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Molecular Detection and Phylogenetic Analysis of Five Honey Bee Viruses from Wet Pollen in Türkiye

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Abstract—Pollen is important for honey bees and humans to maintain a healthy diet and strong immune system. The aim of this study was to determine the prevalence of honey bee viruses in pollen. One hundred pollen samples were collected from different regions of Türkiye. The seven most devastating viral diseases of honey bees (Black Queen Cell Virus (BQCV), Israeli Acute Paralysis Virus (IAPV), Deformed Wing Virus (DWV), Kashmir Bee Virus (KBV), Acute Bee Paralysis Virus (ABPV), Sacbrood Virus (SBV), Chronic Bee Paralysis Virus (CBPV)) were tested for their presence in pollen using the reverse transcriptase-polymerase chain reaction method. 15% of the pollen samples were identified as positive for five viruses (ABPV, BQCV, DWV, CBPV and KBV). While ABPV was the most common virus in the samples, IABV and SBV were not found. Two viruses (ABPV + KBV and ABPV + CBPV) were detected simultaneously in two pollen samples. The results of this study showed that pollen is a reservoir of honey bee viruses. In addition, the use of pollen instead of live bees taken from the hive for regional detection of viral diseases in honeybees is an important choice to reduce the number of live bees used in experiments.

Keywords: pollen, reservoir, RNA viruses, honey bee health

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INTRODUCTION

Honeybees play a very important role in the pollination of flowering plants (Corbert et al., 1991). Thus, the existence of life depends largely on the role of honeybees in pollination (Korkmaz and Aydın, 1999). In particular, it has been found that when beehives are placed in plant production areas, productivity increases between 20 and 60% depending on the plant species (Yılmaz, 2016).

The various viral diseases threaten the health of honey bees (Gisder and Genersch, 2015). To date, more than twenty-five viral pathogens with symptomatic and asymptomatic features have been identified in honey bees (Beaurepaire et al., 2020). The seven most destructive of these diseases are known as (Black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), Deformed wing virus (DWV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV), Sacbrood virus (SBV), Chronic bee paralysis virus (CBPV)) (Tantillo et al., 2015; McMenamin and Flenniken, 2018; Kalaycı et al., 2020). While four of these viruses (ABPV, IAPV, KBV, and BQCV) belong to the family *Dicistroviridae*, two (SBV and DWV) belong to the family *Iflaviridae*, CBPVs have not yet been placed in a family. They are known as unclassified (Qi et al., 2023). Honeybee viruses were found to be similar in their genome sequences in the replicase, polyprotein, helicase, protease, and polymerase regions (Baker and Schroeder, 2008).

Viral diseases can be transmitted from diseased bees to healthy bees in many ways, both horizontally and vertically (Chen and Siede, 2007; De Miranda etal., 2012). One of these pathways is thought to be pollen through oral transmission (Singh et al., 2010; Pereira et al., 2019; Fetters et al., 2022). Oral transmission, which is a direct horizontal transmission route, occurs as a result of healthy honeybees consuming virus-contaminated pollen (Chen et al., 2006a). In addition, the process of pollen packaging also results in the spread of the viral agent among adults. Detection of the virus in the intestinal tissue and faeces of bees also confirmed that oral infectivity is active (Chen et al., 2006a).

In recent years, increasing studies on the detection of viral contamination in pollen have raised concerns about the transmission of pathogens to healthy individuals in the hive (Mutinelli, 2011; Schittny et al., 2020; Balkanska et al., 2023; Salkova et al., 2024). Studies have investigated the transmission capacity of virus-contaminated pollen within the hive and found that the virus replicates and causes disease in the bodies of healthy bees (Mazzei et al., 2014; Schittny et al., 2020). Studies on the detection of viral load in bee products are still limited (Balkanska et al., 2023).

