

Isolation and characterization of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: evidence for diversity due to intra- and inter-species recombination

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We report the isolation, purification, genome-sequencing and characterization of a picorna-like virus from dead bees in Israel. Sequence analysis indicated that IAPV (Israeli acute paralysis virus) is a distinct dicistrovirus. It is most homologous to Kashmir bee virus and acute bee paralysis virus. The virus carries a 9487 nt RNA genome in positive orientation, with two open reading frames separated by an intergenic region, and its coat comprises four major proteins, the sizes of which suggest alternate processing of the polyprotein. IAPV virions also carry shorter, defective-interfering (DI)-like RNAs. Some of these RNAs are recombinants of different segments of IAPV RNA, some are recombinants of IAPV RNA and RNA from another dicistrovirus, and yet others are recombinants of IAPV and non-viral RNAs. In several of the DI-like RNAs, a sense-oriented fragment has recombined with its complement, forming hairpins and stem-loop structures. In previous reports, we have shown that potyviral and IAPV sequences are integrated into the genome of their respective hosts. The dynamics of information exchange between virus and host and the possible resistance-engendering mechanisms are discussed.

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INTRODUCTION

Viral diseases of bees (*Apis mellifera*) are a major economic consideration in apiculture. Bee viruses discovered to date include: acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), deformed wing virus (DWV), sacbrood virus (SBV), black queen cell virus (BQCV), and chronic bee paralysis virus (CBPV) (for example: Allen & Ball, 1996; Ball & Bailey, 1997; Evans & Hung, 2000; Elus & Munn, 2005). Individual bees often harbour more than one species of virus (Anderson & Gibbs, 1988).

Several bee viruses have been fully sequenced, including KBV (de Miranda *et al.*, 2004), ABPV (Govan *et al.*, 2000), SBV (Ghosh *et al.*, 1999), BQCV (Leat *et al.*, 2000), Kakugo virus (KV; Fujiyuki *et al.*, 2004) and DWV (Lanzi *et al.*, 2006). Based on replicase features, they all belong to the *Picornaviridae* superfamily (Koonin & Dolja, 1993). Viruses belonging to the subgroup *Dicistroviridae* (Mayo, 2002) carry two open reading frames (ORFs) for two polyproteins, divided by a short spacer. In dicistroviruses the polyprotein which is processed to the structural proteins is located at the 3'-proximal region of the viral

genome (van Munster *et al.*, 2002). Dicistroviral precursor proteins are processed by a virus-encoded protease, homologous to the 3C protease (3C-pro) of picornaviruses. Other insect-infecting picorna-like viruses include *Drosophila* C virus (DCV; Johnson & Christian, 1998), *Triatoma* virus (TrV; Czibener *et al.*, 2000), cricket paralysis virus (CrPV; Wilson *et al.*, 2000), aphid lethal paralysis virus (ALPV; van Munster *et al.*, 2002), *Homalodisca coagulata* virus-1 (HoCV-1; Hunnicutt *et al.*, 2006), himetobi P virus (HiPV; Nakashima *et al.*, 1999), *Solenopsis invicta* virus 1 (SINV-1; Valles *et al.*, 2004), *Plautia stali* intestine virus (PSIV; Sasaki *et al.*, 1998), *Rhopalosiphum padi* virus (RhPV; Moon *et al.*, 1998), and *Varroa destructor* virus 1 (VDV-1; Ongus *et al.*, 2004).

Recently, severe bee mortality has inflicted heavy losses on Israeli apiculture. Bees exhibited symptoms reminiscent of those inflicted by ABPV, therefore the isolated virus was tentatively named Israeli acute paralysis virus (IAPV).

Recently, Crochu *et al.* (2004) and Tanne & Sela (2005) reported that DNA versions of non-retro RNA viruses is incorporated into the genome of their hosts. In grapevine, it has been indicated that RNA recombination followed by retrotransposition may have led to this integration. Recently, we reported that a segment of IAPV is also incorporated into some of its bee hosts, and that bees

The GenBank/EMBL/DBJ accession number of the IAPV sequence reported in this paper is EF219380.

Supplementary material is available with the online version of this paper.

harbouring the viral segment are resistant to subsequent IAPV infection. We also reported that the exchange of genetic information between virus and host is reciprocal, and segments of a host gene were found embedded in a viral defective-interfering (DI)-like RNA (Maori *et al.*, 2007). The presence of subviral RNAs due to recombination has been well documented (e.g. Nagy & Simon, 1997).

In this paper, we characterize IAPV as a new member of the *Dicistroviridae* family. We sequenced the viral RNA, analysed its two polyproteins and the processed capsid proteins, and report on the presence of recombinant IAPV-related DI-like RNAs within encapsidated virions.

METHODS

Purification of IAPV. Healthy-looking bee larvae were inoculated with homogenate of a single dead bee. The inoculated larvae died within 4 days. The dead larvae were homogenized in detergent-containing buffer (0.01 M Na-phosphate, pH 7.6; 0.2% Na-deoxycholate, 2% Brij 58) and subjected to two cycles of differential centrifugation (10 000 g for 20 min then 100 000 g for 3 h). The final pellet was suspended in CsCl (0.6 g ml⁻¹). Following 24 h centrifugation at 100 000 g, a clear band appeared at a density of 1.33 g ml⁻¹. This band was collected and dialysed.

IAPV inoculation. IAPV was purified as described by Maori *et al.* (2007). Bees were inoculated by injection into larvae or pupae and by feeding virus-contaminated food to adult workers. Purified virus (1 µl, containing 1 µg of virus) was injected into the abdominal inter-segment space of 20 to 50 individual healthy-looking larvae taken from an apparently non-affected hive. Following a 4 day incubation at 35 °C, the infected, dead larvae were counted. Non-inoculated, as well as buffer-injected larvae served as controls. Larvae injected with tobacco mosaic virus (TMV) served as an additional negative control. Similar injection inoculations were carried out with pupae (Maori *et al.*, 2007). In other experiments, adult worker bees were fed on a 'cake' made of 66% sugar powder, 33% honey and 1% starch. Groups of approximately 150 to 200 healthy-looking bees were kept in separate cages at room temperature. Some groups were fed on IAPV-infested cake (approx. 1 µg virus per gram cake). Other groups were fed on virus-free cakes and another control group was fed on TMV-infested cake (1 µg virus per gram cake).

RNA extraction and electrophoresis. Purified virions were RNase-treated. Addition of RNasin was following proteinase K digestion. The viral RNA was extracted by phenol or TRI Reagent (Ambion) according to the manufacturer's protocol. RNA was electrophoresed on 1% agarose gels.

