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DNA polymerase gene sequences indicate western and forest tent caterpillar viruses form a new taxonomic group within baculoviruses☆

Cydney B. Nielsen,[a] Dawn Cooper,[b] Steven M. Short,[c] Judith H. Myers,[d] and Curtis A. Suttle[e,*]

[a] *Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada V6T 1Z4*
[b] *Department of Zoology, University of British Columbia, Vancouver, BC, Canada V6T 1Z4*
[c] *Department of Botany, University of British Columbia, Vancouver, BC, Canada V6T 1Z4*
[d] *Department of Zoology, University of British Columbia, Vancouver, BC, Canada V6T 1Z4*
[e] *Departments of Earth and Ocean Sciences, Microbiology and Immunology, and Botany, University of British Columbia, Room 1461 Biological Sciences, 6270 University Blvd., Vancouver, BC, Canada V6T 1Z4*

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Abstract

Baculoviruses infect larval lepidopterans, and thus have potential value as microbial controls of agricultural and forest pests. Understanding their genetic relatedness and host specificity is relevant to the risk assessment of viral insecticides if non-target impacts are to be avoided. DNA polymerase gene sequences have been demonstrated to be useful for inferring genetic relatedness among dsDNA viruses. We have adopted this approach to examine the relatedness among natural isolates of two uncharacterized caterpillar-infecting baculoviruses, *Malacosoma californicum pluviale* nucleopolyhedrovirus (McplMNPV) and *Malacosoma disstria* nucleopolyhedrovirus (MadiMNPV), which infect two closely related host species with little to no cross-infectivity. We designed two degenerate primers (BVP1 and BVP2) based on protein motifs conserved among baculoviruses. McplMNPV and MadiMNPV viral DNA was obtained from naturally infected caterpillars collected from geographically distinct sites in the Southern Gulf Islands and Prince George regions of British Columbia, Canada. Sequencing of 0.9 kb PCR amplicons from six McplMNPV and six MadiMNPV isolates obtained from a total of eight sites, revealed very low nucleotide variation among McplMNPV isolates (99.2–100% nucleotide identity) and among MadiMNPV isolates (98.9–100% nucleotide identity). Greater nucleotide variation was observed between viral isolates from the two different caterpillar species (only 84.7–86.1% nucleotide identity). Both maximum parsimony and maximum likelihood phylogenetic analyses support placement of McplMNPV and MadiMNPV in a clade that is distinct from other groups of baculoviruses.

Keywords: *Malacosoma californicum pluviale* NPV (McplMNPV); *Malacosoma disstria* NPV (MadiMNPV); *Malacosoma californicum pluviale* (Dyar); *Malacosoma disstria* (Hubner); Nuclear polyhedrosis virus; Phylogenetic; Diversity; Polymerase; PCR

1. Introduction

Baculoviruses are the largest and most widely studied group of viruses that infect insects (Frederici, 1986). Characterized by a large (80–230 kbp), supercoiled, circular, double-stranded DNA genome, baculoviruses have spurred interest because of their potential use as biological insecticides. Traditionally, classification of baculoviruses has been based on morphology (Francki et al., 1991; Matthews, 1982) and their nomenclature based on the host from which the virus was first isolated (Tinsley and Kelly, 1985). Relatedness among baculoviruses has been assessed by a variety of methods, including the degree of genomic hybridization (Kislev,

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* Corresponding author. Fax: +604-822-6091.
E-mail address: csuttle@eos.ubc.ca (C.A. Suttle).

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1985; Smith and Summers, 1982), serological techniques (Hohmann and Faulkner, 1983; Knell et al., 1983; Volkman, 1985), and restriction enzyme analysis (Miller and Dawes, 1978; Vlak and Smith, 1982). The strength of these methods lies in their ability to differentiate among related baculoviruses and thus they have immediate applications in monitoring the distribution and ecological impact of wild-type and genetically modified baculoviruses within natural communities. However, these techniques do not assess baculovirus taxonomy in a systematic way. More recently, the availability of genetic sequence data has allowed for the construction of phylogenetic trees with reasonable resolution and thus enabled a more rigorous assessment of baculovirus relatedness (Bulach et al., 1999; Cowan et al., 1994; Rohrmann, 1986; Volkman, 1985). An understanding of the genetic relatedness among baculoviruses may provide insights into virus host range, in turn assisting in the design of future bio-pesticides (Cowan et al., 1994).

Typically, gene sequences used to assess baculovirus relatedness should be relatively conserved, but sufficiently varied to allow for meaningful comparisons. The baculovirus family includes the genera *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). Each GV occlusion body contains a single, enveloped nucleocapsid (virion) in a granulin protein matrix, whereas the NPV occluded form consists of multiple virions embedded in a polyhedrin protein matrix (Miller, 1996). Much baculovirus phylogenetic analysis is based on occlusion protein sequences (polyhedrin and granulin), primarily because of the abundance of data. This method of classification has drawbacks. Occlusion proteins are small and provide limited sequence data for analysis. The fact that more than half the residues are invariant, and thus offer few sites for phylogenetic estimates, exaggerates the problem (Bulach et al., 1999). It is also challenging to define an appropriate out-group criterion on which to root the phylogenetic tree. As a result, different tree construction procedures produce conflicting branch placements (Cowan et al., 1994; Zanotto et al., 1993).

Bulach et al. (1999) attempted to solve this problem by using DNA polymerase gene sequences for phylogenetic analysis of baculoviruses. DNA polymerase genes have relatively large coding regions with ca. 3000 nucleotides (Pellock et al., 1996) and the similarity to entomopox and other poxvirus polymerase genes provide reasonable out-group taxa for rooting the phylogenetic tree (Braithwaite and Ito, 1993). The results of Bulach et al. (1999) illustrate a relatively robust statistical significance of DNA polymerase trees compared to occlusion protein trees, confirming that DNA polymerase has significant advantages over occlusion proteins for resolving deep branching taxonomic relationships. In addition, DNA polymerase gene sequences have been used to determine the taxonomic placement of viruses infecting microalgae, and are concordant with trees based on total genomic DNA hybridization (Chen and Suttle, 1996). Ecdysteroid UDP-glucosyltransferase (*egt*) genes and gp41 structural protein genes have also been used for phylogenetic analysis of baculoviruses. However, some *egt* tree constructs do not resolve deep branches with high assurance (Clarke et al., 1996; Faktor et al., 1995; Popham et al., 1997) and in the case of gp41 tree constructs, analysis is somewhat limited by the lack of available data (Liu and Maruniak, 1999).

Given the success of using DNA polymerase sequence data to determine relatedness among baculoviruses, we used sequence data from this gene to assess the nucleotide variation among baculoviruses isolated from geographically distinct populations of two closely related caterpillar species. We also examined the variation among viruses infecting the same host species. The interest in examining genotypic variation and its role in the biology of baculoviruses has been motivated by an interest in understanding baculovirus and host evolution, and by the demand to identify more effective virus strains for the biological control of insects (Cherry and Summers, 1985; Shapiro et al., 1991). Though genotypic variation among geographic isolates of a single baculovirus species has been documented by restriction fragment length polymorphism (RFLP) analysis, there are few data on nucleotide variation at this level (Allaway and Payne, 1983; Cherry and Summers, 1985; Crawford et al., 1986; Gettig and McCarthy, 1982; Loh et al., 1982; Shapiro et al., 1991). Two uncharacterized baculoviruses are of particular interest for our analysis, *Malacosoma californicum pluviale* NPV (McplMNPV) and *Malacosoma disstria* NPV (MadiMNPV). McplMNPV infects the western tent caterpillar, *Malacosoma californicum pluviale* (Dyar) whereas MadiMNPV infects the forest tent caterpillar, *Malacosoma disstria* (Hubner). Both caterpillars are common defoliators of deciduous trees in British Columbia, with western tent caterpillars feeding primarily on red alder, *Alnus rubra*, and forest tent caterpillars feeding on trembling aspen, *Populus tremuloides*. RFLP analysis has shown that viruses from these closely related hosts are genetically distinct (Cooper et al., 2003), but their genetic relatedness to each other and to other baculoviruses remained unknown. We use DNA polymerase nucleotide sequence data to quantify the variation within these viruses, and to place them within a phylogenetic tree of other baculoviruses. The sequence data were obtained using two degenerate primers designed from conserved regions in the DNA polymerase amino acid sequences of characterized baculoviruses. The simplicity and power of this degenerate primer system makes it an attractive tool for rapidly obtaining nucleotide sequence data from uncharacterized baculoviruses.

