



## Research Paper

Molecular Characterization and Phylogenetic  
Analysis of Deformed Wing Virus in Honey Bees (*Apis  
Mellifera*), North of Iran

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## ABSTRACT

**Introduction:** Deformed wing virus (DWV) is one of the major bee viruses that can be transmitted by the *Varroa destructor* mite and is a key contributor to honey bee colony collapse. To date, three main variants of the DWV (DWV-A, DWV-B, DWV-C) have been described.

**Materials & Methods:** In the present study, bee samples showing signs of DWV infection and *Varroa* infestation were collected from two different farms in Alborz Province, northern Iran. The samples were screened for the presence of DWV genome and also other bee viral pathogens including chronic bee paralysis virus, acute bee paralysis virus, Kashmir bee virus, and sac brood virus, using polymerase chain reaction (PCR) assay.

**Results:** PCR results indicated that among collected samples, two samples were positive for DWV, while all samples were negative for other viral disease. Phylogenetic analysis revealed that the detected DWVs clustered within the DWV-A genotype. Homology analysis of isolate UT-SHN07 showed approximately 99% similarity to isolate PA and DWV-A-OR-OCT2018-EVR23 from USA, as well as to DWV-Iraq-2023 and the reference sequence NC004830 from Iraq and Italy, respectively. For the other isolate, UT-SHN62, homology analysis showed 100% similarity with isolate PA from USA and 99% similarity with DWV-A-OR-OCT2018-EVR23, DWV-Iraq-2023, NC004830 and Gotland-2 sequences from the USA, Iraq, Italy and Sweden, respectively.

**Conclusion:** Iran is ranked among top 10 honey-producing countries, and continuous monitoring of circulating viruses and *Varroa* mites, the key contributors to colony losses, is essential. Further molecular and phylogenetic studies are recommended to better understand the current status of honey bee viral disease in Iran.

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## 1. Introduction

Since the beginning of 21<sup>st</sup> century, substantial honey bee losses have been reported across the Northern Hemisphere [1]. It's estimated that nearly one-third of the typical Western diet depends on bee pollination. The honey bee, *Apis mellifera*, is recognized as the primary insect responsible for pollinating a wide range of crops, including fruits, nuts, vegetables and oilseeds, with an estimated annual added market value exceeding 15 billion dollars [2, 3]. In addition to pollination, honey bees contribute to the economy by producing various hive products such as honey, beeswax, propolis, royal jelly, etc [4]. Over the past 15 years, severe winter colony losses have been reported from different regions worldwide, raising growing concern about the health status of bee colonies. Nevertheless, over the past 60 years, the number of managed bee colonies has increased all over the world [5]. Like many other species, honey bees are susceptible to a variety of pathogens that can cause considerable colony losses. Among these, viruses are considered one of major threats to honey bee health and survival [4]. Viruses that infect honey bees are primarily classified within the order Picornavirales and are positive-sense single-stranded RNA (+ssRNA) viruses. Common bee viruses include members of the Dicistroviridae, such as Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV) and Black queen cell virus (BQCV); the Iflaviridae such as deformed wing virus (DWV), Kakugo virus, *Varroa destructor* virus-1 (also known as DWV-B), Sacbrood virus (SBV), and Slow bee paralysis virus (SBPV); and a taxonomically unclassified viruses Chronic bee paralysis virus (CBPV) and Lake Sinai viruses (LSVs) [6].

DWV, transmitted by the ectoparasite mite *V. destructor*, causes wing deformities in honeybees and is considered one of the main viruses associated with the colony collapse, premature death and reduced performance in asymptomatic bees [7, 8]. Transmission of DWV by *V. destructor* to pupae can result in clinical symptoms such as pupal death, the emergence of adult bees with deformed wings, bloated and shortened abdomen, and discoloration. However, in the absence of *V. destructor* mites, DWV infection appears to cause no visible symptoms or detectable impact on host fitness [7]. Although infection with DWV in honey bees is strongly associated with *V. destructor* mite infestation, it can also exist as a covert (symptomless) infection in colonies where the mite is absent [9]. DWV is a picorna-like virus classified within the family Iflaviridae. Its genome encodes a poly protein that is subsequently cleaved by viral and/or

cellular enzyme into structural and non-structural proteins required for the viral replication cycle [10]. Mature Virions of the family Iflaviridae are spherical, without envelope, and measuring 22-30 nm in diameter. They contain three major structural proteins (VP1, VP2 and VP3) and a smaller capsid protein VP4, which is found in some species [11].

