

Short Communication

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Genetic characterization of slow bee paralysis virus of the honeybee (*Apis mellifera* L.)

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Complete genome sequences were determined for two distinct strains of slow bee paralysis virus (SBPV) of honeybees (*Apis mellifera*). The SBPV genome is approximately 9.5 kb long and contains a single ORF flanked by 5'- and 3'-UTRs and a naturally polyadenylated 3' tail, with a genome organization typical of members of the family *Flaviviridae*. The two strains, labelled 'Rothamsted' and 'Harpenden', are 83% identical at the nucleotide level (94% identical at the amino acid level), although this variation is distributed unevenly over the genome. The two strains were found to co-exist at different proportions in two independently propagated SBPV preparations. The natural prevalence of SBPV for 847 colonies in 162 apiaries across five European countries was <2%, with positive samples found only in England and Switzerland, in colonies with variable degrees of *Varroa* infestation.

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Slow bee paralysis virus (SBPV) is one of several honeybee (*Apis mellifera*) viruses linked to high mortality of colonies infested with the ectoparasitic mite *Varroa destructor* (Carreck *et al.*, 2010; Martin *et al.*, 1998). SBPV was discovered fortuitously in England in 1974, during propagation experiments involving bee virus X. It induces paralysis of the

anterior two pairs of legs about 10 days after injection into the abdomen of adult bees (Bailey & Woods, 1974), with high virus accumulation in the head, the hypopharyngeal, mandibular and salivary glands, the fat body, crop and forelegs, but less in the hindlegs, midgut, rectum and thorax (Denholm, 1999). Like most honeybee viruses, SBPV persists naturally as a covert infection, most likely through oral transmission (Bailey & Ball, 1991). However, SBPV can be transmitted readily among adult bees and to pupae by *Varroa* mites (Santillán-Galicia *et al.*, 2010), with lethal consequences at the individual bee and colony levels (Carreck *et al.*, 2010). Overt SBPV infection can also be induced in adult bees (but not in pupae) by injection with inert fluids, especially the

The GenBank/EMBL/DDBJ accession numbers for the complete genome sequences of SBPV strains Rothamsted and Harpenden are EU035616 and GU938761, respectively.

A supplementary table showing primers and performance indicators of several universal and strain-specific RT-qPCR assays is available with the online version of this paper.

anticoagulant apyrase, and the proportion of bees with inducible covert SBPV peaks during mid-summer (Denholm, 1999).

SBPV has a 30 nm icosahedral particle containing an RNA genome and three major capsid proteins, of 46, 29 and 27 kDa (Bailey & Ball, 1991). The virion profile, induction in pupae and mite-mediated transmission are key characteristics that SBPV shares with the other picorna-like viruses associated with *Varroa*-induced colony mortality (Ribi re *et al.*, 2008). However, unlike these other viruses, SBPV appears to be extremely rare, having been identified positively only in Britain, Fiji and Western Samoa (Allen & Ball, 1996; Anderson, 1990; Carreck *et al.*, 2010; Martin *et al.*, 1998), despite being included in surveys of Australia (Hornitzky, 1987), New Zealand (Todd *et al.*, 2007), Scandinavia (Nordstr m *et al.*, 1999) and Poland (Topolska *et al.*, 1995). Only in Britain has it ever been associated with colony mortality (Carreck *et al.*, 2010).

Two SBPV preparations were used for genetic characterization, both derived from the original English SBPV isolate (Bailey & Woods, 1974); one was produced in 1994 in Canada and the other in 2006 in Sweden. SBPV was propagated in 50 pupae and purified by using either CsCl (Canada) or sucrose (Sweden) gradient centrifugation (Bailey & Ball, 1991; Stoltz *et al.*, 1995). Samples from both purifications were resolved by SDS-PAGE and electroblotted to PVDF membranes for N-terminal sequencing of the individual proteins (Alphalyse, Denmark) and, for Western blots, probed with IgG of the original SBPV antiserum (Bailey & Woods, 1974) and developed with 250 ng alkaline phosphatase-conjugated protein A ml⁻¹ (Harlow & Lane, 1988). The Canadian preparation contained seven different proteins (Fig. 1a, b). However, several of these are of uncertain origin, as the Swedish preparation lacked P4 and P6, whilst P1 appeared only in the lighter fractions (Fig. 1c). Those proteins present in both preparations (P2, P3, P5 and P7) are comparable in size to those described in the original reports (Bailey & Ball, 1991). Only P2 and P3 reacted with the SBPV antiserum (Fig. 1b). As their N termini were also identical (DNPPDP), they are essentially the same protein, most likely processed differently at the C terminus. The P5 and P6 N-terminal sequences were ambiguous, whereas those of P4 and P7 were blocked. There was progressive enrichment of P2 relative to P3 in the lighter gradient fractions (Fig. 1b, c, lanes 4–5), suggesting that the P2–P3 size difference affects virion density.

