

Short communication

Development of a rapid and sensitive RT-PCR method for the detection of deformed wing virus, a pathogen of the honeybee (*Apis mellifera*)

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Deformed wing virus (DWV) is a honeybee pathogen, transmitted to larvae by the ectoparasitic mite *Varroa destructor*. It is an insect picorna-like, positive-stranded RNA virus and recently the complete nucleotide sequence of the ssRNA genome of DWV has been determined. Larvae infected with DWV either develop to adult bees showing a reduction in emergence size and characteristic morphological deformities, or die during pupation (DeJong et al., 1982; Bowen-Walker et al., 1999). Bee deformity includes malformed appendages, shortened abdomens, and miscolouring (Fig. 1). Bees infected with DWV also have a reduction in life span (Kovac and Crailsheim, 1988). DWV is considered one of the most serious secondary pathogens vectored by *V. destructor*. Secondary infections with DWV add to the pathology of *V. destructor* and play a major role in colony collapse in the course of Varroa infestation (Martin, 2001).

Until now, identification of DWV has been based on traditional methods such as electron microscopy and ELISA techniques (Bowen-Walker et al., 1999; Nordstrom, 2003). Culture methods cannot be used since no honeybee cell lines are available. The traditional techniques are low in sensitivity and specificity, thus hampering molecular analysis of the incidence and

pathogenicity of DWV and of investigations concerning host–vector–pathogen relationship. Here, we describe for the first time an RT-PCR based method for the rapid and sensitive detection of DWV in honeybee samples.

Based on the complete nucleotide sequences of the genome of DWV (accession no. NC 004830, Lanzi and Rossi, unpublished; accession no. AY292384, de Miranda et al., unpublished) a panel of unique PCR primers within a 6500 bp region from the 3' end of the genome (Table 1) was designed to amplify seven fragments ranging from 600 to 400 bp (Fig. 2). One-step-RT-PCR reactions were performed according to standard protocols (One-step-RT-PCR kit, Qiagen) using the following temperature scheme: 30 min at 50 °C, 15 min at 95 °C followed by 35 cycles with 30 s at 94 °C, 1 min at 54.3 °C, 30 s at 72 °C, each, including a final elongation step for 10 min at 72 °C. Total RNA was extracted from heads of crippled bees collected from a Varroa infested colony. Total RNA from heads of asymptomatic bees was used as negative control. RNA extraction was performed using standard methods (RNeasy Kit, Qiagen).

To evaluate the usefulness of the primers designed for the detection of DWV, 20 crippled bees were analyzed by RT-PCR for the presence of DWV sequences. Each of the seven primer pairs generated a single amplicon. As negative control, twenty healthy-looking bees from two colonies differing in their grade of Varroa infestation were collected. No amplicons were

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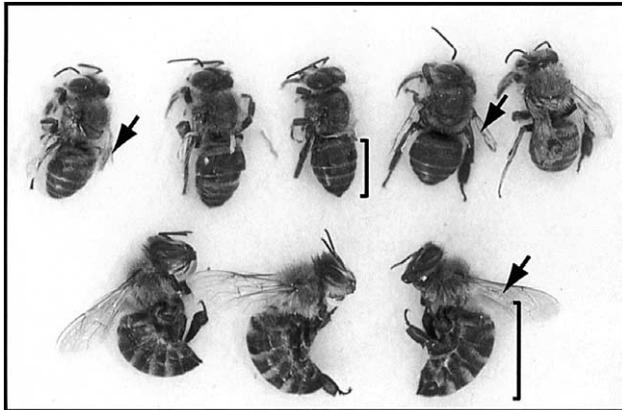


Fig. 1. Crippled and healthy worker bees. In the upper row five crippled worker bees are depicted which show the characteristic symptoms of DWV-infection: deformed wings (arrows) and a shortened abdomen (bracket). In the lower row three asymptomatic, healthy-looking bees with normal wings (arrow) and normal size of abdomen (bracket) are shown.

generated in any of these probes (Fig. 3). Electrophoretic mobility of the amplicons correlated with the expected size, suggesting that the amplicons are specific. Specificity of the amplicons was further verified by sequencing (Medigenomix). In total, the seven PCR target regions covered 33% of the entire 10,131 nt DWV genome. After sequencing, it was possible to align 3139 nt covering 31% of the total genome. In total, the sequences of the amplicons revealed a homology of 99.2% with the sequences published. Nucleotide sequence identities ranged from 98.9% to 100%. Amplicons generated with primer pairs F1/B1 and F4/B5 were identical to the available sequences, whereas amplicons generated with primer pairs F2/B3, F6/B8, F7/B11, F10/B16, and F15/B23 revealed 99.5%, 99.0%, 99.2%, 99.7% and 98.9% homology, respectively, as compared to the