Three main variants of DWV have been described: DWV-A, DWV-B and DWV-C, with DWV-A and DWV-B being the most prevalent globally in honey bee colonies [12]. More recently, however, a newly identified virus named Egypt bee virus (EBV) has been proposed as a potential fourth variant of DWV. EBV appears to be more closely related to DWV-C than to either DWV-A or DWV-B [13]. The DWV-A variant is associated with colony loss. However, the effects of DWV-B on colony health remain a subject of debate. While DWV-B has been detected in healthy colonies, it has also been suggested that this variant may offer protective benefits to colonies through a mechanism known as superinfection exclusion [14].

According to FAOSTAT 2023 report, Iran ranked third among the top ten natural honey-producing countries, with an annual production of approximately 100,000 tons of honey [15]. It has been reported that, in Iran, pesticides and diseases, particularly *Varroa* mite infestations, are among the primary causes of colony losses and reduction honey production. Consequently, viruses transmitted by this mite are believed to contribute significantly to these losses. However, limited information is available regarding viral infections and their relationship with *Varroa* mites and colony losses in Iran [16]. DWV was first reported in Iran by Ghorani et al. in 2017, and since then, several studies have confirmed the presence and circulation of this virus among bee colonies and mites in the country [16-20]. Since Iran ranks among the top ten honey-producing countries, monitoring the presence and circulation of viruses and mites in bee colonies is of great importance, as these factors can significantly affect colony health and, consequently, colony performance. In this study, we investigated the simultaneous presence of DWV and *Varroa* mites in honey bee colonies located in Alborz Province, northern Iran.

## 2. Material and Methods

### 2.1. Sample collection

Samples were collected from honey bee colonies showing signs of wing deformities at two separate farms located in Alborz Province, northern Iran. A total of 24

colonies were sampled from first farm (farm A) and 15 colonies from the second farm (farm B). The sampled colonies showed darkened brood cells, deformed and atrophied wings, and, in some cases, symptoms suggestive of wing paralysis. Samples from each farm were first screened for *V. destructor* infestation, then pooled and homogenized. All specimens were submitted to the Virology Laboratory of the Faculty of Veterinary Medicine, University of Tehran, and stored at  $-20^{\circ}\text{C}$  until further processing.

## 2.2. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Samples from each colony were pooled to generate a single representative sample per farm. The pooled samples were then crushed and homogenized using a ceramic mortar with diethylpyrocarbonate (DEPC) -treated water. The homogenates were centrifuged in  $20,000\times g$  for 1 minute, and 200  $\mu\text{L}$  of the supernatant was used for total RNA extraction. RNA extraction was performed using Sinapure™ RNA Extraction Kit according to the manufacturer's instructions. The extracted RNA was eluted into 50  $\mu\text{L}$  of RNAase free water and stored at  $-20^{\circ}\text{C}$  until cDNA synthesis. For cDNA synthesis, 1  $\mu\text{L}$  of random hexamer primer was added to 10  $\mu\text{L}$  of total RNA. The mixture was incubated at  $70^{\circ}\text{C}$  for 5 minutes, followed by immediate cooling at  $5^{\circ}\text{C}$  for 1 minute. In the next step, 9  $\mu\text{L}$  of cDNA master mix containing 1  $\mu\text{L}$  of MMLV reverse transcriptase enzyme, 4  $\mu\text{L}$  of 5X RT-buffer (Yekta Tajhiz Azma, Iran), 1  $\mu\text{L}$  of dNTPs (SinaClone, Iran), and 2  $\mu\text{L}$  of DEPC-treated water were added, resulting in a final reaction volume of 20  $\mu\text{L}$ . In the second step, the mixture kept at  $25^{\circ}\text{C}$  for 5 minutes, after than incubated at  $42^{\circ}\text{C}$  for 1 hour, followed by heating at  $85^{\circ}\text{C}$  for 5 minutes and cooling to  $5^{\circ}\text{C}$  for 1 minute.

