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Deformed wing virus associated with *Tropilaelaps mercedesae* infesting European honey bees (*Apis mellifera*)

Eva Forsgren · Joachim R. de Miranda · Mats Isaksson · Shi Wei · Ingemar Fries

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Abstract Mites in the genus *Tropilaelaps* (Acari: Laelapidae) are ectoparasites of the brood of honey bees (*Apis* spp.). Different *Tropilaelaps* subspecies were originally described from *Apis dorsata*, but a host switch occurred to the Western honey bee, *Apis mellifera*, for which infestations can rapidly lead to colony death. *Tropilaelaps* is hence considered more dangerous to *A. mellifera* than the parasitic mite *Varroa destructor*. Honey bees are also infected by many different viruses, some of them associated with and vectored by *V. destructor*. In recent years, deformed wing virus (DWV) has become the most prevalent virus infection in honey bees associated with *V. destructor*. DWV is distributed world-wide, and found wherever the *Varroa* mite is found, although low levels of the virus can also be found in *Varroa* free colonies. The *Varroa* mite transmits viral particles when feeding on the haemolymph of pupae or adult bees. Both the *Tropilaelaps* mite and the *Varroa* mite feed on honey bee brood, but no observations of DWV in *Tropilaelaps* have so far been reported. In this study, quantitative real-time RT-PCR was used to show the presence of DWV in infested brood and *Tropilaelaps mercedesae* mites collected in China, and to demonstrate a close quantitative association between mite-infested pupae of *A. mellifera* and DWV infections. Phylogenetic analysis of the DWV sequences recovered from matching pupae and

E. Forsgren (✉) · J. R. de Miranda · I. Fries
Department of Ecology, Swedish University of Agricultural Sciences,
PO Box 7044, 75007 Uppsala, Sweden
e-mail: eva.forsgren@ekol.slu.se

J. R. de Miranda
School of Biological Sciences, Queen's University Belfast,
97 Lisburn Road, Belfast BT9 7BL, Northern Ireland

M. Isaksson
The National Veterinary Institute, Ulls väg 2 B, 75189 Uppsala, Sweden

S. Wei
Institute of Apiculture, Chinese Academy of Agricultural Sciences,
Xian Shan, 10000 Beijing, China

mites revealed considerable DWV sequence heterogeneity and polymorphism. These polymorphisms appeared to be associated with the individual brood cell, rather than with a particular host.

Keywords *Tropilaelaps mercedesae* · *Apis mellifera* · Deformed wing virus · qRT-PCR

Introduction

Deformed wing virus (DWV) is a virus with a single positive-stranded RNA genome belonging to the genus *Iflavirus* (Lanzi et al. 2006). Overt DWV infections are a major cause for colony collapse of honey bees (*Apis mellifera*) worldwide. Virus infections (i.e. DWV) in association with infestation by the Asian honey bee mite *Varroa destructor* have devastating effects on European honey bees (Ball 1989). A colony can collapse when mite populations become too large and many bees emerge with symptoms of DWV infection, i.e. malformed or missing wings and shortened abdomens (Bowen-Walker et al. 1999). The mites' feeding behaviour impairs the immune system of the bees (Yang and Cox-Foster 2005) and overt DWV infections are triggered and vectored by the infesting mites (Shen et al. 2005). DWV not only infects honey bees, since recent data indicate that DWV can also replicate within the *Varroa* mite (Ongus et al. 2004; Yue and Genersch 2005) and that bumble bees may also become infected, exhibiting symptoms of crippled wings (Genersch et al. 2006).

