

## ORIGINAL ARTICLE

# Diversity in a honey bee pathogen: first report of a third master variant of the Deformed Wing Virus quasispecies

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**Treatment of emerging RNA viruses is hampered by the high mutation and replication rates that enable these viruses to operate as a quasispecies. Declining honey bee populations have been attributed to the ectoparasitic mite *Varroa destructor* and its affiliation with Deformed Wing Virus (DWV). In the current study we use next-generation sequencing to investigate the DWV quasispecies in an apiary known to suffer from overwintering colony losses. We show that the DWV species complex is made up of three master variants. Our results indicate that a new DWV Type C variant is distinct from the previously described types A and B, but together they form a distinct clade compared with other members of the *Iflaviridae*. The molecular clock estimation predicts that Type C diverged from the other variants ~319 years ago. The discovery of a new master variant of DWV has important implications for the positive identification of the true pathogen within global honey bee populations.**

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## Introduction

RNA viruses have rapid replication and high error rates, leading to immense diversity within each viral replication cycle (Domingo and Holland, 1997). As a result, many RNA viruses are highly genetically heterogeneous and exist within infected population structures known as quasispecies. It has been proposed that this gives these viral pathogens an increased ability to shift to a new environmental niche, such as a new host, as a suitable mutant is more likely to already exist if this opportunity arises. However, if the diversity within a quasispecies becomes too high, deleterious mutations can accumulate leading to loss of overall fitness (Clarke *et al.*, 1993). The adaptability and host range of a virus are a function of the level of diversity found within a quasispecies (termed the quasispecies swarm size) (Schneider and Roossinck, 2001). As well as a randomly generated swarm of mutants around one variant, quasispecies can also exist as a number of master variants, each with their own swarm of random mutations (Palacios *et al.*, 2008; Lauring and Andino, 2010). Determining the extent of genetic heterogeneity in virus populations thus has

important implications for predicting and preventing emerging viral diseases.

The global decline in honey bee populations over the past few decades has been attributed to the ectoparasitic mite *Varroa destructor* and its affiliation with Deformed Wing Virus (DWV) type A variant (Dainat *et al.*, 2012; Martin *et al.*, 2012; Schroeder and Martin, 2012; Francis *et al.*, 2013). The role of honey bees as pollinators is vital to the environment and economy, as bees are a key pollinator species for agriculture. The economic value of honey bees is estimated to be more than 225 billion US dollars worldwide (Gallai *et al.*, 2009). DWV, a member of the single-stranded positive-sense RNA genus *Iflavirus* (Lanzi *et al.*, 2006), exists as a group of closely related viruses, often considered as variants of the same species complex (Ryabov *et al.*, 2014). The ICTV (The International Committee on Taxonomy of Viruses) database categorises DWV type A as two variants, DWV (Lanzi *et al.*, 2006) and *Kakugo virus* (KV) (Fujiyuki *et al.*, 2004). A second closely related virus *Varroa destructor virus-1* (VDV-1) is also part of the genus *Iflavirus*, which is now designated DWV type B (Martin *et al.*, 2012; Mordecai *et al.*, 2016). DWV type B was designated a separate species based on a nucleotide identity to DWV type A of ~84% that, according to demarcation criteria, is sufficient dissimilarity to warrant the creation of a new master variant (Fauquet *et al.*, 2005). VDV-1 was originally isolated from *V. destructor* but has since been

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reported to replicate in honey bees (Ongus *et al.*, 2004; Zioni *et al.*, 2011) where it has been shown to cause wing deformities in bees (Zioni *et al.*, 2011). DWV type B was also recently found to dominate the DWV population in honey bees from an isolated apiary in Swindon, UK (Mordecai *et al.*, 2016); to the apparent exclusion of any other master variants. In honey bee populations that have never been exposed to *Varroa* mites, DWV exists in a very large variant swarm with numerous master variants (Martin *et al.*, 2012). However, transmission of DWV by *Varroa* reduces variant diversity to one master variant (Martin *et al.*, 2012). Therefore, DWV exists as an endlessly mutating swarm of variants with these master variants constituting part of this quasispecies and sharing a recent common ancestor (Baker and Schroeder, 2008; Martin *et al.*, 2012). The dominance of one master variant over another will lead to ultimately different life histories for the colony, that is, death if DWV type A dominates (Martin *et al.*, 2012) or health if DWV type B dominates (Mordecai *et al.*, 2016).

