Original Article https://doi.org/10.47748/tjvr.1500593

TJVR 2024; 8 (2): 129-136

Turkish Journal of Veterinary Research

https://dergipark.org.tr/tr/pub/tjvr

e-ISSN: 2602-3695

The genetic characterization of *Lake Sinai Virus* in colony losses apiaries in Türkiye

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Received: 13.06.2024 Accepted: 09.08.2024

ABSTRACT

Objective: Honeybees (Apis mellifera) have a unique role in natural pollination and maintaining biodiversity in the ecosystem. The alarming increase in unexpected colony losses, unexpected bee deaths, and the tragic extinction of entire colonies (Colony Collapsed Disorder- CCD) have sounded a global alarm, demanding immediate attention and collaborative action to address these critical challenges in bee breeding. Diseases, parasites, and pathogens significantly threaten colony health. Türkiye is a significant honey producer, providing an ideal environment for beekeeping due to its unique eco-geographical features. Unexpected colony losses and bee deaths are also questions of concern for beekeepers in Türkiye.

Materials and Methods: This study investigated honey bee viruses in apiaries experiencing sudden bee death losses and CCD-like symptoms in Türkiye (Bursa, Edirne, Kocaeli Mersin, Tekirdağ and Zonguldak), involving genetic analysis of the *Lake Sinai Virus* (LSV) RdRp gene region. The honeybee samples were obtained from 52 colonies in 26 apiaries complaining of unexpected bee deaths and CCD-like symptoms between May 2021 and September 2023.

Results: The results showed a high *Deformed wing virus* (DWV), *Black queen cell virus* (BQCV), and LSV prevalence, respectively. The sampled apiaries were infested mild-moderate- high grade with Varroa mites. Following PCR results, DWV, BQCV, LSV, *Israel acute bee paralysis virus*, *Chronic bee paralysis virus* and *Sacbrood virus* positivity was detected at 69.2% (n=18), 50% (n=13), 38.46% (n=10), 26.9% (n=7), 19.2% (n=5) and 3.8% (n=1), respectively. High rates of multiple virus coexisting and high varroa infestation were noted in colonies with heavy losses and CCD-like complaints. The RdRp gene from two LSV samples (TrLSV-6474, TrLSV-6517) was sequenced. Turkish LSV samples (TrLSVs) showed a 72.88% homology of each other and clustered LSV4 branches in the phylogenetic tree. Turkish LSV sequences showed a closer similarity rate than reference sequences in GenBank with Asian Korean, Chinese, and Japanese LSV sequences.

Conclusion: Further investigation is needed to comprehend the implications of elevated LSV populations on colony losses. The execution of genetic research with a more extensive sample size can significantly enhance the demonstration of species diversity and provide valuable insights into the influence of LSV variants on honeybee health and the management of diseases.

Keywords: *Honeybee, Virus, Lake Sinai virus, RdRp gene*

INTRODUCTION

Honeybees (*Apis mellifera*) have a unique role in natural pollination, contributing to sustainable biodiversity in the ecosystem. These incredible insects offer a range of highly sought-after products, including invaluable honey, the resinous compound propolis, the nutrient-rich bee bread known as Perga, the potent royal jelly, and the biologically active apilarnil. These products are of significant socioeconomic value and possess

remarkable medicinal properties, making honeybees an invaluable asset to humanity (Klein et al., 2007). However, the ability of honeybee colonies to sustain their productivity is intricately linked to the overall health and well-being of the bees themselves. The alarming increase in unexpected colony losses, mysterious bee deaths, and the tragic extinction of entire colonies (Colony Collapsed Disorder- CCD) have sounded a global alarm, demanding immediate attention and collaborative action to address these critical challenges in bee breeding and ensure the survival of these indispensable pollinators (Genercsh, 2010; Neumann and Carreck, 2010). Diseases, parasites, and pathogens significantly contribute to unexpected honey bee colony losses (Daniat et al., 2012). The *Varroa destructor* mite is a significant threat to honey bees at every stage of their development. The ectoparasitic mites cause extreme damage to colony health by feeding on bees' essential "fat body". Their excessive consumption behavior results in the fatality of developing bees within the confines of brood cells, and it facilitates the transmission of numerous bee viruses, thereby further compromising the colony's resilience (Ramsey et al., 2019). Varroa mite infestation has serious consequences for bee populations. The mites act as vectors for many viruses and put a lot of strain on colonies through their parasitic activities. This leads to a series of negative impacts, exacerbating existing environmental pressures worse and posing a significant threat to the delicate balance of beekeeping practices (Dainat et al., 2012).