Another mite of Asian origin, *Tropilaelaps clareae*, whose natural host is the Giant honey bee (*Apis dorsata*; Delfinado and Baker 1963), can now also be found on the introduced European honey bee (*A. mellifera*) throughout Southeast Asia (de Jong et al. 1982). Heavy colony losses caused by *Tropilaelaps* have been reported (Burgett and Akratanakul 1985; Camphor et al. 2005), although infestations do not always result in colony mortality (Hosamani et al. 2006; Jitender and Sharma 2003). *Tropilaelaps* infestations may be considered more dangerous to *A. mellifera* than *Varroa* infestations, since the reproduction of the latter can be impaired in dual infestations (Rath et al. 1995). The *Tropilaelaps* mite cannot feed on adult bees and dies within a few days without access to brood (Woyke 1987), limiting its expansion to temperate climates with extended brood-free periods. Varying levels of resistance are probably partly responsible for the survival of infested colonies, since *A. mellifera* detects and removes a significant proportion of infested brood (Boecking et al. 1992). This behaviour is also an important reason why this mite does not cause serious damage to *Apis cerana* colonies (Sharma et al. 1992).

Colonies of European honey bees infested by *Tropilaelaps* can occasionally contain bees emerging with crippled wings (Burgett and Akratanakul 1985; Sharma et al. 1994; Sihag and Singh 1991), symptoms generally associated with DWV infections in European honey bee colonies infested by *V. destructor* (Yue and Genersch 2005; Tentcheva et al. 2006). To investigate if DWV could be linked to *Tropilaelaps* infestation of European honey bee colonies, we assayed brood and adult bees from one *Tropilaelaps* infested colony of *A. mellifera* for DWV. Moreover, since recently presented data redefines the *Tropilaelaps* reproducing on *A. mellifera* brood as two separate species (Anderson and Morgan 2007), we used these new species criteria to identify the *Tropilaelaps* in our samples as *T. mercedesae*.

Materials and methods

Sampling

Samples of brood and live adult worker bees were taken from one colony of *Apis mellifera* infested by *Tropilaelaps* in a permanent apiary 20 km south of Haikou, the capital of Hainan, the southern island province of China. Crippled adult worker bees were sampled from a comb with emerging brood and two hundred adult worker bees and 400 worker brood cells were sampled separately. Ten mite infested worker brood cells, infested with 1–6 mites (Fig. 1), seven uninfested pupae (controls), and five crippled and five asymptomatic worker bees were used for DWV analysis and for species determination of the *Tropilaelaps* mites. All bees and worker brood sampled were investigated for the presence of *V. destructor*. The brood and bee samples were kept on ice until they could be frozen, approximately 24 h post sampling. The samples remained frozen until analysed for DWV.

Species identification of *Tropilaelaps*

Ten *Tropilaelaps* mites were identified using both the mtDNA CO-I and nuclear ITS1-5.8S-ITS2 gene restriction enzyme digestion and sequencing analyses as described by Anderson and Morgan (2007).

RNA purification

The worker bees (asymptomatic $N = 5$, crippled bees $N = 5$), infested pupae ($N = 10$), their pooled mite samples ($N = 10$) and uninfested pupae ($N = 7$) were ground individually in liquid nitrogen. The adult bees and pupa were each resuspended in 400 µl RLT

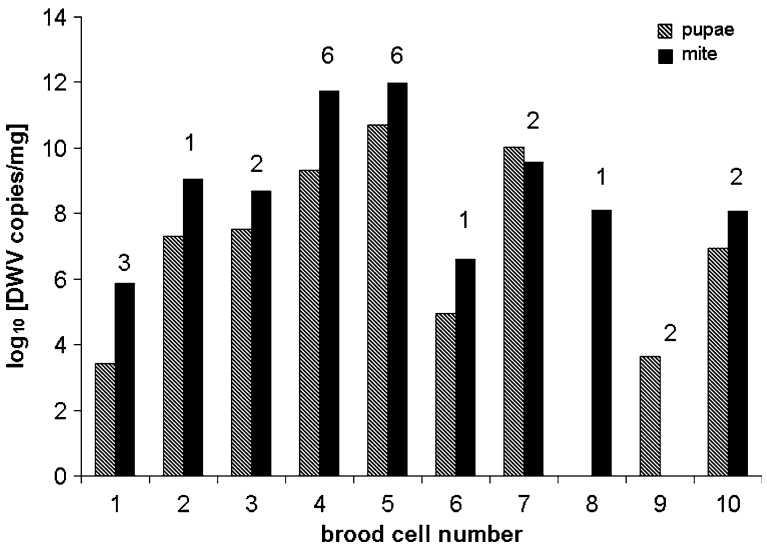

Fig. 1 Virus load ($\log_{10}$(DWV copies)/mg tissue) for pupae and their infesting mite(s) from 10 individual brood cells. The Pearson correlation coefficient was 0.83, $P = 0.003$. The numbers above the *bars* represent number of adult mites in the respective infested brood cell

