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Author(s): Jilian Li, Wenjun Peng, Jie Wu, James P. Strange, Humberto Boncristiani, and Yanping Chen
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Cross-Species Infection of Deformed Wing Virus Poses a New Threat to Pollinator Conservation

JILIAN LI,[1] WENJUN PENG,[1] JIE WU,[1] JAMES P. STRANGE,[2] HUMBERTO BONCRISTIANI,[3] AND YANPING CHEN[3,4]

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ABSTRACT The Deformed wing virus (family *Iflaviridae*, genus *Iflavirus*, DWV), one of the most prevalent and common viruses in honey bees, *Apis mellifera* L., is present in both laboratory-reared and wild populations of bumble bees, *Bombus huntii* Greene. Our studies showed that DWV infection spreads throughout the entire body of *B. huntii* and that the concentration of DWV is higher in workers than in males both collected in the field and reared in the laboratory, implying a possible association between the virus infection and foraging activities. Further results showed that gut tissue of *B. huntii* can support the replication of DWV, suggesting that *B. huntii* is a biological host for DWV, as are honey bees. Bumble bees and honey bees sometimes share nectar and pollen resources in the same field. The geographical proximity of two host species probably plays an important role in host range breadth of the virus.

KEY WORDS honey bee, bumble bee, virus, host expansion

Bumble bees (*Bombus* spp.) are the most important native pollinators in temperate and subarctic ecosystems throughout the Northern Hemisphere (Bingham and Orthner 1998) and South America (Velthuis and van Doorn 2006). In comparison with managed honey bees, *Apis mellifera* L., bumble bees are relatively more active at lower temperatures and light intensity and less dependent on weather conditions for their foraging activities (Winter et al. 2006). Currently, bumble bees are used extensively for greenhouse pollination of a variety of high-value crops, such as sweet pepper, *Capsicum annuum* L., and tomatoes, *Solanum lycopersicum* L. (Velthuis and van Doorn 2006). The pollination services provided by bumble bees not only contribute to the productivity of agricultural crops but also to the survival and reproduction of many native plants. Many species of bumble bees, including *Bombus terrestris* (L.), *Bombus huntii* Greene, *Bombus lucorum* (L.), *Bombus occidentalis*, *Bombus ignitus*, and *Bombus impatiens* (Cresson) have been reared and used for commercial crop pollination worldwide (Velthuis and van Doorn 2006, Li et al. 2008).

Approximately, ≈70% of crops grown for direct human consumption are dependent on bee pollination (Klein et al. 2007). In a recent study, it was reported that the services provided by pollinators worldwide are worth US$216 billion (€153 billion) a year, which represents 9.5% of the entire world agricultural production used for human food in 2005 (Gallai et al. 2009). However, an extensive reduction in abundance of pollinators has been reported worldwide in recent years and has caused significantly negative impacts in the agricultural ecosystem (Biesmeijer et al. 2006, Fitzpatrick et al. 2007, Kluser and Peduzzi 2007, Kremen and Ricketts 2000, Oldroyd 2007, Stokstad 2007). Like honey bees that have been experiencing diseases and substantial declines, there has been a severe decline in the abundance and distribution of some bumble bee species in the past 30 yr (Cameron et al. 2011), possibly due to the destruction of resources, use of pesticides, and infection of various pathogens (Colla et al. 2006, Otterstatter and Thomson 2008). Several studies suggest that pathogen-caused diseases are major factors negatively impacting the ecology and distribution of bumble bees and their use as commercial pollinators (reviewed in Schmid-Hempel 2001). Thus, the conservation of pollinators has become an integrated part of our biodiversity conservation efforts.

The cross-species infections by pathogens often play a critical role in the emergence of new diseases in which an established pathogen crosses the species barrier and jumps to a new host and then subsequently spreads within new host populations. Previous studies showed that deformed wing virus (family *Iflaviridae*, genus *Iflavirus*, DWV), a common and prevalent honey bee virus that causes wing deformity in honey

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[1] Institute of Apicultural Research, Chinese Academy of Agricultural Science, Beijing 100093, China.

