Nucleotide sequence variations may be associated with virulence of deformed wing virus

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Abstract – Western honey bees (*Apis mellifera*) are key players in crop pollination and in the maintenance of global biodiversity. Their viability is threatened by *Varroa destructor*, which acts as a vector of the deformed wing virus (DWV). Several genetic DWV variants have been reported, but it is unclear whether their virulence differs. We examined the prevalence of *V. destructor* and DWV as well as bee health in two colonies over 21 months and then characterizing DWV variants from each colony using phylogenetics. Colony H showed no signs of disease or mortality, and DWV sequence from this colony clustered with VDV/DWV-B sequences previously reported in healthy colonies. Colony W showed DWV symptoms, and DWV sequence clustered with DWV-A sequences previously reported in colonies with symptoms. These results suggest that nucleotide variations in the DWV genome can affect its virulence. Genotyping DWV variants in colonies may be an effective tool to assess risk and initiate preventive measures early.

deformed wing virus / *V. destructor* / virus virulence / phylogeny / honey bee

1. INTRODUCTION

Deformed wing virus (DWV), a single-stranded, positive-sense RNA virus of the genus *Iflavirus* (Genersch and Aubert 2010; Ongus et al. 2004), has become one of the most challenging honey bee pathogens. Historically, DWV did not represent a serious threat to honey bee colonies because it could persist as a covert infection without causing apparent symptoms. However, when carried by the globally prevalent ectoparasitic mite *Varroa destructor* (Wilfert et al. 2016),

infection easily becomes overt and the honey bees can show wing deformity, shortened abdomen, and reduced life span (de Miranda and Genersch 2010). The combination of *V. destructor* and DWV has contributed to substantial death of honey bee colonies worldwide (Guzmán-Novoa et al. 2010; Martin et al. 2012; Thompson et al. 2014). DWV is capable of replicating within the mite, in such a way that *V. destructor* acts as a biological vector (Martin 2001; Shen et al. 2005). In addition to transmitting viruses such as DWV, the mites externally digest and consume fat body tissue (Ramsey et al. 2019) and feed on honey bee hemolymph, causing weight loss at individual level (Yang and Cox-Foster 2005). At colony level, the mite renders the colony more vulnerable to viral infection and leads to lower vitellogenin levels, which can reduce survival during overwintering (Amdam et al. 2004).

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The mite appears to have created a particularly effective mode of DWV transmission because the mite's feeding behavior means that DWV infects honey bees at the pupal stage, which is more likely to lead to viral loads $> 10^{10}$ genome equivalent copy/bee that are usually associated with morphological DWV symptoms (Gisder et al. 2009; Möckel et al. 2011). Recent studies have shown that *V. destructor* has introduced a new viral transmission route which has transformed the viral landscape, dramatically decreasing DWV diversity (Martin et al. 2012). Recent studies have linked these viral landscape changes with a selection of a virulent recombinant strain of DWV denominated as DWV-A, which replicates to high levels in honey bees only when directly inoculated into hemolymph by *V. destructor* or experimental injection (Kevill et al. 2017; Mordecai et al. 2016b; Ryabov et al. 2014). This virulent recombinant form of DWV may predispose developing larvae and pupae to developing deformed wing symptoms and, consequently, reducing productivity and their life expectancy. For example, a study carried out by Mordecai et al. (2016a) proposed a phenomenon known as superinfection exclusion in honey bees from Swindon, UK (Mordecai et al. 2016a). Since its discovery, DWV-A has been sub-classified into two types, DWV (Lanzi et al. 2006) and Kakugo virus (KV) (Fujiiyuki et al. 2004). According to demarcation criteria, DWV-B, originally called VDV-1 (Mordecai et al. 2016a), shows ~84% nucleotide identity to DWV-A and has been shown to replicate in *V. destructor* and honey bees (Ongus et al. 2004; Zioni et al. 2011). Recently, Mordecai et al. (2016) have reported the DWV-C, which is a third established variant that has been reported to contribute to winter colony losses (Kevill et al. 2017). It also suggested that the DWV type C has not recently emerged, but also is an established DWV variant. However, the virus has a high ability to mutate and recombine, which hampers the analysis of the sequences.

