Phylogenetic and recombination analyses of two deformed wing virus strains from different honeybee species in China

Dongliang Fei [Equal first author, 1, 2], **Yaxi Guo** [Equal first author, 1], **Qiong Fan** [3], **Haoqi Wang** [1], **Jiadi Wu** [1], **Ming Li** [1], **Mingxiao Ma** [Corresp. 1]

[1] Institute of Biological Sciences, Jinzhou Medical University, Jinzhou, Liaoning, China

[2] College of Veterinary Medicine, Northeast Agricultural University, Haerbin, Heilongjiang, China

[3] Jinzhou Animal Disease Prevention and Control Center, Jinzhou, Liaoning, China

Corresponding Author: Mingxiao Ma
Email address: mamingxiao@jzmu.edu.cn

Background. Deformed wing virus (DWV) is one of many viruses that infect honeybees and has been extensively studied because of its close association with honeybee colony collapse that is induced by *Varroa destructor*. However, virus genotypes, sequence characteristics, and genetic variations of DWV remain unknown in China. **Methods.** Two DWV strains were isolated from Jinzhou and Qinhuangdao cities in China, and were named China1-2017 (accession number: MF770715) and China2-2018 (accession number: MH165180), respectively, and their complete genome sequences were analyzed. To investigate the phylogenetic relationships of the DWV isolates, a phylogenetic tree of the complete open reading frame (ORF), structural protein VP1, and non-structural protein 3C+RdRp of the DWV sequences was constructed using the MEGA 5.0 software program. Then, the similarity and recombinant events of the DWV isolated strains were analyzed using recombination detection program (RDP4) software and genetic algorithm for recombination detection (GARD). **Results.** The complete genomic analysis showed that the genomes of the China1-2017 and China2-2018 DWV strains consisted of 10,141 base pairs (bp) and 10,105 bp, respectively, and contained a single, large ORF (China1-2017: 1,146–9,827 bp; China2-2018: 1,351–9,816 bp) that encoded 2,894 amino acids. The sequences were compared with 20 previously reported DWV sequences from different countries and with sequences of two closely related viruses, Kakugo virus (KV) and *V. destructor* virus-1 (VDV-1). Multiple sequence comparisons revealed a nucleotide identity of 84.3%–96.7%, and identity of 94.7%–98.6% in amino acids between the two isolate strains and 20 reference strains. The two novel isolates showed 96.7% nucleotide identity and 98.1% amino acid identity. The phylogenetic analyses showed that the two isolates belonged to DWV Type A and were closely related to the KV-2001 strain from Japan. Based on the RDP4 and GARD analyses, the recombination of the China2-2018 strain was located at the 4,266-7,507 nt region, with Korea I-2012 as an infer unknown parent and China-2017 as a minor parent, which spanned the entire helicase ORF. To the best of our knowledge, this is the first study to the complete sequence of DWV isolated from *Apis cerana* and the possible DWV recombination events in China. Our findings are important for further research of the phylogenetic relationship of DWVs in China with DWV strains from other countries and also contribute to the understanding of virological properties of these complex DWV recombinants.

Title page

Ms. Ref. No.:

Title: Phylogenetic and recombination analyses of two deformed wing virus strains from different honeybee species in China

Author: Dongliang Fei [1,2], Yaxi Guo[1#], Qiong Fan[3], Haoqi Wang[1], Jiadi Wu[1], Ming Li[1], Mingxiao Ma[1*]

*** Corresponding Author:** Mingxiao Ma **E-mail:** mamingxiao@ jzmu.edu.cn

[#] **Co-author:** Yaxi Guo

Author's Institution: 1. Institute of Biological Sciences, Jinzhou Medical University, Jinzhou, Liaoning, China; 2.College of Veterinary Medicine, Northeast Agricultural University, Haerbin, Heilongjiang, China; 3. Jinzhou Animal Disease Prevention and Control Center, Jinzhou, Liaoning, China

Tel.: +86-18841648216/13841636358

Fax: +86-04164673528

Phylogenetic and recombination analyses of two deformed wing virus strains from different honeybee species in China

ABSTRACT

Background. Deformed wing virus (DWV) is one of many viruses that infect honeybees and has been extensively studied because of its close association with honeybee colony collapse that is induced by *Varroa destructor*. However, virus genotypes, sequence characteristics, and genetic variations of DWV remain unknown in China.

Methods. Two DWV strains were isolated from Jinzhou and Qinhuangdao cities in China, and were named China1-2017 (accession number: MF770715) and China2-2018 (accession number: MH165180), respectively, and their complete genome sequences were analyzed. To investigate the phylogenetic relationships of the DWV isolates, a phylogenetic tree of the complete open reading frame (ORF), structural protein VP1, and non-structural protein 3C+RdRp of the DWV sequences was constructed using the MEGA 5.0 software program. Then, the similarity and recombinant events of the DWV isolated strains were analyzed using recombination detection program (RDP4) software and genetic algorithm for recombination detection (GARD).

Results. The complete genomic analysis showed that the genomes of the China1-2017 and China2-2018 DWV strains consisted of 10,141 base pairs (bp) and 10,105 bp, respectively, and contained a single, large ORF (China1-2017: 1,146–9,827 bp; China2-2018: 1,351–9,816 bp) that encoded 2,894 amino acids. The sequences were compared with 20 previously reported DWV sequences from different countries and with sequences of two closely related viruses, Kakugo virus (KV) and *V. destructor* virus-1 (VDV-1). Multiple sequence comparisons revealed a nucleotide identity of 84.3%–96.7%, and identity of 94.7%–98.6% in amino acids between the two isolate strains and 20 reference strains. The two novel

isolates showed 96.7% nucleotide identity and 98.1% amino acid identity. The phylogenetic analyses showed that the two isolates belonged to DWV Type A and were closely related to the KV-2001 strain from Japan. Based on the RDP4 and GARD analyses, the recombination of the China2-2018 strain was located at the 4,266-7,507 nt region, with Korea I-2012 as an infer unknown parent and China-2017 as a minor parent, which spanned the entire helicase ORF. To the best of our knowledge, this is the first study to the complete sequence of DWV isolated from *Apis cerana* and the possible DWV recombination events in China. Our findings are important for further research of the phylogenetic relationship of DWVs in China with DWV strains from other countries and also contribute to the understanding of virological properties of these complex DWV recombinants.

BACKGROUND

The honeybee is one of the most important pollinators and plays a crucial role in agricultural ecology. However, over the last decades, honeybee populations have rapidly decreased, which has led to a pollination crisis that seriously threatens global agricultural production (Martin, 2001). This dramatic decline of honeybee colonies was suggested to be the result of interactions between parasites and pathogens, including viruses, fungi, mites, bacteria, microsporidia, and other pests. Among the effects of pathogens, viral diseases are considered a major threat to apiculture, and 12–20 kinds of single-stranded positive sense “picorna-like” RNA viruses (Berényi et al., 2007; Baker and Schroeder, 2008; Reddy et al., 2013) have been confirmed to infect honeybees. Among these viruses, the deformed wing virus (DWV) is the most important honeybee virus, causing colony collapse disorder (Vanengelsdorp et al., 2009) owing to an interaction effect with *Varroa destructor*. The global prevalence of DWV is the presumed driver of the substantial frequency of honeybee colony collapse; thus, DWV is regarded as the most destructive honey bee virus infecting *A. mellifera* and *A. cerana*, thereby threatening food safety and the equilibrium of various ecosystems.