2. Materials and methods

2.1. Sources of baculovirus DNA

Naturally infected caterpillars were collected in June 1998 and June 1999 from various sites on the Southern Gulf Islands (*M. californicum pluviale* (Dyar) specimens) and in regions near Prince George (*M. disstria* (Hubner) specimens), British Columbia, Canada (Fig. 1). Baculovirus genomic DNA was extracted from McplMNPV and MadiMNPV as follows: infected larvae were suspended in 500 μl of dH_2O and then ground with plastic pestles. A series of low-speed centrifuge spins (62*g* for 35 s) and washes with dH_2O were used to separate occlusion bodies (OBs) from insect debris. OBs were then pelleted with a high-speed centrifuge spin (12,100*g* for 20 min). The pellet was washed two times with dH_2O, re-suspended in 1 ml of dH_2O, and heated to 65 °C for 30 min to denature any DNases. Virions were released from OBs by treatment by alkali lysis (1 M Na_2Co_3, 150 mM NaCl, and 0.1 mM

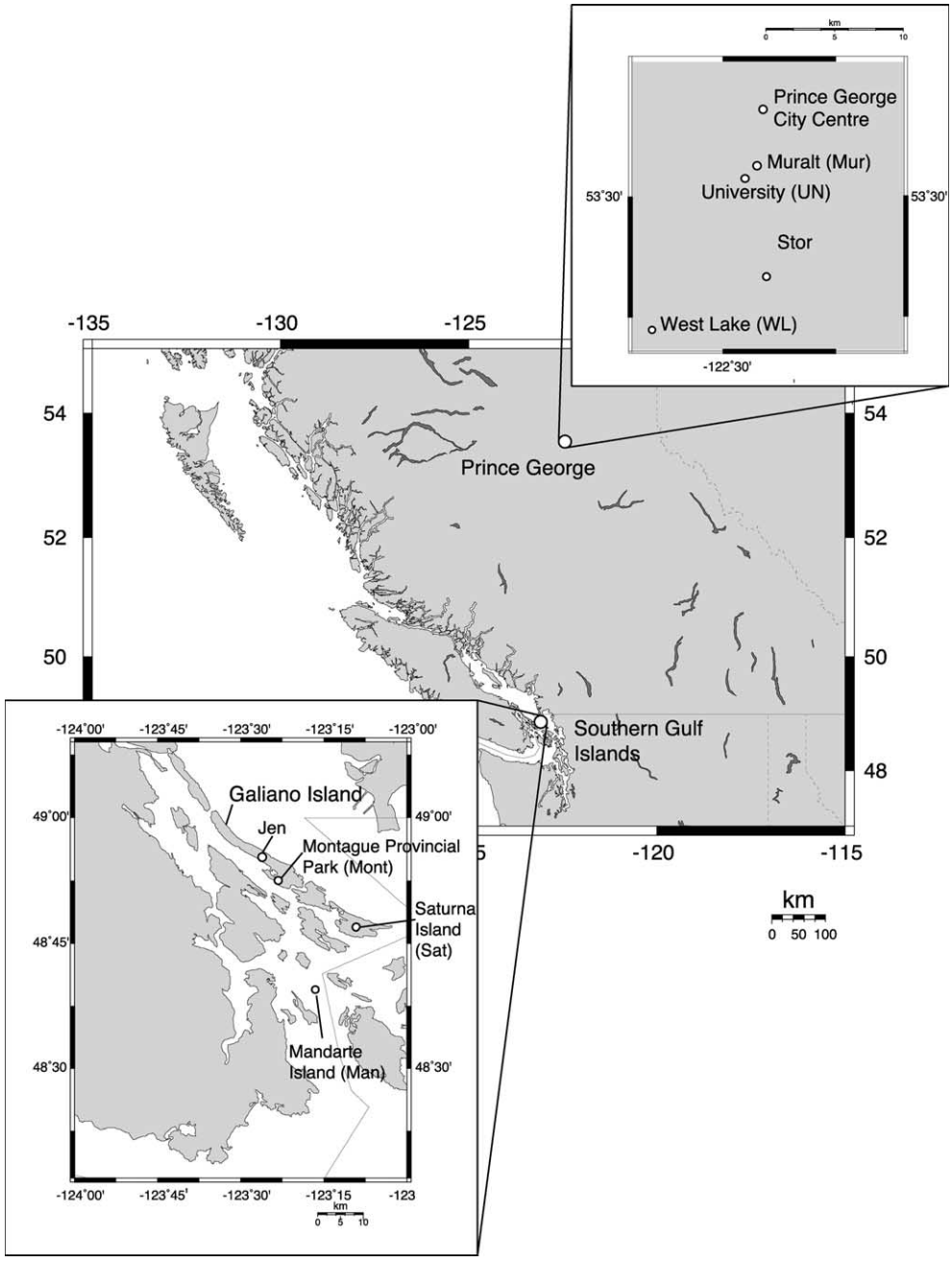

Fig. 1. Map of infected caterpillar collection sites. Six MadiMNPV isolates from sites near Prince George, BC were used in this study: 2 from Mur, 2 from UN, 1 from Stor, 1 from WL. Six McplMNPV isolates from sites on the Southern Gulf Islands, BC were used in this study: 1 from Jen, 1 from Man, 2 from Mont, 2 from Sat.

EDTA, pH 10.8) at 37 °C for 60 min followed by a high-speed centrifuge spin (12,100*g* for 30 min).

DNA was released from the virions by re-suspending the pellet in 500 μl of proteinase K buffer (10 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl, and 0.4% SDS) and 20 μl of proteinase K enzyme (20 mg ml^{-1}) overnight at 37 °C. Proteins were removed using a protein precipitation solution (Puregene). Ethanol (100%) and sodium acetate (3 M, pH 8.0) were used to precipitate DNA (−20 °C for 3 h). DNA was pelleted with a high-speed spin (12,100*g* for 15 min), followed by a wash with 70% ethanol for 1.5 h. The DNA was then pelleted at 12,100*g* for 10 min and re-suspended in 65 μl of TE buffer at room temperature. All DNA was stored at 4 °C.

2.2. Primer sequences and synthesis

Two degenerate primers were used in this study, baculovirus primer 1 (BVP1) and baculovirus primer 2 (BVP2). BVP1 (5′-GAI CCI TA[C/T] TT[C/T] TT[C/T] AA[C/T] AA-3′) and BVP2 (5′-[A/G]TA IGG IAT IC[G/T] [A/G]TC ICC-3′) correspond to nucleotide positions 1366–1385 and 2542–2559, respectively, of the AcMNPV DNA polymerase gene (GenBank Accession No. M20744.1) numbering from the first ATG. Deoxyinosine (I), which will pair with any nucleotide, was incorporated at positions of complete degeneracy to reduce the size of the oligonucleotide pool. BVP1 is a forward primer corresponding to the amino acid sequence [D/E]PYFFNK, while BVP2 is a reverse primer corresponding to the reverse complement of the gene sequence encoding GDRIPY. These primers have degeneracies of 16 and 8, respectively, indicating the size of each primer pool. Calculated melting temperatures generated using dPrimer v1.0 (Chen and Zhu, 1997) range from 39.2 to 48.3 °C for BVP1 and from 32.7 to 60.6 °C for BVP2. Both primers were synthesized by solid phase chemistry and obtained from a commercial source (GibcoBRL).

2.3. PCR and cloning

Viral DNA (20–50 ng) was added to a 25 μl PCR mixture containing 0.375 U Platinum Taq DNA polymerase (GibcoBRL), manufacturer's buffer, 3.0 mM $MgCl_2$, 0.16 mM each deoxyribonucleoside triphosphate, and 60 pmol of each primer. For all PCR reactions, negative controls contained all reagents except template DNA. Mineral oil (30 μl) was added to each reaction tube to prevent evaporation. Reactions were carried out in a Minicycler (MJ Research) using the following cycle parameters: 95 °C denaturation (1.5 min), 30 cycles of 95 °C (30 s), 45 °C (45 s), 72 °C (1.0 min), followed by a final 72 °C extension (4 min). PCR products were electrophoresed on 1.5% SeaKem LE Agarose (FMC BioProducts) in 0.5× TBE buffer (0.045 M Tris–borate, 1 mM EDTA [pH 8.0]). Products were examined by ethidium bromide staining. Agarose gel images were recorded on a digital camera and contrast enhanced for clarity.

PCR products were TA cloned (Marchuk et al., 1991) into *Eco*RV digested, T-tailed pGem5Zf(+) plasmid (Promega), and transformed into *Escherichia coli* JM109 (Promega). Preparation of competent cells, ligation, transformation, and selection of recombinant plasmids were done by standard methods outlined by the supplier (Promega). Plasmids were purified using Qiagen's QIAprep Spin Miniprep Kit according to manufacturer's recommendations (Qiagen, Valencia, CA).

2.4. DNA sequencing and analysis

All sequencing was done using a conventional dideoxy chain termination method (Sanger et al., 1977), using M13 forward and M13 reverse primers and AmpliTaq FS BIGDYE Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA) according to manufacturer's recommendations. Excess Dye-Terminators were removed from the completed sequencing reactions using CENTRI-SEP spin columns (Princeton Separations, Adelphia, NJ). Once purified, all sequencing reactions were run in ABI Model 373 Stretch or ABI Prism 377 automated sequencers (Applied Biosystems) at the University of British Columbia sequencing facility.

BioEdit v 5.0.7 (Hall, 1999) was used for sequence editing, translation, and generation of pair-wise DNA identity matrices. ClustalW (Thompson et al., 1994) was used with default parameter settings and the BLOSUM62 amino acid substitution matrix (Henikoff and Henikoff, 1992) to generate amino acid alignments. Consensus amino acid sequences used as the basis of degenerate primer design were selected from an alignment of all seven complete baculovirus DNA polymerase protein sequences available at the time of analysis (Table 1). Primer sequences were subject to similarity searches against major international databases using BLAST with a reduced word size (7), no filtering, and reduced gap penalty (from –3 to –1) (Altschul et al., 1990). The maximum parsimony phylogenetic tree based on amino acid sequence data was constructed using SEQBOOT, PROTPARS, and CONSENSE programs, PHYLIP package 3.573 (Felsenstein, 1993). A threshold parsimony value of 11 was used with PROTPARS. The maximum likelihood phylogenetic tree was constructed using TREE-PUZZLE version 5.0 (Strimmer and von Haeseler, 1996) using the BLOSUM62 amino acid substitution matrix (Henikoff and Henikoff, 1992), and otherwise default parameter settings. CbEPV was used as an outgroup for both tree construction methods. Phylogenetic trees were drawn and visualized using the program TreeView version 1.6.1 (Page, 1996).