Several previous studies have established that genomic variations in DWV can affect tropism, pathogenicity, and epidemiology (Gisder et al. 2018; Möckel et al. 2011). This implies that genetic analysis of DWV in honey bee colonies may allow the early identification of colonies at risk of

damaging infection and timely implementation of preventive measures. Data on the prevalence of DWV variants in Spain is lacking, although government data suggest the presence of the virus in 83% of colonies and 99% of apiaries (Ministerio de agricultura y pesca 2017).

In the present study, we identified two DWV variants in southern Spain and examined whether genomic differences between them may influence their virulence. DWV load and *V. destructor* infestation were monitored in 10 colonies of an experimental apiary in Andalusia, Spain, over a 21-month period. Of the ten colonies evaluated, two of them were selected based on the following criteria: health status, viral load, and *V. destructor* infestation. One DWV-positive sample from each selected colony was sequenced, and the results were compared with complete DWV genome sequences from the GenBank. Additionally, a follow-up study was based on RT-qPCR of the three DWV master variants (ABC assay) (Kevill et al. 2017). Therefore, the main objective of this study was to determine if the virus sequence was related to the virus virulence. On the remaining colonies, ABC assay (Kevill et al. 2017) was also applied in order to evaluate the distribution of the DWV master variants on the apiary. A secondary objective was to determine what variants of the DWV are present in the South of Spain, which is one of the most important Autonomous Communities regarding beekeeping.

2. MATERIAL AND METHODS

2.1. Bee sampling and colony health determination

Bees were collected from 10 colonies of an experimental apiary at the Reference Centre for Beekeeping, University of Cordoba, Spain. All colonies were similarly managed. These colonies were studied from March 2015 to January 2017, except for July and August 2015, when sampling was not possible. Colonies were treated against *V. destructor* using Apitraz® in March and September. Samples of adult bees were taken from the hive entrance of each colony, frozen at -80°C until analysis, and then analyzed for DWV load

and *V. destructor* infestation level. A total of 149 samples were collected.

Every month, the beekeeping technician inspected all colonies and determined the number of bee, brood, pollen, and honey combs, as well as the presence of DWV symptoms. Colonies with signs of poor population (low activity in the entrance of the colony) or fewer than five bee and brood combs were categorized as having a “poor population.” Otherwise, colonies were categorized as having an “adequate population” if they showed high activity level in the entrance of the colony and more than 6–7 frames covered with bees and 2–3 frames covered with capped brood, considering the beekeeping managing and the time of the year. Health-related events were also recorded in the colonies, such as viral symptoms (deformity in wings and nervous symptoms), symptoms of bacterial disease, and mortality.

2.2. *V. destructor* load determination

V. destructor load was quantified in all colonies throughout the study except for July and August 2015. Mite presence was assessed at each monthly sampling. Mite load was quantified using the soapy water method described in “Standard methods for varroa research” in the COLOSS BEEBOOK (Dietemann et al. 2012).

2.3. DWV load determination

DWV load was determined in samples by homogenizing 10 whole bees with mortar and pestle in 5 ml of phosphate-buffered saline (PBS). This amount of starting material should allow detection of DWV if present in more than 25% of bees with a detection probability of 99% at the colony level (Pirk et al. 2013). RNA was extracted using the column-based Nucleospin II Virus® kit (Macherey Nagel, Düren, Germany) following the manufacturer’s instructions. Total RNA was suspended in RNase- and DNase-free water and stored at -80°C . This RNA served as template in one-step real-time reverse transcription polymerase chain reaction based on SYBR Green detection as described (Kukielka et al. 2008).

Two colonies were selected based on health status, *V. destructor* infestation level, and viral

load, which was classified into the four infection categories defined by Amiri et al. (2015). Health status was defined based on the population size, as assessed from the number of bee/honey/pollen/brood combs, on the presence of DWV symptoms (deformed wings, shortened and rounded abdomens, paralysis), and mortality. Colony H (healthy colony) showed no DWV symptoms and survived until the end of the study. A sample from the colony in September 2015 was used for phylogenetic analysis as described below. Colony W (weak colony) showed deformed wings and mortality during the study and collapse in November 2016. A sample collected in October 2015 was used for phylogenetic analysis.