Viral RNA was extracted from both SBPV preparations with the RNeasy system (Qiagen), converted to random-primed double-stranded cDNA, cloned and sequenced, resulting in a series of ‘footprints’ covering the entire genome. The intervening regions were amplified by RT-PCR using sequence-specific primers. The 3′ terminus was obtained by RT-PCR with anchored oligo-dT, priming the natural SBPV poly(A) tail, and a sequence-specific upstream primer. The 5′ terminus was approximated by

semi-nested 5′ RACE, using the SMART-Oligo protocol (Clontech). All RT-PCR fragments were cloned and at least three independent clones were sequenced for each fragment (ten for the 5′ RACE). During this process, a number of clones were identified that were highly divergent from the principal sequence, but consistent among each other, indicating the presence of a distinct strain of SBPV. Strain-specific primers were used to amplify between successive clones of this second strain and these PCR products were sequenced directly. With no obvious biological, geographical or historical criteria to distinguish them, these strains were labelled ‘Rothamsted’ and ‘Harpenden’, respectively, after the Rothamsted Research Institute in Harpenden, England, where SBPV was first described. The overall sequencing redundancy for both strains was 3.64 genome equivalents.

The SBPV genome is approximately 9500 nt long, contains a 2964 aa ORF flanked by approximately 300 nt of 5′-UTR and approximately 270 nt of 3′-UTR, and is terminated by a natural 3′ poly(A) tail. The structural proteins are encoded in the N-terminal part of the ORF and the non-structural proteins in the C-terminal part (Fig. 2). This genome organization is typical of members of the *Iflaviridae*, an insect-specific virus family also containing *Deformed wing virus* (DWV), *Varroa destructor virus-1* (VDV-1) and *Sacbrood virus* (SBV). The polyprotein is processed into functional units by proteolysis. The P2/P3 N-terminal sequence matches the start of VP1 (Fig. 2), thus connecting the RNA sequence to the SBPV antiserum. As only P2 and P3 react with the SBPV antiserum, VP1 harbours most of the virion’s antigenicity, similar to the DWV/VDV-1 VP1 (Lanzi *et al.*, 2006; Ongus *et al.*, 2004; B. V. Ball, unpublished data), suggesting that VP1 is exposed on the virion surface, with VP2–VP3 located more internally. The N-terminal sequences also identify an autocatalytic protease activity, associated with RNA packaging during the final stages of virion maturation (Nakashima & Uchiumi, 2009). The SBPV-encoded 3C protease provides the remaining protease activity, with an AxPE/M consensus cleavage sequence. This is identical to the DWV/VDV-1 3C protease-cleavage sequence (Lanzi *et al.*, 2006) and typical for viral 3C proteases, which cut at glutamine (Q) or glutamate (E), with methionine (M), proline (P) and alanine (A) also common in the shown positions (Palmenberg, 1990). The predicted sites are ideally located for releasing the functional proteins (Fig. 2), with excellent agreement between the predicted and observed VP1–VP3 molecular masses.

Nineteen stably conserved amino acid regions (a–s) were identified by CORE analyses of M-Coffee consensual sequence alignments (Moretti *et al.*, 2007; Wallace *et al.*, 2006) involving members of the families *Iflaviridae*, *Dicistroviridae* and *Picornaviridae*. The functional domains were identified by using InterProScan (Quevillon *et al.*, 2005) and the CATH protein database (Pearl *et al.*, 2003). These included two picornavirus capsid protein domains (a–b and c–d), the three helicase domains (f–h), the 3C protease domains (j–k),