To detect DWV, a pair of primers targeting the poly-protein- coding region were used: DWVF: 5'-CT-TACTCTGCCGTCGCCCA-3' and DWVR: 5'-CCGT-TAGGAATCATTATCG-3'). These primers amplify a 160 bp fragment of the DWV genome. The PCR conditions were as follows: initial denaturation at  $94^{\circ}\text{C}$  for 1 minute; 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 1 minute, annealing at  $56^{\circ}\text{C}$  for 1 minute, and extension at  $72^{\circ}\text{C}$  for 1 minute; followed by a final extension at  $72^{\circ}\text{C}$  for 10 minutes. To evaluate PCR products, 5  $\mu\text{L}$  of each reaction was loaded onto a 1.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet (UV) light using a transilluminator.

## 2.3. Sequencing and phylogenetic analysis

Positive samples were submitted for Sanger sequencing by Codon Genetic Group (Tehran, Iran). The quality of the obtained sequences was evaluated using NCBI BLAST and FinchTV software (version 1.4.0). Phylogenetic analysis was performed using MEGA 7 software with the maximum likelihood method based on the general time reversible (GTR) model with 1000 bootstrap replicates. For this analysis, the sequences obtained in the present study were compared with previously submitted DWV sequences available in the NCBI database. The sequences of the current isolates were submitted to GenBank and available under accession numbers: PV975901 (isolate UT-SHN07) and PV975902 (isolate UT-SHN62).

## 3. Results

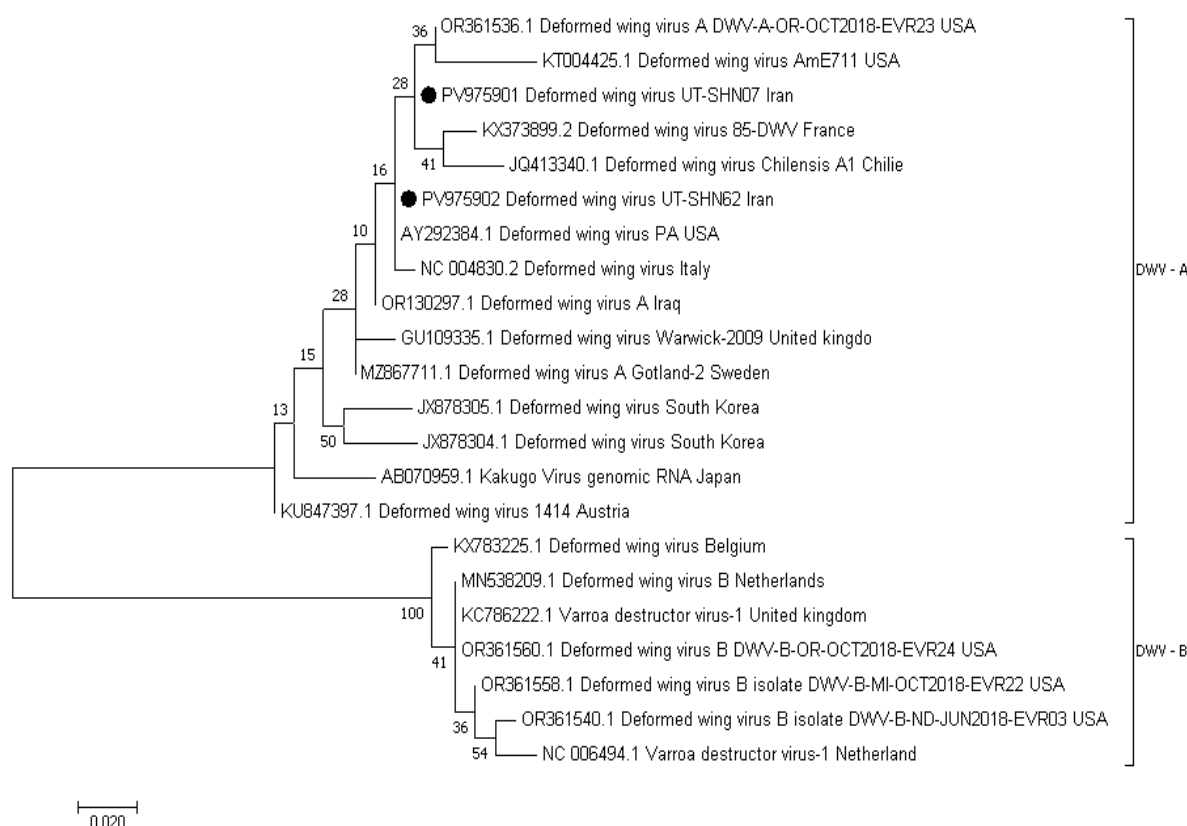
Phylogenetic analysis of the detected DWV isolates revealed that they belong to DWV variant A (DWV-A) (Figure 1). Homology analysis showed that the sequence UT-SHN07 from the present study share approximately 99% nucleotide identity with sequences AY292384 and OR361536 from the USA and OR130297 and NC004830 from Iraq and Italy, respectively. The results of homology analysis for the other isolate in this study, UT-SHN62, indicated 100% similarity between the sequence of this isolate and sequence AY292384 isolated from USA. The sequence of UT-SHN62 has also showed almost 99% similarity with sequences OR361536 (USA), OR130297 (Iraq), NC004830 (Italy), and MZ867711 (Sweden) (Table 1). Investigation of *Varroa* infestation demonstrated that 13 out of 24 colonies (54%) from farm A and 3 out of 15 (20%) from farm B were infested with mites.