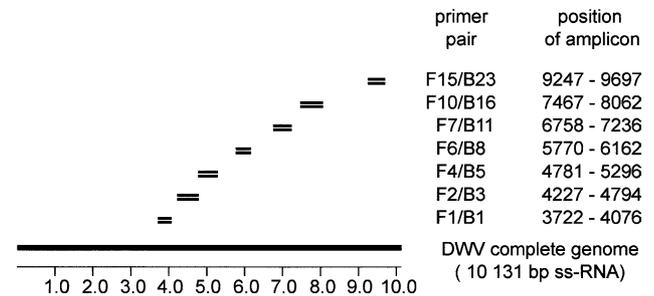


Fig. 2. Location of the amplified PCR products (amplicons) within the DWV genome. The location and position of the RT-PCR products amplified with the primer pairs F1/B1, F2/B3, F4/B5, F6/B8, F7/B11, F10/B16, and F15/B23 are shown. Positions refer to the published DWV sequence (Lanzi and Rossi, accession no. NC 004830).

deposited sequences. Since the two DWV-sequences published by Lanzi and Rossi (acc. no. NC 004830) and de Miranda et al. (acc. no. AY292384) also show some variation, distinct genetic lineages of DWV based on geographic origin are likely, as already described, e.g., for sacbrood virus of the honeybee (Grabensteiner et al., 2001).

While classification of DWV has been carried out, very limited information on DWV is available at the molecular level. With the development of the RT-PCR-protocol presented in this study, we now have a means to tackle all possible questions concerning different molecular aspects of DWV infection of honeybees, pathogenicity, and virulence. In general, all seven DWV-primer pairs employed in this study are suitable for direct, specific and sensitive detection of DWV in honeybee samples. In addition, all seven RT-PCR primer pairs described here could be used in principle for phylogenetic analysis of DWV; however primer pairs F1/B1 and F4/B5 might be less useful.

Table 1
Primers selected for DWV RT-PCR

Primer ^a	Sequence (5'–3')	Opt. Ann. Temp.	Length of amplicon (bp)
F1	CCTGCTAATCAACAAGGACCTGG	54.2–54.4	355
B1	CAGAACCAATGTCTAACGCTAACCC		
F2	ATCGTAGACTGGAAGGATGGTCC	54.3–54.5	568
B3	GAGAAGACATTTGCTTGAACCTCC		
F4	GCAATGTCTTCTCACTGGTGTCTC	54.1–54.2	516
B5	TGCTTTCAAAATCTCAGGCTCG		
F6	TTTCCAGGTCCATTCCCCTATC	53.4	393
B8	TCATTGCGCTTACGACGGTTAG		
F7	TCATCTTCAACTCGGCTTTCTACG	52.6–52.9	479
B11	CGAATCATTTTCACGGGACG		
F10	TGCCAGTTACTACTAAGCCTCAGGG	53.8–53.9	596
B16	CGAACCACAAACACCATCGC		
F15	TCCATCAGGTTCTCCAATAACGG	54.1–54.3	451
B23	CCACCCAAATGCTAACTCTAACGC		

^aF, forward; B, reverse.

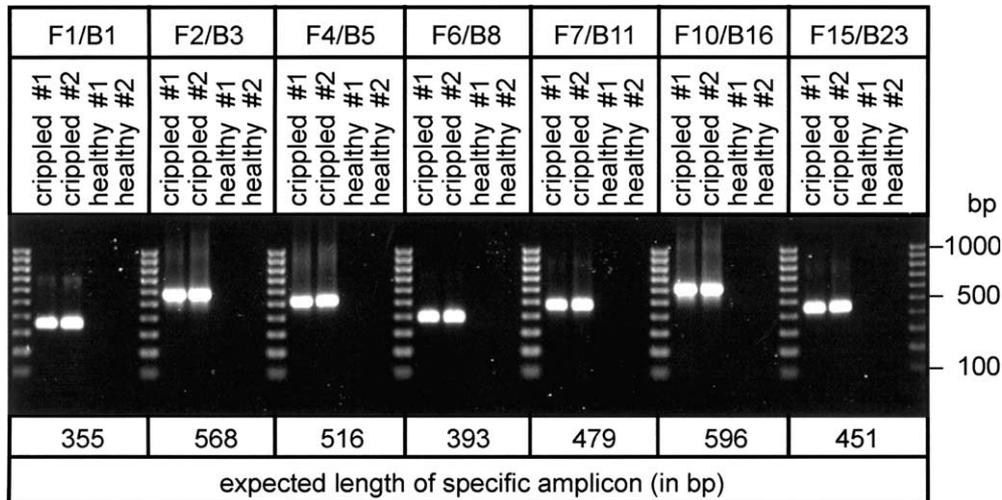


Fig. 3. Detection of DWV in honeybees by RT-PCR amplifying seven different regions of the DWV-genome. The amplification products were separated in a 0.8% agarose gel, stained with ethidium bromide and visualized under UV-light. Expected length of specific amplicons and size of marker bands are given. For each primer pair, representative results of two crippled and two healthy bees are shown. Whereas in control bees no DWV sequences were found, amplicons were obtained from crippled bees with all seven primer pairs tested.

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