

Preliminary study of Lake Sinai virus in honey bee samples from İzmir and Muğla, Türkiye

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ABSTRACT

Lake Sinai virus (LSV) is an RNA virus that affects honey bee health and is considered a significant contributor to colony losses. In this study, the prevalence of LSV was investigated in adult bees and capped broods collected from 32 apiaries in İzmir and Muğla, Türkiye. Using RT-PCR, LSV was detected in 68.7% of adult bees and 46.8% of capped broods, indicating a widespread presence in the studied regions. Phylogenetic analysis was conducted to determine the genetic diversity of LSV strains. In the variant typing study, LSV-2 was the most commonly detected variant, while LSV-4 was identified at a lower frequency. However, LSV-1, LSV-3, and LSV-5 were not detected in the analyzed samples. According to the phylogenetic analysis, high genetic heterogeneity was observed among LSV strains, with no distinct clustering by geographical distribution. These findings suggest that LSV is prevalent in Türkiye and exhibits genetic diversity independent of its location. This study provides RdRp-based phylogenetic data on honey bee LSV from Türkiye, contributing valuable molecular evidence to ongoing efforts to understand LSV distribution and evolution. Further research is necessary to assess the pathogenicity of different LSV variants and their potential impact on colony health.

Keywords: Honey bees, Lake Sinai virus, LSV-2, LSV-4, Phylogenetic analysis.

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Introduction

The annual contribution of insects to biodiversity and agricultural products through pollination is estimated to be around \$175 billion worldwide (14). Honey bees are the principal pollinators of agricultural products in both the United States and Europe, contributing over \$17 billion annually (7, 21). Honey bees are essential for both economic value and agricultural sustainability (18).

As with any living creature, there are a variety of factors that influence the health of a honey bee colony. Many studies have found that a variety of abiotic and biotic variables influence colony health (12, 36). Although colony losses are rarely due to a single factor, pathogens (bacteria, parasites, and viruses) are frequently mentioned as a cause of colony losses (8, 9, 20). The development of

new molecular diagnostic techniques and the use of next-generation sequencing methods have enabled the detection of colony losses and their causative agents (19). This has allowed the discovery of many new viruses, such as Lake Sinai virus (LSV) (11, 15, 25, 35).

LSV is a 5.6 kb positive polarity RNA virus classified in the family *Sinhaliviridae* within the order *Nodamuvirales* (16, 30, 37). LSV was first identified between 2008 and 2009 as two different variants, LSV-1 and LSV-2, as a result of the increase in colony losses of commercial beekeepers around Lake Sinai, located in the state of South Dakota, USA (30). The presence of different variants, such as LSV 3-8 was shown in subsequent studies (15, 27, 29, 32). Although its pathogenicity and symptoms remain unknown, it is known that colonies with

high losses have a higher LSV viral load than colonies with low losses (8).

The LSV genome encodes three genes: ORF1, RdRp, and capsid (3). The RdRp gene encoding an RNA-dependent RNA polymerase has been reported to be a conserved region in different LSV strains (27). Therefore, the RdRp gene region is a suitable target for phylogenetic analysis and grouping of different LSV strains (31, 32).

Viruses affecting bee health in Türkiye have been examined in previous studies, and have been reported to adversely affect colony health. In studies, DWV (deformed wing virus), SBV (sacbrood virus), BQCV (black queen cell virus), ABPV (acute bee paralysis virus) and CBPV (chronic bee paralysis virus) are frequently detected in colonies (5, 17, 20, 35). Although the presence of LSV has been reported in Türkiye (6, 35), only a limited number of studies have examined its molecular characterisation to date (23).

This study, aimed to investigate the prevalence of LSV variants in adult bees and capped larvae in Turkish beehives (Muğla and İzmir), and to reveal its phylogeny and molecular characterisation.

