ISOLATION AND CHARACTERIZATION OF TWO IMPORTANT HONEYBEE-KILLING VIRUS SPECIES, DEFORMED WING VIRUS (DWV) AND BLACK QUEEN CELL VIRUS (BQCV) FROM *MESSOR CONCOLOR* ANTS (HYMENOPTERA: FORMICIDAE)

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Abstract. Pathogenic organisms such as viruses can infect multiple host species from different trophic levels. Honeybee, *A. mellifera* is naturally infected by many viruses. Understanding the mechanism underlying virus spread in honeybee colonies highly depends on the pathogenic ability of honeybee killing viruses in other insects such as ants, *Messor concolor* (Hymenoptera: Formicidae). The purpose of this study was to investigate the existence and prevalence of two significant viruses, deformed wing virus (DWV) and black queen cell virus (BQCV) of honeybees in ants from the nests around different honeybee apiaries in Southeast Turkey by using reverse transcription polymerase chain reaction (RT-PCR). Both viruses were present in two nests while the former existed in another hive. Some ant nests were uninfected. This study is the first to report the molecular detection of the honeybee infecting DWV and BQCV in ants. Further studies should be devoted to the mechanisms underlying virus spread between honeybee and other interacting insects especially the Hymenopterans.
Keywords: *honeybee virus, new host, foodborne transmission, Southeast Turkey*

Introduction

Insects are host to a wide range of viruses that have significant effects on their nature. Honeybee viruses are probably the best-known insect viruses due to important economic losses (Smith et al., 2013; McMenamin and Genersch, 2015). Presence of more than twenty different virus species infecting honeybee, most of which are single-stranded positive RNA viruses, have been reported so far (de Miranda et al., 2013). Understanding the transmission mechanism of honeybee viruses in a hive and apiary is necessary to reveal population dynamics of bee viruses as an important threat. The deformed wing virus (DWV) (Iflaviridae) and black queen cell virus (BQCV) (Dicistroviridae) are the most commonly found virus species (de Miranda et al., 2013).

Deformed wing virus (DWV) is probably the most commonly encountered virus species infecting honeybees (Bailey and Ball, 1991; Lanzi et al., 2006; Berényi et al., 2007). Deformed wing, shortened abdomens, discoloring, and a decrease in the life span of bees are the clinical indicators noticed under heavy DWV infection. Deformed wing virus infection mostly does not result in clear symptoms when transmitted by other vectors than the ectoparasitic mite, *Varroa destructor* (Mesostigmata: Varroidae) (de Miranda and Genersch, 2010). DWV transmits both vertically and horizontally and also isolated from all cast (Queen, Drone, Worker) and life stage (egg, larva, pupae, and adult) of honeybee. Commercial and wild bumble bees can be infected by DWV. *Bombus terrestris* and *Bombus pascorum* shows wing deformities, (Genersch et al., 2006), and serology in *Apis cerana* and *Apis florea* (Allen and Ball, 1996; Ellis and Munn, 2005). DWV was also recorded in other insects such as small hive beetle, *Aethina tumida* (Coleoptera;

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Nitidulidae) and the wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae) which are in interaction with honeybee (Eyer et al., 2009; Traiyasut et al., 2016).

BQCV, one of the most common honeybee virus, multiplies in adult bees, particularly when ingested with the microsporidian parasite spores *Nosema apis* (Bailey et al., 1983; Tapaszti et al., 2009). The infection ability of these viruses is not restricted to *A. mellifera*. Previous reports indicated that wild species of bees, *Apis dorsata* and *A. florea* (Zhang et al., 2012), and *B. terrestris* (Choi et al., 2015) were also infected by DWV and BQCV. Understanding and monitoring the global dispersal of DWV and BQCV are crucial for predicting their epidemy and successfully controlling the aforementioned viruses (Freiberg et al., 2012). Previous studies revealed presence of some honeybee viruses infecting other Arthropoda and, especially, ant species. For example, the earliest bee virus detected in an ant species (*Camponotus vagus*) is the chronic bee paralysis virus (CBPV) which is both viral and replicative (Celle et al., 2008). In another study, beside BQCV and DWV, some other viruses such that Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), and sacbrood virus (SBV) were detected in the species of 11 non-Apis hymenoptera and pollen pellets of forager bees (Singh et al., 2010). Further research approved the existence of honeybee viruses in some ant species such as Camponotus sp. and *Tetramorium caespitum* (Levitt et al., 2013). In addition, the Argentine ant (*Linepithema humile*) was also reported being infected by DWV, BQCV, and KBV (Gruber et al., 2017). Moreover, Lake Sinai viruses (LSVs) were detected in *Messor concolor, M. capitatus* and *M. barbarous* (Bigot et al., 2017). Caged ants (*Myrmica rubra*) fed with infected *A. mellifera* pupae can be infected by DWV (Schläppi et al., 2019). In total, 57 samples belonging to 13 different ant genera collected in apiaries were analyzed and 51 (89%) of the samples were found infected at least by one of the following honeybee viruses; DWV, BQCV, IAPV, SBV, KBV, and Acute bee paralysis virus (ABPV) (Payne et al., 2020).