Semiquantitative RT-PCR assays. The amount of each type of RNA was determined by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). A mixture containing 90% RNA from an IAPV preparation and 10% TMV RNA was submitted to reverse-transcription with a mixture of hexamer primers (Applied Biosystems). The resultant cDNA was amplified with specific primers (Supplementary Table S1, available with the online version of this paper). Samples were drawn every three cycles and electrophoresed. The appearance of the first visible band in each preparation was indicative of the amount of the respective template relative to other templates. Correlation between PCR thresholds and actual amount of RNA was drawn from a calibration curve with TMV-RNA.

Molecular procedures and cloning of full-length viral RNA.

Standard molecular procedures such as Northern and Western blots, PCR and sequencing were carried out according to Sambrook & Russell (2001). The longest RNA from a gel was extracted and served as the template for cDNA cloning. First and second DNA strands were prepared from the viral RNA according to the kit provider protocol (Stratagene). First, the RT step was primed with oligo(dT), and the PCR stage was performed with that primer and random primers. The longest product was 1905 bp in length, and all smaller products were of sequences found within the longer one, confirming that they had all reacted with the same template. At later stages, an IAPV-specific 20-base-long anchor was added to the oligo(dT) primer (Supplementary Table S1). The resultant DNA fragments were cloned into the vector λZap Express (Stratagene) and their sequence was determined from the rescued plasmids. The longest fragment thus obtained was 1905 bases long. BLAST analysis indicated that it is homologous (75.4% identity) to the 3' section of KBV, carrying a segment of an ORF for the viral structural polyprotein and its 3' untranslated region (UTR). Primers for further RT-PCR assays were designed from the innermost section of this sequence, gradually advancing towards the 5' end using the SMART procedure (Clontech). The 5' rapid amplification of cDNA ends (RACE) was repeated several times and always resulted in the same 5' sequence. The sequence was determined by aligning overlapping fragments. The sequence was further confirmed by a series of PCRs with different sets of primers. Primer descriptions and designations are given in Supplementary Table S1.

Protein analyses. IAPV capsid proteins were analysed following electrophoresis on SDS-polyacrylamide gels. Extraction of bee proteins and Western blot analyses were performed according to Sambrook & Russell (2001). Edman degradation procedure for the determination of N termini was performed by the Biological Services unit of the Weizmann Institute of Science (Rehovot, Israel). IAPV proteins were first resolved on denatured gels, transferred onto a membrane, and each band was analysed for the first 3–6 amino acids at the peptide's N terminus. The resolution of gels performed according to protocols specified for Edman degradation analysis was better than that of gels electrophoresed according to the protocol for Western blot analysis. Serological relationships with other bee viruses were tested by immunodiffusion assay (Mansi, 1958). Instructions and antibodies were provided by Dr Brenda Ball, and the tests were carried out at the Rothamsted Research Laboratory, England.

Isolation and determination of virion-associated DI-like RNAs.

Only a single fragment of IAPV was found integrated into the bee genome. In addition, a DI-like fragment, fused to a segment of the host gene, was isolated from IAPV virions by PCR with primers designed for flanking sequences of the genome-inserted fragment (Maori *et al.*, 2007). We therefore assumed that the ends of these fragments serve as 'hot spots' for recombination. DI-like RNAs residing within virions were extracted from RNase-treated, CsCl-purified virions, to eliminate any contamination on the outside of the virions. RT-PCR was carried out with the aforementioned primers (Supplementary Table S1, available with the online version of this paper), and products deviating from the expected size were gel-purified, TA-cloned and sequenced.

Sequence analyses. Protein molecular masses were computed from their actual sequence by the 'compute pI/Mw' program. Post-translational protein modifications were determined by the following programs: NetOGlyc, NetNGlyc, NetPhos and SUMOplot. 3C-pro cleavage sites were determined by NetPicoRNA. All the aforementioned programs can be found in the ExPASy Proteomic Tool package (www.expasy.org/tools). RNA folding was predicted by the program 'RNA secondary structure prediction' (www.genebee.msu.su).

RESULTS

Isolation of IAPV

Dead bees were collected from a cluster of hives near Alon Hagalil, Israel. A virus was isolated by differential centrifugation followed by equilibrium gradient centrifugation as described in Methods. Electron micrographs showed icosahedral particles with a mean diameter of 27 nm (Fig. 1b).

Purified virus is the causative agent of the disease

The percentage mortality of injected and IAPV-fed bees is given in Table 1.

The virus purified from the injected larvae was identical in sequence and infectious. Injection with IAPV RNA (100 µg per larva) resulted in the death of over 80 % of the injected bees. The virus was purified from RNA-injected larvae and found free of other known viruses by differential PCR.

IAPV-injected bees died within 4 days. Bees fed on IAPV-infested cakes gradually developed symptoms and died

within 10 days. Early on, the only indication of infection was darkening of the abdomen tip. Between the 3rd and 6th day of infection, the thorax darkened as well, and the bees were unsettled: they were constantly going around in circles, and barely flew or ate. Between the 7th and 10th days, the bees' abdomen and thorax became dark (dark brown to black), and the thorax became hairless. The bees stopped flying, barely moved, underwent periods of spasms, and eventually died.

Viral genome

Nucleic acids were extracted from RNase-treated purified viral preparations and electrophoresed. A band of approximately 9 kb was observed, which was DNase-insensitive and RNase-sensitive. In addition to the full-length band, several strong, but shorter bands appeared under denaturing conditions (Fig. 1a). These shorter RNA forms were considered to be DI-like RNAs and are described in detail further on.

The viral RNA was cloned and sequenced (GenBank accession no. EF219380). It is 9487 nt long (excluding the poly-A tail) and carries two ORFs, both coding for polyproteins. The 5' proximal ORF, translating to 1900 aa, codes for proteins involved in RNA replication and protein processing. The ORF near the 3' end, translating to 908 aa, codes for a polyprotein which is processed to the various capsid proteins. Since the protein initiation site may not be AUG (Johnson & Christian, 1998; Domier & McCoppin, 2003; Nishiyama *et al.*, 2003), the N termini of both polyproteins may differ (by being slightly shorter) from the ones presented here. The two ORFs are separated by a 184-nucleotide-long intergenic region. A schematic illustration of the IAPV genome is given in Fig. 1(c).

Based on homology and genomic structure, IAPV belongs to the family *Dicistroviridae* (Mayo, 2002).