3. Results and discussion

3.1. PCR primer design

Our degenerate primers correspond to conserved baculovirus DNA polymerase protein sequences, allowing amplification of uncharacterized baculoviruses such as MadiMNPV and McplMNPV. A ClustalW alignment of seven available complete, baculovirus DNA polymerase amino acid sequences (Table 1) was used to identify conserved regions of the protein. Two consensus sites of 7 and 6 amino acids and ca. 1200 bp apart, made suitable PCR primer targets. These sites flank a characteristic DNA polymerase motif, YGDTDS (Fig. 2), that is the most highly conserved motif among divergent B family (α-like) DNA-dependent DNA polymerases (Wong et al., 1988). Furthermore, it has been observed in DNA and RNA-dependent RNA and DNA polymerases of humans, yeast and viruses (Argos, 1988; Braithwaite and Ito, 1993; Illana et al., 1996; Iwasaki et al., 1991; Pizzagalli et al., 1988). The functional importance of this motif for polymerase activity has been demonstrated in the HSV DNA polymerase, where single changes in the 'GDTD' sequence destroyed polymerization activity (Dorsky and Crumpacker, 1990), and this motif is proposed to be involved in metal binding and catalysis (Argos, 1988; Bernad et al., 1990). For these reasons, the presence of this motif in the inferred amino acid sequence of PCR amplicons from uncharacterized baculoviruses, would provide strong evidence that the amplified fragments were from DNA polymerase genes. BLAST searches revealed that significant similarity to both consensus sites under consideration were only found in baculovirus DNA polymerase genes, suggesting that these primers are baculovirus specific. The degenerate primers BVP1 (upstream) and BVP2 (downstream) were designed to target these baculovirus consensus sites, and were predicted to generate a 1194 bp (398 aa) product from AcMNPV (Fig. 2).

Table 1
Abbreviations, host names, and GenBank sources of sequences used for this study

Virus	Host	GenBank database Accession No.	Reference
AcMNPV[a]	*Autographa californica*	M20744.1	Tomalski et al. (1988)
BmNPV[a]	*Bombyx mori*	D16231.1	Chaeychomsri et al. (1995)
BusuNPV[a]	*Buzura suppressaria*	AF068184.1	Bulach et al. (1999)
CfMNPV[a]	*Choristoneura fumiferana*	U18677.1	Liu and Carstens (1995)
CuniNPV	*Culex nigripalpus*	AF403738.1	Afonso et al. (2001)
EppoNPV	*Epiphyas postvittana*	AY043265.1	Hyink et al. (2002)
HearNPV	*Helicoverpa armigera*	AF303045.1	Zhang et al. (2001)
HzSNPV[a]	*Helicoverpa zea*	U11242.1	Bulach et al. (1999)
LdMNPV[a]	*Lymantria dispar*	D11476.1	Bjornson et al. (1992)
MacoNPV	*Mamestra configurata*	U59461.2	Li et al. (2002)
MbMNPV	*Mamestra brassicae*	AF068183.1	Bulach et al. (1999)
OpMNPV[a]	*Orgyia pseudotsugata*	U39145.1	Ahrens et al. (1996)
OranNPV	*Orgyia anartoides*	AF068185.1	Bulach et al. (1999)
SeMNPV	*Spodoptera exigua*	AF169823.1	Ijkel et al. (1999)
SpltNPV	*Spodoptera litura*	AF325155.1	Pang et al. (2001)
CypoGV	*Cydia pomonella*	U53466.2	Luque et al. (2001)
PlxyGV	*Plutella xylostella*	AF270937.1	Hashimoto et al. (2000)
XecnGV[a]	*Xestia c-nigrum*	AF034436.1	Goto et al. (1998)
CbEPV	*Choristoneura biennis*	X57314.1	Mustafa et al. (1991)
FPV	*Gallus gallus*	M31638.1	Binns et al. (1987)
Vac	*Homo sapiens*	M13213.1	Earl et al. (1986)

[a] Baculoviruses used for BVP1 and BVP2 primer design.

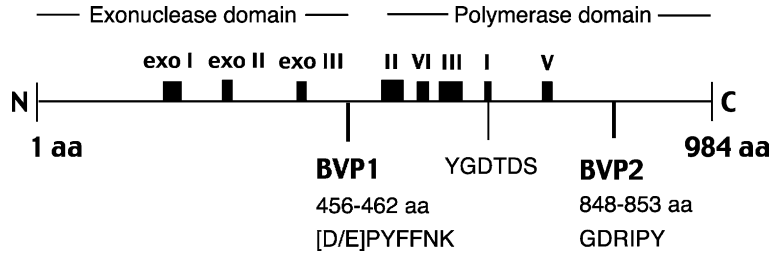

Fig. 2. *Autographa californica* (AcMNPV) DNA polymerase gene. Regions of similarity to ExoI, ExoII, and ExoIII protein motifs (Blanco et al., 1991) and conserved regions I, II, III, V, and VI (Wong et al., 1988) common to B-family (α-like) DNA polymerases are indicated with black boxes. Locations of BVP1 and BVP2 protein motifs are also marked.

3.2. PCR amplification of DNA polymerase genes

PCR amplification using BVP1 and BVP2 produced distinct band patterns for purified AcMNPV, McplMNPV, and MadiMNPV (Fig. 3). However, not all template preparations produced products of equal intensity under these conditions. Increasing the cycle number from 30 to 35 typically resolved the problem of low intensity products. In some cases amplification did not occur, likely because of impurities in the DNA preparation. No amplification was obtained when the $MgCl_2$ was reduced to 1.5 mM (data not shown).

The expected dominant ca. 1.2 kb band was amplified from the AcMNPV template (Fig. 3, lane 3). An additional ca. 2.0 kb band was also observed, but unlike the dominant 1.2 kb band, it was not produced at a more stringent annealing temperature of 47 °C (data not shown) and it was amplified by BVP1 alone (Fig. 3, lane 4). Extensive BLAST searches of all possible BVP1 sequences against the AcMNPV genome (GenBank

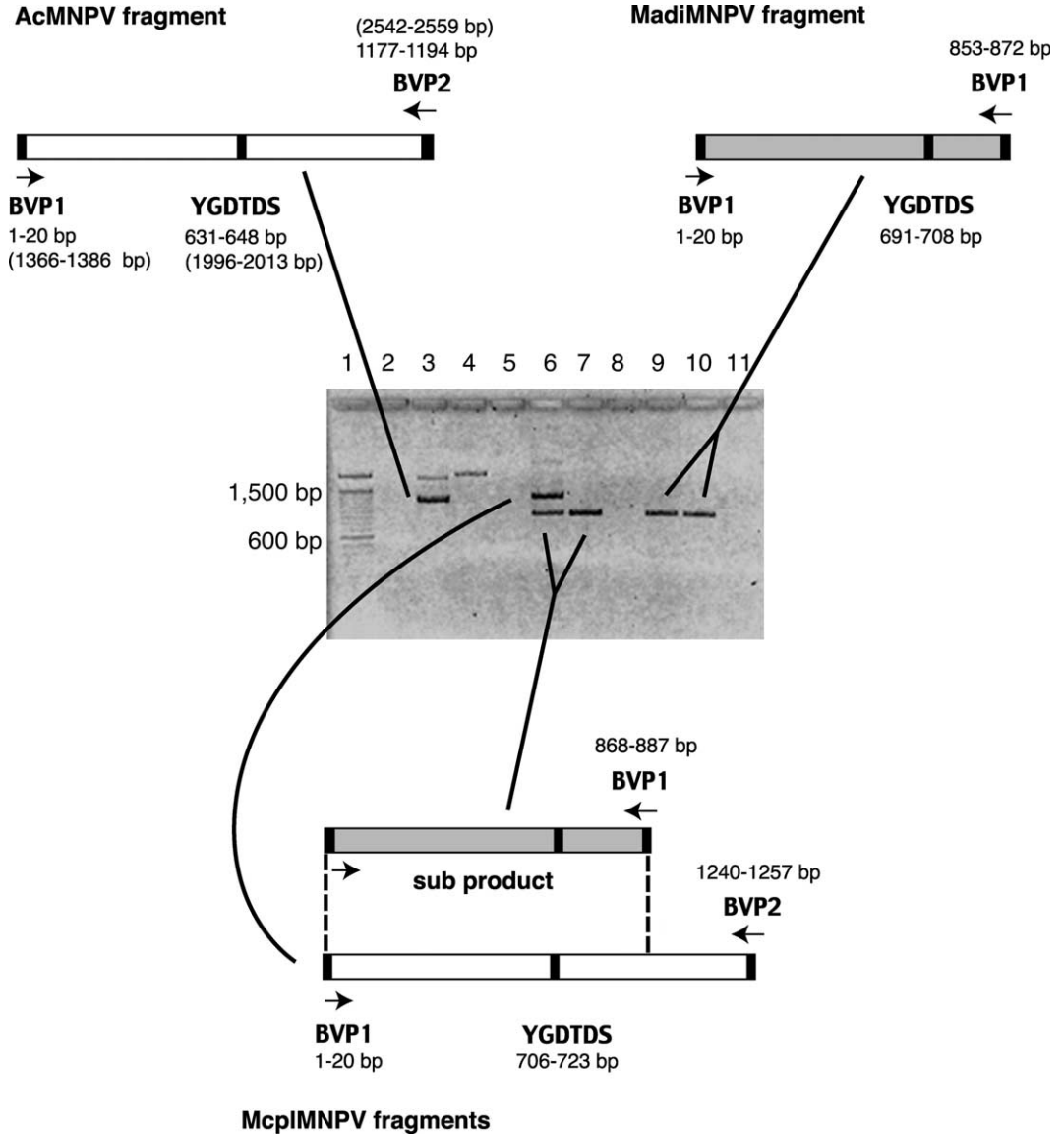

Fig. 3. Analysis of BVP1 and BVP2 PCR products by agarose gel electrophoresis. PCR product diagrams illustrate nucleotide positions of BVP1 and BVP2 primer binding sites relative to the YGDTDS conserved motif in AcMNPV, MadiMNPV, and McplMNPV products (black boxes indicate primer sites and the YGDTDS motif; arrows indicate primer directionality). Positions of the primer sequences relative to the start of the protein coding region are provided in parentheses for AcMNPV. BVP1-only products are shaded in gray. Gel lane descriptions: Lane 1, 100 bp molecular weight ladder (GibcoBRL); Lane 2, negative control; Lane 3, AcMNPV template with 1.2 μM each BVP1 and BVP2; Lane 4, AcMNPV template with 1.2 μM BVP1; Lane 5, AcMNPV template with 1.2 μM BVP2; Lane 6, McplMNPV template with 1.2 μM each BVP1 and BVP2; Lane 7, McplMNPV template with 1.2 μM BVP1; Lane 8, McplMNPV template with 1.2 μM BVP2; Lane 9, MadiMNPV template with 1.2 μM each BVP1 and BVP2; Lane 10, MadiMNPV template with 1.2 μM BVP1; Lane 11, MadiMNPV template with 1.2 μM BVP2. McplMNPV template was from Sat1 isolate. MadiMNPV template was from WL3 isolate.

