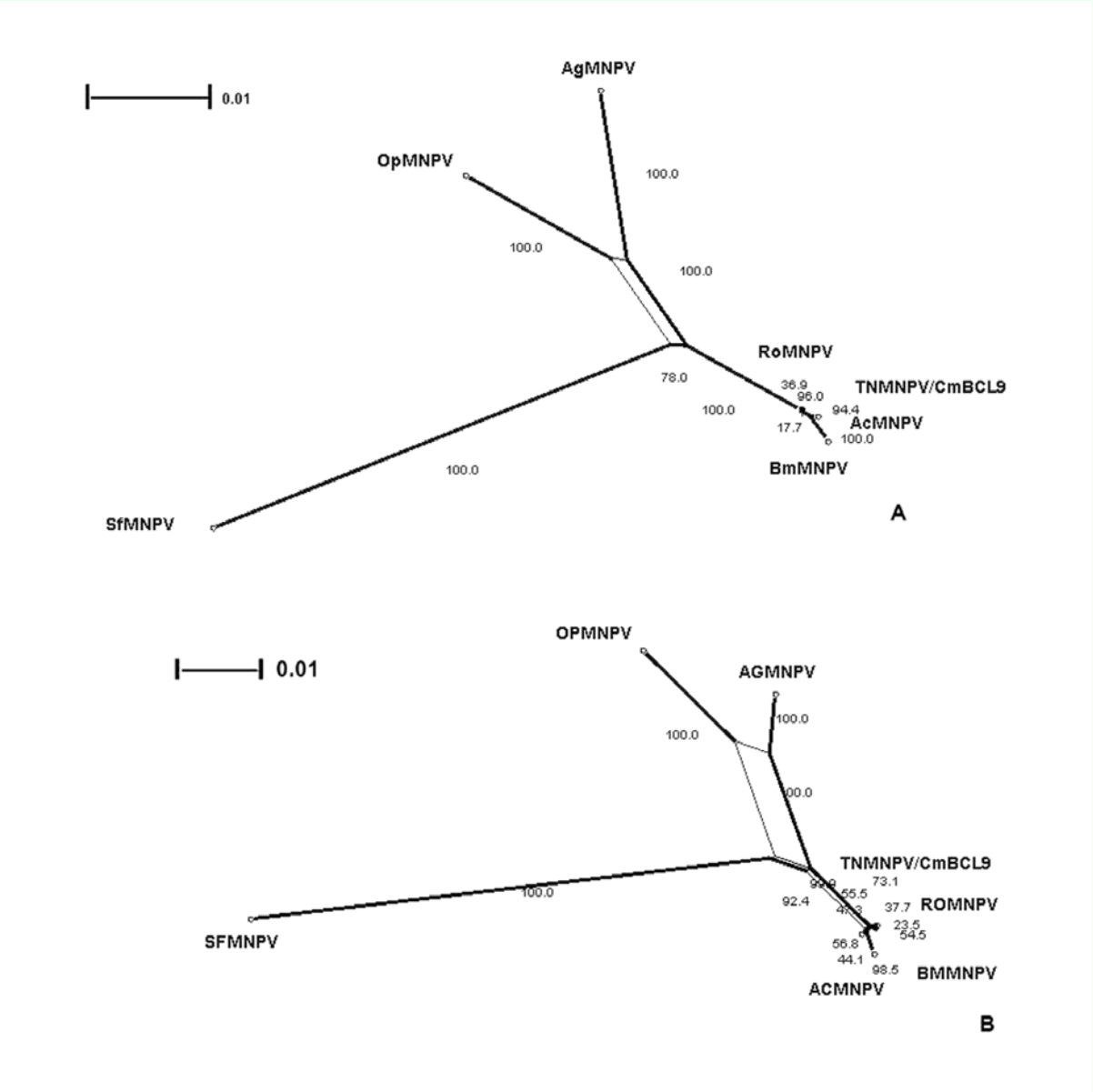

Figure 8. The split-graph of aligned *egt* DNA sequences from seven baculovirus 33 including TNMNPV/CmBCL9. This network is based on a logDet character transformation matrix and split-decomposition distance transformation. The fit index 35 indicates that 95% of the original distance is represented by the split-graph. (B) The split-graph of the aligned *egt* protein translated DNA sequences from seven 37 baculovirus including TNMNPV/CmBCL9. The split index is 98.1%. The splits were calculated employing the ProteinMLdist character transformation and split- 39 decomposition distance transformation.

Vail PV, Hoffmann DF, Streett DA, Manning JS, Tebbets JS. 1993. Infectivity of a nuclear polyhedrosis virus isolated from *Anagrapha falcifera* (Lepidoptera: Noctuidae) against production and postharvest pests and homologous cell lines. *Environmental Entomology* 22(5): 1140-1145.

Vinson SB, Iwantsch GF. 1980. A. Host regulation by insect parasitoids. *Quarterly Review of Biology* 55: 143-165.