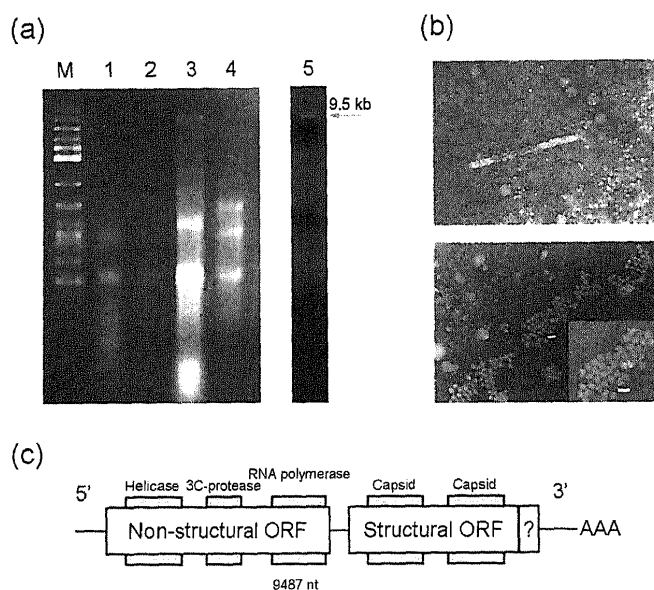


Fig. 1. (a) Electrophoretic patterns of RNA isolated from RNase-treated, CsCl-purified IAPV virions. Electrophoresis was performed under denaturing conditions as described in Methods. The different lanes exhibit patterns of various independent virus preparations. Lane 5 shows the electrophoresis pattern of a gel-extracted full-length RNA. Lane M shows size markers. The arrow points to the putative full-size viral RNA. (b) Electron micrograph of clarified crude sap from infected bee haemolymph (top) and of a purified virus preparation (bottom). TMV (17×300 nm) was added to the top micrograph as a size marker. The bar in the bottom micrograph represents 100 nm. (c) Schematic illustration of the IAPV genome. White frames represent the two ORFs. The vertical line near the right end marks the position of a stop codon, immediately followed by a methionine codon.

The non-structural polyprotein

This polyprotein carries motifs for helicase, protease and RNA-dependent RNA polymerase (RdRp) found in non-structural polyproteins of the picornaviridae (Koonin & Dolja, 1993). The helicase domain carries three signature sequences. Motif A is the nucleotide-binding motif GxxGxGK (⁵¹²GESGVGK⁵¹⁸ in IAPV). This motif, responsible for nucleotide binding and hydrolysis (Walker *et al.*, 1982), has been found in all hitherto sequenced dicistroviruses. Motif B, the catalytic core WDGY (⁵⁵⁷WDNY⁵⁶⁰ in IAPV) followed by [E/Q]x5D[D/E] (⁵⁶³QNVVVYDD⁵⁷⁰ in IAPV), is also present in all dicistroviruses (motif alignments are presented in Fig. 5 of Hunnicutt *et al.*, 2006). The protease domain carries two signature sequences as described by Koonin & Dolja (1993): the classical signature of cysteine proteases, GxCG (¹³⁰¹GDCG¹³⁰⁴ in IAPV), and the putative substrate-binding motif GxHxxG (¹³²⁰GIHVAG¹³²⁵ in IAPV). The

Table 1. Bee mortality following administration of IAPV

Percentage mortality following feeding with:				Percentage mortality following injection with:			
Buffer	TMV	IAPV (purified)	Non-injected	Buffer	TMV	IAPV (crude)	IAPV (purified)
13.1	8	80	6.25	12	25	80	98

RdRp domain of subgroup 1 of RdRp carries eight conserved regions (Koonin & Dolja, 1993; Fig. 2 in Hunnicutt *et al.*, 2006). IAPV carries the ¹⁵⁶⁴TLKDER¹⁵⁶⁸ of conserved region I, ¹⁵⁷⁹KTRVFS¹⁵⁶⁴ of conserved region II, ¹⁶¹⁹NVY¹⁶²¹ of conserved region III, DFxxFDG (¹⁶⁴³DFSTFDG¹⁶⁴⁹ in IAPV) of conserved region IV, THS[Q/I/L]PSG[N/C/H][P/E/Y] (¹⁶⁹⁹THSQPSGNP¹⁶⁷⁷ in IAPV) of conserved region V, YGDD (a signature sequence of RdRp) of conserved region VI, TDExK (¹⁷⁸³TDELK¹⁷⁸⁷ in IAPV) and ¹⁸⁰⁵LKR¹⁸⁰⁷ of conserved region VII and APLx₁₀W (¹⁸²⁰APLCMDTILEMPNW¹⁸³³ in IAPV) of conserved region VIII. In addition to linear motifs, two aspartic acid residues situated five residues apart and an asparagine located 69 residues downstream of the second aspartic acid (D¹⁶⁴³, D¹⁶⁴⁸ and N¹⁷¹⁷, respectively) are required for magnesium-binding and discrimination between ribonucleotides and 2' deoxyribonucleotides (Hansen *et al.*, 1997; Love *et al.*, 2004). The exact same distances for this triad have been reported for HoCV-1 (Hunnicutt *et al.*, 2006). Alignment of the RdRp sequences of several dicistroviruses indicated conservation of the aforementioned motifs (Fig. 2a). However, as expected from the homology determinations, ABPV, KBV and IAPV were set off as a separate, most closely related group (Fig. 2b).

The structural polyproteins

As already mentioned, the precise location of the N terminus of the polyproteins may not be the first methionine. In IAPV, a stop codon is located near the C terminus of the structural protein ORF, immediately followed by a methionine codon. A read-through may occur, producing a protein of 944 aa (rather than the postulated 908 aa residues).

The patterns of dicistroviruses' mature capsid proteins differ from one species to the next. In several papers, three major proteins have been demonstrated, along with an additional minor protein (or proteins), and possibly a short peptide (C4, less than 10 kDa), believed to be cleaved off after virion assembly (for example, Sasaki *et al.*, 1998). Four capsid proteins, of approximately 9.5, 24, 33 and 35 kDa, have been identified in ABPV and KBV, the two closest relatives of IAPV. Electrophoretic patterns of denatured IAPV indicated that the major IAPV capsid proteins are approximately 17, 26, 33 and 35 kDa and three additional minor proteins of approximately 19, 30 and 46 kDa were also observed (Fig. 3a). Western blot analysis (of a somewhat lower resolution gel) exhibited the same general pattern (Fig. 3b). The IAPV antibodies did not