[2] USDA–ARS Pollinating Insects–Biology, Management and Systematic Laboratory, BNR 255, Utah State University, Logan, UT 84322-5310.

[3] USDA–ARS Bee Research Laboratory, BARC-East Bldg., 476, Beltsville, MD 20705.

[4] Corresponding author, e-mail: judy.chen@ars.usda.gov.

bees (Allen and Ball 1996, Ellis and Munn 2005), could cause infection in two species of bumble bees, *B. terrestris* and *Bombus pascuorum* (Scapoli) (Genersch et al. 2006). To gain a deep insight into the cross-species infection of pathogens between populations of honey bees and bumble bees, DWV infection in laboratory-raised and wild-caught *B. huntii*, one of the most common bumble bee species in North America (Williams and Osborne 2009), was investigated in the current study.

Materials and Methods

Insects. Three groups of *B. huntii* were include in this study: 1) male *B. huntii* reared in the laboratory, 2) worker *B. huntii* reared in the laboratory, and 3) male *B. huntii* collected from the field. Laboratory-reared males and workers came from a colony originated from a queen collected in the spring. The colony was fed sugar syrup (25% sucrose, glucose, and fructose) and pollen collected from honey bee colonies, until the colony reached a size exceeding 100 individuals. Individual bees were then removed from the colony and mailed alive in cages provisioned with sugar syrup to the USDA-Bee Research Lab, Beltsville, MD, for molecular analysis. Field-collected males were collected in the gardens at the USDA-Pollinating Insect Research Unit, Logan, UT, and captured with an entomological aerial net while foraging on raspberry (*Rubus* sp.). No female *B. huntii* were observed during the field collection, probably due to the seasonality of worker production in bumble bees. Captured bees from the field were transferred directly to a separate shipping cage also provisioned with sugar syrup. All bees were express shipped the day they were captured.

Tissue Dissection. Each live bumble bee was restrained on the wax top of a dissecting dish with steel insect pins. Under a dissecting microscope, ≈20 μl of hemolymph was collected from each bee with a micropipette tip by making a small hole on the roof of the bee's thorax with a needle to make it bleed. After hemolymph collection, the legs, wings, antennae, and eyes were cut off with a pair of fine scissors. The body was opened by cutting the dorsal body wall with scissors along the dorsal mid-line from the head to the tip of the abdomen. Tissues of the brain, fat bodies, salivary gland, gut, nervous system, trachea, and hypopharyngeal gland were individually removed using a pair of fine forceps under a dissecting microscope. To prevent possible contamination with hemolymph, all tissues were rinsed three times in 1× phosphate-buffered saline and twice in nuclease-free water. The washing solution was changed every time for each tissue to prevent possible cross-contamination. In total, 12 tissues were collected from each bee, and 10 bees in total were dissected for each group of *B. huntii*. For each group, the same tissues of 10 bees were pooled together for subsequent RNA extraction and analysis.