To date, numerous viruses have been identified in bee colonies. Some viruses threaten sustaining colony health (Martin et al., 2013; McMenamin and Genersch, 2015). *Acute bee paralysis virus* (ABPV), *Black queen cell virus* (BQCV*), Chronic bee paralysis virus* (CBPV), *Deformed wing virus* (DWV), *Kashmir bee virus* (KBV), *Lake Sinai Virus* (LSV), and *Sacbrood virus* (SBV) are frequently detected in apiaries. These viruses have a single-stranded positive-sense RNA genome. ABPV, BQCV, KBV, and IAPV belong to the *Dicistroviridae* family in the *Picornavirales* order, while DWV and SBV are classified in the *Iflaviridae* family within the same order. CBPV is an unclassified RNA virus, sharing similarities with the *Nodaviridae* and *Tombusviridae* families. Bee viruses can remain in a bee's body without causing clinical symptoms through latent (covert) infection. Some viruses can only become harmful due to specific environmental stress factors, such as pesticides, food scarcity, and climate change (Genercsh and Aubert, 2010; Daniat et al., 2012).

Although viruses generally exist in the colony with latent (cover) infections, they can lead to epidemics (overt) disease when the colony is subjected to stress factors. However, highly virulent and contagious viruses can lead to unexpected colony losses, sudden deaths, and detrimental effects on colony health and productivity (Genersch and Aubert, 2010). The presence of co-pathogens and mixed infections can impact losses, and the pathogenic effects of many viruses, especially DWV, have been identified (Muz and Muz, 2023a; Muz and Muz, 2023b).

LSV was initially detected in honeybee colonies during a metagenomic survey in America (Runckel et al., 2011), there has been a notable increase in reports of LSV in colonies in recent years (Daughenbaugh et al., 2015; Ravoet et al., 2015; Tozkar et al., 2015). LSV has a single-stranded positive-sense RNA genome and is classified in the *Sinhaliviridae* family within the *Nodamuvirales* order (Walker et al., 2020). It demonstrates genetic diversity based on classifying the viral RNA-dependent RNA polymerase (RdRp) gene, LSV1-8 (Daughenbaugh et al., 2015). There is still a lack of information regarding the pathogenesis and clinical characteristics of LSV in bees. However, its presence has also been observed in colony losses and weak and collapsed colonies (Runckel et al., 2011; Cornman et al., 2012; Daughenbaugh et al., 2015; Glenny et al., 2017).

Türkiye is a significant honey producer, providing an ideal environment for beekeeping due to its unique eco-geographical features. Reports of colony losses, sudden deaths, and cases resembling CCD have been linked to pathogens, viruses, and Varroa mites (Muz and Muz, 2009; 2017; 2022; 2023b; Giray et al., 2010; Kalaycı et al., 2020). The occurrence of LSV has been reported by based PCR method in some studies in apiaries in Türkiye (Tozkar et al., 2015; Çağırgan et al., 2022; Mayack and Hakanoğlu, 2022; Muz and Muz, 2022; 2023a). There is still insufficient information about the genetic diversity of LSV field isolates, their virulence properties, and their effects on bee deaths and colony losses. In this study, we investigated several honey bee viruses in apiaries that had experienced unexpected colony losses between 2021 and 2023. The research involved the molecular characterization and phylogenetic

analysis of the LSV RdRp gene region obtained from these apiaries.