By existing as a diverse swarm of variants, viruses are able to co-occupy several biological niches. Certain biological traits may allow a virus to infect one cell type over another, known as cell tropism (Koyanagi *et al.*, 1987). Whether or not a virus is able to infect a susceptible cell depends firstly on recognition of a cellular receptor on the cell surface and secondly on intracellular host factors that dictate whether the host cell is permissive to virus replication. Therefore, amino acid substitutions caused by nucleotide mutations in the structural or nonstructural region of the virus genome can affect both the host range and cell tropism of a virus. When categorising viruses based on a phylogenetic relationship it is important to note that a single amino acid change can have a substantial effect on the phenotypic traits of a virus. Therefore, when a virus exists as a collection of variants or quasispecies, although the phylogeny and ancestry of the viruses may be similar, the host range, tropism, pathogenicity and epidemiology of the variants may differ greatly (Domingo *et al.*, 2012). In addition, recombination between these variants is a source of further variation (Moore *et al.*, 2011).

There are numerous biological implications of quasispecies occupying large amounts of sequence space that challenge the treatment and control of both established and emerging infectious diseases (Gomez *et al.*, 1999). For example, quasispecies theory has been used to describe how viruses such as *Hepatitis C virus* and HIV are able to escape host immune responses (Pavio and Lai, 2003; Woo and Reifman, 2012). In addition, effective vaccines are yet to be developed for both these viruses because of the rapid emergence of resistant mutants under vaccine selection pressure (Gaschen *et al.*, 2002; Law *et al.*, 2013). For similar reasons, the highly divergent nature of RNA viruses has been implicated in the emergence of antiviral drug-resistant infections in AIDS (Metzner

*et al.*, 2009), hepatitis C (Halfon and Locarnini, 2011), hepatitis B (Nishijima *et al.*, 2012) and influenza (Boivin *et al.*, 2002).

Conventional methods to define and analyse variance within RNA viruses include RNA ‘fingerprinting’ (Domingo *et al.*, 1978) and reverse transcriptase-PCR amplification using specifically designed primers (see, for example, Highfield *et al.*, 2009). Clone libraries and Sanger sequencing were used to identify the DWV type A as being associated with *Varroa* infestation and colony collapse (Martin *et al.*, 2012). Although these techniques are valid for identifying known variants, primer-based methods are prone to missing unidentified variants and are biased towards overrepresented sequences (Gomez *et al.*, 1999). PCR-based methods are less appropriate to determine the extent of variation in a quasispecies that is not normally distributed that is, where multiple variants exist, each with their own spectrum of mutants (Gomez *et al.*, 1999). In these instances deep sequencing methods such as Illumina platforms are more suited to discovering new variants as well as to diversity analysis (Wood *et al.*, 2014).

Illumina sequencing is advantageous for samples with large amounts of genetic variation because of its depth of sequencing, although significant raw read analysis is required. Reference-based assembly methods can overlook biological variants because of inaccurate read alignments and loss of data (Archer *et al.*, 2010; Iqbal *et al.*, 2012; Yang *et al.*, 2012), although former studies using high-throughput next-generation sequencing of DWV have used these methods (Moore *et al.*, 2011; Ryabov *et al.*, 2014).

A previous study (Highfield *et al.*, 2009) found that despite controlling *Varroa* populations, high DWV loads were associated with overwintering colony losses (OCL). Historical losses due to OCL of ~10% were normal; these have now risen to ~20% since the establishment of *Varroa*. This suggests that a ‘new’ non-*Varroa*-transmitted DWV master variant may be circulating in some colonies during the winter causing OCL. To investigate this hypothesis we used a bespoke *de novo* assembly pipeline (Mordecai *et al.*, 2016). The Vicuna ([broadinstitute.org/scientific-community/science/projects/viral-genomics/vicuna](http://broadinstitute.org/scientific-community/science/projects/viral-genomics/vicuna)) *de novo* assembler was used as it is designed to assemble highly heterogeneous viral populations and is well suited to the computational challenge that the DWV quasispecies present (Yang *et al.*, 2012). As well as assembling the DWV type A master variant, a third DWV master variant (Type C) was assembled and confirmed to be distinct through phylogenetic inference.

## Materials and methods

### *Sequencing and assembly*

Total RNA was sequenced without an amplification step. Illumina (San Diego, CA, USA) Hi-seq (2 × 100)

pair-end sequencing was carried out by TGAC (The Genome Analysis Centre) and at the University of Exeter on four samples from Highfield *et al.* (2009); Late June, Early October and Late October from GD1, and early October from GD2 (Supplementary Table S1). The samples were originally collected from Devon in the southwest of England (Highfield *et al.*, 2009). Twenty asymptomatic bees were pooled for each sample before RNA extraction. RNA extractions were prepared as in Highfield *et al.* (2009) followed by a complementary DNA amplification step before sequencing.

A Bioinformatics pipeline was developed to accommodate the large amount of variation found within the DWV species complex. First, the quality of the raw reads was verified using FastQC (Babraham Bioinformatics, Cambridge, UK). Samples were then converted from fastq to fasta using the `fastq_to_fasta` script that is part of the FASTX-toolkit (Hannon Lab) ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)).