buffer (RNEasy™ kit, Qiagen). RNA was extracted, using the RNEasy™ protocol (Qiagen), from 100 µl of each suspension. This corresponds to about 25 mg of tissue, since one individual adult bee or pupa weighs approximately 100 mg. Mites collected from the same brood cell were pooled and crushed and the ground tissue was resuspended in 100 µl RLT buffer before RNA extraction. Since each *Tropilaelaps* mite weighs approximately 0.1 mg and the number of mites per cell varied from one to six, the weight of the extracted tissue was 0.1–0.6 mg. RNA was eluted into 50 µl nuclease-free water and the nucleic acid concentration was determined using NanoDrop™ (NanoDrop). The number of mites analysed per brood cell is shown in Fig. 1.

qRT-PCR DWV detection

For quantitative detection of DWV, a Taqman® probe real-time qRT-PCR assay was designed, targeting the RNA dependent RNA polymerase gene of the virus. This is a highly conserved region of the DWV genome, and for both PCR primers, the 3′ nucleotide avoids the third codon position of the DWV open reading frame, minimising the risk of miss-amplification due to natural virus variation. The targeted fragment consists of 136 nucleotides, and the DWV-specific primers were: DWV-fwd (5′-TTCATTAAAGCCACC TGGAACATC-3′), DWV-rev (5′-TTTCCTCATTAACTGTGTCGTTGA-3′) and DWV-probe (5′ (6-FAM) TCAAGT (Dabcyl-dT) CGGGACGCATTCCACGC (Phosphate) 3′). A single-tube RT-PCR assay was performed containing 10 µl BioRad®iScriptP™MP master mix (2×), 0.4 µM of each primer and 0.2 µM DWV-probe (FAM reporter). Two microlitres of sample RNA were added, and the final reaction volume adjusted to 20 µl with HPLC grade water. The amplification and data acquisition were carried out using a Bio-Rad® MiniOpticon real-time PCR machine, under following cycling conditions: cDNA synthesis, 50°C for 10 min; reverse transcriptase inactivation, 95°C for 5 min; followed by 40 cycles of template denaturation at 95°C for 10 s and annealing and extension at 60°C for 30 s. Fluorescence was measured at 60°C for each cycle. An in vitro-transcribed recombinant RNA template was used for calibrating viral concentration, using serial dilutions ranging from 10^{10} to 10^{3} copies per ml as quantitation standards in every run. The quality of the RNA extracted from the bee samples was determined by amplifying the mRNA of the honey bee internal control gene RP49, as described by de Miranda and Fries (2008). Quantitative RNA controls have not yet been developed for *Tropilaelaps*. The quantitative data was normalised to milligram of adult bee or pupal tissue or to milligram of the corresponding pooled mite tissue.

Phylogenetic analyses

The DWV-Lp gene was chosen for phylogenetic studies, since it is one of the most variable regions of the DWV genome (Lanzi et al. 2006) and therefore well suited for investigating natural genetic diversity within DWV populations. The 694 nucleotide fragment was amplified separately from four individual pupae and from their corresponding mites (cells 2, 4, 5 and 6) using 3 µl pupal or mite RNA, the BioRad iScript™ One-Step RT-PCR kit, a cycling profile of cDNA synthesis at 50°C for 10 min; reverse transcriptase inactivation, 95°C for 5 min; followed by 40 cycles of template denaturation at 95°C for 10 s and annealing and extension at 55°C for 30 s. The assay was performed using 0.4 µM each of primers DWV-F1153 (ATTAAAAATGGCCTTTAGTTG) and DWV-B1806 (CTTTTCT AATTCAACTTCACC). These primers are highly conserved, allowing the recovery of most sequence variants within the DWV/VaDV-1 species complex (Lanzi et al. 2006;

Ongus et al. 2004). The products were purified on QiaQuick™ columns (Qiagen) and sequenced directly with the DWV-F1153 and DWV-B1806 primers. The sequences were analysed with SeqScape v2.5 (Applied Biosystems; Le et al. 2008) to identify instances of genetic polymorphism within the DWV sequences, edited manually to resolve conflicts and analysed phylogenetically as a minimum evolution phylogenetic tree using MEGA-4 (Tamura et al. 2007).