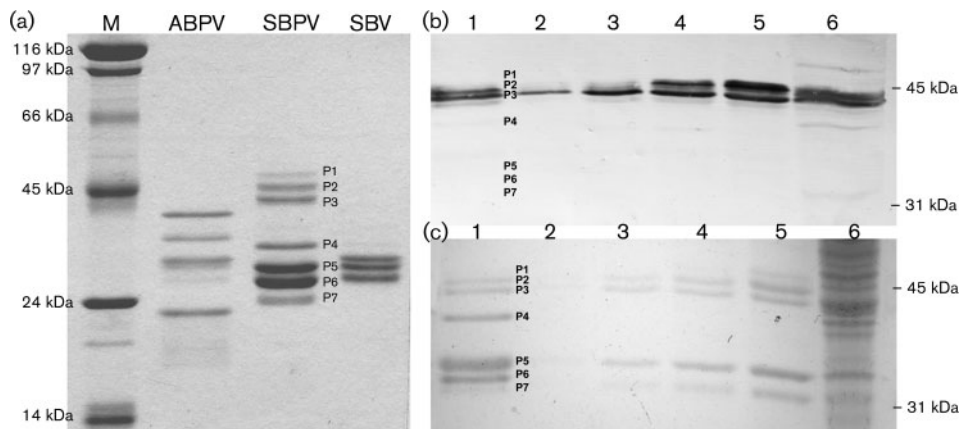


Fig. 1. (a) SDS-PAGE of the Canadian SBPV preparation, flanked by purified acute bee paralysis virus (ABPV) (43.0, 35.0, 33.0, 24.0 kDa), SBV (29.5, 30.5, 31.5 kDa) and molecular mass markers (M). (b, c) Western blot (b) and SDS-PAGE (c) of purified Canadian SBPV (lane 1), alternate Swedish SBPV gradient fractions (lanes 2–5) and crude SBPV propagation extract (lane 6).

including the GxCG²³⁷⁹ cysteine-protease motif (Ryan & Flint, 1997) and the GxHxxG²⁴⁰¹ substrate-binding motif (Gorbalenya *et al.*, 1989), and the eight RNA polymerase domains (o–s), including the DxxxxxD²⁷⁰⁹, GxxxTxxxN²⁷⁸³ and YGDD²⁸²³ motifs involved in NTP binding and catalysis (Gorbalenya *et al.*, 2002; Ng *et al.*, 2008). The functions of

the other conserved regions (e, i, l, m and n) are currently unknown. Signal peptide and transmembrane regions were identified at the helicase N terminus, prior to the 3C protease–RNA-dependent RNA polymerase (RdRp) complex, and in the unidentified protein between VP2 and the helicase. These observations suggest that genome replication,

