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Evidence of circulating recombinants between deformed wing virus and *Varroa destructor* virus-1 in honey bee colonies in Türkiye

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Research Paper

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Abstract

Deformed wing virus (DWV), which is an important honey bee virus transmitted by *Varroa destructor (V. destructor)*, causes colony losses in honey bee colonies. This study aimed to investigate the prevalence and genetic diversity of DWV in honey bees in Türkiye and to determine the role of *V. destructor* in the transmission of the genetic variants of DWV. Honey bee samples were collected from 62 apiaries, by simple random sampling, during March 2022 and April 2023. The presence of *V. destructor* in collected bee samples was examined using a stereo microscope. Real-time RT-PCR was used for the detection of DWV-A and DWV-B (*Varroa destructor* virus-1 (VDV-1)) viruses. Genetic characterisation of the positive samples was conducted by sequencing polyprotein genomic region. Considering the *V. destructor* infestation rate of 3% as relevant, out of the 62 apiaries examined, 17 (27.4%) were positive. However, DWV-A and VDV-1 specific RNA was not detected in *V. destructor* samples. VDV-1 specific RNA was detected in 6.5% (4/62) of the apiaries, whereas DWV-A was not detected in the sampled apiaries. Phylogenetic analysis showed that isolates detected in this study were located in a separate cluster from previously characterised DWV-A and VDV-1 isolates. According to RDP4 and GARD analyses, DWV-VDV-1 recombination breakpoints were detected in field isolates. To the best our knowledge, this is the first report of the presence of VDV-1-DWV recombinants in Türkiye. Further studies are needed to determine the impact of VDV-1-DWV recombinants and their virological and antigenic properties.

Introduction

Honey bees are crucial pollinators that play a vital role in sustaining global agricultural production. However, massive colony losses in honey bee populations have been reported worldwide (Highfield *et al.*, 2009; McMenamin and Genersch, 2015). Viral infections were recognised as the main cause of colony collapse disorder (Martin *et al.*, 2013; McMenamin and Genersch, 2015). Mostly viruses cause in-apparent infections in honey bee colonies. However, clinical symptoms can be observed when colonies are stressed (Chen and Siede, 2007). The presence of *Varroa destructor* (*V. destructor*) in bee colonies significantly affects colony stress and increases the incidence of colony losses due to viral infections (Martin *et al.*, 2013; McMenamin and Genersch, 2015).

Deformed wing virus (DWV) is reported to be the most common viral agent in honey bee colonies infested with *Varroa* mites (Yue and Genersch, 2005; Schroeder and Martin, 2012; Chen *et al.*, 2021). DWV has a positive, single-strand RNA genome, and it belongs to the *Iflavirus* genus of the *Iflaviridae* family (Ongus *et al.*, 2004; Lanzi *et al.*, 2006). The viral genome encodes four major structural proteins (VP1, VP2, VP3 and VP4) and four non-structural proteins (an RNA helicase, a genome-linked viral protein, a 3C-like cysteine protease and an RNA-dependent RNA polymerase) (Škubník *et al.*, 2017). DWV is classified into four main genetic variants: DWV-A, DWV-B (*Varroa destructor* virus-1 (VDV-1)), DWV-C and DWV-D (de Miranda *et al.*, 2022). Recent studies have reported that recombination events frequently occurred between DWV-A and DWV-B variants (Moore *et al.*, 2011; Ryabov *et al.*, 2017). DWV can cause subclinical infection in honey bees. However, wing deformity, paralysis and reduced body size can be seen in infected bees (Schroeder and Martin, 2012). VDV-1 causes wing deformities and shortened life expectancy in infected adult bees (Benaets *et al.*, 2017; Brettell *et al.*, 2017).

Beekeeping is important for both ecological and economic reasons in Türkiye. Türkiye has around 8.1 million bee colonies and ranks second position on the list of the world's largest honey producers (TEPGE, 2021). However, honey production per colony is considerably lower than those of countries such as China, Brazil, the USA and Canada (FAOSTAT, 2021). The reasons why beekeeping has not yet reached its full potential in Türkiye remain

unclear, as there are no comprehensive regular disease monitoring programmes. However, bee diseases are expected to play a significant role in honeybee losses in Türkiye as they have contributed to significant honey bee losses worldwide (Oğuz *et al.*, 2017; Karapınar *et al.*, 2018; Muz and Muz, 2018). The presence of DWV in honey bees has been reported in Türkiye (Muz and Muz, 2017; Karapınar *et al.*, 2018; Mayack and Hakanoğlu, 2022). Yet, there is limited information about the genetic variants of DWV circulating in Türkiye. Monitoring and determining the prevalence of viral infections in honey bees are important to ensure the continuity of beekeeping. Therefore, in this study, it was aimed to investigate the prevalence and genetic diversity of DWV in honey bees and to determine the role of *V. destructor* in the transmission of genetic variants of DWV.