During routine surveys, I found that infected honeybee individuals were taken by ants to their nests which could be a possible route of transmission for honeybee infecting viruses from bees to ants. Thus, the aim of this study was first to demonstrate whether two important honeybee infecting viruses, BQCV and DWV exist in *M. concolor*. Further, the genome variability of a partial sequence of DWV and BQCV were evaluated.

Material and Methods

Survey

Samples from 7 ant nests were collected from Southeast Turkey (Hakkari and Şırnak provinces), in June 2016 (*Figure 1*). From each nest, four ant samples were collected. This samples were placed in a freezer at -80 ºC till the laboratory analysis. The collected samples were transported to the laboratory for diagnostic studies and RNA isolations. Samples were stored in 70% ethyl alcohol for diagnosis at -80 for RNA isolation.

RNA extraction

For preparing homogenates from ant samples, liquid nitrogen was used in a sterile mortar. All homogenized ant samples were placed into the sterile Eppendorf tubes. The RNA was prepared using a modified silica-capture method (Foissac et al., 2001). Four ants (*M. concolor*) were extracted for each ant nest. Total RNA was resuspended in free RNases and DNases water and kept at 20 ºC temperature. Total RNAs were placed in a freezer at –20 ºC till for laboratory analysis.

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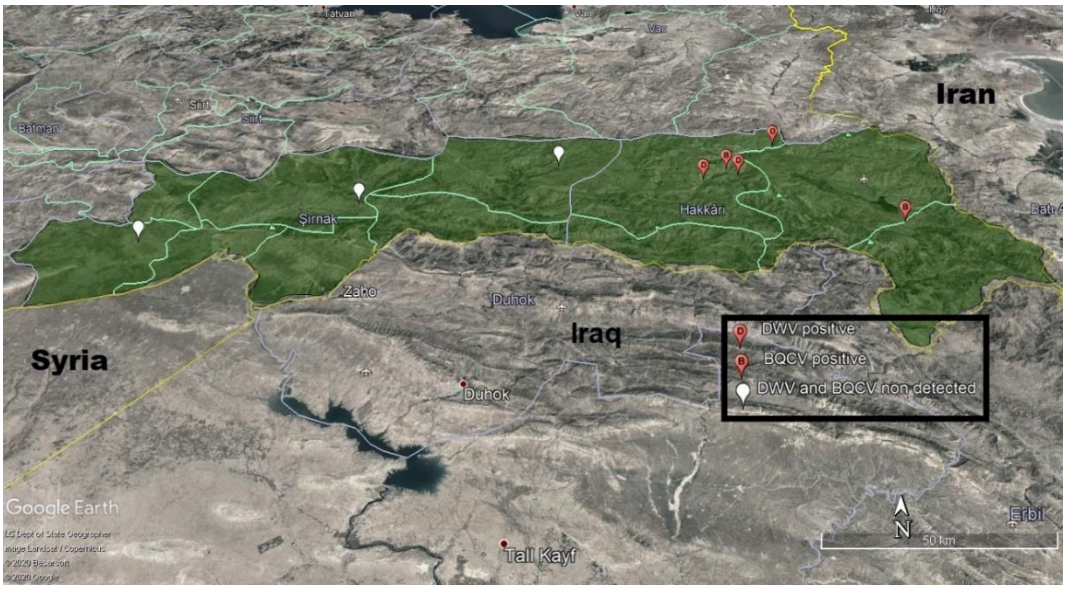

Figure 1. *The map of the collected ant sample is the green area. Each symbol shows the initial letter of the virus isolated from the collected ant sample*