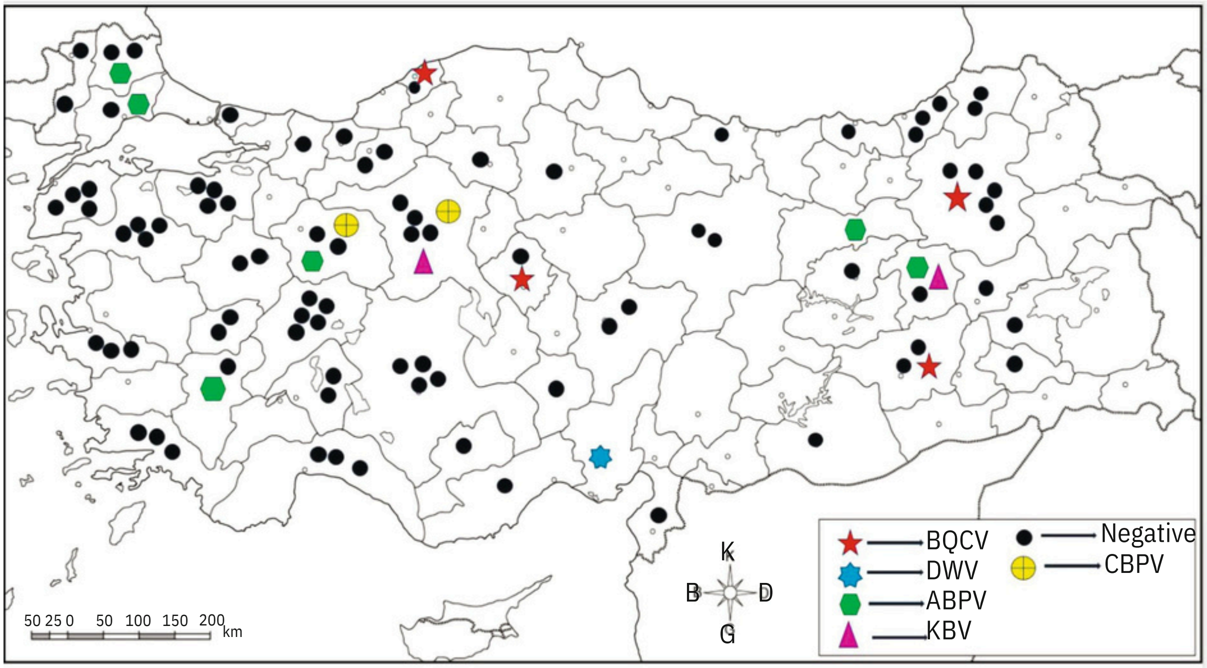

Fig. 1. Türkiye map showing the location of collected pollen and the location of virus-positive samples.

Since Türkiye is a country where beekeeping is common, many studies have been conducted on viral bee diseases (Selvitopi and Eroğlu, 2023; Öz et al., 2023; Utkan and Eroğlu, 2023; Akpinar et al., 2024; Altay et al., 2024). However, despite all these efforts, it has not yet been clarified whether there is virus contamination in bee products in our country. This study aims to investigate the frequency of the seven most lethal honey bee viruses (BQCV, IAPV, DWV, KBV, ABPV, SBV and CBPV) in pollen collected from different regions of Turkey. Thus, the contamination of pollen with honey bee viruses in our country has been clarified for the first time in Turkey. In addition, the nucleotide sequences of the viruses that we molecularly detected in pollen were used together with the sequences of viruses detected in honeybees in our country and in the world to construct phylogenetic trees.

MATERIALS AND METHODS

Pollen Collection

A total of 100 pollen samples were collected from beekeepers in spring and summer in 7 regions of Türkiye in 2023 and 2024 (Fig. 1). The pollen was collected by the beekeepers by placing pollen traps at the entrance of the hives. A small amount of pollen was collected from each hive so as not to interfere with the pollen needs of the honeybees. The pollen collection method was carefully applied so as not to cause any physical harm or stress to the bees. The amount collected from different hives in each apiary was recorded as a sample. The pollen was kept in a cold chain to prevent fungal and bacterial contamination until it was taken to the laboratory.