MATERIALS and METHODS

Sampling area and sample collection

The research focused on apiaries experiencing colony losses from six locations in Türkiye: Bursa, Edirne, Kocaeli, Mersin, Tekirdağ, and Zonguldak (Figure 1). The research sampling took place between May 2021 and September 2023. Sampling occurred in 26 apiaries where colony loss and unexpected bee death complaints were reported. In each apiary, 52 colonies were sampled, including one colony with symptomatic bees (deformed wings, inability to fly, trembling, paralysis, blackened bees) and one colony without symptomatic bees. Worker bees (n=10) and mite samples were collected from each colony. Moreover, it examined climatic data, breeding type, and feeding patterns when colony losses occurred. The bee samples designated for molecular testing were carefully stored at -80 °C until they were ready for analysis.

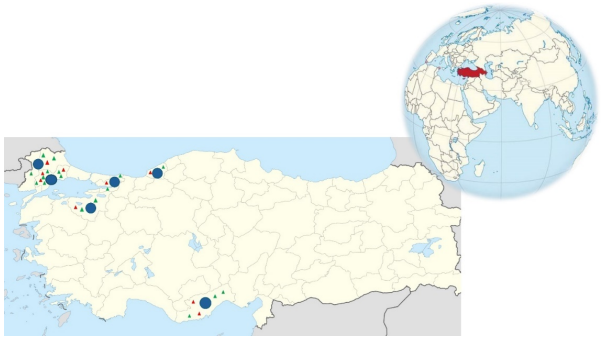

Figure 1. The map indicates the sampling provinces by blue circles. The sampled apiaries are denoted by triangles, and apiaries testing positive for LSV are marked in red.

Varroa destructor detection

The assessment of *Varroa destructor* infestation levels was conducted using two primary methods. Firstly, a sample of one hundred live honey bees was collected and introduced into a jar. Subsequently, 70% ethyl alcohol was added to the jar. The jar's contents were then thoroughly agitated to remove Varroa mites from bees. After a settling period, a meticulous count of the Varroa mites settled at the bottom of the jar was performed.

In addition, twenty sealed brood cells were carefully opened within each bee colony to inspect for the presence of Varroa mites visually. This two-pronged approach allowed for a comprehensive evaluation of mite infestation levels.

Based on the collected data, a classification system was employed to categorize the severity of infestation

* **High Infestation: Colonies exhibiting more than seven mites per one hundred bee sample.

* **Moderate Infestation: Colonies exhibiting four to six mites per one hundred bee sample.

* **Mild Infestation: Colonies exhibiting one to three mites per one hundred bee sample.

RNA extraction, Reverse Transcriptase-PCR and PCR

A total of 52 colonies from 26 apiaries were tested. Five bee pools were homogenized in PBS for a colony, and RNA was extracted from bee samples for molecular analysis. According to the kit's protocol, RNA samples were prepared using a commercial kit (GeneJET RNA Purification Kit, Thermo). RT-PCR was achieved using a cDNA Synthesis Kit (RevertAid First Strand, Thermo) according to the suggested protocol. PCR tests were applied to the cDNA samples obtained to detect honeybee viruses.

The reaction mixture was prepared, including 5u Taq polymerase, 10x Taq buffer, 3mM MgCl2, 300 pmol of dNTP mix, and sterile water in a 30 µl final volume. Virus-specific primer pairs for ABPV, BQCV, CBPV, DWV, IAPV, KBV, LSV, SBV were used for the mixture based on previously reported (Table 1). The reaction conditions involved previously reported Muz and Muz (2023a). After amplifying the PCR products, agarose gel electrophoresis was performed and the results were visualized using a UV transilluminator. To perform sequence analysis, positive DNA bands from the gel were purified using an extraction kit (GeneJet Gel Extraction Kit, Thermo) and then analyzed using an Applied Biosystems DNA analyzer. The sequences were examined using the BLAST tool, BioEdit program (v.7.2.5), and MEGA XI program. It was made with other strains deposited in the GenBank nucleotide database to compare and align sequences. The phylogenetic analysis utilized the neighbor-joining method with Kimura's two-parameter model (Kimura, 1980), and 1000 replicates were used for bootstrapping purposes (Tamura et al., 2021).

Table 1. The primer pairs utilized in the RT-PCR protocols within this study, along with the targeted genes and relevant literature information, are detailed in the provided table.