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AcMNPV          DPYFFNKNDLSIISGQF------------------------KADKAAAGISNLKRKLIP
BmNPV           DPYFFNKNDLSIISGQF------------------------NADKAAGGISNLKRKLTP
CfMNPV          DPYFFNKDDLGRMSSK-------------------------DAGFCAG-MSRLQRKRIP
OpMNPV          DPYFFNKDDLGLMSSKG------------------------SAGLTTG-MSRLRRRRIP
EppoNPV         DAYFFNKNDLGFMSCKN------------------------GKGMAG-----LQRKLIP
McplMNPV        EPYFFNKWDLNVTVNRKRTWNKSNAADDGAELSIPPNDDGVMTSDINTIDFTKLKRTPID
MadiMNPV        EPYFFNKWDLNVTVNRKRTWDKGNAAVVADNDTFSDIG-----SDMNTIDFTKLKRTPID
HearNPV         EPYFFNKHDLSVTSGRKRNAAGDSVD---------------DQQMVDLSLLQRRPVP
HzSNPV          EPYFFNKHDLSVTSGRKRNAAGDSVD---------------DQQMVDLSLLQRRPVP
SpltNPV         DPYFFNKSDLNVTSGR----SGGGGD---------------RQIVDLTQLNRKCIVP
MacoNPV         DPYFFNKYDLNVTSGRKRNYD--------------------GDSVDSQNVDLTKLQRKPIP
MbMNPV          DPYFFNKYDLSVTSGRKRNYD--------------------GDSVDSQNVDLTQLHRKPIP
SeMNPV          DPYFFNKYDLSITSGQKRTFDERNNNDGD-----------GVVVDSQVVDLTQLKRKPIS
BusuNPV         DPFIFNKNDLNITSGKRN---TTFDNSNNRN--------SVNETRTGFVNLDLLKRQPVP
OranNPV         DPYIFNKNDLHITSGRWSNKVADYENSSSSN--------GISSNKPGAVDLTLWNRTPVP
LdMNPV          DPYFFNKYDLSVTSGASAPSTSRPAN---------------------AIDLSQLKRTPVD
XecnGV          DPYFFNKFDLNKMVIRKPIAQSPESDSDS----------DEKKDEGDPMDLTLLLRRRVP
PlxyGV          DSYFFNKFDLNKMCKKIDDGN-------------------------DEVDLTKLARCRVP
CypoGV          DPYFFNRSDLSKIMGGNKRFAADEKED------------KEVENEGELVDFTVLGRPPVP
Vac             ARYCIHDACLCQYLWEYYGVETKTDAGASTYVLP-------QSMVFEYRASTVIKGPLLK
FPV             ERYCIHDACLCKYIWDYYRVPSKINAASSTYLLP-------QSLALEYRASTLIKGPLLK
CbEPV           AYYCTHDTVLCNCIFKYDMIHDKVIAFSNEYLLP-------QYMSFKYKSTTNISGLLLK
CuniNPV         -DSLRNKLSPEVLGGRRQRYDAEILNISFKRAIE--------EERKRSPERQDELDDLER
                     :

AcMNPV          LKNIPKDAINLGPANQTVKYKGGKVLKP-RAGIYKNAFSLDFNSLYLTIMIAICACLSN-
BmNPV           LKNIPKDAINLGPANQTVKYKGGKVLQP-RASIYKNAFSLDFNSLYLTIMIAICACLSN-
CfMNPV          LKDLPANSIRLGAINEIVNYEGGKVLQP-RAGVYEFAFSLDFNSLYLTIMIDICACLTN-
OpMNPV          LKDVPATAIRLGAIDENVKYEGGKVLQP-RAGVYEFAFSLDFNSLYLTIMIDICACLTN-
EppoNPV         QKDVPTNALCLGAINASIKYEGGKVLQP-RAGVYEYAFSLDFNSLYLTIMIDICACLTN-
McplMNPV        VALLPENATRLCPAIGKCLYKGGKVLTP-IPGCHENSFTLDFNSLYLRIMVLHTICLTN-
MadiMNPV        VALLPDNATKLCPAIGKCLYKGGKVLTP-VPGCHDNSFTLDFNSLYLRIMVLHTICLTN-
HearNPV         VDMIPSNAVKLCGKRQRCVYKGGKVLEP-QPGFKQWVVTLDFNSLYLSIMMYEGICLSN-
HzSNPV          VDMIPSNAVKLCGKRQRCVYKGGKVLEP-QPGFKQWVVTLDFNSLYLSIMMYEGICLSN-
SpltNPV         TNMIPQTAIKLCARRERCVYKGGKVIAP-IPGMYRSVVTLDFNSLYLNIMKNEGICLSN-
MacoNPV         IGEVPNDAVKLCHQNQKCVYTGGKVLAP-KPGYKKWVVTLDFNSLYLSIMMQEGICLSN-
MbMNPV          MSQVPIDAVKLCHQNQKCVYTGGKVLSP-IPGYKKWVVTLDFNSLYLSIMMQEGICLSN-
SeMNPV          VADIPCDAVKLCHQKQKCVYTGGKVLSP-NPGFKKWVVTLDFNSLYLSIMMQEGICLSN-
BusuNPV         IEKIPSEAVKLCTTRPICNYKGGKVLAP-KTGLQKWVVTLDFNSLYLTIMMYEGACFSN-
OranNPV         SAAIPPDAIKLCTTRSVCNYEGGKVLSP-QSGFKKWVVTFDFNSLYPTIMMYEGVCFSN-
LdMNPV          AARIPPSAVKLCSTRQSCTYKGGKVLSP-KPGFNRWVATLDFNALYPTIMMWEGVCMSSN
XecnGV          EHEIPSNAVRLCPLKCNIKYTGGMVLSP-VPGYYGLTFTLDFSQLYTSIMIAESACLSN-
PlxyGV          EHHIPKDAVKLCPLKCAINYTGGKVLAP-KPGYYELLFTLDFSQLYTSIMIAETACLSN-
CypoGV          TSQIPADAIPLCDLKTRIKYIGGKVLSP-KPGYYRKTFTLDFSQLYTSIMIFFTTCLSN-
Vac             LLLETKTILVRSETKQKFPYEGGKVFAPKQKMFSNNVLIFDYNSLYPNVCIFGNLSPETL
FPV             LLLEERVIYTRKITKVRYPYIGGKVFLPSQKTFENNVMIFDYNSLYPNVCIYGNLSPEKL
CbEPV           TLFCNRSMIVSGNLEFEK-FEGGYVLEPKQKYIDSITAVFDFNSEYPSNIIEANLSPEKV
CuniNPV         KSLFRISTAQQTWGDQGNTKIGGGLVLPTVPGLHEHLAIMDFNSLYPSIMLAERVCPNN-
                                ** :. *           :*:.  *         .  .