Materials and Methods

Sample Collection: A total of 32 apiaries, located in İzmir (38°25'27.9"N, 27°08'34.5"E) and Muğla (37°14'49.5"N, 28°21'25.5"E) in western Türkiye (Figure 1), were randomly selected, sampling took place between April and June 2020. In total, 12 samples were collected from İzmir and 20 from Muğla. From each apiary, approximately 300

adult bees and larvae were collected from three different hives (one colony per hive) and immediately transported to the laboratory under cold-chain conditions. Adult bees and larvae from these 32 apiaries were crushed in sterile porcelain containers, 5 ml of Eagle's Minimum Essential Medium (Sigma Aldrich, UK) was added to them and they were transferred to 15 ml centrifuge tubes. The homogenates were centrifuged for 30 minutes at 4°C and 5000 rpm. It was kept at -80°C until RNA extraction.

RNA Extraction: For RNA extraction, 200 µl of supernatant was taken. RNA extraction was performed using the High Pure Viral RNA Kit (Roche, Germany) following the manufacturer's protocol. Extracted RNA was stored at -80°C until testing.

Detection of LSV by RT-PCR Method: Primers (LSV 1765-F:5'-TCA AYC TKG AGC GAT TTC GTG CTG-3', LSV 2368-R: GAG GTG GCG CSA GAT AAA GT-3') were used to detect all LSV variants by targeting the conserved ORF1 RdRp gene region, amplifying a 603-bp fragment (27). Amplification was performed using the Xpert One-Step RT-PCR Kit (Grisp Research Solutions, Porto, Portugal). The total reaction volume was 25 µl, and the final concentration of the primer was 0.4 µM. The following temperature profile was used: 15 min at 45°C (reverse transcription), 1 min at 95°C (initial denaturation), followed by 40 cycles of 10 s at 95°C (denaturation), 10 s at 60°C (annealing), and 10 s at 72°C (elongation), and finally an extension at 72°C for 15 s.