Statistics

The viral load data from infested pupa and their mites (per mg of extracted tissue) was tested for normal distribution and analysed by correlating the data sets (Pearson correlation).

Results

The restriction enzyme analyses clearly identified all investigated mites as *T. mercedesae* (data not shown), and results from sequencing both the mtDNA and nuclear gene are consistent with the results presented by Anderson and Morgan (2007) for *T. mercedesae*. No *V. destructor* mites could be found in the investigated brood or bee samples.

The method developed for DWV detection successfully allows quantification of the number of virus copies present in the investigated samples, using external RNA calibration standards. Standard curves were generated by duplicate amplifications of serial dilutions of recombinant RNA template, plotting the C_t values against copy number. The resulting calibration curves indicated an RT-PCR efficiency of between 96.8 and 98.2% and a linear detection range of between 10^3 and 10^{11} copies. The level of detection in the quantitative RT-PCR assay was estimated by assaying 10 replicates of samples containing 10, 10^2 and 10^3 copies of the RNA standard per reaction. One thousand copies of the standard RNA were detected in all 10 replicates. This method uses external recombinant RNA standards to calculate the number of viral copies in a sample, in preference to the use of PCR amplicons as external quantitation standards (Tentcheva et al. 2006) or to normalising virus loads relative to an exogenously added reference RNA (Tentcheva et al. 2006) or to normalising relative to the steady-state mRNA expression of a honeybee 'housekeeping' gene (Chen et al. 2005; de Miranda and Fries 2008). The RP49 mRNA control gene was successfully amplified from all honey bee RNA samples, confirming that the RNA in these samples was intact.

In 8 of the 10 mite-infested brood cells, both the pupae and matching mite samples were heavily infected with DWV. In brood cell-8 there was no detectable virus in the pupa even though the one infesting mite contained high titres of DWV. In brood cell-9 the converse was true; the pupa was infected but not the (two) infesting mites. The DWV load in mites, i.e. the number of viral copies detected in 1 mg of the pooled mite samples, was strongly correlated to the DWV load in the corresponding pupae, i.e. the number of viral copies detected in 1 mg of bee tissue (Pearson correlation 0.832, $P = 0.003$; Fig. 1). Furthermore, the mite DWV titre is generally far higher than the corresponding bee DWV titre, often by several orders of magnitude (Fig. 1). None of the seven uninfested pupae contained detectable viral loads. There was on average about 1,000 times more DWV in crippled adult bees ($N = 5$; $5.0 \pm 2.3 \times 10^8$ copies DWV/mg bee) than in asymptomatic adult bees ($N = 5$; $5.7 \pm 1.7 \times 10^5$ copies DWV/mg bee).

The DWV-Lp gene was sequenced for four brood cells of matching pupae and mites. For one of the brood cells (cell 6), both the pupal and the mite sample produced a single homogenous DWV sequence. However, the pupal and mite DWV sequences for the other three brood cells (cells 2, 4 and 5) were all heterogeneous. An example of this heterogeneity is shown in Fig. 2a, with the alternative sequence base-calling indicated above the primary sequence. Matching heterogeneous nucleotides were found on both the forward and reverse sequences in each case, indicating that this heterogeneity is due to a natural DWV sequence polymorphism, rather than due to sequencing artefacts. Furthermore, the