Virus	Amplified Region	Primer Sequence (5′-3′)	Length (bp)	Reference
ABPV	*Capsid protein*	F: GTGCTATCTTGGAATACTAC R: AAGGYTTAGGTTCTACTACT	618	Berenyi et al. 2006
BQCV	*Nonstructural polyprotein*	F: AGTAGTTGCGATGTACTTCC R: CTTAGTCTTACTCGCCACTT	472	Berenyi et al. 2006
CBPV	*RdRp*	F: TGTCGAACTGAGGATCTTAC R: GACCTGATTAACGACGTTAG	315	Berenyi et al. 2006
DWV	*Helicase*	F: ATCAGCGCTTAGTGGAGGAA R: TCGACAATTTTCGGACATCA	702	Chen et al. 2004
IAPV	*IGR*	F:GGTTGGCTGTGTGTCATCAT R:CGATGAACAACGGAAGGTTT	767	Palacios et al. 2008
KBV	*Nonstructural polyprotein*	F: GATGAACGTCGACCTATTGA R: TGTGGGTTGGCTATGAGTCA	415	Stoltz et al. 1995
LSV	*RNA dependent RNA polimerase*	F: CGTGCGGACCTCATTTCTTCATGT R: CTGCGAAGCACTAAAGCGTT	152	Daughenbaugh et al. 2015
SBV	*Structural protein*	F: ACCAACCGATTCCTCAGTAG R: CCTTGGAACTCTGCTGTGTA	487	Berenyi et al. 2006

RESULTS

As PCR results, in 26 apiaries tested, positivity for BQCV, DWV, CBPV, IAPV, LSV, SBV of the eight viruses investigated was detected. All apiaries were found negative for ABPV and KBV (Figure 2). The presence of DWV was detected in 18 (69.2%) of the apiaries with the highest positivity rate. Following this, BQCV, LSV, IAPV, CBPV, and SBV positivity was 50% (n=13), 38.46% (n=10), 26.9% (n=7), 19.2% (n=5) and 3.8% (n=1), respectively. LSV was detected during the experiment period.

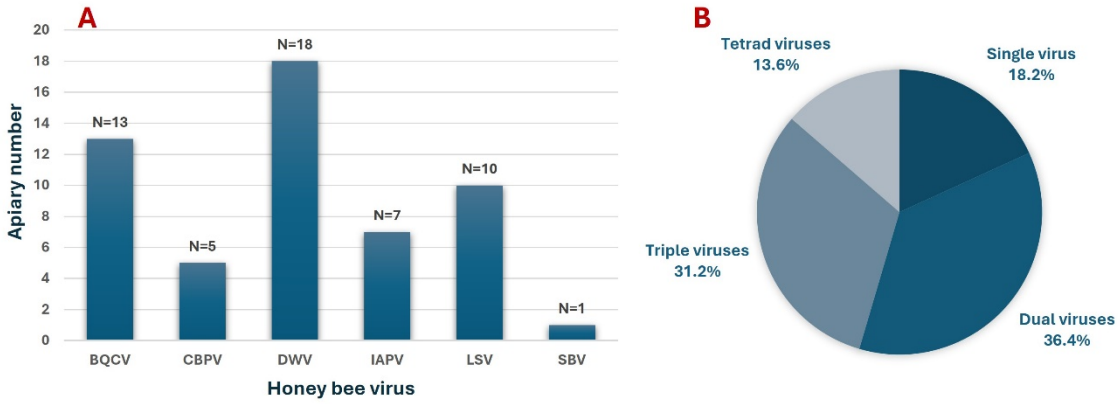

Figure 2. A: The honeybee virus positivity rates of the sampled apiaries are shown. B: The distribution and percent of single and multiple viruses coexist are shown.