2.4. Whole transcriptome amplification

Total RNA was extracted from the colony H and colony W samples mentioned above and was selected to perform the phylogenetic analysis after amplification using TransPlex Whole Transcriptome Amplification (Sigma-Aldrich). This kit provides a rapid method for preparing amplified cDNA from total RNA for downstream RNASeq applications. It employs a single primer isothermal amplification (SPIA) method to amplify total RNA into double-stranded cDNA and depletes rRNA without preselecting mRNA. In brief, sample homogenate (600 μl) was centrifuged for 10 min at 1792g, the supernatant was passed through at 0.45- μm membrane filter, and RNA was isolated using the column-based Nucleospin II Virus® kit (Macherey-Nagel) following the manufacturer’s instructions. Total RNA was quantified using the Nanodrop system (Thermo Fisher Scientific, Wilmington, USA) and amplified as cDNA using the TransPlex Whole Transcriptome Amplification Kit (Sigma-Aldrich, San Luis, USA) according to the manufacturer’s instructions, except that each reaction contained 3 μl of template RNA and the amplification involved 30 cycles. Each sample was split into three and processed in parallel. Amplified cDNA was purified using the High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer’s protocol. Concentration of amplified cDNA was measured

using the Nanodrop (128–297 ng/μl), and the ratio of absorbance at 260/280 and 260/230 nm (Table I). A 260/280 ratio of ~1.8 is generally accepted as “pure” for DNA; expected 260/230 values are commonly in the range of 2.0–2.2. Therefore, the obtained values indicated reasonably pure DNA (Wilfinger et al. 1997).

2.5. PCR amplification and DNA sequencing

The DWV genomes in the samples from colony H and colony W were sequenced nearly completely using the primer walking approach with 48 PCR primer pairs (Table II), which were designed based on the genomes of DWV (NC_004830.2) and VDV-1 (AY251269.2). PCR was performed using the high-fidelity PrimeSTAR® HS DNA Polymerase (Takara, Saint-Germain-en-Laye, France) in reactions (25 μl) containing 15 μl of 5x PrimeSTAR Buffer, 1 μl of forward primer, 1 μl of reverse primer, 2 μl of cDNA template, and 6 μl of RNase- and DNase-free water. Reactions were subjected to 35 cycles of denaturation at 94 °C for 2 min, annealing at 62 °C for 30 s, and extension at 70 °C for 5 min in a T3000 thermocycler (Biomtra, Göttingen, Germany).

The amplified PCR products were analyzed using 1% agarose gel electrophoresis in 45 mM Tris borate (pH 8.0) and 2.5 mM EDTA (0.53 TBE) containing 0.5 mg/ml ethidium bromide; DNA products were visualized by transillumination with a long-wave

UV light box. PCR products were purified using a PCR Purification Kit (Qiagen, Germantown, USA) and >700 bp (excluding primers) were sequenced using the Sanger method on an ABI Prism 3730 (Applied Biosystems, Foster City, CA, USA). The sequencing primers were the same as those used for amplification.

Colony H and colony W sequences were edited and assembled into nearly complete DWV genomes using MEGA 6 software (Tamura et al. 2013). These sequences were aligned with published sequences using ClustalW. One alignment contained 16 complete genomes of DWV, KV, and VDV (Supplementary data 2), together with the sequences from colony H and colony W. Another alignment was based on the variable RNA-dependent RNA polymerase (RdRp) region in 39 DWV genomes, including the sequences from colony H and colony W. Alignments were edited by hand where necessary based on conserved protein domains as a guide. The two final alignments were considered adequate because the first was associated with an average amino acid p-distance (1—amino acid identity) of 0.074 and the second with an average p-distance of 0.069. These values are within the acceptance threshold of <0.8 (Thompson et al. 1999; Ogden and Rosenberg 2006). From these alignments, phylogenetic trees were constructed using the maximum likelihood method and SPR algorithm and bootstrap testing of 2000 replicates.