Viral RNA Extraction

60 mg of pollen samples were weighed, transferred to 2 mL sterile tubes and numbered. A steel ball and 800 μL phosphate buffer solution (PBS) were added to each tube and homogenised in a tissue lyser (Qiagen) for 4 min at a speed of 50 strokes. The samples were then centrifuged at 5000 rpm for 4 min at 4°C and 350μL of the supernatant was transferred to a new tube. Total nucleic acid extraction of viruses likely to be present in pollen samples was performed according to the manufacturer's instructions using the Indimag Pathogen Kit (Indical Bioscience, Cat. no.: SP947457). After measuring the purity and quantity of the extracted RNAs in the microdrop, they were stored at –80°C until they were converted into complementary DNA (cDNA).

cDNA Synthesis and RT-PCR Reactions

The Intron Maxime™ RT PreMix Kit was used for cDNA synthesis. 8 μL dH2O and 2 μL RNA were added to the premix solution containing random primers and incubated in the thermal cycler at 45°C for 60 min and 95°C for 5 min. The primers listed in Table 1 were used for RT-PCR (Rüstemoğlu and Sipahioğlu, 2016; Rüstemoğlu and Sipahioğlu, 2019). The reaction mixture was prepared by adding 12.5 μL Ecotaq 2× PCR master mix, 1 μL of each primer, 1 μL

Table 1. Primers used for RT-PCR detection of viruses in pollen samples

Family	Vir us	Target region	Sequences	Base pair	*T*m	References
Dicistroviridae	ABPV	Capsid protein	Forward: 5'-GTATGGAAGTGGGCTGAGGA-3' Reverse: 5'-CGCGGTACTAAAAAGCTACGA-3'	476 bp	55°C	Rüstemoğlu and Sipahioğlu, 2016
	IAPV		Forward: 5'-TTGGCGTGCAACTATGTGTT-3' Reverse: 5'-TCTTCTGCCCACTTCCAAAC-3'	402 bp		Rüstemoğlu and Sipahioğlu, 2019
	KBV		Forward: 5'-CACATTCCGAACAATAA-3' Reverse: 5'-GCGATAGGAATTTTGCGGTA-3'	339 bp		
	BQCV		Forward: 5'-GACAGCGTGCCAAAGAGAG-3' Reverse: 5'-GCGAACCCGTCCAATACTTA-3'	567 bp		
If laviridae	SBV		Forward: 5'-TATTCAGGGGGACGCTACAC-3' Reverse: 5'-AGTGCTGCTTGAAACCCTGT-3'	429 bp		
	DWV	Non-structural protein	Forward: 5'-TTGGTATGCTCCGTTGACTG-3' Reverse: 5'-ATTCCTCAGAAGTTGGTTTCG-3'	488 bp		
Unclassified	CBPV	RNA-dependent RNA polymerase	Forward: 5'-GCAAACTGCCCACCAATAGT-3' Reverse: 5'-TGGTACGGAAGGTGTGTCAA-3'	500 bp		

cDNA, and 4.5 μL dH2O. RT-PCR conditions were as follows 30 s at 98°C, 35 cycles of 10 s at 94°C, 15 s at 55°C, 45 s at 72°C and a final extension of 1min at 72°C. To observe the presence of bands after the reaction, a 0.8% agarose gel containing ClearBand SAFE DNA Gel Stain Solution (Ecotech Biotechnology, Cat. No. SDGS1) was run at 80 V for 40 min and imaged under UV light. Samples with bands of the expected size were sent to the bioinformatics company for one-way sequence analysis (Sentebiolab, Türkiye).