All sampled apiaries were positive for varroa mites with changing intensity (mild, moderate, high infested) (Table 2). A mild varroa infestation was found in 12 apiaries, a moderate varroa infestation in 7 apiaries, and a high varroa infestation in 7 apiaries. Multiple viruses were also detected in apiaries with high varroa infestation. Complaints of bee deaths and colony loss were reported in all sampled apiaries. CCD-like symptoms were observed in 6 apiaries. High varroa infestation and multiple viruses were identified in apiaries with CCD-like symptoms. The existence of mixed infections, where more than one virus coexists, is noteworthy. 22 out of 26 sampled apiaries tested positive for at least one virus, which accounts for 84.61% of the total. The four (18%) apiaries were

positive for a single virus. These positivities were for DWV (n=3) and BQCV (n=1). In positive apiaries, dual, triple and tetrad virus positivity was detected in 8 (36.4%), 7 (31.2%) and 3 (13.6%) cases, respectively. Multiple virus positivity was also detected for at least two viruses in LSV-positive apiaries (n=10) (Table 2, Figure 2B).

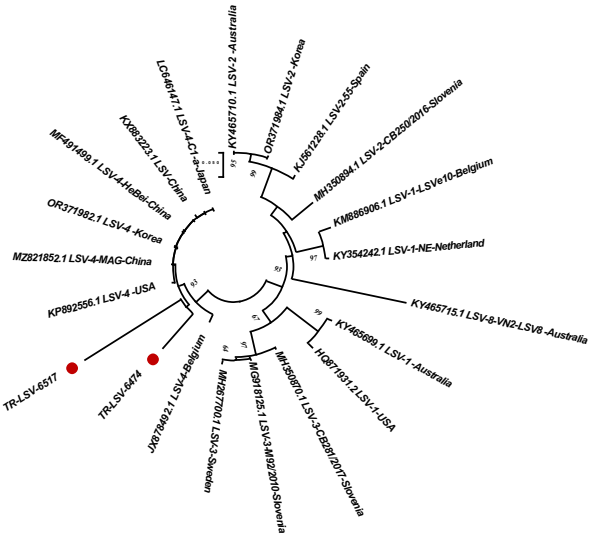

Figure 3. The phylogenetic tree of the LSV partial RdRp gene region was constructed using the neighbor-joining method and Kimura's two-parameter model with a 1000 bootstrap value. The tree highlights the TrLSVs identified in our study and is marked in red.

The RdRp gene from two samples tested positive for LSV through PCR (TrLSV-6474, TrLSV-6517) and was subjected to sequencing. These samples were taken from colonies that were experiencing colony losses. The sequences obtained from these Turkish LSV samples (TrLSVs) showed a 72.88% similarity. Furthermore, these TrLSVs were compared with sequences from Europe, America, and Asia deposited in GenBank. The TrLSVs exhibit similarities to other LSV sequences, ranging from 61.53% to 89.74%. Specifically, TrLSVs show higher (75.21-89.74%) similarity with LSV-4 sequences. For instance, TrLSV-6517 demonstrates a homology of 78.63% with Asian LSV-4 (Japanese-LC646147, Chinese- MF491499, MZ821852, Korean-OR371982) sequences, 75.21% with the Belgium sequence (JX878492), and 77.77% with the USA sequence (KP892556). Similarly, TrLSV-6474 displays 89.74%, 87.17%, and 88.88% homology with Asian (Japanese-LC646147, Chinese-MF491499, MZ821852, Korean- OR371982), European (Belgium-JX878492), and American LSV-4 (KP892556) sequences, respectively. In the phylogenetic tree analysis, the two TrLSV sequences cluster with LSV-4 sequences, using LSV1, LSV2, LSV3, LSV4, and LSV8 sequences from GenBank as reference (Figure 3).

Table 2. Detected viruses and varroa mite density in apiaries with unexpected colony losses and CCD-like symptoms. Varroa infestation intensity was graded (+: Mild infestation, ++: Moderate infestation, +++: High infestation).

