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The Acute bee paralysis virus–Kashmir bee virus–Israeli acute paralysis virus complex

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ABSTRACT

Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV) are part of a complex of closely related viruses from the Family Dicistroviridae. These viruses have a widespread prevalence in honey bee (*Apis mellifera*) colonies and a predominantly sub-clinical etiology that contrasts sharply with the extremely virulent pathology encountered at elevated titres, either artificially induced or encountered naturally. These viruses are frequently implicated in honey bee colony losses, especially when the colonies are infested with the parasitic mite *Varroa destructor*. Here we review the historical and recent literature of this virus complex, covering history and origins; the geographic, host and tissue distribution; pathology and transmission; genetics and variation; diagnostics, and discuss these within the context of the molecular and biological similarities and differences between the viruses. We also briefly discuss three recent developments relating specifically to IAPV, concerning its association with Colony Collapse Disorder, treatment of IAPV infection with siRNA and possible honey bee resistance to IAPV.

1. Introduction

Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV) are closely related viruses from the Family Dicistroviridae that can be best analysed as a complex of related species, using their similarities and differences to identify and link the important components of genetic and biological variation. Apart from their close genetic relationship they share a number of biological characteristics, such as the principal routes of transmission, the primary host life stage, and a low but widespread prevalence with a predominantly sub-clinical etiology that contrasts sharply with the extremely virulent pathology encountered at elevated titres, either artificially induced or encountered naturally. The viruses are naturally highly variable, complicating both reliable diagnosis and classification.

2. History and distribution

The history of the discovery, distribution, pathology and seasonality of ABPV and KBV has been reviewed in great detail recently (Ribière et al., 2008), while IAPV is a more recent addition to this group. These viruses were all discovered in a similar manner; as a consequence of virus propagation in white-eyed honey bee pupae. This technique involves injecting a small volume of purified or crude extract between the integuments of white-eyed pupae, and was initially developed for producing enough virus for raising antibodies. However, it soon became evident that occasionally unrelated viruses were amplified this way (Bailey et al., 1963). ABPV was thus discovered as an unintended by-product during transmission studies with chronic bee paralysis virus (CBPV) (Bailey et al., 1963; Ribière et al., 2010). KBV was similarly discovered in 1974 as a contaminant in preparations of Apis iridescent virus from the Asian hive bee (*Apis cerana*) that multiplied to high titres when injected or fed to adult *Apis mellifera* (Bailey et al., 1976, 1979). IAPV was initially purified in 2002 after propagating the extract of a single bee from a cluster of dead bees found in front of failing hives near Alon Hagalil in Israel (Maori et al., 2007a,b).

The original host of ABPV is probably *A. mellifera*, although it has also been detected by infectivity tests in five bumble bee species, but not in several non-hymenopteran insects (Bailey and Gibbs, 1964; Allen and Ball, 1996; Ribière et al., 2008). The host origin of KBV is more obscure. It has been detected in *A. cerana* from Kashmir (Bailey and Woods, 1977), India (Bailey et al., 1979) and Papua New Guinea (Allen and Ball, 1996) as well as in *A. mellifera*

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populations from around the world (Ball and Bailey, 1997; Allen and Ball, 1995, 1996), in bumble bees from New Zealand and European wasps (*Vespula germanica*) from Australia (Anderson, 1991). IAPV was characterised only recently (Maori et al., 2007a,b) and its phylogenetic position in relation to ABPV and KBV suggests that it may well have been classified previously as a variant of KBV. So far, the only known host of IAPV is *A. mellifera* (Maori et al., 2007a,b; Palacios et al., 2008; Chen and Evans, 2007).

ABPV, KBV and IAPV have a worldwide distribution (Allen and Ball, 1996; Ellis and Munn, 2005). Fig. 1 indicates only where these viruses have been detected. Countries which have not completed surveys (in grey, *e.g.* large parts of Africa, Central Asia and South America) should therefore not be presumed virus free. The prevalence, regional distribution and seasonal incidence of these three viruses across apiaries are variable according to the virus and the area of study. Generally speaking, ABPV appears to be the most common of these viruses in Europe (Ball and Allen, 1988; Kulinčević et al., 1990; Varis et al., 1992; Topolska et al., 1995; Békési et al., 1999; Nordström et al., 1999; Bakonyi et al., 2002a,b; Siede and Büchler, 2006; Berényi et al., 2006; Tentcheva et al., 2004; Siede et al., 2005; Gauthier et al., 2007; Ward et al., 2007; Baker and Schroeder, 2008; Blanchard et al., 2008b; Nielsen et al., 2008) and South America (Antunez et al., 2005, 2006; Weinstein-Teixeira et al., 2008), KBV in North America (Bruce et al., 1995; Hung et al., 1996b,c, 2000; Cox-Foster et al., 2007) and New Zealand (Todd et al., 2007) and IAPV in the Middle East and Australia (Maori et al., 2007a; Palacios et al., 2008). Seasonally, ABPV and KBV tend to increase in prevalence and titre as the season progresses, with ABPV peaking a little earlier (late summer) than KBV (autumn; Bailey et al., 1981; Ball and Allen, 1988; Bailey and Ball, 1991; Tentcheva et al., 2004; Siede and Büchler, 2006; Gauthier et al., 2007). This seasonal fluctuation in ABPV/KBV prevalence and titre is similar to that of their closest genetic relative, Solenopsis invicta virus (SInV-1; Valles et al., 2004, 2007), also a virus of a social hymenopteran (the fire ant), where viral titre is thought to be related to the growth rate of the colony (Valles et al., 2007).

3. Pathology and transmission

The pathology of ABPV, KBV and IAPV is quite similar at the level of the individual bee and the colony (Ribière et al., 2008). Like most dicistroviruses (Christian and Scotti, 1998; Valles et al., 2007), they normally persist at low titres as rather common, presumably covert infections within the colony, with no obvious symptoms at the individual or colony level. However, they are extremely virulent when injected into pupae or adults (Bailey et al., 1963; Dall, 1985, 1987; Bailey and Ball, 1991; Ribière et al., 2008), with less than 100 particles required to cause death within a few days, and the same effect can also be achieved by feeding around 10^{11} virus particles per bee (Bailey et al., 1963; Bailey and Woods, 1977; Bailey and Ball, 1991; Nordström, 2000; Maori et al., 2007a; Ribière et al., 2008). For ABPV and IAPV, but not KBV (Ribière et al., 2008; Maori et al., 2007a), the death of lethally infected adults is preceded by a rapidly progressing paralysis, including trembling, inability to fly and the gradual darkening and loss of hair from the thorax and abdomen (Bailey et al., 1963; Ribière et al., 2008; Maori et al., 2007a). Paralysis is also a symptom of other dicistroviruses, such as cricket paralysis virus (CrPV; Scotti et al., 1981; Christian and Scotti, 1998) and aphid lethal paralysis virus (ALPV; van Munster et al., 2002). One paradox is that this paralysis at the individual level only rarely translates to evidence of mass paralysis at colony level (e.g. Maori et al., 2007a), even in cases where the virus is known to have caused the death of the colony (Bailey et al., 1979; Békési et al., 1999; Ribière et al., 2008). One possible explanation is that rapid progression from paralysis to death prevents the accumulation of sufficient live paralytic adults for such effects to be noticed at the colony level. This may be why colony-level symptoms of mass paralysis are usually associated with chronic bee paralysis virus (CBPV; Bailey et al., 1963; Ribière et al., 2008; Ribière et al., 2010), which develops more slowly and retains the paralytic behaviour for longer. One observation associated with severe ABPV and KBV infection is a sharp decline in the adult bee population (Ball and Allen, 1988; Hung et al., 1995, 1996c; Ribière et al., 2008; Todd et al., 2004),

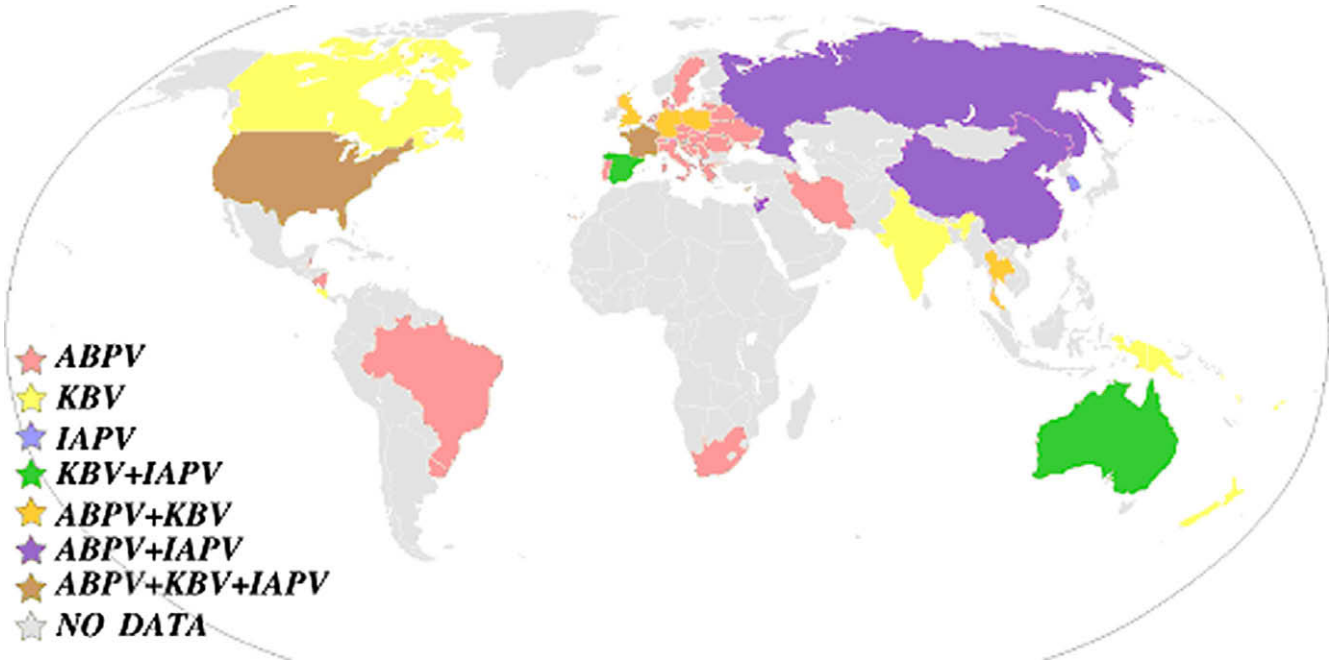

Fig. 1. World distribution of ABPV, KBV and IAPV by country. Data were compiled from Allen and Ball (1996), Ellis and Munn (2005), recent publications and from published and unpublished GenBank database entries, identified by geography in the database and analysed for genetic affiliation by phylogeny. The colour codes for the presence of single or multiple viruses are shown.

resulting in the appearance of diseased larvae and pupae due to the lack of adults to tend the brood.

ABPV accumulates in the brain and hypopharyngeal glands of the adult bee host (Bailey and Milne, 1969), and ABPV and KBV can also be readily detected in faeces (Ribière et al., 2008; Hung, 2000) implying several oral transmission routes involving adults, larvae, cannibalised brood, contaminated food and/or faeces (Chen et al., 2006a; Chen and Siede, 2007). This strong association of the virus with the alimentary canal is also a very common feature of the dicistro- and iflaviruses (Christian and Scotti, 1998; Valles et al., 2007). ABPV has also been detected in semen (Yue et al., 2006) and KBV has been detected in surface-sterilised eggs (Shen et al., 2005a; Chen et al., 2006b), although not in the ovaries of the corresponding queens (Chen et al., 2006b). The absence of KBV in the semen studies in Europe (Yue et al., 2006) and of ABPV in the queen studies in the USA (Chen et al., 2006b) may be more related to the natural geographic distribution of these viruses, than to their inability to infect these tissues. Although the viruses can also infect the larval and pupal stages (Hornitzky, 1987; Ball and Allen, 1988; Bailey and Ball, 1991; Brødsgaard et al., 2000; Shen et al., 2005a; Ribière et al., 2008) especially in lethally infected colonies, they appear to be naturally more prevalent in adult bees, with ABPV the most likely of the three to also be detected in the brood (Tentcheva et al., 2004; Gauthier et al., 2007; de Miranda, Tournaire, Paxton and Gauthier unpublished). Such observations of relative prevalence between the different life stages of bees should be interpreted cautiously, however, and within the context of the colony population dynamics, because diseased brood is normally rapidly removed or cannibalised by the adult population, thereby escaping detection. In failing colonies with a dwindling adult population such hygienic behaviour is compromised, making it possible to identify diseased larvae before the bees remove them.