RNA Extraction. All tissue samples were subjected to RNA extraction after tissue dissection. Each tissue was ground manually in a 1.5-ml tube by using a pestle that fits into a 1.5-ml Eppendorf tube. Total RNA was extracted from each tissue by using TRIzol reagent according to the manufacturer's instructions (RNA extraction kit, Invitrogen, Carlsbad, CA). The resultant RNA pellets were resuspended in UltraPure DNase/RNase-free water (Invitrogen) in the presence of ribonuclease inhibitor (Invitrogen). The quantity and purity of RNA were measured using a spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA samples were stored at −80°C until used.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR). To determine the presence, distribution, and quantity of DWV infection within the body of *B. huntii*, SYBR-Green real-time quantitative (q)RT-PCR was performed for detection and quantification of DWV in different tissues of *B. huntii*. qRT-PCR was performed using an Mx3005P real-time PCR system operated by MxPro qPCR software (Stratagene, La Jolla, CA). A pair of primers (forward, 5′-CGAAACCAACTTCTGAGGAA-3′ and reverse, 5′-GTGTTGATCCCTGAGGCTTA-3) was used for amplification of a 174-bp DWV-specific fragment. The expression of a housekeeping gene, β-actin, in each sample also was measured for normalization of real time qRT-PCR results using a pair of primers (forward, 5′-AGGAATGGAAGCTTGCGGTA-3′ and reverse, 5′-AATTTTCATGGTGGATGGTGC-3′) under the same conditions as described for DWV. The β-actin primers were expected to amplify a 181-bp PCR fragment. The qRT-PCR was carried out in a 50-μl reaction volume containing 25 μl of 2× Brilliant SYBR Green qRT-PCR Master Mix (Stratagene,), 0.4 μM each of forward and reverse primer, and 200 ng of template RNA. The thermal profile parameters consisted of one cycle at 50°C for 30 min, one cycle at 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s. After amplification, a melting curve analysis was performed to determine the specificity of the PCR products. The PCR products were incubated for 1 min at 95°C, ramping down to 55°C at a rate of 0.2°C/s. The dissociation curve was constructed using 81 complete cycles of incubation where the temperature was increased by 0.5°C/cycle, beginning at 55°C and ending at 95°C. Negative controls (no reverse transcriptase and no template) were included in each run of the reaction. The positive control was purposely not included in the reaction to avoid any potential chances of contamination. The expected size of the PCR product was confirmed by 1.5% agarose gel electrophoresis and subsequent visualization with ethidium bromide. Individual PCR bands were cut out and purified using Wizard PCR Prep DNA purification system (Promega, Madison, WI) and sequenced to confirm the specificity of the qRT-PCR assay. The RNA extraction, PCR reaction mixture, PCR amplification, and post-PCR products were handled in different areas to reduce the risk of contamination. The nucleotide sequences of PCR products were determined and compared with sequences published at GenBank, National Center for

Biotechnology Information, National Institutes of Health, Bethesda, MD.

Statistics and Data Interpretation. The level of the virus and β-actin was quantified based on the value of the cycle threshold (Ct) that represents the number of cycles needed to generate a fluorescent signal above a predefined threshold and therefore is inversely proportional to the concentration of the initial target that has been amplified. qRT-PCR was replicated three times for each sample to address the variability of the analysis process. The values obtained for DWV and β-actin were averaged individually, and the data are represented as means ± SE. The comparison of the relative amount of DWV in different tissues of *B. huntii* was conducted by using the comparative Ct method ($2^{-\Delta\Delta Ct}$ Method). The mean value and standard deviations of triplicate measurements of DWV in each tissue was normalized using the Ct value corresponding to the triplicate measurements of endogenous control, β-actin following the formula $\Delta Ct = (\text{avg } Ct_{DWV}) - (\text{avg } Ct_{\beta\text{-actin}})$. The tissue that had the minimal virus level of DWV was chosen as a calibrator. The ΔCt value of each tissue was subtracted by ΔCt value of the calibrator to yield ΔΔCt. The concentration of DWV in each tissue was calculated using the formula $2^{-\Delta\Delta Ct}$ and expressed as the fold change.