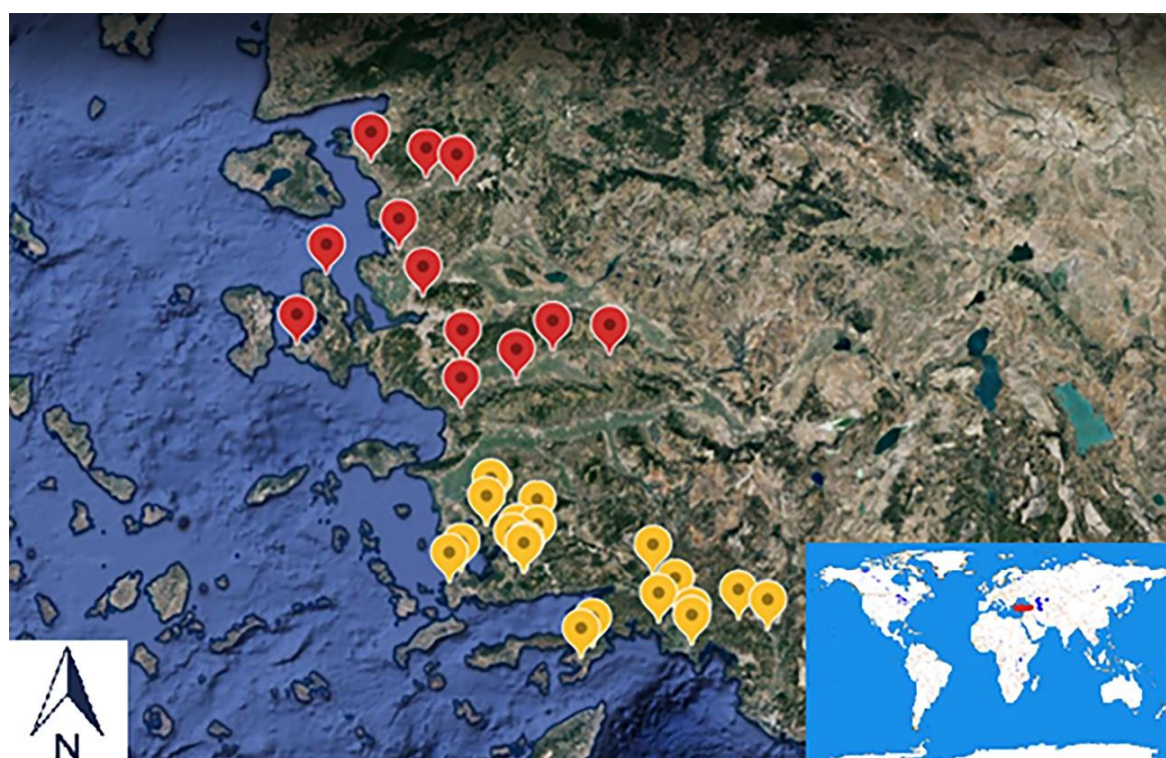


Figure 1. Türkiye's geopolitical location and the regions of the sampled apiaries. Muğla is indicated by yellow, whereas İzmir is indicated by red.

Primers targeting the ORF1 RdRp region designed by Runckel et al. (30) (LSV 1, 2) and Daughenbaugh et al. (11) (LSV 3, 4, 5) in their respective studies, were used to determine variants in LSV positive samples. The following temperature profile was used: 15 min at 45°C (reverse transcription), 1 min at 95°C (initial denaturation), followed by 40 cycles of 10 s at 95°C (denaturation), 10 s at 55–60°C (annealing) and 10 s at 72°C (elongation), and finally an extension at 72°C for 15 s. The obtained PCR products were separated by 1.5% agarose gel electrophoresis (containing ethidium bromide) and visualised under UV light.

Phylogenetic Analysis: The PCR products were sequenced in both forward and reverse directions by Microsynth AG (Balgach, Switzerland). Phylogenetic analysis of the ORF1 RdRp gene region (1774–2328 bp) was performed using 52 LSV sequences obtained from other geographic regions. These sequences were retrieved from GenBank. The nucleotide sequence results were analyzed and combined in the DNA Dynamo software. Consensus sequences and other sequences obtained from GenBank were aligned using the MEGA 11. Phylogenetic analyses were generated in MEGA 11 using the Maximum Likelihood (ML) method with the Kimura 2-parameter (K2+G) model and 1,000 bootstrap replicates to determine genetic distances between nucleotide sequences (24, 33). GenBank accession numbers were obtained in this study for two strains (LSV2/Bor1/bee/Türkiye/2020 and LSV4/Bor2/bee/Türkiye/2020), MZ355920 and MZ355921, respectively.

Results

Among the 32 sampled apiaries, adult bees tested positive for LSV in 22 (68.7%), whereas larvae tested positive in 15 (46.8%). In every apiary where larvae tested positive, the corresponding adult bee samples were also positive; accordingly, seven apiaries had only adult bees positive, 15 had both adult bees and larvae positive, and none showed larvae-only positivity. Of the LSV-positive adult bee apiaries, 20 were positive for LSV-2 and two were positive for LSV-4; no apiary harbored both LSV-2 and LSV-4 (Table 1). LSV-1, LSV-3, and LSV-5 were not detected.

Based on the partial RdRp region, Turkish sequences clustered within the LSV-2 and LSV-4 lineages, consistent with variant typing. In this study, grouping was performed as described by Šimenc et al. (32). The phylogenetic tree did not segregate according to geographical distribution and instead formed five distinct clusters (Figure 2). Within-lineage pairwise nucleotide similarity ranged from 90.7% to 96.7% (Table 2). The Turkish strain MZ355921 in LSV lineage 4, clustered with the Belgian (KM886903) and Chinese (KX883223)

strains. It was 96.8% similar to the Belgian strain KM886903 and 94.7% similar to the Chinese strain KX883223. The Turkish strain MZ355920 included in LSV lineage 2 clustered with the Australian strain isolated in 2013 (KY354243) and Slovenian strains isolated between 2016 and 2017. While the Turkish LSV-2 strain was 93% similar to the Australian strain, it showed 96.5–97.7% similarity to Slovenian strains. Two Turkish isolates from two different variants showed 83% nucleotide similarity. Nucleotide similarities between variants (groups) are shown in Table 3.

Table 1. LSV was detected in adult honey bees and capped larvae in each apiary.

