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The Complete Genome Sequence of *Perina nuda* Picorna-like Virus, An Insect-Infecting RNA Virus with a Genome Organization Similar to That of the Mammalian Picornaviruses

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Perina nuda picorna-like virus (PnPV) is an insect-infecting RNA virus with morphological and physicochemical characters similar to the *Picornaviridae*. In this article, we determine the complete genome sequence and analyze the gene organization of PnPV. The genome of PnPV consists of 9476 nucleotides (nts) excluding the poly(A) tail and contains a single large open reading frame (ORF) of 8958 nts (2986 codons) flanked by 473 and 45 nt noncoding regions on the 5′ and 3′ ends, respectively. Northern blotting did not detect the presence of any subgenomic RNA. The PnPV genome codes for four structural proteins (CP1–4), and determination of their N-terminal sequences by Edman degradation, showed that all four are located in the 5′ region of the genome. The 3′ part of the PnPV genome contains the consensus sequence motifs for picornavirus RNA helicase, cysteine protease, and RNA-dependent RNA polymerase (RdRp) in that order from the 5′ to the 3′ end. In all of these characters, the genome organization of PnPV resembles the mammalian picornaviruses and two other insect picorna-like viruses, infectious flacherie virus (IFV) of the silkworm and Sacbrood virus (SBV) of the honeybee. In a phylogenetic tree based on the eight conserved domains in the RdRp sequence, PnPV formed a separate cluster with IFV and SBV, which suggests that these three insect picorna-like viruses might constitute a novel group of insect-infecting RNA viruses.

Key Words: Perina nuda; PnPV; picornavirus; insect; complete genome sequence.

INTRODUCTION

Positive-strand RNA viruses are assigned to supergroup I, II, or III on the basis of sequence alignments of their RNA-dependent RNA polymerase (RdRp) (see Strauss *et al.,* 1996). The picorna-like viruses, all of which share a conserved array of replicative proteins, i.e., H-P-Rep (Helicase-Protease-Replicase) (Koonin and Dolja, 1993), represent a single lineage within RdRp supergroup I, and presently comprise the five established families *Picornaviridae*, *Caliciviridae*, *Sequiviridae*, *Comoviridae*, and *Potyviridae* (Goldbach and Haan, 1994; Gromeier *et al.,* 1999). Four of these families (*Picornaviridae*, *Sequiviridae*, *Comoviridae*, and *Potyviridae*) form the picornavirus "superfamily," all the members of which utilize their genomic RNA as an exclusive message for a single polyprotein with all proteins being produced as the result of processing. By contrast, although the *Caliciviridae* are also picorna-like viruses, they are excluded from the picornavirus superfamily because they produce subgenomic RNA. In addition, many small RNA viruses from various insect species have picornavirus-like biophysical properties (Moore *et al.,* 1985; Minor *et al.,* 1995), but their relationships with established members of the picorna-like virus lineage have only recently begun to be established. Surprisingly, the insect-infecting viruses appear to form distinct taxonomic clusters outside of the family *Picornaviridae* (Christian *et al.,* 1999), and the Seventh Report of the International Committee on Taxonomy of Viruses (ICTV VII) assigns five of these insect picorna-like viruses [*Cricket paralysis virus* (CrPV), *Drosophila C virus* (DCV), *Rhopalosiphum padi virus* (RhPV), *Plautia stali intestine virus* (PSIV), *Himetobi P virus* (HiPV)] to a new genus, the "*Cricket Paralysis-like Viruses* " (or "CrPV-like viruses"; Christian *et al.,* 1999). In this genus, the gene order of the nonstructural proteins is the same as in the picorna-like viruses, but unlike the picornavirus superfamily, CrPV-like viruses produce their capsid proteins through internal initiation of translation from the genomic length RNA. In the case of PSIV and CrPV, this phenomenon has been demonstrated to be dependent on an internal ribosome entry site (IRES) (Sasaki and Nakashima, 1999; Wilson *et al.,* 2000). Nucleotide sequences highly similar to the IRES of PSIV were also found in RhPV and DCV (Sasaki and Nakashima, 1999), suggesting the presence of an IRES for these viruses as well. Furthermore, the CrPV-like viruses resemble the caliciviruses in that their nonstructural proteins are encoded in the 5′ part of the genome, and their capsid proteins are encoded in the 3′ part (Johnson and

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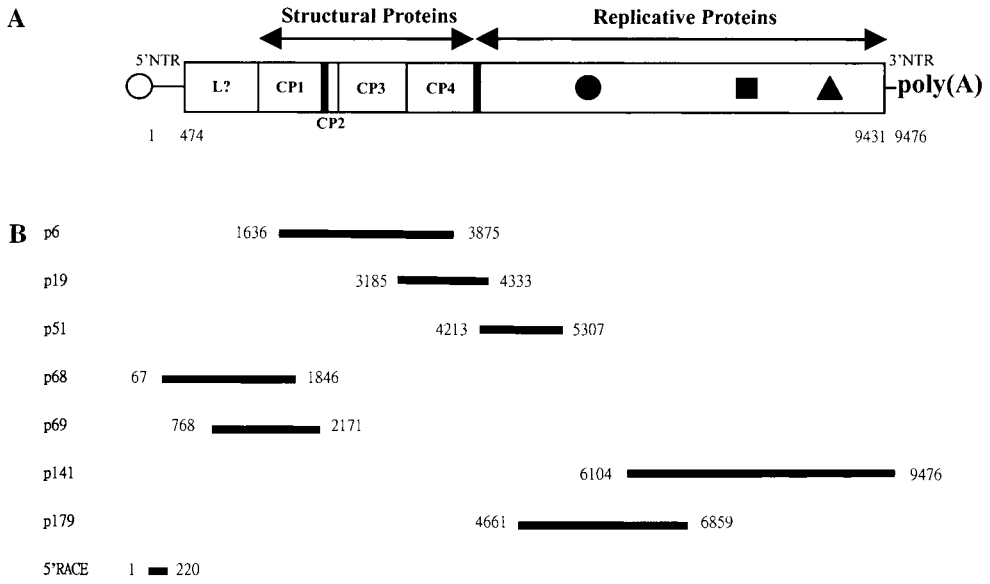


FIG. 1. (A) Schematic diagram of the PnPV genome. The ORF corresponds to the entire open box. Numbers indicate nucleotide positions. The approximate positions of the nonstructural proteins were identified by sequence similarity as follows: ●, helicase; ■, protease; ▲, RdRp. L? denotes the probable location of the leader peptide, and the open circle of the 5′ end represents the VPg (if present). Solid lines indicate where cleavages are known to occur in the polyprotein, and solid rectangles indicate the positions of 2A-like sequences. CP1–4 denote the four capsid proteins of PnPV as described in Table 2. (B) The alignment of PnPV cDNA clones produced by genomic cDNA cloning (p6, p19, p51, p68, p69, p141, and p179) and 5′-RACE analysis (see Fig. 2). Numbers indicate nucleotide positions. The horizontal scale is the same in both parts of this figure.