Strand-Specific RT-PCR for Analysis of DWV Replication. To determine whether different tissues of bumble bees were able to serve as biological hosts to support replication of DWV, RNA samples identified with DWV infection by qRT-PCR were further analyzed for the presence of the negative-strand RNA of DWV, a replicative intermediate, by using strand-specific reverse transcription coupled with PCR. The cDNA that is complementary to the negative-strand RNA of DWV was synthesized from total RNA extracted from different tissues of *B. huntii* with Tag-DWV-sense primer (5′-agcctgcgcacgtggATCAGCGCTTAGTGGAGGAA-3′) and SuperScript III Reverse transcriptase (Invitrogen). The cDNA that is complementary to the positive-strand RNA of DWV also was synthesized using Tag-DWV-antisense primer (5′-agcctgcgcacgtggTCGACAATTTTCGGACATCA-3′) by using SuperScript III reverse transcriptase (Invitrogen). The sequence of Tag is shown in lowercase and was published by Yue and Genersch (2005). The synthesized cDNAs by both Tag-DWV-sense primer and Tag-DWV-antisense primers were then purified individually using MinElute PCR purification kit and MinElute Reaction Clean kit to remove short fragments of oligonucleotides and residues of enzymatic reagents to prevent amplification of nonstrand-specific products (Boncristiani et al. 2009), respectively. The PCR amplification of cDNA for negative-strand RNA of DWV was conducted with a Tag (3′-AGCCTGCGCACCGTGG-5′) and DWV-antisense primer (5′-TCGACAATTTTCGGACATCA-3′). The PCR amplification of cDNA for positive-strand RNA of DWV was conducted with DWV-sense primer (5′-ATCAGCGCTTAGTGGAGGAA-3′) and the same Tag primer. The following thermal cycling profile was used: 94°C for 2 min; 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min; and an extension at 72°C for 10 min. A PCR fragment of 718 bp was expected to be generated. Positive control (plasmid DNA with DWV fragment inserted into pCR 2.1 vector [Invitrogen]) and negative control (no reverse transcriptase and no template) were included in each run of the reaction. The PCR products were electrophoresed on ethidium bromide-containing agarose gel and the specificity of PCR products were purified and confirmed by sequencing analysis as described above.

Phylogenetic Analysis. The sequences of DWV amplified from the region of RNA-dependent RNA polymerase (RdRp) domain by RT-PCR in the current study were compared with the sequences of different geographic strains of DWV retrieved from GenBank and identified in honey bees from Pennsylvania, USA (GenBank accession AY292384); Maryland. USA (GenBank accession FJ347142); France (GenBank accession AY224602); United Kingdom (GenBank accession GU109335); Korea (GenBank accession EU836051); and Taiwan (GenBank accession GU108222). The sequence of *Varroa destructor* virus-1 (GenBank accession AY152713) was used as an outgroup to root the tree. The sequences were aligned using MegAlign (Lasergene software program, DNASTAR, Madison, WI). The sequences that could not be aligned unambiguously at both 3′ and 5′ ends were truncated. Phylogenetic analysis was conducted in MEGA5 by using ClustalW. Maximum parsimony under a heuristic search was used to construct the phylogenetic relationship. The reliability of the phylogenies was assessed by bootstrap replication (N = 500 replicates). Numbers in the nodes correspond to bootstrap values and bootstrap values of >50% were regarded as providing evidence for the phylogenetic grouping.

Results

The results of the qRT-PCR assay showed the detection of DWV in different tissues of *B. huntii.* For males collected from the field, DWV was detected in 10 examined tissues including wings, legs, antennae, fat bodies, gut, hemolymph, salivary gland, nervous system, trachea, and hypopharyngeal gland but not in the brain and compound eyes (Fig. 1). Males reared in the laboratory had a similar pattern of tissue infection (Fig. 2) except that virus was detected in the brain, but not in the hemolymph. Workers reared in the laboratory, had detectable DWV in eleven tissues and only lacked virus in the compound eyes (Fig. 3). In fact, no DWV signal was detected in the compound eyes of any group of *B. huntii* (Table 1).