To isolate the DWV complex sequence reads from the host and other contaminating sequences, the BLASTn (Altschul *et al.*, 1990) tool was used. The reads were searched against a custom BLAST database containing the DWV, VDV-1 and KV genomes, with an *e*-value of  $10e-5$ . BLAST was carried out against Read 1 of the Illumina data. The `ncbi-blast-parser_perl` script (<http://www.bioinformatics-made-simple.com/2012/07/ncbi-blast-parser-extract-query-and.html>) was then used to parse and read the top hit of the BLAST output.

Next, 'sed' and 'awk' scripts were used to delete empty lines and the reads that contained 'nohits'. The corresponding BLAST hits were extracted from the Read 2 raw reads using QIIME (Caporaso *et al.*, 2010). The paired reads were balanced using a custom script written in R version 3.2.0 (R Core Team, 2015). Finally, the balanced DWV family reads were assembled using the Vicuna assembler that was developed to generate consensus assemblies from genetically heterogeneous populations, specifically RNA viruses. (Yang *et al.*, 2012).

Vicuna contigs > 200 bp in length were imported into Geneious (Version 7.04, created by Biomatters, Auckland, New Zealand) and the 'Map to Reference tool' was used to align the contigs with the DWV and VDV-1 reference genomes. For several of the samples the Vicuna assembly yielded full-length contigs that covered the whole genome, whereas for others consensus scaffolds were created from two or more contigs. The ends of the contigs were then trimmed of any assembly or sequencing artefacts.

A second novel variant was also assembled by Vicuna. In order to create a consensus sequence of the novel variant, the contigs from three samples were realigned against the DWV genome. Any contigs containing sequence other than the novel variant were removed. If a contig contained the novel variant as well as sequence belonging to the type A DWV genome because of recombination or *in silico*

recombination, the type A DWV regions were trimmed and deleted. A consensus genome of the novel variant was created from these sorted and trimmed contigs. Accordingly, the same steps were carried out on the type A sequence in order to assemble the type A genome consensus from Devon. The DWV scaffolds were then aligned with full-length genome sequences from the NCBI (National Center for Biotechnology Information) database using the MUSCLE alignment tool (Edgar, 2004) within Geneious and the full-length genome was obtained. Full genome comparisons were visualised in mVISTA (Frazer *et al.*, 2004).

To quantify the number of reads attributed to each DWV variant using BLAST (Supplementary Table S1), the novel Type C variant genome sequence was added to the custom DWV family database and the BLAST for each sample was carried out a second time. This allowed the Type C variants to be categorised correctly by BLAST rather than attributed to the closets hit to the three other reference genomes.

To validate the quantification of reads attributed to each variant, the balanced read 1 and 2 BLAST hits were aligned competitively to three reference genomes using the 'Map to Reference Tool' within Geneious.

Consensus genomes of type A and Type C were translated using the 'live annotation' tool within Geneious. Because of repetitive regions in the genome, assembly error took place in several sites, leading to an incomplete open reading frame. These assembly errors were corrected by using the Vicuna analysis tool to pull out individual reads covering these regions, and the correct sequence was determined. Finally, the RDP4 programme (Martin *et al.*, 2010) was used to determine whether any recombination took place between the sequences. Assembled genomes are available from the European Nucleotide Archive under the accession numbers ERS657948 (type A) and ERS657949 (Type C).

#### *Amino acid-based phylogeny*

To establish the phylogenetic relationship of DWV subtypes and closely related Iflaviruses, we reconstructed the phylogeny of the conserved *RdRp* amino acid sequences for seven DWV subtypes, spanning all three types that were either sequenced and assembled from the Devon hive or available from genbank (type A: NC\_005876.1, NC\_004830.2; type B: KC\_786222.1, NC\_006494.1, JQ\_413340), as well as *Formica exsecta* Virus 1 (NC\_023022.1) and Sacbrood Virus (NC\_002066.1). We used a Bayesian approach using MrBayes (v. 3.1.2) (Huelsenbeck and Ronquist, 2001). We assumed a fixed rate model of protein evolution and reconstructed the phylogeny using a model jumping method. This method allows for different models of amino acid substitution to be used in the Markov chain Monte Carlo (MCMC) procedure, with all models contributing to the final

result weighted according to their respective posterior probability. We ran two runs of four chains for 4 000 000 MCMC generations, sampling trees every 1000 generations. All trees were drawn using FigTree v.1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