Christian, 1998; Sasaki *et al.,* 1998; Moon *et al.,* 1998; Nakashima *et al.,* 1999; Wilson *et al.,* 2000). The CrPV-like viruses, which have two ORFs, are also bicistronic. In addition to the CrPV-like viruses, there are also other insect picorna-like viruses that are not included in the *Picornaviridae*. Similar to the CrPV-like viruses, Acyrthosiphon pisum virus (APV) is both monopartite and bicistronic, and the order of the ORFs for its nonstructural and structural proteins is the same. However, in APV, the two ORFs overlap slightly, with the 3′-proximal ORF thought to be translated by a −1 ribosomal frameshift (van der Wilk *et al.,* 1997). APV is also unlike the CrPV-like viruses in that it produces a large amount of subgenomic RNA, which was detected in purified virus particles and APV-infected aphids (van der Wilk *et al.,* 1997). By contrast, the genomes of two other insect picorna-like viruses, Infectious flacherie virus (IFV) and Sacbrood virus (SBV), are both monopartite and monocistronic, and they resemble the mammalian picornaviruses in that their structural proteins are encoded in the 5′ part of the genome and their nonstructural proteins are encoded in the 3′ part (Isawa *et al.,* 1998; Ghosh *et al.,* 1999).

The present study investigates the *Perina nuda* picorna-like virus (PnPV), an insect picorna-like virus that was originally isolated from flacherie-infected larvae of the ficus transparent wing moth, *P. nuda* Fabricius (Lepidoptera: Lymantriidae). Flacherie is a lethal disease that occurs frequently from spring to early summer every year in Taiwan, and it is considered a mixed infection of *P. nuda* nucleopolyhedrovirus (PenuNPV) and PnPV (Wang *et al.,* 1998, 1999). The PnPV viral genome is composed of one single-strand RNA molecule with a length of 10 kb and a poly(A) tract, and the virus particle consists of three major and three minor structural proteins (Wang *et al.,* 1999). The viral particles of PnPV exhibit icosahedral symmetry, are approximately 30 nm in diameter, have no envelope and no distinct surface structure, and have a buoyant density of 1.381 g/ml in cesium chloride, and it was on the basis of these biophysical properties that PnPV was first tentatively identified as an insect picorna-like virus (Wang *et al.,* 1999). Here we report the complete nucleotide sequence and gene organization of the PnPV genome, including the coding regions of the capsid proteins, which were mapped by determining their N-terminal sequences. The transcript species were determined by Northern blotting, and the phylogenetic relationships between PnPV and other picornaviruses are also explored.

RESULTS AND DISCUSSION

Nucleotide sequence and open reading frame analysis

The PnPV virion contains a single-stranded RNA molecule, about 10 kb in length (Wang *et al.*, 1999). Except for the 5′ end, the sequence of the genomic RNA from PnPV was constructed by compiling sequences from a series of seven overlapping cDNA clones (see Fig. 1). Both strands of each of the cDNA clones were completely sequenced. The 5′ end of the viral genome was cloned by 5′ rapid amplification of cDNA ends (RACE), and the 5′-terminal nucleotides were determined by comparison

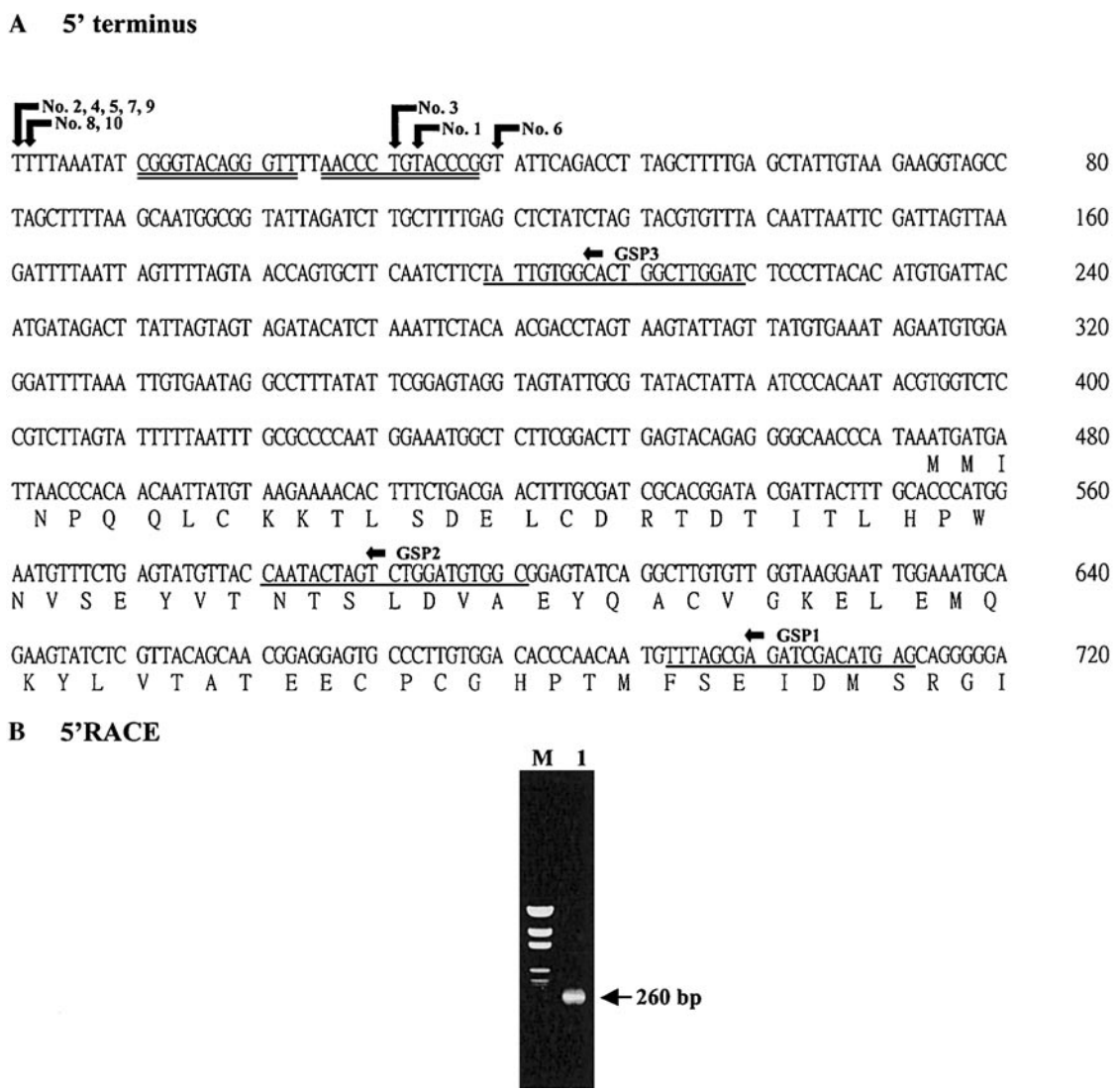


FIG. 2. (A) Determination of the 5′-terminus of the PnPV genome. GSP1, GSP2, and GSP3 are three antisense primers used for 5′-RACE analysis. The bent arrows indicate the location of the 5′-termini of 10 5′-RACE clones (1–10). A complementary region (double underlines, nucleotide positions 11–38) of the PnPV genome appears to form a secondary structure that may interfere with the cDNA synthesis during reverse transcription. (B) The product of 5′-RACE analysis in agarose gel. M, pGEM DNA Markers (Promega).