Phylogeny

All sequences were aligned in the Bioedit 7.7 program, and the sequences were trimmed in the Mega 11 software at the end and head to prepare for phylogenetic analysis (Tamura et al., 2021). For phylogenetic analysis, the sequences obtained from the database were used together with the sequences of the Turkish isolates. Phylogenetic trees were drawn separately for each virus based on the amplified gene regions (capsid protein (cp), RNA-dependent RNA polymerase (RdRp), and nonstructural protein). The phylogenetic tree was constructed using the Tamura-Nei model of the Maximum Likelihood Neighbor-Joining method with 1000 bootstraps (Tamura et al., 2004).

Data Analysis

The prevalence graph of honey bee viruses in pollen was plotted using GraphPad Prism 9.5.1.

The Pearson chi-square test was used for the statistical analysis of the viral contamination of the pollen samples. The results were statistically analyzed using IBM SPSS Statistics 29. The prevalence of pathogens in each geographical region of Türkiye was determined using the Pearson chi-squared test at $p < 0.05$ using a contingency table and a two-way frequency table.

RESULTS

Regional Distribution of Viruses

While the presence of five tested viruses was detected in the pollen samples (ABPV, KBV, DWV, BQCV, and CBPV), the presence of IAPV and SBV was not detected. The presence of mixed viruses was also detected in 2 pollen samples. 15% of the pollen was virus-positive (Table 2). The prevalence rates of bee viruses detected in pollen were ABPV 6/100, 6%, BQCV 4/100, 4%, KBV and CBPV 2/100, 2%, and DWV 1/100, 1%. Statistical results were obtained for ABPV ($\chi2 = 16.314$, $n = 100$, df = 2, $p = 0.04$), BQCV ($\chi2 = 14.244$, $n = 100$, df = 2, $p = 0.04$), KBV and CBPV ($\chi2 = 6.204$, $n = 100$, df = 2, $p = 0.04$) and DWV ($\chi2 = 4.006$, $n = 100$, df = 2, $p = 0.04$). In the samples collected and analyzed from seven different regions, the presence of viruses was found in all regions. The prevalence of the viruses studied varied significantly between the regions (Fig. 2, Table 2).

Among the regions of Türkiye, the two regions with the most diverse virus contamination in pollen were Central Anatolia (BQCV, CBPV, KBV, ABPV) and Eastern Anatolia (BQCV, KBV, ABPV). In addition, the presence of contamination was detected in these regions. These results show that pollen collected from these regions of the country shows a high diversity in virus contamination (Fig. 2).

Phylogeny

The resulting sequences were deposited in the GenBank database, National Center for Biotechnol-

Table 2. Type, and name of infection, and locality information of virus-positive pollens

Regions	Virus contaminated pollen, %	Virus name	Contamination
Black Sea	13	BQCV	S
Aegean	17	ABPV	S
Mediterranean	8	DWV	S
Marmara	21	ABPV	S
Central Anatolian	23	KBV, CBPV, BQCV, ABPV + CBPV	S S S M
Eastern Anatolian	13	ABPV, BQCV, ABPV + KBV	S S M
South Eastern Anatolian	5	BQCV	S
Total 100%			

* S: Single, M: Multiple.

ogy Information (NCBI), and accession numbers are given in Fig. 3. We successfully sequenced six positive samples for the partial cp gene of ABPV (97–98%), four for the partial cp gene of BQCV (89–93%), two for the partial cp gene of KBV (98%) and the partial RdRp gene of CBPV (99%), and one for the partial