react with any protein of healthy bees (Maori *et al.*, 2007), thus they are specific to viral proteins only. Edman degradation of gel-extracted bands determined five N-terminal sequences. (i) SVL, corresponding to ¹²⁶SVL¹²⁸ of the translated sequence. (ii) SQPKTS, corresponding in five out of its six amino acids to ³³⁹SQKSTS³⁴⁴ as determined from the translated IAPV sequence. (iii) SKP, corresponding to ⁴⁰⁰SKP⁴⁰² of the translated sequence. In IAPV this latter sequence resides within the ³⁹⁸GWSKP⁴⁰² motif. This motif, G[F/W]SKP, has been identified as a CP4/CP2 cleavage site, approximately residing between residues 300 and 400 in the structural polyproteins of ALPV, RhPV, DCV, CrPV, BQCV, PSIV, TrV, ABPV and Taura syndrome virus (TSV) (summarized in van Munster *et al.*, 2002) and in KBV. (iv) SVP, corresponding to ⁴²⁷SVP⁴²⁹ of the translated sequence. (v) INIGNK, identical to the translated IAPV sequence (⁷⁰¹INIGNK⁷⁰⁶) and similar to the INLSNK cleavage site in KBV (GenBank accession no. NC_004807). In addition, *in silico* analysis identified two strong potential 3C-pro cleavage sites: ²⁰⁸YASFQEAYD²¹⁶ and ²⁷⁰DIVKQGASR²⁷⁸. Analysis of cleavage sites and comparison to the capsid-protein profile indicated that alternate protein processing and sequential processing may have taken place. In this scenario, the polyprotein is first cleaved at the exposed sites, skipping other cleavage sites. The cleaved products then present new accessible sites and are further cleaved at those newly exposed sites (discussed further on). The minor bands probably represent remnants of the primary processed peptides which were then further processed at a later stage.

Alignment of the IAPV structural polyprotein with other dicistroviruses is shown in Fig. 4(a). Analysis of only IAPV, KBV and ABPV, as in Fig. 2(b), reiterated the close relationship among the latter three viruses (data not shown).

IAPV in relation to other dicistroviruses

Phylogenetic analysis of various parts of the IAPV genome indicated that IAPV clusters together with KBV and ABPV. Fig. 4(b) shows the phylogenetic relationship among dicistroviruses, based on the amino acid sequence of the RdRp domain of the non-structural polyprotein. Similar relationships were obtained with amino acid and nucleotide sequences of other parts of the genomes. While most of the ORFs' sequences are highly homologous to KBV and ABPV, the homology of IAPV UTRs to those of KBV and ABPV is weak (Supplementary Table S2, available with the online version of this paper). However, IAPV could be

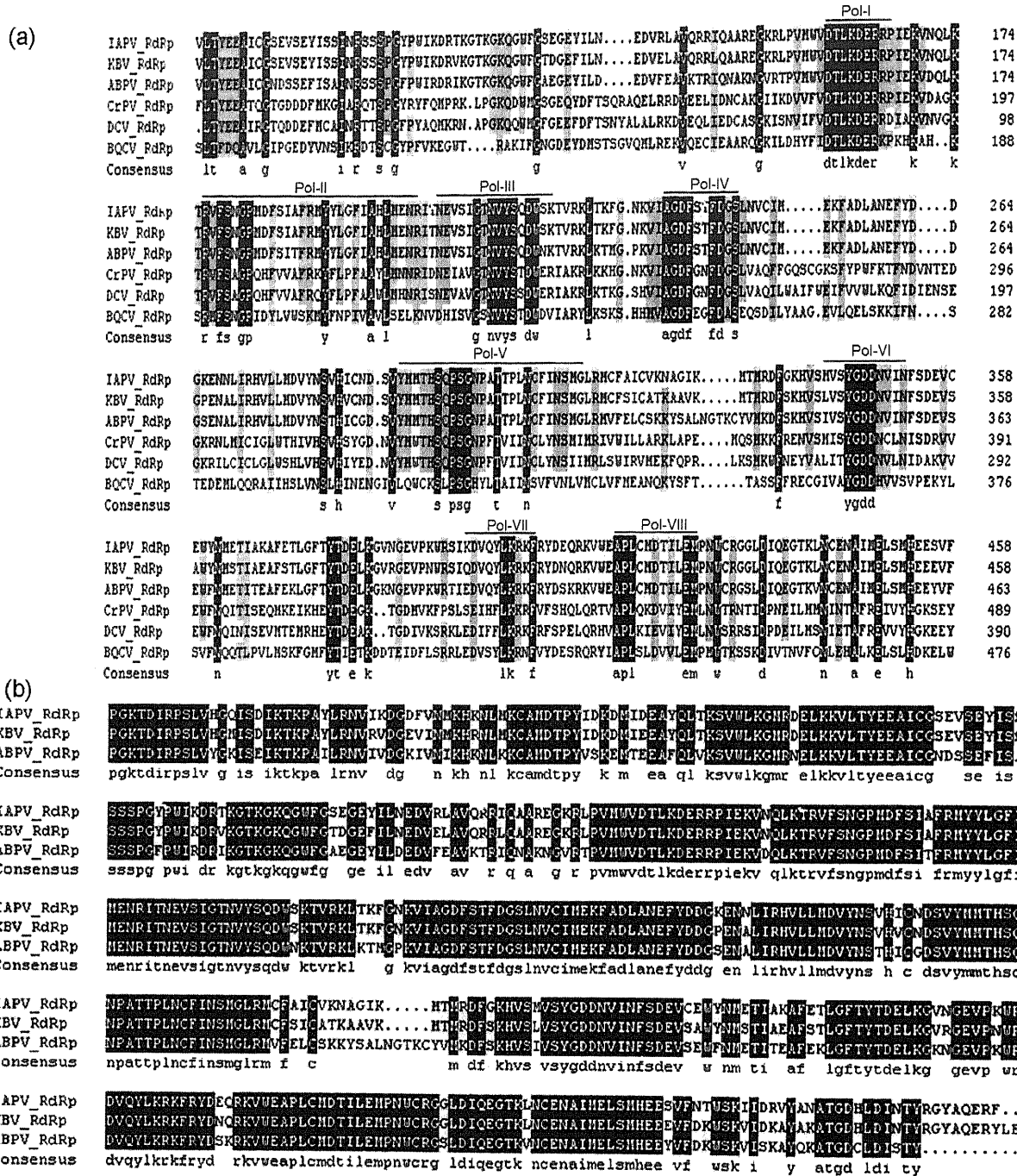


Fig. 2. (a) Alignment of the RdRp domain of several dicistroviruses. (b) Same alignment comparing only IAPV, KBV and ABPV. Horizontal lines mark the positions of the eight conserved motifs.

easily discerned from its closest relatives, KBV and ABPV, by differential PCR assay, even when RNAs of two of these viruses were present in the same RNA preparation (Supplementary Figure S1, available with the online version of this paper).

Differential PCR primers distinguished all three viruses (Supplementary Figure S1). Bees can harbour more than one virus, and the ability to distinguish IAPV from the other viruses in mixed infections indicated that IAPV is a

unique viral entity. The structural polyprotein of IAPV is most related to KBV and ABPV, yet IAPV did not react with antibodies against KBV, ABPV, DWV, BQCV, slow paralysis virus (SPV), SBV, cloudy wing virus (CWV) or CBPV. In addition, the homology of IAPV UTRs to its closest relatives is very weak (Supplementary Table S2, available with the online version of this paper). Another indication that IAPV varies from other dicistroviruses is its different pattern of capsid proteins, suggesting different cleavage sites in these highly homologous polyproteins.