of the sequences of 10 clones (see Fig. 2). Analysis of the sequences of the cDNA clones confirmed the presence of a poly(A) tail on the 3′ end of the PnPV genome. The PnPV genome was found to have 9476 nucleotides (nts), excluding the 3′ poly(A) tail. Similar to other insect picornaviruses, the sequence is A/U rich (28.29% A, 27.55% U, 19.17% C, and 24.98% G). A computer-aided analysis of the PnPV nucleotide sequence showed that the genomic RNA contains a single, large open reading frame (ORF) oriented from the 5′ to the 3′ end. This large ORF accounts for 94.5% of the PnPV genome (8958 nts), while the other 5.5% consists of untranslated regions (UTR) (518 nts). The 5′UTR (473 nts) is considerably shorter than those of other picornaviruses (610–1200 nts) (Stanway, 1990). No large ORFs were found in the inverse orientation of the PnPV genome, and this finding confirmed that PnPV is a positive-strand RNA virus.

Mapping of the coding region of the capsid proteins

The three major (31.5, 29.7, 28.4 kDa) and three minor (27.0, 24.5, 4.0 kDa) structural proteins of the purified PnPV particles are shown in Fig. 3. The N-terminal sequences of these proteins were GDEDTPAGELSIEQDTHKNT (31.5 kDa), DRPQNIIEPTNFYLQQNTSL (29.7 and 28.4 kDa), GDERREPHTV (27.0 and 24.5 kDa), and PFLSGLLGTV (4.0 kDa). The N-terminal sequence of the 29.7-kDa protein was the same as that of the 28.4-kDa protein, indicating that they had the same origin, with the smaller protein probably being formed via degradation of the C-terminal part of the larger protein. A similar expla-

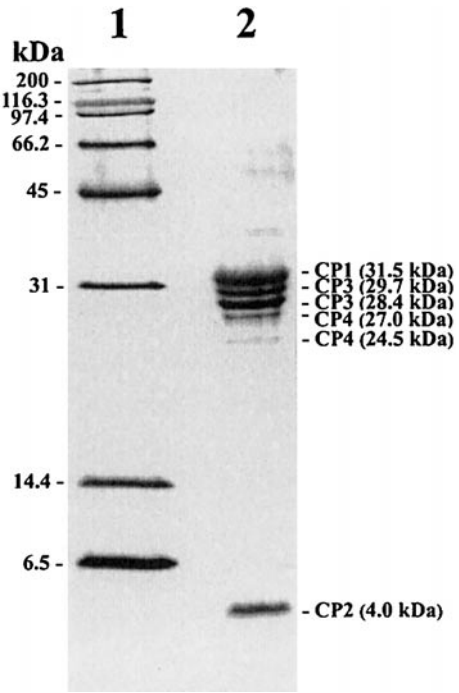


FIG. 3. Structural proteins of PnPV in a 16% SDS–polyacrylamide gel. Lane 1, broad-range protein marker (Bio-Rad); lane 2, structural proteins of PnPV.

nation applies for the 27.0- and 24.5-kDa proteins. The sequences for the 31.5-, 29.7- (or 28.4 kDa), 27.0- (or 24.5 kDa), and 4.0-kDa proteins were serially found encoded from the 5′ end of the PnPV genome at predicted amino acid residues 320–339, 638–657, 907–916, and 574–583, respectively, and the structural proteins were therefore named CP1 (31.5 kDa), CP2 (4.0 kDa), CP3 (29.7 or 28.4 kDa), and CP4 (27.0 or 24.5 kDa) based on their proximity to the N-terminus of the deduced amino acid sequence of PnPV's ORF (Table 1). Their locations are shown in Fig. 1.

The deduced amino acid sequence of the capsid proteins of PnPV was compared with entries in protein sequence databases by using BLAST. The greatest overall similarity was observed against the structural proteins of insect-infecting RNA viruses: RhPV (15% identity, ID; 31% similarity, SI), PSIV (14% ID; 31% SI), DCV (14% ID; 31% SI), IFV (13% ID; 35% SI), SBV (12% ID, 31% SI), HiPV (12% ID; 30% SI), and CrPV (12% ID; 29% SI). Interestingly, although PnPV, IFV, and SBV share a picornavirus-like gene order, they have low homology to each other. Furthermore, the structural proteins of PnPV share relatively low overall similarity to those of mammalian picornaviruses listed in Table 2 (8–11% ID; 22–26% SI), and also to those of the plant picorna-like viruses PYFV (8% ID; 25% SI) and CPMV (5% ID; 23% SI). Multiple alignments (Fig. 4A) showed that the amino acid residues 420–492, which are part of the coding region of PnPV's CP1, exhibit not only a high similarity to the VP2 structural protein of several mammalian picornaviruses (Table 3), but also to the VP1 of DCV (38-kDa protein) and PSIV (33-kDa protein), the VP2 of RhPV (28-kDa protein), the VP3 of IFV (31-kDa protein), and the amino acid residues 257–326 of SBV polyprotein. A second region, located at the amino acid positions 741–812 (i.e., part of the coding region of PnPV's CP3), is similar to the VP3-like proteins of mammalian picornaviruses (Table 3) and also to the structural

TABLE 1

Summary of PnPV's Structural Proteins

Designation	N-terminal sequence[a]	Position of N-terminus[b]	Cleavage sites[c]	Size (aa)[d]	Molecular mass (kDa)	
					From deduced amino acid sequence	From SDS–PAGE
CP1	**GD**EDTPAGEL SIEQDTHKNT	320–339	VTAQ/**GD** (L/CP1)	254	28.1	31.5
CP2	**P**FLSGLLGTV	574–583	NPG/**P** (CP1/CP2)	64	6.0	4.0
CP3	**DR**PQNIIEPTN FYLQQNTSL	638–657	KKDM/**DR** (CP2/CP3)	269	29.3	29.7 or 28.4
CP4	**GD**ERREPHTV	907–916	VTAM/**GD** (CP3/CP4) NPG/P (CP4 C-terminus)[d]	284	31.6	27.0 or 24.5

[a] Determined by Edman degradation.

[b] Relative to the deduced amino acid sequence of PnPV's ORF.

[c] The scissile bond at cleavage site is represented by "/".

[d] Determined from the deduced amino acid sequence of PnPV's ORF and based on the assumption that each structural protein sequence is terminated at the amino acid just before the N-terminus of the following structural protein. CP4's sequence was assumed to terminate at the cleavage site of the 2A-like sequence.