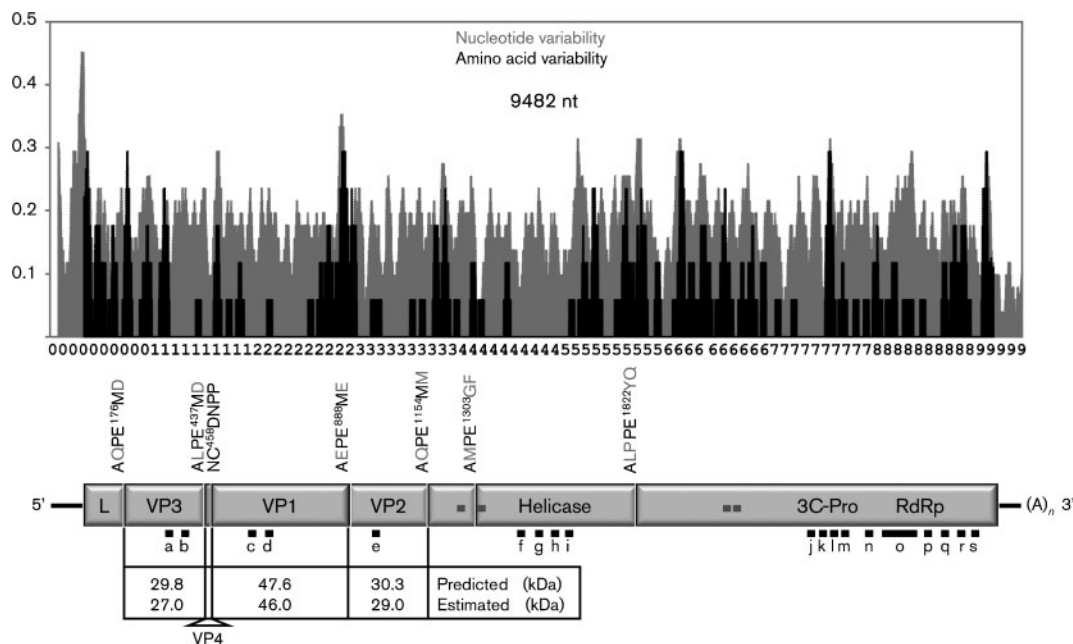


Fig. 2. SBPV genome organization and distribution of SBPV^{Roth}/SBPV^{Harp} variation, calculated over 51 nt or 17 aa intervals, with the digits marking 100 nt intervals. Indicated are those proteins with identified function, the protease-cleavage sites (consensus amino acids in black font), the estimated and predicted VP1–VP3 molecular masses, four transmembrane/signal peptide domains (grey bars) and 19 highly conserved regions (black bars) covering aa 290–310 (a), aa 336–344 (b), aa 576–609 (c), aa 628–647 (d), aa 986–998 (e), aa 1473–1493 (f), aa 1513–1536 (g), aa 1565–1587 (h), aa 1598–1605 (i), aa 2376–2381 (j), aa 2393–2401 (k), aa 2406–2414 (l), aa 2461–2473 (m), aa 2544–2574 (n), aa 2622–2721 (o), aa 2757–2795 (p), aa 2811–2857 (q), aa 2864–2878 (r) and aa 2887–2905 (s).

translation and proteolysis are synchronized, membrane-bound processes (Salonen *et al.*, 2005). The L-protein is quite variable at the amino acid level and is highly basic (pI=9.1), whereas the four capsid proteins are all acidic (pI=5.3–5.8), traits that are common to all honeybee-infecting iflaviruses. Picornavirus L-proteins are associated with virulence and pathology, as they affect host and virus RNA translation and protease activity (Glaser *et al.*, 2001; Guarné *et al.*, 1998; Hinton *et al.*, 2002). Not identified, although expected to be present, was a viral genome-linked protein (VPg), a highly heterogeneous class of small proteins bound covalently to the 5' terminus of most RNA viruses (including iflaviruses) and involved in RNA stability, genome replication, translation and movement (Hébrard *et al.*, 2009; Ng *et al.*, 2008; Steil & Barton, 2009). These processes also involve the 5'- and 3'-UTRs, as well as numerous host factors (Belsham, 2009). Translation is probably initiated by an internal ribosome entry site (IRES) in the 5'-UTR (Ongus *et al.*, 2006; Roberts

& Gropelli, 2009), thus avoiding the host's cap-dependent translation (Belsham, 2009). The first amino acid is probably methionine (Belsham, 2009), although this is not obligatory for IRES-mediated translation (Jan, 2006).

The two SBPV strains are 83% identical at the nucleotide level (94% identical at the amino acid level), whilst internally, each strain is >99% identical. Eighty-two per cent of the nucleotide differences were C–U or A–G transitions, which are common for RNA viruses, due to the relative stability of G–U bonds during replication (Roossinck, 1997). The variation is distributed unevenly over the genome, with the 5'-UTR being particularly variable, containing significant insertions/deletions just before the ORF. The extent and distribution of the variation resemble those for DWV/VDV-1 (de Miranda & Genersch, 2010; Lanzi *et al.*, 2006) and SBV. The relationship of SBPV to other viruses was determined through phylogenetic