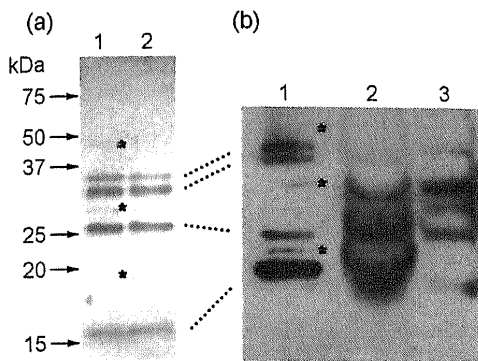


Fig. 3. Electrophoretic analyses of IAPV capsid proteins. (a) Coomassie blue-stained gel, taken for Edman degradation. Lanes 1 and 2, proteins from two different IAPV preparations. (b) Western blot analysis with antibodies to IAPV. Lane 1, virion proteins; lanes 2 and 3, protein extracts from IAPV-infected bees. Position of size markers are shown on left. Dashed lines connect peptides of the same size in both gels. Asterisks denote the position of minor bands. The three first N-terminal amino acids of the major bands (top to bottom) are SQP, SKP, INI and SVP.

IAPV-related RNA fragments resembling DI-RNA

IAPV RNA was extracted from RNase-treated, CsCl-purified virions. All viral preparations were purified from subsequent passages of the original purified IAPV isolate. Electrophoretic analysis indicated that various viral preparations carry shorter than full-length intra-virion RNAs alongside the full-length viral genome (Fig. 1). Northern blots (Fig. 5) and sequence analyses indicated that the encapsidated RNAs carried IAPV sequences, albeit fused to viral and non-viral sequences. It appears that some DI-like RNAs carried signals enabling their encapsidation. This agrees with similar previous findings, and explains the appearance of a multitude of viral-related RNA species in IAPV-infected tissues (Fig. 2 in Maori *et al.*, 2007). Apparently, recombination events take place quite frequently, but only a small proportion of the generated DI-like RNAs are capable of encapsidation. The composition of DI-like RNAs in any given virion depends on what recombination events have taken place in the infected cells prior to virus assembly.

Intra- and inter-species recombination of viral and viral-non-viral sequences

A DNA version of a segment of IAPV RNA has been found integrated into the bee genome, and reciprocally, a host sequence has been found embedded in an IAPV-related DI RNA (Maori *et al.*, 2007). Figs 1 and 5 indicate the presence, within the virions, of RNAs carrying IAPV sequences which are smaller than full-length. Looking for recombinants, we carried out Northern blot analyses with RNA extracted from two different viral preparations. Two membranes, each carrying the same RNAs, were prepared: one was reacted with a probe corresponding to the 5' IAPV

sequence and the other with that of the 3' end. Fig. 5 indicates that besides the full-length RNA, shorter bands reacted with one or the other of these probes. Some reacted with both probes, a priori indicating middle-sequence deletions, and some reacted with only one of the probes. The Northern blot patterns of the two viral preparations were similar, but not identical, indicating ongoing recombination during infection.

Only a single IAPV fragment has been found integrated into the bee genome (Maori *et al.*, 2007), and we therefore tentatively assumed that it carries sequences serving as 'hot spots' for recombination. We amplified RNA from virions (using primers flanking the ends of the integrated IAPV segment) and sequenced them. In addition, to identify DI-like RNAs in which the middle portion of the IAPV genome had been deleted, RT-PCR was carried out with primers corresponding to the 5' and 3' ends of IAPV RNA. Sequence analysis revealed four types of DI RNAs. (i) Deleted DIs: DI-like RNAs in which part of the middle section of the viral RNA has been deleted. An example of a deleted DI is shown in Fig. 6(c), its sequence is shown in Supplementary Figure S2(a), available with the online version of this paper. Positioning of this deleted DI sequence within the IAPV genome reveals that it is flanked by two inverted repeats, and it is predicted to fold as shown in Fig. 6(b). The middle portion of the IAPV genome has been looped-out, and the possibility of replicase crossing from one proximal IAPV sequence to the opposite proximal sequence is strongly supported by this model. (ii) Inverted repeats of segments of the IAPV genome: a segment of IAPV RNA folds on an (almost) inverted repeat of itself (Fig. 6d, e), the sequence is shown in Supplementary Figure S2(b). (iii) A sequence of IAPV and an inverted sequence which is most highly homologous to KBV, indicating inter-viral recombination. An example of the organization and predicted folding pattern of such a sequence are shown in Fig. 6(f, g), the sequence is shown in Supplementary Figure S2(c). (iv) IAPV-derived DI RNAs carrying non-viral (host?) sequences (an example is shown in Fig. 6f). A case in which a non-viral sequence originates from the host is demonstrated in Maori *et al.* (2007).

Quantitative analysis of a recombinant within virions

Semiquantitative RT-PCR (qRT-PCR) assays were carried out in order to estimate the proportion of DI-like RNAs within a viral preparation. The absence of mixed viruses in the viral preparation tested was determined by RT-PCR discerning IAPV from other dicistroviruses.

For qRT-PCR analysis, a calibration curve was first drawn for TMV RNA. The relative amount of one prominent deleted DI-like RNA (the one depicted in Fig. 6) in this particular viral preparation was determined. RNA isolated from the viral preparation (1008 ng) was mixed with TMV RNA (112 ng), such that TMV RNA constituted 10% of the total and served as an internal control. Portions of this

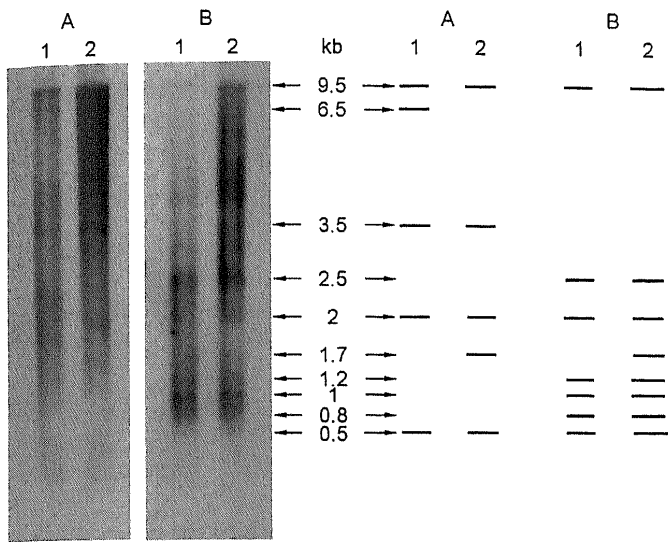


Fig. 5. Northern blot analysis of virion-extracted RNA (two left frames) and a schematic illustration of the bands detected (two right frames). A, Membrane reacted with a probe to the 5' end of IAPV; B, membrane reacted with a probe to the 3' end of IAPV. 1 and 2 designate two different viral preparations.