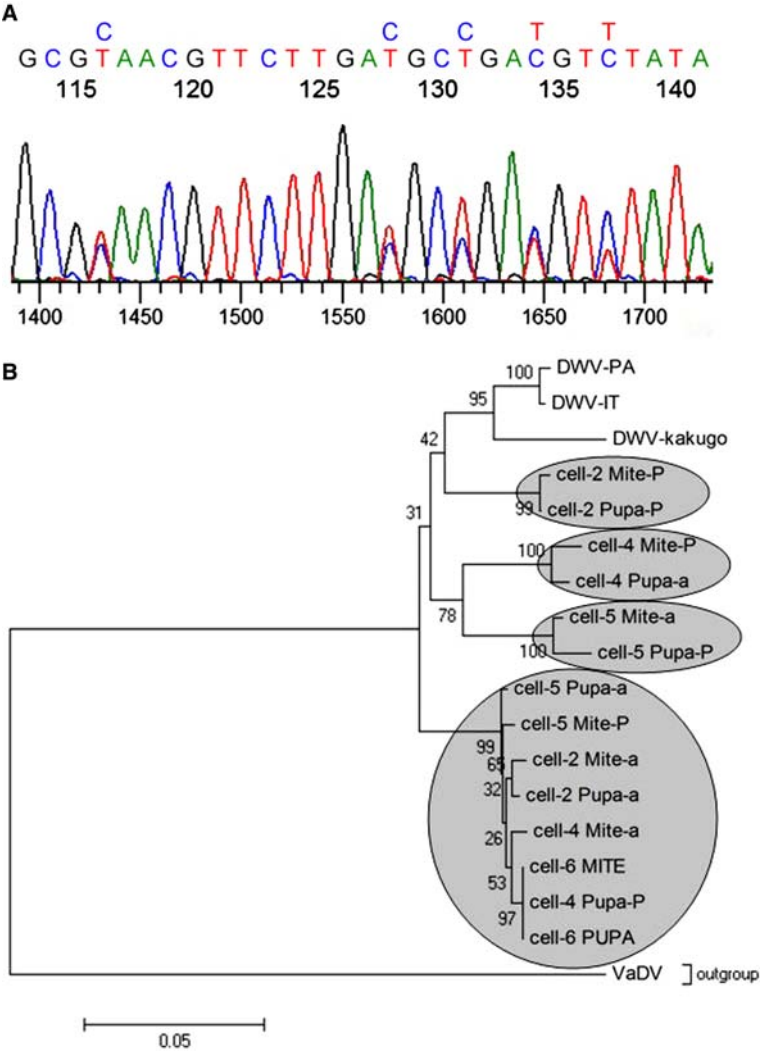

Fig. 2 **a** Illustration of sequence heterogeneity in an electropherogram. The types of substitution and triplet pattern suggest viral polymorphism. **b** Phylogenetic analysis of the polymorphic DWV sequences from four individual capped brood cells with *Tropilaelaps* mites and corresponding *Apis mellifera* pupae. The sequences are marked as primary (P) or alternate (a) according to the initial sequence reads. The statistical strength of the nodes is shown as the percentage of correct partitions in a 1,000 replicate bootstrap analysis

heterogeneous nucleotides were overwhelmingly located in the third codon positions of the DWV-Lp gene, and they usually involved transitions (C–U or A–G changes), both of which are well known features of natural variation in RNA virus populations (Roossinck 1997) including DWV (Lanzi et al. 2006).

By scanning the electropherograms for such heterogeneous nucleotides, using the SeqScape software (Le et al. 2008), and matching the forward and reverse sequences of both the primary ('P') and alternate ('a') sequences for each sample, the polymorphism in each sample could be translated into two sequences. The (few) discrepancies between the forward and reverse 'P' or 'a' sequences for each sample were resolved conservatively, i.e. towards the consensus sequence for all samples combined.