Materials and methods

Honey bee (Apis mellifera) sampling

This study was conducted from March 2022 to April 2023 in the Hatay Province (36°12′12.1′N, 36°09′31.4′E) (fig. 1). Hatay Province has 15 districts. However, five districts (Antakya, Arsuz, Dörtyol, Kırıkhan and Samandağ) were randomly selected for sampling due to budget limits. The selected five districts had 593 apiaries (54.0% of the apiaries in the Hatay Province) (TUIK, 2021). In this study, a two-stage sampling was used to select the apiaries and bees in the apiaries for the detection of DWV strains that were circulating in bees and *Varroa* mites. First, the method specified by Thrusfield (2007) was used to determine the number of apiaries required for sampling. It was determined that 62 apiaries were required with a 90% confidence level, 10% acceptable error and 50% expected apiaries level DWV prevalence. The list of apiaries in the selected districts was obtained from the Hatay Province Beekeepers Association. From the list, 62 apiaries were randomly selected using the randomise tool in Microsoft Excel software (Microsoft Corporation, USA), and contacted with apiaries owners. The contacted apiary owners agreed to participate in this study. The method described by Pirk *et al.* (2013) was used to determine the minimum number of bees to be examined within each colony, and it was determined that 25 bees should be sampled in each colony with 95% probability. Adult bee samples were obtained from four randomly selected colonies in each apiary according to the signs of the diseases. The swollen abdomen, wing deformations, discoloration, shrinking body, crawling on the ground and flightlessness were observed in the sampled bees. The collected bees were placed into dry ice and transported to the laboratory where they were kept frozen at −80°C until analysis.

V. destructor determination in honey bee samples

The presence of *V. destructor* in adult honey bee samples was examined morphologically using a stereo microscope (Motic, Wetzlar, Germany) with 30× magnification (OIE, 2021). A total of 100 adult bees from each apiary were tested. The apiaries which had infestation rates ≥3 mites per 100 adult bees were considered as *Varroa* infested (Jack and Ellis, 2021). Detected *Varroa* mites were separated by considering the apiaries where they were detected, and pools of *Varroa* mites were created using sterile phosphate buffer saline (PBS).

Nucleic acid extraction

The frozen bees from each colony in each apiary were pooled and were considered as one single sample. Furthermore, *V. destructor* from each colony in each apiary were also pooled. The samples were homogenised in sterile falcon tubes (50 ml) containing sterile PBS by using the TissueRuptor (Qiagen, Hilden, Germany). The falcon tubes then were centrifuged for 30 min at 5000 rpm at 4°C, and 50 µl of supernatant sample was taken for RNA extraction. Nucleic acid extraction was performed using a commercial kit (High Pure Viral Nucleic Acid Kit, Roche, Germany) following the manufacturer's instructions. The obtained extracts were stored at −80°C until analysis.

DWV-A and VDV-1 screening by real-time RT-PCR

For the detection of DWV-A and VDV-1, separate real-time RT-PCR assays were performed using VP3 gene region-specific probes and primers described by Schurr *et al.* (2019) with a commercial master mix kit (AgPath-ID One-Step RT-PCR, ThermoFisher Scientific, MA, USA). For DWV-A amplification, 25 µl reaction mix containing primers and probe (350 nM, F: 5′-GCGGCTAAGATTGTAAATTG-3′, R: 5′-GTGACTAGCATA ACCATGATTA-3′, and probe 100 nM (6-Fam) 5′-CCTTGAC CAGTAGACACAGCATC-3′ (Tamra), targeting the region between nucleotides 4258 and 4329 of the DWV-A genome)

Figure 1. The location of the study area. The red colour represents the location where the study took place.

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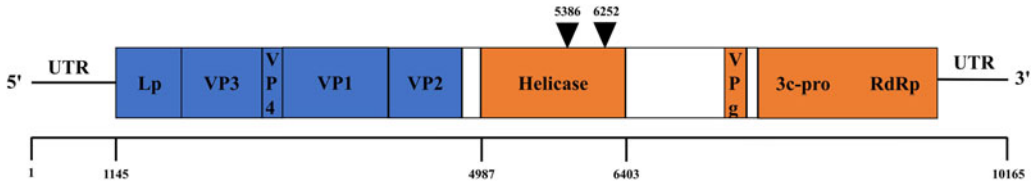

Figure 2. Schematic of the DWV genome showing the sequenced region (at positions 5386–6252) in this study denoted by black triangles. Structural genes are represented by blue while non-structural genes are represented by orange.

and 5 µl of extracted RNA was prepared, while 25 µl reaction mix containing primers and probe (1200 nM, F: 5′-GGTCTGA AGCGAAAATAG-3′ and R: 5′-CTAGCATATCCATGATTAT AAAC-3′, and probe 400 nM (6-Fam) 5′-CCTTGTCCA GTAG ATACAGCATCACA-3 (Tamra), targeting the region between nucleotides 4218 and 4290 of the VDV-1 genome) and 5 µl of extracted RNA was prepared for VDV-1 amplification. The cycling conditions were: reverse transcription at 45°C for 10 min, followed by initial PCR activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 1 min at 60°C. Positive DWV RNA obtained from the Department of Parasitology (Veterinary Faculty, Hatay Mustafa Kemal University, Antakya, Türkiye) was used as positive control, whereas nuclease-free water was used as negative control in all analyses. The cycle threshold value (Ct) equal or lower than 35 was considered as positive (De Miranda *et al.*, 2013).