#### Evolutionary rate

To estimate the evolutionary rate of DWV and its subtypes, we collated three independent data sets with temporal information for at least one population: (A) partial *lp*-gene; 10 populations,  $n=78$ , 328 nt (1323–1650 bp) (Genbank AJ489744, AY292384, GU109335, HM162355, JF346615–JF346620, JF346624–JF346629, JF346633–JF346639, KF164292, KF164293, KJ437447, KP734726, KP734738, KP734747, KP734765–KP734770, KP734774–KP734787, KP734817–KP734825, KP734827–KP734846); (B) partial *capsid*-gene; 1 population,  $n=167$ , 1215 nt (2634–3848 bp) (Genbank AY292384, HQ655502–HQ655561, KF314827–KF314932); (C) partial *RdRp*-gene; 1 *B. terrestris* population,  $n=145$ , 508 nt (8016–8522 bp) (Genbank KP734326–KP734470). To test whether these fragments contain a molecular clock signal, we estimated the root-to-tip divergence using Path-o-gen v.1.4 (<http://tree.bio.ed.ac.uk/software/pathogen/>) to estimate how much genetic variation can be explained by the sampling date. We constructed maximum likelihood phylogenetic trees without the assumption of a molecular clock in Phylip v. 3.695 (evolution.genetics.washington.edu; Felsenstein, 1989) and tested whether the regression between root-to-tip distance in these maximum likelihood trees and the age of the samples indicated a clock-like signal. In addition, we tested for a significant temporal signal by randomising the temporal information across each data set 100 times and compared the resulting random evolutionary rates from BEAST with the real data set. A temporal signal is supported if there is a significant difference between the real data set and the randomised data sets (Ramsden *et al.*, 2009; Alizon and Fraser, 2013). Both analyses support a temporal signal in these data sets (Supplementary Figure S3 and Supplementary Table S4).

#### Model selection and BEAST runs

To determine the appropriate molecular clock models for each data set, we used the path sampling maximum likelihood estimator implemented in BEAST 1.8 (Baele *et al.*, 2012, 2013). As this method is very computationally intensive, we first used a range of simpler tests to limit the number of models to be compared by this method as suggested by Alizon and Fraser (2013), and Drummond and Bouckaert (2014). For the three large temporal data sets, we used jModelTest v.2.1.1 (<https://github.com/ddarriba/jmodeltest2>; Posada, 2008) to compare substitution models based on the Bayesian Information Criterion (Alizon and Fraser 2013); the resulting

substitution models were the Tamura-Nei (TnR) model (*lp*-fragment) and the Hasegawa, Kashino and Yano (HKY) model (*vp3*- and *RdRp*-fragments), both with Gamma variation. For the DWV-subtype data set, we compared the general time-reversible model and the SRD06 model (Shapiro *et al.*, 2006) using path sampling. We partitioned substitution rates between the first and second and third codon positions as, for all data sets, the third codon position had a significantly higher rate. We tested whether a strict clock rate can be excluded by running models with a lognormal relaxed clock; if the relaxed clock's coefficient of variation statistic abuts the zero boundary, a strict clock cannot be excluded (Gray *et al.*, 2011). We ran models with exponential population growth for all models; if the exponential growth rate was significantly higher than zero, a constant population size can be excluded. We then used path sampling to distinguish between clock models (exponential and lognormal relaxed clock) and demographic models (constant population size, exponential population growth or a Gaussian Markov random field (GMRF skyride); Drummond *et al.*, 2002; Minin *et al.*, 2008) as indicated for each fragment by the initial analyses. Based on these analyses, we chose an exponential relaxed clock and exponential growth prior for the *lp*- and *capsid*-fragments and a lognormal relaxed clock and constant growth prior for the *RdRp*-fragment as well as for the DWV-subtype analyses.

To generate a genome-wide estimate for the evolutionary rate in DWV, we calculated the mean of the relaxed clock means for the individual fragments (*lp*-fragment:  $9.097 \times 10^{-4}$  (95% highest posterior density:  $4.412 \times 10^{-4}$ – $1.394 \times 10^{-3}$ ), *capsid*-fragment:  $1.845 \times 10^{-3}$  ( $1.159 \times 10^{-3}$ – $2.569 \times 10^{-3}$ ), *RdRp*-fragment:  $1.278 \times 10^{-3}$  ( $4.131 \times 10^{-4}$ – $2.513 \times 10^{-3}$ ), resulting in a mean evolutionary rate of  $1.346 \times 10^{-3}$  ( $5.41 \times 10^{-4}$ – $2.627 \times 10^{-3}$ ) substitutions/site/year. We implemented this evolutionary rate as an uncorrelated lognormal clock prior for the DWV-subtype analysis with a lognormal distribution with a mean of  $1.35 \times 10^{-3}$  in real space and a log s.d. of 0.4. We used the default priors in BEAST v.1.8.1. We ran models with 2 runs each of 50 million MCMC generations, sampling every 5000 generations with a burn-in of 5 million generations to obtain effective sample sizes >200. We examined traces for convergence using Tracer v.1.6 (<http://www.tree.bio.ed.ac.uk/software/tracer/>) and used TreeAnnotator v.1.8 (beast.bio.ed.ac.uk/downloads) to produce a Maximum Clade Credibility tree for the DWV-subtype analysis. We used the method from Xia *et al.* (2003), as implemented in DAMBE, to confirm that the alignment had not reached substitution saturation.

