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Medlar (*Mespilus germanica*), a novel natural host for Hop stunt viroid (HSVd)

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Abstract: Hop stunt viroid (HSVd) infects various plants such as citrus, hop, almond, grapevine, pear, plum, peach, mulberry, fig, and pistachio. Medlar trees in an orchard in Malatya province of Türkiye were surveyed for the presence of HSVd in 2021. Twenty leaf and flower samples were collected and tested by RT-PCR methods using pathogen-specific primers. HSVd was found in five of the twenty medlar samples showing novel sequence variations. Two of the five HSVd variations were chosen at random and registered in GenBank. Both Turkish HSVd isolates had genomes that were 300 nucleotides long. The complete genome sequence of these variations was compared to isolates in GenBank. The nucleotide sequences of HSVd isolates exhibited 89.7–100% similarity with HSVd isolates found in various crops worldwide. Analysing the alignment of multiple sequences and conducting phylogenetic analyses revealed that identified HSVd variants clustered with citrus Türkiye (MZ995256), citrus Italy (KC584022), citrus Iran (GQ260203) and citrus Japan (X06719) isolates with 100% similarity rate and citrus China (FJ716172) and citrus Spain (AF213503) isolates with 99.5% and 98.0% similarity rates, respectively. To our knowledge, this is the first report of medlar serving as a natural host for HSVd. HSVd infection in medlar could be a problem in the future, and additional study is needed. The infection appears to be latent, but it might be a source of infection for susceptible plants.

Keywords: medlar; Hop stunt viroid; phylogenetic analysis; polymerase chain reaction; Türkiye

The common medlar (*Mespilus germanica* L.) is one of two species in the *Mespilus* genus that is becoming increasingly intriguing and appealing owing to the unique features of its fruits. It is used in traditional medicine (fruits, leaves, bark, and bud flowers) for a number of disorders or medical problems, as well as in gastronomy and a variety of dishes (traditional/local cuisines). Fruits, leaves, bark, or bud flowers had significant antioxidant chemicals (polyphenols and flavonoids), carotenoids, vitamins, minerals, etc. Highlighting the composition and qualities of medlar fruits is critical for rediscovering this unique fruit tree and stimulating its cultivation and consumption (Voaides et al. 2021).

Viroids are the smallest pathogens infecting plants (di Serio et al. 2014). Hop stunt viroid (HSVd) is the type species of the genus Hostuviroid, which belongs to the Pospiviroidae family and has a genome length of 294–305 nucleotides (Astruc et al. 1996). HSVd was originally identified in hops in Japan (Yamamoto et al. 1973). HSVd has been associated with various diseases, including hop stunt, citrus cachexia, cucumber pale fruit, and distortion in peach, plum, apple and apricot (Bove 1995; Reanwarakorn & Semancik 1999; Amari et al. 2001; Ragozzino et al. 2002; Sano 2003a; Elbeaino et al. 2012). It spreads mechanically and via infected seeds (Marquez-Molins et al. 2021). Having the most diverse host range of any viroid,

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HSVd infects plums, citrus, apricots, grapevine, peach, almond, pear, mulberry, and cucumber (Sano et al. 1989; Shikata 1990; Astruc et al. 1996; Polivka et al. 1996; Canizares et al. 1999; Sano 2003b; Sano 2003c; Li et al. 2006; Yang et al. 2006; Zhou et al. 2006; Elbeaino et al. 2011). Conversely, in certain other host plants like grapevine, almond, jujube, and pomegranate, the infection remains latent, as stated by Astruc et al. (1996), Kawaguchi-Ito et al. (2009), Zhang et al. (2009) and Gorsane et al. (2010). That suggests that the vast majority of HSVd-infected hosts are asymptomatic. HSVd isolates exhibit categorisation into five distinct groups, including three prominent groups and two lesser groups. The initial three classifications, namely 'plum-type', 'hop-type', and 'citrus-type', consist of isolates obtained from a small number of host plants, as described by Kofalvi et al. (1997). The second group is believed to have emerged through occurrences of recombination among members of the primary groups, as proposed by Amari et al. (2001).

The current study intended to detect HSVd in medlar, a new natural host for this viroid and characterise the molecular features of Turkish HSVd variants.