Considering the extreme virulence of these viruses upon injection into the bee haemolymph, it is not surprising that all have been strongly associated with *Varroa destructor*, a recently acquired parasitic mite of *Apis mellifera* that feeds on the haemolymph of adults and pupae (Bailey and Ball, 1991; Ribière et al., 2008). ABPV has been heavily implicated in varroa induced colony losses, primarily in Europe in the 1980s–1990s (Ball, 1985; Ball, 1987; Allen et al., 1986; Ball and Allen, 1988; Bailey and Ball, 1991; Faucon et al., 1992; Békési et al., 1999; Berényi et al., 2006). Varroa can transfer ABPV among adults and pupae with 50–80% efficiency, depending on the sensitivity of the detection method used (Wiegers, 1988; Ball, 1989). This efficiency drops with successive transfers and there is no noticeable latent period between acquisition and transmission, which suggests that there is no virus replication in the mite (Wiegers, 1988). A similar relationship exists between varroa and KBV, including a similar transmission efficiency (Chen et al., 2004a; Shen et al., 2005b), detection of KBV in varroa saliva (Shen et al., 2005a), and implication in varroa-associated colony losses (Bailey et al., 1979; Hung et al., 1995; Hung et al., 1996c; Todd et al., 2007; Ribière et al., 2008). These interactions have been modelled, to predict the complex seasonal dynamics of bee and mite population growth, and colony winter survival, as affected by ABPV transmission by mites and bees (Martin, 2001; Sumpter and Martin, 2004).

Little is known specifically about the transmission routes of IAPV, although much historical evidence may be obscured by the likelihood that IAPV may have been classified as a strain of KBV during these earlier studies, especially those from Australia where IAPV appears to be particularly prevalent (e.g. Hornitzky, 1987; Dall, 1985, 1987; Anderson and Gibbs, 1988; Anderson and East, 2008). Nearly 80% of adult bees infected orally with IAPV die within a week, with little difference across a 1000-fold range of inocu-

Table 1
Summary of recorded large-scale colony lossess from 950 to 2006. After Underwood and vanEngelsdorp (2007).

Year	Location	Citation
950, 992, 1443	Ireland	Flemming (1871)
1868	Kentucky, Tennessee	Anonymous (1869)
1872	Australia	Beuhne (1910)
1906	Isle of Wight	Rennie et al. (1921) and Bullamore (1922)
1910	Australia	Beuhne (1910)
1915	Portland, Oregon	Root and Root (1923)
1915	Florida to California	Tew (2002)
1917	United States	Root and Root (1923)
1917	New Jersey, Canada	Carr (1918)
1945–1946	Baden, Germany	Gnädinger (1984)
1960's	Louisiana, Texas	Williams and Kauffeld (1974)
1960's	Louisiana, Texas	Kauffeld (1973)
1960's	Louisiana	Roberge (1978)
1962–1963	Baden, Germany	Gnädinger (1984)
1963–1964	Louisiana	Oertel (1965)
1964	California	Foote (1966)
1970's	Mexico	Mraz (1977)
1970's	Seattle, Washington	Thurber (1976)
1970's	USA survey	Wilson and Menapace (1979)
1972–1973	Baden, Germany	Gnädinger (1984)
1974	Texas	Kauffeld et al. (1976)
1975	Australia	Olley (1976)
1977	Mexico	Kulinčević et al. (1984)
1978	Florida	Kulinčević et al. (1982)
1984–1985	Baden, Germany	Gnädinger (1984)
1990	France	Faucon et al. (1992)
1990's	USA	Shimanuki et al. (1994)
1995–1996	Pennsylvania	Finley et al. (1996)
1999–2000	France	Faucon et al. (2002)
1998–2000	Hungary	Békési et al. (1999) and Bakonyi et al. (2002b)
2002	Alabama	Tew, 2002
2002–2003	Northern Europe	Svensson (2003)
2004–2006	Austria	Berényi et al. (2006)
2006–2009	USA	vanEngelsdorp et al. (2007, 2008) and vanEngelsdorp and Meixner (2010)

lum concentrations (Maori et al., 2009). This rapid adult mortality, described independently for ABPV, KBV and IAPV, as well as their relative absence in the larval stages, is interesting in the light of the identification of IAPV as a major risk indicator for Colony Collapse Disorder (CCD; Cox-Foster et al., 2007). This condition is characterised by the rapid depletion of adult bees from otherwise apparently healthy colonies, leaving just the queen with a small number of young workers and often large areas of unattended brood. Food stores are generally unaffected by robbing bees or honey bee comb pests such as wax moths or small hive beetles for several weeks after the collapse (Underwood and vanEngelsdorp, 2007; vanEngelsdorp et al., 2007, 2008).

Large-scale bee losses with apparently cryptic causes have been reported throughout history, starting with "The great mortality of bees" in Ireland in 950, 992 and 1443 (Flemming 1871). There is increasing documentation of extensive colony losses worldwide from the late 19th century onwards, many with symptoms similar to CCD (Underwood and vanEngelsdorp, 2007; vanEngelsdorp and Meixner, 2010; Table 1). Each documented decline sparked animated debates across the scientific community discussing the potential causes (Rennie et al., 1921; Bailey, 1964; Oertel, 1965; Foote, 1966; Kauffeld, 1973; Olley, 1976; Thurber, 1976; Wilson and Menapace, 1979; Shimanuki et al., 1994; Tew, 2002; Svensson, 2003; Anderson, 2004), generally without a clear-cut resolution. This response is also true for CCD, with parasites, viruses, pollen, nectar, pesticides and stress all implicated (Cox-Foster et al., 2007; Stokstad, 2007a,b; Oldroyd, 2007; Anderson and East, 2008; Cox-Foster et al., 2008). One highly diagnostic feature of CCD, and other historic "disappearing" disorders, is the absence of dead adult bees in or near the hive (vanEngelsdorp et al., 2007, 2008; Cox-Foster et al., 2007) or signs of diseased brood, which is highly atypical for a monocausal infectious disease and more symptomatic of acute poisoning or possibly a prolonged brood-free spell that upsets the age distribution of the bees. However, neither direct poisoning nor brood rearing problems were associated with CCD (vanEngelsdorp et al., 2007, 2008; Cox-Foster et al., 2007). More recent data suggested that KBV, rather than IAPV, was a significant marker of colonies displaying CCD symptoms (Pettis, 2008). In central Europe, prior to CCD, ABPV was believed to be a significant co-factor in the unexplained depopulation of hives, usually in spring (Berényi et al., 2006). Further work is, therefore, required to elucidate the precise role(s) ABPV, KBV or IAPV play in this syndrome (Anderson and East, 2008; Cox-Foster et al., 2007, 2008; vanEngelsdorp et al., 2008), including the interchangeability of these closely related viruses as risk indicators, and the possible existence of other environmental, biological or behavioural cues that could have precipitated the dramatic, widespread and simultaneous collapses seen with CCD. A detailed review of CCD and other honey bee declines can be found elsewhere in this issue (vanEngelsdorp and Meixner, 2010).

4. Genetics and variation

The basic genome organisation of ABPV, KBV and IAPV is typical for the Dicistroviridae: a single positive strand RNA containing two open reading frames (ORF), separated by an intergenic region (IGR) and flanked by non-translated regions (Fig. 2). The larger ORF is located in the 5′ half of the genome and encodes the non-structural proteins involved in virus replication and processing. The shorter ORF is located towards the 3′ end of the genome and encodes the structural capsid proteins found in the viral particle, and the genome is naturally poly-adenylated at the 3′ end (Govan et al., 2000; de Miranda et al., 2004; Maori et al., 2007a). The major functional domains associated with the helicase, 3C-protease (3C-pro) and RNA-dependent RNA polymerase (RdRp; Gorbalenya and Koonin, 1989; Koonin and Dolja, 1993) can be readily identified, as well as two capsid protein domains, and these have been described in detail for all three viruses (Govan et al., 2000; de Miranda et al., 2004; Maori et al., 2007a). The helicase domains include the putative NTP-binding residues (GxxGxGKS and DD; Gorbalenya and Koonin, 1989) in domains A and B, respectively. The 3C-protease contains both the putative substrate binding motif (GxHxxG) and the cysteine protease motif (GxCG), with the cysteine forming a catalytic triad with a histidine and either an aspartate or glutamate (Gorbalenya et al., 1989; Koonin and Dolja, 1993; Ryan and Flint, 1997), while the RNA-dependent RNA polymerase contains all eight recognised domains, including the universal polymerase mo-

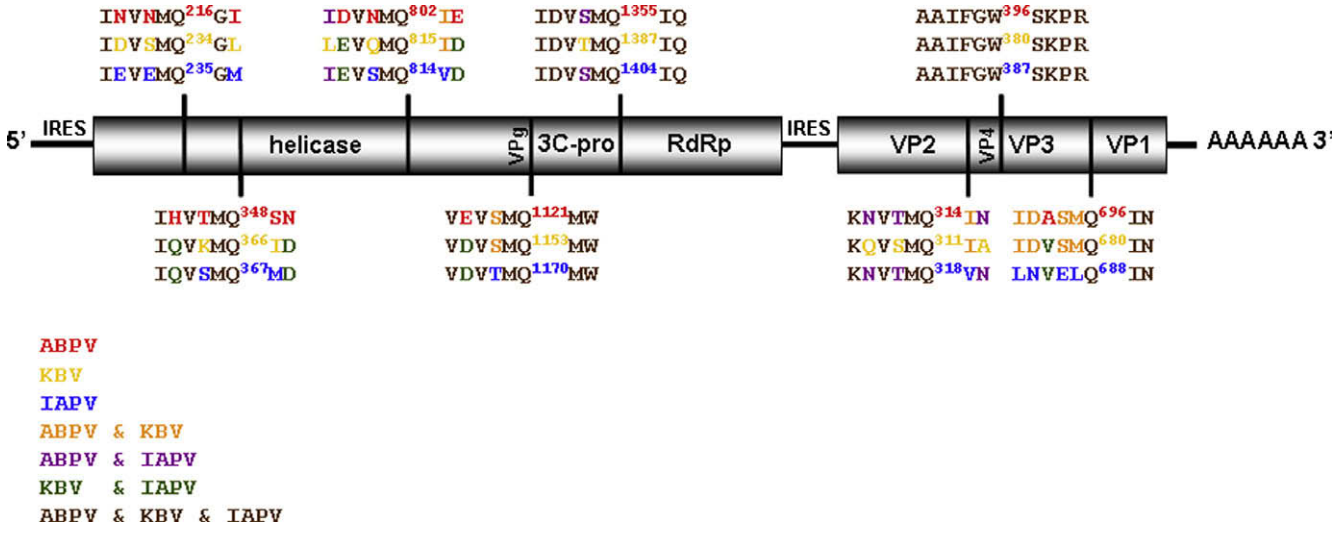

Fig. 2. Organisation of the ABPV–KBV–IAPV genomes. The identified functional domains are the helicase, 3C-protease (3C-pro) and the RNA-dependent RNA polymerase (RdRp) in the non-structural open reading frame, followed by an Internal Ribosome Entry Site (IRES) in the intergenic region, and the four capsid proteins (VP1–VP4) in the structural open reading frame. An IRES is also expected in the 5′ untranslated region (5′UTR). The order of the capsid proteins is according to the current convention for dicistroviruses (Roberts and Gropelli, 2009; Nakashima and Uchiumi, 2009). The genomic RNA is naturally poly-adenylated. Also shown are the experimentally determined and inferred proteolytic processing sites, with the position of the splicing site in the peptide sequence indicated for each virus. The consensus non-structural polyprotein translational starting positions were used for IAPV (Palacios et al., 2008) and ABPV (Bakonyi et al. 2002b). The colour codes for unique, partially or universally conserved amino acid residues in these sites are indicated.

tif (YGDD) in the pol-VI domain (Koonin and Dolja, 1993). Prior to the 3C-protease domain there are one or more signatures for the genome-linked viral protein (VPg) (Fig. 2; Nakashima and Shibuya, 2006), a small protein common to most positive strand RNA viruses that stabilises the 5′ end of the genome and is involved in genome replication, translation and movement (Hébrard et al., 2009). The two polyproteins are processed by protease digestion to produce the functional proteins, with the virus-encoded 3C-protease providing most of the protease activity (Fig. 2; Gromeier et al., 1999; van Munster et al., 2002). The general functionality of the genome, as well as the number and sizes of the capsid proteins and the resultant particle shape and size, is similar to that of other picorna-like viruses (Gromeier et al., 1999).