mixture were taken for qRT-PCR (the equivalent of 123 ng RNA per reaction). IAPV RNA amplification was carried out with primer pairs specific to the 5' end of IAPV, and to the 3' end of the viral RNA, to ensure that the quantitative determination was of the full-length IAPV RNA and not of the DI-like RNA. As shown in Fig. 7, IAPV amplification products were first observed at cycle 18, those of TMV RNA at cycle 21, and those of the DI-like RNA at cycle 24. Calibration against TMV RNA indicated that the relative amount of IAPV RNA in the viral RNA preparation was 88.9% and that of the tested DI-like RNA, 0.99%. The remaining 10.11% probably represents other DI-like RNAs. Indeed, the weak bands of Fig. 7(c) were isolated and sequenced and found to be forms of 'deleted DIs' of IAPV demonstrating strong 'hot spots' between bases 9100 and 9300 of IAPV.

DISCUSSION

IAPV closely relates to KBV and ABPV, but is sufficiently different to be discerned by PCR and serology. The two ORFs translate to 1899 (non-structural polyprotein) and 908 aa (structural polyprotein). However, ATG appears immediately adjacent to the structural protein's TAG stop codon, and the possible appearance of a read-through protein of 944 aa cannot be ruled out. Two potential 3C-pro cleavage sites were identified in the structural polyprotein and a sequence of a well-established cleavage site (GW/SKPRNQ) was determined. N termini of five of the gel-resolved peptides were determined. The peptide pattern suggests that initially, cleavage takes place only at a few exposed sites. The resultant primary products are then

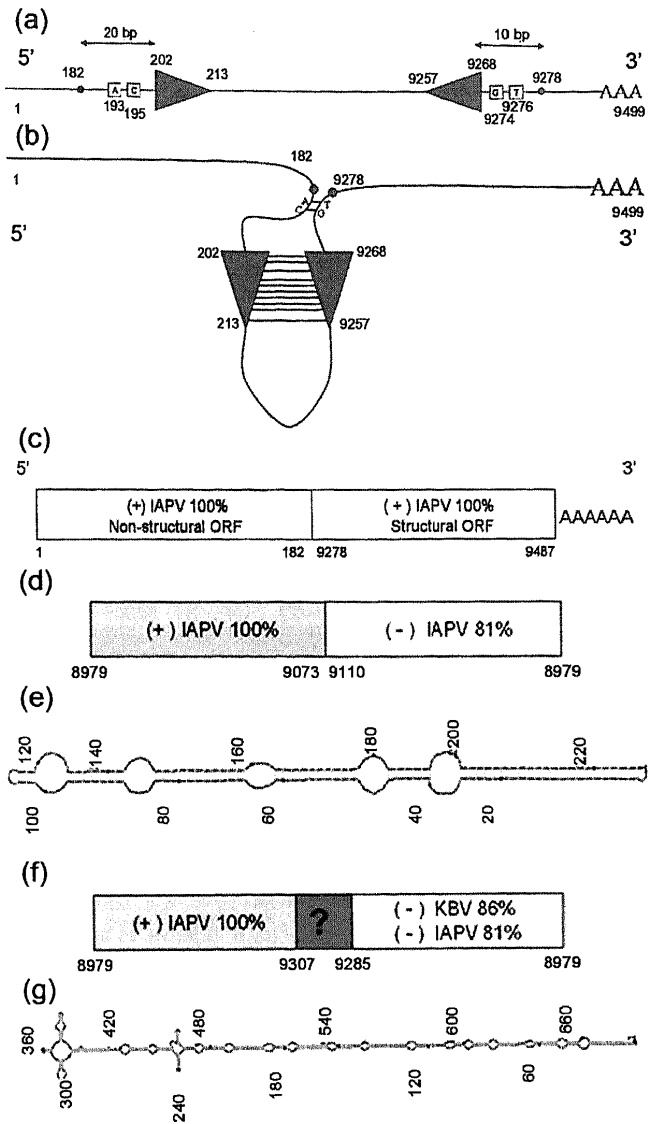


Fig. 6. (a-c) A DI-like sequence suggesting a proposed model for looping-out of a portion of the viral sequence. (a) Schematic illustration of the IAPV RNA sequence and the relevant repeats to the deleted DI. (b) Folding of IAPV RNA suggesting a looping out of viral sequences, facilitating replicase crossing from one part of the viral RNA to another. (c) Illustration of the obtained deleted DI. (d-g) Illustrations of inverted-repeats-carrying DI-like RNAs. (d, e) Represent the organization and predicted folding of an inverted repeat segment of IAPV RNA. (f, g) Organization and predicted folding of the sequence depicted in (b). (+) and (-) represent sequences of viral and complementary orientations, respectively. (?) represents a sequence which could not be annotated.

further cleaved due to exposure of other sites, causing a reduction of the primary products and the generation of a number of short (undetectable) peptides. *In silico* analysis, taking into account the various processing possibilities, suggested end products compatible with the gel patterns. One can speculate that the structural polyprotein is first cleaved alternately at ¹⁷⁷SVL¹⁷⁹, at the 3C-pro site ²⁷⁰DIVKQASR²⁷⁸, at ³⁹⁸GWSKP⁴⁰² and at ⁷⁰¹INIGNK⁷⁰⁶.

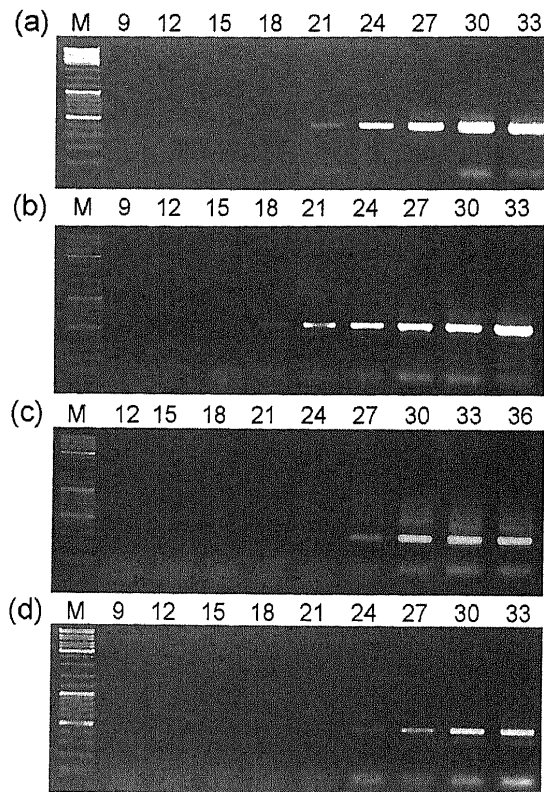


Fig. 7. Estimation of the relative amount of a DI-like RNA within virions of an IAPV preparation. TMV RNA (10%) was added to the same IAPV preparation described in Fig. 6 to serve as an internal control. The mixture of IAPV RNA and TMV RNA was submitted to semiquantitative RT-PCR assays. (a) PCR profile obtained with a primer-pair specific to the 3' proximal part of IAPV. (b) PCR profile obtained with a primer-pair specific to the 5' proximal part of IAPV. (c) PCR profile obtained with a primer-pair designed according to sequences near both ends of the DI RNA. (d) PCR profile of TMV RNA. Primer details are given in Supplementary Table S1. Note that the cycle numbers in (c) differ from those in (a, b and d).