## Results and discussion

Illumina Hi-seq (2 × 100) pair-end sequencing of asymptomatic honey bees from Devon was carried

out on colonies that either survived (GD2) or collapsed (GD1) because of OCL (Highfield *et al.*, 2009). To assess DWV diversity within the samples the raw reads were searched against a custom DWV family database (including KV and VDV-1 genomes) and reads matching to any of the DWV genomes were extracted. The coverage for the number of reads that matched to a custom DWV BLAST database was estimated using the Lander/Waterman equation (that is, the depth of sequencing). Genome coverage depth ranged from 457 to 165 927 × (average coverage was 86 838 ×) (Supplementary Table S1).

*De novo* assembly of the Illumina reads yielded a complete genome of a type A variant as well as of a novel DWV variant that we named Type C (Supplementary Table S2). Competitive alignment to the now three master variants revealed that out of the ~30 million DWV blast positive reads, ~27 million were assembled to one of three genomes (Supplementary Table S3 and Supplementary Figure S1). Around 3 million assembled to type A, ~3.5 thousand to type B and ~24 million to Type C. As expected, more reads align to the 3' region of the genome than the 5'; an artefact of the reverse

transcription 3' bias (Brooks *et al.*, 1995). Although reads aligned to the whole of the Type C genome, the depth of coverage was lower at the 5' region (Supplementary Figure S1). In addition, coverage of the 5' region of the type A variant was unusually high, indicating that this disproportionate coverage in the 5' regions of type A and C could be the evidence of recombination between the two variants. Because of the low read depth and coverage for type B in our samples, a full genome could not be assembled (Supplementary Table S3 and Supplementary Figure S1). Nonetheless, closer examination of 3' region where the RNA-dependent RNA polymerase (*RdRp*) gene is located (Baker and Schroeder, 2008) revealed that the Devon type B variant shared 100% identity to VDV-1 type B genome in this region (Supplementary Figure S2). The sequence identity of our newly assembled type A and C variants was compared with other members of the DWV complex (Table 1 and Figure 1). Both type B and C differ from the type A nucleotide sequence in similar regions of the genome. However, type B and C share only 79% nucleotide identity in the polyprotein encoding region of the genome and 89% identity in the amino acid sequence.

Phylogenetic analysis showed the relationship of the novel type A and C variants from Devon to previously sequenced viruses (Figures 2 and 3). The type A genome clustered with other type A variants, whereas Type C formed a distinct and separate branch, thereby confirming the originality and thus new master variant assignment of DWV Type C. Given that type A and C can recombine with each other, we screened for any genomes with evidence of recombination and excluded them from the analysis in order to comply with the assumptions underlying phylogenetic reconstruction.

The Type C genome codes for a full-length polyprotein and translation of the genome permitted the amino acid sequence to be compared with more distant members of the genus *Iflavirus* (Figure 2). The relationship of the DWV complex with

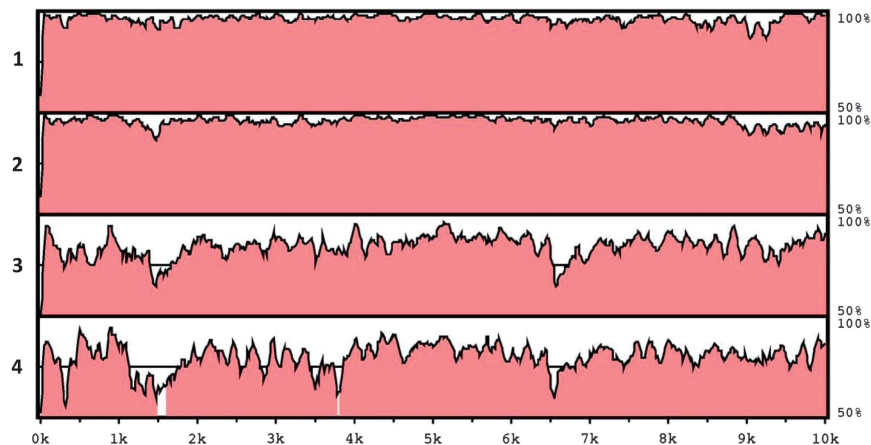
**Table 1** Percent identity matrix of Deformed Wing Virus (DWV) variants amino acid (top half) and nucleotide alignments (bottom half)

	DWV	Devon type A	Type B	Devon Type C
Type A		98.8	95.2	89.5
Devon type A	98.8		95.2	89.3
Type B	95.2	95.2		89.1
Devon Type C	89.5	89.3	89.1	