Vinson SB, Iwantsch GF. 1980. B. Host suitability for insect parasitoids. *Annual Review of Entomology* 25: 397-419.

Webb BA, Strand MR. In: Gilbert LL, Latrou K, Gill SS, editors. 2005. The biology and genomics of polydnaviruses. *Comprehensive Molecular Insect Science* 6: 323-360. Elsevier.

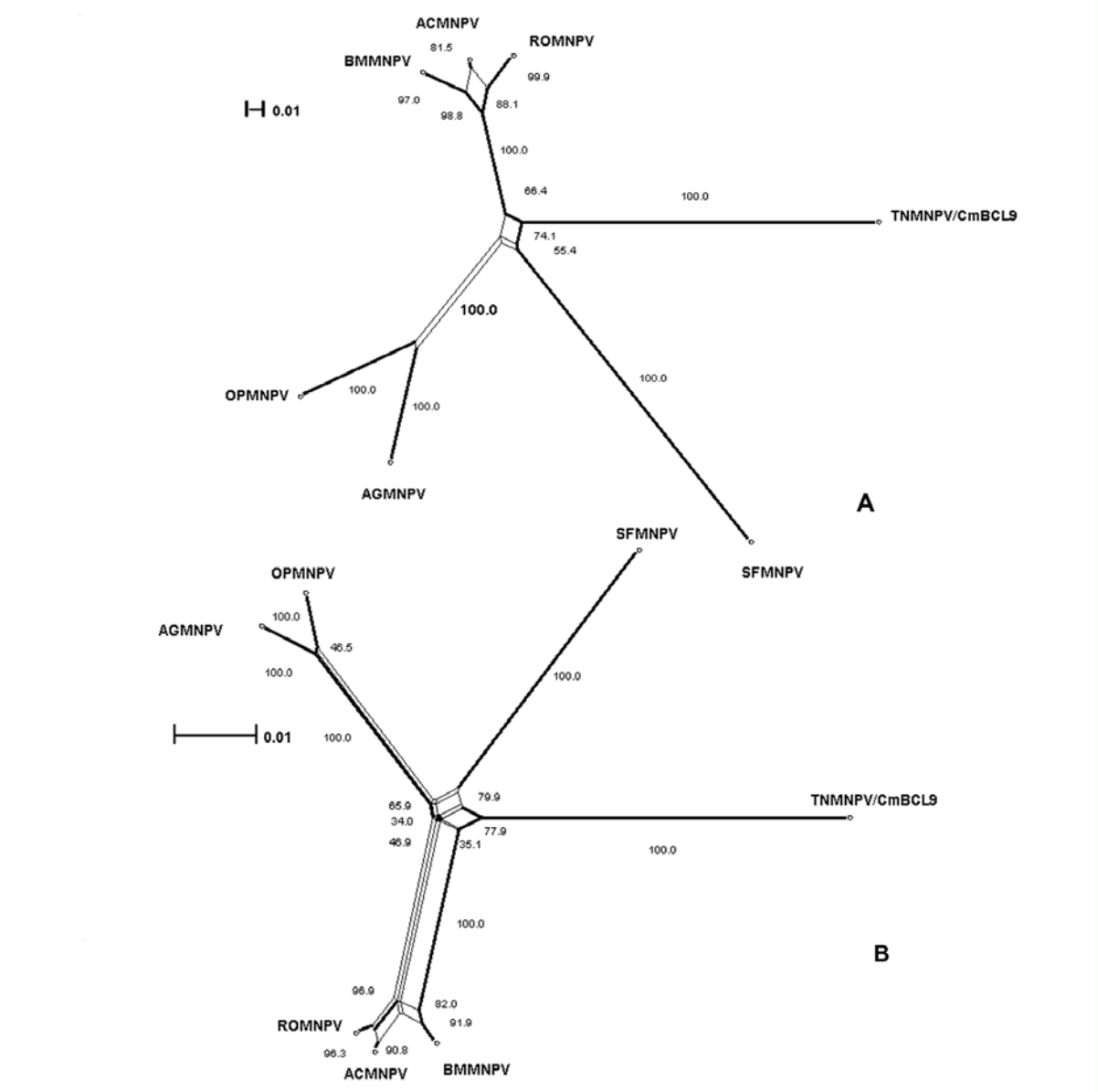

Figure 9. The split-graph of aligned *p10* DNA sequences from seven baculovirus including TNMNPV/CmBCL9. This network is based on a uncorrected_P character 43 transformation matrix and split-decomposition distance transformation. The fit index is 13 indicates that 90.4% of the original distance is represented by the split-graph. (B) 1 The split-graph of the aligned *p10* protein translated DNA sequences from seven baculovirus including TNMNPV/CmBCL9. The split index is 98.7%. The splits were 3 calculated employing the uncorrected_P character transformation and splitdecomposition distance transformation.