DWV is a member of the picorna-like insect virus family *Iflaviridae* and consists of a 30 nm icosahedral particle with a single positive strand RNA genome. This

genome is about approximately 10 kb in length with a large open reading frame (ORF), which encodes a 2,894 amino acid polyprotein. After cleavage by viral proteases, polyproteins produce structural and non-structural proteins, and the major structural proteins are composed of four proteins, VP1, VP2, VP3, and VP4. The non-structural proteins include a helicase, a genome-linked viral protein (VPG), a 3C-protease (3C-pro), and an RNA-dependent RNA polymerase (RdRp). Furthermore, similar to other *Iflaviruses*, DWV structural and non-structural proteins are at the N-terminal end and C-terminal end of the polyprotein, respectively (Lanzi et al., 2006; Berényi et al., 2007).

The clinical symptoms of DWV infection are atrophy of the wings, smaller body size, discoloration and paralysis in adult bees, and a generally shortened life span (Kovac and Crailsheim, 1988; Prisco et al., 2011). Although DWV infections in adult bees often produce no clinical symptoms, it is a serious threat to honeybee colonies (Miranda et al., 2010). The virus was originally isolated from infected adult bees in Japan in the 1980s, and since then has been distributed globally, including throughout Asia, Europe, Africa, North America, South America, and the Middle East (Bailey et al., 1981; Kovac and Crailsheim, 1988; Calderon et al., 2003; Ellis and Munn, 2005) . DWVs can be categorized into two master variant strains, DWV-A and DWV-B (Moore et al., 2011; Mcmahon et al., 2016; Mordecai et al., 2016). DWV-A comprises the original DWV strain and the Kakugo virus (KV) (Mcmahon et al., 2016). *Varroa destructor* virus-1 (VDV-1) was classified as DWV-B, with an overall RNA genome of 84% to those of the classic DWV-A. In addition, the first 1455 nt of the ORF encoding the lower molecular mass structural proteins shows the greatest diversion from those of the classic DWV-A, with an RNA identity of 79%, and translates to a polypeptide of 485 aa with an identity of 90% (Ongus JR et al., 2004; Ryabov EV et al., 2014). Recent studies have shown that recombination events have frequently occurred between these two main variants (Mordecai et al., 2015; Zioni et al., 2011). Thus far, the complete genome sequences of approximately 20 strains have been sequenced, with isolates mostly obtained from *A. mellifera* and *Vespa crabro*; however, there are no complete genome sequences of DWV isolates from *A. cerana* (Reddy et al., 2013; Lamp et al., 2016; Forzan et al., 2017). The

epidemiology of DWV in *A. cerana* and *A. mellifera* has been investigated in China, however, virus genotypes, sequence characteristics and genetic variations of DWV remain unknown (Zheng et al., 2015; Chao et al., 2017).

In the present study, we isolated two DWV strains (China1-2017 and China2-2018) from *A. mellifera* and *A. cerana*, respectively, and produced the complete nucleotide sequences of DWV from *A. cerana*. The sequences of the China1-2017 and China2-2018 isolates were analyzed and compared to the reference nucleotide sequences of DWV genotypes from other countries. Furthermore, we analyzed molecular biological characteristics and phylogenetic relationship of DWV structural and non-structural polyprotein regions of the China1-2017 and China2-2018 strains and reference strains. Moreover, we performed a recombination analysis of DWV from Chinese isolates using recombination detection program (RDP4) software and a genetic algorithm for recombination detection (GARD).

METHODS

Ethics statement and legal agreement

This research was approved by the Experimental Animal Ethics Committee of Jinzhou Medical University (No. 20180016) and the Agricultural and Rural Comprehensive Service Center of Jinzhou (No. 20180619).

Sample collection

Samples were collected from two different regions in China. A total of 257 *A. mellifera* samples originating form Jinzhou of Liaoning Province (41°14′34″ N, 121°8′15″ E) were collected in March 2016, and 463 *A. cerana* worker bee samples originating from Qinhuangdao of Hebei Province (40°3′26″ N, 119°33′4″ E) were collected in April 2017. All sampled individuals exhibited typical deformed wing symptoms. Upon collection, the bees were stored on ice and immediately transported to the laboratory, where they were kept frozen at -80 °C until analysis.

Virus isolation

Virus isolation was performed according to previously published methods (Ying et al., 2016; Mingxiao et al., 2011; Jakubowska et al., 2016) Briefly, 50–60 adult bees were completely homogenized with a mortar and pestle in 10 mL of phosphate-buffered saline (pH 7.4), containing 0.5% Nonylphenol ethoxylate, then the homogenate was incubated for 30 min at 20 °C. After this, the homogenate was centrifuged at 5,000 × *g* for 20 min at 4 °C. Large debris was removed, and the supernatant was again centrifuged at 8000 × *g* for 30 min at 4 °C. After discarding the precipitate, the supernatant was placed in an ultracentrifuge tube and centrifuged at 82,000 × *g* for 1 h at 4 °C. The resulting pellet was resuspended in 2 mL STE buffer (10 mM Tris-HCl, 0.1 M NaCl, and 1 mM EDTA; pH 7.3). The viral strains of *A. mellifera* and *A. cerana* were named China1-2017 and China2-2018, respectively.

Viral RNA isolation and DWV screening by PCR

Total RNA was extracted from the purified virus of China1-2017 and China2-2018 strains using a TIANamp Virus DNA/RNA Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. Total RNA was eluted in 30 μL of diethyl pyrocarbonate-treated water and stored at -80 °C until further analyses. To determine the presence of DWV in *A. mellifera* and *A. cerana*, a RT-PCR assay was performed using a PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China), according to the manufacturer's instructions.
The primers DWV-F (5′-TTTGCAAGATGCTGTATGTGG-3′) and DWV-R (5′-GTCGTGCAGCTCGATAGGAT-3′) were used to amplify a 395 bp fragment of the DWV RdRp gene (accession number: AY292384). The PCR amplification was carried out under the following conditions: 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 5 min. A sample of 6 μL of PCR product was loaded on a 1.2% agarose gel containing GelStainand analyzed using a Tanon 2500 Digital Gel Image Analysis System. The PCR products were sequenced commercially (Shanghai Biotech Co., Ltd.).

RT-PCR amplification and genome sequencing

Viral genomic RNA of China1-2017 and China2-2018 were reverse-transcribed to cDNA with an oligo (dT) primer (Sambrook and Russell, 2001) (TransGen Biotech), according to the manufacturer's recommendations. Seventeen primer pairs were designed to amplify the complete genome sequence of China1-2017 and China2-2018, using the complete DWV sequence of the USA and KOR strains (accession number: AY292384 and JX878304; Table 1) (Lanzi et al., 2006; Reddy et al., 2013) . PCR amplifications were performed in Eppendorf tubes, and the cycling protocol for RT-PCR amplification was as follows: 45 min at 42 °C (reverse transcription), followed by 30 cycles at 95 °C for 60 s, 50–55 °C for 30 s, and 72 °C for 60 s. The 3′ and 5′ termini of the China1-2017 and China2-2018 strains were obtained by employing a rapid amplification of cDNA ends (RACE) technique, using a SMARTer RACE 5′/3′ kit (Clontech). PCR products were electrophoresed and purified, and subsequently sequenced commercially (Sangon Biotech Co., Ltd.). The nucleotide sequences of all fragments were assembled to compile the genomic sequences of the China1-2017 and China2-2018 strains using Lasergene software (DNASTAR), based on published complete DWV sequences (accession number: AY292384, JX878304, and JX878305).