MATERIAL AND METHODS

Collecting medlar samples. Medlar flower and leaf samples were collected in the spring of 2021 from a collection orchard that comprises several fruits, including peach, persimmon, apple, pear, and almond, all of which are 15 years old in Malatya province, which is located in eastern Türkiye (Figure 1). Twenty medlar trees were in the orchard (Figure 2A), and samples were collected from each one and tested individually (Figures 2B and 2C). The collected plant samples were transported in a cold chain at 4 °C to the virology lab for viroid testing and further studies.

Total RNA extraction and RT-PCR tests. Medlar leaf and flower samples were subjected to total RNA extraction using a commercial purification kit for genomic RNA, following the instructions provided by the manufacturer (GeneJET Plant RNA Purification Kit, Thermo Fisher Scientific, USA). RT-PCR for HSVd testing was conducted using specific primers VP20 (5' CGC CCG GGG CAA CTC TTC TCA GAA TCC3') and VP19 (5' GCC CCG GGG CTC CTT TCT CAG GTA AG3'), as outlined by Amari et al. (2001). To a total volume of 12 µL cDNA reaction mix including 1 µL of 20 pmol/mL genome-specific reverse primer, 1 µL of 10 mM dNTP, and 7 µL of RNAase free water, 3 µL of RNA was added. The mixture was heated for 5 minutes at 65 °C before being cooled on ice. Then four microliters of 5X first-strand cDNA buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM MgCl2), 2 µL of DTT, and 1 µL of Moloney murine leukaemia virus reverse transcriptase (Promega, Madison, WI, USA) was added to the reaction mixture and incubated at 42 °C for 50 min. The re-

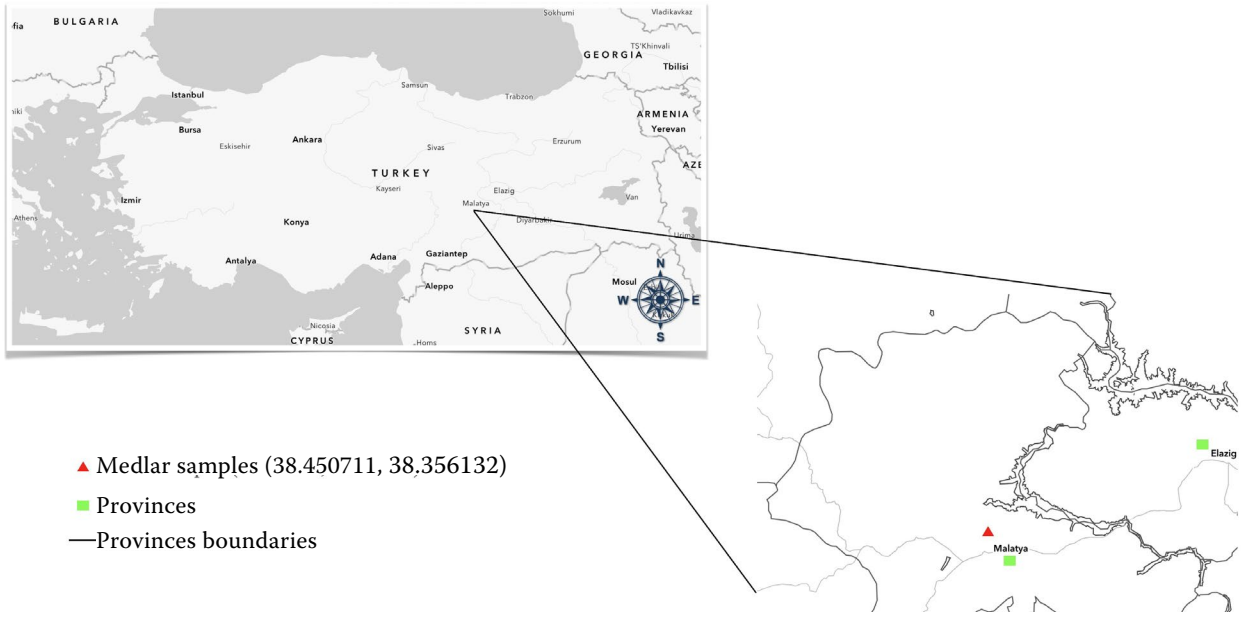

Figure 1. Map of Türkiye and Malatya province where medlar samples were collected