Apiary number	Adult	Capped larvae	LSV-2	LSV-4
1	+	-	+	-
2	-	-	-	-
3	+	+	+	-
4	+	+	+	-
5	+	+	+	-
6	-	-	-	-
7	+	-	+	-
8	+	+	-	+
9	+	-	+	-
10	+	+	+	-
11	+	+	+	-
12	+	+	+	-
13	-	-	-	-
14	+	-	+	-
15	-	-	-	-
16	+	+	-	+
17	+	-	+	-
18	-	-	-	-
19	+	+	+	-
20	+	+	+	-
21	-	-	-	-
22	+	+	+	-
23	+	+	+	-
24	+	-	+	-
25	+	-	+	-
26	-	-	-	-
27	+	+	+	-
28	-	-	-	-
29	-	-	-	-
30	+	+	+	-
31	+	+	+	-
32	-	-	-	-

Samples positive in both adult bees and capped larvae are shown in green, whereas those positive only in adult honey bees are shown in blue.

Table 2. Within-lineage nucleotide similarity (%) of RdRp sequences based on Kimura 2-parameter (K2P) distances.

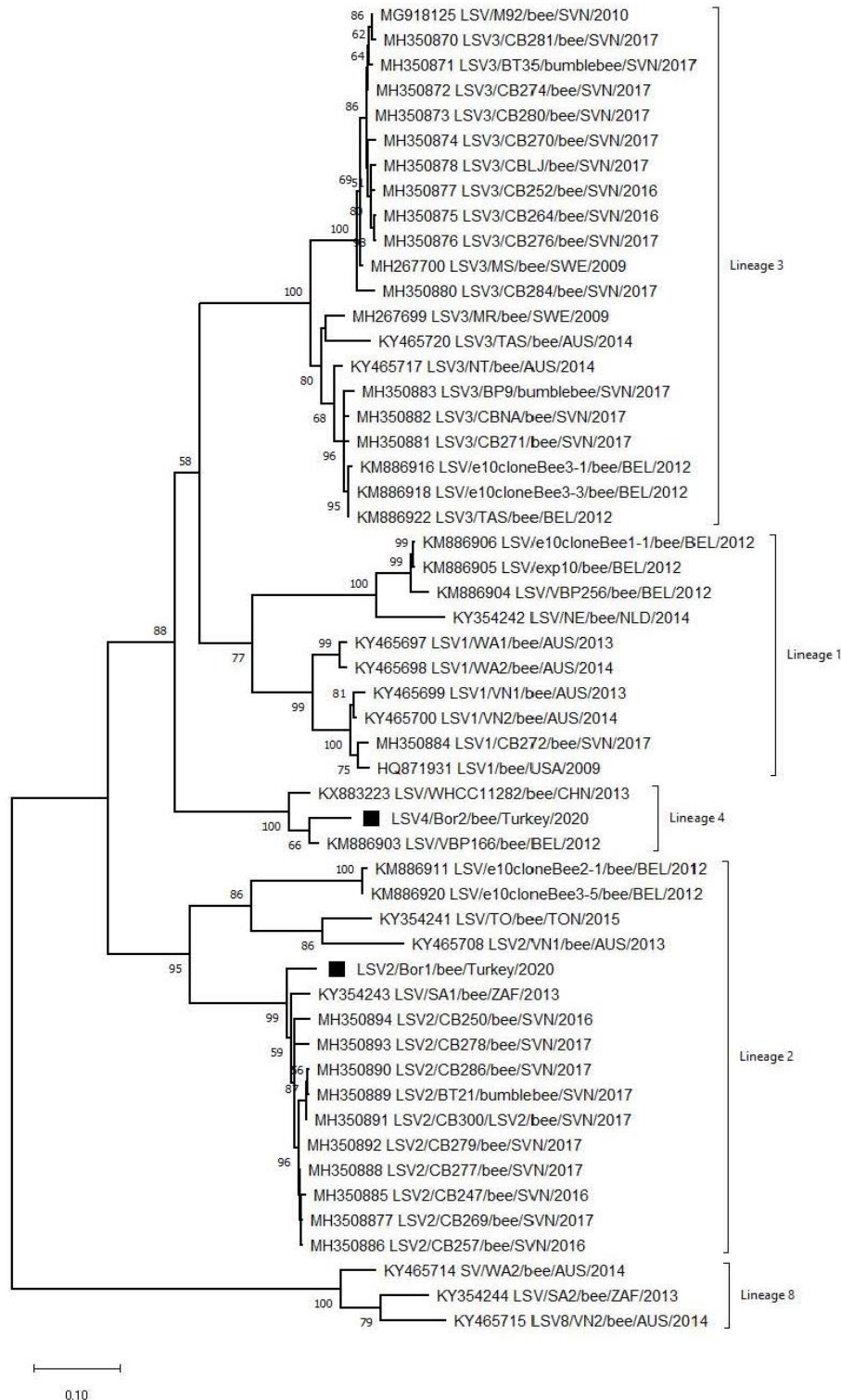
Lineage	Within-group nt similarities (%)
Lineage 3	96.7
Lineage 1	90.7
Lineage 2	93.0
Lineage 4	96.1
Lineage 8	92.4