Sequence and phylogenetic analyses

To research the DWV genome sequence characteristics of the two novel strains, 20 complete genome sequences of DWV were obtained online from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) (Table 2). Multiple alignments of nucleotides and amino acid analyses were performed using the ClustalW program in the MegAlign softwareprogram (DNASTAR, Madison, USA). At the same time, the conserved domains of the nonstructural proteins in the C-terminal portion of the polyprotein were analyzed with reference to the functional domains of mammalian picornaviruses. To investigate the genetic variation and evolutionary characteristics of the isolates, DWV complete genome sequences of the classic Type A and Type B strains from 2001 to 2018 and two closely related viruses, KV (Fujiyuki et al., 2004) and VDV-1 (Ongus et al., 2004) in the GenBank database were selected for further analysis, including 4 isolates in Asia, 13 in Europe, 3 in America, and 1 in Oceania. Then,

phylogenetic trees of ORF, VP1 and 3C+RdRp were reconstructed by the maximum likelihood method using MEGA5. The best-fitting nucleotide substitution model (GTR+I+G) was used for the all alignment datasets, which was determined by the lowest BIC score in MEGA 5.0 (Tamura et al., 2011). A total of 1,000 replicates of bootstrap resampling were used to ensure the reliability of individual nodes in each phylogenetic tree.

Recombination analysis

Recombination analysis was performed using eight full-length genomes of DWV from Japan (KV-2001, AB070959), Korea (Korea I-2012, JX878304; Korea II-2012, JX878305), China (China-2017, MF036686; China1-2017, MF770715; China2-2018, MH165180), England (VDV-2013, KC786222), and the Netherlands (VDV-2004, AY251269), and the detection of inter-strain recombination, identification of closest parental sequences and localization of possible recombination break points were assessed using RDP4 software, which comprises RDP, GeneConv, Bootscan, MaxChi, Chimaera, SiScan, and 3SEQ algorithms. The standard settings of these algorithms were used with the default values of RDP4. The likelihood of recombination events was significant in at least four algorithms at $P<$ 1.0E-6 or recombination consensus scores (RCS) above 0.6, based on the RDP4 analysis. Recombination events were considered possible when the P-value of at least three algorithms was below 0.05, and the RCS was between 0.4 and 0.6, and the likelihood of recombination events was considered insignificant when the RCS was under 0.4 with $P < 0.05$ (Wang et al., 2015;Lee et al., 2017; Gao et al., 2018). Moreover, the recombination events were further verified by GARD implemented in the Datamonkey web interface (Delport et al., 2010) and the credibility of the recombination breakpoints was assessed by the KH test.

Nucleotide sequence accession number

The DWV nucleotide sequences of the strains China1-2017 and China2-2018 are accessible on GenBank (accession number: MF770715 and MH165180).

RESULTS

RT-PCR detection of DWV samples

After virus isolation, specific primer pairs were used to detect DWV in samples from Jinzhou and Qinhuangdao by RT-PCR, and fragments of approximately 395 bp were produced (Figure 1). After sequencing and alignment, the nucleotide sequence identity exceeded 97% by BLAST, thus we successfully isolated two novel DWV strains from *A. mellifera* and *A. cerana* in China.

Nucleotide sequence analysis of DWV isolates

The nucleotide sequences of China1-2017 and China2-2018 were 10,141 nt and 10,105 nt, respectively. The whole nucleotide sequences of China1-2017 and China2-2018 are enriched in A/U (China1-2017: A-29.17%, U-32.17%, G-22.76%, C-15.90%; China2-2018: A-29.09%, U-32.00%, C-16.16%, G-22.74%). The isolates contained a single major ORF from the 5′–3′ end, which was composed of 8,682 nt (ORF position in China1-2017: 1,146–9,827 bp; China2-2018: 1,135–9,816 bp), encoding 2,894 amino acids. Multiple sequence comparisons showed that the sequences of China1-2017 and China2-2018 were similar to those of previously reported DWVs/VDV-1 strains. Furthermore, compared to other DWV isolates, the nucleotide sequence identity and deduced amino acid sequence identity of China1-2017 and China2-2018 ranged from 84.3% to 96.7% and 94.8% to 98.6%, and 84.3% to 96.7% and 94.7% to 98.4%, respectively (Table S1).

Amino acid sequence analysis of DWV isolations

As previously reported for mammalian picornaviruses, the ORF of China1-2017 and China2-2018 strains encoded a 2,894-amino-acid polyprotein, with structural proteins at the N-terminus and non-structural proteins at the C-terminus (Lanzi et al., 2006; Organtini et al., 2016). The structural and non-structural proteins were positioned in the genomes as follows: VP3 (China1-2017: 1,800–2,537 nt, China2-2018: 1,789–2,526 nt), VP1 (China1-2017: 2,601–3,848 nt, China2-2018: 2,590–3,837 nt), VP2 (China1-2017: 3,849–4,622 nt, China2-2018: 3,838–4,611 nt), helicase (China1-2017: 5,010–6,428 nt, China2-2018: 4,999–6,417 nt), and 3C-RdRp-protease (China1-2017: 7,686–9,827 nt, China2-2018:

7,675–9,816 nt). Moreover, six conserved domains in the helicase, 3C-pro, and RdRp were identified from China1-2017 and China2-2018. Three conserved helicase regions were found in the deduced amino acid sequences of the China1-2017 and China2-2018 ORF, ranging from 1,472 to 1,575, namely, domain A (^{1472}**G**xx**G**x**GKS**^{1479}), domain B (^{1518}**Q**x$_5$**DD**^{1525}), and domain C (^{1561}**KK**x$_4$**P**x$_5$**NTN**^{1575}). The 3C-pro conserved domains included the cysteine protease motif (^{2305}**G**x**CG**^{2308}) and the putative substrate-binding motif (^{2322}**G**x**H**xx**G**^{2327}). The highly conserved RdRp region, ^{2495}**TS**x**G**x**P**^{2500}, was recognized between the deduced amino acid positions 2,495 and 2,500.

Phylogenetic relationships of the DWV isolates

To assess the genetic relationships of the DWVs, three phylogenetic trees were constructed based on the VP1, 3C+RdRp segments, and ORF gene sequences. The results showed that the ORF and VP1 groups better explained the geographical distribution of DWV, and the 3C+RdRp-coding region better explained the genotype and diversity of DWVs.

In the ORF gene phylogenetic tree, the 24 DWV isolates (including China1-2017 and China2-2018) were divided into two groups (lineage A and B; Figure 2A). The first group contained two master lineages (lineage A1 and A2), one of which included eight isolates from America and Europe; the second lineage included six isolates from Asia. China1-2017 and China2-2018 belonged to lineage A2. Lineage B contained six isolates from Europe. We found that the phylogenetic tree of the ORF among DWV isolates correlated with the geographical distribution.