AcMNPV          LILCEDGN-------------------------------------VYLNHNSRAIVVKLL
BmNPV           LVLCEDGN-------------------------------------VYLNHNSRAIVVKLL
CfMNPV          LILCEDGN-------------------------------------VYLNQDKQAINVQLL
OpMNPV          LILCEDGN-------------------------------------VYLNQDKQAINVQLL
EppoNPV         LILCQDGN-------------------------------------VYLNQDKNAINVKLL
McplMNPV        LVMGNDGY-------------------------------------VYLINNPKAPTVQLL
MadiMNPV        LVMGSDGY-------------------------------------VYLINNPKAPTVQLL
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Fig. 4. ClustalW alignment of inferred amino acid sequences for the BVP1-BVP1 region from McplMNPV (Man4 isolate), MadiMNPV (UN3 isolate), 18 other baculoviruses, and 3 poxviruses. McplMNPV and MadiMNPV sequences are shown in bold. Conserved residues are indicated by asterisks (identity), colons (high chemical similarity), and periods (low chemical similarity). McplMNPV and MadiMNPV amino acid sequences are inferred from actual BVP1 PCR product sequences. The BVP1-BVP1 regions of all other viral sequences were extracted from GenBank DNA polymerase gene records by searching for the BVP1 conserved motifs or by alignment. Note that the terminal BVP1 site for McplMNPV and MadiMNPV is inferred from incorporated BVP1 primer. Given that the 5′ end of BVP1 does not bind perfectly to this site, the last few amino acids are unlikely to be the same as those translated by the genomic sequence.

```
HearNPV         VFVAQDDN-----------------------------------VYLHKDLDAVNPKLL
HzSNPV          VFVAQDDN-----------------------------------VYLHKDLDAVNPKLL
SpltNPV         VFIASDNN-----------------------------------VYLNKNRDAVNPKLL
MacoNPV         VFVADDCN-----------------------------------VYLVKYREAINPKLL
MbMNPV          VFVADDCN-----------------------------------VYLVKYREAINPKLL
SeMNPV          VFVAEDGF-----------------------------------VYLIKNREAINPKLL
BusuNPV         LLIGSDNN-----------------------------------VYLVKDSNAINPKLL
OranNPV         LFVADDDC-----------------------------------VYLHNNMDATNPKLL
LdMNPV          VFIASDGN-----------------------------------VYLDKNVNAVNPKLL
XecnGV          LFFGSDGY-----------------------------------LYLQKNKNAITTKFL
PlxyGV          LFFGTDGY-----------------------------------LYLQKNDNAITTKFL
CypoGV          LFVGANKK-----------------------------------LYLQINQNAITTKFL
Vac             VGVVVSTN-RLEEEINNQLLLQKYPPPRYITVHCEPRLPNLISEIAIFDRSIEGTIPRLL
FPV             VCILLNSN-KLESEINMRTIKSKYPYPEYVCVSCESRLSDYYSEIIVYDRREKGIIPKLL
CbEPV           EKVIKLQDDEYAVDIVENYLKEKYPYPDYCYMLIKK---DKTYKFIVMDRRKPGIITQMI
CuniNPV         IAVESNGD-----------------------------------LLLVPMPKENLTSSII
                  .                                                      ::

AcMNPV          LKLLSERCKFKKNRDNQSESAFLYDLYDQKQNSVKRTANSIYGYYG-----IFYKVLANY
BmNPV           LKLLSERCKFKKNRDNQSESAFLYDLYDQKQNSVKRTANSIYGYYG-----IFYKVLANY
CfMNPV          LQLLKQRSELKKCRDSQTDSEFLYDLYDQMQNLSKRTANSIYGYYG-----IFCKLLANY
OpMNPV          LQLLKQRSELKKCRDSQTESEFLYDLYDQMQNLSKRTANSIYGYYG-----IFCKLLANH
EppoNPV         LELLKQRCELKKRRDDQAESEFLYDLYDQMQNLSKRTANSIYGYYG-----IFCKALANY
McplMNPV        NKLFALRSSYKKLRDNYEVGTFQYNLYDKLQNAIKRIANSIYGYYG-----IFFKPIANY
MadiMNPV        NKLFALRSSYKKLRDNYEVGTFQYNLYDKLQNAIKRIANSIYGYYG-----IFFKPIANY
HearNPV         RELLDLRAKYKNRRDKHEPGTFQYNLNDKIQNAVKRIANSIYGYFG-----IFFKPLANY
HzSNPV          RELLDLRAKYKNLRDKHEPGTFQYNLNDKIQNAVKRIANSIYGYFG-----IFFKPLANY
SpltNPV         EELLHLRSVYKDSRDRYPKTSFKYNLYDKMQNAVKRIANSIYGYFG-----IYFKPLANF
MacoNPV         KTLLDLRTFYKNKRDKFEPGSFQYNLYDKTQNAVKRIANSIYGYFG-----IFFKPLANY
MbMNPV          KTLLDLRTFYKNKRDKFEPGSFQYNLYDKTQNAVKRIANSIYGYFG-----IFFKPLANY
SeMNPV          KTLLDLRTMYKKKRDNFDVNSFLYNVYDKTQNAVKRIANSIYGYFG-----IFFKPLANY
BusuNPV         ATLLNLRTTYKNKRDIHEKNSFLYNLYDTLQNAVKRIANSIYGYFG-----IYFKILANY
OranNPV         RNLMTLRQKYKDMRDTHPVGSFLYNLYDTLQNAVKRIANSIYGYFG-----IYFKVLVNY
LdMNPV          KTLSEMRVRYKGLRDQCEYNSFYYKLYDKIQNALKRIANSIYGYYG-----IFFKPLANY
XecnGV          QEQAGKRAGWKAEMKKFPNNSFMYFLFDSWQNAAKLNCNSQYGWFG-----LFCKALANH
PlxyGV          REMATKRAEWKADMKKNANNPFMYSLYDSWQNAAKLICNSQYGWFG-----MYCKALANH
CypoGV          KEMANNRVLYKQEMKKYEPDSFEYQMYDSWQNAAKLVCNSQYGWFG-----LCCKPLANF
Vac             RTFLAERARYKKMLKQATSS-TEKAIYDSMQYTYKIVANSVYGLMGFRNSALYSYASAKS
FPV             EMFIGKRKEYKNLLKTASTT-IESTLYDSLQYIYKIIANSVYGLMGFSNSTLYSYSSAKT
CbEPV           DKGMKSKNEYKNLKNINKNNPVLYNYYTSALYSKKITINSLYGLLGSERFDFNSPYCAEY
CuniNPV         KEALKNRKFHRSEAAKYDKDSYEYMMHDAAQGAYKITANSVYGFFA-----LRFPPLGNF
                      :   :                        *   ** **  .     :      :

AcMNPV          ITRVGRNQLRLAISLIEGLSNDPE----------------------ILEKFNLGSITFK
BmNPV           ITRVGRNQLRRAISLIEGLSNDPE----------------------ILKKFNLNSIGFK
CfMNPV          ITRVGREKLTAAIGMIEGLSNDAE----------------------LLSKFNLSTLTFR
OpMNPV          ITRVGREKLTAAIGTVEGLSNDPD----------------------LLREFGLSTLTFK
EppoNPV         ITKIGREKLTAAIEIIESLVDDPE----------------------LLQSFNLSSLSFK
McplMNPV        ITKIGRQMLSKAIKKIEGMSDNRE----------------------VCEKFGIDSMTLK
MadiMNPV        ITRIGRQMLSKAIKKIESMSDNRE----------------------VCDKFGIDSMSLK
HearNPV         ITKIGREKLTEAIVRIQAMSNRAD----------------------ILKDFNLSRINFR
HzSNPV          ITKIGREKLTEAIVRIQAMSNRAD----------------------ILKDFNLSRINFR
SpltNPV         VTHIGREKLSDAIKKIESMSNDAT----------------------ILEDFGLSRIKFR
MacoNPV         VTKIGRDKLMEAIEKIEATSDDAD----------------------ILRDFNLSSIKFQ
MbMNPV          VTKIGRDKLMEAIEKIEATSDDAD----------------------ILQDFNLSSIKFK
SeMNPV          ITKIGREKLMEAIEKIEATSDDED----------------------ILKRFNLSTVRFK
BusuNPV         ITKIGRKKLMEAIKKIEAMSQDDD----------------------IRAKFHLSNIEFR
```

Fig. 4. *(continued)*

Accession No. NC_001623.1) were undertaken to find a potential BVP1 primer binding site ca. 2.0 kb downstream of the targeted BVP1 site. No significant match with a conserved 3′ end was found in the downstream region. However, a few candidate pairs of imperfect matches were observed in other genomic regions, and though it is difficult to predict the degree of mismatch tolerated by our chosen PCR conditions, we speculate that one such pair of sites was responsible for the observed ca. 2.0 kb product under the less stringent

```
OranNPV         VTKMGRTKLIEAVRRIEAMSDNAD----------------------IKKKYNLSKINFK
LdMNPV          ITKMGRGKLKEVVGKVEAMSDDPR----------------------ILREFGLSKINFS
XecnGV          ITFIGRENLTDAIAKIEALSNDER----------------------IMKKWNLTKMTLK
PlxyGV          ITAIGRQRLDEAKRKIESLSDSEP----------------------VMRKWGLKTFKLE
CypoGV          ITAQGRSKLEEAQRIITELSENEA----------------------IKTKWNLSKMKLE
Vac             CTSIGRRMILYLESVLNGAELSNGMLRFANPLSNPFYMDDRDINPIVKTSLPIDYRFRFR
FPV             CTTIGRNMITYLDSIMNGAVWENDKLILADFPRNIFSGETMFNKELEVP--NMNESFKFR
CbEPV           CTALGQKCIKYIKNLVDKSRYIDNNLYLN-EQNNPFSNEP----VITRYSGNLDVNFTFY
CuniNPV         ITKTGRGKVRSAVNYTTEYWNEQYG-------------------------------LCAF
                 *  *:  :                                              .

AcMNPV          VVYGDTDSTFVLPTFNYN----EISNET--DTLKQICTHVETRVNNSFTDG--YKMAFEN
BmNPV           VVYGDTDSTFVLPTFNYN----EIFDET--DTLKQICTHVETRVNSSFTDG--YKMAFEN
CfMNPV          VLYGDTDSTFVLPTFKRD----EIPEESCMVTLTRICAAVETRVNGLFANG--YKMAFEN
OpMNPV          VLYGDTDSTFVLPVFRRE----EIPEEGRMATLGRICAAVEARVNGLFTNG--YKMAFEN
EppoNPV         VLYGDTDSTFVLPVFKHD----EIPEDQRMAILKSICATVECRVNSLFTNG--YKMAFEN
McplMNPV        VIYGDTDSTFVKINVKSD----DKVDNDKIKSILNYIVATLN----PCLEG--HNMALEN
MadiMNPV        VIYGDTDSTFVKIDVKSK----HEIDNEKIKNILNYIVEMLN----PSLEG--HNMALEN
HearNPV         VIYGDTDSSFIQVDFEKT----DIPIKDQHNTIKTIVNDYVLKTLNSSWNG--YKMALEN
HzSNPV          VIYGDTDSSFIQVDFEKT----DIPIKDQHNTIKTIVNDYVLKTLNSSWNG--YKMALEN
SpltNPV         VVYGDTDSSFIQVDYESN----EIDETLRHETVERIVNGYVLKKLNASWDG--YKMALEN
MacoNPV         VIYGDTDSSFIQVLFNEN----EIAG-NVESVIRTIINDYVLKKLNSGWVG--YKMALEN
MbMNPV          VIYGDTDSSFIQVVFNEN----EICG-NVESVIRTIINDYVLKKLNSGRVG--YKMALEN
SeMNPV          VIYGDTDSSFIQVLFDEN----EIQGDNVENVIRDIINEHVLKKLNAGWVG--YKMALEN
BusuNPV         VIYGDTDSSFIQVLFKED----EIAADMRHEIIKKIVNDHVLKKLNDSWDGKGYKMALEN
OranNPV         VIYGDTDSSFIQVDFNED----EIEPQLRFATIKTIVQNEVLTRLNASWHGKGYKMALEN
LdMNPV          VIYGDTDSCFIRVLFDEA----EWRRTAARPRSAPSCRTTCAKRSTTLWCG--YKMSLEN
XecnGV          VVYGDTDSTFVNIKMDEN----ELQAMG-DAKLRQMIMEEIVIPVNDGWHG-DYKMELEN
PlxyGV          VVYGDTDSNFVSITLSG-------AQLN-MDELRKLILDDILSPVNACWNG-AYRMELEN
CypoGV          VVYGDTDSNFVSIDLERE----EFERMGGDVGLRRLIMEDIMKPLNDTWKG-AFKMELEN
Vac             SVYGDTDSVFTEIDSQ--------DVDKSIEIAKELERLIN---NRVLFNN--FKIEFEA
FPV             SVYGDTDSIFSEISTK--------DIEKTAKIAKHLEHIIN---TKILHAN--FKIEFEA
CbEPV           IIYGDTDSLFINIKFDNKFDNKEDLVNKSHECFQFLSNIINDEKNIILSKN--FNFEYEK
CuniNPV         FVYGDTDSIMFKFMQPTS------GVTVDPNAFPGLIEHFSAGLAKTVGVG--YNMALEL
                 :****** :                                          .  ..:  *

AcMNPV          LMKVLILLKKKK
BmNPV           LMKVLIILKKKK
CfMNPV          LMSVLILLKKKK
OpMNPV          LMSVLILLKKKK
EppoNPV         LMNVLILLKKKK
McplMNPV        IIPRLILLKKYG
MadiMNPV        IIPRLILLKKYG
HearNPV         VMLSLILLKKKK
HzSNPV          VMLSLILLKKKK
SpltNPV         VMQSLILLKKKK
MacoNPV         VMSSLILLKKKK
MbMNPV          VMSSLILLKKKK
SeMNPV          VMSSLILLKKKK
BusuNPV         VMSNLILLKKKK
OranNPV         VMSSLILLKKKK
LdMNPV          IMLSLILLKKKK
XecnGV          IMRCMLIKGKKS
PlxyGV          IIKNMLIKGKKS
CypoGV          IMDCMLIKGKKS
Vac             VYKNLIMQSKKK
FPV             IYTQLILQSKKK

CbEPV           MYIWMLLLAKKK
CuniNPV         IVRSSVFTHRKK
                :    ::  :
```

