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Molecular characterization and phylogenetic analysis of deformed wing viruses isolated from South Korea

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ABSTRACT

Deformed wing virus (DWV) is one of the most common viral infection in honeybees. Phylogenetic trees were constructed for 16 partial nucleotide sequences of the structural polyprotein region and the RNA helicase region of South Korean DWVs. The sequences were compared with 10 previously reported DWV sequences from different countries and the sequences of two closely related viruses, Kakugo virus (KGV) and *Varroa destructor* virus-1 (VDV-1). The phylogeny based on these two regions, the Korean DWV genomes were highly conserved with 95–100% identity, while they also shared 93–97% similarity with genotypes from other countries, although they formed a separate cluster. To investigate this phenomenon in more detail, the complete DWV genome sequences of Korea-1 and Korea-2 were determined and aligned with six previously reported complete DWV genome sequences from different countries, as well as KGV and VDV-1, and a phylogenetic tree was constructed. The two Korean DWVs shared 96.4% similarity. Interestingly, the Korea-2 genome was more similar to the USA (96.5%) genome than the Korea-1. The Korean genotypes highly conserved with USA (96%) but low similarity with the United Kingdom3 (UK3) genome (89%). The end of the 5′ untranslated region (UTR), the start of the open reading frame (ORF) region, and the 3′ UTR were variable and contained several substitutions/transitions. This phenomenon may be explained by intramolecular recombination between the Korean and other DWV genotypes.

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1. Introduction

Honeybees, *Apis mellifera*, are important pollinators with huge ecological and agricultural importance throughout the world (Martin, 2001; Winston, 1987). However, the health and vigor of honeybees worldwide is compromised by various disease agents including viruses, bacteria, fungi, protozoa, parasitic mites, and nematodes (Bailey and Ball, 1991; Ellis and Munn, 2005). Among these, viral diseases of bees are a major economic consideration in Apiculture.

Deformed wing virus (DWV) is one of the most common and widespread of the 19 viruses known to infect honeybees (Neumann and Carreck, 2010; Van Englsdorp et al., 2010). DWV was originally isolated from diseased adult bees during the early 1980s in Japan (Bailey and Ball, 1991). It has a worldwide distribution and has been

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reported from Europe, North America, South America, Africa, Asia, and the Middle East (Allen and Ball, 1996; Ellis and Munn, 2005; Antunez et al., 2006). Generally, DWV appears in coexistence with the ectoparasitic mite *Varroa destructor* (Ball, 1997; Hung et al., 1996; Nordstrom et al., 1999; Benjeddou et al., 2001), which is highly effective vector of DWV transmission among bees, and the virus is able to replicate in the mite (Bowen-Walker et al., 1999; Ongus et al., 2004; Shen et al., 2005). According to Martin et al. (2012), millions of honey bee colonies have been killed due to the worldwide spread of the Varroa mite and its interaction with DWV. DWV comprises an encapsulated, non-enveloped, icosahedral capsid that measures about 30 nm in diameter (Bailey and Ball, 1991; Lanzi et al., 2006). It contains a positive-sense, single-stranded, polyadenylated, and monocistronic RNA genome of 10,144 nucleotides (nt). The monopartite genome encodes one large, uninterrupted open reading frame (ORF), which is flanked by a long 5′ untranslated region (5′ UTR) and a short, highly conserved 3′ UTR that terminates with a 3′ poly-A tail (Lanzi et al., 2006; Berenyi et al., 2007). The 5′ and 3′ UTRs have important functions in regulating the replication and translation of the genome (Belsham, 2009; Nakashima and Uchiumi, 2009). The N-terminal end of the polyprotein starts with a leader peptide (L protein), which is followed by the structural proteins VP3, putative VP4, VP1, and VP2. The C-terminal part of the polyprotein contains nonstructural proteins, conserved motifs of the RNA helicase, the putative VPG (genome-linked viral protein), the C protease, and the RNA-dependent RNA polymerase (RdRp), which were predicted from the deduced amino acid sequence (Lanzi et al., 2006; de Miranda and Genersch, 2010). Based on its virion structure and genome organization, DWV has been assigned to the genus *Iflavirus* in the picorna-like family *Iflaviridae* (Chen et al., 2011; Lanzi et al., 2006).