RT-PCR of CP and RdRp genes

The DWV and BQCV viruses were detected using the procedure described in Rüstemoğlu and Sipahioğlu (2019). Genome-specific primers amplifying the 488 bp and 567 bp fragments of RNA depended RNA polymerase (RdRp) and partial coat protein (CP) genes were characterized by molecular cloning and sequencing for DWV and BQCV, respectively. The reverse transcription polymerase chain reaction was performed using a purified RNA from the RevertAid First Strand cDNA kit in accordance with the instruction of the producer company (Vilnius, Thermo-Fermentas Lithuania). The final volume was completed to 25 µl for PCR reaction. The solution comprised of 2.5 µl of 10 × reaction buffer (200 mM Tris–HCl pH of 8.4 and 500 mM KCl), 1 µl of cDNA, 0.5 µl of dNTPs (20 mM each), 1.5 µl of $MgCl_2$ (25 mM), 0.5 µL of each primer (100 pmol), 0.2 µL of Taq DNA polymerase and 18.3 µl of nuclease free water. Following, the partial CP and RdRp gene were amplified using thermal cycling diagram in a RT-PCR. The thermal cycle diagram was as follow; 2 minutes 35 cycles in a minute at 94 ºC temperature, 30 seconds at 57 ºC temperature, 45 seconds at 72 ºC temperature and finally incubated for 10 minutes at 72 ºC temperature. The amplified products at the end of the thermal cycling were separated using 1.5% agarose gel and stained with ethidium bromide to visualize (Sambrook et al., 1989).

Molecular cloning and sequencing

The virus isolates identified were cloned and sequenced separately. The isolation of amplified pieces was carried out in 1% agarose gel and recovered by using a GeneJET Gel Extraction Kit based on the prescription provided by the producer company (Thermo Scientific). The pGEM®-T Easy vector (Promega) was used to purify the DNA fragments. The plasmid was employed to turn competent cells of Escherichia coli JM 109 to ampicillin resistance using electroporation (BioRad, USA). Blue-white selection on X-gal medium plate was used to choose the transformants harboring the DNA of

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DWV-Ant-1 and BQCV-Ant-1 isolate and a colony PCR was employed to screen as positive clones. One clone, named DNA of DWV-Ant-1 and BQCV-Ant-1 was selected for the sequencing of DNA. Automated DNA sequences of Refgen Research and Biotechnology Company (İstanbul, Turkey) was used in sequencing the clones of cDNA.

Phylogenetic analysis

The sequences of DWV and BQCV used in comparisons were procured from the database of Genbank. The unreleased isolates for Turkish sequences of DWV and BQCV isolates were also used in the phylogenetic analysis. Multiple sequence alignments and phylogenetic reconstructions were performed for sequence similarity and phylogenetic analysis using the software of CLC Main Workbench and MEGA-X (Kumar et al., 2018). The stability of phylogenies was deduced by 1000 bootstrap replication using the method of maximum likelihood described in Tamura and Nei (1993).

Results

PCR results indicated that DWV (3/7) and BQCV (2/7) were found in the *M. concolor* nests (*Figure 2*). All positive samples were detected within the borders of Hakkari province (*Figure 1*). The sequence of DWV (GenBank Accession No. MT831948) isolates obtained from surveys had %99-95 similarity when compared with other DWV isolate records from different countries. The DWV sequence obtained from this study shows the most nucleotide similarity with KX373900.1 (France) (99.02%), MH267695.1 (Sweden), KT215905.1 (United Kingdom), KF840795.1 (Lithuania), HM067438.1 (United Kingdom) (98.77%) and KP835214.1 (Turkey), KF840794.1 (Lithuania), (98.53%) isolates, respectively. The BQCV sequence (GenBank Accession No. MT338252) obtained indicated the highest similarity to four isolates MK431882.1 (99.78%), MK431880.1 (99.10%), MK431881.1 (98.65%) and KP835213.1 (98.24%), respectively. These four BQCV isolates were obtained from *A. mellifera* in Turkey (Hakkari and Muğla). Besides these four isolates in the phylogenetic tree, BQCV (*M. concolor*) was clustered in a common branch with South Korean isolates JQ434132.1 (Ac), KP835213.1 (Am) and JQ434135.1 (Am). Phylogenetic trees were used to illustrate the relationships between the sequences. The phylogenetic trees were constructed with the confidence values for each node based on 1000 bootstrap replicates (*Figure 3* and *Figure 4*).