The relationships between these sequences are represented as a minimum evolution phylogenetic tree, with the confidence values associated with each node based on 1,000 bootstrap replicates (Fig. 2b). The DWV-Lp sequences from these pupae and *Tropilaelaps* mites are quite variable, and fall clearly between the DWV sequences from Japan ('Kakugo'; Fujiyuki et al. 2004), Pennsylvania (PA) and Italy (IT; Lanzi et al. 2006) and the VaDV-1 sequence from the Netherlands (Ongus et al. 2004). There is one universal sequence variant that is present in all pupae and mite samples, including those from cell 6 which produced only homogenous sequences for pupa and mite. These sequences constitute the larger *Tropilaelaps* DWV clade. This clade is well separated from the three smaller clades, each of which represents a unique polymorphic DWV sequence variant found in the pupal and mite samples of a single brood cell. The short branch lengths and low bootstrap values for some of the interior nodes are a reflection of the uncertain ancestry between the three smaller *Tropilaelaps* DWV clades, and among the essentially clonal sequences of the larger clade. In two of the three polymorphic brood cells, the primary sequence ('P') that predominates in the pupa is the alternate ('a'), minor sequence in the mite, i.e. there is a slight shift in the ratio of the universal and unique polymorphic sequences between the pupal samples and their corresponding mite samples.

Discussion

The results presented here strongly suggest that the presence of *T. mercedesae* in European honey bees is linked to infections of DWV in the host, as demonstrated previously with *Varroa* infestations. We find that the DWV viral load of mite infested pupae is positively correlated to the viral load of the corresponding infesting mites (Fig. 1). This clearly links DWV infection to *Tropilaelaps* infestations. The high virus titres in crippled bees ($\sim 10^8$ copies/mg bee) compared to the levels in asymptomatic bees ($\sim 10^5$ copies/mg bee) indicates that high DWV levels are also correlated with wing deformity in bee colonies parasitized by *Tropilaelaps*, much as they are for *Varroa* infested colonies (Yue and Genersch 2005; Tentcheva et al. 2006). The relatively high DWV infection rate detected in asymptomatic adult bees in the sampled colony, especially when compared to the absence of detectable DWV in non-infested pupae, can have several causes. Since *Tropilaelaps* does not feed on adult bees, direct virus vectoring by the mites between adult bees can be excluded. However, not all pupal DWV infections lead to crippled bees (Yue and Genersch 2005), producing a significant proportion of DWV infected asymptomatic adults that can be correlated to the high infestation rate of the investigated brood (77%). Furthermore, the combination of oral DWV transmission between adult bees (Chen et al. 2006), primed by the removal and cannibalism of mite-infested pupae (Boecking et al. 1992) will also help to maintain detectable DWV levels among asymptomatic adult bees,

again related to brood infestation rates. In summary, it is highly probable that the damages in *A. mellifera* associated with infestations of *T. mercedesae* are due to virus infections (DWV infections in particular), similar to the interaction between DWV, *A. mellifera* and *V. destructor* (Martin et al. 1998) and that reported heavy losses of European honey bees infested by *Tropilaelaps* mites (Burgett and Akratanakul 1985; Camphor et al. 2005) are also driven by virus infections. One of the investigated pupae contained DWV, whereas its two infesting mites lacked detectable virus titres. There are several possible explanations for this. It may be that this mite RNA sample was not intact, or contained RT-PCR inhibiting material. This is something we could not ascertain, due to the absence of *Tropilaelaps* mRNA internal control primers. Another explanation is that this pupa acquired DWV through an alternative route of infection. This is in slight contradiction to the apparent absence of DWV in the seven non-infested pupae tested, although the existence of such alternative DWV transmission routes in *A. mellifera* is well established (Yue and Genersch 2005; Chen et al. 2006; Yue et al. 2007; de Miranda and Fries 2008). The high DWV titres in the infesting mites, compared to those of the corresponding pupae (Fig. 1), strongly suggest viral replication in the mites. Direct evidence of DWV replication in *Tropilaelaps* mites has been shown in an independent study of DWV in *Tropilaelaps* infested *A. mellifera* colonies (Dainat et al. 2008). Again, this appears to be similar to the *V. destructor*—DWV association, where there is also direct evidence for virus replication in the mite host (Ongus et al. 2004; Yue and Genersch 2005). In the *V. destructor* mite, virus replication in mites was correlated with wing deformity in bees (Yue and Genersch 2005). High DWV levels in *Varroa* mites are generally associated with high levels of DWV in corresponding pupae (Bowen-Walker et al. 1999; Nordström 2000). This general correlation was also observed in this study (Fig. 1). Even so, one of the pupae had no detectable DWV infection although it was infested with a *Tropilaelaps* mite with high virus levels, and one DWV infected pupa was infested with apparently DWV-free mites. These observations show that, despite an overall close correlation between the virus levels in mite infested pupae and infesting mites, DWV infected mites do not always transmit a virus infection to the pupal host, nor does an infesting mite inevitably acquire detectable virus by feeding on an infected pupa.