The phylogenetic tree based on the VP1 segment produced two groups (lineages C and D; Figure 2B) and was similar to the ORF tree (Figure 2A). The first group (lineage C) was further divided into two sub-groups (lineages C1 and C2): ten isolates from America, Europe, and Oceania formed lineage C1, whereas eight isolates formed lineage C2. The second group (lineage D) contained six isolates from China, Korea, Japan, and Europe.

The phylogenetic tree based on the 3C+RdRp segments produced two distinct groups (lineages E and F; Figure 2C). The 22 isolates in lineage E belonged to DWV Type A, which included variants from America, Europe, Oceania, and Asia.

Lineage F belonged to the classic DWV Type B, which only contained two isolates from Belgium and the Netherlands.

Recombination analysis of the DWV isolates

To explore potential recombination signals in the DWV isolates from Asia, recombination signals were assessed using the RDP4 software. Using seven algorithms, nine recombination events were detected in Asian strains (Figure 3B and Table 3). In all potential recombination events, three recombination events (events 2, 3, and 5) had a high degree of certainty based on the RDP4 software standard (Table 3). However, GARD analyses indicated that only one isolate (event 3), China2-2018, was identified as a recombinant at the breakpoint in the positions 4266 and 7507 nt with a high level of confidence (LHS, RHS *P*-values < 0.01). Based on the above analysis, event 3 was identified as the real recombination event. In event 3, the recombination of strain China2-2018 was located at the 4266–7507 nt region, with Korea I-2012 as an infer unknown parent and China-2017 as a minor parent (Figure 4A-C), which spanned the entire helicase ORF (Figure 3).

DISCUSSION

DWV is one of the most prevalent, pathogenic honeybee viruses in the world, and has been directly linked to colony collapse disorder (Organtini et al., 2016; Kevill et al., 2017). Despite the importance of DWV as a honeybee virus, only a limited number of complete genome sequences for DWV are available. In the present study, we determined the entire genome sequences for two DWV isolates collected from *A. mellifera* and *A. cerana* in China. Generally, DWV is only prevalent in *A. mellifera* and *V. destructor* and is not common in *A. cerana* (Tentcheva et al., 2004; Xie et al., 2016; Zhang and Han, 2018). However, we obtained the complete genome sequence from the China2-2018 strain *A. cerana* for the first time in the present study, which helps to investigate the host range of DWV. The comparison of China1-2017 and China2-2018 showed 96.7% identity of nucleotide sequences and 98.1% identity of amino acids. Although the identity of the two isolates was relatively high, they did not belong to the same strain. The isolated strain sequences of similarities with 20 reference strains ranged from 84.3%

to 96.4%, and the highest sequence identity was assigned to strain UK-2009 (GU109335), whereas Belgium-2016 (KX783225) and VDV-2004 had the lowest sequence identities. Therefore, the two novel isolates were significantly different from DWV Type B. The amino acid identity ranged from 94.8% to 98.6%, and the highest similarity belonged to the strain VDV-2004 and the lowest to Chile-2012 (JQ413340). As shown in Table 1S, virus strains from the same continents or from the same countries showed higher levels of identity of the nucleotides and amino acids, including the novel isolates; therefore, these viruses have been present in the honeybee populations for a long time and the viruses have evolved more or less independently. Moreover, six highly conserved motifs were identified from the two novel isolates, featuring the typical characteristics of iflaviruses.

Based on the phylogenetic analyses of ORF, VP1, and 3C+RdRp segments, the two novel isolates from China clustered within the same clade as other DWV strains form Asia; therefore, the novel isolates had a closer relationship with other Asian strains, and the DWV strains from Asia might have originated from the ancestor KV-2001 (AB070959). Furthermore, the phylogenetic analysis of DWV based on ORF and VP1 revealed numerous different geographically determined clades and the phylogenetic tree of VP1 also presented a clearer pattern regarding the geographical distribution; therefore, the strains with the closer relationship had different evolutionary rates in different environments and hosts. Moreover, the phylogenetic tree based on the VP1 sequences confirmed that China1-2017 and China2-2018 were more closely related to the isolates from Korea than to those from Japan. In RNA viruses, 3C-pro and RdRp genes tend to be highly conserved; therefore, 3C-pro and RdRp genes have usually been used to distinguish subtype classification of RNA viruses (Baker and Schroeder, 2008; Geng et al., 2014; Kevill et al. 2017). In the present study, the phylogenetic analysis of 3C+RdRp showed that the two novel isolates all belonged to DWV Type A. With a difference in the ORF and VP1 tree, the China2-2018 (MH165180) strain was more closely related to the Korea-2012 strain (JX878304) than to the China1-2017 strain (MH770715). We suspect that the VP1 and 3C+RdRp genes had generated at a different evolutionary rate in different environments, which led to the diversity of

359 the phylogenetic trees, because the structural proteins genes (VP1, VP2, and VP3)
360 are more likely to mutate in the picornavirus (Amin SA et al., 2014;Hu Y et al.,
361 2016).

362 Natural recombination is an important strategy for viruses to adapt to new
363 environmental conditions and hosts. Recombination events have been observed in
364 DWVs (Seo et al., 2009; Lian et al., 2013; Dalmon et al., 2017); however, the
365 recombination events of Chinese DWVs have never been described. Based on
366 geographical location, we used all DWV strains from Asia , the classic DWV Type
367 B and two novel isolates from China in the present study to analyze the potential
368 recombination events using RDP4 software and GARD, and the recombination
369 event of the China2-2018 strain was confirmed (Table 3 and Figure 4). In this
370 recombination event, the strains from China and Korea were mainly involved,
371 including the novel isolates; therefore, the DWV recombination widely exists in
372 honeybee colonies of East Asia. In general, irregular and complicated
373 recombination patterns indicate that the recombination events are usually random,
374 although a detailed understanding of the mechanism involved in such
375 recombination phenomenon must be clarified. When compared with the DWV
376 recombinant isolated by Dalmon et al., we found that all recombinant sites located
377 in the region encoded non-structural proteins. The apiculture characteristic of
378 China is probably the greatest factor that led to the DWV epidemic in *A. cerana*
379 colonies. In China, there are a large number of *A. mellifera* and *A. cerana* colonies
380 found in the same region during the nectar collecting season, which could promote
381 the transmission of DWV from *A. mellifera* to *A. cerana* by pollen. In this
382 interaction among the virus, host, and infectious vector, the recombination strains
383 continuously appear. Future studies are required to discover more recombination
384 phenomenon and the occurrence of recombination events may contribute to the
385 high levels of genetic diversity and viral adaptability to host in DWVs, which may
386 increase the potential of this virus to threaten successful beekeeping.

387 **CONCLUSIONS**

In summary, we found two novel DWV isolates from China and reported the first complete genome sequence of DWV from *A. cerana*. Based on phylogenetic trees, the novel DWV isolates from China were confirmed to be the closest related to the strain from Korea. Furthermore, the recombinant phenomenon was discovered in the novel isolates of China2-2018, which is the first description of DWV recombination in China. Our study has not only revealed the presence of novel DWV recombinants in China but also provides information that may be useful for further research on the phylogenetic origins of Chinese DWV strains.

REFERENCES

Amin SA, Sasmita U, Nick JK, Donald PK, David JP, Mana M. 2014. Novel antibody binding determinants on the capsid surface of serotype O foot-and-mouth disease virus. *Journal of General Virology* **95:** 1104–1116**.**

Bailey L, Carpenter JM, Woods RD. 1981. Properties of a filamentous virus of the honey bee (*Apis mellifera*). *Virology* **114(1):** 1-7.