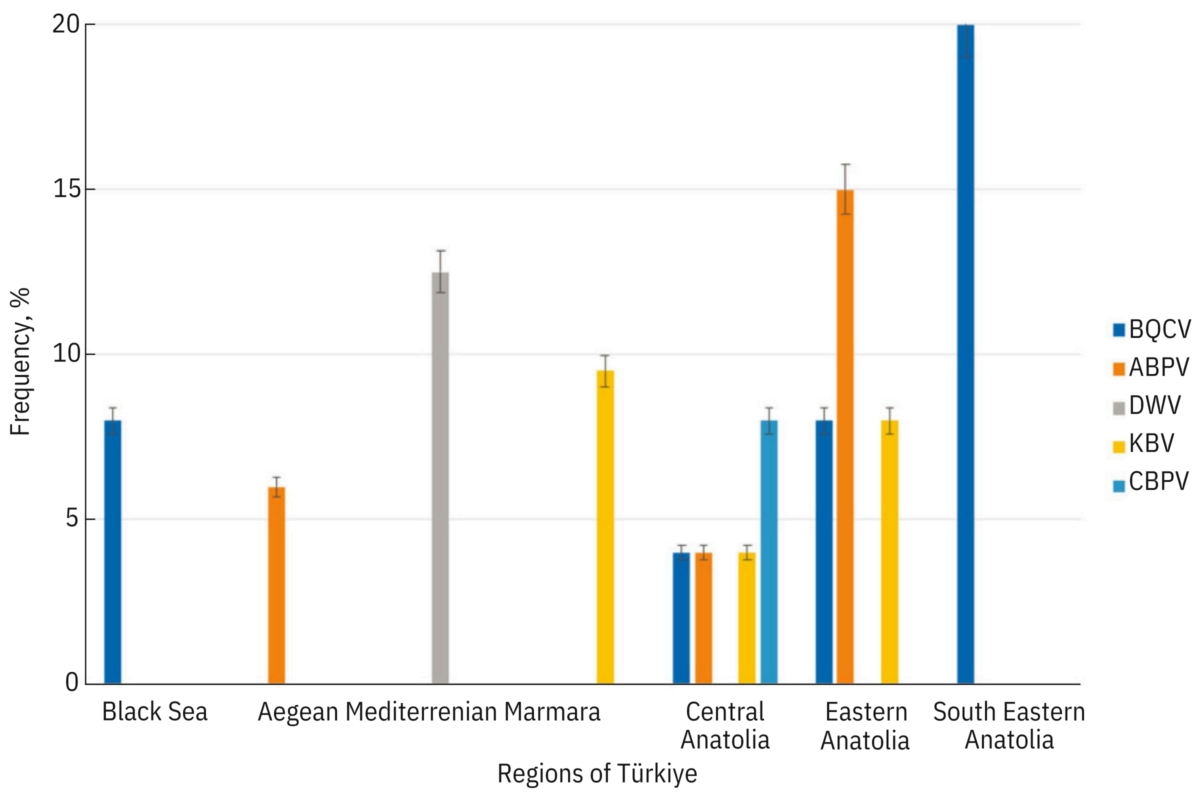

Fig. 2. Frequency of virus positivity (DWV, KBV, ABPV, BQCV, CBPV, and SBV) in pollen samples collected from seven regions of Türkiye.

nonstructural protein gene of DWV (99%). The phylogenetic analysis also included virus sequences isolated from other countries in the database that sequenced the same gene regions. The phylogeny of a DWV detected in pollen from Türkiye is shown in Fig.3a. According to this analysis, it clustered close to DWVs previously isolated from Türkiye and Uzbekistan. The phylogeny of two CBPV isolates detected in pollen in this study is shown in Fig.3b. The monophyletic clustering of the isolates isolated in this study and the isolates from Türkiye in different studies suggests a homogeneous population in the country. While the 4 BQCVs detected in pollen clustered together with the Turkish isolates isolated from the eastern Anatolian region, they clustered far away from the viruses iso- lated from the western part of the country. This situa- tion indicates that the strains of Turkish BQCV iso- lates show genetic diversity (Fig. 3c). Figure 3d shows the phylogeny of Turkish ABPV isolates obtained from pollen samples. The sequences obtained in this study clustered with the other isolates isolated from Türkiye in the database, forming a Turkish clade. Figure 3e shows that the KBVs detected in pollen clustered close to the French isolate.