## 4. Discussion

The global population of honeybees (*A. mellifera*) is under pressure due to habitat loss, environmental stress, and specially pathogens such as viruses that can cause lethal epidemics [21]. Honeybees play a vital role in pollination and contribute billions of dollars in added value to agriculture [22]. Although many viral infections can be asymptomatic, they are of great concern as they can harm honeybees at various developmental stages, including egg, larva, pupa, adult, worker, drone, and queen [22]. Among these viruses, DWV, along with its vector, the *V. destructor* mite, appears to be a major global threat to honeybee populations [21].

**Table 1.** Nucleotide sequence variation for DWV of isolates in present study compared with previous sequences retrieved from NCBI GeneBank

| No. | Isolate (GenBank Accession No.)                                    | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   | 13   | 14   | 15   | 16   | 17   | 18   | 19   | 20   | 21 |
|-----|--------------------------------------------------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|----|
| 1   | UT-SHN07 (PV975901)                                                |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 2   | UT-SHN62 (PV975902)                                                | 99.3 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 3   | PA/USA (AY292384.1)                                                | 99.3 | 100  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 4   | DWV - A - O R - O C T 2 0 1 8 - E V R 2 3 / U S A (OR361536.1)     | 99.3 | 98.6 | 98.6 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 5   | DWV-Iraq-202/Iraq (OR130297.1)                                     | 98.6 | 99.3 | 99.3 | 98   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 6   | DWV reference sequence/Italy (NC_004830.2)                         | 98.6 | 99.3 | 99.3 | 97.9 | 98.6 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 7   | Gotland-2/Sweden (MZ867711.1)                                      | 98   | 98.6 | 98.6 | 97.2 | 99.3 | 98.6 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 8   | 85-DWV/France (KX373899.2)                                         | 97.9 | 97.2 | 97.2 | 97.2 | 96.5 | 97.2 | 97.2 |      |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 9   | Chilensis_A1/Chille (JQ413340.1)                                   | 97.2 | 96.5 | 96.5 | 96.5 | 95.7 | 96.4 | 96.5 | 97.2 |      |      |      |      |      |      |      |      |      |      |      |      |    |
| 10  | Warwick-2009/United Kingdom (GU109335.1)                           | 96.5 | 97.2 | 97.2 | 95.8 | 97.9 | 97.2 | 98.6 | 96.5 | 96.5 |      |      |      |      |      |      |      |      |      |      |      |    |
| 11  | Austria 1414/ Austria (KU847397.1)                                 | 96.5 | 97.2 | 97.2 | 95.8 | 96.5 | 97.2 | 97.3 | 95.8 | 95   | 95.8 |      |      |      |      |      |      |      |      |      |      |    |
| 12  | AmE711/USA (KT004425.1)                                            | 95.7 | 95   | 95   | 96.5 | 94.3 | 94.2 | 93.5 | 93.4 | 94.2 | 91.8 | 93.4 |      |      |      |      |      |      |      |      |      |    |
| 13  | Kakugo virus/ Japan (AB070959.1)                                   | 94.3 | 95.1 | 95.1 | 93.6 | 94.3 | 95   | 95.1 | 93.6 | 92.7 | 93.5 | 96.5 | 89.4 |      |      |      |      |      |      |      |      |    |
| 14  | Korea1/ South Korea (JX878304.1)                                   | 94.1 | 94.9 | 94.9 | 93.4 | 95.7 | 94.9 | 96.4 | 93.4 | 94.2 | 94.9 | 91   | 92.6 |      |      |      |      |      |      |      |      |    |