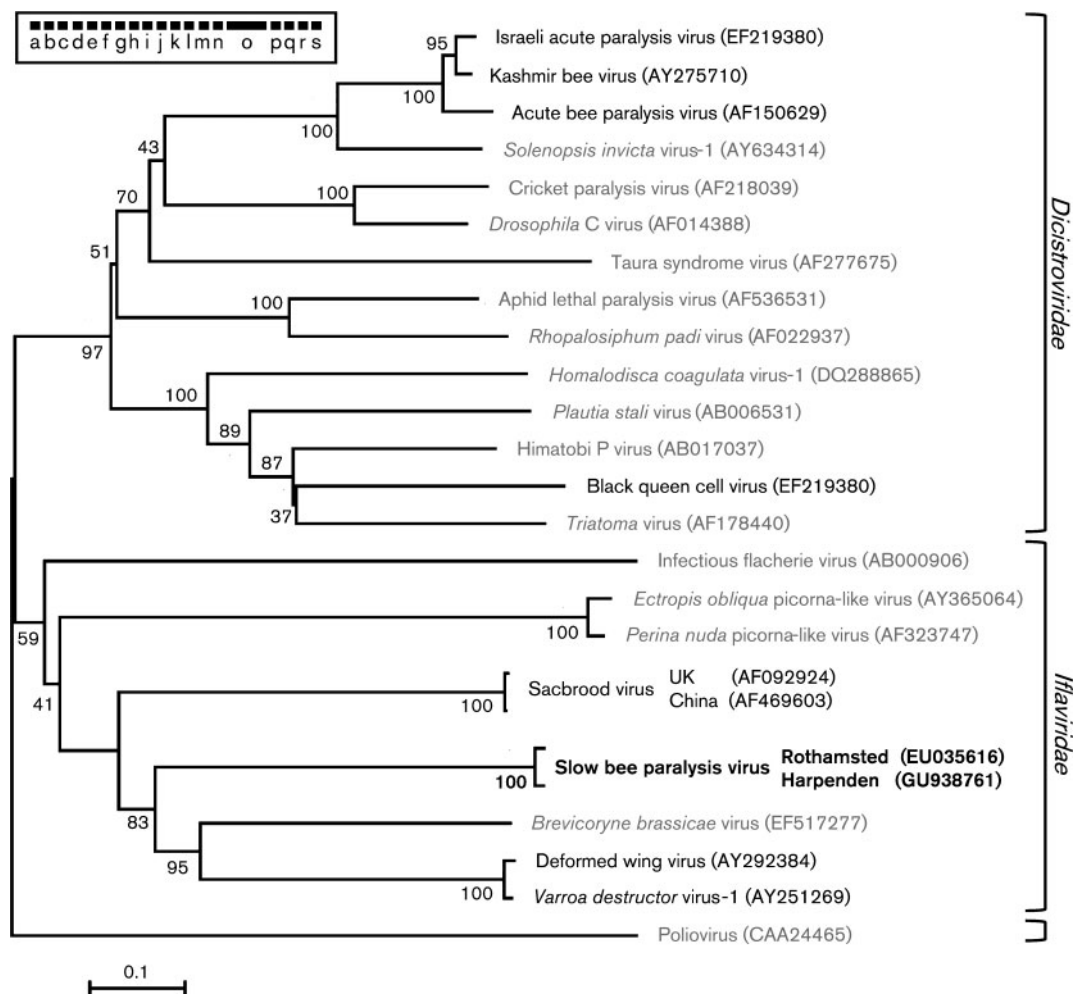


Fig. 3. Minimum evolution phylogram of members of the families *Iflaviridae* and *Dicistroviridae*, using poliovirus (family *Picornaviridae*) as outgroup, based on conserved regions a–s (Fig. 2). The percentage bootstrap support for each partition is based on 500 replicates. Bar, 0.1 amino acid substitutions per site. Viruses infecting honeybees are in black font. GenBank accession numbers are shown in parentheses.

analyses of the 19 conserved amino acid regions, using minimum evolution criteria as implemented in MEGA4 (Tamura *et al.*, 2007). Despite the conservative M-Coffee approach to identifying consistent phylogenetic characters with strong positional homology, the relationships between the taxa remained unstable, as shown by the deep, clustered nodes with low bootstrap support (Fig. 3). The inclusion/exclusion of certain viruses or genomic regions had a profound effect on the perceived relationships between the taxa, and the most reliable phylogenies were obtained with the helicase (f-h) and RdRp I-IV (o) domains. Such 'star' phylogenies are common for viruses and are due to the extreme variability and adaptability of RNA viral genomes, which include extensive cross-species recombination, evolutionary convergence and other forms of genetic mosaicism (Koonin *et al.*, 2006, 2008; Roossinck, 2002).