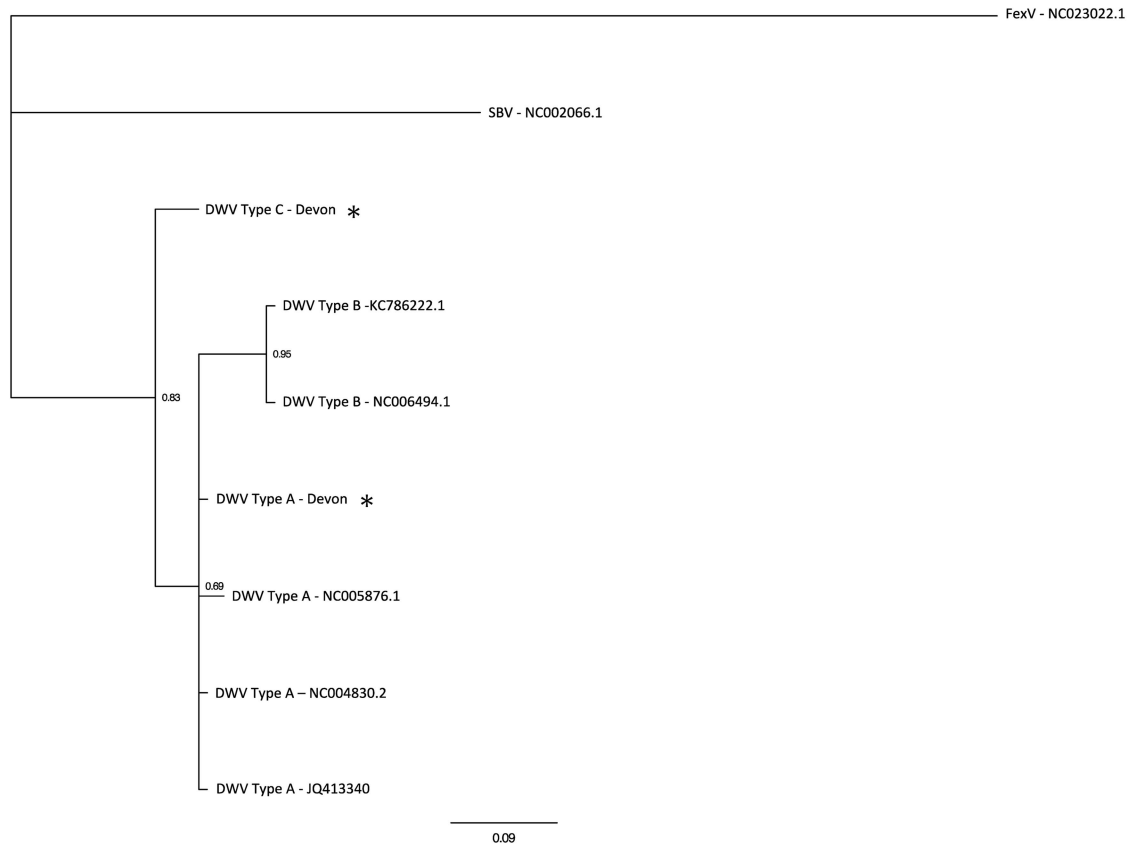
  

	DWV	Devon type A	Type B	Devon Type C
Type A		98.2	84.4	79.1
Devon type A	98.2		84.4	79.2
Type B	84.4	84.4		78.9
Devon Type C	79.1	79.2	78.9	

Dark Green > 98%, Light Green 90–98%, Yellow 85–90%, Orange 80–85%, Red < 80%.



**Figure 1** Plot showing the percentage identity across the whole genome of (1) Devon DWV type A (ERS657948), (2) KV type A (NC0058762), (3) VDV-1 type B (AY251269) and (4) Devon Type C (ERS657949) compared with the DWV type A (NC004830) reference genome. Plot created by mVISTA (Frazer *et al.*, 2004).