The genomes sequenced from colony H and colony W were analyzed for recombination using the Recombination Detection Program 4.1 (Martin et al. 2015), with its algorithms GENECONV (Padidam et al. 1999), BootScan (Martin et al. 2005), MaxChi (Smith 1992), CHI-MAERA (Posada and Crandall 2001), SIScan (Gibbs et al. 2000), and 3Seq (Boni et al. 2007). Default parameters and Bonferroni's correction for multiple comparisons were used. $P < 0.05$ was regarded as statistically significant. Only recombination events that more than four algorithms identified as statistically significant were included in further analysis.

Table I. Amounts and purity assessment of cDNA after whole transcriptome amplification

Sample	Amount (ng/μl)	280/260 ratio	230/260 ratio
Colony H-1	170	1.74	2.15
Colony H-2	159.4	1.81	1.9
Colony H-3	177.5	1.86	2.15
Colony W-1	214.8	1.89	2.26
Colony W-2	202.3	1.80	1.58
Colony W-3	297.1	1.84	2.10

Table II. Matrix of percent identity from alignment of DWV variants. Colors are used to indicate percent identity: dark blue, > 95%; light blue, 90–95%; orange, 85–90%; yellow, 80–85%

Sequence	DWV type A	VDV-1	VDV-1/DWV	DWV Colony H	DWV Colony W
DWV type A	100%	84%	92.2%	97.19%	97.19%
VDV-1	84%	100%	90.2%	97%	83.85%
VDV-1/DWV	92.2%	90.2%	100%	88.5%	91.79%
DWV Colony H	85.3%	97%	88.5%	100%	85.1%
DWV Colony W	97.19%	83.85%	91.79%	85.1%	100%

2.6. RT-qPCR ABC assay

For further investigation to the DWV strain distribution in colony H and colony W, as well as within the remaining colonies of the study, ABC assay (Kevill et al. 2017) was performed. In the case of colony H and colony W, all samples collected after the sampling used for sequencing were evaluated using ABC assay. In the remaining colonies, three samples from each colony were analyzed, according to the following criteria: if the colony died during the study (called Dx), samples from the three last samplings before collapsing were selected. If the colony did not die during the study (called Sx), samples from the last three samplings were selected.

One-step, real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad), SYBR Green detection, cycling protocols, and primers for DWV-A, DWV-B, and DWV-C previously published (Kevill et al. 2017). Cycling protocol was slightly amended: RT step occurred at 45 °C for 15 min and annealing at 61 °C for 15 s.

Load of positive samples was determined by absolute quantification based on a standard curve constructed using serial 10-fold dilutions of known amounts of PGemT® TA plasmid (Promega, Madison, USA) containing the target

genes (RdRp region) of DWV master variant types A, B, and C. Standard curves were fitted with lines showing correlation coefficients of 0.99 (data not shown).

3. RESULTS

3.1. DWV and *V. destructor* loads in selected samples

Colony H showed no DWV symptoms and survived until the end of the study. A sample from the colony in September 2015 was used for phylogenetic analysis as described above. This sample showed a DWV load of 1.70×10^9 GEC and *V. destructor* infestation level of 10.68%.

Colony W showed deformed wings and mortality during the study and collapse in November 2016. A sample collected in October 2015 was used for phylogenetic analysis. This sample showed DWV load of 6.70×10^7 GEC and *V. destructor* infestation of 1.45%.

3.2. Colony health during the study

Two DWV genomes from colonies in southern Spain were sequenced for this study. Both genomes were 9031 nt long, excluding 3' polyadenylated tails (GenBank accession MK262742 and MK262743). One genome came from a colony with good health status, reflected in high population and adequate numbers of bee/honey/

pollen/brood combs. This colony H showed high DWV load in 10 of 21 monthly samplings. *V. destructor* infestation rate was higher at the beginning and end of the study (Figure 1). Despite high DWV and mite levels, the beekeeping technician reported the population to be adequate and stable throughout the study period. Anti-mite treatments successfully decreased mite levels and DWV load. Pearson's correlation analysis showed a positive correlation between the two pathogens ($r = 0.591$, $P = 0.015$). No DWV symptoms were detected in this colony throughout the study.