RT-PCR amplification, sequence and phylogenetic analyses

The samples found positive by real-time RT-PCR assay (cut-off for positivity was Ct value ≤ 35) were subjected to one-step RT-PCR using primers (F: 5′-TGACTTAACGGCTGAAATGA ATCA-3′ and R: 5′-TTCATTTCCTCCACTAAGCGCTGATT-3′, targeting an 866-bp long sequence spanning nucleotide positions 5386–6252 of DWV genome) (fig. 2) described by Wang *et al.* (2013). The reaction mix was prepared in a final volume of 50 µl, containing primers (400 nM) and 5 µl of extracted RNA, with a commercial master mix kit (MegaFi Pro One-Step RT-PCR, ABM, Canada). The amplification conditions were as follows: 15 min at 60°C, 30 s at 98°C, followed by 45 cycles with 20 s at 95°C, 20 s at 55°C, 30 s at 72°C and a final extension cycle 10 min at 72°C. PCR products were separated by gel electrophoresis in 1.5% agarose gel containing GelRed (Biotium, USA). PCR products of 866 bp were purified from gel using a commercial purification kit (HibriGen Gel Extraction Kit, Kocaeli, Türkiye), and sequenced in reverse and forward directions by BM Laboratory (Ankara, Türkiye). Nucleotide sequences were analysed using the Bioedit software (version 7.0.5.3), and compared with sequences obtained from the GenBank. Sequences from the GenBank were selected based on the criteria of the information available regarding the genetic variants, the location and the year of isolation. The phylogenetic tree of polyprotein gene was constructed by the maximum likelihood method with Kimura's two-parameter substitution model using MEGA software (version 11.0). The lowest BIC score in MEGA software was used to determine the best-fitting nucleotide substitution model for the alignment of datasets (Fei *et al.*, 2019).

Nucleotide sequence accession numbers

The nucleotide sequences of the VDV-1 isolates detected in this study are available in the GenBank database under accession numbers OQ849766 and OQ849767.

Recombination analysis

Recombination analysis of the field isolates detected in this study was performed using RDP4 software (Martin *et al.*, 2015). The following algorithms were used in the RDP4 analysis: (1) RDP with a window size of 40, (2) Geneconv (default settings), (3) Bootscan with a window size of 300, step size = 20, bootstrap = 100 and Jin and Nei model selected for the Neighbour-Joining tree, (4) χ^2 using variable sites per window = 60, (5) Chimaera using variable sites per window = 60, (6) Siscan with a window size of 300 and (7) 3SEQ (default settings). The presence of recombination event was considered when the *P* value of at least three algorithms was below 0.05 and the recombination consensus score was between 0.4 and 0.6 (Fei *et al.*, 2019). Furthermore, the presence of recombination was confirmed by the genetic algorithm for recombination detection (GARD) analysis available in the Datamonkey web interface (Delport *et al.*, 2010).

Results

V. destructor frequencies in apiaries

V. destructor was detected in 46 (74.2%) of the 62 apiaries, whereas the number of apiaries that achieved ≥3% of infestation with *V. destructor* was 17 (27.4%) (table 1).

Table 1. The prevalence and district distribution patterns of *V. destructor* mite, DWV-A and VDV-1 in Hatay Province

District	Sampled apiaries (*n*)	Apiaries with *Varroa mites* detected (*n*)	Apiaries with *Varroa* mites infestation level ≥3% (*n*, %)	DWV-A positive apiaries *n* (%)	VDV-1 positive apiaries *n* (%)
Antakya	14	10	9 (64.3%)	–	2 (14.3%)
Arsuz	7	5	–	–	1 (14.3%)
Dörtyol	30	21	3 (10%)	–	–
Kırıkhan	4	4	4 (100%)	–	1 (25%)
Samandağ	7	6	1 (14.3)	–	–

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Table 2. Nucleotide changes in the helicase coding region of the field isolates detected in this study compared to Netherlands VDV-1