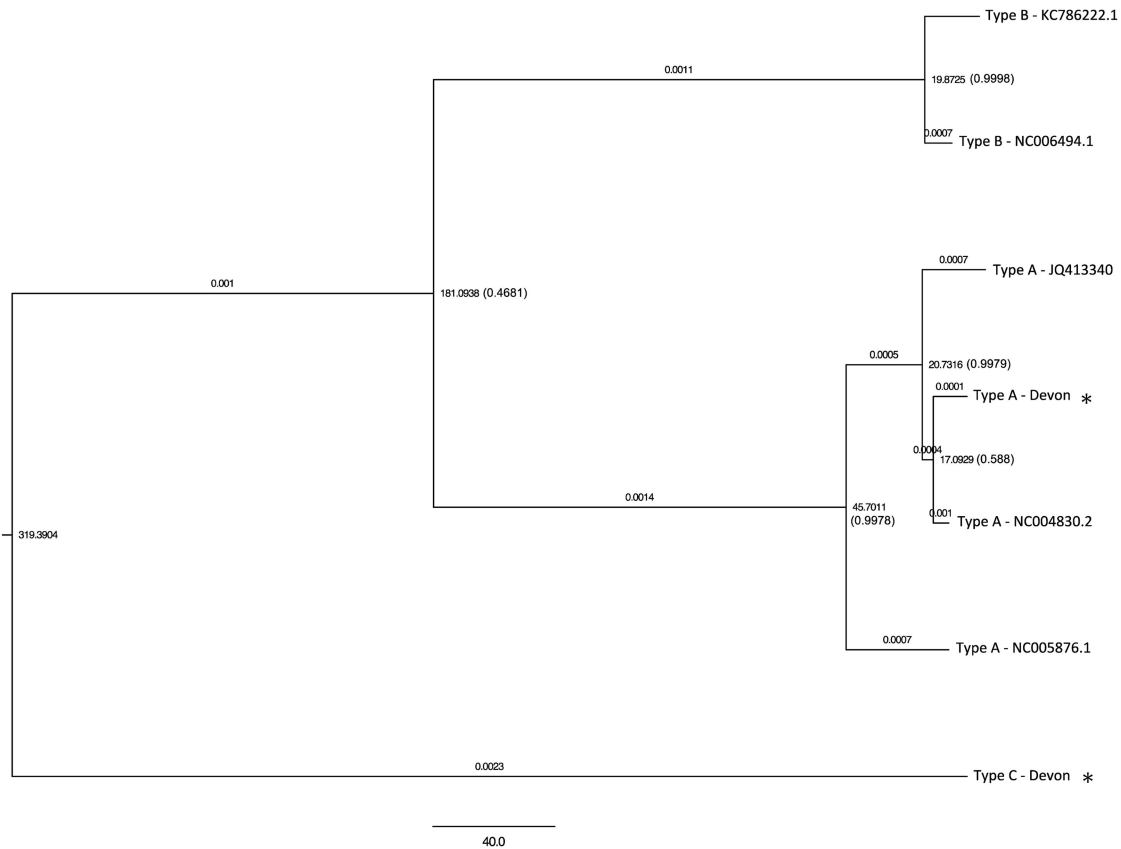
**Figure 2** Bayesian inference of phylogeny based on a conserved region of amino acid sequence encoding for the RNA-dependent RNA polymerase, with the Bayesian support values shown on the nodes. Samples from this study are labelled with an asterisk (\*). Bar represent number of nucleotide substitutions per site.

*F. exsecta* virus 2 and Sacbrood virus was analysed using a Bayesian inference of phylogeny of a conserved region of the RdRp amino acid sequence (Figure 2). The DWV sequences were attributed to three main groups (type A, B and C). The nucleotide consensus sequences created by Vicuna for type A was found to be 98.2% identical to the type A reference DWV genome. The analysis suggested that the new Type C genome is clearly related to type A and B, and forms a distinct clade to other members of the family *Iflaviridae* (for example, Sacbrood virus) that are all only distantly related to a dicistrovirus outgroup, *F. exsecta* virus.

In addition, a Bayesian analysis of the polyprotein encoding region of the sequences was carried out using an MCMC model, permitting a molecular clock model to be run within BEAST v1.8.1 (Figure 3) (Drummond *et al.*, 2012). Divergence times were calculated based on a tip-dated coalescent model, with an evolutionary rate prior based on three independent tip-dated fragments of DWV type A. The samples in the tree span 11 years (2000–2011), and the samples used for estimating the evolutionary rate span 11, 13 and 22 years for the *RdRp*-, *capsid*- and *lp*- fragments, respectively. The Bayesian tree of the nucleotide sequences had a similar structure to the amino acid tree, showing that the Type C viral

variant is distinct from type A and B. The molecular clock estimation predicts that Type C diverged from the other DWV variants 319 years ago (57–1010 95% highest posterior density), and type A and B disassociated from each other 181 years ago (38–497 95% highest posterior density). This estimate is unlikely to be biased by substitution saturation, as there is no evidence for saturation in our data set (Xia *et al.*, 2003).

The phylogenetic analysis concludes that the DWV quasispecies is made up of three distinct master variants. However, the number of variants is not exhaustive and it is conceivable that more variants will be added in the future as the use of NGS becomes increasingly prolific. The result further suggests that Type C has not recently emerged, but rather is an established DWV variant. Moreover, using reverse transcriptase-PCR to amplify a region of the DWV *RdRp* gene, Martin *et al.* (2012) attributed a novel variant to the type B swarm of variants, although it is now clear that this novel variant was in fact Type C. The presence of Type C in Hawaii implies that this variant is widespread and not specific to the United Kingdom. It also confirms that it has not emerged recently as the Hawaiian sample originates from Kauai that has yet to be colonised by *Varroa* (Martin *et al.*, 2012),



**Figure 3** Bayesian phylogeny of the polyprotein encoding nucleotide sequence using an MCMC model (BEAST). Node labels show the age and the posterior probability in brackets. The branches are labelled with the clock rate as a relaxed clock rate was used. Genomes sequenced from this study are labelled with an asterisk (\*) and are available from the European Nucleotide Archive under the accession numbers ERS657948 (type A) and ERS657949 (Type C). Scale bar represents years based on a relaxed molecular clock.

suggesting Type C is part of the wild-type DWV quasispecies.