Baker AC, Schroeder DC. 2008. The use of RNA-dependent RNA polymerase for the taxonomic assignment of Picorna-like viruses (order Picornavirales) infecting *Apis mellifera L.* populations. *Virology Journal* **5(1):** 1-10.

Berényi O, Bakonyi T, Derakhshifar I, Köglberger H, Topolska G, Ritter W, Pechhacker H, Nowotny N. 2007. Phylogenetic analysis of deformed wing virus genotypes from diverse geographic origins indicates recent global distribution of the virus. *Applied & Environmental Microbiology* **73(11):** 3605-3611.

Calderon RA, Veen JV, Arce HG, Esquivel ME. 2003. Presence of deformed wing virus and Kashmir bee virus in Africanized honey bee colonies in Costa Rica infested with *Varroa destructor*. *Bee World* **84(3):** 112-116.

Chao YZ, Cui XP, Wang Y, Wang HF, Liu SC, XU BH, Guo XQ. 2017. Investigation on the Occurrence of Deformed Wing Virus of Honeybees in Shandong Province. *Journal of Bee* **3:** 19-22.

Dalmon A, Desbiez C, Coulon M, Thomasson M, Le Conte Y, Alaux C, Vallon J, Moury B. 2017. Evidence for positive selection and recombination hotspots in Deformed wing virus (DWV). *Scientific Reports* **7:** 41045.

De Miranda JR, Genersch E, Genersch E, Evans JD, Fries I. 2010. Deformed wing virus. *Journal of Invertebrate Pathology* **103(1):** S48-61.

Ellis JD, Munn PA. 2005. The worldwide health status of honey bees. *Bee World* **86(4):** 88-101.

Forzan M, Felicioli A, Sagona S, Bandecchi P, Mazzei M. 2017. Complete Genome Sequence of Deformed Wing Virus Isolated from *Vespa crabro* in Italy. *Genome Announc* **5(40):** e00961-00917.

Fujiyuki T, Takeuchi H, Ono M, Ohka S, Sasaki T, Nomoto A, Kubo T. 2004. Novel Insect Picorna-Like Virus Identified in the Brains of Aggressive Worker Honeybees. *Journal of Virology* **78(3):** 1093-1100.

Gao F, Du Z, Shen J, Yang H, Liao F. 2018. Genetic diversity and molecular evolution of Ornithogalum mosaic virus based on the coat protein gene sequence. *PeerJ* **6(11):** e4550.

Geng P, Li W, Lin L, de Miranda JR, Emrich S, An L, Terenius O. 2014. Genetic characterization of a novel Iflavirus associated with vomiting disease in the Chinese oak silkmoth *Antheraea pernyi*. *PLoS One* **9(3):** e92107.

Hu Y, Fei DL, Jiang L, Wei D, Li FB, Diao QY, Ma MM. 2016. A comparison of biological characteristics of three strains of Chinese sacbrood virus in *Apis cerana*. *Scientific Reports* **6:** 37424.

Jakubowska AK, Murillo R, Carballo A, Williams T, van Lent JW, Caballero P, Herrero S. 2016. *Iflavirus* increases its infectivity and physical stability in association with baculovirus. *PeerJ* **4:** e1687.

Kevill JL, Highfield A, Mordecai GJ, Martin SJ, Schroeder DC. 2017. ABC Assay: Method Development and Application to Quantify the Role of Three DWV Master Variants in Overwinter Colony Losses of European Honey Bees. *Viruses* **9(11):** 314.

Kovac H, Crailsheim K. 1988. Lifespan of Apis Mellifera Carnica Pollm. Infested by *Varroa Jacobsoni Oud.* in Relation to Season and Extent of Infestation. *Journal of Apicultural Research* **27(4):** 230-238.

Lamp B, Url A, Seitz K, Eichhorn J, Riedel C, Sinn LJ, Indik S, Köglberger H, Rümenapf T. 2016. Construction and Rescue of a Molecular Clone of Deformed Wing Virus (DWV). *PLoS One* **11(11):** e0164639.

Lanzi G, de Miranda JR, Boniotti MB, Cameron CE, Lavazza A, Capucci L, Camazine SM, Rossi C. 2006. Molecular and biological characterization of deformed wing virus of honeybees (*Apis mellifera L.*). *Journal of Virology* **80(10):** 4998-5009.

Lee SH, Kim WK, No JS, Kim JA, Kim JI, Gu SH, Kim HC, Klein TA, Park MS, Son, JW. 2017. Dynamic Circulation and Genetic Exchange of a Shrew-borne Hantavirus, Imjin virus, in the Republic of Korea. *Scientific Reports* **7:** 44369.

Lian S, Lee JS, Cho WK, Yu J, Kim MK, Choi HS, Kim KH. 2013. Phylogenetic and recombination analysis of tomato spotted wilt virus. PLoS One 8(5): e63380.

Martin SJ. 2001. The role of Varroa and viral pathogens in the collapse of honeybee colonies: a modelling approach. *Journal of Applied Ecology* **38(5)**: 1082–1093.

Ma MM, Li M, Cheng J, Song YJ, Wang SD, Li PF. 2011. Molecular and Biological Characterization of Chinese Sacbrood Virus LN Isolate. *Comparative & Functional Genomics* **2011(3):** 409386.

Mcmahon DP, Natsopoulou ME, Doublet V, Fürst M, Weging S, Brown MJ, Gogol-Döring A, Paxton, RJ. 2016. Elevated virulence of an emerging viral genotype as a driver of honeybee loss. *Proceedings of the Royal Society B Biological Sciences* **283(1833):** 20160811.

Moore J, Jironkin A, Chandler D, Burroughs N, Evans DJ, Ryabov EV. 2011. Recombinants between Deformed wing virus and *Varroa destructor* virus-1 may prevail in *Varroa destructor*-infested honeybee colonies. *Journal of General Virology* **92(1):** 156-161.

Mordecai GJ, Brettell LE, Martin SJ, Dixon D, Jones IM, Schroeder DC. 2015. Superinfection exclusion and the long-term survival of honey bees in Varroa-infested colonies. *Isme Journal* **10(5):** 1182-1191.

Mordecai GJ, Wilfert L, Martin SJ, Jones IM, Schroeder DC. 2016. Diversity in a honey bee pathogen: first report of a third master variant of the Deformed Wing Virus quasispecies. *Isme Journa*l **10(5):** 1264-1273.

Ongus JR, Peters D, Bonmatin JM, Bengsch E, Vlak JM, van Oers MM. 2004. Complete sequence of a picorna-like virus of the genus Iflavirus replicating in the mite Varroa destructor. *Journal of General Virology* **85(Pt12):** 3747-3755.

Organtini LJ, Shingler KL, Ashley RE, Capaldi EA, Durrani K, Dryden KA, Makhov AM, Conway JF, Pizzorno MC, Hafenstein S. 2016. Honey Bee Deformed Wing Virus Structures Reveal that Conformational Changes Accompany Genome Release. *Journal of Virology* **91(2):** e01795-16.