Netherlands VDV-1	Pos. nt	5608 G	5656 C	5662 C	5674 T	5701 C	5728 T	5836 T	5845 T	5860 T	5878 T
TUR/Hatay/Kirikhan K1		A*	T			T		A	G	C	C
TUR/Hatay/Antakya A3				T*	C*	T	C*				
Israel VDV-1			T								
United Kingdom VDV-1										C	
USA VDV-1 California-2016											
United Kingdom VDV-1-DWV-No-5[a]			T			T		A	G		C
United Kingdom DWV-VDV-1-DWV[a]			T			T		A	G		C
United Kingdom VDV-1-DWV Centre-No4[a]			T			T		A	G		C
Netherlands VDV-1	Pos. nt	5896 A	5917 A	5926 C	5932 A	5935 T	5944 G	5947 T	5949 A	5965 T	5974 A
TUR/Hatay/Kirikhan K1		T	G	T	G		A	G	G	A	G
TUR/Hatay/Antakya A3			G	T	G	C*	A	G	G	A	G
Israel VDV-1											
United Kingdom VDV-1											
USA VDV-1 California-2016											
United Kingdom VDV-1-DWV-No-5[a]		T	G	T	G		A	G	G	A	G
United Kingdom DWV-VDV-1-DWV[a]		T	G	T	G		A	G	G	A	G
United Kingdom VDV-1-DWV Centre-No4[a]		T	G	T	G		A	G	G	A	G
Netherlands VDV-1	Pos. nt	5986 T	5987 A	5992 A	5995 C	6013 A	6016 A	6019 T	6037 C	6047 C	6049 T
TUR/Hatay/Kirikhan K1			G	T	T	T	G	G	T		
TUR/Hatay/Antakya A3		C*		T	T	T	G	G	T	A	G
Israel VDV-1											
United Kingdom VDV-1											
USA VDV-1 California-2016											
United Kingdom VDV-1-DWV-No-5[a]			G	T	T	T	G	G	T	A	G
United Kingdom DWV-VDV-1-DWV[a]			G	T	T	T	G	G	T	A	G
United Kingdom VDV-1-DWV Centre-No4[a]			G	T	T	T	G	G	T	A	G

Netherlands VDV-1	Pos. nt	6055 T	6064 G	6073 T	6076 A	6079 T	6082 G	6091 G	6098 T
TUR/Hatay/Kirikhan K1		A	A	C	G	C	A	A	A
TUR/Hatay/Antakya A3		A	A	C	G	C	A	A	A
Israel VDV-1									
United Kingdom VDV-1									
USA VDV-1 California-2016									
United Kingdom VDV-1-DWV-No-5[a]		A	A	C	G	C	A	A	A
United Kingdom DWV-VDV-1-DWV[a]		A	A	C	G	C	A	A	A
United Kingdom VDV-1-DWV Centre-No4[a]		A	A	C	G	C	A	A	A

[a]VDV1-DWV recombinant strains; Pos. nt, nucleotide position in the polyprotein gene.
*Mutations found exclusively in the field isolates detected in this study and never reported in other strains included in this study.

Detection of DWV-A and VDV-1

DWV-A specific viral RNA was not detected, whereas VDV-1 specific RNA was detected in four (6.5%) of the 62 apiaries. Each VDV-1 positive apiary was in the Antakya ($n = 2$), Arsuz ($n = 1$) and Kırıkhan ($n = 1$) districts in the Hatay Province. However, DWV-A and VDV-1 specific RNA was not detected in *V. destructor* pools. In this study, positive samples showed Ct values ranging between 18.51 and 34.48.

By using a RT-PCR method, two strong amplicon bands of about 860 bp were obtained from two of the four samples that were found positive by VDV-1 real-time RT-PCR. This situation can be explained by the low viral load in other real-time RT-PCR positive samples (Ct value > 30).

Nucleotide and amino acid sequence analyses of VDV-1 isolates

Analysis of the polyprotein gene revealed that the nucleotide homology between the isolates detected in this study was 97.8% when compared with each other, and 91.2–98.4% when compared with DWV-A and VDV-1 isolates from other countries, the lowest with a Chinese DWV isolate (MF770715) and the highest with United Kingdom VDV-1-DWV recombinants (HM067437, HM162354 and HM162360).

The amino acid homology between the isolates detected in this study was 99.4%, whereas homology with the previously characterised DWV-A and VDV-1 isolates ranged between 97.9 and 100.0%.

In this study, a total of 38 nucleotide substitutions were detected in field isolates when polyprotein gene region of the field isolates was compared with the Netherlands VDV-1 (NC_006494), a reference isolate. Six of the 38 nucleotide substitutions have not been reported in other strains included in this study (table 2). Furthermore, 26 of the 38 nucleotide substitutions were non-synonymous substitutions. Three of the 26 amino acid substitutions have not been previously reported in other strains included in this study (table 3).

Phylogenetic relationships of the VDV-1 isolates

The phylogenetic tree based on the polyprotein gene revealed that VDV-1 isolates detected in this study were clustered separately from the previously detected DWV-A and VDV-1 isolates (fig. 3). Furthermore, the field isolates detected in this study formed separate branches from the VDV-1-DWV recombinants that were previously detected in the United Kingdom.