The second genome came from a colony (colony W) that presented deformed wings, mortality, and smaller population than the other colonies in the apiary. In March 2015, near the beginning of the study, the colony

showed high DWV load, which decreased slightly over time (Figure 2). *V. destructor* was present from the beginning of the study but decreased after anti-mite treatment. Toward the end of the study, viral load and *V. destructor* infestation rate increased. This colony died in November 2016, 2 months before the end of the study. Before death, the colony showed high DWV load (2.4×10^7 GECs), small population, and the DWV symptom of deformed wings.

In both colonies, *V. destructor* infestation rate and, to a lesser extent, DWV load varied seasonally, with pathogen levels highest during the summer and at the beginning of the fall. Both colonies showed high levels of both pathogens but responded differently to them.

Figure 1 DWV load and *V. destructor* infestation rate in colony H during the study period. The asterisks mark months when anti-*V. destructor* treatment was applied.

Figure 2 DWV load and *V. destructor* infestation rate in colony W during the study period. The asterisks mark months when anti-*V. destructor* treatment was applied.

3.3. Phylogenetic analysis based on complete DWV genome sequences

Specific primer pairs were applied to both DWV-positive colonies in order to sequence the complete DWV genome (Supplementary data 1). The complete DWV genome sequences from colony H and colony W were aligned with the 16 complete genomes of DWV or VDV-1 available in GenBank (Supplementary data 2). The alignment was 9045 nt long, corresponding to genome sequences determined here (9031 nt) as well as some gaps. The sequences determined here covered 88.5% of the DWV reference genome (NC_004830.2). Alignment of both sequences showed 7088 of 9031 nt (78.5%) to be identical. The nucleotides showed low divergence among the DWV reference genomes used for the alignment.

Two thousand one hundred seventeen variable nucleotide positions were identified across the entire genome. Among segregating sites, the average pairwise nucleotide diversity between sequences was $\pi = 0.073$, and the Tajima D test statistics rejected the neutrality hypothesis ($D = 0.146$). The colony H genome exhibited 85.3% similarity to the DWV-A reference genome (NC_004830.2), 97% similarity to the VDV-1 reference genome (AY251269.2), and 88.5% similarity to the recombinant VDV-1/DWV genome (KX373900.1). The corresponding similarity percentages for the colony W genome were 89% similarity to the DWV type A, 77.1% to the VDV-1, and 84% to the VDV-1/DWV recombinant.

Evolutionary relationships among DWV genomes were inferred using maximum likelihood based on the general time reversible model (Figure 3) (Kumar 2000). The analysis

involved 18 nucleotide sequences, including 1st, 2nd, 3rd, and noncoding codon positions. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. In the end, 9031 positions were analyzed. The bootstrap consensus tree inferred from 2000 replicates (Felsenstein 1985) was taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches were collapsed if the corresponding partitions occurred in fewer than 50% of bootstrap replicates. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using maximum composite likelihood and then selecting the topology with better log-likelihood value. Differences in rate of evolution among different sites were modeled using a discrete gamma distribution (2 categories, +G, parameter = 0.2136).

Phylogenetic analysis showed that the colony H genome clustered with genomes identified

from honey bee colonies without DWV symptoms. These other genomes included one from an experimental apiary in Belgium, the VDV-1 reference genome, and two recombinant VDV-1/DWV genomes from the UK. In contrast, the colony W genome clustered with genomes from colonies with DWV symptoms, mainly deformed wings (Supplementary data 2). The most closely related sequence was from an Austrian colony with losses.