492 **Prisco DG, Zhang X, Pennacchio F, Caprio E, Li J. 2011.** Dynamics of
493 Persistent and Acute Deformed Wing Virus Infections in Honey Bees, *Apis*
494 *mellifera*. *Viruses* **3(12):** 2425-2441 DOI: 10.3390/v3122425.
495 **Reddy KE, Jin HN, Yoo MS, Kim YH, Kim NH, Doan HTT, Ramya M, Jung**
496 **SC, Dong VQ, Kang SW. 2013**. Molecular characterization and
497 phylogenetic analysis of deformed wing viruses isolated from South Korea.
498 *Veterinary Microbiology* **167(3-4):** 272-279.
499 **Ryabov EV, Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D. 2014.** A
500 virulent strain of DeformedWing Virus (DWV) of honeybees (*Apis mellifera*)
501 prevails after *Varroa destructor*-mediated, or in vitro, transmission. *PLoS*
502 *Pathog* **10(6):** e1004230.
503 **Sambrook J, Russell D. 2001.** Molecular Cloning: A Laboratory Manual (3-
504 Volume Set). *Immunology* **49(1):** 895–909.
505 **Seo JK, Ohshima K, Lee HG, Son M, Choi HS, Lee SH, Sohn SH, Kim KH.**
506 **2009.** Molecular variability and genetic structure of the population of
507 soybean mosaic virus based on the analysis of complete genome sequences.
508 *Virology* **393(1):** 91-103.
509 **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011.**
510 MEGA5: molecular evolutionary genetics analysis using maximum
511 likelihood, evolutionary distance, and maximum parsimony methods. *Mol*
512 *Biol Evol* **28(10):** 2731-2739.
513 **Tentcheva D, Gauthier L, Zappulla N, Dainat B, Cousserans F, Colin ME,**
514 **Bergoin M. 2004.** Prevalence and seasonal variations of six bee viruses in
515 A*pis mellifera L.* and *Varroa destructor* mite populations in France. *Applied*
516 *& Environmental Microbiology* **70(12):** 7185-7191.

Vanengelsdorp D, Evans JD, Saegerman C, Mullin C, Haubruge E, Nguyen BK, Frazier M, Frazier J, Coxfoster D, Chen Y. 2009. Colony Collapse Disorder: A Descriptive Study. *Plos One* **4(8):** e6481.

Wang D, Yu C, Wang G, Shi K, Li F, Yuan X. 2015. Phylogenetic and recombination analysis of Tobacco bushy top virus in China. *Virology Journal* **12(1):** 111.

Xie X, Huang ZY, Zeng Z. 2016. Why do Varroa mites prefer nurse bees? *Scientific Reports* 6: 28228.

Zioni N, Soroker V, Chejanovsky N. 2011. Replication of *Varroa destructor* virus 1 (VDV-1) and a *Varroa destructor* virus 1–deformed wing virus recombinant (VDV-1–DWV) in the head of the honey bee. *Virology* **417(1):** 106-112.

Zheng Y, Yang Q, Song ZY, Wang ZG, Li MS. 2015. Progress on Deformed Wing Disease in Honey Bees. *Progress in Veterinary Medicine* **36(3):** 96-101.

Zhang Y, Han R. 2018. A Saliva Protein of Varroa Mites Contributes to the Toxicity toward Apis cerana and the DWV Elevation in *A . mellifera.* *Scientific Reports* **8:** 3387.

Table 1(on next page)

Table 1 Primers designed for overlapping sequences to ensure the complete sequencing of the selected DWV genotypes.

F: forward primer; **R:** reverse primer. Nucleotide positions refer to the published complete DWV genome sequence with GenBank accession number AY292384.

Primer name	Sequence(5′-3′)	Nucleotide position	Amplicon size(bp)
DWV1F	TCCATAGCGAATTACGGTG	8-26	741
DWV1R	GTCCCAGCTCTATCGCAGAAA	729-749	
DWV2F	GAA GTG ACT AGC AAT CAT GGA	605-625	716
DWV2R	ATG TCG YCT GGT YAT AGA CG	1320-1301	
DWV3F	TCT GTY GCC YAT GCA CCT C	1191-1210	691
DWV3R	GCG CTG GAA TAG ATG TAC TAG	1881-1861	
DWV4F	ACC CTA ATC CAG GAC CTG AT	1780-1799	657
DWV4R	AGG TAG TTG GAC CAG TAG CAC	2436-2416	
DWV5F	AACAAGAATTGTGCCAGA	2333-2350	716
DWV5R	GTTGCAAAGATGCTGTCA	3045-3028	
DWV6F	CCG TGG GTG TAG TAT CTA G	3011-3030	640
DWV6R	GCG AGC TCG TTC AGC ATT AT	3650-3631	
DWV7F	AGC AAG CTG CTG TAG GAA CTC	3547-3567	739
DWV7R	TGA CCA GTA GAC ACA GCA TC	4285-4266	
DWV8F	ACA TCG ACC GGA TCG TAG A	4211-4229	720
DWV8R	AGT AAC CGC WTG ACT ACA GT	4930-4911	
DWV9F	GAA GAC AGT TGC TTG GGC GA	4832-4851	796
DWV9R	AGG AGT ACG ACT CGC ACG T	5627-5609	
DWV10F	GAT ATG CAT GTG TGG TGC ATC	5540-5560	754
DWV10R	GTG TAC GCT CCT TAA ATG CCT	6294-6274	
DWV11F	AATCAGCGCTTAGTGGA	6249-6265	636
DWV11R	ATCAGTCAACGGAGCATAC	6866-6884	
DWV12F	GCR TGA ACG TTC ATC TTC AAC	6752-6772	720
DWV12R	AAT CTA TGG ATT CTA GGT GCC	7471-7451	
DWV13F	TCACCAGGAATGGCAA	7395-7411	746
DWV13R	ATC CTT CAG TAC CAG CAA CA	8140-8121	
DWV14F	CATGTTGCTGGTACTGAAGGA	8109-8129	483
DWV14R	TCCAGGCACACCACATACAGC	8571-8591	
DWV15F	GTGTGCCTGGWTTAGATGGG	8581-8600	622
DWV15R	GCT AAR ATC TCT TGC GCC AT	9202-9183	
DWV16F	GATTCTGATGTTGCAGCTTC	9081-9100	618
DWV16R	CCGAATGCTAACTCTAGCGC	9698-9678	
DWV17F	GCATCCAACTAGACCCGTGT	9576-9595	532
DWV17R	AGGACGCATTACCACTAGTTGA	10085-10107	

1

Table 2(on next page)

Table 2 DWVs strains used in this study.