TABLE 2

Summary of the Viruses Used in the Phylogenetic Analysis

Group[a]	Viral strain/abbreviation (GenBank Accession No.)	Host	Genomic organization: Number of ORFs/sgRNA[b]	Genomic organization: Locations of the capsid proteins/nonstructural proteins	Reference
I	Human rhinovirus 1B/HRV (D00239)	Mammals	1/A	5′ part/3′ part	Hughes *et al.,* 1998
	Human poliovirus 1, strain Mahoney/PV (V01149)	Mammals	1/A	5′ part/3′ part	Racaniello and Baltimore, 1981
	Foot-and-mouth disease virus, strain 01/FMDV (AF189157)	Mammals	1/A	5′ part/3′ part	[c]
	Encephalomyocarditis virus/EMCV (X87335)	Mammals	1/A	5′ part/3′ part	Nelsen-Salz *et al.,* 1996
	Hepatitis A virus, strain HM-175, wild-type/HAV (M14707)	Mammals	1/A	5′ part/3′ part	Cohen *et al.,* 1987
	Echovirus 22/HPeV (S45208)	Mammals	1/A	5′ part/3′ part	Hypiä *et al.,* 1992
II	Feline calicivirus, strain F9/FCV (P27409)	Mammals	3/P	3′ part/5′ part	Carter *et al.,* 1992
	Rabbit haemorrhagic disease virus/RHDV (AAB02225)	Mammals	2/P	3′ part/5′ part	Gould *et al.,* 1997
III	Cowpea mosaic virus/CPMV (X00206)	Plant	2[d]/A	RNA 2/RNA 1[c]	Lomonossoff and Shanks, 1983
IV	Parsnip yellow fleck virus/PYFV (D14066)	Plant	1/A	5′ part/3′ part	Turnbull-Ross *et al.,* 1992
V	Potato virus A/PVA (AJ131403)	Plant	1/A	3′ part/5′ part	Rajamaeki *et al.,* 1998
VI	Southern cowpea mosaic virus/SCPMV (M23021)	Plant	4/P	3′ part/5′ part	Wu *et al.,* 1987
VII	Drosophila C virus, strain EB/DCV (AF014388)	Insect	2/A	3′ part/5′ part	Johnson and Christian, 1998
	Himetobi P virus/HiPV (AB017037)	Insect	2/A	3′ part/5′ part	Nakashima *et al.,* 1999
	Triatoma virus/TrV (AF178440)	Insect	2/A	3′ part/5′ part	Czibener *et al.,* 2000
	Rhopalosiphum padi virus/RhPV (AF022937)	Insect	2/A	3′ part/5′ part	Moon *et al.,* 1998
	Plautia stali intestine virus/PSIV (AB006531)	Insect	2/A	3′ part/5′ part	Sasaki *et al.,* 1998
	Black queen-cell virus/BQCV (AF183905)	Insect	2/A	3′ part/5′ part	Leat *et al.,* 2000
	Acute bee paralysis virus/ABPV (AF150629)	Insect	2/A	3′ part/5′ part	Govan *et al.,* 2000
	Cricket paralysis virus/CrPV (AF218039)	Insect	2/A	3′ part/5′ part	Wilson *et al.,* 2000
VIII	Infectious flacherie virus/IFV (AB000906)	Insect	1/A	5′ part/3′ part	Isawa *et al.,* 1998
	Sacbrood virus/SBV (AF092924)	Insect	1/A	5′ part/3′ part	Ghosh *et al.,* 1999
IX	Acyrthosiphon pisum virus/APV (AF024514)	Insect	2/P	3′ part/5′ part	van der Wilk *et al.,* 1997

[a] I: *Picornaviridae;* II: *Caliciviridae;* III: *Comoviridae;* IV: *Sequiviridae;* V: *Potyviridae;* VI: *Sebomovirus* (genus; no assigned family); VII: "Cricket Paralysis-like Viruses" (genus; no assigned family); VIII: unassigned "Insect Picorna-like" viruses; IX: unassigned insect picorna-like viruses.

[b] Present (P) or absent (A) of subgenomic RNA (sgRNA).

[c] L. Benvenisti and Y. Stram, 1999, direct submission.

[d] CPMV has a bipartite genome; the other viruses in the table have a monopartite genome.

proteins of insect picorna-like viruses such as the VP2 of DCV (33.3-kDa protein) and PSIV (30-kDa protein), the VP3 of RhPV (29-kDa protein), the VP1 of IFV (35-kDa protein), and the amino acid residues 528–602 of SBV polyprotein (Fig. 4B).

The order of PnPV's capsid proteins more closely resembles the other insect picorna-like viruses (IFV, CrPV, PSIV, and BQCV) in that the smallest capsid protein (i.e., CP2 in PnPV and the VP4-like proteins in IFV, CrPV, PSIV, and BQCV) is in all cases located near the center of the capsid protein precursor. This is in contrast to the mammalian picornaviruses, in which VP4 is located at the N-terminus of the capsid protein precursor. There is also increasing evidence that in deriving VP4 and VP3 from VP0, the insect picorna-like viruses might follow a different posttranslational processing pathway from the mammalian picornaviruses (Choi *et al.,* 1992; Isawa *et al.,* 1998; Tate *et al.,* 1999); if so, then PnPV would also presumably process VP0 similar to the other insect picorna-like viruses. However, the correspondence between PnPV's CP2 and the VP4-like proteins of the insect picorna-like viruses has not been firmly established and it should be noted that PnPV's CP2 shows only a low similarity to these VP4-like proteins. Furthermore, no conserved sequence (GF/SKP in CrPV-like viruses) has been found at the CP2/CP3 cleavage site of PnPV.

Leader polypeptide

The coding region for the structural proteins (CP1–CP2–CP3–CP4) starts at deduced amino acid position 320, which suggests that the PnPV genome may code for a leader polypeptide (L) of 36.7 kDa prior to the coding region. At the N-terminus of IFV, SBV, aphthoviruses (a