One particular feature of the Dicistroviridae is the presence of an IGR harboring a highly conserved Internal Ribosome Entry Site (IRES; Fig. 3). IRESs have also been identified in the 5′ untranslated region (5′UTR) of picornaviruses (Fernández-Miragall et al., 2009; Belsham, 2009) as well as in other dicistroviruses (Jan, 2006). They are thought to be a means for the virus to avoid, and possibly disrupt, the host's CAP-dependent mRNA translation mechanism (Carter and Generscch, 2008; Belsham, 2009), since IRES-mediated translation requires far fewer host factors than CAP-dependent translation (Pestova et al., 2004; Pestova and Hellen, 2006). The presence of an IRES in the dicistrovirus IGR re-emphasises this strategy, and also provides a means for the virus to differentially regulate the translation of its two ORFs (Wilson et al., 2000; Carter and Generscch, 2008).

The relevance of this with respect to analysing the differences and similarities within the ABPV–KBV–IAPV complex is whether each virus is able to replicate, translate and process exclusively its own genome and proteins, and thus retain its unique identity. Although ABPV, KBV and IAPV are closely related, they are not identical. Despite considerable cross-reaction, they can be distinguished by serology (Allen and Ball, 1995; Stoltz et al., 1995; Maori et al., 2007a), capsid protein profiles (Bailey et al., 1979; Allen and Ball, 1995; Todd et al., 2007) and by RT-PCR (Stoltz et al., 1995; Evans, 2001; de Miranda, 2008; see later). The reason for these unique patterns in otherwise overlapping diagnoses appears to be the considerable scope within the genomes of these viruses for generating variation at both nucleotide and amino acid level (Allen and Ball, 1995; Bakonyi et al., 2002a; de Miranda et al., 2004; Palacios et al., 2008). Moreover, this innate variability appears to be greater for KBV and IAPV than it is for ABPV (Bailey et al., 1979; Allen and Ball, 1995; Bakonyi et al., 2002b; de Miranda et al., 2004; Palacios

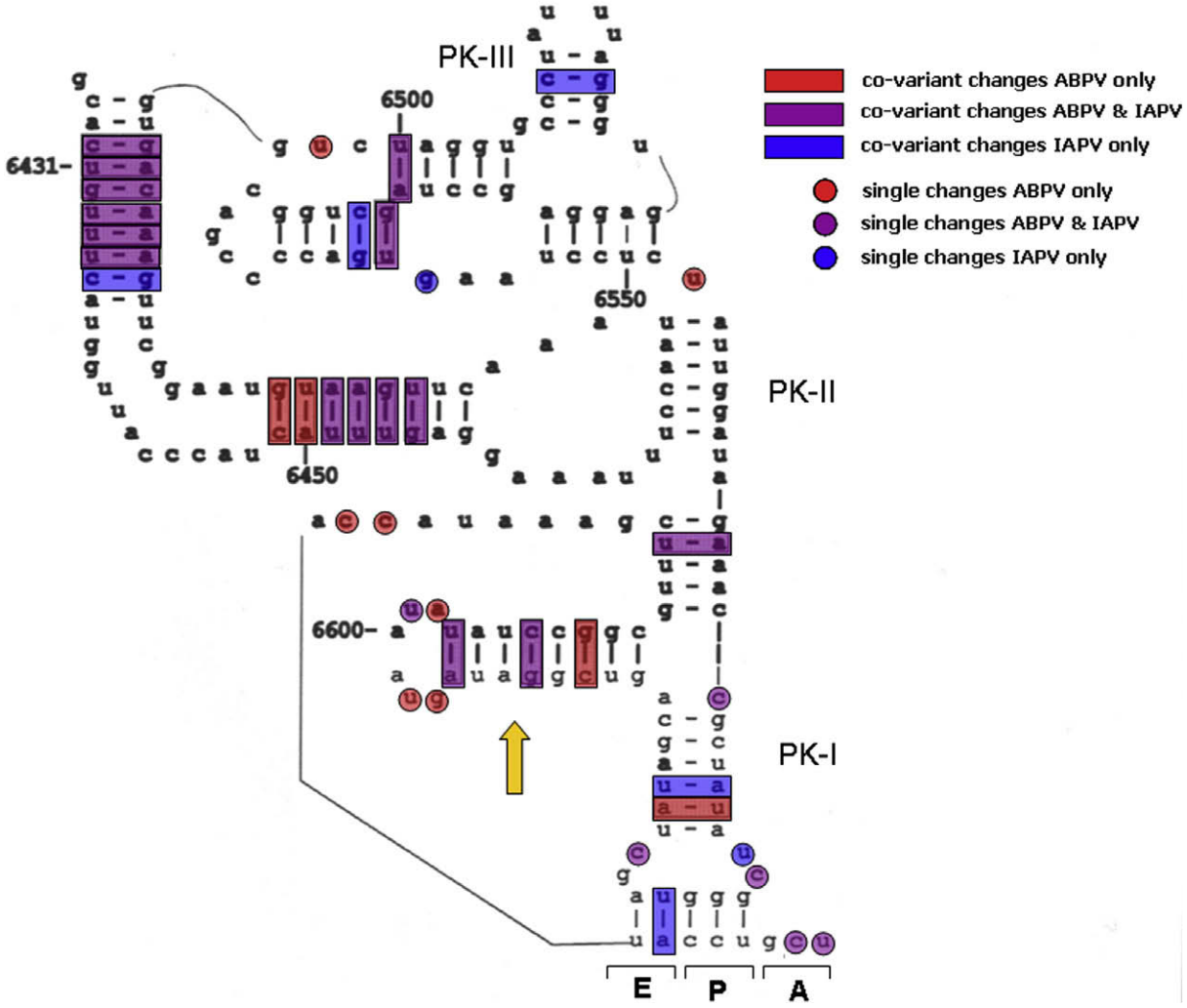

Fig. 3. Schematic representation of the secondary-tertiary structural conformation of the KBV intergenic region Internal Ribosome Entry Site (IRES). The colour scheme highlights the nucleotide positions where there are co-variant or single changes in the corresponding ABPV and/or IAPV IRES. PK-I, PK-II and PK-III refer to the three pseudoknots. A, P and E mark the sites where the IRES interacts with the corresponding ribosomal entry point for new Aminoacyl-tRNA (A), the site where the Peptidyl-tRNA (P) attaches to the growing peptide chain and the Exit site (E) for the newly deacylated tRNA. The yellow arrow highlights a variable stem-loop structure in the dicistrovirus intergenic IRES. Original KBV fold courtesy of Christopher U.T. Hellen, State University of New York, Brooklyn, New York, USA.

et al., 2008), and it appears to be most pronounced at the 5′ end of the genome (de Miranda et al., 2004; Palacios et al., 2008).

The effect of this variability at the functional level is illustrated by Figs. 2 and 3. Fig. 2 compares the putative 3C-protease sites of ABPV, KBV and IAPV. The templates for identifying the predicted sites are the experimentally determined sites, obtained from the N-terminal sequences of the VP2 and VP3 structural proteins of ABPV (Govan et al., 2000), KBV (de Miranda, unpublished) and IAPV (Maori et al., 2007a), as well as of several non-structural proteins of related dicistroviruses (Nakashima and Nakamura, 2008). There are two items of interest here; the relative positions of the protease sites in the three viruses and the degree of amino acid similarity, both at each site and between sites. For the non-structural ORF, the position of the sites is taken relative to the first consensus in-frame methionine residue (Govan et al., 2000; de Miranda et al., 2004; Palacios et al., 2008). For the structural ORF, the positions of the protease sites were taken relative to the first amino acid predicted by the IRES analysis (Fig. 3; Palacios et al., 2008) rather than the first AUG, as originally published (Govan et al., 2000; de Miranda et al., 2004; Maori et al., 2007a). Fig. 2 shows that the relative positions of the protease sites change dramatically over the course of each ORF. For the non-structural ORF there is a difference of 19 amino acids between ABPV and IAPV in the location of the first two protease sites, decreasing to 12 amino acids for the C-terminal helicase site and then increasing to 49 amino acids for the 3C-pro and RdRp sites. Similarly, while the KBV and IAPV sites are practically in the same positions for the first three sites, they differ by 17 amino acids for the last two sites. For the structural ORF the most dramatic change is the 13 amino acid increase in the relative position of the ABPV sites between the VP2–VP4 and the VP4–VP3 junctions. The corresponding increase in the size of the VP4 protein is readily noted on SDS protein gels (Stoltz et al., 1995). These changes in the relative positions of the protease sites are indicative of insertions and deletions in the amino acid sequence, and the extent to which this occurs between otherwise closely related viruses reflects the ease with which such major, protein-level variational changes are generated. The second analysis concerns the pattern of conservation between the protease sites, both between viruses and between sites. The brown coloured amino acids are conserved between the three viruses for each site. The most impressive degree of conservation is for the VP4–VP3 cleavage site (AAIFGW/SKPR). This site is also highly conserved between different dicistroviruses (van Munster et al., 2002) and is most likely processed autocatalytically (*i.e.* without a protease) from a VP4–VP3 precursor, inside the mature virion (Liljas et al., 2002). The remaining proteolytic sites conform to the basic requirements for viral 3C-proteases (Palmenberg, 1990; Gromeier et al., 1999). Between all sites the general 3C-protease recognition sequence is IxVxMQ, with the proteolysis occurring right after the glutamine (Q) residue. There are deviations from this sequence at individual sites, consensual changes even (for example, VxVxMQ at VPg–3C-pro, or KxVxMQ at VP2–VP4), reflecting perhaps the relative importance of these conserved variants for functionality, or a means for the virus to regulate polyprotein processing through the variable efficiency of the 3C-protease at different sites. The remaining positions in the protease sites, both upstream and downstream of the splicing site, have varying degrees of conservation within each site, but little between the sites, which may also reflect the need for differential proteolytic efficiency at the different sites. The final observation concerns the relationship between the three viruses, as revealed by these protease sites. For the first four sites of the non-structural ORF, the KBV and IAPV sites tend to be more similar (green amino acids), reflecting the overall phylogenetic relationship between ABPV, KBV and IAPV (Fig. 4, Table 2). However, the 3C-pro–RdRp and VP2–VP4 sites are more closely related between ABPV and IAPV (purple) while the VP3–VP1 site is much more closely related between ABPV and KBV (orange). There are three possible explanations for these observations. The differences are accidental, reflecting their (lack of) importance to the protease-site interaction. Alternatively, the changing affiliations reflect relationship by descent, with recombination between the viruses explaining the current composition of the viruses. In this scenario, recombination between ABPV and IAPV explains the similarity of their 3C-pro–RdRp and VP2–VP4 sites, and between ABPV and KBV the similarity of their VP3–VP1 sites. This theory has support from the considerable degree of intra-specific recombination found for many RNA viruses (Nagy and Simon, 1997; Lukashev et al., 2003), including the ABPV–KBV–IAPV complex (Maori et al., 2007a,b; Palacios et al., 2008). Finally, the changing affiliations of the protease sites can reflect a functional relationship, in which case they are due to evolutionary convergence, *i.e.* where different lineages converge towards a common amino acid pattern, driven by functional selection pressures. In this hypothesis, the ABPV protease would be able to process the KBV VP3–VP1 site, but not the VP2–VP4 site, and *vice versa* for the corresponding IAPV sites. The differential ability of the three viruses to cross-process each others' polyproteins would provide each with a measure of competition (disrupting each others' proteolytic processing patterns) and hence a unique identity within an otherwise highly related genetic environment (Ryan and Flint, 1997).