DISCUSSION

Studies over the past two decades have reported the presence of many viruses in pollen and other in-hive honey bee foods, including ABPV, BQCV, CBPV, DWV, IAPV, KBV, and SBV (Chen et al., 2006a, 2006b; Shen et al., 2005). Asymptomatic or covert-persistent infected honeybees contaminate pollen through various secretions. Because honeybees are highly social and colonial, the disease can spread horizontally in a short time (Li et al., 2014).

The presence of some viruses in pollen has been reported in the literature, and they have even been reported to cause infection in healthy honeybee individuals (Schittny et al., 2020). Viral particles are transferredtoyounglar vae through feeding, as a result of ABPV-infected bees coming into contact with pollen. This has been demonstrated by the detection of ABPV in the pharyngeal gland and feces of young larvae (DeMiranda and Genersch, 2010; Ribiere et al., 2008; Benjeddou et al., 2001). In addition, contamination of pollen, the food source in the colony, with KBV has been demonstrated by the presence of KBV in honey bee faeces, suggesting that the infection is transmitted orally (Hung, 2000; De Miranda et al., 2010). DWV can be transmitted by exposure to viruses deposited on flowers or by contact with contaminated pollen carried by bees (Singh et al., 2010; Koch et al., 2017; Grozinger and Flenniken, 2019). Singh et al. (2010) were the first to detect BQCV, SBV, and DWV in pollen collected from worker bees using molecular detection methods. Their studies showed that the queen, fed with contaminated pollen and honey stored in the hive, was infected with these viruses and laid infected eggs. The viruses were found to infect not only honeybees but also another important group of pollinators, bumblebees, and wasps. The results suggest that honeybee viruses have a broad host spectrum and threaten the health of other organisms in the ecosystem. Mazzei et al. (2014) collected pollen from flowers visited and not visited by honeybees and examined the virus load in the pollen and whether these viruses were infectious. They found the presence of DWV in pollen from flowers visited by honeybees. They also found that when they injected the virus into healthy honeybees, it replicated and caused active infection. Schittny et al. (2020) noted that their studies of the ability of DWV-contaminated hive products to transmit the virus to healthy bee individuals showed a positive relationship between DWV concentration and mortality in honey and pollen. Thus, they demonstrated that virus transmission is possible via honey and pollen. Balkansa et al. (2023) detected the presence of two honey bee viruses (DWV and SBV) in pollen and perga samples collected in Bulgaria using molecular methods. Salkova et al. (2024) reported the presence of four honey bee viruses (DWV, BQCV, ABPV, and IAPV) in honey bee products (pollen, bee bread, and royal jelly). In this study, the five most lethal honey bee viruses (DWV, ABPV, CBPV, KBV, and BQCV) in pollen were collected from different regions of Türkiye (Fig. 1). The presence of all viruses detected in pollen in this study is the first record for Türkiye. Although DWV was previously detected predominantly in honey bee products (Singh et al., 2010; Balkanska et al., 2023; Koch et al., 2017; Grozinger and Flenniken, 2019), the most abundant virus in our samples were ABPV and BQCV, respectively. In addition, IAPV and SBV were not detected in any of the samples. CBPV and KBV were equally distributed among the pollen samples. DWV was the least frequent. The prevalence rates of bee viruses detected in pollen were ABPV 6/100, 6%, BQCV 4/100, 4%, KBV and CBPV 2/100, 2% and DWV 1/100, 1%. Statistical results were obtained for ABPV ($\chi2$ = 16.314, n = 100, df = 2, p = 0.04), BQCV ($\chi2$ = 14.244, n = 100, df = 2, p = 0.04), KBV and CBPV ($\chi2$ = 6.204, n = 100, df = 2, p= 0.04) and DWV ($\chi2$ = 4.006, n = 100, df = 2, p=0.04).

While the two most common viruses detected in honey bees in our country so far are BQCV and DWV (Akpinar et al., 2024), it is noteworthy that the first most common virus in pollen in this study was ABPV. BQCV was the second most common virus found in pollen.