3.4. Recombination breakpoints

We examined recombination events in the entire dataset of complete genomes, without any assumption of putative parental sequences. A total of 17 recombination events were detected (Supplementary data 3). This analysis suggested that the colony H genome showed three recombination events. The first recombination event (between VDV-1/DWV-B and DWV-A) was between a genome from Belgium (JX783225.1), which came from a colony that lacked DWV-specific symptoms but showed

Figure 3 Maximum likelihood phylogenetic analysis of complete DWV genomes. A total of 16 complete DWV, VDV-1, and KV genome sequences were obtained from the GenBank database and aligned with the two nearly complete genomes reported here. Red circles indicate previously published sequences from colonies with symptoms (deformed wings, mortality, low population); green circles indicate previously published sequences from colonies without any symptoms; purple circles mark the two sequences obtained in the present study.

relatively short lifespan, and the DWV reference genome (NC_004830.2), which came from a colony that had deformed wings. It was located at 1045–1685 nt, encoding the VP2 and VP3. The second recombination (VDV-1/DWV-B and DWV-A) was located at 4163–4703 nt, encoding the VP3 and the helicase. The third recombination event (between DWV-A and DWV-A) was located at 8895–9026 nt, encoding the N-terminal region.

The analysis suggested that the colony W genome showed a recombination event between a genome from INRA in Avignon, France (KX373899.1), which came from a colony that showed deformed wings, and a genome from South Korea (JX878304.1), which came from a colony that lacked DWV-specific symptoms.

3.5. Phylogenetic analysis based on RdRp regions of the DWV genome

In order to explore phylogenetic diversity based on the variable RdRp region (nucleotide position 9265–9594 in the DWV reference complete genome), we performed a second phylogenetic tree. Analysis based on 37 DWV RdRp sequences in Genbank showed a cluster including DWV and KV genomes and another including VDV-1 genomes. The colony W genome fell within the DWV-KV cluster, while the colony H genome fell within the VDV-1 cluster (Figure 4).

3.6. Detection of DWV master variants using the ABC assay

In colony H, ABC assay revealed that DWV-B was the most prevalent variant, although DWV-A was also present, but to a lesser extent. DWV-B load was higher than DWV-A load in this colony. In colony W, DWV-A was detected in all the samples; meanwhile, DWV-B was detected on a smaller number of samples and at lower load (Supplementary data 4).

In the remaining colonies, ABC assay was applied to the 3 months before collapsing (if the colony died) or to the 3 months before the end of the study (if the colony did not died). Five of

the eight remaining colonies died before the end of the study period (Supplementary data 5). RT-qPCR results (ABC assay) showed that DWV-A and DWV-B variants were detected in honey bee samples selected from the other eight colonies; meanwhile, DWV-C was not detected in any sample. DWV type A was detected in the majority of samples tested, being the dominant variant in colonies that collapsed. On the other hand, DWV type B was also quite prevalent and achieved higher load in surviving colonies.

4. DISCUSSION

Positive-strand ssRNA viruses are associated with honey bee colony losses; in particular, the combination of DWV and *V. destructor* has been related to strong virulence and severely colony mortality (Dainat et al. 2012). Field evidence have demonstrated that the presence of DWV and *V. destructor* is an important factor contributing to the current colony losses, but how both pathogens interact so effectively to trigger colony losses requires further study. Phylogenetics suggests that the mite may be driving changes in the DWV genome (Mordecai et al. 2016a; Mordecai et al. 2016b; Ryabov et al. 2014), but it is unclear whether such genetic changes affect viral virulence and therefore risk of colony loss (McMahon et al. 2016; Natsopoulou et al. 2017). The present study assessed whether nucleotide variations in the DWV genome can influence virulence. Our results suggest that different DWV variants at the same load can trigger different effects on colonies in the same apiary that are exposed to the same conditions and show similar *V. destructor* infestation. These findings suggest that nucleotide differences in the DWV strain can make the difference between colony health and collapse, implying that DWV genotyping of colonies may facilitate early identification of colonies at greater risk of collapse. At the same time, our study provides the first nearly complete sequences of DWV isolates from southern Spain, where the beekeeping sector is more professional than in other parts of the country.

Figure 4 Maximum likelihood phylogenetic analysis based on the variable RdRp region in the DWV genome. A total of 35 RdRp sequences from DWV, VDV-1, and KV in GenBank were compared with the two RdRp sequences determined here.