Recombination breakpoints

Recombination analyses indicated that field isolates, TUR/Hatay/Antakya A3 and TUR/Hatay/Kirikhan K1, were determined as recombinants at the breakpoint in the positions 5923–6244 nt and 5908–6202 nt, respectively, with high level of confidence (table 4). The identified breakpoints were located in the helicase-coding region of the genome. The potential parental isolates of TUR/Hatay/Antakya A3 were Netherlands VDV-1 (AY251269) and New Zealand DWV (MN538208), whereas potential parental isolates of TUR/Hatay/Kirikhan K1 isolate were United Kingdom VDV-1 (KC786222) and Chile DWV A1 (JQ413340) (fig. 4). Two field isolates, TUR/Hatay/Antakya A3 and TUR/Hatay/Kirikhan

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Table 3. Amino acid changes in the helicase coding region of the field isolates detected in this study compared to Netherlands VDV-1

Netherlands VDV-1	Pos. aa	1870 A	1886 R	1888 P	1901 R	1910 C	1960 S	1966 S	1973 T	1976 R	1978 N
TUR/Hatay/Kirikhan K1		T*	C		C		P	C	A	C	D
TUR/Hatay/Antakya A3				S*	C	R*			A	C	D
Israel VDV-1			C								
United Kingdom VDV-1											
USA VDV-1 California-2016											
United Kingdom VDV-1-DWV-No-5[a]			C		C		P	C	A	C	D
United Kingdom DWV-VDV-1-DWV[a]			C		C		P	C	A	C	D
United Kingdom VDV-1-DWV Centre-No4[a]			C		C		P	C	A	C	D
Netherlands VDV-1	Pos. aa	1982 G	1989 W	1992 T	1998 T	1999 H	2006 N	2007 F	2013 H	2017 L	2019 S
TUR/Hatay/Kirikhan K1		S	R	A	S	Y	D	V	Y	V	T
TUR/Hatay/Antakya A3		S	R	A	S	Y	D	V	Y	V	T
Israel VDV-1											
United Kingdom VDV-1											
USA VDV-1 California-2016											
United Kingdom VDV-1-DWV-No-5[a]		S	R	A	S	Y	D	V	Y	V	T
United Kingdom DWV-VDV-1-DWV[a]		S	R	A	S	Y	D	V	Y	V	T
United Kingdom VDV-1-DWV Centre-No4[a]		S	R	A	S	Y	D	V	Y	V	T
Netherlands VDV-1	Pos. aa	2022 V	2026 N	2027 Y	2028 V	2031 V	2033 V				
TUR/Hatay/Kirikhan K1		M	D	H	M	M	D				
TUR/Hatay/Antakya A3		M	D	H	M	M	D				
Israel VDV-1											
United Kingdom VDV-1											
USA VDV-1 California-2016											
United Kingdom VDV-1-DWV-No-5[a]		M	D	H	M	M	D				
United Kingdom DWV-VDV-1-DWV[a]		M	D	H	M	M	D				
United Kingdom VDV-1-DWV Centre-No4[a]		M	D	H	M	M	D				

[a]VDV1-DWV recombinant strains; Pos. aa, amino acid position in the helicase gene.
*Mutations found exclusively in the field isolates detected in this study and never reported in other strains included in this study.