The symptoms of the disease caused by DWV infection include shrunken, crumpled wings, reduced body size, and discoloration in adult bees, as well as reduction in the life span (Bailey and Ball, 1991; Ball and Bailey, 1997; Kovac and Crailsheim, 1988; de Miranda and Genersch, 2010). The most severe symptoms of DWV infections are associated with infections with the parasitic mite, *Varroa destructor* (Bowen-Walker et al., 1999; Tentcheva et al., 2004; Shen et al., 2005). Among *Iflaviruses*, DWV is one of the most effected and high prevalence virus, and associated with pathological conditions in honey bees (Lanzi et al., 2006; de Miranda and Genersch, 2010). Despite the worldwide distribution and frequency of honeybee virus strains, so far only a few studies have focused on the genetic diversity of these viruses. Direct sequencing of the amplicons and phylogenetic analyses of the sequences provide insights into the genetic relationships between different virus strains. This approach may even allow the prediction of virus taxonomies, which has already been achieved in many cases (Bakonyi et al., 2002; Berenyi et al., 2007; Tapaszti et al., 2009; Reddy et al., 2013b).

The aim of this study was to be the first to analyze and report the complete nucleotide sequences of two DWV genomes from South Korea and to compare the nucleotide sequence diversity of DWV genotypes with those reported previously from other countries. We also analyzed the phylogenetic relatedness of the partial nucleotide sequences of the DWV structural polyprotein region and RNA helicase region from the different regions of South Korean isolates, and to assess the genetic relationship between DWV strains of various geographic origins.

2. Materials and methods

2.1. Sample collection

Adult honeybees, *A. mellifera*, were collected from different regions in South Korea between March and August 2012. A total of 68 samples were collected, and each sample contained ~100 honeybees from each colony. All of the colonies were in healthy condition during the sampling period. The samples were collected by beekeepers and immediately sent to our laboratory by express mail, and they were stored at −80 °C until use.

2.2. Extraction of viral RNA

From each sample, five honeybees were homogenized in 3 ml of sterile PBS to yield a 10% (W/V) solution using a mortar and pestle. The homogenates were centrifuged for 3 min at 16,000 × *g* and the supernatant was used for RNA extraction with an RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. Total RNA was eluted in 30 μl of elution buffer and stored at −80 °C until use.

2.3. RT-PCR amplification of viral RNA

The RNA was used to synthesize cDNA with an oligo (dT) primer by reverse transcriptase method (Invitrogen, USA), according to the manufacturer's protocol. Two pairs of oligonucleotide primers were designed to amplify partial sequences in the structural polyprotein gene and RNA helicase enzyme regions using an IDT oligo analyzer program (Integrated DNA technologies, version 3.1), based on the USA reference genome (AY292384). Seventeen sets of primers were designed to amplify overlapping PCR products comprise the complete genome sequence of selected DWV genotypes. The sequences, orientations, and locations of the primers, as well as the expected product sizes, are shown in Tables 1 and 2. The oligo nucleotides were synthesized by Bioneer Corporation Ltd. (Daejeon, Korea). PCR was conducted using the gene-specific primer sets described in our previous study (Reddy et al., 2013a). The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. Bands were photographed using a Kodak Digital Science Electrophoresis documentation and Analysis System. Fragment sizes were determined with reference to a 100-bp ladder (Enzynomics, Korea).

2.4. Nucleotide sequencing and computational analysis

Specific amplicons were excised from the agarose gel and extracted using a QIAquick Gel Extraction Kit (Qiagen, Germany), according to the manufacturer's instructions.

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