We were able to sequence nearly the entire DWV genomes from the two colonies in our study, including the internal ribosome entry site, the variable RdRp sequence, and the sequences encoding the L protein, structural proteins, and helicase. We were unable to sequence two open reading frames of 949 and 161 nt, flanked respectively by the 5' or 3' non-translated region, or the poly(A) tail. The colony H genome (accession number MK262742), from a healthy colony, showed recombination at three breakpoints between the DWV reference genome from Italy (Lanzi et al. 2006) and a DWV sequence from Belgium (Benaets et al. 2017). The colony H genome clustered with sequences that mainly came from healthy colonies, including the VDV-1 reference genome (97% similarity) and recombinant VDV-1/DWV genomes from the UK (Moore et al. 2011). Despite the fact that the colony H genome mainly clustered with VDV-1/DWV-B genomes, it was a recombinant between VDV-1/DWV-B and DWV-A. Therefore, both DWV-A and DWV-B wild-type genomes must have been present simultaneously. For this to be the case, the DWV-A genome has had to diverge significantly enough to mainly cluster with VDV-1/DWV-B and not with the other DWV-A/DWV-B recombinants. It showed three recombination events throughout its genome. The first recombination event was located in a similar region of the genome as reported by Mordecai et al. (2016a), between the Lp and the VP2. However, the second recombination event was located in the VP3 and the helicase region. Therefore, it is, to our knowledge, the first report of a recombination between DWV-B and DWV-A in this region of the genome. The colony W genome (accession number MK262743), from a weak colony with DWV symptoms, showed one recombination event between the same sequence from Belgium and an isolate from South Korea (Reddy et al. 2013). The colony W genome clustered with sequences from colonies with symptoms and mortality. It also clustered with the KV reference genome. Similar clustering results for the colony H and colony W isolates were obtained in phylogenetics analyses based only on the variable RdRp sequence.

Despite not all the nucleotides of the DWV sequences from the Spanish apiary were sequenced, our primer walking approach covered part of the IRES, L protein, and the structural proteins, as well as the helicase and the RdRp, in order to study recombination events in these regions of the viral genome. These results show that substantial genetic variation can be found in DWV strains affecting the same apiary. Indeed, the isolates from colony H and colony W showed only 85.5% similarity. This variation may help explain differences in viral virulence (Mordecai et al. 2016a; Mordecai et al. 2016b) and therefore colony outcomes such as in the present study. Our results are consistent with previous reports that VDV-1, DWV-B, and their recombinants are less virulent than DWV-A and KV (Mordecai et al. 2016a). Our findings justify further work into the potential association between DWV nucleotide sequence and virulence.

While differences in DWV virulence on their own may explain why colony H survived with good health despite high viral load and *V. destructor* infestation rate, another potential factor is the so-called superinfection exclusion (Folimonova 2012). According to this concept, an established virus infection can interfere with secondary infection by the same or a closely related virus. This exclusion may benefit the established virus because it reduces competition for resources and stabilizes its genome by reducing the risk of recombination with other viral genomes in the same cell. This exclusion may help explain how the mite can decrease DWV diversity by making certain DWV variants dominate over others (Mordecai et al. 2016a). Superinfection exclusion may protect the honey bee colony if an established infection with a weakly virulent DWV strain (e.g., transmitted by the mite) prevents secondary infection with virulent DWV-A. Under the assumption that DWV-B can be regarded as weakly virulent, and taking into account that colony H genome was a DWV-B/DWV-A recombinant, our results provide the first supporting evidence of superinfection exclusion at the colony level by a recombinant DWV-A/DWV-B (more similar to DWV-B). One of the issues that emerges from this finding is if the region of the genome where recombination occurs

plays a key role in determining DWV virulence, since the recombinant DWV from colony H protected the colony. This should be also carefully confirmed in light of reports that DWV-B may be correlated with losses in worker number over the overwintering period, as it was suggested by a recent study carried out by Natsopoulou et al. (2017). McMahon et al. (2016) also probed, under laboratory conditions, that DWV-B could be more virulent than the DWV-A variant. In this experiment, the authors observed higher mortality in the DWV-B-infected group, compared to the mock-infected control group. However, these experiments need to be replicated in honey bees from different geographical locations, at different viral loads, and under field conditions to test this conclusion. All of these variables make it difficult to understand the current role of variant DWV-B.