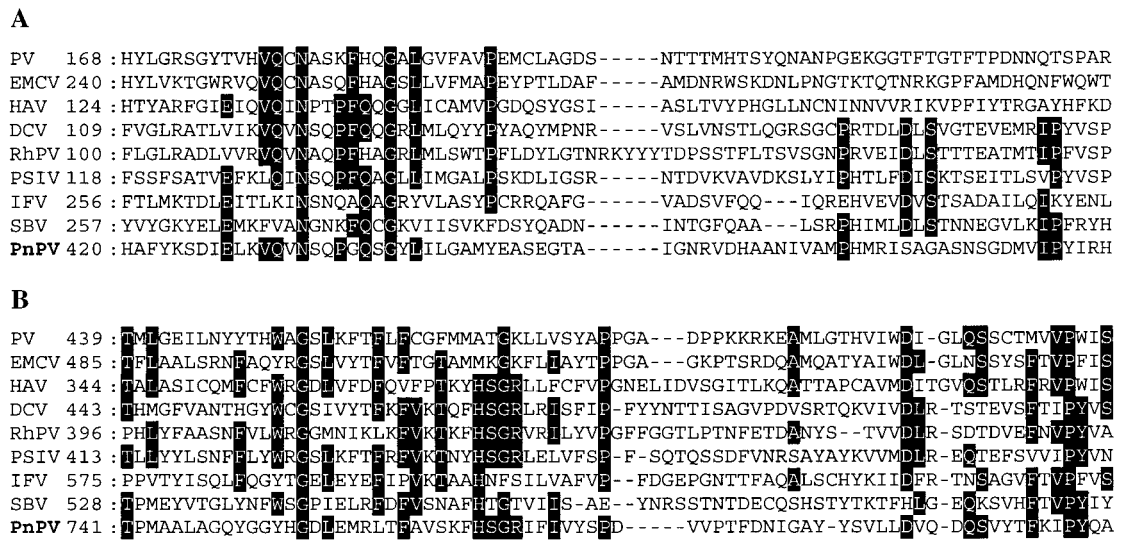


FIG. 4. Comparison of the deduced amino acid sequence of capsid proteins between PnPV and other viruses. (A) Alignment of the amino acid sequences of the CP1 of PnPV with VP2 of PV, EMCV, and HAV, VP1 of DCV (38-kDa protein) and PSIV (33-kDa protein), VP2 of RhPV (28-kDa protein), VP3 of IFV (31-kDa protein), and the amino acid residues 257–326 of SBV polyprotein. (B) Alignment of the amino acid sequences of CP3 of PnPV with VP3 of PV, EMCV, and HAV; VP2 of DCV (33.3-kDa protein) and PSIV (30-kDa protein), VP3 of RHPV (29-kDa protein), VP1 of IFV (35-kDa protein), and the amino acid residues 741–812 of SBV polyprotein. Numbers on the left indicate residue number for the N-terminus. Residues identical in at least half of the viruses are shown in inverse typeface.

genus under *Picornaviridae*), and cardioviruses (a genus under *Picornaviridae*), a (sometimes putative) leader protein (L), precedes the structural protein (Isawa *et al.,* 1998; Ghosh *et al.,* 1999; Stanway, 1990). In aphthoviruses, the L protein amino acid sequence contains the conserved cysteine-tryptophan motif, which includes the histidine moiety required for protease activity in the L protein of FMDV (foot-and-mouth disease virus, which is also a member of the *Picornaviridae*) (Gorbalenya *et al.,* 1991; Piccione *et al.,* 1995; Roberts and Belsham, 1995). However, this motif was not found in the L protein of PnPV nor in the L proteins of IFV and SBV (Isawa *et al.,* 1998; Ghosh *et al.,* 1999), which suggests that these proteins are not subject to proteinase.

"2A-like" sequences

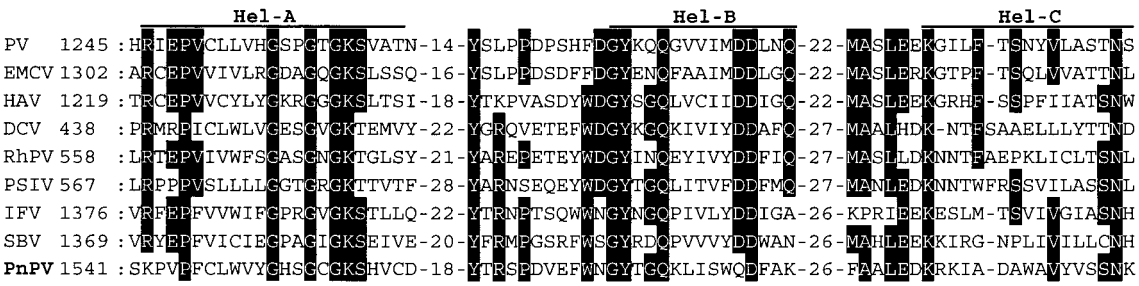
The conserved "2A-like" "cleavage" motif -DxExNPGP- was originally identified by alignment of cardio- and aphthovirus 2A sequences (Hahn and Palmenberg, 1996; Donnelly *et al.,* 1997). Interestingly, there were two 2A-like sequences found within the PnPV polyprotein at amino acid residues 555–574 (AQGWVPDLTVDG**D**V**E**S-**NPGP**) and 1172–1191 (IGGGQKDLTQDG**D**I**E**S**NPGP**). As Fig. 1 shows, the first sequence is located inside the structural protein-coding region and spans the CP1/CP2 junction. Its activity was confirmed by N-terminal sequencing of the CP2 protein (Table 1). The second motif is located near the downstream end of the PnPV struc-

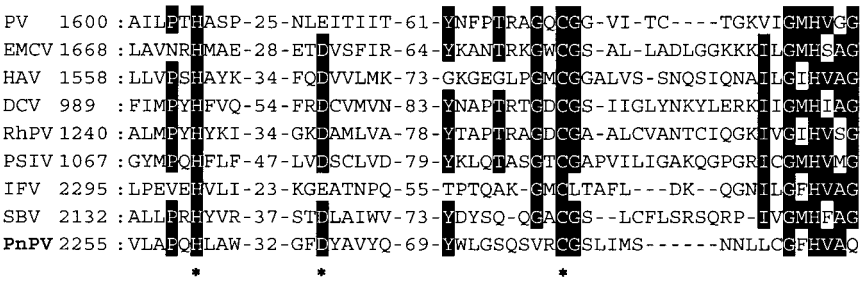
TABLE 3

Pairwise Comparisons of Amino Acid Sequences of PnPV's CP1 (Upper Right Triangle) and PnPV's CP3 (Lower Left Triangle) with the Corresponding Regions of Picornaviruses and Picorna-Like Viruses[a]

	PnPV	SBV	IFV	PSIV	RhPV	DCV	HAV	EMCV	PV
PnPV	—	21 (53)	17 (42)	22 (50)	18 (39)	24 (41)	16 (32)	14 (32)	13 (29)
SBV	24 (48)	—	22 (52)	21 (40)	17 (41)	21 (45)	9 (31)	6 (24)	9 (22)
IFV	18 (42)	23 (52)	—	21 (45)	21 (41)	21 (47)	14 (31)	13 (32)	6 (25)
PSIV	29 (58)	20 (53)	20 (49)	—	29 (58)	35 (58)	20 (37)	13 (31)	16 (29)
RhPV	25 (51)	20 (44)	23 (46)	34 (65)	—	46 (65)	15 (34)	15 (34)	16 (30)
DCV	24 (47)	28 (42)	23 (48)	45 (67)	39 (61)	—	21 (32)	10 (33)	13 (32)
HAV	21 (48)	16 (44)	26 (53)	31 (54)	25 (49)	21 (51)	—	21 (37)	20 (28)
EMCV	25 (52)	21 (46)	33 (52)	25 (55)	21 (43)	25 (47)	30 (56)	—	31 (44)
PV	19 (42)	18 (41)	17 (41)	25 (49)	15 (40)	22 (44)	30 (46)	41 (59)	—

[a] The percentages of amino acid sequence identity (or similarity) were calculated using GeneDoc (score table: Blosum 35) for the alignments shown in Fig. 4.