This would result in primary products spanning positions M¹ to S¹⁷⁷ (19 666 Da), M¹ to D²⁷⁰ (30 007 Da), M¹ to W³⁹⁹ (43 987 Da), S⁴⁰¹ to Q⁷⁰⁰ (33 349 Da) and I⁷⁰¹ to P⁹⁰⁸ (C-terminal; 26 745 Da). Some of these peptides are then submitted for further cleavage at ⁴²⁷SVP⁴²⁹, ³³⁹SQKSTS³⁴⁴ and the second HC-pro site ²⁰⁸YASFQEAYD²¹⁶, cleaving the 43 987 Da primary product to a peptide between ¹²⁶SVL¹²⁸ and ⁴²⁷SVP⁴²⁹ (27 072 Da), and a number of small peptides. In addition, an unidentified cleavage site around position 605 of the polyprotein is suggested. Cleavage between S³³⁹ and this site is expected to result in a peptide of approximately 35 kDa, and between S⁴²⁸ and the postulated site, a 17 kDa peptide. A site around position 605 is also compatible with the position of a cleavage site between VP2 and VP3 of KBV (GenBank accession no. NC_004807). Most cleavage sites of picorna-like viruses are determined by only one pair of amino acids (QG in most picornaviruses), and the surrounding sequence contributes to the rate at which cleavage occurs

at a particular site. Hence, even a point mutation in the polyprotein may result in a totally different pattern following its processing from very similar precursor polyproteins.

A pronounced protein of approximately 30 kDa (not detected in the virions) appears among the bee-extracted proteins. It may represent a modified capsid protein that is unable to encapsidate and probably plays some role in virus infection, or virus–host interaction. Indeed, the N-terminal part of the IAPV capsid protein precursor carries two strong SUMO signals (¹²⁷LKAG¹³⁰ and ²⁷³VKQG²⁷⁶). SUMO proteins affect protein modification, cleavage and folding (for example, Ulrich, 2005; Bossis & Melchior, 2006), and it is conceivable that SUMO-bound proteins cannot encapsidate.

We were previously able to isolate and sequence DI-like RNAs from virions. We also demonstrated that RNAs, shorter than the full-length IAPV, are present in IAPV-infected tissues (Maori *et al.*, 2007). Here we demonstrate the abundant accumulation of shorter-than-full-length RNAs within virions. The shorter RNAs appear to be recognized by the viral replicase and replicate efficiently, and some carry assembly recognition signals as well, and are therefore found encapsidated within virions. Several of the DI-like RNAs carry sequences of the 5' and 3' viral ends, but the middle sequences are deleted. The viral genome carries inverted repeats flanking the joining point of the two segments of the deleted DI-like RNA, causing looping out of the middle sequences and enabling the replicase to cross over from one segment to a distant segment of the same RNA. Several other DI-like RNAs are comprised of a segment of a viral sequence in its sense orientation followed by a short spacer and the almost identical viral sequence in an antisense orientation, suggesting template switching of the replicase from a viral-oriented RNA to a nascent complementary RNA. Viral recombinants are known mostly between segments of the same virus, but recombination among related viruses has been previously recorded (Nagy & Simon, 1997). Here, we demonstrate a similar case of an IAPV–KBV 'hybrid', but in opposite orientations. Sense:antisense DI-like RNAs fold into a largely double-stranded RNA structure (Fig. 6).

Although RNA recombination requires certain structural features, by and large, it is a random occurrence. Therefore, the amount and characteristics of encapsidated DI-like RNAs may change from one cycle of infection to the next, and viral populations may differ from each other in this respect. We showed different profiles in different viral preparations (Fig. 5). Therefore, the reported quantitative values apply to that particular viral preparation, and may differ when other preparations are tested.

In many instances, bee viruses are found as non-apparent infections in their host. This may result from competition between the viral RNA and the abundant DI-like RNAs, but also from the abundance of double-stranded RNA structures carrying viral sequences. In the latter case, the

balance between host factors silencing viral sequences and the virus-induced silencing suppressors may shift in favour of viral silencing. Another postulated consequence inferred from the various types of DI-like RNAs is the modification of proteins resulting from deletions, engendering deleted or frame-shifted proteins. Thus, RNA recombination may elicit protein divergence with obvious evolutionary impact. Furthermore, a reciprocal exchange between host DNA and viral RNA (or a DNA version of a recombinant viral RNA) has been demonstrated (Tanne & Sela, 2005; Maori *et al.*, 2007). Therefore, RNA recombination may engender divergence in host genes, and the evolution of both virus and host may be interrelated and linked to the very same eliciting process.