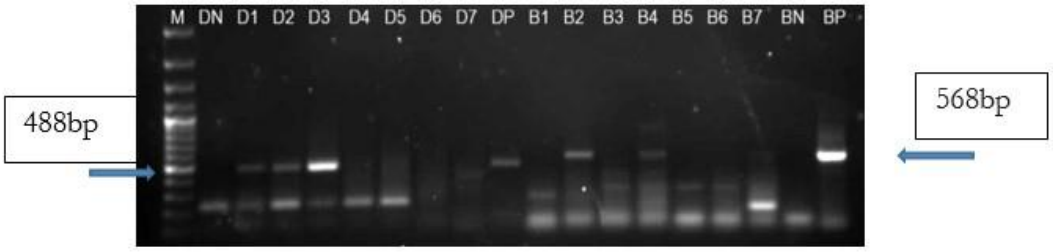

Figure 2. *Electrophoresis PCR products of DWV(D) and BQCV(B) in ants (M. concolor). Lane M: 100bp plus (thermo). Lane DN: DWV negative control. Lane DP: DWV positive control (488 bp). Lane D1-D3: DWV Positive amplification DWV (488 bp). Lane D4-D7: none-amplification DWV. Lane B2, B4: Positive amplification of BQCV (568 bp). Lane B1, B3, B5, B6, B7: none-amplification BQCV. Lane BN: BQCV negative control. Lane BP: BQCV positive control (568 bp)*

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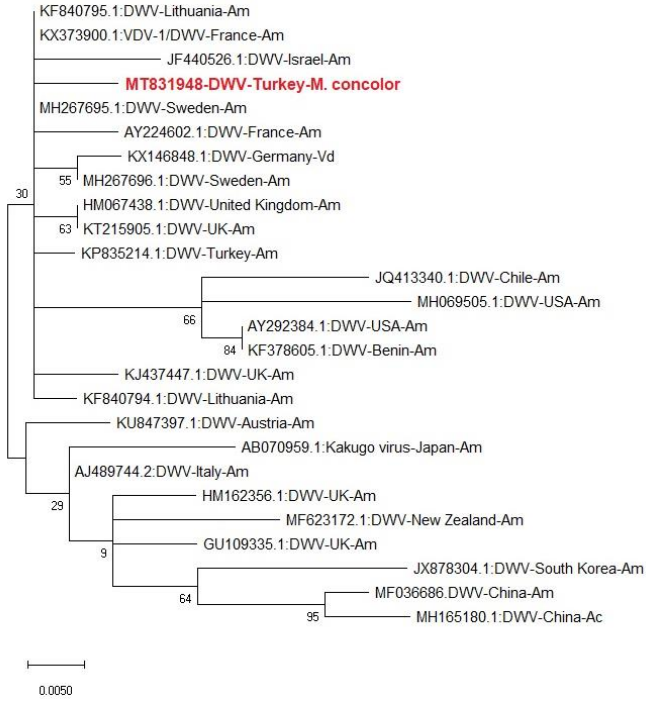

Figure 3. *Phylogram of the MT831948-DWV- Turkey-M. concolor isolated based on sequences of polyprotein gene created using the maximum likelihood method in MEGA X. The ratio of correct partition in a 1000-replicate bootstrap analysis can be observed in the statistical base of the nodes. Am = Apis mellifera, Ac = Apis cerana, Vd= Varroa destructor*