The polymorphisms detected in the DWV-Lp gene in matching samples of *A. mellifera* pupae and *T. mercedesae* mites are not surprising. The DWV-Lp gene is one of the most variable regions of the DWV genome (Lanzi et al. 2006) and therefore well suited for investigating natural genetic diversity within DWV populations. Phylogenetic analysis of the polymorphic DWV-Lp sequences from several pupae and their associated mites revealed some interesting observations. The first is that these sequences fall well outside the group of known DWV-Lp sequences, from *Apis mellifera* in Japan, Italy and the USA, widening the sequence diversity for this virus and raising the possibility that other variants could be found to bridge the sequence space between DWV and VaDV-1. The second observation is that there is considerable sequence variation within such a limited sample range (four individual brood cells from a single colony), even without taking the polymorphic nature of the samples into account (i.e. comparing only the primary sequences). Both these observations contrast with two previous DWV phylogenetic studies, where the DWV sequences were largely clonal with the USA/Italy sequences, in the world-wide geographic survey of Berenyi et al. (2007), or with the Japan 'Kakugo' sequence, in the worker bee tissue and function survey of Fujiyuki et al. (2006). Berenyi et al. (2007) attributed the lack of divergence to the world-wide trade in bees and bee products, while Fujiyuki et al. (2006) ascribed it to sub-strains of a single 'Kakugo' quasi-species in their colonies. Both these groups used primers designed to amplify only within the DWV

sequence space, as defined by the USA, Italy and Japan 'Kakugo' sequences, and so may have missed strains that lie outside that space, such as the ones found in this study. The use of bulk versus individual samples is another possible explanation for the differences between the studies in sequence divergence and variation. Had we pooled our pupae and mite samples prior to sequencing, as Berenyi et al. (2007) and Fujiyuki et al. (2006) did, all of the polymorphic sequences unique to each brood cell would have disappeared in the background, leaving only the universal sequence common to all samples, which is far more uniform (Fig. 2b). Indeed, Fujiyuki et al. (2006) also obtained heterogeneous sequences when analysing individual, rather than bulk bee samples. This means that the variability of the DWV quasi-species perhaps can be accessed better through individual samples than by bulk samples. The similar polymorphisms of matching pupae and mites suggest that the engine for variation is the individual brood cell, rather than the differences between the pupal and mite hosts/vectors. The effect of different hosts is at best expressed as a minor shift in the polymorphism in each brood cell. Through the events in individual brood cells, and transmission by the mites, both the universal and unique polymorphic sequences can be efficiently maintained within the DWV quasi-species. The absence of significant differences between bee and mite derived virus sequences has also been found for *Varroa* mites, for both DWV (Fujiyuki et al. 2006) and Kashmir bee virus (Hung et al. 2000; Chen et al. 2004). Finally, these results are consistent with the broader picture of honey bee virus diversity, with new variants filling the sequence space of the ABPV-KBV-IAPV species complex (de Miranda et al. 2004; Todd et al. 2007; Bakonyi et al. 2002; Maori et al. 2007; Chen and Evans 2007), SBV (Grabensteiner et al. 2001), and to a lesser degree for CBPV (Blanchard et al. 2008). Qualitative, genetic differences within the DWV population such as the one described here were inferred by Yue and Genersch (2005) to explain the correlation between DWV replication in *Varroa* mites and pathology in bees. It remains to be determined whether replication of DWV in *T. mercedesae* mites is also specifically correlated with wing deformities in *A. mellifera*.

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