Apiary No	Virus Positivity	Varroa Density	Colony Loss / CCD-like symptom
1	DWV, BQCV, IAPV	+++	+/+
2	BQCV, IAPV, LSV	++	+/-
3	-	+	+/-
4	-	+	+/-
5	DWV, BQCV, LSV	+++	+/-
6	BQCV	+	+/-
7	IAPV, LSV	+	+/-
8	-	+	+/-
9	DWV, BQCV, CBPV	+++	+/+
10	DWV, BQCV, CBPV	+++	+/+
11	IAPV, LSV	++	+/-
12	DWV, LSV	++	+/-
13	DWV, BQCV, CBPV, LSV	+++	+/+
14	DWV, BQCV, IAPV, LSV	+++	+/+
15	DWV, BQCV	+	+/-
16	DWV, CBPV, IAPV	++	+/-
17	DWV, IAPV	+	+/-
18	DWV, BQCV, LSV	+	+/-
19	DWV	++	+/-
20	DWV, BQCV, CBPV, LSV	+++	+/+
21	DWV, LSV	++	+/-
22	DWV	+	+/-
23	DWV	+	+/-
24	DWV, BQCV	++	+/-
25	-	+	+/-
26	DWV, BQCV	+	+/-

DISCUSSION

Türkiye with its diverse eco-geographic features, serves as a vital bridge connecting the Asian, European, and African continents. The country's seven geographical regions, each with distinct climate characteristics, contribute to the ecosystem's sustainable dynamics of living organisms. Changes in environmental conditions, animal and human population movements, and habitat alterations can impact the species diversity of living populations. Honeybees significantly contribute to species diversity within the ecosystem (Klein et al., 2007). Colony losses, unexpected bee deaths, and CCD may also occur in apiaries due to the presence of many bee viruses, parasites, and pathogens. The spread of

pathogens, variations in virulence, and the compounding effects of multiple stress factors negatively impact colony health, leading to a rise in colony losses (Genersch, 2010; McMenamin and Genersch, 2015). This issue has developed into a global crisis (Muz, 2008). Apiculture is facing a formidable challenge due to honey bee colony losses, with diseases, parasites, and pathogens significantly contributing to this alarming phenomenon (Daniat et al., 2012). The presence of various viruses in colonies of young honeybees can lead to complications in their field activities and other biological functions. These complications may manifest as wing deformities, paralysis, and flight issues (Muz et al., 2019). Additionally, a lack of timely or sufficient feed, like nectar and pollen, can cause a decrease in population density towards the end of winter, resulting in missed opportunities to capitalize on the advantages of early spring. Since LSV has recently been identified in honey bees, its effect on colonies as a bee pathogen has been intriguing. Knowledge of the pathogenicity, genetic diversity, and effect of LSV on colony losses is still insufficient. More detailed information on bee pathogens is one of the strategies to be used in planning colony management to prevent losses in apiaries. This study investigated LSV positivity and genetic structure in apiaries with colony losses, unexpected bee deaths and CCD-like complaints. It was also analyzed regarding common viruses, including DWV, BQCV, CBPV, IAPV, KBV, ABPV, LSV, and SBV, and mite infestation in apiaries. The findings revealed that varroa infestation was widespread in all apiaries, with viruses detected in 84.61% of the cases. Apiaries with heavy losses and CCD-like complaints showed elevated rates of both multiviruses and high varroa. Furthermore, DWV and BQCV were identified as the most prevalent viruses, followed by LSV the apiaries.

Apiaries that suffered colony losses and displayed symptoms similar to CCD were found to have been affected by multiple viruses and high varroa mite infestation. Varroa mites feed on bees' tissue fluids, fatty tissue, and hemolymph, which can diminish the flight performance, sperm quantity, and quality of male honey bees (Rinkevich et al., 2017). Furthermore, high varroa infestations threaten colony health by transmitting viruses (Muz 2008; Ramsey et al., 2019). Research indicates that due to the high mite infestation of honey bees engaged in field activities, the offspring's ability in orientation flights and worker bees' ability to return to the hive are impaired. As a result, affected bees require more time and food to return to the colony, or they may be unable to return (Peck et al., 2016). These factors may contribute to CCD-like symptoms.