Several RT-PCR assays were developed for SBPV detection and quantification (see Supplementary Table S1, available in JGV Online). All assays were optimized for primer concentration and annealing temperature, whilst the strain-specific assays A^{Roth} and A^{Harp} were also optimized for the absence of cross-amplification, using cloned SBPV^{Roth} and SBPV^{Harp} templates. Assays A, A^{Roth}, A^{Harp} and B contained 0.2 µM of each primer in 20 µl Bio-Rad SYBR-green OneStep RT-qPCR mixture. The template was either approximately 2 ng random-primed cDNA, produced according to Tentcheva *et al.* (2004), or approximately 200 ng RNA purified from bulk homogenate with the RNeasy system (Qiagen) and converted to cDNA for 10 min at 50 °C prior to qPCR. The qPCR profile was 5 min at 95 °C plus 40 cycles of 10 s at 95 °C, 30 s at 58 °C, 5 s fluorescence reading. The C_q values were determined by Opticon Monitor3 (Bio-Rad) at a fluorescence threshold of 0.025, after global minimum baseline subtraction. Product specificity was determined by melting-curve analysis of 60 s at 95 °C, 60 s at 55 °C, followed by fluorescence reading at 0.5 °C increments from 55 to 95 °C (Bustin *et al.*, 2009). For assay C, RNA was purified from bulk homogenate with the RNeasy system and amplified with the RT-qPCR cycling profile of Chantawannakul *et al.* (2006). Assay D is qualitative, based on the RNA polymerase region.

Quadruplicate runs of assays A, A^{Roth} and A^{Harp} were used to determine that SBPV^{Roth} and SBPV^{Harp} constituted 86.0 and 13.5% ($\pm 2.6\%$) of the Canadian preparation, and 66.3 and 32.8% ($\pm 7.8\%$) of the Swedish preparation, respectively. This means that SBPV^{Roth} and SBPV^{Harp} co-exist as a major strain polymorphism within these propagated preparations. This is analogous to the sequence polymorphisms found naturally within the DWV/VDV-1 and acute bee paralysis virus (ABPV)/Kashmir bee virus/Israeli acute paralysis virus complexes (de Miranda & Genersch, 2010; de Miranda *et al.*, 2010) and to the serological similarities between bee viruses X and Y, Arkansas and Berkeley bee virus, and Egypt bee virus/DWV/VDV-1 (Bailey & Ball, 1991). Such co-existing strain complexes may therefore be a common feature of honeybee viruses.

The natural prevalence of SBPV in France (2002), Switzerland (2007, 2008), Sweden (2008) and England/Wales (2008) was determined. The French survey comprised 360 colonies in 36 apiaries throughout France, all actively managed for *Varroa* control, sampled in spring, summer and autumn of 2002 for 100 adult bees, 30 pupae and 100 *Varroa* mites per colony, per occasion (Tentcheva *et al.*, 2004). SBPV was not detected in any sample with assay A, using cDNA template. The Swiss 2007 survey comprised 87 colonies in three apiaries, actively managed for *Varroa* control, sampled in April 2007 for 100 adult bees per colony. The Swiss survey in 2008 comprised 29 colonies in two apiaries, all without *Varroa* control, heavily infested with mites and dying, sampled in November 2008 and January 2009 for 100 adult bees per colony. SBPV was not detected in 2007, but five dying colonies in November 2008 and three dying colonies in January 2009 contained SBPV, as detected with assay B and confirmed with assay A, using cDNA template. The Swedish survey comprised nine colonies in one apiary, heavily *Varroa*-infested and unmanaged, sampled in August 2008 for 30 adult bees, five mite-infested pupae, the five corresponding mites and five non-infested pupae per colony. SBPV was not detected in any sample with assay A, using RNA template. The English/Welsh survey comprised 360 colonies in 120 apiaries throughout England/Wales with varying degrees of *Varroa* management, sampled during summer 2008 for 40 brood-chamber adult bees per colony, three colonies per apiary and pooled by apiary. SBPV was detected with assay C and confirmed with assay D, using RNA template, in four apiaries with variable degrees of *Varroa* infestation and inconsistent colony-level disease symptoms. The positive RT-PCR products from Switzerland and England/Wales were sequenced directly. These were >98% identical to SBPV^{Roth}, the dominant strain and therefore likely to mask any evidence of SBPV^{Harp}. These surveys confirm the low natural prevalence of SBPV across a large part of Europe (Carreck *et al.*, 2010). Although *V. destructor* is probably critical for SBPV-induced colony mortality (Carreck *et al.*, 2010; Santillán-Galicia *et al.*, 2010), these studies suggest that SBPV can persist in colonies with a range of *Varroa* infestation levels and disease symptoms.

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