Most likely all these mechanisms are or have been important in shaping the genomes within this species complex. However, recombination and convergence have the additional effect of severely disrupting reconstruction of the phylogenetic relationships between the viruses, *i.e.* the history of their descent and origins. The effect of this is noted primarily in the instability of the branching patterns, indicated by weak statistical (bootstrap) support (Fig. 4). If recombination or convergence is rare, or limited to close relatives, their effect on phylogenetic reconstruction will be buffered by more conventional patterns of evolution elsewhere in the genome. However, frequent recombination and convergence may provide the main explanation for the often difficult and unstable phylogenetic reconstructions between these viruses (Evans, 2001; Bakonyi et al., 2002a; de Miranda et al., 2004; Cox-Foster et al., 2007; Todd et al., 2007; Palacios et al., 2008; Blanchard et al., 2008b). The phylogram in Fig. 4 concerns a 349 nucleotide section of the RdRp gene, one of the more conserved sections of the genome and a fragment for which still the largest number of taxa are available across all three viruses, mostly in the KBV–IAPV groups. The well-supported separation between ABPV, KBV and IAPV is also seen when other sections of the genome are analysed (de Miranda et al., 2004; Cox-Foster et al., 2007; Palacios et al., 2008; Blanchard et al., 2008b), including the peculiar 'floating' isolate from Hungary, positioned between ABPV and KBV–IAPV (de Miranda et al., 2004; Palacios et al., 2008). Branch support weakens noticeably within each virus. Branch support does not improve significantly when including more characters (larger genome sections) or analysing amino acid sequences (neutralising the effect of codon redundancy). This may, therefore, reflect the influence of recombination and convergence on the generation and selection of variation. This pattern of variable branch support across the phylogeny is also seen for other bee viruses (Grabensteiner et al., 2001; Forsgren et al., 2009; Blanchard et al., 2008a; de Miranda and Genersch, 2010). Even so, internally conserved sequence groups can still be identified at the terminal branches, particularly for IAPV, indicating that these isolates are relatively stable genetic entities. There are 2 to 4 such genetic groups for IAPV, depending on the isolates used, the genomic region analysed and the phylogenetic criteria employed (Cox-Foster et al., 2007; Chen and Evans, 2007; Blanchard et al., 2008b; Palacios et al., 2008). These generally include a largely EurAsian group typified by the Israeli isolates and North American and Australian groups that each may include

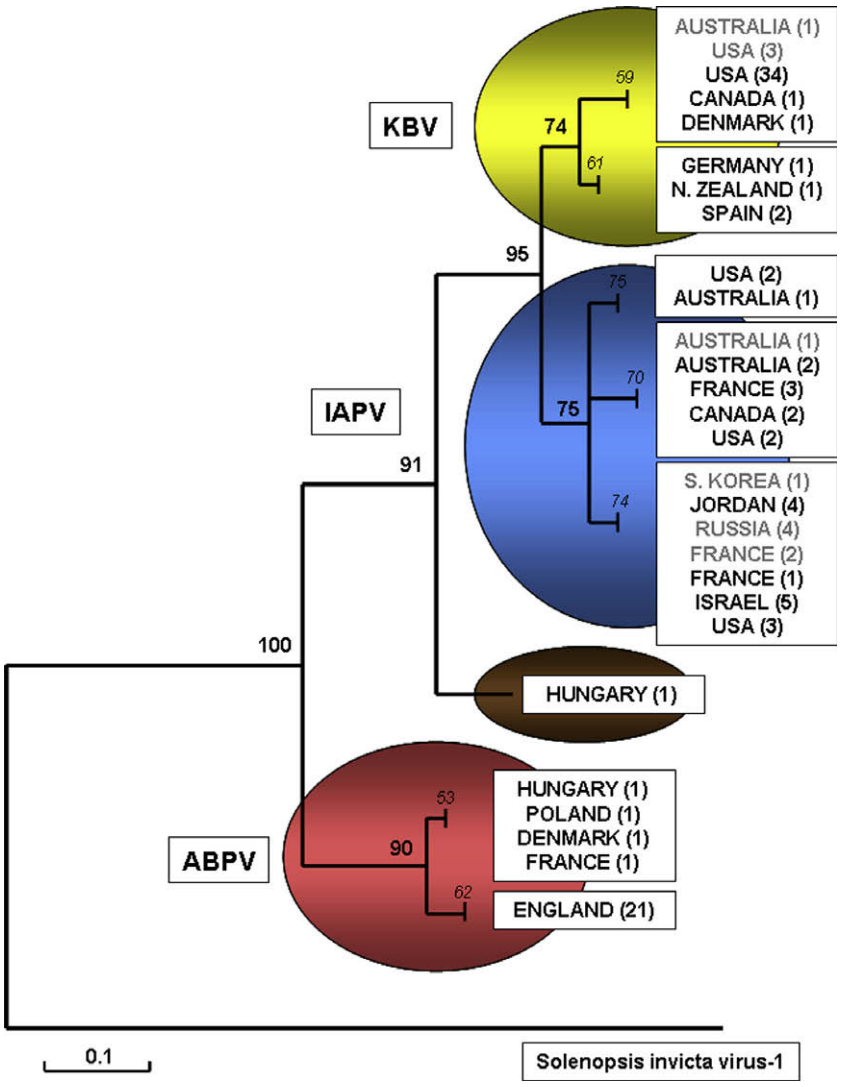

Fig. 4. Phylogram depicting the relationships between KBV, IAPV and ABPV isolates, as inferred from a 349 nt section within the polymerase region, corresponding to nt 5454–5802 of the KBV genome (de Miranda et al., 2004), using Solenopsis invicta virus-1 (SnIV-1) as outgroup. The isolates were classified according to their geographic origin, and their details are given in Table 1. The original phylogram was constructed by MEGA-4 (Tamura et al., 2007), using Minimum Evolution criteria. The statistical strength of the nodes is shown as the percentage of correct partitions in a 1000 replicate bootstrap analysis, and is shown in **bold** for each internal branch, and in *italic* for the terminal node leading to each taxonomic genogroup. Branches with less than 60% bootstrap support were collapsed. The IAPV and KBV isolates currently misclassified in the public DNA databases are shown in grey type. The isolates in black type are correctly classified. After de Miranda, Tournaire, Paxton and Gauthier (unpublished).

representatives from Australia and Europe/North America, respectively. Similarly vague and uncertain geographic assignations can be observed for the ABPV and KBV internal sequence groups (de Miranda et al., 2004). The most obvious explanation for this limited and variable geographic identity of otherwise distinct sequence groups is that the isolates have been spread around the continents by the long distance transport of live bees, such as the queen and bee-package trade and migratory beekeeping (de Miranda et al., 2004; Cox-Foster et al., 2007). From other phylogenies using different genome regions, a large number of American (Cox-Foster et al., 2007; Chen and Evans, 2007; Palacios et al., 2008), French (Blanchard et al., 2008b), and Chinese IAPV isolates can be added to top, middle and bottom IAPV groups in Fig. 4, as can a number of Central European and North American ABPV isolates (Bakonyi et al., 2002b; de Miranda et al., 2004) to the top and bottom ABPV sequence groups, respectively. Fig. 5 shows how the ABPV–KBV–IAPV complex relates to other dicistroviruses and to the iflaviruses, a closely related group of insect viruses several of which also infect honey bees (Carter and Genersch, 2008; Chen and Siede, 2007; de Miranda and Genersch, 2010).

Further evidence of the high variability of the ABPV–KBV–IAPV virus complex can be found when analysing the nucleotide substitution pattern of the open reading frames. Usually the vast majority of nucleotide substitutions between related viruses occur in the third codon position, followed by the second and then the first codon position, reflecting the pattern of redundancy in amino acid coding. However, within the RdRp segment analysed in Fig. 4, there are nearly 2.5 times as many variations in the first codon position as there are in the second codon position, both when comparing between viruses and within each virus, which greatly increases the chance that amino acid changes are associated with the nucleotide variation. This concurs with previous analyses of the internal variability across the entire genome of a single KBV isolate, where nearly 50% of all nucleotide substitutions resulted in amino acid changes (de Miranda et al., 2004).

A second component of the genome where genetic variation can be analysed within a functional context are the structural conformations of the genomic RNA in the non-coding regions at the 5′ and 3′ ends and in the IGR, which have important functions in regulating the replication and translation of the genome (Gromeier

Table 2
Virus isolates used in the phylogenetic analysis of Fig. 4. Shown are the correct virus assignation, the geographic origin of the isolate (including regional information where available), the year of isolation (inferred for some unpublished isolates), the GenBank accession number of the sequence and the original reference. After de Miranda, Tournaire, Paxton and Gauthier (unpublished).

Virus	Country	Year	Accession	Reference
	USA (CA)	1994	AF085478–AF085479	Hung et al., 2000
	USA (CA)	1998	AF052566; AF052567; AF093457–AF093460	Hung et al., 2000
	USA (CA)	1998;1999	AF083239; AF117953	Hung et al., 2000
	USA (CA)	1999	AF135852–AF135861	Hung et al., 2000
	USA (ME)	1997	AF035359	Hung et al., 2000
	USA (PA)	2002	AY275710	de Miranda et al., 2004
	USA (MD)	2000	AF177935	Evans and Hung, 2000
KBV	USA (MD)	2000	AF233366–AF233367	Evans, 2001
	USA (MD)	1997	AF037591; AF027125	Hung and Shimanuki, 1999
	USA	1995	AF232007	Hung (1999b) (unpublished)
	USA	1999	AF200331–AF200336	Hung (1999b) (unpublished)
	USA (MD)	1999	AF034543	Hung (1999) (unpublished)
	USA (TX)	2007	EU436460–EU436461	Palacios et al. (2008)
	Australia	2007	EU436457	Palacios et al. (2008)
	Canada (Ontario)	2001	AF034542	Hung et al. (2000)
	Denmark	2007	EF570891	Nielsen et al. (2008)
	Germany (Hesse)	2004	AY787143	Siede and Büchler (2004)
	New Zealand	2004	–	Siede and Büchler (2004)
	Spain	2004	AY821562–AY821563	Esperon et al. (2004) (unpublished)
	USA (PA; TX)	2007	EU436462; EU436464	Palacios et al. (2008)
	Australia	2007	EU436468	Palacios et al. (2008)
	Australia (NSW)		AF034541	Hung et al. (2000)
	Australia	2007	EU436456; EU436469	Palacios et al. (2008)
	France	2002		de Miranda et al. (2009) (unpublished)
	Canada	2007	EU436458–EU436459	Palacios et al. (2008)
IAPV	USA (PA)	2007	EU436423; EU436463	Palacios et al. (2008)
	Israel	2000	EF219380	Maori et al. (2007)
	Israel	2007	EU436455; EU436470–EU436472	Palacios et al. (2008)
	Jordan	2007	FJ225116–FJ225119	Al-Abbadi et al. (2008) (unpublished)
	South Korea	2008	EU770972	Ju et al. (2008) (unpublished)
	Russia	1999	AF197905–AF197908	Hung (1999a) (unpublished)
	France	2002	AY669845-AY669846;	Tentcheva et al. (2004)
	France	2002		de Miranda et al. (2009) (unpublished)
	USA (TX; CA–PA)	2007	EU436465–EU436467	Palacios et al. (2008)
?	Hungary	1996–2000	AF468967	Bakonyi et al. (2002a)
	Hungary	1996–2000	AF486072	Bakonyi et al. (2002a)
	Poland	1996–2000	AF486073	Bakonyi et al. (2002a)
	Denmark	2007	EF570888	Nielsen et al. (2008)
ABPV	France	2002	AY669853	Tentcheva et al. (2004)
	England		AF150629	Govan et al. (2000)
	England (Devon)	2007	DQ434968–DQ434990	Baker and Schroeder (2008)
SnIV-1	USA (FL)	2004	AY634314	Valles et al. (2004)