The LSV was initially discovered in the USA through a metagenomic analysis (Runckel et al., 2011), and subsequent reports have shown its widespread prevalence in honeybee populations across various continents in subsequent years (Corman et al., 2012; Daughenbaugh et al., 2015; Amakpe et al., 2016; Robert et al., 2017; Simenc et al., 2020; Kitamaru et al., 2022; Nguyen et al., 2024). Its genetic variants and global prevalence suggest a longstanding and stable relationship with *A. melifera*. While the precise role of LSV in colony collapses and its pathogenesis in bees remains incompletely understood, numerous studies have suggested its association with weakened colonies (Glenny et al., 2017; Faurot Daniels, 2020). Furthermore, its high prevalence has been linked to losses (Cepero et al., 2014), with LSV2 abundance specifically associated with weaker and collapsed colonies (Corman et al., 2012; Daughenbaugh et al., 2015; Faurot-Daniels et al., 2020). Although LSV2 and LSV3 have been reported as more prevalent in Korean apiaries, the presence of LSV2, LSV3, and LSV4 has been established. LSV has been identified as the virus with the highest prevalence among other viruses in severe winter losses in Korean apiaries (Nguyen et al., 2024). Conversely, a study in Belgium reported no association with winter deaths. Additionally, research conducted in China (Hue et al., 2023) suggested that the seasonal prevalence of LSV is irregular and may exhibit seasonal patterns alongside other honeybee pathogens. Our own findings indicate that in apiaries experiencing colony losses and showing symptoms reminiscent of CCD, the higher diversity of viruses, including LSV, suggests a possible role of virulence in these instances after DWV and BQCV.

The prevalence of LSV in apiaries in this study was recorded at 38.46%. Colony losses and varroa infestations were also documented. Previous studies in Türkiye have reported LSV prevalence ranging from 40% to 56.5% (Çağırgan et al., 2022; Mayack and Hakanoğlu, 2022; Muz and Muz, 2022). However, a ten-year study across different regions of Türkiye reported a lower prevalence of 30% (Muz and Muz, 2023a). Globally, the prevalence of LSV varies between countries (Simenc et al., 2020; Cukanova et al., 2022; Hue et

al., 2023; Nguyen et al., 2024) and is considered a common honey bee virus. Stress factors can influence honeybee viruses' seasonal dynamics, diversity, and burden (D'Alvise et al., 2019). Similar to DWV genotypes, Varroa mites can contribute to increased viral load and genetic diversity of viruses through vectoring. This can potentially lead to greater damage to bee colonies. However, the prevalence of DWV, BQCV, and CBPV has increased mite's existence (Ryabov et al., 2014; Robert et al., 2017), but no evidence of LSV has been demonstrated. The impact of mite infestation on LSV population expansion remains uncertain. Environmental factors such as migratory beekeeping and abundant food resources are also effective bee pathogens and disease transmission (Hue et al., 2023). Further comprehensive research will help fully understand the effects of high LSV populations on colony losses.

Analyzing a wide range of LSV variants is crucial for identifying strains that affect honeybee health. Our study identified LSV-4 in apiaries where colony losses and unexpected bee deaths occurred, based on RdRp gene region sequences. Turkish LSV sequences showed a closer relationship with Asian Korean, Chinese, and Japanese LSV sequences. Although a metagenomic study reported the presence of LSV1 and LSV2 (Tozkar et al., 2015) from Türkiye, further classification and characterization of LSV field variants are needed.

CONCLUSION

It is crucial to gather more information about genetic diversity and the pathogenesis of field viruses to develop strategies to manage colony losses. Conducting genetic research with a larger sample size can help demonstrate species diversity more effectively, shedding light on the diversity and impact of LSV variants on honeybee health and disease management. Further investigation is needed to comprehend the implications of elevated LSV populations on colony losses. The execution of genetic research with a more extensive sample size can significantly enhance the demonstration of species diversity and provide valuable insights into the influence of LSV variants on honeybee health and the management of diseases.

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ACKNOWLEDGMENTS

Author contributions: DM, MNM designed the study. DM and MNM performed laboratory analysis. DM and MNM participated in drafting and revising the manuscript. DM: Dilek Muz, MNM: Mustafa Necati Muz

Financial Disclosure: The authors declared that this study has received no financial support.

Conflict of Interests: The authors declared that there is no conflict of interests.

Additional information: All authors have read and agreed to the published version of the manuscript Correspondence and requests for materials should be addressed to DM.

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