The amplification efficiencies of the SYBR-Green real-time qRT-PCR assay for both DWV and β-actin were compared and validated to be approximately equal (data not shown); therefore, the comparative Ct method for relative quantification of DWV used in the current study was valid. For males collected from the field, hemolymph had the lowest level of DWV concentration among 12 examined tissues and was chosen as a calibrator. In relation to the DWV in hemolymph,

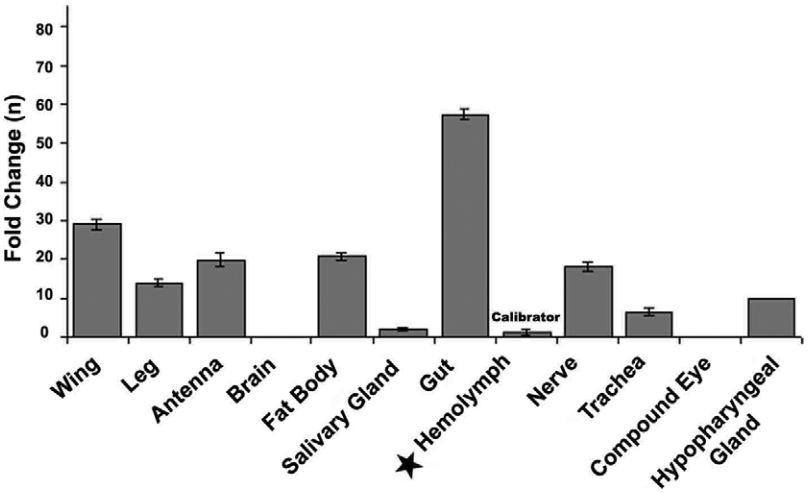

Fig. 1. Relative abundance of DWV RNA genome copies in different tissues of *B. huntii* males collected from the field. The tissue of hemolymph had the minimal level of DWV and therefore was chosen as a calibrator. The concentration of DWV RNA in other tissues was compared with calibrator and expressed as n-fold change. The y-axis depicts fold change relative to the calibrator.

the concentration of DWV in the other tissues ranged from 1.94 to 57.8-fold difference with gut harboring the highest concentration of DWV RNA. The concentration of DWV descended from gut in the following order: gut > wing >fat body > antennae > nervous system > leg > hypopharyngeal gland > trachea > salivary gland > hemolymph (Fig. 1). In laboratory-reared *B. huntii* males, the gut had the highest concentration of DWV among all examined tissues. The concentration of DWV in the gut was 10-fold higher than that in nerve tissues that had the lowest level of DWV concentration in the group. However, the DWV concentrations in all tissues of *B. huntii* male reared in the laboratory were relatively lower than that in tissues of males collected from the field (Figs. 1 and 2). The tissues of *B. huntii* females reared in the laboratory had the highest concentrations of DWV among the three experimental groups. Among tissues within the same group, nerve tissue also had the lowest level of DWV concentration and therefore was used as a calibrator for the calculation of relative concentration of the virus in the group. As in the other two groups, gut had the highest concentration of DWV among all examined tissues (Fig. 3).

The results of the strand-specific RT-PCR assays were consistent with the results of qRT-PCR (Table 1). For males collected from the field, except for the tissues of the compound eyes and the brain, the spe-

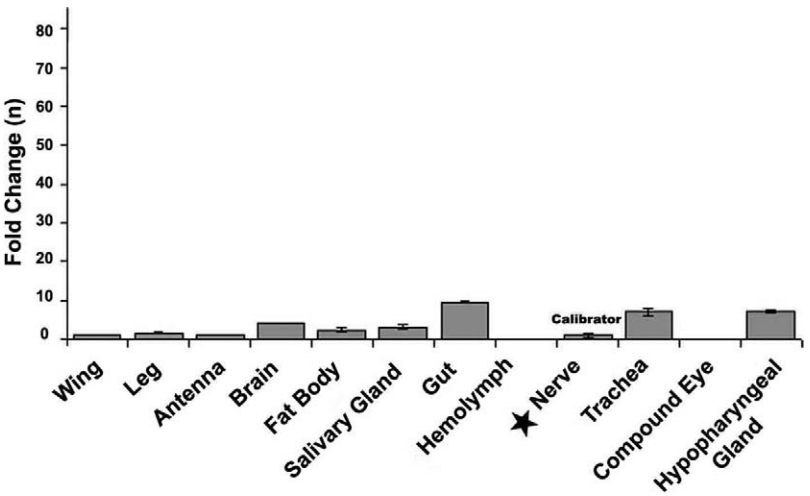

Fig. 2. Relative abundance of DWV RNA genome copies in different tissues of *B. huntii* males reared in the laboratory. The tissue of nerve had the minimal level of DWV and therefore was chosen as a calibrator. The concentration of DWV RNA in other tissues was compared with calibrator and expressed as n-fold change. The y-axis depicts fold change relative to the calibrator.