1

No.	Name in this study	Accession Number	Geographic origin	Length (nucleotides)	Host species	Submitted year
D1	Italy-2002(AJ489744)	AJ489744	Italy	10140	*Apis mellifera*	2002
D2	USA-2003(AY292384)	AY292384	USA	10135	*Apis mellifera*	2003
D3	USA-2015(KT004425)	KT004425	USA	10137	*Apis mellifera*	-
D4	France-2016(KX373899)	KX373899	France	10143	-	2016
D5	UK-2009(GU109335)	GU109335	UK	10140	*Apis mellifera*	2009
D6	Italy-2017(KY909333)	KY909333	Italy	10104	*Vespa crabro*	2017
D7	Chile-2012(JQ413340)	JQ413340	Chile	10140	-	2012
D8	Austria-2016(KU847397)	KU847397	Austria	10164	*European honeybee*	2016
D9	China-2017(MF036686)	MF036686	China	9838	*Apis mellifera*	2017
D10	KV-2001(AB070959)	AB070959	Japan	10152	*Apis mellifera*	2001
D11	Korea-2012(JX878305)	JX878305	Korea	10096	*Apis mellifera*	2012
D12	Korea-2012(JX878304)	JX878304	Korea	10094	*Apis mellifera*	2012
D13	VDV-2004(AY251269)	AY251269	Netherlands	10112	*Varroa destructor mites*	2004
D14	Belgium-2016(KX783225)	KX783225	Belgium	10112	*Apis mellifera*	2016
D15	France-2016(KX373900)	KX373900	France	10103	-	2016
D16	UK-2010(HM067438)	HM067438	UK	10127	*Apis mellifera*	2010
D17	UK-2015(KT215905)	KT215905	UK	10264	*Apis mellifera*	2015
D18	UK-2014(KJ437447)	KJ437447	UK	10140	*Apis mellifera*	2014
D19	UK-2010(HM067437)	HM067437	UK	10126	*Apis mellifera*	2010
D20	UK-2015(KT215904)	KT215904	UK	10264	*Apis mellifera*	2015
D21	China1-2017(MF770715)	MF770715	China	10141	*Apis mellifera*	2017
D22	China2-2018(MH165180)	MH165180	China	10105	*Apis cerana*	2018

2

3

Table 3(on next page)

Table 3 Summary of possible recombination events in DWV isolates from Asian identified by RDP4

[a]Detection methods used in RDP4: R, RDP; G, GENECONV; B, BOOTSCAN; M, MaxChi; C, CHIMAERA; S, SISCAN; T, 3SEQ. Statistical signifcance is indicated according to the code described in "Materials and methods". NS: not significant

Event number	Recombinant Sequence (s)	Parental sequence (s)	Breakpoint position	Recombinant score	P-Value for the seven detection methods in RDP4 [a]						
		Major / Minor	Begin/End		R	G	B	M	C	S	T
1	Korea-2012(II)	KV-2001/Unknown	10130-10168	0.548	1.369E-7	1.513E-7	NS	NS	NS	NS	9.926E-4
2	KV-2001	Unknown/China2-2018	7500-8908	0.621	5.316E-2	NS	NS	8.604E-7	2.184E-5	4.671E-3	3.816E-2
3	China2-2018	Unknow/China-2017	4266-7507	0.49	5.309E-3	1.302E-7	2.551E-7	4.347E-8	5.820E-3	4.424E-12	3.675E-2
4	China-2017	China1-2017/China2-2018	973-1314	0.393	8.010E-2	NS	NS	4.723E-4	4.14E-3	2.76E-2	NS
5	China-2017	KV-2001/China1-2017	1315-1878	0.606	3.239E-7	NS	7.157E-6	4.662E-3	3.695E-3	NS	NS
6	China1-2017	China2-2018/KV-2001	2950-3624	0.461	NS	NS	NS	6.260E-4	1.273E-3	NS	NS
7	China1-2017	KV-2001/VDV-2013	991-1232	0.594	4.668E-3	NS	NS	3.228E-3	NS	NS	NS
8	Korea-2012(II)	KV-2001/ China2-2018	1222-1264	0.410	1.217E-2	3.589E-1	1.330E-2	5.172E-3	NS	4.554E-4	NS
9	KV-2001	Korea-2012(I)/ China2-2018	838-1202	0.423	7.182E-4	1.959E-2	5.385E-4	1.031E-3	NS	1.357E-5	NS

Figure 1

Figure 1 RT-PCR detection results

M: DNA marker DL2000; A: China1-2017 detection results B: China2-2018 detection results; C: Negative control (Healthy worker bee cDNA)

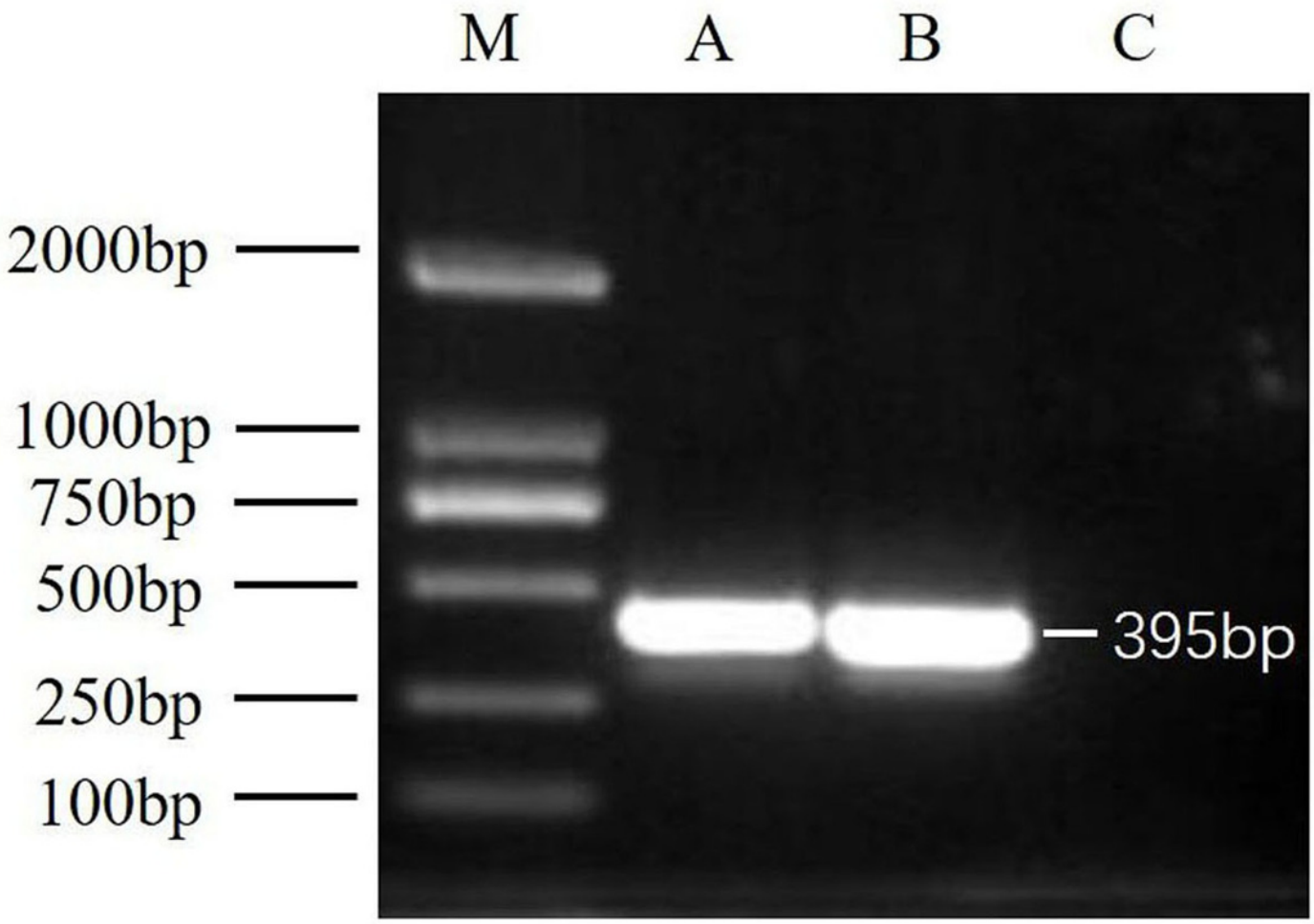

Figure 2

Figure 2 Phylogenetic trees of DWV isolates

A Phylogenetic tree based on the ORF-coding nucleotide sequence of DWV. **B** Phylogenetic tree based on the VP1 segment of DWV. **C** Phylogenetic tree based on the 3C+RdRp segment of DWV.