When the viruses detected in pollen and honey bee samples were compared as phylogenetic, virus isolates from pollen generally had the highest similarity to Turkish virus isolates previously detected in honeybees (Fig. 3). As a result of the phylogenetic tree constructed using sequences obtained from DWV-contaminated pollen, our samples clustered with isolates

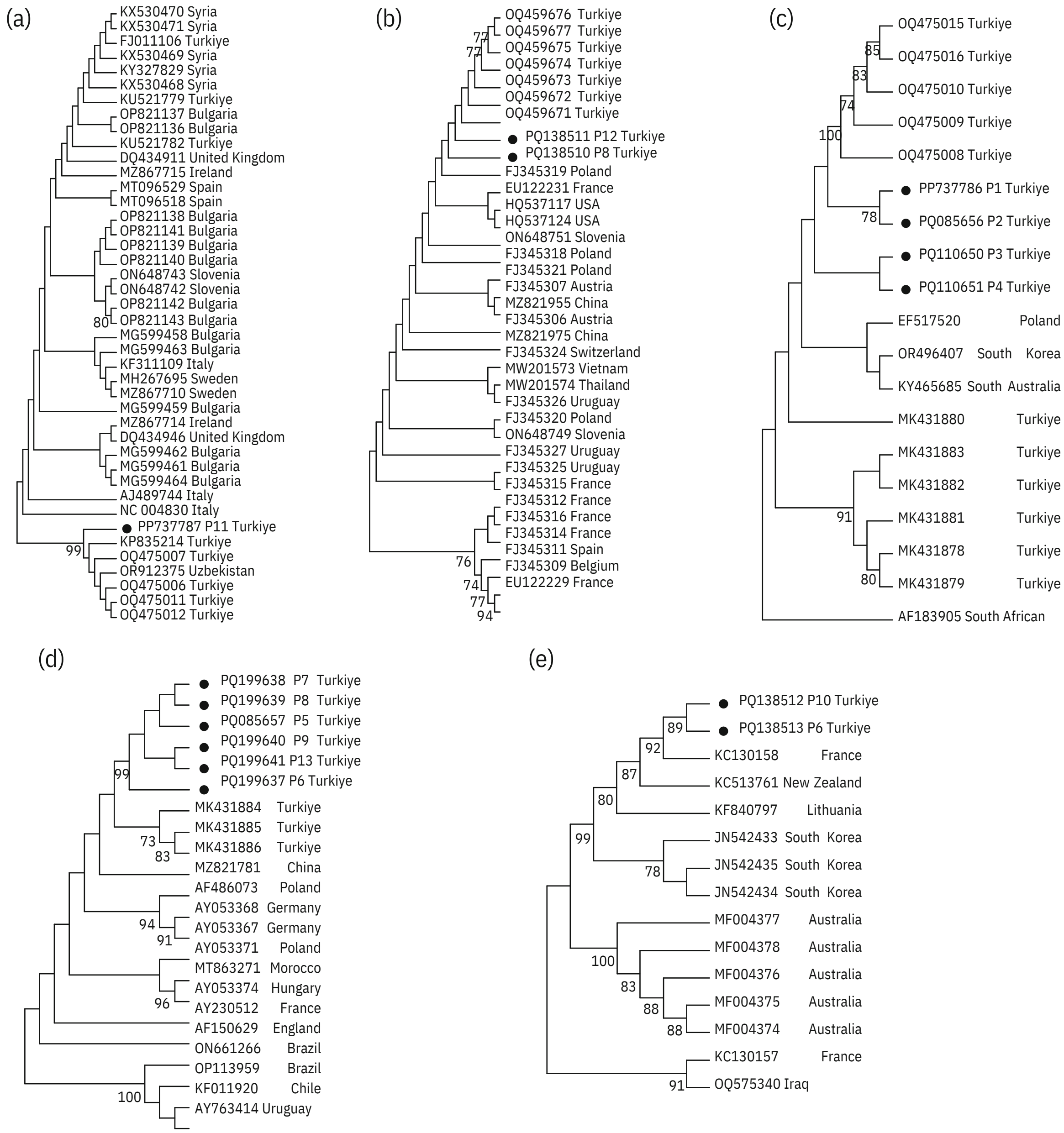

Fig. 3. Phylogenetic tree of nonstructural protein, RdRp, cp genes nucleotide of DWV, CBPV, BQCV, ABPV, and KBV viruses obtained from the GenBank and Türkiye. Phylogenetic trees were constructed by the Mega11 software using the Maximum Likelihood method and Tamura-Nei model, and a bootstrap value of 1000 replicates. Branches marked indicate the sequences obtained from the work. (a) Phylogeny tree of DWV as per nonstructural protein, (b) Phylogeny tree of CBPV as per RdRp gene, (c) Phylogeny tree of BQCV as per cp gene, (d) Phylogeny tree of ABPV as per cp gene, (e) Phylogeny tree of KBV as per cp gene. Country names and corresponding GenBank accession numbers are indicated for each known and new sequence.

obtained from DWV-infected honeybees in Türkiye and Uzbekistan (Fig. 3a). Akpinar et al. (2024) obtained DWV isolates from Türkiye and found that their samples clustered on a common branch with other European countries in the tree they constructed on this basis. The sequences of our samples with CBPV-contaminated pollen clustered very closely with the sequences of samples from Turkish honey bees infected with CBPV, previously identified by Utkan and Eroğlu (2023) (Fig. 3b). This suggests that the viruses contaminating pollen are transmitted from the honey bee host. However, different and distant clustering was observed between the west and east of the country for BQCV isolates (Fig. 3c). Avcı et al. (2022) collected BQCV-infected honey bees from the intensive beekeeping regions of Central Anatolia and the Mediterranean areas of Türkiye. Phylogenetic analysis showed that Turkish BQCV strains clustered with Australian and European strains. They reported that this result showed differences between continents without any geographical distinction. Akpinar et al. (2024) reported that phylogenetic