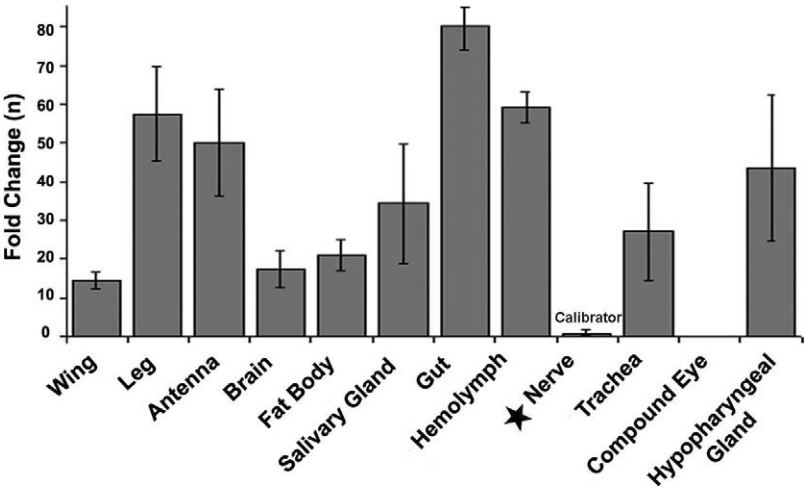

Fig. 3. Relative abundance of DWV RNA genome copies in different tissues of *B. huntii* workers reared in the laboratory. The tissue of nerve had the minimal level of DWV and therefore was chosen as a calibrator. The concentration of DWV RNA in other tissues was compared with calibrator and expressed as n-fold change. The y-axis depicts fold change relative to the calibrator.

cific signal of positive-strand RNA of DWV was detected in all the tissues. For males reared in the laboratory, except for the compound eyes, the signal of positive-strand RNA of DWV was detected in all examined tissues (Fig. 4A). For workers reared in the laboratory, the tissues of the compound eyes, hemolymph, and hypopharyngeal gland were negative for positive-strand RNA of DWV, and the other tissues were positive for the positive-strand RNA of DWV. Detection of negative-strand RNA of DWV was done simultaneously in all tissues. However, the signal of negative-strand RNA of DWV was detected only in the tissues of the gut for each group of *B. huntii* (Fig. 4B).

A phylogenetic tree based on the partial sequences of RdRp domain illustrated the phylogenetic relationship of the DWV from *B. huntii* collected in Utah with the DWV from its original host, *A. mellifera*, from different regions of the world (Fig. 5). The DWVs from *B. huntii* collected from the field and reared in the laboratory clustered into a monophyletic clade that was most closely related to the DWV isolate from *A. mellifera* in Pennsylvania. The DWV from *B. huntii* seemed to be the most distantly related to the strain of DWV from Asia, including Taiwan and Korea in the phylogenetic tree.

Table 1. Presence of strand-specific RNA of DWV in the tissues of the *B. huntii*

Tissue	Males from field		Males reared		Workers reared	
	Positive strand	Negative strand	Positive strand	Negative strand	Positive strand	Negative strand
Wing	+	−	+	−	+	−
Leg	+	−	+	−	+	−
Antenna	+	−	+	−	+	−
Brain	−	−	+	−	+	−
Fat body	+	−	+	−	+	−
Salivary gland	+	−	+	−	+	−
Gut	+	+	+	+	+	+
Hemolymph	+	−	+	−	+	−
Nerve	+	−	−	−	+	−
Trachea	+	−	+	−	+	−
Compound eyes	−	−	−	−	−	−
Hypopharyngeal gland	+	−	+	−	+	−