et al., 1999; Wilson et al., 2000; Belsham, 2009; Nakashima and Uchiumi, 2009; Roberts and Groppelli, 2009). The 3′ UTR and intergenic region nucleotide sequences are highly conserved between the three viruses, with ~80% nucleotide identity (de Miranda et al., 2004; Palacios et al., 2008), which presumably also extends to their predicted secondary and tertiary RNA structures. By contrast, the 5′ UTR is highly divergent between the viruses, with numerous gaps and low (~40%) nucleotide identity in those regions that can be aligned (de Miranda et al., 2004; Chen and Evans, 2007; Palacios et al., 2008). However, this low nucleotide identity is irrelevant if the secondary and tertiary RNA conformations, and hence the functionality of these structures, remain constant (Nakashima and Uchiumi, 2009; Kieft, 2009). How this can be achieved is illustrated in Fig. 3, with respect to the IGR IRES of KBV. The vast majority of nucleotide changes with respect to ABPV (red), IAPV (blue) or both (purple) are co-variant changes in the stem structures, *i.e.* ones that do not affect the IRES structure. Moreover, the changes generally avoid the most critical components of the IRES, the pseudoknots PK-I, PK-II and PK-III. These pseudoknots converge to mimic the presence of deacylated tRNA in the E-site and Met-tRNA^{Met} in the P-site, leaving the A-site unoccupied and ready to accept the first AA-tRNA^{AA} of the ORF (Fig. 3; Pisarev et al., 2005; Pestova and Hellen, 2006; Kieft, 2009). This is an Alanine for KBV and ABPV, and a Glycine for IAPV (Palacios et al., 2008). The three pseudoknots structures are universally conserved among all dicistrovirus intergenic IRESs, despite nucleotide variations (Jan, 2006; Kieft, 2009). However, the presence, size and shape of the stem-loop structure near nucleotide 6600 (highlighted by the yellow arrow) varies between different dicistroviruses. It is present in ABPV–KBV–IAPV, Solenopsis invicta virus-1 and Taura syndrome virus, but absent in black queen cell virus, cricket paralysis virus and Plautia stali virus, among others (Jan, 2006; Nakashima and Uchiumi, 2009). Of interest in Fig. 3 is how the sequence differences between the viruses translate into potential functional differences. Most of the co-variational changes in the stem structures are in positions where there are variants in both ABPV and IAPV, relative to the KBV sequence shown. Often the type of change is different for ABPV and IAPV, and there is also internal variability within ABPV and IAPV in these positions (Bakonyi et al. 2002b; Palacios et al. 2008). None of these materially affect the structure, although the strength of the bonds may be altered. The only sequence changes where a corresponding alteration in function could be contemplated are those in the loop of the unique stem-loop structure, which could cause several of the loop nucleotides to be-

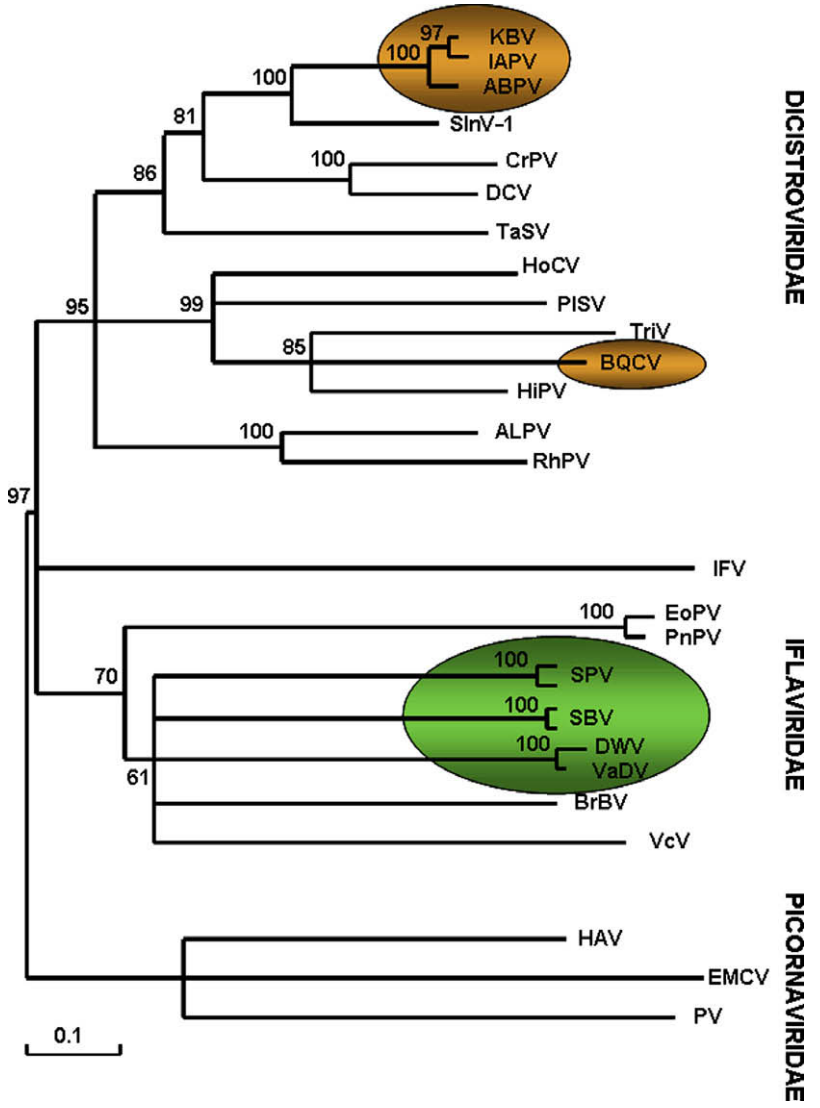

Fig. 5. Phylogram relating ABPV, KBV and IAPV to other dicistroviruses, iflaviruses and picornaviruses, based on conserved amino acid domains in the capsid proteins, helicase, 3C-protease and RNA-dependent RNA polymerase. The virus abbreviations are: ABPV, KBV, IAPV, SInV-1 (Solenopsis invicta virus), CrPV (cricket paralysis virus), DCV (Drosophila C virus), TaSV (Taura syndrome virus), HoCV (Homalodisca coagulata virus), PlSV (Plautia stali intestine virus), TriV (Triatoma virus), BQCV (black queen cell virus), HiPV (Himetobi P virus), ALPV (aphid lethal paralysis virus), RhPV (Rhopalosiphum padi virus), IFV (infectious flacheri virus), EoPV (Ectropis obliqua picorna-like virus), PnPV (Perina nuda picorna-like virus); SPV (slow paralysis virus), SBV (sacbrood virus), DWV (deformed wing virus), VaDV (Varroa destructor virus), BrBV (Brevicoryne brassicae picorna-like virus), VcV (Venturia canescens virus), HAV (hepatitis A virus), EMCV (Encephalomyocarditis virus), PV (polio virus). The phylogram was constructed by MEGA-4 (Tamura et al., 2007), using Minimum Evolution criteria. The statistical strength of the nodes is shown as the percentage of correct partitions in a 1000 replicate bootstrap analysis. Branches with less than 60% bootstrap support were collapsed. Viruses infecting bees are highlighted in orange (dicistroviruses) and green (iflaviruses). After de Miranda, Dainat, Stoltz, Neumann and Ball (unpublished).

come part of the stem structure. The relatively high degree of structural conservation in the IRES elements is not surprising, because these have significant interaction with host proteins and ribosomes. For this reason a structurally conserved IRES is also expected in the 5′ UTR (Roberts and Groppelli, 2009; Nakashima and Uchiumi, 2009). These host-related reasons for sequence-structure conservation in the virus genome do not apply for the virus replication signals in the 5′ and 3′ UTR however, because here the interaction is primarily with the virus-encoded RdRp and helicase. In this context, the high degree of variation between ABPV, KBV and IAPV in their 5′ UTR's may be a means for the rival RNA polymerases and helicases to identify the correct viral genomic RNA for replication, during mixed infections (de Miranda et al., 2004). The contrastingly low variability of the 3′ UTR suggests that this may apply more to the production of positive-strand genomic RNA than for the negative-strand replicative intermediates.

5. Diagnostics

Diagnostics for dicistroviruses have evolved rapidly over the last 10–15 years, moving from serology-based approaches, which detect surface antigens on virus particles, to molecular protocols that target virus genetic material. Each approach offers specific advantages, strengths and biases when detecting closely related RNA viruses with a propensity for high mutation rates and the presence of quasispecies. Optimal sampling strategies are essential to minimise error when developing a diagnostic strategy for dicistroviruses. Considerations for these steps are covered in detail by other reviews (de Miranda, 2008).

The generation of species-specific antibodies has been hampered by the lack of *in vitro* culturing methods for honey bee viruses (Stoltz et al., 1995). Virus propagation has generally been achieved by injecting purified virus into honey bee pupae

(Anderson and Gibbs, 1988; Bailey and Woods, 1977; Allen and Ball, 1995; Stoltz et al., 1995). However, honey bee colonies are rarely virus free and may already contain viruses which contaminate the culture. Furthermore, the virions of such viruses may have similar physical properties, making them impossible to separate from the dicistrovirus(es) under study (Bailey and Ball, 1991). Polyclonal antibodies generated from such material risk showing cross-reaction with co-purified contaminating viruses. Bergem et al. (2006) reported methods to maintain honey bee cell lines for more than 3 months before significant degeneration occurred. Such cell lines could be employed in the future for the production of pure, uncontaminated virus, from either natural or cloned sources.

The majority of antibodies generated for the detection of discistroviruses have been polyclonal, a preparation containing multiple antibodies with a range of epitope specificities and affinities (Anderson, 1984; Allen et al., 1986; Allen and Ball, 1995). Species-specific polyclonal antibodies have been raised against gel-purified VP4 proteins of ABPV and KBV (Stoltz et al., 1995) and against purified expressed KBV coat proteins (Shen et al., 2005a; de Miranda, 2008). Monoclonal antibodies, which comprise a single antibody with a defined specificity and affinity, have not been generated to ABPV, KBV or IAPV. Monocolonal antibodies may prove valuable for the most sensitive serology-based detection, however, the epitope detected by each antibody must be stable and be represented in all virus strains to avoid false negatives. Polyclonal antibodies have also been generated against IAPV (Maori et al., 2007a), although the specificity of these antibodies is not known.

Several diagnostic methods have been explored using antisera including immunodiffusion (Anderson, 1984) and Enzyme-linked immunosorbent assay (ELISA) (Anderson, 1984; Allen et al., 1986; de Miranda, 2008). Immunodiffusion was used to demonstrate that strains of KBV from Canada and Spain were serologically more closely related to each other than to Australian KBV strains (Allen and Ball, 1995). In addition, immunodiffusion demonstrated that IAPV is closely related to ABPV and KBV, but is sufficiently different to be discerned using serology (Maori et al., 2007a). Whilst immunodiffusion is useful to determine serological relatedness between viruses, the method is considerably less sensitive than formats like ELISA (Anderson, 1984; Allen et al., 1986; de Miranda, 2008). ELISA is a useful method for screening large numbers of samples cheaply and easily, with sufficient sensitivity to detect viruses at sub-clinical levels, and has been used widely in the 1980's–1990's to screen environmental samples, before nucleic acid-based methods became available.

The advent of the molecular age and the accessibility of RT-PCR has revolutionised dicistrovirus diagnostics. The limiting factor for any such protocol is the availability of nucleotide sequence data. The number of available nucleotide sequences for these viruses has increased exponentially since the first sequence of KBV was published in 1995 (Stoltz et al., 1995). Nucleotide sequence databases now contain a wealth of genetic information, comprising complete genome coverage for all three viruses and over 300 partial sequences. However, the information contained within online databases is not always accurate, especially when considering species designation. A multitude of RT-PCR protocols are available for the detection of structural and functional genes of ABPV, KBV and IAPV (Table 3). RT-PCR protocols offer a means of detecting viruses, and when coupled with sequencing, can be used to confirm diagnoses and obtain phylogenetic information on the viruses under study (Bakonyi et al., 2002b; Palacios et al., 2008; de Miranda et al., 2004;).