Fig. 4. *(continued)*

condition. No products were generated with BVP2 alone (Fig. 3, lane 5).

Various McplMNPV isolates also generated a ca. 1.2 kb product, in addition to a ca. 0.9 kb product (Fig. 3, lane 6). MadiMNPV templates generated only a ca. 0.9 kb product (Fig. 3, lane 9). Interestingly, the 0.9 kb McplMNPV and MadiMNPV products were generated when only BVP1 was provided (Fig. 3, lanes 7 and 10, respectively). As with the AcMNPV template, no products were generated with McplMNPV or Madi-

MNPV templates when only BVP2 was present in the reaction mixture (Fig. 3, lanes 8 and 11, respectively).

Selected 1.2 and 0.9 kb PCR amplicons were subsequently cloned. Colony PCR screens revealed that the cloned 1.2 kb McplMNPV fragment generated both 1.2 and 0.9 kb PCR products (data not shown), indicating that the 0.9 kb amplicon is a sub-fragment of the larger 1.2 kb amplicon. This was confirmed by sequencing.

3.3. Sequencing of DNA polymerase gene fragments

To verify the identity of the PCR amplicons, we cloned and sequenced representatively intense PCR products from a McplMNPV isolate (Sat1), a MadiMNPV isolate (WL3), and AcMNPV as a control. As expected, the sequenced AcMNPV fragment matched the GenBank sequence (Accession No. M20744.1) used in the original sequence alignment. An exact match to the McplMNPV 0.9 kb sequence was found at the 5′ end of the 1.2 kb McplMNPV fragment, followed by 370 bp of additional sequence including the BVP2 primer site at the 3′ end (Fig. 3). This confirmed that the 0.9 kb product is a sub-fragment of the 1.2 kb amplicon. All 1.2 and 0.9 kb sequences contained the YDGTDS catalytic motif, verifying that these are DNA polymerase gene fragments. Inferred amino acid sequence for McplMNPV (Sat1) and MadiMNPV (WL3) 0.9 kb BVP1-BVP1 sequences are shown in Fig. 4.

The sequenced primer site corresponds to the primer incorporated into the product during PCR. BVP1 binds non-perfectly downstream of the BVP1 target site, resulting in amplification of a 0.9 kb BVP1-BVP1 product, and thus the sequenced primer at this second site may not correspond to the genomic sequence. In this work, the sequencing of the McplMNPV 1.2 kb product (Sat1 isolate) revealed the genomic sequence corresponding to the nested BVP1 reverse site. The genomic sequence translates to LLKKKK, and not LLKKYG as obtained from the reverse complement of BVP1 (Fig. 5). This discrepancy resulted from a mismatch between the 3′ end of the genomic sequence and the 5′ end of BVP1. Because DNA extension by Taq polymerase occurs at the 3′ end of the primer, which in this case is the perfect complement to the genomic sequence, amplification was obtained despite the mismatch. It is interesting that the LLKKKK amino acid sequence is almost perfectly conserved among all currently sequenced nucleopolyhedrovirus DNA polymerase genes (Fig. 5), with the exception of CuniMNPV which is thought to be evolutionarily distant from lepidopteran baculoviruses (Afonso et al., 2001; Moser et al., 2001). This makes BVP1 potentially useful as a universal NPV primer. Although this amino acid sequence is conserved, the AcMNPV template did not generate the 0.9 kb BVP1 product because of different codon usage. Fig. 5 reveals that the 5′ leucine (L) in the AcMNPV BVP1 reverse region is encoded by a CTA codon, not a TTR codon as with most other nucleopolyhedroviruses. The resulting mismatch at the crucial 3′ end of the primer is the likely reason why no BVP1 product was amplified. However, Fig. 5 illustrates that SeMNPV, HearNPV, HzSNPV, MbMNPV, SpltNPV, OranNPV, and EppoNPV se-

		L	L	K	K	K	K	
McplMNPV (Sat1) [a]	/868/	ttg	ttg	aaa	aag	aaa	aag	ta
SeMNPV [b]	/2341/	ttg	ttg	aaa	aag	aaa	aag	ta
HearNPV [b]	/2230/	ttg	ttg	aaa	aag	aaa	aaa	ta
HzSNPV [b]	/2230/	ttg	ttg	aaa	aag	aaa	aaa	ta
MbMNPV [a]	/1816/	ttg	ttg	aag	aaa	aaa	aaa	ta
SpltNPV [b]	/2167/	tta	ttg	aag	aag	aaa	aag	ta
OranNPV [a]	/1900/	ttg	tta	aag	aaa	aaa	aag	ta
EppoNPV [b]	/2149/	ttg	tta	aaa	aag	aaa	aag	ta
OpMNPV [b]	/2158/	ttg	ctg	aaa	aag	aaa	aaa	ta
LdMNPV [b]	/2224/	ttg	ctg	aaa	aag	aaa	aag	ta
BusuNPV [a]	/1867/	tta	ctt	aaa	aag	aaa	aaa	ta
BmNPV [b]	/2164/	ata	tta	aaa	aag	aaa	aaa	ta
CfMNPV [b]	/2155/	ctg	tta	aaa	aag	aaa	aag	ta
AcMNPV [b]	/2164/	cta	tta	aaa	aag	aaa	aaa	ta
MacoNPV [b]	/2233/	ctg	ttg	aag	aaa	aaa	aaa	ta
BVP1 [c]		TTR	TTR	AAR	AAR	TAN	GGN	TC
		L	L	K	K	Y	G	

[a] Complete DNA polymerase sequence unknown. Coordinates given relative to the beginning of the GenBank record (see Table 1 for appropriate accession #).

[b] Coordinates relative to the start of the protein coding region.

[c] Reverse complement to which the BVP1 primer hybridizes.

Fig. 5. Comparison of baculovirus BVP1 reverse binding site sequences. IUPAC code is used to indicate where an equimolar mix of more than one base is present at the position in the population of oligonucleotides (R = A and G; N = A, T, G, and C).

quences match the reverse complement of BVP1 equally well as the McplMNPV Sat1 isolate, which did generate a successful 0.9 kb BVP1 amplicon. Thus, BVP1 alone appears to be a valuable tool for amplifying significant regions of the DNA polymerase gene from diverse nucleopolyhedroviruses.

3.4. Nucleotide diversity among geographically distinct McplMNPV and MadiMNPV wild-type isolates based on DNA polymerase gene fragments

The BVP1 and BVP2 primer set provides a valuable tool for rapidly obtaining DNA polymerase gene fragments from baculovirus isolates. In fact, BVP1 alone was sufficient for the collection of DNA polymerase sequence data from several, geographically distinct isolates of McplMNPV and MadiMNPV. The occurrence of genotypic variants among wild-type isolates of a single baculovirus species has been documented (Allaway and Payne, 1983; Cherry and Summers, 1985; Crawford et al., 1986; Gettig and McCarthy, 1982; Loh et al., 1982; Shapiro et al., 1991). Equally, several studies have revealed variation among baculoviruses of the same species within a single host (Knell and Summers, 1981; Lee and Miller, 1978; Maruniak et al., 1984). Beginning with the work of Lee and Miller over 20 years ago (1978), restriction fragment length polymorphism (RFLP) analysis has been widely used to illustrate variation among virus isolates (Gettig and McCarthy, 1982; McIntosh et al., 1987). However, grouping viral variants by RFLP analysis is difficult (Shapiro et al., 1991). Nucleotide sequence data provide a more conclusive means of identifying genomic variants. Our study is one of the first to examine nucleotide sequence variation among geographically distinct, wild-type, baculovirus isolates of the same species.