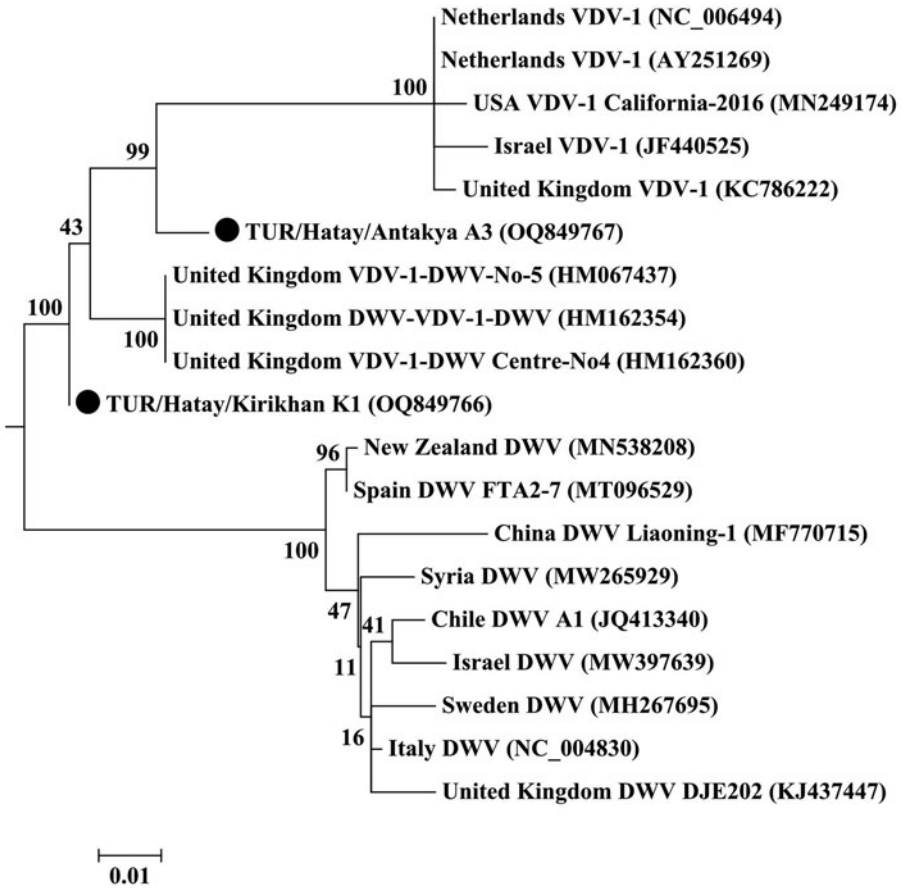

Figure 3. Phylogenetic tree of the VDV-1 polyprotein gene, only values above 50% were reported. The sequences obtained in this study are marked with a round black dot.

K1, were also identified as recombinant by GARD analysis (RHS, LHS *P*-values < 0.05).

Discussion

DWV is one of the most common honey bee viruses in the world (Gülmez *et al*., 2009; Kevill *et al*., 2017; Fei *et al*., 2019; Brasesco *et al*., 2021; de Miranda *et al*., 2022). *V. destructor* plays an important role in the transmission of DWV among honey bees (Piou *et al*., 2022). DWV mostly persists in bee colonies and causes asymptomatic infections. Asymptomatic infections can remain latent and spread, resulting in large-scale colony losses, since colonies are rarely sampled and investigated for infections (Highfield *et al*., 2009). Therefore, this study focused on the prevalence of DWV variants and the role of *V. destructor* in the transmission of DWV variants in Türkiye.

In the present study, considering the *V. destructor* infestation rate of 3% as relevant, out of the 62 apiaries examined, 17 (27.4%) were positive. This rate is lower than the results of the previous studies that reported the rate of *V. destructor* in apiaries ranging between 35.0 and 90.0% in Türkiye (Cakmak *et al*., 2003a, 2003b; Gülmez *et al*., 2009; Gümüşova *et al*., 2010). These differences in *V. destructor* rates in different regions of Türkiye could be explained by the climatic conditions, sampling time and use of drugs against *Varroa* mites (Cobey, 2001). Furthermore, observed lower detection rate can be attributed to the small number of adult bees per colony for the *Varroa* mite laboratory examination and diagnostic methods used in this study. According to the apiary owners' report, all sampled apiaries have been using drugs, containing amitraz, against *Varroa* for a long time, and last *Varroa* treatments were conducted 1–2 months before sampling. However, *V. destructor* mite was detected in these apiaries. This situation may be explained by the ability of mites to develop drug resistance. It has been reported that amitraz resistance can develop in *Varroa* mites due to long-term drug use (El Agrebi *et al*., 2020).

DWV can infect both larvae and adult forms of honey bee, and is very common all over the world (Chen *et al*., 2006; Martin and Brettell, 2019). Reported DWV rates in honey bees were 18.6% in Spain (Antúnez *et al*., 2012), 20.3% in Brazil (Teixeira *et al*., 2008), 57.3% in Denmark (Nielsen *et al*., 2008), 87.0% in Greece (Bacandritsos *et al*., 2010), 91.0% in Austria (Berényi *et al*., 2006) and 97.0% in France (Tentcheva *et al*., 2004). Previous studies conducted in different regions of Türkiye reported that the rate of DWV in apiaries ranged between 25.2 and 74.2% (Kalaycı *et al*., 2020; Cagırgan and Yazici, 2021; Usta and Yildirim, 2022). However, there is no information about the genetic variants of DWV that were detected in Türkiye. DWV-A was not detected in this study. The possible explanations for the difference could be due to the level of beekeepers' knowledge and awareness of DWV, the conditions of care and feeding, types of beekeeping (migratory or stationary) and sampled bees (larvae or adult bees). In this study, only adult bees were investigated for the presence of DWV. Furthermore, the reason for not detecting DWV-A in this study may be related with the sampling method. In this study, apiaries were sampled randomly and bees were randomly collected from each apiary. However, in most of the previous studies with high prevalence, samples were collected from apiaries with signs of disease.

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Table 4. Possible recombination events in VDV-1 isolates described by RDP4

Recombinant sequence	Parental sequences major/minor	Breakpoint position start/end	Recombinant score	*P*-value for the seven algorithms in RDP4						
				R	G	B	M	C	S	T
Antakya A3	Netherlands VDV-1 (AY251269) New Zealand DWV (MN538208)	5923–6244	0.50	4.625×10^{-5}	2.823×10^{-4}	NS	4.639×10^{-9}	7.059×10^{-9}	NS	4.449×10^{-12}
Kirikhan K1	United Kingdom VDV-1 (KC786222) Chile DWV A1 (JQ413340)	5908–6202	0.57	NS	5.858×10^{-5}	NS	9.020×10^{-9}	1.179×10^{-8}	2.279×10^{-11}	9.560×10^{-11}

R, RDP; G, Geneconv; B, Bootscan; M, MaxChi; C, Chimaera; S, Siscan; T, 3Seq; NS, not significant.