IAPV and KBV sequences continue to be misclassified in the literature and the public sequence databases, highlighting problems with both molecular virus diagnosis and the online sequence databases. The original KBV primers were designed in 1995 (Stoltz et al., 1995), as part of the very first RT-PCR protocol to be determined for any honey bee virus. These primers continue to be used widely (Evans, 2001; Yue et al., 2006; Chen et al., 2004a,b; Hung and Shimanuki, 1999; Chen et al., 2005, 2006; Tentcheva et al., 2004; Antunez et al., 2006; Baker and Schroeder, 2008) even when the potential for cross-amplification with related viruses became clear with the subsequent sequencing of the ABPV, KBV and IAPV genomes (Govan et al., 2000; Bakonyi et al., 2002b; de Miranda et al., 2004; Maori et al., 2007a). While important historically, these original KBV primers should now be discontinued. The problem of potential misdiagnosis of virus infections, including false positive and false negative detection and strain misassignment, has been recognised before (Blanchard et al., 2008a; Genersch, 2005; de Miranda, 2008). One excellent way to resolve uncertainties is to base a diagnosis on multiple primer pairs (Evans and Hung, 2000; Siede et al., 2005; Bakonyi et al., 2002b; see also Genersch (2005), Blanchard et al. (2008a) and Grabensteiner et al. (2001) for other bee viruses). Another approach is to augment the RT-PCR assay with diagnostic restriction enzyme digestions (Evans, 2001; Siede and Büchler, 2004; Siede et al., 2005), although this approach can also be sensitive to micro-variation between isolates, if variation is located in the targeted restriction enzyme site, and also offers much reduced sensitivity. Both of these approaches entail increased labour and cost. The simplest and most cost-effective solution is to carefully design virus-specific primers, based on the pattern of sequence conservation and variation within and between the different viruses, taking particular care to avoid locating the 3′ nucleotide of either primer on highly variable nucleotides, *i.e.* the third codon position of the open reading frames (see Table 3; Topley et al., 2005; de Miranda, 2008). Guidelines for designing RNA-virus diagnostic primers and avoiding misdiagnosis can be found in de Miranda (2008). Further excellent instruction on the design of RT-PCR experiments can be found in the MIQE guidelines for the *M*inimum *I*nformation for publication of *q*uantitative real-time PCR *E*xperiments (Bustin et al., 2009), produced by an umbrella group of the leading experts and developers of RT-qPCR technology whose aim is to provide a set of minimum benchmark standards for the correct design, execution and reporting of biological experiments (Burgoon, 2006; Taylor et al., 2008). The problems with virus misdiagnosis highlight the importance of continually updating primers and protocols as new information is obtained, especially given that RNA viruses are highly variable entities that can evolve rapidly (Carter and Genersch, 2008; de Miranda, 2008).

The second problem concerns the redundancy of sequence databases. Many of the misclassified IAPV isolates (*i.e.* from France, Russia and Australia) date from before 2004 (when the first IAPV sequences were made available) and were correctly classified as KBV according to the information available at that time. Unfortunately, these remain misclassified when it is clear that they now belong to a different taxon. More recent misassignments are due to failure to confirm assignation with simple sequence comparison, compounded by the dynamic nature of the classification systems.

Real-time RT-qPCR offers unrivalled sensitivity and specificity for the detection of honey bee viruses, with the additional bonuses of virus quantification and the abolition of PCR product visualisation. Sensitivity comparisons between serological and molecular detection protocols for ABPV, KBV and IAPV are lacking. However, such comparisons for other single-strand, positive-sense RNA viruses suggest that real-time RT-qPCR is 1000 times more sensitive than ELISA and 100 times more sensitive than conventional non-nested RT-PCR (Ratti et al., 2004). Several different real-time chemistries have been utilised for the detection of discistroviruses. Real-time RT-qPCR assays using SYBR-Green, a DNA dye which fluoresces upon binding, have been reported for ABPV, KBV, IAPV (Cox-Foster et al., 2007; Evans, 2007; Kukielka et al., 2008; Palacios et al., 2008; Siede et al., 2008). Palacios et al. (2008) estimated the

Table 3
Published protocols for the diagnosis of ABPV, KBV and IAPV. Indicated are the virus for which the protocol is designed (VIRUS); the genome location of the 3′ end of the forward (F) and reverse (R) primers, according to GenBank accessions AF150629 (ABPV), AY275710 (KBV) and EF219380 (IAPV); the codon position of the 3′ terminus of each primer (#); the forward and reverse PCR primer sequences (primer sequence), using the standard nucleotide codes (A = Adenine; C = Cytosine; G = Guanine; T = Thymidine; I = Inosine; R = A/G; Y = C/T; K = G/T; M = A/C; S = C/G; W = A/T; H = A/C/T; B = C/G/T; V = A/C/G; D = A/G/T; N = A/C/G/T), with lower-case letters representing extensions or tags not derived from virus sequence; the length of the PCR product (Size); the viral gene(s) targeted by the primers (Gene); the potential of the primers to amplify ABPV (A), KBV (K) or IAPV (I) as verified by cross-amplification optimisation assays or if it is clear from sequence comparison that amplification should occur (+), should not occur (–) or is uncertain (?); the incubation temperatures and times for the reverse transcription step ('RT'), the pre-PCR denaturation step ('denat.'), the number of PCR cycles ('C') of denaturation ('denat.'), annealing ('ann.') and extension ('ext.'), where the extension step marked with '*' increases by 5 s every cycle, starting cycle ten; the protocols adapted for real-time RT-PCR (C_q), and the detection chemistry used (HP = hydrolysis probe, or TaqMan®; SG = SYBR-Green); and the original reference for each assay. Table adapted and updated from de Miranda, 2008.

Virus		3′	#	Primer sequence (5′–3′)	Size	Gene	A	K	I	RT	denat.	C	denat.	ann.	ext.	C_q	REFERENCE
ABPV	F	4802	1	AAATGATACCGGTGGGCAGAT	66	RdRp	+	–	–	50 °C	95 °C	40	95 °C	58 °C	72 °C		Gauthier et al. (2007)
	R	4825	3	AAGGTCGTATGTCCGTCTTACCA						60 min	5 min		10 s	30 s	30 s		
ABPV	F	5290	3	TGAGAACACCTGTAATGTGG	452	RdRp	+	?	+	50 °C*	94 °C	35	94 °C	55 °C	72 °C		Tentcheva et al. (2004)
	R	5703	2	ACCAGAGGGTTGACTGTGTG						60 min	2 min		30 s	30 s	60 s		
ABPV	F	5381	1	AATGGGCCTATGGACTTTTCTA	178	RdRp	+	–	–	50 °C	95 °C	40	95 °C	58 °C	72 °C		Siede et al. (2008)
	R	5517	2	AAATCTCCTGCAATAACCTTGG						30 min	15 min		15 s	30 s	30 s		
ABPV	F	6261	2	TATCAGAAGGCCACTGGAGA	722	IGR	+	?	?	50 °C	95 °C	40	95 °C	55 °C	72 °C		Bakonyi et al. (2002b)
	R	6995	1	TCCACTCGGTCATCATAAGG						30 min	5 min		20 s	20 s	60 s		
ABPV	F	6548	1	TCATACCTGCCGATCAAG	197	VP2	+	–	–	50 °C	95 °C	40	95 °C	58 °C	72 °C	SG	de Miranda, Tournaire,
	R	6707	1	CTGAATAATACTGTGCGTATC						10 min	5 min		10 s	30 s	30 s		Paxton Gauthier (unpublished)
ABPV	F	6867	2	TCTTGGACATTGCCTTCAGT	778	VP2	+	–	–	50 °C	95 °C	40	95 °C	55 °C	72 °C		Bakonyi et al. (2002b)
	R	7607	1	ATACCATTCGCCACCTTGTT						30 min	5 min		20 s	20 s	60 s		
ABPV	F	7466	1	TGCAGTTCCAGAAGTTAAGA	686	VP4	+	–	+	50 °C	95 °C	40	95 °C	55 °C	72 °C		Bakonyi et al. (2002b)
	R	8114	1	ATAGTRGCTCGCCAATATGA						30 min	5 min		20 s	20 s	60 s		
ABPV	F	7947	2	GTGCTATCTTGGAATACTAC	619	VP3	+	?	?	50 °C	95 °C	40	95 °C	55 °C	72 °C		Bakonyi et al. (2002b)
	R	8527	3	AAGGYTTAGGTTCTACTACT						30 min	5 min		20 s	20 s	60 s		
ABPV	F	7947	2	GTGCTATCTTGGAATACTAC	619	VP3	+	?	?	50 °C	95 °C	40	94 °C	55 °C	72 °C		Berényi et al. (2006)
	R	8527	3	AAGGYTTAGGTTCTACTACT						30 min	15 min		30 s	50 s	60 s		
ABPV	F	8134	3	CATATTGGCGAGCCACTATG	398	VP3	+	?	?	42 °C	94 °C	40	94 °C	55 °C	72 °C		Bakonyi et al. (2002a)
	R	8493	2	CCACTCCACACAACTATCG						60 min	3 min		60 s	60 s	60 s		
ABPV	F	8134	3	CATATTGGCGAGCCACTATG	398	VP3	+	?	?	50 °C	95 °C	35	94 °C	49.5 °C	72 °C		Yue et al. (2006)
	R	8493	2	CCACTCCACACAACTATCG						30 min	15 min		30 s	30 s	30 s		
ABPV	F	8134	3	CATATTGGCGAGCCACTATG	398	VP3	+	?	?	50 °C	95 °C	40	95 °C	55 °C	72 °C		Siede and Büchler (2006)
	R	8493	2	CCACTCCACACAACTATCG						30 min	5 min		20 s	20 s	60 s		
ABPV	F	8145	2	AGCCACTATGTGCTATCGTAT	202	VP3	+	?	?	50 °C	95 °C	40	94 °C	55 °C	72 °C		Grabensteiner et al. (2007)
	R	8311	3	ATGGTGACCTCTGTGTCATTA						30 min	2 min		30 s	30 s	60 s		
ABPV	F	8232	2	TCCTATATCGACGACGAAAGACAA	65	VP3	+	–	–	48 °C	95 °C	40	95 °C	–	60 °C	HP	Chantawannakul et al. (2006)
	R	8251	3	GCGCTTTAATTCCATCCAATTGA						30 min	10 min		15 s	–	60 s		
ABPV	F	8137	3	ATTGGCGAGCYACTATGTGC	858	VP1	+	–	–	50 °C	95 °C	40	95 °C	55 °C	72 °C		Bakonyi et al. (2002b)
	R	8957	1	CGCGGTAYTAAGAAGCTACG						30 min	5 min		20 s	20 s	60 s		
ABPV	F	8484	2	TTATGTGTCCAGAGACTGTATCCAI	900	VP1	+	–	–	50 °C	94 °C	35	94 °C	60 °C	72 °C		Benjeddou et al. (2001)
	R	9336	–	GCTCCTATTGCTCGGTTTTTCGGTI						30 min	2 min		30 s	30 s	30 s		
ABPV	F	8484	2	TTATGTGTCCAGAGACTGTATCCAI	900	VP1	+	–	–	58 °C	94 °C	35	94 °C	63 °C	68 °C*		Topley et al. (2005)
	R	9336	–	GCTCCTATTGCTCGGTTTTTCGGTI						30 min	2 min		30 s	30 s	60 s		
ABPV	F	8484	2	TTATGTGTCCAGAGACTGTATCCA	900	VP1	+	–	–	50 °C	95 °C	40	94 °C	55 °C	72 °C		Antunez et al. (2005, 2006)
	R	9336	–	GCTCCTATTGCTCGGTTTTTCGGT						30 min	15 min		60 s	60 s	60 s		
ABPV	F	8697	2	TCTGATGATGCTGAAGAGAGAAA	500	VP1	+	–	–	42 °C	95 °C	35	95 °C	54 °C	72 °C		Weinstein-Teixeira et al. (2008)
	R	9172	3	AATCATCATTGCCGGCTCTA						50 min	2 min		30 s	60 s	60 s		
ABPV	F	8713	3	GGAACATGGAAGCATTATTG	687	VP1	+	–	–	50 °C	95 °C	40	95 °C	55 °C	72 °C		Bakonyi et al. (2002b)
	R	9362	–	AATGTCTTCTCGAACCATAG						30 min	5 min		20 s	20 s	60 s		
ABPV	F	?	?	CAGTGTAGCTAGTTTAAAAGCCAATG	?	?	?	?	?	50 °C	95 °C	44	95 °C	–	60 °C	SG	Cox-Foster et al. (2007)
	R	?	?	AACATGCAGATTGAGACAGTTGA						10 s	2 min		15 s	–	60 s		
KBV	F	3029	3	ATGACGATGATGAGTTCAAG	290	3C-pro	–	+	–	42 °C	94 °C	35	94 °C	50 °C	72 °C		Shen et al. (2005a)
	R	3282	1	AATTGCAAGACGGCATC						60 min	5 min		20 s	20 s	60 s		
KBV	F	4428	1	CAAACTGCTGAATCAATGTCAAAAT	122	3C-pro	?	+	+	50 °C	95 °C	44	95 °C	–	60 °C	SG	Cox-Foster et al. (2007)
	R	4502	3	ACATGCCTCTACTTTGTCACATTCA						10 s	2 min		15 s	–	60 s		