Pairwise K2P distances were calculated in MEGA 11 from the trimmed RdRp alignment (positions 1774–2328; 555 nt) and converted to percent similarity using % similarity = (1 – distance) × 100.

Table 3. Between-lineage nucleotide similarity (%) of RdRp sequences based on Kimura 2-parameter (K2P) distances

	Lineage 3	Lineage 1	Lineage 2	Lineage 4	Lineage 8
Lineage 3	-	84.2	81.0	84.4	75.7
Lineage 1	84.2	-	81.5	83.8	75.8
Lineage 2	81.0	81.5	-	83.0	76.4
Lineage 4	84.4	83.8	83.0	-	75.8
Lineage 8	75.7	75.8	76.4	75.8	-

Pairwise K2P distances were calculated in MEGA 11 from the trimmed RdRp alignment (positions 1774–2328; 555 nt).

**Figure 2.** Phylogenetic tree inferred by the Maximum-Likelihood method in MEGA 11. Node support values represent 1,000 bootstrap replicates.

Discussion and Conclusion

The first reports of LSV in bee colonies emerged in 2009 when two variants (LSV-1 and LSV-2) were detected in the USA (39). In Türkiye, LSV was initially reported in a metagenomic study, without phylogenetic or detailed molecular characterisation (35), and was later detected in *Varroa* spp. from Turkish apiaries (6). Muz et al. (23) subsequently reported phylogenetic analyses of LSV from Türkiye. In this study, we investigated LSV prevalence and variant distribution in apiaries from Muğla and İzmir and characterised circulating variants based on the RdRp region in the context of globally available sequences. Given previous associations between LSV and weak colonies (11) and colony losses (8), our findings provide additional regional data to ongoing efforts to elucidate the epidemiology and genetic diversity of LSV.

Tozkar et al. (35) reported that LSVs are quite common in Turkish colonies. Reported prevalence varies across studies and regions; for example, one survey reported a prevalence of 3.57% in African colonies (2), despite the generally high prevalence and broad distribution reported elsewhere (4, 28, 29, 30, 31). LSV-2, together with LSV-1, have been described as the most common variants (30). In the study by Runckel et al. (30), LSV-2 was the predominant component of the honey bee microbiome. LSV-2 levels have been reported to be lower in strong colonies with larger populations, and associations between LSV and colony health have been noted in some studies (13). Tozkar et al. (35) indicated that LSV-1, LSV-2, and LSV-4 were prevalent; however, in this study LSV-2 was the dominant variant in the colonies, LSV-4 was detected only in adult bees and their larvae in two colonies, and LSV-1 was not detected. This pattern may reflect differences in sampling period, seasonal fluctuations, sampling strategy, and colony strength. Prior work has shown that the prevalence of bee viruses varies seasonally (30, 34). In this study, sampling occurred from April to June, which may explain the high prevalence observed. Consistent with our findings, D'Alvise et al. (10) reported high prevalence of LSV in samples collected during the summer in Germany, and another study from Argentina emphasized the importance of seasonal dynamics (4). In addition to prevalence-based surveys, a recent Türkiye study that focused on apiaries reporting sudden bee deaths and CCD-like complaints detected LSV in a substantial proportion of affected apiaries and, where sequencing was performed, placed Turkish RdRp sequences within the LSV-4 clade (23).