analysis of honey bees infected with the virus they collected from all over Türkiye showed that Turkish isolates were generally similar. Still, there were isolates from different coun- tries of origin in common branches and Turkish iso- lates interacted with isolates from other countries, and this situation posed significant risks to bee health in Türkiye. Çağirgan and Yazici (2021) detected ABPV in bees collected from the Eger region of Turkey and stated that these samples clustered close to the Polish isolate as a result of phylogenetic analysis. In this study, the six ABPV sequences most frequently found in pollen from Türkiye clustered together and clus- tered close to the Turkish ABPV isolates detected in the Cagirgan and Yazici studies (Fig. 3d). In this study, the KBV isolates detected in pollen were compared with sequences from different countries, as the KBV isolate reported from Türkiye was not included in the database and clustered close to the French isolate (Fig. 3e).

Data from phylogenetic analyses show that sequences from virus-contaminated pollen have a high degree of overlap with sequences from honeybee-host viruses, suggesting that viral sequences from different hosts are highly conserved.

CONCLUSIONS

Virus detection in hive products provides a non-invasive approach to detecting viral diseases in honey bees. Rapid detection of the presence of diseases in pollen entering the hive will provide a significant advantage in developing strategies to prevent the spread of these diseases within the hive. Honey bees are social creatures and many factors can lead to colony losses, particularly viruses, which are thought to play an important role in colony decline. This study molecularly identified the five most lethal honey bee viruses in pollen, suggesting that pollen may serve as a virus reservoir and has the potential for non-invasive virus monitoring.

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AUTHOR CONTRIBUTION

Yasemin Aş: Conceptualization, methodology, and data curation, Gözde Büşra Eroğlu: Investigation, writing original draft, and supervision.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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