KBV	F	5437	1	CGTCGACCTATTGAAAAAGTTAATCA	69	3C-pro	?	+	+	50 °C	95 °C	40	95 °C	58 °C	72 °C		Gauthier et al. (2007)
	R	5458	2	TGAGAAGTCCATTGGTCCATTTG						60 min	5 min		10 s	30 s	30 s		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	?	+	+	37 °C*	94 °C	35	94 °C	57 °C	72 °C		Stoltz et al. (1995)
	R	5800	2	TGTGGGTTGGCTATGAGTCA						60 min	10 min		60 s	60 s	60 s		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	?	+	+	50 °C	94 °C	35	94 °C	57 °C	68 °C*		Hung et al. (1996a, 2000) and Hung (2000)
	R	5800	2	TGTGGGTTGGCTATGAGTCA						30 min	2 min		30 s	30 s	45 s		Hung and Shimanuki (1999, 2000)
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	?	+	+	50 °C	94 °C	35	94 °C	50 °C	68 °C*		Evans and Hung (2000) and Evans (2001)
	R	5800	2	TGTGGGTTGGCTATGAGTCA						30 min	2 min		30 s	30 s	45 s		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	?	+	+	50 °C*	94 °C	35	94 °C	55 °C	72 °C		Tentcheva et al. (2004)
	R	5800	2	TGTGGGTTGGCTATGAGTCA						60 min	2 min		30 s	30 s	60 s		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	?	+	+	50 °C	95 °C	35	94 °C	61 °C	72 °C		Siede and Büchler (2004)
	R	5800	2	TGTGGGTTGGCTATGAGTCA						30 min	5 min		60 s	60 s	60 s		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	?	+	+	48 °C	95 °C	40	95 °C	55 °C	68 °C		Chen et al. (2004a,b)
	R	5800	2	TGTGGGTTGGCTATGAGTCA						45 min	2 min		30 s	60 s	120 s		Chen et al. (2005, 2006a)
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	?	+	+	50 °C	95 °C	35	94 °C	50.5 °C	72 °C		Yue et al. (2006)
	R	5800	2	TGTGGGTTGGCTATGAGTCA						30 min	15 min		30 s	30 s	30 s		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	?	+	+	50 °C	95 °C	40	94 °C	55 °C	72 °C		Berényi et al. (2006)
	R	5800	2	TGTGGGTTGGCTATGAGTCA						30 min	15 min		30 s	50 s	60 s		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	?	+	+	50 °C	95 °C	40	94 °C	55 °C	72 °C		Antunez et al. (2006)
	R	5800	2	TGTGGGTTGGCTATGAGTCA						30 min	15 min		60 s	60 s	60 s		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	?	+	+	50 °C	95 °C	45	95 °C	–	60 °C		Cox-Foster et al. (2007)
	R	5800	2	TGTGGGTTGGCTATGAGTCA						2 min	10 min		15 s	–	60 s		
KBV	F	5561	3	ATGAAGTGTCTATTGGAACG	550	RdRp	–	+	?	50 °C	94 °C	35	94 °C	57 °C	68 °C*		Evans and Hung (2000)
	R	6069	1	ttcgaaCCCTCGCCTCCAACTCC						30 min	2 min		30 s	30 s	45 s		
KBV	F	6216	1	GCCTAATTGGTGTCGAGGAG	769	RdRp	?	+	?	50 °C	95 °C	45	95 °C	–	60 °C		Cox-Foster et al. (2007)
	R	6966	1	GCTTTTCCACCAGCTTTCAA						2 min	10 min		15 s	–	60 s		
KBV	F	6639	1	CCATACCTGCTGATAACC	200	VP2	–	+	–	50 °C	95 °C	40	95 °C	58 °C	72 °C	SG	de Miranda, Tournaire,
	R	6801	1	CTGAATAATACTGTGCGTATC						10 min	5 min		10 s	30 s	30 s		Paxton Gauthier (unpublished)
KBV	F	7568	3	gggatccGTTTCTATGCAAATCGCA	282	VP4	–	+	–	42 °C	94 °C	30	94 °C	59 °C	72 °C		Todd et al. (2007)
	R	7806	1	aagctTCCAGGCACATTCTG						30 min	2 min		30 s	30 s	30 s		
KBV	F	8269	2	ACCAGGAAGTATTCCCATGGTAAG	69	VP3	–	+	–	48 °C	95 °C	40	95 °C	–	60 °C	HP	Chantawannakul et al. (2006)
	R	8304	3	TGGAGCTATGGTTCCGTTCAG						30 min	10 min		15 s	–	60 s		
IAPV	F	92	–	CGACATTAGTTAAGTTACAATTACACG	998	5′NTR	–	–	+	50 °C	95 °C	45	95 °C	–	60 °C	SG	Palacios et al. (2008)
	R	1042	3	TTTCTTCAACATCTCCTGAAAGG						2 min	10 min		15 s	–	60 s		
IAPV	F	5473	3	AATGGACCAATGGATTTYTCWATWGCT	136	RdRp	–	?	+	50 °C	95 °C	44	95 °C	–	60 °C	SG	Palacios et al. (2008)
	R	5554	3	CGAACAGTTTTACTCCAGTCYTGAGARTAC						10 s	2 min		15 s	–	60 s		
IAPV	F	5418	2	CGTCGACCCATTGAAAAAGT	403	RdRp	+	+	+	50 °C	95 °C	45	95 °C	–	60 °C	SG	Cox-Foster et al. (2007) and
	R	5783	1	GGTTGGCTGTGTGTCATCAT						2 min	10 min		15 s	–	60 s		Palacios et al. (2008)
IAPV	F	6146	1	CGATGAACAACGGAAGGTTT	766	IGR	?	?	+	50 °C	95 °C	45	95 °C	–	60 °C	SG	Cox-Foster et al. (2007) and
	R	6874	3	ATCGGCTAAGGGGTTTGTTT						2 min	10 min		15 s	–	60 s		Palacios et al. (2008)
IAPV	F	6627	1	CCATGCCTGGCGATTCAC	203	VP2	–	–	+	50 °C	95 °C	40	95 °C	58 °C	72 °C	SG	de Miranda, Tournaire,
	R	6792	1	CTGAATAATACTGTGCGTATC						10 min	5 min		10 s	30 s	30 s		Paxton Gauthier (unpublished)
IAPV	F	7910	1	CGAACTTGGTGACTTGAAGG	110	VP3	–	–	+	50 °C	95 °C	44	95 °C	–	60 °C	SG	Cox-Foster et al. (2007)
	R	7985	1	GCATCAGTCGTCTTCCAGGT						10 s	2 min		15 s	–	60 s		
IAPV	F	7987	3	CCAGCCGTGAAACATGTTCTTACC	225	VP3	–	?	+	50 °C	95 °C	44	95 °C	–	60 °C	SG	Palacios et al. (2008)
	R	8164	3	ACATAGTTGCACGCCAATACGAGAAC						10 s	2 min		15 s	–	60 s		
IAPV	F	7795	3	GGTCCAAACCTCGAAATCAA	839	VP3	–	?	+	50 °C	95 °C	45	95 °C	–	60 °C	SG	Palacios et al. (2008)
	R	8596	3	TTGGTCCGGATGTTAATGGT						2 min	10 min		15 s	–	60 s		
IAPV	F	8880	2	AGACACCAATCACGGACCTCAC	135	VP1	–	?	+	42 °C	95 °C	40	92 °C	62 °C	72 °C		Maori et al. (2007a,b)
	R	8976	2	GAGATTGTTTGAGAGGGGTGG						60 min	10 min		30 s	30 s	30 s		
IAPV	F	8880	2	AGACACCAATCACGGACCTCAC	475	3′NTR	–	–	+	42 °C	95 °C	40	92 °C	65 °C	72 °C		Maori et al. (2007a,b)
	R	9336	–	AGATTTGTCTGTCTCCCAGTGCACAT						60 min	10 min		30 s	30 s	30 s		
ABPV, KBV, IAPV	F	7259	1	ggatcCAGTCTATATGTGGT	543	VP4	+	+	+	42 °C	94 °C	30	94 °C	59 °C	72 °C		Todd et al. (2007)
	R	7763	1	aagctTCCAGGCACATTCTG						30 min	2 min		30 s	30 s	30 s		
ABPV, KBV, IAPV	F	8140	3	GGCGAGCCACTATGTGCTAT	401	VP1	+	+	+	50 °C	94 °C	35	94 °C	50 °C	68 °C*		Evans (2001)
	R	8507	1	ATCTTCAGCCCACTT						30 min	2 min		30 s	30 s	45 s		

number of copies of IAPV in honey bee samples from the USA, but these results were not fully explored. Siede et al. (2008) used real-time RT-qPCR using SYBR-Green chemistry to demonstrate a positive correlation of APBV titre with winter mortalities in Germany. In addition, this work considered the experimental error which accumulates when quantifying using real-time PCR. Such high variation requires a large number of replicates in order to detect treatment differences. Chantawannakul et al. (2006) presented protocols for the detection of KBV and ABPV using hydrolysis-probe chemistry in *Varroa* mites from Thailand. The addition of a dual-labelled fluorogenic probe means hydrolysis-probe based RT-qPCR negates problems with detecting non-target specific binding, such as primer-dimers. However, this increase in assay specificity heightens the chances of false negatives when dealing with sequence variants. The selection of real-time chemistry influences Type 1 or Type 2 error, and as with conventional PCR, assay specificity should be reviewed in the light of new sequence data before each study to minimise such errors. Detailed instructions for the correct design, execution and reporting of RT-qPCR experiments can be found in the MIQE guidelines (Bustin et al., 2009).

5.1. Multi-target screening

Microarrays have been used for studies of honey bee gene expression to provide insight into chemical response (Kucharski and Maleszka, 2005), worker bee sterility (Thompson et al., 2008), caste differentiation (Barchuk et al., 2007), behavioural maturation (Adams et al., 2008) and post mating changes (Kocher et al., 2008). This technology is particularly suited to screening for multiple targets within the same sample. However, high production costs of standard glass-slide formats have limited the availability of this technology for diagnostics of honey bee pathogens. Low density arrays, measuring colorimetric change, offer many of the benefits of glass-slide formats with far lower set-up and running costs. Müller et al. (2009) reported the simultaneous detection of multiple RNA viruses using such low density microarrays, demonstrating the suitability of this method for dicistrovirus detection. In addition, certain low-density platforms are applicable for the detection of protein and peptide targets (Ehricht et al., 2009) which allows target detection in genomic regions with conserved amino acid sequence, even when nucleotide variation is high.

Suites of quantitative PCR-based tests offer an alternative to microarrays for monitoring multiple targets in the same sample. Evans (2007) described the development of a quantitative-PCR array to accurately measure the transcript abundance of 48 honey bee and pathogen genes in parallel. Whilst useful, such formats require access to high throughput real-time PCR machines. Microfluidics solve such throughput problems by repeating up to 96 reactions against 96 different samples (a total of 9216 reactions) in a single 3–4 h run. Such platforms allow the direct transfer of existing, validated real-time RT-qPCR assays to facilitate multi-target screening and accurate quantification. Digital arrays, also using microfluids, repeat the same test on multiple aliquots of a single sample, offering single-molecule detection and the accurate quantification of rare targets (Bhat et al., 2009).