Discussion

Cross-species infections by pathogens are often responsible for new emerging and reemerging diseases and therefore of significant concern. Here, we provide the evidence that DWV, one of the most prevalent honey bee viruses, causes infection in both laboratory-reared and field-collected bumble bees, *B. huntii.* Although infected *B. huntii* did not show overt signs of illness, the infection of the DWV had spread throughout the tissues of the host body. Among all the tissues that harbored the DWV, the guts contained the highest concentration of the virus. The observation of the highest concentration of the DWV found in the gut together with invasion of the virus in various tissues suggests that the gut was the initial site of the virus infection and that the replication and multiplication of the virus occurred at the site of entry and then spread into different tissues. However, it is unclear whether the nondetection of DWV in the compound eyes of the three experimental groups was due to the presence of PCR inhibitors in the compound eyes (Boncristiani et al. 2011) or whether DWV does not attack the tissue of compound eyes.

Our previous study (Chen et al. 2006) demonstrated that DWV could be found in comb-stored pollen in honey bee colonies and suggested that DWV could be spread throughout the body of honey bees via a food-borne transmission pathway. In nature, bumble bees share nectar and pollen resources with honey bees, and honey bees were observed in

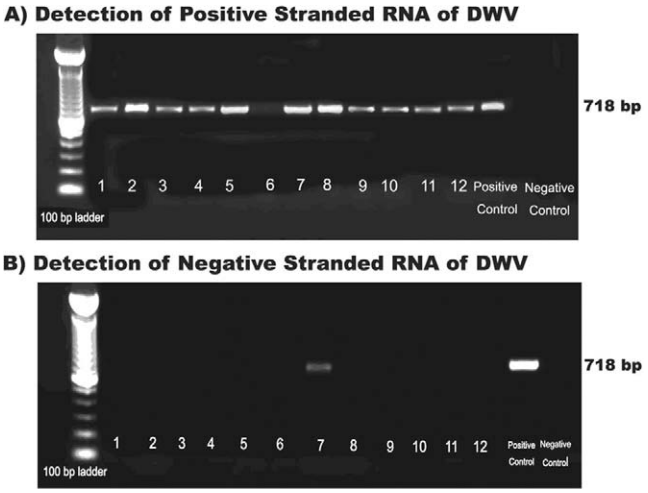

Fig. 4. Detection of positive-strand (A) and negative-strand (B) RNA of DWV in different tissues of *B. huntii* male reared in the laboratory. Strand-specific RT-PCR was performed in 12 different tissues: 1) wings, 2) legs, 3) antennae, 4) brain, 5) salivary gland, 6) compound eye, 7) gut, 8) hypopharyngeal gland, 9) hemolymph, 10) nerve, 11) trachea, and 12) fat body. The 718-bp bands on the right side of the gels indicate the presence of both positive- and negative-strand RNA of DWV. Negative (H_2O) and positive controls were included in the RT-PCR. A 100-bp DNA ladder was included in the first lane of the gels.

the garden when bumble bees from the current study were collected. This geographic and ecological overlap creates opportunity for cross-species transmission of pathogens. If food is a source of virus transmission, then it is likely that bumble bees pick up the virus from flowers that have previously been visited by infected honey bees, bring virus-contaminated food into the colonies and the virus is spread within the colony via consumption of virus-contaminated food. The observation of the significantly higher concentration of DWV in the gut together with the factor that the colony used in the study was fed with pollen collected from honey bee colonies suggests that transmission of DWV in *B. huntii* probably is occurring via a food-borne transmission pathway. The close phylogenetic distance of DWV from the U.S. populations of *B. huntii* with DWV from U.S. populations of *A. mellifera* further supports the conclusion that geographical proximity between two hosts plays a role in horizontal transfer of the virus. Furthermore, the fact that commercially reared bumble bees are fed pollen collected from honey bee colonies (Velthuis and van Doorn 2006), presents a direct pathway for DWV into commercial production facilities if the pollen is contaminated by the virus.