| 15  | Korea2/ South_Korea (JX878305.1)                                   | 93.4 | 94.2 | 94.2 | 92.6 | 95   | 94.1 | 95.7 | 92.6 | 93.5 | 94.1 | 95.7 | 90.3 | 93.5 | 94.9 |      |      |      |      |      |      |    |
| 16  | DWV-B-MI-OCT2018-EVR22/ (OR361558.1)                               | 70.7 | 71.8 | 71.8 | 71.9 | 70.7 | 73.4 | 70.7 | 70.7 | 66   | 70.3 | 72.9 | 68.9 | 66.8 | 65.3 | 67.3 |      |      |      |      |      |    |
| 17  | Leuven-dw1/ Belgium (KX783225.1)                                   | 69.8 | 71   | 71   | 71.1 | 69.8 | 72.6 | 69.8 | 69.8 | 65.2 | 69.5 | 72.1 | 68.1 | 68.7 | 64.5 | 66.4 | 97.9 |      |      |      |      |    |
| 18  | DWV-B-OR-OCT2018-EVR24/ (OR361560.1)                               | 69.4 | 70.6 | 70.6 | 70.7 | 69.4 | 72.2 | 69.4 | 69.4 | 64.6 | 69   | 71.6 | 67.5 | 68.2 | 63.9 | 65.9 | 99.3 | 98.6 |      |      |      |    |
| 19  | DWV_B_Netherlands/ (MN538209.1)                                    | 69.4 | 70.6 | 70.6 | 70.7 | 69.4 | 72.2 | 69.4 | 69.4 | 64.6 | 69   | 71.6 | 67.5 | 68.2 | 63.9 | 65.9 | 99.3 | 98.6 | 100  |      |      |    |
| 20  | VDV-1_Ox/ United Kingdom (KC786222.1)                              | 69.4 | 70.6 | 70.6 | 70.7 | 69.4 | 72.2 | 69.4 | 69.4 | 64.6 | 69   | 71.6 | 67.5 | 68.2 | 63.9 | 65.9 | 99.3 | 98.6 | 100  | 100  |      |    |
| 21  | DWV-B-ND-JUN2018-EVR03/ (OR361540.1)                               | 68.2 | 69.4 | 69.4 | 69.5 | 68.2 | 71   | 68.2 | 68.2 | 63.3 | 67.8 | 70.5 | 66.3 | 64.2 | 62.6 | 64.6 | 98.6 | 96.5 | 97.9 | 97.9 |      |    |
| 22  | V. destructor virus-1 reference sequence/ Netherland (NC_006494.1) | 67.1 | 68.3 | 68.3 | 68.4 | 69.5 | 70   | 69.5 | 67.1 | 62.1 | 69.1 | 69.4 | 65.2 | 63.1 | 64   | 66   | 98   | 97.2 | 97.3 | 97.3 | 97.3 | 98 |



**Figure 1.** Phylogenetic analysis of DWV

Note: Phylogenetic analysis was performed with MEGA 7 software, using maximum likelihood method based on general time reversible model with 1000 bootstrap. Black circles represent for isolates in this study. According to this phylogenetic tree, current isolates are clustered with isolates in genotype A of DWV.

The RT-PCR method developed to amplify a specific nucleotide sequence located in virus genome present in sample is considered as valuable tool for diagnosis of viral disease in honey bees as it is fast, reliable, and specific, especially when target-specific primers are used [17]. In addition to molecular detection, phylogenetic analysis serves as a useful approach for defining the diversity and differences among nucleotide sequences of various virus strains. These differences are affected by different ways, including the replication cycles, the accuracy of replication, and the phenotypic effects of mutations. The quantity of these variations is evaluated using phylogenetic algorithms to define the probable genetic relationships among isolates [23]. Colony losses have been associated with infestation by the exotic mite, *V. destructor*, which feeds on honey bee hemolymph and can harbor different honeybee viral pathogens, specially DWV. Severe DWV infection can result in pupal death, wing deformities, shortened abdomen, and cuticle discoloration in adult bees, which often die shortly after pupation, causing colony collapse [21]. In colonies that don't show the symptoms of DWV disease, *V. destructor* mites are

either absent or present at low levels. The severity of DWV infection has been shown to be directly correlated with the prevalence and intensity of *V. destructor* infestation within the colony [16].