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REFERENCES

- Allen, M. & Ball, B. (1996). The incidence and world distribution of honey bee viruses. *Bee World* 77, 141–162.
- Anderson, D. L. & Gibbs, A. J. (1988). Inapparent virus-infections and their interactions in pupae of the honey bee (*Apis mellifera* Linnaeus) in Australia. *J Gen Virol* 69, 1617–1625.
- Ball, B. V. & Bailey, L. (1997). Viruses. In: *Honey Bee Pests, Predators and Diseases*, 3rd edn, pp. 11–31. Edited by R. A. Morse & K. Flottum. Medina, OH: A. I. Root.
- Bossis, G. & Melchior, F. (2006). SUMO: regulating the regulator. *Mol Cell* 21, 349–357.
- Crochu, S., Cook, S., Attoui, H., Charrel, R. N., de Chesse, R., Belhoucet, M., Lemasson, J.-J., de Micco, P. & de Lamballerie, X. (2004). Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of *Aedes* spp. mosquitoes. *J Gen Virol* 85, 1971–1980.
- Czibener, C., La Torre, J. L., Muscio, O. A., Ugalde, R. A. & Scodeller, E. A. (2000). Nucleotide sequence analysis of Triatoma virus shows that it is a member of a novel group of insect RNA viruses. *J Gen Virol* 81, 1149–1154.
- de Miranda, J. R., Drebot, M., Tyler, S., Shen, M., Cameron, C. E., Stoltz, D. B. & Camazine, S. M. (2004). Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus. *J Gen Virol* 85, 2263–2270.
- Domier, L. L. & McCoppin, N. K. (2003). *In vivo* activity of *Rhopalosiphum padi* virus internal ribosome entry sites. *J Gen Virol* 84, 415–419.
- Elus, J. D. & Munn, P. A. (2005). The worldwide health status of honey bees. *Bee World* 86, 88–101.
- Evans, J. D. & Hung, A. C. (2000). Molecular phylogenetics and the classification of honey bee viruses. *Arch Virol* 145, 2015–2026.
- Fujiyuki, T., Takeuchi, H., Ono, M., Ohka, S., Sasaki, T., Nomoto, A. & Kubo, T. (2004). Novel insect picorna-like virus identified in the brains of aggressive worker honeybees. *J Virol* 78, 1093–1100.
- Ghosh, R. C., Ball, B. V., Willcocks, M. M. & Carter, M. J. (1999). The nucleotide sequence of sacbrood virus of the honey bee: an insect picorna-like virus. *J Gen Virol* 80, 1541–1549.
- Govan, V. A., Leat, N., Allsopp, M. & Davison, S. (2000). Analysis of the complete genome sequence of acute bee paralysis virus shows that it belongs to the novel group of insect-infecting RNA viruses. *Virology* 277, 457–463.
- Hansen, J. L., Long, A. M. & Schultz, S. C. (1997). Structure of the RNA-dependent RNA polymerase of poliovirus. *Structure* 5, 1109–1122.
- Hunnicut, L. E., Hunter, W. B., Cave, R. D., Powell, C. A. & Mozoruk, J. J. (2006). Genome sequence and molecular characterization of *Homalodisca coagulata* virus-1, a novel virus discovered in the glassy-winged sharpshooter (Hemiptera; Cicadellidae). *Virology* 350, 67–78.
- Johnson, K. N. & Christian, P. D. (1998). The novel genome organization of the insect picorna-like virus *Drosophila* C virus suggests this virus belongs to a previously undescribed virus family. *J Gen Virol* 79, 191–203.
- Koonin, E. V. & Dolja, V. V. (1993). Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Crit Rev Biochem Mol Biol* 28, 375–430.
- Lanzi, G., de Miranda, J. R., Boniotti, M. B., Cameron, C. E., Lavazza, A., Capucci, L., Camazine, S. M. & Rossi, C. (2006). Molecular and biological characterization of deformed wing virus of honeybees (*Apis mellifera* L.). *J Virol* 80, 4998–5009.
- Leat, N., Ball, B., Govan, V. & Davison, S. (2000). Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees. *J Gen Virol* 81, 2111–2119.
- Love, R. A., Maegley, K. A., Yu, X., Ferre, R. A., Lingardo, L. K., Diehl, W., Parge, H. E., Dragovich, P. S. & Fuhrman, S. A. (2004). The crystal structure of the RNA-dependent RNA polymerase from human rhinovirus: a dual function target for common cold antiviral therapy. *Structure* 12, 1533–1544.
- Mansi, W. (1958). Slide gel diffusion precipitation test. *Nature* 181, 1289.
- Maori, E., Tanne, E. & Sela, I. (2007). Reciprocal sequence exchange between non-retroviruses and hosts leading to the appearance of new host phenotypes. *Virology* 362, 342–349.
- Mayo, M. A. (2002). A summary of taxonomic changes recently approved by ICTV. *Arch Virol* 147, 1655–1656.
- Moon, J. S., Domier, L. L., McCoppin, N. K., D'Arcy, C. J. & Jin, H. (1998). Nucleotide sequence analysis shows that *Rhopalosiphum padi* virus is a member of a novel group of insect-infecting RNA viruses. *Virology* 243, 54–65.
- Nagy, P. D. & Simon, A. E. (1997). New insights into the mechanisms of RNA recombination. *Virology* 235, 1–9.
- Nakashima, N., Sasaki, J. & Toriyama, S. (1999). Determining the nucleotide sequence and capsid-coding region of Himetobi P virus: a member of a novel group of RNA viruses that infect insects. *Arch Virol* 144, 2051–2058.
- Nishiyama, T., Yamamoto, H., Shibuya, N., Hatakeyama, Y., Hachimori, A., Uchiyumi, T. & Nakashima, N. (2003). Structural elements in the internal ribosome entry site of *Plautia stali* intestine virus responsible for binding with ribosomes. *Nucleic Acids Res* 31, 2434–2442.
- Ongus, J. R., Peters, D., Bonmatin, J.-M., Bengsch, E., Viak, J. M. & van Oers, M. M. (2004). Complete sequence of a picorna-like virus of

- the genus *Flavivirus* replicating in the mite *Varroa destructor*. *J Gen Virol* **85**, 3747–3755.
- Sambrook, J. & Russell, D. W. (2001).** *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sasaki, J., Nakashima, N., Saito, H. & Noda, H. (1998).** An insect picorna-like virus, *Plautia stali* intestine virus, has genes of capsid proteins in the 3' part of the genome. *Virology* **244**, 50–58.
- Tanne, E. & Sela, I. (2005).** Occurrence of a DNA sequence of anon-retro RNA virus in the host plant genome and its expression: evidence for recombination between viral and host RNAs. *Virology* **332**, 614–622.
- Ulrich, H. D. (2005).** Mutual interactions between the SUMO and ubiquitin systems: a plea of no contest. *Trends Cell Biol* **15**, 525–532.
- Valles, S. M., Strong, C. A., Dang, P. M., Hunter, W. B., Pereira, R. M., Oi, D. H., Shapiro, A. M. & Williams, D. F. (2004).** A picorna-like virus from the red imported fire ant, *Solenopsis invicta*: initial discovery, genome sequence, and characterization. *Virology* **328**, 151–157.
- van Munster, M., Dulleman, A. M., Verbeek, M., van den Heuvel, J. F. J. M., Clerivet, A. & van der Wilk, F. (2002).** Sequence analysis and genomic organization of aphid lethal paralysis virus: a new member of the family *Dicistroviridae*. *J Gen Virol* **83**, 3131–3138.
- Walker, J. E., Saraste, M., Runswick, M. J. & Gay, N. J. (1982).** Distantly related sequences in the α -subunits and β -subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* **1**, 945–951.
- Wilson, J. E., Powel, M. J., Hoover, S. E. & Sarnow, P. (2000).** Naturally occurring dicistronic cricket paralysis virus RNA is regulated by two internal ribosome entry sites. *Mol Cell Biol* **20**, 4990–4999.