VDV-1 is one of the most significant infectious agents causing colony losses (Ryabov *et al.*, 2017). In this study, VDV-1 was detected in four (6.5%) of 62 apiaries. In this study, VDV-1 detection rate is lower than the results of previous studies. A previous study conducted in the United States revealed that detection rate of VDV-1 was 66.0% (Ryabov *et al.*, 2017). Another study conducted in Argentina reported that the rate of VDV-1 was 47.0% (Brasesco *et al.*, 2021). The possible explanations for the difference could be due to the types of beekeeping, the conditions of care and feeding and level of beekeepers' knowledge and awareness. Furthermore, results of the study suggest that detection of low rate of *V. destructor* in the study area may contribute to the low rate of VDV-1 in sampled apiaries when considering the role of *Varroa* mites in the transmission of VDV-1 (Ongus *et al.*, 2006).

In this study, VDV-1 was detected in apiaries where *V. destructor* was detected, whereas DWV-A was not detected. This result is consistent with the results of previous studies that reported VDV-1 is the most common variant in honey bee colonies (Kevill *et al.*, 2021; Piou *et al.*, 2022). This situation could be explained by the fact that VDV-1 multiplies more efficiently and with higher viral load in the *V. destructor* than DWV-A (Gisder and Genersch, 2021; Piou *et al.*, 2022).

Comparison of TUR/Hatay/Kirikhan K1 and TUR/Hatay/Antakya A3 sequences showed that they had 97.8 and 99.4% nucleotide and amino acids homology, respectively. Although the homology of the two isolates was relatively high, they do not belong to the same strain (fig. 3). Furthermore, analysis of the polyprotein gene region showed that three amino acid substitutions detected in filed isolates had not been previously reported in other isolates (table 3). One of these mutations (A1870T (hydrophobic to hydrophilic)) was detected in the TUR/Hatay/Kirikhan K1 isolate, and two mutations (P1888S (hydrophobic to hydrophilic) and C1910R (hydrophilic to hydrophilic)) were detected in the TUR/Hatay/Antakya A3 isolate. Further studies are needed to investigate the functional roles of these amino acid changes in viral replication and virulence.

The phylogenetic tree based on polyprotein gene showed that TUR/Hatay/Kirikhan K1 and TUR/Hatay/Antakya A3 isolates had a closer relationship with VDV-1-DWV recombinants which were detected in the United Kingdom than other DWV and VDV-1 isolates (fig. 3). This situation could be explained by recombination between different genetic variants due to factors such as queen bee imports from different countries. Recombination is a strategy for viruses to adapt to new environmental conditions and hosts. Also, recombination is a mechanism that can facilitate the transmission of viruses from *Varroa* mites to bees (Mordecai *et al.*, 2016). Recombination events between DWV-A and VDV-1 viruses have been reported in the United Kingdom and the United States (Moore *et al.*, 2011; Ryabov *et al.*, 2017). Furthermore, Zioni *et al.* (2011) have reported recombinant events in Israel, a flight distance of approximately 380 km from Hatay Province, where this research was carried out. A recombination event between DWV-A and VDV-1 has not been previously reported in Türkiye. To the best our knowledge, this is the first report of VDV1-DWV recombinants in honey bees in Türkiye. It has been reported that VDV-1-DWV recombinants detected in the United Kingdom were associated with mite transmission (Moore *et al.*, 2011). In this study, *V. destructor* was also detected in apiaries where VDV-1-DWV recombinants were determined.

In this study, recombination points were detected in the helicase gene. This result is consistent with the results of previous