Note: All phylogenetic trees were constructed by Maximum-likelihood method (ML) method with bootstrap resampling (1000 replicates). The number at each branch of phylogenetic tree represents the bootstrap value (1000 replicates). The different colors triangles indicate the different clusters of DWV isolates.

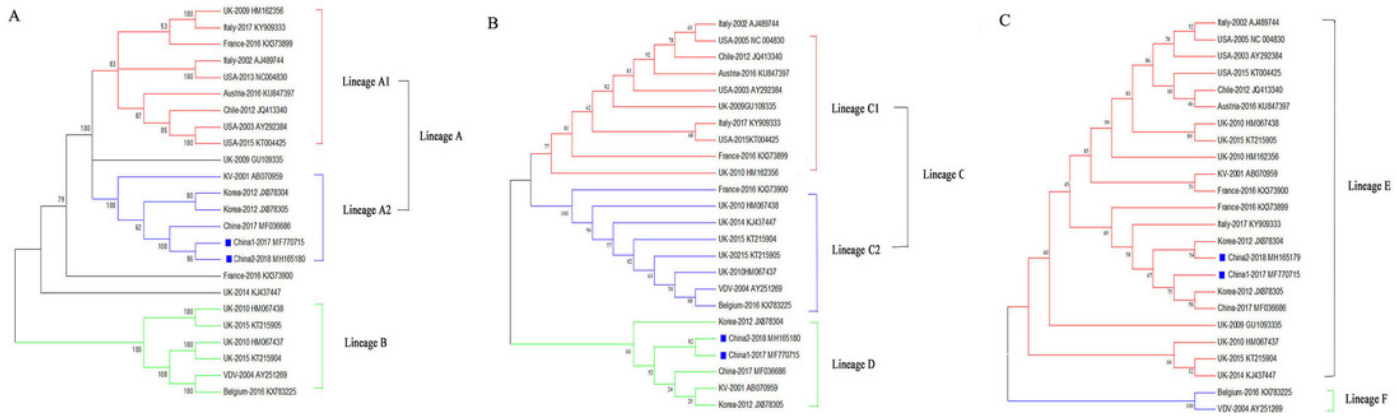

Figure 3

Figure 3 Analysis of possible recombination in different DWV isolates from Asia

A Genomic organization of DWV. **B** Summary of potential recombination in different isolates of DWV. Two events in China-2017 (Event 4, 5), three events in China1-2017(Event 6, 7), one event in China2-2018(Event 3), two events in Korea II -2012(Event 1, 8), two events in KV-2001(Event 2, 9). Dark bars in Fig. 4b indicating recombination regions with breakpoint positions and minor parent shown. Dot lines indicating breakpoints. Detailed information of recombination assay is provided in Table 4.

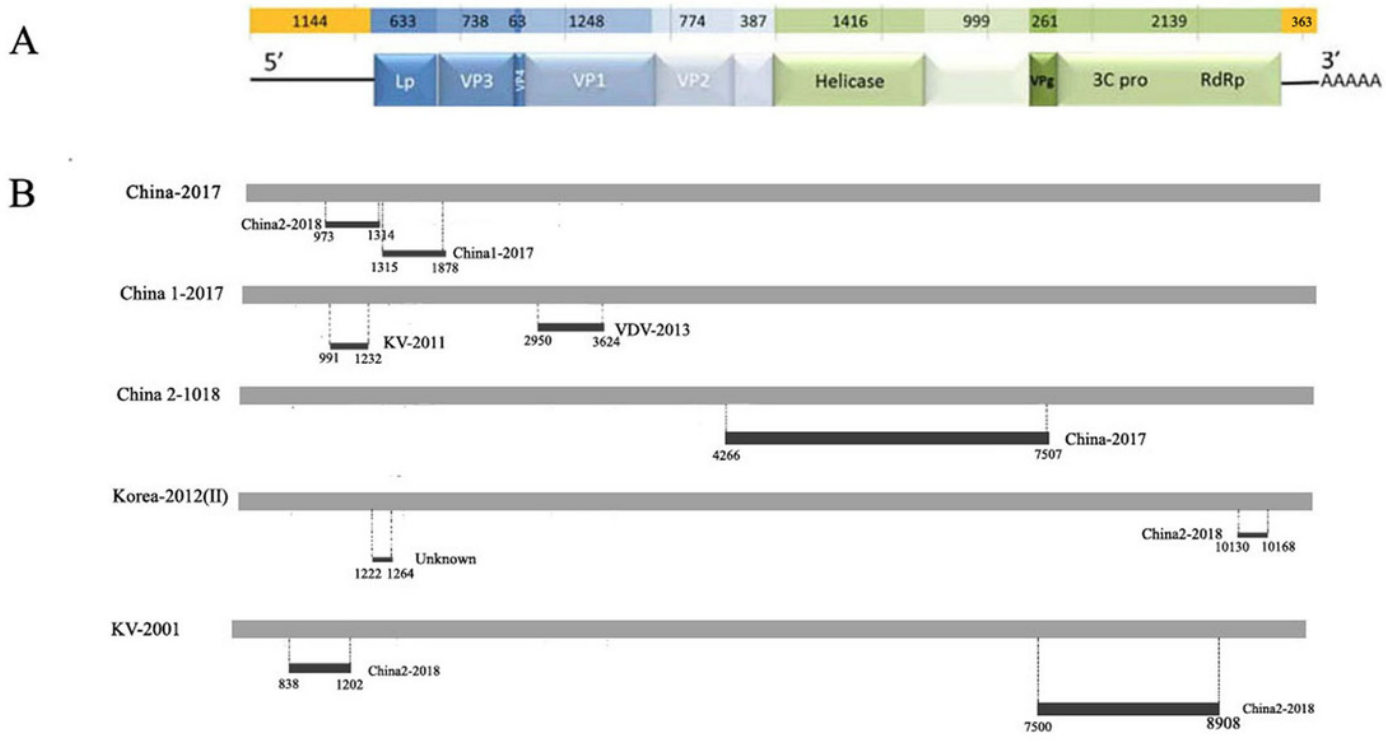

Figure 4

Figure 4 Recombination analysis of full-length DWV genome sequences of China-2017 and China2-2018 isolations by RDP4

An alignment of the 8 DWV genomes from Asia and Europe was analysed using the RDP4 software. Two potential recombination events were identifed for Chinese strains (illustrated by A–F, respectively). The left part illustrates theresults of RDP analyses (A and D). The right part presents phylogenetic analyses based on the full-length genome (excluding one of the terminal direct repeats) excluding the region of recombination (B and E) or based on the recombination region only (C and F) using UPGMA in MEGA5.0 with 1000 replicates. Values on internal branches refer to the percentage of bootstrap replicates in which the branch was found; only values greater than 50% are shown. The scales illustrate the number of substitutions per nucleotide. The color code used is described at the top.