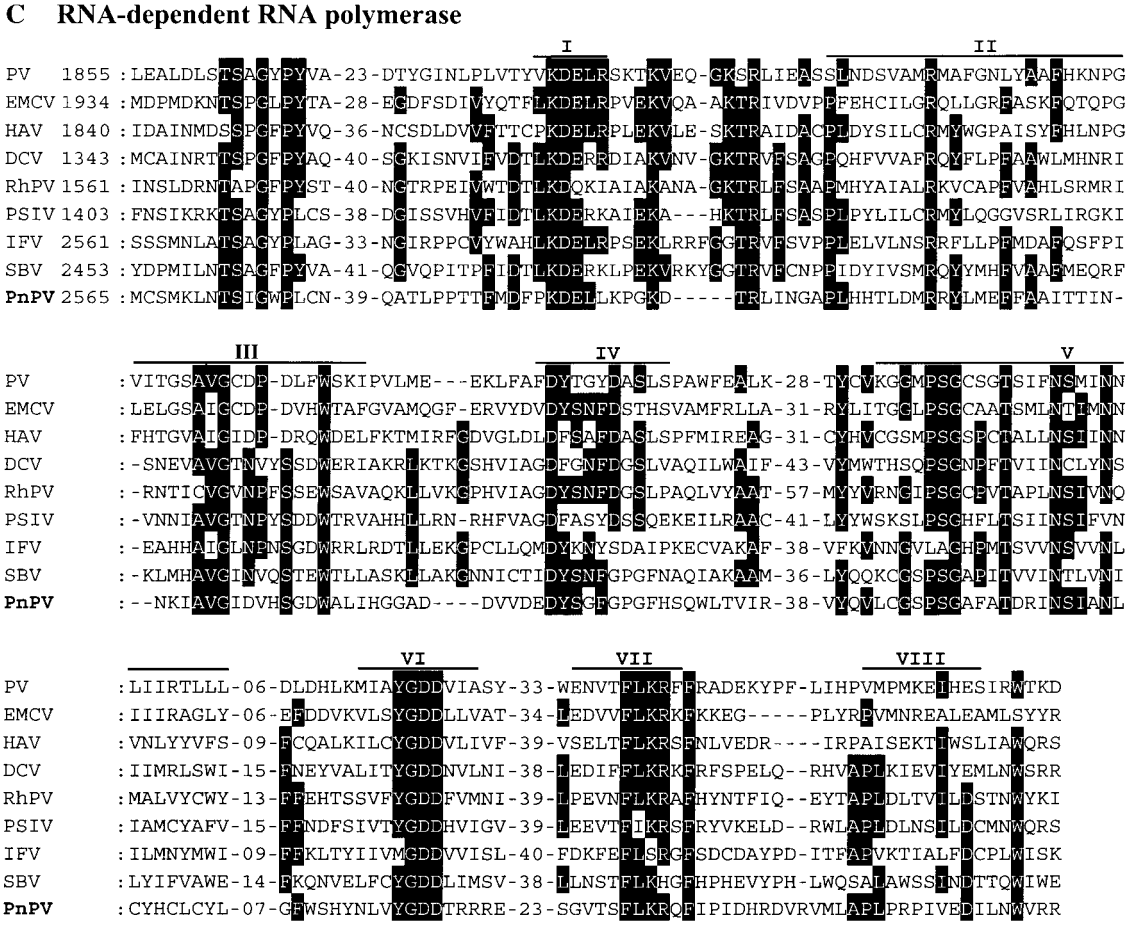


FIG. 5. Comparison of deduced amino acid sequences of nonstructural proteins of PnPV and other picorna-like viruses. (A) Alignment of the conserved regions of the putative RNA helicase regions from PnPV with PV, EMCV, HAV, DCV, RhPV, PSIV, IFV, and SBV (full names and references for these viruses are given in Table 2). Residues identical in at least half of the viruses are shown in inverse typeface. The motifs identified by Koonin and Dolja (1993) are labeled Hel-A, Hel-B, and Hel-C. Numbers on the left show the amino acid position of the aligned sequences. (B) Alignment of the putative protease domain of PnPV with those of other viruses. The residues that should form the catalytic triad (Koonin and Dolja, 1993) are marked with asterisks (*). (C) Alignment of the putative RNA-dependent RNA polymerase domain of PnPV with those of other viruses. The motifs identified by Koonin and Dolja (1993) are labeled I–VIII.

tural protein-coding region, and its proximity to the predicted C-terminal end of the CP4 protein suggests that it might also be functionally active.

Recently, in a study of the aphthovirus 2A/2B polyprotein cleavage mechanism, it was proposed that the activity of this site depends not on a proteolytic reaction, but on a novel translational effect that involves a putative ribosomal "skip" from one codon to the next without the formation of a peptide bond (Donnelly *et al.,* 2001a). Some 2A-like sequences have already been identified at various locations within the ORFs of insect virus polyproteins (reviewed by Donnelly *et al.,* 2001b). In IFV, a presumably functional 2A-like sequence was found near the downstream end of the structural protein-coding region and probably functions as it does in mammalian picornaviruses (Isawa *et al.,* 1998). In DCV, ABPV, and CrPV, there are conserved 2A-like sequences in the N-terminal region of the replicative ORF1 (Johnson and Christian, 1998; Govan *et al.,* 2000; Wilson *et al.,* 2000). In the *Thosea asigna* virus (*Tetraviridae*), a 2A-like sequence is present within the capsid protein precursor (Pringle *et al.,* 1999). In PnPV, the N-terminal sequence of CP2 suggests the possible functionality of the first 2A-like motif (aa 555–574) (Table 1). The second PnPV 2A-like sequence (aa 1172–1191) has a similar location to the 2A-like motif in IFV (i.e., at the downstream end of the structural protein-coding region) and it also appears to be functionally active. Thus PnPV appears to have two active 2A-like cleavage sites, a characteristic that has never been seen before in any picornaviruses.

Nonstructural proteins

Analysis of the 3′ part of the PnPV genome revealed that three distinct regions correspond to the conserved motifs of the helicase, protease, and RNA-dependent RNA polymerase (Fig. 1). These nonstructural proteins are also found in the same order in the picorna-like viruses (Koonin and Dolja, 1993). In Fig. 5, the amino acid sequences of the conserved region of PnPV's putative helicase, protease, and RdRp protein are aligned with those of eight other viruses: mammalian picornaviruses (PV, EMCV, HAV), CrPV-like viruses (DCV, RhPV, PSIV), and insect picorna-like viruses (IFV, SBV).