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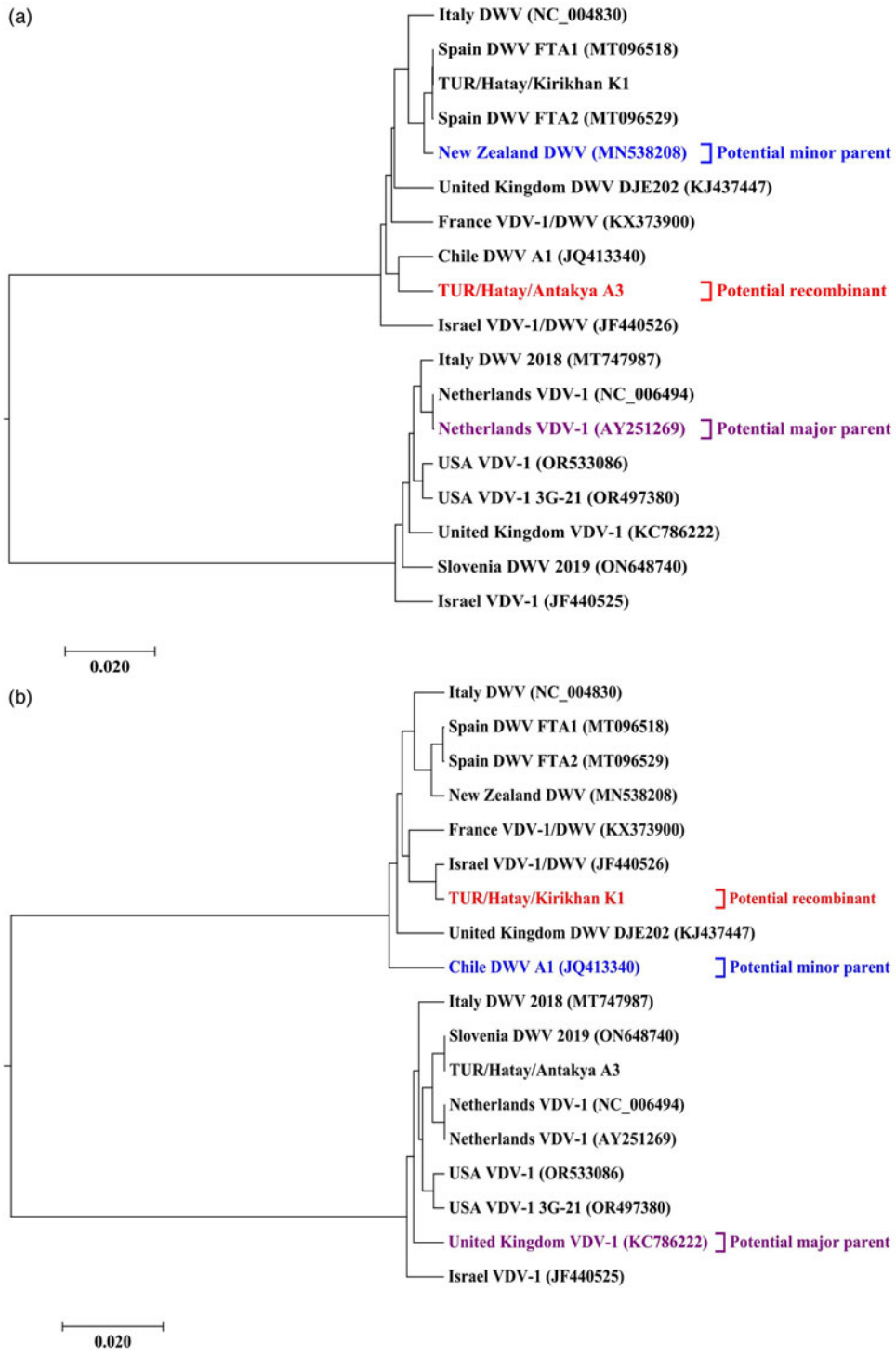

Figure 4. Phylogenetic analyses based solely on the recombination site using UPGMA in RDP4. (A) TUR/Hatay/Antakya A3 and (B) TUR/Hatay/Kirikhan K1. The colour code used is explained in the figure.

studies that reported helicase gene region contains recombinant points (Moore *et al.*, 2011; Dalmon *et al.*, 2017). A high degree of similarity in parental sequences can encourage recombination events (Kirkegaard and Baltimore, 1986). It has been reported that helicase coding region has the highest identity among virus variants (Dalmon *et al.*, 2017). Therefore, the helicase gene region can be considered as recombination 'hot spots', and associated with the emergence of new virus variants. In this study, only partial genome sequences of the helicase gene were used to identify potential recombination events. The findings of the study are limited by the lack of whole genome sequencing of field isolates. Further studies are needed to determine other recombinants and recombination breakpoints among the circulating DWV-A and VDV-1 isolates in Türkiye.

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Conclusions

Türkiye has a significant geographical position that connects Asia and Europe, and ranks first position on the list of the Europe's honey producers. Therefore, it is very important to understand the prevalence of honey bee viruses in Türkiye. This study underlines the presence of VDV-1-DWV recombinants in honey bees in Türkiye. VDV-1-DWV recombinants detected in this study showed a number of specific nucleotide and deduced amino acid motifs that are not observed in other DWV and VDV-1 isolates. VDV-1-DWV recombinants have been reported to be highly virulent in the United Kingdom (Ryabov *et al*., 2014). The occurrence of recombination events may threaten beekeeping by contributing to high levels of genetic diversity and increased host susceptibility. Therefore, further studies are necessary to determine the impact of VDV-1-DWV recombinants and their virological and antigenic properties.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions. A. Z. and M. Y. collected samples. A. Z. and I. E. isolated the RNA. M. Ş. performed the molecular analyses. M. Ş. wrote the original draft. All authors read and approved the final manuscript.

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Competing interests. None.

Ethical standards. This study was approved by the Experimental Animal Ethics Committee of Hatay Mustafa Kemal University (No. 2021/02-02).

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