5.2. Non-targeted approaches

Pyrophosphate-based sequencing technology (pyrosequencing) is a method of generating nucleotide sequence information without any *a priori* knowledge of primer binding sites. The method has recently become sufficiently miniaturised to generate massive datasets from picolitres of nucleic acid preparations. This method has been used to identify previously cryptic pathogens in large-scale colony losses in the United States (Cox-Foster et al., 2007). Whilst metagenomics is one application, pyrosequencing offers several other potential uses for the study of dicistroviruses. Pyrosequencing has been used to effectively characterise non-dicistrovirus populations within a single infected host, including quantifying variants of low prevalence within a quasispecies (Eriksson et al., 2008; Margeridon-Thermet et al., 2009). Such data may provide insights into viral evolutionary dynamics and be informative for primer design for more traditional targeted methods of detection, like PCR.

The ideal diagnostic method should be sensitive, accurate, robust, simple, rapid and cheap. Clearly technological advances in diagnostics have made progress towards more valuable methods. There are many such advances that could further our understanding of dicistrovirus biology, distribution and evolution that have yet to be employed to study these viruses. However, all diagnostic methods are sensitive to temporal shifts in the knowledge base, and as such, the merits of each published method should be carefully evaluated before use.

6. Resistance and treatment

Apart from the documentation of higher or lower prevalence of certain virus diseases in different honey bee races (e.g. Rinderer et al., 1975) or with respect to the mating frequency of queens (Tarpy and Seeley, 2006), there has been very little examination of the honey bee germplasm for genetic resistance to honey bee viruses (Page and Guzmán-Novoa, 1997; Moritz and Evans, 2008). In this context the recent reports of frequent, reciprocal exchange of genetic material between IAPV and the honey bee genome, and in particular the correlation of such genome-integrated IAPV with resistance to IAPV infection (Maori et al., 2007a,b) requires serious consideration.

The natural integration of virus sequences into eukaryotic host genomes is a well established phenomenon for most subfamilies within *Retroviridae* and viruses with dsDNA genomes (e.g. Doucet et al., 2007; Gundersen-Rindal and Lynn, 2003). However, for RNA viruses with no intermediary DNA forms, such as the dicistroviruses, integration of viral genetic material into the host genome is a far less frequent occurrence; essentially a rare, unusual evolutionary event (Crochu et al., 2004). Originally proposed as a possible evolutionary mechanism in 1975 (Zhdanov, 1975), genomic integration of an RNA virus was first confirmed in mammals, where persistent DNA forms of *Lymphocytic choriomeningitis virus* (LCMV) were observed in mouse spleen cells (Klenerman et al., 1997). It was subsequently demonstrated that endogenous retrovirus retrotransposons, commonly present in the mouse genome, promoted the reverse transcription of LCMV and subsequent integration of non-retroviral genetic material into the host genome (Geuking et al., 2009). A similar mechanism of integration was also reported for *Potato virus Y*, whose viral coat-protein sequences were integrated into the genome of grape, flanked by retrotransposon-like repeat elements (Tanne and Sela, 2005). These findings are supported by the discovery of flavivirus-like genetic material naturally integrated into the mosquito genome (Crochu et al., 2004). Whilst the majority of integrated coding regions are silenced by insertions or deletions, in one case the integrated segment did retain a conserved coding region, likely leading to the production of functional viral enzymes. Maori et al. (2007b) reported a similar sequence exchange between IAPV and *Apis mellifera*. Virus-host recombination events were surprisingly common, with 30% of IAPV-infected bees testing positive for integrated viral sequences. Although the honey bee genome does have higher than average recombination rates (Beye et al., 2006), very few transposons and no active retrotransposons have been reported (Honey Bee Genome Sequencing Consortium, 2006). Crucially, no sequences resembling IAPV or any other honeybee RNA virus can be found in the completed honeybee genome sequence.

Whilst RNA virus recombination into the honey bee genome is distinctly possible, the suggested high frequency contrasts sharply with the sparse reports of such events in the wider literature, and further independent study by the wider research community is required for confirmation of this potentially important finding.

If genome-integrated IAPV fragments do exist, then the second claim, that such integrated viral material promotes resistance to virus infection (Maori et al., 2007b), has much better support from the literature. It has been demonstrated repeatedly that transgenic plants carrying a portion of a viral genome (generally the coat protein gene) are often resistant to the virus in question (El-Borollosy et al., 2008; Kertbundit et al., 2007; Pedersen et al., 2007). This effect is generally due to interference by the host-expressed viral RNA (or sometimes the translated proteins) with the replication and translation of the infectious virus (Ratcliff et al., 1997, 1999). The effect is analogous to that of defective-interfering RNAs (DI-RNAs; non-viable viral deletion mutants produced by intra-molecular recombination), and satellite RNAs (molecular parasites of the virus), both of which monopolise RNA polymerase activity, thereby suppressing virus replication (White and Morris, 1999; Simon et al., 2004). The effect is also related to RNAi (RNA interference; see later) and gene silencing, which are natural mechanisms for controlling mRNA expression and degradation through the binding of sense RNA with short antisense complements, followed by digestion of the double-stranded RNA segment with specific RNA-ases (Ratcliff et al., 1997, 1999).

The third claim, that IAPV-integrated honey bees are "naturally transgenic" (Maori et al., 2007b) represents an unfortunate use of terminology, in that it links the data to a highly controversial industry that too often tries to justify its commercial aims by finding parallels with naturally occurring processes. This obscures the significant difference between instant transgenic evolution by Man, and similar natural accidents scattered throughout the evolutionary history of the entire biosphere covering millions of years.

Another recent development involving IAPV concerns a possible treatment, using RNAi technology (Maori et al., 2009). Treatment against virus infections has never been seriously considered in the beekeeping and research community, even though anti-viral treatments have been used successfully in human and veterinary settings (Vignuzzi et al., 2005, 2006). However, the devastating consequences of CCD and the link to IAPV and related viruses may change this. One of the most promising developments in this field is the use of small interfering RNA (siRNA), which takes advantage of the gene silencing or RNA interference (RNAi) pathway for the post-transcriptional control of mRNA levels, that is present in practically all eukaryotic organisms (Grassmann and Jang, 2008; van den Berg et al., 2008). This pathway is activated by the molecular detection of double-stranded RNA by Dicer-2, a type-III RNAse, and subsequent cleavage of the dsRNA into small ~22 bp fragments. These fragments are loaded into an RNA-induced Silencing Complex (RISC), which then drives the specific degradation of the corresponding mRNA through complementarity with the siRNAs (van den Berg et al., 2008). Given that RNA viruses pass through a double-stranded RNA phase during replication they are natural targets for degradation by the RNAi pathway, and it is possible that the RNAi pathway may have originated as a mechanism for degrading foreign nucleic acids and molecular parasites (Obbard et al., 2009). The RISC can also be loaded with micro RNAs (miRNAs), which are small hairpin dsRNAs transcribed by the host genome and processed by Dicer-1, for post-transcriptional control of mRNA levels. By introducing synthetic siRNA molecules into the cell (either through transgene expression or cellular uptake), RISC is primed to degrade specific RNA species, thus 'silencing' the expression of a gene (van den Berg et al., 2008). This ability to specifically silence individual genes has been used extensively to study gene expression pathways and has been quickly adopted for the treatment (gene therapy) of a number of genetic disorders, cancers and diseases (Campbell and Choi, 2005; Pfister et al., 2009; Aigner, 2006). However, the application of siRNA for the control of viral infections is more problematic (Leonard and Schaffer, 2006). Viruses have evolved a number of strategies to evade the RNAi machinery (Grassmann and Jang, 2008; Shi et al., 2008) and even produce miRNAs themselves for regulating their own gene expression and disrupting host gene expression (Schütz and Sarnow, 2006), resulting in an miRNA "arms-race" between virus and host (Shi et al., 2008; Obbard et al., 2009). One particular difficulty with siRNA anti-viral strategies is the requirement for near-perfect complementarity between siRNA and target RNA for RISC to function. This means that the virus can easily avoid RNAi degradation by mutating out of range (Stram and Kuzntzova, 2006; Grassmann and Jang, 2008) which, given the high variability of the ABPV–KBV–IAPV complex, may be a particular risk for effective and long-term treatment of IAPV, KBV and ABPV. This places extra emphasis on the accurate design and performance prediction of siRNAs (Li and Cha, 2007). The other main challenge is the optimisation of the delivery of therapeutic siRNA to the target tissues. It has become obvious that siRNA does not easily cross membrane boundaries (White, 2008), at least not without the help of host transmembrane functions (Aronstein et al., 2006). This observation also suggests that the anti-IAPV effect of orally administered siRNA observed by Maori et al. (2009) may reside primarily in the cells lining the alimentary canal, a likely site of replication for the large amount of virus found in the gut lumen, and less in systemically infected sites in the brain or reproductive tissues. The key to reaching these tissues is to link the siRNA to agents that are absorbed specifically by the targeted tissues and to protect the siRNA from degradation during transport (White, 2008; Jeong et al., 2009).

7. Future developments

Honey bee virology has developed rapidly over the last decade, and the public and political interest in honey bee and pollinator health generated by the CCD phenomenon has provided a healthy impetus to the field. Thus far, most of the research has concentrated on the viruses, including characterisation, diagnosis, incidence and transmission. This research has set the stage for more dynamic investigation of the relationship of the pathogen with its host(s) and other pathogenic agents, including the molecular, physiological, immunological and epidemiological mechanisms underlying the pathology at the individual bee and colony levels. It remains to be determined whether ABPV, KBV, IAPV and other variants within this complex are autonomous viruses meriting species status, or strains of single species (van Regenmortel et al., 2000). Since there are no clear geographical, temporal, or ecological separation between ABPV, KBV and IAPV, species designation will depend heavily on the unique biological and molecular characteristics of these viruses, such as the specificity of viral replication, protease processing and encapsidation, to support their clear phylogenetic separation. This close genetic relationship between the viruses is also a useful resource for mapping any biological differences between the viruses on the viral genome. An additional area to develop is the search for genetic resistance and cures to viral diseases. Due to the far-reaching implications it is essential that the stable integration of virus sequences in the honey bee genome is confirmed by the bee research community, as well as their possible interference with virus replication and pathology. Similarly, the potential universal applicability of RNAi technology to provide virus-specific solutions to infection and pathology requires further investigation, primarily to determine the mechanism of action, possible secondary effects and limitations, in order to optimise the procedure and applicability.

The rapid progress of our diagnostic capabilities is likely to continue apace. The challenge for the honey bee research community is to exploit emerging diagnostic platforms to fill considerable knowledge gaps in dicistrovirus biology. Methods which offer multi-target screening, using hybridisation (microarrays) or real-time (RT) PCR 'macroarrays' are powerful tools that can be applied to monitor viral populations within a single host or even host responses to virus challenge (e.g. Evans, 2007). The collection of host transcription data could lead to the recognition of effective 'biomarkers' to help monitor the impact of virus infection. Suites of biomarkers could help build a picture of virus disease signatures, thus providing additional evidence to determine whether ABPV, KBV and IAPV merit species designation based on differing biological properties.

Current diagnostics simply offer beekeepers confirmation of viral presence without informing management practice. Basic management information such as actual virus impact, likely routes of infection onto the apiary (e.g. bees, food, wax and equipment) and effective methods of eradication (once infection occurs) are lacking. The future challenge is to develop efficient diagnostics within a decision support system that would allow beekeepers to make informed management decisions based on the result of the diagnostic test(s) employed. The development of onsite viral diagnostics, like lateral flow devices (Tomkies et al., 2009), could empower beekeepers to deal with virus infection within such a framework, ensuring the impact of diagnostics is maximised, whilst the colony losses incurred by dicistroviruses are minimised.

Conflicts of interest

There are no conflicts of interest to be declared.

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