Helicase. Three conserved helicase regions recognized by Koonin and Dolja (1993) were found in the deduced amino acid sequence of PnPV's ORF from 1541 to 1655 (Fig. 5A). The highly conserved consensus sequence within the first domain, GXXGXGKS (Gorbalenya *et al.,* 1990), occurs in the PnPV sequence between amino acids 1552 and 1559. The last two domains deviate somewhat from the consensus. The highly conserved amino acids in these two domains are QX_5DD and KGX_4SX_5STN, while the PnPV equivalents are QX_5QD and KRX_4AX_5SSN, respectively (Fig. 5A).

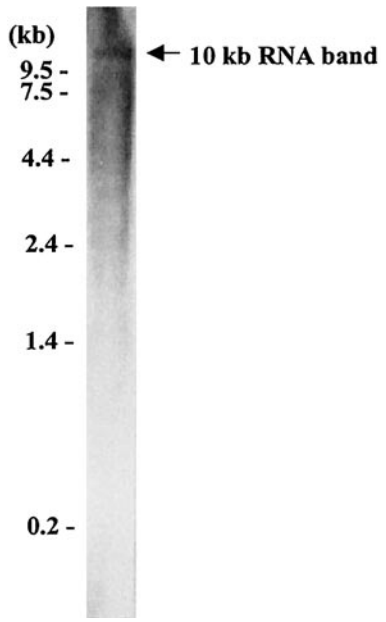


FIG. 6. Northern blot analysis of PnPV RNAs accumulated in infected PN cells (3 days postinfection) showing that no subgenomic RNA was synthesized.

Protease. The deduced amino acid sequence of PnPV's ORF from 2255 to 2399 is similar to the protease sequences of other picornaviruses (Fig. 5B). The GXCG motif was found at 2380–2383, although valine was substituted for glycine at the first position of this motif. Multiple alignment suggested that the H^{2260}, D^{2298}, and C^{2382} of PnPV's protease sequence should form the catalytic triad. However, although we were unable to find any alternative putative protease site, the dependence of PnPV's protease activity on these three amino acids has not yet been confirmed experimentally. The inclusion of aspartic acid in this triad suggests that PnPV belongs to the lineage B group of viruses (Ryan and Flint, 1997). This lineage includes members of the cardioviruses, aphthoviruses, and parechoviruses. By contrast, the lineage A group, which includes the enteroviruses and human rhinoviruses, all have glutamatic acid in the triad instead of the aspartic acid residue (Ryan and Flint, 1997).

RNA-dependent RNA polymerase. Eight conserved domains in RdRps corresponding to those recognized by Koonin and Dolja (1993) were found between amino acids 2565 and 2875 on the deduced amino acid sequence of PnPV (Fig. 5C). The conserved motifs in RdRp of positive-strand RNA viruses, SGX_3TX_3N, YGDD, and FLKR (Koonin, 1991), are located at amino acids 2765–2774, 2804–2807, and 2841–2844, respectively (Fig. 5C).

Northern blot analysis

Northern blot analysis of the RNA produced during PnPV infection of PN cells confirmed that PnPV does not produce subgenomic RNA (Fig. 6). No hybridization was seen in uninfected PN extracts (data not shown).

PnPV's taxonomic status

PnPV's genomic organization [5′NTR–putative leader protein–structural proteins–nonstructural proteins–3′NTR–poly(A) tail; see Fig. 1], the fact that it does not produce subgenomic RNA (see Fig. 6), and the fact that its genomic RNA contains a single large ORF, all argue that PnPV should be a member of the picorna-like superfamily. Accordingly, we propose that PnPV should be grouped with the unassigned "insect picorna-like" viruses IFV and SBV (group VIII), as shown in Table 2.

Phylogenetic analysis

The highly conserved fragments of RdRp amino acid sequences encompassing motifs I to VIII in RdRps (approximately 300 amino acid residues) of the picornaviruses and picorna-like viruses (Koonin and Dolja, 1993) were used in the phylogenetic analysis. Both the neighbor-joining (NJ) and the Quartet Puzzling trees generated similar results, but since the NJ tree revealed finer structures within major phylogenetic clades, only the NJ tree is shown here. The results reflect the current systematic assignment of the viruses. As Fig. 7 shows, all the members of *Picornaviridae* form a clade (bootstrap value 89) and a second clade includes all the members of *Cricket paralysis-like viruses* [note that since ICTV VII, three new members have been added to this genus: Triatoma virus (TrV; Czibener *et al.,* 2000); Black queen-cell virus (BQCV; Leat *et al.,* 2000); and Acute bee paralysis virus (ABPV; Govan *et al.,* 2000)] (bootstrap value 96). A third clade of insect picorna-like viruses (PnPV, SBV, and IFV) was formed, though with less bootstrap support (bootstrap value 45). A fourth clade of two caliciviruses (FCV and RHDV) was also formed (bootstrap value 69). Notably, the insect picorna-like viruses do not belong to the picornavirus clade (all the members of which are mammalian-infection viruses). This suggests that despite the similarity in genomic organization (i.e., the members of both clades contain just one ORF, they do not express subgenomic RNAs, and their capsid and nonstructural protein sequences are respectively located in the 5′ and 3′ region, see Table 2), the insect picorna-like viruses and the *Picornaviridae* are distinct evolutionary entities. Therefore, we suggest that PnPV, SBV, and IFV might constitute a novel group of insect-infecting viruses.

MATERIALS AND METHODS

Virus purification and viral RNA extraction

PnPV was originally isolated from its natural host, *P. nuda* Fabricius (Lepidoptera: Lymantriidae) and propagated in its homologous cell line, NTU-PN-HH (Wang *et al.,* 1996). The viral particles were purified from the infected PN cells as previously described by Wang *et al.* (1999). Genomic PnPV RNA was extracted from purified viral particles using TRIzol reagent (Gibco BRL) based on the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) and then precipitated with ethanol according to the manufacturer's recommendations.

cDNA synthesis, cloning, and nucleotide sequencing

Standard molecular biological procedures, essentially as described by Sambrook *et al.* (1989), were used to clone PnPV cDNAs. Briefly, cDNA synthesis was performed using a cDNA synthesis kit (Gibco BRL), reverse transcription of PnPV RNA being primed by the combination of oligo(dT_{12-18}), and six random residue primers. Second-strand synthesis, *Eco*RI (*Not*I) adaptor ligation, and size fractionation of cDNA were performed according to the manufacturer's recommendations. The synthesized cDNAs were ligated into the *Eco*RI site of pUC19 plasmid molecules and transformed into *Escherichia coli* JM109 cells. The large cDNA inserts (>1000 bp) were selected by *Eco*RI digestion. Seven overlapping cDNA clones containing almost the entire PnPV genome except for the extreme 5′ end were obtained. They were sequenced in both directions commercially (Mission Biotech, Taiwan) using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (ABI PRISM) and a 373A sequencing system (Perkin–Elmer).