In Iran, different studies have reported the presence and circulation of bee viruses among colonies. In 2017, Ghorani et al. examined 89 Iranian honey bee samples showing signs of depopulation, sudden collapse, paralysis, or dark coloring. Using the RT-PCR method with DWV- specific primers targeting the structural protein-encoding gene, they found that 16 samples (17.97%) were positive for DWV, making it the most prevalent virus detected in this study [17]. In 2018, Moharrami et al. screened 156 apiaries for DWV infection using the RT-PCR method with specific primers targeting a 435 bp fragment of the polyprotein- encoding gene. DWV was detected in 34 apiaries (21.8%), with 21.8% of adult bees and 20.51% of pupae testing positive [24]. In another study by Moharrami et al. in 2018, 160 adult bee samples from 23 provinces were analyzed, and DWV was detected in 34 samples (21.8%), ranking it as the



second most prevalent virus [20]. In a more recent study by same group (2023), 45 samples were tested for six major bee viruses, with DWV emerging as the most prevalent, being detected in 37.7% of samples [19]. Additionally, Shojaei et al. screened 30 apiaries using RT-PCR with primers targeting partial VP2, VP4, VP1, and partial RNA helicase gene. Their findings showed that 36.6% of apiaries were infected with DWV, including 8, 0, and 3 positive samples from capped larvae and workers, and *Varroa* mites, respectively [8]. Altogether, these studies suggest that DWV is one of the most prevalent honey bee viruses currently circulating among honeybees in Iran.

In addition to Iran, neighboring and nearby countries have also reported the presence of DWV in their honey bee colonies. In a study conducted by Çağırman et al. in 2021, a total of 111 samples were collected from seven provinces in Turkey to screen for seven major honey bee viruses. Among the detected viruses, 22 out of 111 samples tested positive for DWV, making it the most prevalent virus in this study (19.8%) [25]. In another study by Karapinar et al., samples were collected from 26 hives, of which 18 hives (69.23%) were positive for DWV infection [26]. Haddad et al. investigated the distribution of DWV in Middle Eastern and North African (MENA) countries. For this purpose, 111 adult worker bee samples were collected from 12 countries, including Lebanon, Syria, Iraq, Palestine, Jordan, Egypt, Libya, Tunisia, Algeria, Morocco, Yemen, and Sudan. According to the results, the highest prevalence was observed in Syria and Lebanon, with lower rates reported in Palestine, Jordan, and Iraq [27].

In the present study, homology analysis revealed 100% and 99% sequence similarity of isolates UT-SHN62 and UT-SHN07, respectively, with the PA/USA isolate reported from the United States. The genome of this isolate (PA/USA) was completely studied, and phylogenetic analysis revealed that it clustered within genotype A of DWV [28].

## 5. Conclusion

Besides their vital role in ecosystem, honey bees contribute significantly to the economy through products like honey, beeswax, propolis, and royal jelly. Viruses, particularly DWV, pose a serious threat to bee health and colony sustainability. Present study is performed to detect and characterize the phylogeny of DWV isolated from 2 different farms in Alborz Province, north of Iran. According to this study, both isolates were clustered within genotype A of DWVs. Monitoring common

circulating viruses is necessary to identify new ways to prevent and control viral bee disease and, subsequently, prevent colony losses and reduced production. Constant monitoring can help to improve production quality and increase the exportation potential of honey bee products. Further studies are recommended to genotype circulating DWVs in Iran.

## Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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## Authors' contributions

Conceptualization, methodology and supervision: Arash Ghalyanchilangeroudi; Sample collection: Shabnam Jozghasemi; Formal analysis and software: Zahra Ziafati Kafi and Soroush Sarmadi; Writing the original draft: Fahimeh Jamiri, Zahra Ziafati Kafi, Soroush Sarmadi, Alireza Bakhshi, and Nazanin Sarvian; Review and editing: Arash Ghalyanchilangeroudi and Shabnam Jozghasemi.

## Conflict of interest

The authors declared no conflict of interest.

## Data availability

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

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