Although LSVs are common in colonies, their pathogenicity and transmission routes remain unresolved. Shared flowers may facilitate spillover from wild bees to managed bees (1). LSV variants have been detected in wild solitary bees (26), bumble bees (32), and the mite *Varroa destructor* (6, 27, 31). LSV has also been found in

nurse bees and pollen, and vertical transmission to offspring has been suggested (27). In this study, LSV was detected in 68.7% of adult bees and 46.8% of capped larvae, supporting both horizontal (via flowers/pollen and mites) and vertical transmission routes. The higher positivity in adults is consistent with substantial horizontal circulation, while detection in larvae could be compatible with vertical carryover. For comparison, Shojaei et al. (31) reported 83.3% positivity among adult bees and 10% among larvae.

LSV was first described in honey bees in 2009 (30), and its epidemiology and clinical relevance are still being clarified. Current evidence suggests that LSV may be associated with colony weakness and losses in some settings (8, 11, 13), but its pathogenic role and routes of transmission remain incompletely understood. Genetic diversity analyses and phylogenetic reconstruction are therefore useful to place newly detected sequences in an evolutionary context and to support hypotheses about virus spread and population structure.

In this study, we performed an RdRp sequence-based phylogenetic analysis of two Turkish LSV strains obtained from Muğla and İzmir. Despite the limited number of Turkish sequences analysed here, the two strains showed notable nucleotide divergence from each other, indicating that genetically distinct LSV variants may circulate in Turkish apiaries. High heterogeneity of LSV has also been reported in other locations, including on Norfolk Island (22), and surveys from Slovenia have identified multiple LSV lineages and strains (e.g., LSV-1, LSV-2, and LSV-3), which is consistent with substantial diversity in honey bee colonies (32). Because RdRp is relatively conserved and may provide limited resolution compared with more variable genomic regions, sequencing additional genomic regions and increasing sample size will be important to better resolve circulating diversity and to assess whether further variants are present in Türkiye. Consistent with this, Muz and Muz (23) reported two Turkish RdRp sequences that clustered within LSV-4 but showed relatively low nucleotide similarity to each other, reinforcing the possibility that multiple genetically distinct LSV lineages (including LSV-4) may be co-circulating in Türkiye.

It has been suggested that LSV heterogeneity is not related to any geographical distribution (27). In a phylogenetic study in Iran, Hamedan-1, Hamedan-4 and Hamedan-5 clustered with USA and Chinese strains, whereas Hamedan-2 and Hamedan-3 clustered with European strains from Belgium and Spain (31). Our two Turkish strains from İzmir and Muğla, shared 83% nucleotide similarity, and their placement in the phylogenetic tree did not reflect their geographic origins. Similarly, the two Turkish LSV-4-clustered sequences reported by Muz and Muz (23) did not support a simple

geographic structuring pattern, further suggesting that LSV lineages may spread across regions through mechanisms that blur local phylogeographic signals.

This study was carried out to investigate LSV infection in adult bees and their larvae in honey bee colonies and to reconstruct their phylogeny. According to our data, LSV is prevalent in both adult bees and larvae in Türkiye, and only LSV-2 and LSV-4 variants are present. In addition, the phylogenetic study showed heterogeneity among LSV variants and clustering in the tree did not correspond to geographic distribution. Finally, migratory beekeeping practices in Türkiye may facilitate movement of viruses between regions; therefore, broader surveillance and sequencing-based studies are warranted to monitor genetic diversity and detect emerging variants.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

The authors declare that they have contributed equally to the article. All authors provided significant contributions by giving feedback and helping to shape the manuscript.

Data Availability Statement

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

Declaration of Generative Artificial Intelligence

The authors declare that generative artificial intelligence (AI) and AI-assisted technologies were not used in the writing of this manuscript.

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