5′-RACE analysis

The sequence of the extreme 5′ end of the PnPV genome was obtained by using a 5′-RACE system (Gibco BRL). Briefly, total RNA was prepared from PnPV-infected cells and treated by proteinase K (400 μg/ml) at 37°C for 60 min prior to use as a template for cDNA synthesis. This procedure ensured that the VPg of PnPV genomic RNA was removed to avoid interference in cloning the 5′ end as described by Johnson and Christian (1998). Three antisense primers, GSP1 (nt 713–694), GSP2 (nt 602–581), and GSP3 (nt 220–200), were synthesized and 5′-RACE was performed as per the manufacturer's instructions. First-strand cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase, and then the dC-tailed cDNA was amplified by polymerase chain reaction (PCR). All PCR products (approximately 260 bp) were ligated into the pGEM-T Easy vectors (Promega), prepared according to the manufacturer's protocol. The inserts of 10 clones were sequenced.

SDS–PAGE and N-terminal sequencing of viral structural proteins

Viral structure proteins were electrophoresed in 16% SDS–polyacrylamide gels using the Laemmli (1970) buffer system. Proteins were further stained by a silver stain or transferred onto a PVDF membrane as described by Moos *et al.* (1988). The membrane was stained with 0.1% amido black solution. The stained bands were excised and analyzed directly with an automated Edman

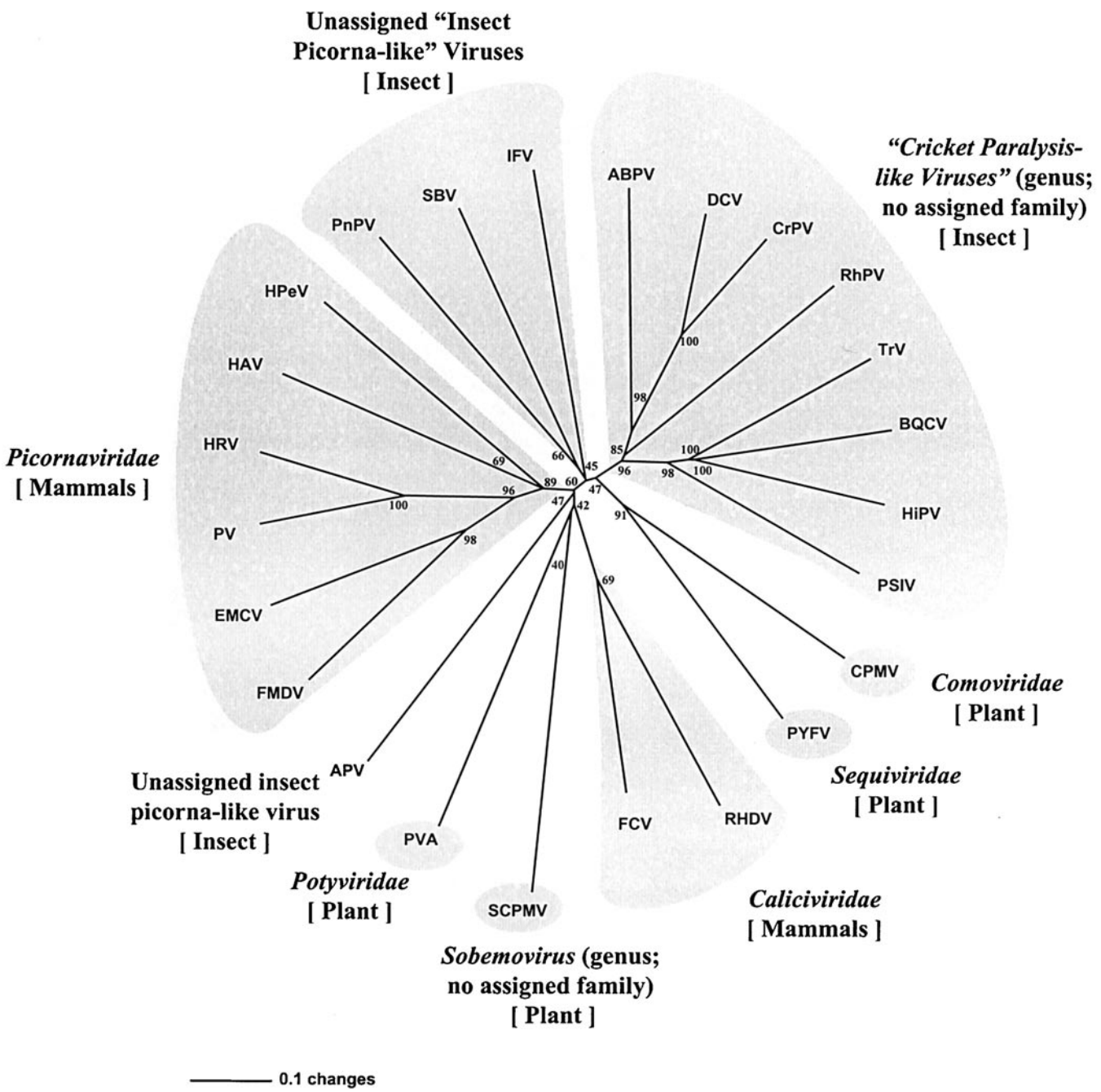


FIG. 7. Phylogenetic analysis of putative RdRp domains. The neighbor-joining trees were produced and bootstrapped (1000 replicates) using PAUP* 4.0b software. Viruses and references are as given in Table 2.

degradation sequencer (Applied Biosystems, Model 477A/120A).

Northern blot analysis

The general procedure of Sambrook *et al.* (1989) was used for Northern blot hybridizations. Briefly, total RNA was extracted from PnPV-infected (3 days postinfection) and uninfected PN cells, electrophoresed in a 1% agarose gel containing formaldehyde, and blotted onto a nylon membrane (Hybond-N^+, Amersham). Two species of DIG-RNA probes were prepared from *in vitro* transcription with a commercial kit (DIG-RNA Labeling Kit, Roche). These probes corresponded to the coding regions of a putative leader protein (nt 581-1092) and the helicase domain (nt 4850–5463) of the PnPV sequence, and they were used simultaneously in the Northern analysis.

Nucleotide sequence analysis and comparison

PnPv's genome cDNA sequences were assembled and analyzed using three computer programs (Genework

Version 2.5.1, GCG release 9.0, and Neural Network). The DNA and deduced amino acid sequences were compared with the latest GenBank/EMBL databases using FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.,* 1990), respectively. Multiple alignments of amino acid sequences were obtained with CLUSTALX (Thompson *et al.,* 1997) and edited in Genedoc (Nicholas and Nicholas, 1997). We used the deduced amino acid sequences of RNA-dependent RNA polymerase to construct phylogenetic trees of 24 viruses (see Table 2) using the neighbor-joining method (Saitou and Nei, 1987) and the Quartet Puzzling method as implemented in the PAUP* 4.0b program (Swofford, 1998). The statistical significance of branch order was estimated by performing 1000 replications of bootstrap resampling of the original aligned amino acid sequences.

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