The high levels of heterogeneity within viral RNA populations mean that viruses are able to occupy large areas of sequence space and consequently are able to exist in multiple hosts (Domingo and Holland, 1997). DWV is a 'generalist' known to infect bumblebees (Genersch *et al.*, 2006; Fürst *et al.*, 2014), *V. destructor* (Ongus *et al.*, 2004) and other insects (reviewed in Manley *et al.*, 2015). Further work is required to ascertain whether Type C causes acute wing deformities in honey bees or its presence is because of viral 'spill over' from another host. Three of the four samples in this study were taken from a hive (GD1) that went on to collapse from OCL. However, as type A and the type A–C 5' recombinant were also present, it remains unclear which variant was responsible for OCL. It is worth noting however that between 85% and 98% of the reads in hive GD1 were Type C that make up the Type C or the A–C recombinant genomes (Supplementary Table S1). The second hive (GD2) that survived OCL contained roughly equal levels of type A and C reads (which includes reads that make up the A–C recombinant); yet, the coverage indicates that the viral load was much lower in this hive. Moreover, the low abundance of DWV type B in these Devon colonies

appears to confirm the observation made in the colonies from Swindon (Mordecai *et al.*, 2016); that is, DWV type B is not present at sufficient levels to protect the honey bee from the virulent type A, or possibly C variants (as well as any recombinants between A and C). In addition, *Varroa* mites in the Devon colonies were controlled using chemical methods, thereby potentially preventing the mites from transmitting type B into the honey bees; as observed in the Swindon Apiary (Mordecai *et al.*, 2016).

A recombinant between DWV type A and B has been reported previously (Moore *et al.*, 2011), and found to be hypervirulent as it was more efficient at replicating than other variants when co-injected directly into the haemolymph of honey bee larvae (Ryabov *et al.*, 2014). As it is presently unclear whether the Type C variant or the A–C recombinant lead to disease, further direct manipulation experiments such as those carried out by Ryabov *et al.* (2014) could be crucial in determining whether the novel Type C genome and any of its recombinants are hypervirulent.

The large number of DWV Type C and A–C recombinant reads in the Devon samples (Supplementary Table S1 and Supplementary Figure S1) suggests that these variants are able to

replicate in honey bees. The capacity of DWV to exist as a swarm of recombining variants and occupy large amounts of sequence space may be one of the factors contributing to its capability to maintain a persistent infection. Defining DWV as a quasispecies with discrete master variants is comparable with the current categorisation of several other RNA viruses. The genetic variability within *Hepatitis C virus* has been classified into four hierarchical strata: genotypes, sub-genotypes, isolates and finally background variation of the quasispecies (Farci and Purcell, 2000). A total of seven *Hepatitis C virus* genotypes exist to date, differing from each other by >15% over their complete coding region (Smith et al., 2014). Similarly, Japanese encephalitis virus is currently classified into five genotypes based on sequence identity that differ in replication efficiency in a range of hosts (Han et al., 2014). In order to further our understanding of DWV and the impact of the virus on honey bees as well as other hosts, a more progressive classification of the DWV quasispecies would be prudent, that is, the new classification of DWV as a quasispecies with at least three master variants that can recombine with each other.

Bioinformatics pipelines designed for eukaryotes and prokaryotes are not necessarily suited to studying viral systems. For example, pipelines incorporating reference assemblers could hinder the discovery of novel virus variants. The genome sequences presented here are representative of the different variants found within a sample; however, the Vicuna pipeline is not suited to analysing the further level of diversity around these variants, for which diversity models are better suited, such as those developed by Wood et al. (2014).

As well as being of significance to globally important honey bee health, the ability to study highly heterogeneous virus genomes is of wider importance. Cross-species virus transmission and emergence of new epidemic diseases such as severe acute respiratory syndrome, Ebola and influenza are major threats to public health (Parrish et al., 2008). Exploring the extent of viral diversity in RNA quasispecies, of which DWV may be a suitable model, may offer insight into the mechanisms by which viruses are able to transmit between different hosts as well as how viruses are able to develop resistance to antiviral therapies (Domingo et al., 2012). Further study of the DWV quasispecies may help to explain how DWV is able to exist as multiple variants in many hosts, and may elucidate mechanisms by which it establishes a persistent infection among several hosts but only proves pathogenic in some.

## Conflict of Interest

The authors declare no conflict of interest.

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