When exploring RdRp region by constructing a phylogenetic tree, sequence from colony H clustered with VDV-1 sequences and sequence from colony W clustered with DWV and KV sequences. These results confirmed the similarity between the sequences from this study and the stipulated DWV variants.

ABC assay on colonies H and W revealed that the distribution of DWV-A and DWV-B variants was relatively constant over the study period. DWV-A was detected in both colonies, but high DWV-B loads may have protected colony H from DWV-A, since DWV-A was present at lower levels and this colony survived the study period and did not show any symptoms. On the contrary, colony W showed higher DWV-A loads and lower DWV-B loads, which may have contributed to its collapse. DWV-A titres over 10^7 GEC were detected in colony W the months before collapsing; meanwhile, DWV-B was not detected in some samplings or it was present at low levels. This result may be explained by the fact that DWV-B was not in sufficiently high levels to protect the colony from the virulent DWV-A.

Additionally, ABC assay on colonies that died revealed that DWV-A was the dominant variant in the 3 months before collapsing. By contrast, colonies that survived showed lower DWV-A load and higher DWV-B load, which could have had a protective effect on those colonies. These findings are consistent with those of other studies (Kevill

et al. 2017; Martin et al. 2012; Mordecai et al. 2016a; Schroeder and Martin 2012) and support the hypothesis that DWV-A can be more virulent but high DWV-B titres may have a protective effect on colonies. However, these data must be interpreted with caution because of the low sample size, and further study of the potential protective effect of DWV-B should be done. Surprisingly, DWV-C was not detected in any sample, which may reveal that this variant is not established in southern Spain yet. Therefore, DWV-C implications on colony health could not be assessed.

Our study indicates the presence of at least two DWV strains in southern Spain: VDV-1/DWV-B recombinant with DWV-A (more similar to DWV-B) (DWV-SpB) and DWV-A (DWV-SpA). These findings extend our limited understanding of the global distribution of DWV variants (Cornman et al. 2013; Kielmanowicz et al. 2015). Further work, which considers these results, should examine the prevalence of variants in Spain and more broadly in Europe, since Europe has been reported to be a critical source for the global spread of the DWV variants.

Recombination breakpoints were identified in the colony H genome at 1095–1685 nt in the regions encoding VP2 and VP1, as well as at 4163–4703 nt in the region encoding the VP3 and the helicase. These two breakpoints were derived from the DWV-A reference sequence. A third breakpoint was identified at 8895–9026 nt in the N-terminal region, derived from the DWV sequence from Belgium. The colony W genome showed a recombination breakpoint at 3828–4408 nt overlapping with the region encoding the helicase and derived from a South Korean isolate showing deformed wing symptoms. These results clearly show that honey bee colonies can be infected by a mixture of DWV and its recombinants, which may result from multiple modes of virus transmission.

In this study, we report the nearly complete genome sequences of two DWV isolates from an apiary in southern Spain and show that they correspond to two DWV variants. We show evidence of genetic diversity in DWV populations and for an association between genomic sequence and viral virulence. Genetic changes in DWV may help it adapt to its host and its vector (*V. destructor*). In

conclusion, our findings add evidence to a growing body of literature on the genetic study of DWV and emphasize the importance of understanding pathogen genetic diversity when investigating causes of honey bee losses.

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AUTHOR CONTRIBUTIONS

SB, MVR, and JSV designed experiments, FP helped in the interpretation of the data, SB and FMR performed experiments and analysis, and SB wrote the paper. All authors read and approved the manuscript.

Des variations de séquence nucléotidique peuvent être associées à la virulence du virus de l'aile déformée

virus de l'aile déformée / *V. destructor* / phylogénie / abeille mellifère

Variationen der Nukleotidsequenz sind möglicherweise mit der Virulenz des Flügeldeformationsvirus verknüpft

flügeldeformationsvirus / *V. destructor* / virusvirulenz / phylogenie / honigbiene

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