Viral DNA was purified from infected caterpillars collected from four sites on the Southern Gulf Islands (*M. disstria* (Hubner) specimens) and four sites in the Prince George region (*M. californicum pluviale* (Dyar) specimens) in British Columbia, Canada (Fig. 1). Purified viral DNA was PCR amplified with BVP1, and six representative 0.9 kb PCR products from each of McplMNPV and MadiMNPV were cloned. A single representative colony from each PCR cloning experiment was selected for plasmid purification and sequencing. Comparison of the resulting nucleotide sequence data revealed little to no variation between McplMNPV isolates, with pair-wise sequence identity ranging from 99.2 to 100% at the nucleotide level, and 99.5 to 100% at the amino acid level. There was also little to no variation between MadiMNPV isolates, with 98.9 to 100% sequence identity at the nucleotide level, and 99.1 to 100% at the amino acid level. However, there was notable variation between the McplMNPV and MadiMNPV sequences, with pair-wise sequence identities between isolates of the two species ranging from 84.7 to 86.1% at the nucleotide level, and 86.3 to 87.0% at the amino acid level. Note that degenerate primer sequences were removed from the pair-wise sequence identity analysis, as the sequence variation in the primer regions may not reflect the actual variation in the corresponding genomic region due to non-perfect primer hybridization.

The small degree of nucleotide variation among isolates of McplMNPV and MadiMNPV from geographically separated locations may seem surprising given RFLP analysis has shown genomic variation among the same isolates (Cooper et al., 2003) and among isolates of other single baculovirus species isolated from different locations (Allaway and Payne, 1983; Cherry and Summers, 1985; Crawford et al., 1986; Gettig and McCarthy, 1982; Loh et al., 1982; Shapiro et al., 1991). Although such variation has been mapped to specific genomic regions, only a few of these regions are known to affect the biological activity of purified clones (Stiles and Himmerich, 1998). DNA polymerase may not be the best locus for assessing genotypic variation among closely related geographic isolates. B family (α-like) DNA polymerases from phylogenetically distant species have been shown to share highly conserved amino acid sequences (Ito and Braithwaite, 1991; Wong et al., 1988). This observation led to postulations that α-like DNA polymerases from prokaryotes and eukaryotes share a common ancestor, and thus can be used as markers to determine evolutionary relationships among organisms (Jung et al., 1987; Kamer and Argos, 1984). However, the conserved nature of this gene may not allow resolution of genotypic variation among closely related isolates. This may explain the low sequence divergence between McplMNPV and MadiMNPV DNA polymerase gene fragments. The ecdysteroid UDP-glucosyltransferase (*egt*) gene may serve as a better indicator of genotypic variation among closely related baculoviruses. There is evidence that variation in this gene may influence virus efficacy, and it has been mapped as a region responsible for generating different RFLP profiles (Stiles and Himmerich, 1998). Deletion of the *egt* gene often reduces the time required to kill the target insect, and reduces the food consumption of the infected larvae (O'Reilly and Miller, 1991; Slavicek et al., 1999).

3.5. DNA polymerase gene phylogeny

Investigation of the phylogenetic relationships among baculoviruses is motivated by an interest in the relationships between phylogenies of hosts and their viral pathogens. We assessed the phylogenetic relationships of McplMNPV and MadiMNPV to other baculoviruses using DNA polymerase gene fragment sequences. One representative 0.9 kb BVP1 product sequence was chosen for each of McplMNPV (selected a Man4 isolate)

and MadiMNPV (selected a UN3 isolate) due to the high degree of similarity among isolates from a given location.

Fig. 6 shows a maximum parsimony tree constructed from DNA polymerase protein fragments using PROTPARS software (Phylip package 3.573). The bootstrap values at each internal branch indicate the number of times per 100 bootstrap replicates that branch (i.e., the group which consists of the species to the right (descendents) of that internal branch) appeared in trees estimated by random re-sampling of the data. The topology of our maximum parsimony tree is consistent with previously published maximum parsimony (PAUP software) DNA polymerase trees (Bulach et al., 1999; Herniou et al., 2001; Moser et al., 2001) indicating that our translated BVP1 fragment is comparable to larger or complete DNA polymerase gene sequences in its ability to resolve the phylogenetic relationships

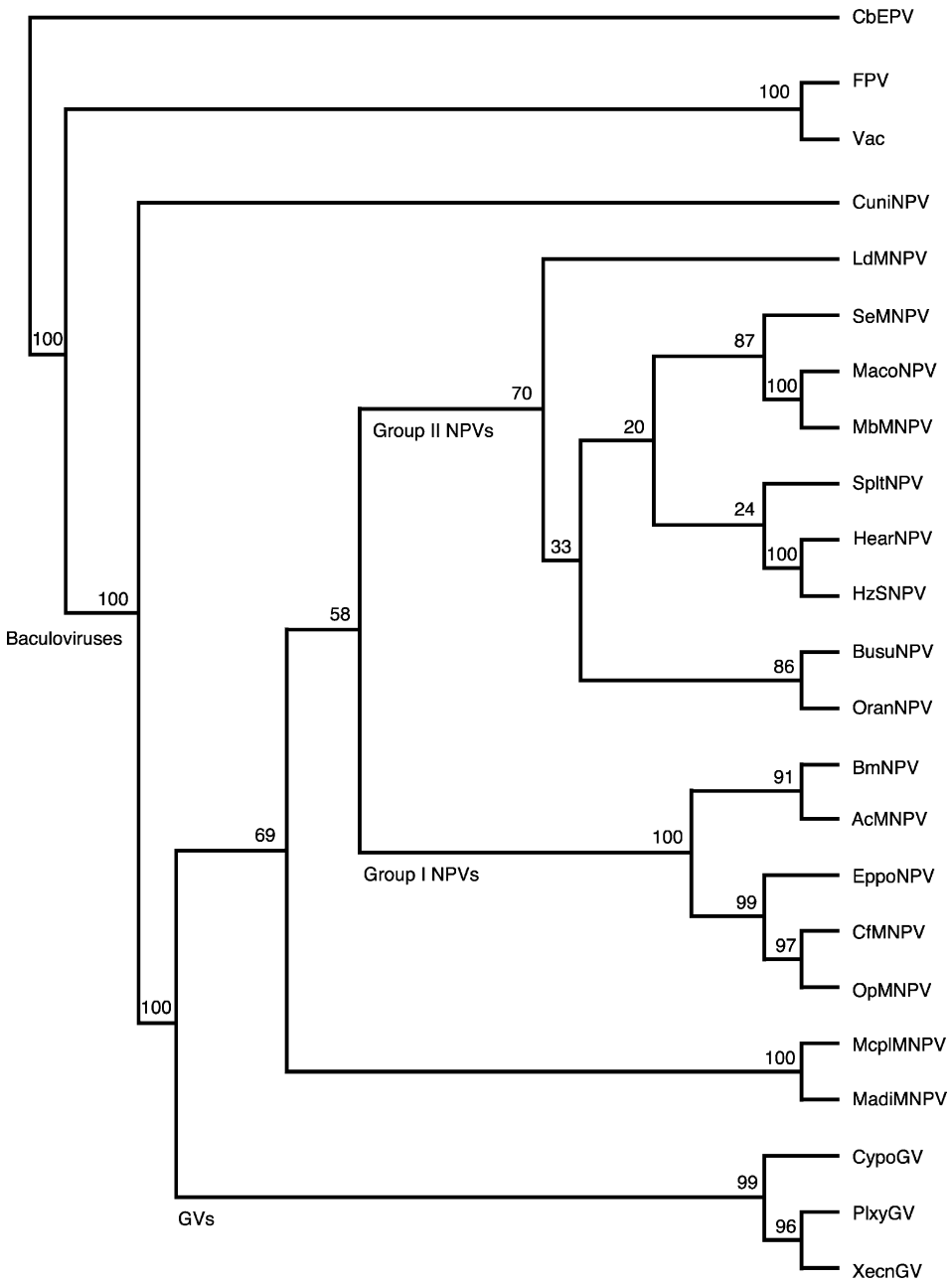

Fig. 6. Maximum parsimony analyses based on amino acid sequence data performed using SEQBOOT, PROTPARS, and CONSENSE programs (PHYLIP package 3.573) and a threshold parsimony value of 11. BVP1-BVP1 sequence regions are from McplMNPV (Man4 isolate), MadiMNPV (UN3 isolate), 18 other baculoviruses, and 3 poxviruses. CbEPV was used as the out-group. The bootstrap values at each internal branch indicate the number of times per 100 bootstrap replicates that branch appeared in trees estimated by random re-sampling of the data. Branch lengths are arbitrary, only the branching pattern is important.

among baculoviruses. In particular, the baculoviruses form a distinct cluster from the poxviruses with 100% bootstrap support. CuniNPV forms a separate group from the other baculoviruses with 100% bootstrap support, consistent with reports by Moser et al. (2001). Among the other baculoviruses, individual clusters have high bootstrap support. The three granuloviruses (GVs) form a distinct sub cluster with a 99% bootstrap value, and there is a clear division of the nucleopolyhedroviruses (NPVs) into Group I (100% support) and Group II (70% support), as initially illustrated by Zanotto et al. (1993). Li et al. (2002) used whole genome analysis to illustrate that the recently sequenced MacoNPV is most similar to SeMNPV in terms of gene content and gene rearrangement. Our DNA polymerase maximum parsimony tree is consistent with this observation, clustering MbMNPV in the same group as SeMNPV with 87% bootstrap support, but interestingly MacoMNPV appears most closely related to MbMNPV (100% bootstrap support), a virus for which the full genome sequence is not yet available. The low bootstrap values for branches within the Group II NPV cluster are consistent with reports that branches within the Group II NPV clade in DNA polymerase trees are not resolved with high fidelity (Chen, 2001). The low bootstrap values may result from maximum parsimony's forced bifurcation in cases where multifurcated branching patterns would be more appropriate.