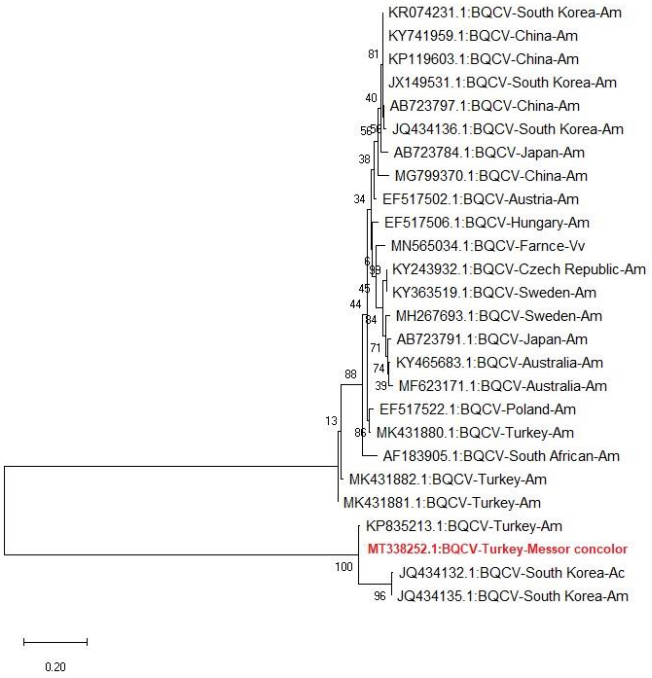

Figure 4. *Phylogram of the MT338252.1. BQCV- Turkey-M. concolor isolated based on sequences of Capsid Protein (CP) created using the Maximum likelihood method in MEGA X. The ratio of correct partition in a 1000-replicate bootstrap analysis can be observed in the statistical base of the nodes. Am = Apis mellifera, Ac = Apis cerana, Vv= Vespa velutina*

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Discussion

This study clearly revealed that the isolates of both viruses, BQCV and DWV extracted from same hosts do not cluster (*Figures 3 and 4*). This may indicate that these two honeybee viruses have not undergone any specific changes to their hosts. The DWV and BQCV sequences obtained from *M. concolor* were mostly identical with nucleotide sequences found *A. mellifera* from same geographic origin.

The earliest bee viruses identified in ants (*Camponotus vagus* and *Formica rufa*) was the Chronic bee paralysis virus (CBPV) (Celle et al., 2008), followed by BQCV, DWV, IAPV, and SBV (Levitt et al., 2013). The DWV, BQCV, and KBV were detected in in Argentine ant (*Linepithema humile*) (Gruber et al., 2017). Lake Sinai viruses (LSV) as a honeybee virus detected in *Messor concolor*, *M. capitatus* and *M. barbarous* (Bigot et al., 2017). ABPV and DWV were detected from the ants (*Lasius platythorax*) collected from the apiary (Schläppi et al., 2020). Further the efforts on honeybee virus in ants revealed the presence of at least one of the DWV, BQCV, IAPV, ABPV, KBV, and SBV in Brachymyrmex, Forelius, Linepithema, Solenopsis, Nylanderia, Pheidole, Camponotus, Aphaenogaster, Crematogaster, Pogonomyrmex, and Pseudomyrmex ant genera (Payne et al., 2020). BQCV and DWV can infect other Apis species (*Apis florea* and *Apis dorsata*) (Zhang et al., 2012), some of bumble bee species *(Bombus terrestris, B. pascuorum, B. huntii, B. impatiens, B. vagans, B. ternarius*) (Genersch et al., 2006; Singh et al., 2010; Li et al., 2011; Peng et al., 2011), and isolated from the eastern carpenter bee (*Xylocopa virginica*), mining bees (*Andrena* sp.), yellow jackets (*Vespula vulgaris*), and sand wasp (*Bembix* sp.), pollen pellets from forager bees (Singh et al., 2010). Herein, the existence of DWV and BQCV in *M. concolor*, a carnivore ant species is reported for the first time.

The phylogenetic analysis showed that viruses isolated from the different host can cluster each other, for both BQCV and DWV, the sequences isolated from the ants clustered together with previous sequences from the *A. mellifera*. According to the phylogenetic tree, the hosts from the same geographical origin likely cluster each other, and besides during this the survey, it was observed that ants attack the honeybees, especially which have disabilities and cannot enter the hive because of wing deformations. These results suggest that BQCV and DWV might have transmitted from *A. mellifera* to *M. concolor*. *Messor concolor* could be another vector of these viruses as a possible spread route. In apiaries, any virus infected organism could pose a danger for honeybee populations especially, the hymenopteran insects sharing same sources with honeybees. On the other hand, since the infected honeybee individuals are dismissed from the hives and preyed by ants, the virus density in hives may reduce. Otherwise, infected honeybee individuals could be a greater threat for hives via conataminating healthy individuals by topical contact (Amiri et al., 2014, 2019; Coulon et al., 2018). Further, the genome variability of a partial sequence of DWV and BQCV were evaluated and it does not appear to be separated by the host, but rather moving between species.

Conclusion

In summary, this study reports the presence of two significant honeybee infecting virus species, DWV and BQCV from *M. concolor*, which is carnivore ant species, under natural conditions. These findings suggest that other trophic levels could be involved in spread of honeybee viruses. However, it is not clear how such an involvement could affect honeybee population dynamics. The presence of the honeybee infecting viruses should

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be checked across other arthropod communities around apiaries which could help a better understanding of virus transmission routes.

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