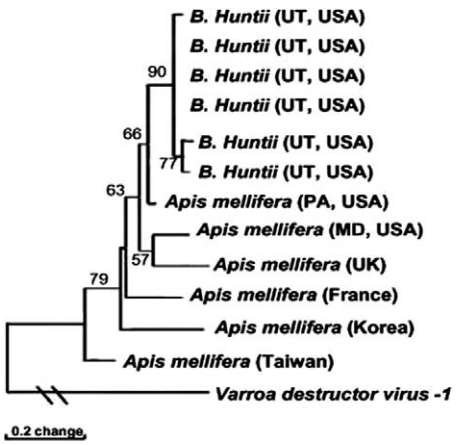

Fig. 5. Phylogenetic tree showing the relationship between DWV from *B. huntii* and *A. mellifera* from different geographic locations. The partial sequences of RdRp domain of DWV from *B. huntii* in the current study were compared with the sequences from honey bees retrieved from GenBank and originating from different countries were used to produce the phylogenetic tree. The sequence of *V. destructor* virus-1 was used as an outgroup to root the tree. Phylogenetic analysis was conducted using ClustalW and neighbor-jointing algorithm. Numbers in the nodes correspond to bootstrap values.

DWV contains a positive-stranded, polyadenylated, and monopartite monocistronic RNA genome (Lanzi et al. 2006). DWV was first isolated from diseased adult bees in Japan (Bailey and Ball 1991), and now the occurrence and distribution of DWV are worldwide (Allen and Ball 1996, Ellis and Munn 2005). Like other honey bee viruses, DWV generally persists as an inapparent infection in honey bee colonies (reviewed in Chen and Siede 2007), and severe DWV infection could lead to honey bee wing deformities (Chen et al. 2004). A previous study showed that the DWV was very pathogenic to two species of bumble bee (*B. terrestris* and *B. pascuorum*) by causing wing deformities (Genersch et al. 2006). However, in the current study where DWV was found in both laboratory-reared and field-collected *B. huntii*, no symptoms were observed in either individuals or at the colony level. These direct transfers of pathogens between two host

species involve not only the opportunity of exposure but also the acquisition of variations that allow pathogens to overcome host species barriers to establish the infection in the new hosts. Due to the high mutation rate and short generation time, RNA viruses have been reported to the group of pathogens that are the most likely to cross the species barriers and adapt into a new environment (Pedersen et al. 2005). The high mutation rate of the RNA virus in conjunction with the geographic proximity between the bumble bees and honey bees and the high contact rate of foragers at food sources would provide high adaptability for host shift of DWV in nature.

The long-term consequences of a host range breach can range from transient pathogen "spillover" to persistence of infections. The presence of positive-strand RNA of DWV in different tissues provided evidence of the virus invasion and adherence in the host. The replication of DWV in infected *B. huntii* was demonstrated by the detection of negative-sense molecules in gut tissue. The replication event observed in the study indicates an established infection in the host. However, compared with the widespread distribution of positive-strand RNA of DWV within the body of *B. huntii*, the active replication of the virus seemed to be limited to the gut. It is unclear whether the failure in detection of negative-strand RNA of DWV in other tissues was due to the presence of undetectable or *low copy* number of the virus replicates or the existence of host mechanisms to regulate the pattern of the virus replication. Further investigation to confirm if tissues that harbored significant amounts of DWV can serve as biological compartments for the virus replication will be necessary.

In sum, the current study demonstrated that a honey bee virus, DWV, attacks the bumble bee species *B. huntii* in both wild and laboratory-reared bees, and suggests that cross-species infection of pathogens may be frequent events across populations of honey bees and bumble bees. The pathogens in honey bees likely pose the biggest risk of the host shift to bumble bees due to the close relatedness, foraging behavior, and geographical proximity of the two bee species.

Acknowledgements

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