McplMNPV and MadiMNPV are more closely related to each other than to any other baculovirus, clustering together with 100% bootstrap support. These two viruses group with the other 14 NPVs with 69% bootstrap support, and the bootstrap value for the NPV cluster is low (58%). Together these values are inconsistent with the 97% bootstrap values reported by Bulash et al. (1999) for a subset of these viruses. These low bootstrap values result from the variable placement of our new sequences and of the GVs relative to the other NPVs in the 100 bootstrap trees. Individual clusters (GVs, Group I NPVs, Group II NPVs, McplMNPV–MadiMNPV) have high bootstrap support as discussed above, however the relative placement of these clusters cannot be resolved. We experimented with various maximum parsimony tree building parameters such as using alignments built with the Gonnet substitution matrix (Gonnet et al., 1992) instead of the BLOSUM matrix (Henikoff and Henikoff, 1992), utilizing different sets of sequences, and imposing different threshold parsimony values (8–12). The resulting trees illustrated several topologies as predicted by the low confidence values in Fig. 6, while the sub-clusters discussed in the above paragraph remained intact. The low bootstrap values found in Fig. 6 reflect the tendency of our maximum parsimony method to force bifurcation of branches without sufficient support. In summary, the MadiMNPV and McplMNPV sequences fall outside of previously published GV, Groups I and II NPV clusters, though their placement relative to these clusters remains unclear.

It has been shown that when the substitution model assumed to generate the data becomes more complex and realistic, the probability that maximum parsimony recovers the true topology, and especially its performance relative to that of maximum likelihood, generally deteriorates (Yang, 1996). For this reason, we chose to also construct a maximum likelihood tree for our sequences, using TREE-PUZZLE software, version 5.0 (Strimmer and von Haeseler, 1996). TREE-PUZZLE implements a quartet puzzling algorithm, which draws on the fact that although maximum-likelihood procedures are typically slow for the case of n sequences, the maximum-likelihood tree based on four sequences can be readily determined. Quartet puzzling involves three steps. First, all possible quartet maximum-likelihood trees are reconstructed, second, the quartet trees are repeatedly combined to generate an overall tree, and third, the majority rule consensus of all intermediate trees is computed to give the final quartet puzzling tree.

The overall topology of our maximum likelihood (Fig. 7) and maximum parsimony (Fig. 6) trees are consistent, although some subtrees collapsed with maximum likelihood analysis. Fig. 7 gives a summary of all groups that occur in the majority of intermediate trees, and thus appears multifurcated in cases where the internal branching pattern cannot be completely resolved (Strimmer and von Haeseler, 1996). Flattening the tree in this manner correctly disallows low confidence branching, in contrast to the maximum parsimony method discussed above. It appears that the clusters among the baculoviruses were evaluated as being sufficiently different from each other to prevent any superclustering, such as formation of a NPV cluster that includes both Group I and Group II.

It is interesting that McplMNPV and MadiMNPV do not cluster with the other NPVs, but form their own group in the maximum likelihood tree (Fig. 7). The value at each internal branch in Fig. 7 gives the percentage of times the corresponding cluster was found among 1000 intermediate trees. These suggested reliability values are an intrinsic result of the quartet-puzzling algorithm (Strimmer and von Haeseler, 1996), and should not be confused with bootstrap values, which can be generated by an external procedure. However, both measures are highly correlated (Strimmer and von Haeseler, 1996). CuniNPV appears distinct among the baculoviruses, forming its own cluster with 86% reliability. Among the other baculoviruses, the GVs form a cluster with 80% reliability, while Group I and II NPV clusters have reliability values of 92 and 72%, respectively. McplMNPV and MadiMNPV appear to be more closely related to each other than to any other baculovirus in the tree, clustering together with 97% reliability.

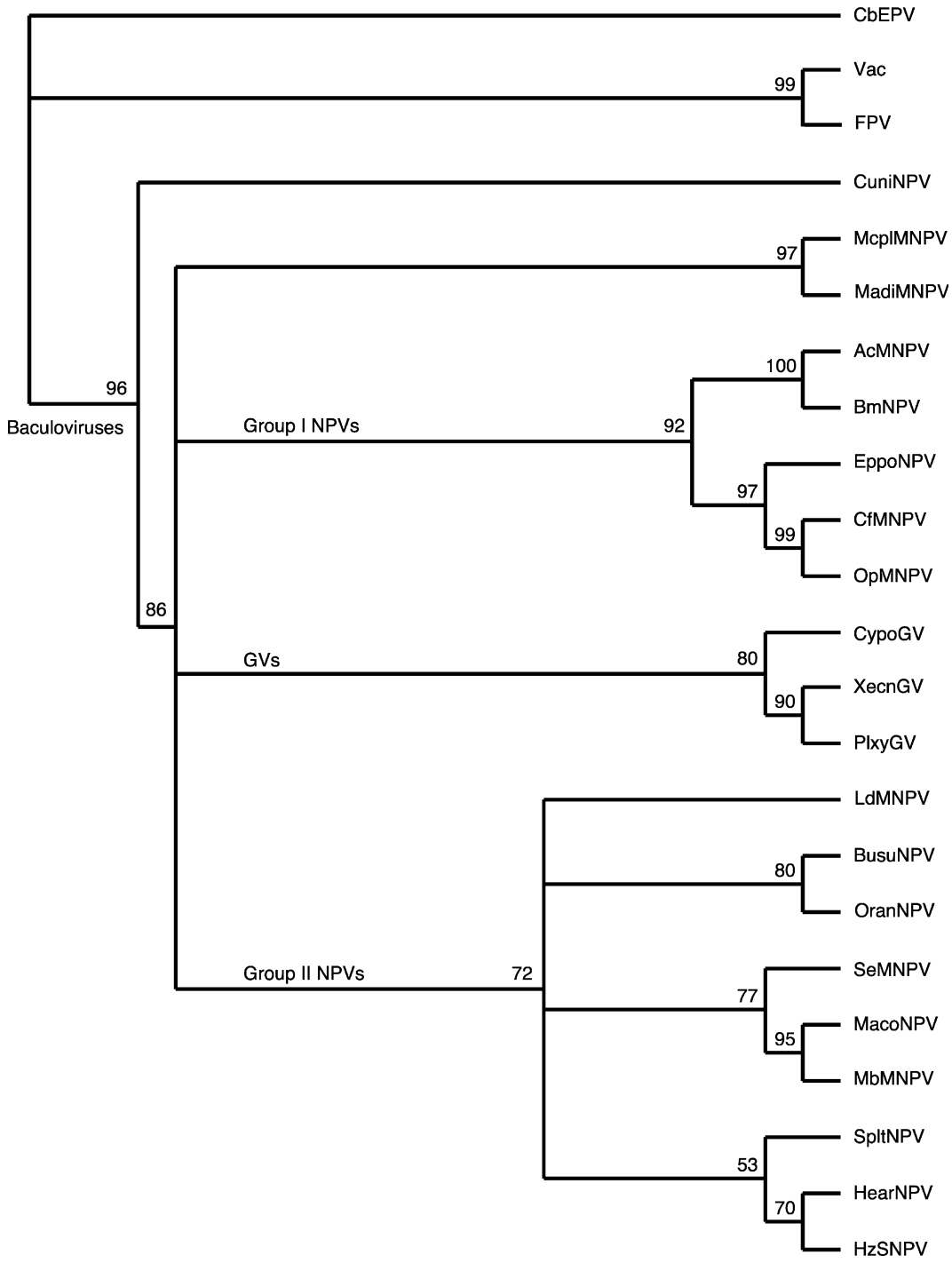

Fig. 7. Maximum likelihood analyses (TREE-PUZZLE version 5.0) using BVP1-BVP1 sequence regions from McplMNPV (Man4 isolate), MadiMNPV (UN3 isolate), 18 other baculoviruses, and 3 poxviruses. CbEPV was used as the out-group. The reliability value of each internal branch indicates in percent how often the corresponding cluster was found among 1000 intermediate trees. Branch lengths are arbitrary, only the branching pattern is important.

The high reliability values of the GV cluster, Group I NPVs cluster and Group II NPVs cluster indicate that it is unlikely that McplMNPV and MadiMNPV fall into any of these groups. This conclusion, based on maximum likelihood, mirrors our earlier conclusion based on maximum parsimony.

The combined results of our maximum parsimony and maximum likelihood DNA polymerase phylogenetic analyses allow us to draw several conclusions. First, McplMNPV and MadiMNPV appear to be more closely related to each other than to any other sequenced baculovirus. Second, they are more closely related to

other baculoviruses than to CuniNPV. Third, because maximum parsimony only weakly groups them with other NPV sequences, and maximum likelihood assigns them to a unique cluster, we conclude that McplMNPV and MadiMNPV form a distinct clade among the baculoviruses.

The appearance of a unique cluster indicates that much of the genotypic variation in baculoviruses has yet to be sampled. The sequence data show very little genetic variation among baculovirus isolates from geographically separated areas, however, these sequences are sufficiently distinct from other known baculovirus DNA polymerase genes to support formation of a separate phylogenetic clade. As more sequence data are collected, additional groups among the NPVs will likely emerge. This suggests we are only beginning to reveal the degree of genetic diversity among baculoviruses. Molecular tools, such as the PCR approach presented in this study, will allow future exploration of baculovirus diversity in natural environments.

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