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(A)

(B)

(C)

Figure 2. (A) surveyed orchard; (B) sample photographed on the tree and; (C) sample photographed in the laboratory before testing

action mixture was incubated at 70 °C for 15 min to inactivate the reverse transcriptase enzyme before being kept at –20 °C until use. A negative control with DNase-free water was provided. For PCR, a reaction mixture of 25 µL was prepared, containing 0.5 µL of Taq DNA Polymerase, 2.5 µL of 10X GoTaq Green Buffer (Promega, Madison, WI, USA), 1.5 µL of 25 mM MgCl, 1.5 µL of dNTP (20 mM each), 1 µL of primers (10 mM each), 14.5 µL of RNase-free water, and 3 µL of cDNA. The PCR was performed using a Thermo Scientific Arktik Thermal Cycler (Waltham, MA, USA), with the cycling parameters: 94 °C for 2 min or initial denaturation, 30 cycles of 94 °C for 1 min for denaturation, 56 °C for 1 min for annealing, 72 °C for 1 min for extension, and 72 °C for 10 min for a final extension. Electrophoresis of each PCR product (15 µL) was performed on a 2% agarose gel in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The visualisation was achieved by staining with Pronasafe nucleic acid staining solution (CondaLab, Spain). A 100bp DNA ladder from Thermo Scientific was used as a molecular weight marker.

Sequencing, phylogenetic analysis and prediction of the most stable secondary structure. The DNA fragments were bidirectionally sequenced by a commercial firm (BM Labosis, Ankara/Türkiye) using the dideoxy chain termination reaction, and these sequences were analysed by searching the NCBI database. HSVd variant sequences produced in this work, as well as sequences from quite similar species recovered (Table 1) from the NCBI GenBank database, were included in different files in the Geneious Prime (GP) software (version 2023.0.1). Each dataset was individually aligned on the Geneious alignment, and discrepancies were manually corrected. The same software was utilised for each dataset, using Maximum Likelihood analysis (ML) with 1 000 bootstrap replicates to perform phylogenetic inference. Geneious tree builder was used for phylogenetic analysis. The tree was built using the neighbour-joining method and the Tamura-Nei genetic distance model. Peach latent mosaic viroid (PLMVd, accession number MZ289070) was used as an out-

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Table 1. Similarity rates of HSVd isolates with other isolates from throughout the world

Country	Agent	Host	Accession No.	Similarity rate (%)	Reference
Japan	HSVd reference sequence	Citrus	NC_001351	100.00	Sano et. al. (1988)
Brazil	Hop stunt viroid	Grapevine	MF774866	93.06	Fajardo et al. (2018)
China	Hop stunt viroid	Citrus	FJ716172	99.50	Wang et al. (2010)
China	Hop stunt viroid	Jujube	FJ771017	89.73	Zhang et al. (2009)
China	Hop stunt viroid	Peach	EF08057	89.73	Zhou et al. (2006)
Cyprus	Hop stunt viroid	Apricot	AJ297832	91.06	Amari et al. (2001)
Cyprus	Hop stunt viroid	Apricot	AJ297831	91.39	Amari et al. (2001)
Germany	Hop stunt viroid	Grapevine	X87924	95.34	Polivka et al. (1996)
Germany	Hop stunt viroid	Grapevine	X15330	93.37	Puchta et.al. (1989)
Greece	Hop stunt viroid	Apple	GQ249348	95.01	Kaponi et al. (2009)
Iran	Hop stunt viroid	Citrus	GQ260203	100.00	Hashemian et al. (2013)
Iran	Hop stunt viroid	Grapevine	KF916041	93.37	Khezerpour (2014) unpublished
Italy	Hop stunt viroid	Citrus	KC584022	100.00	Loconsole et al. (2013)
Italy	Hop stunt viroid	Peach	Y08437	91.72	Kofalvi et al. (1997)
Italy	Hop stunt viroid	Hibiscus	KC137266	91.72	Luigi et al. (2013)
Japan	Hop stunt viroid	Citrus	X06719	100.00	Sano et al. (1988)
Lebanon	Hop stunt viroid	Fig	HE662805	99.00	Elbeaino et al. (2013)
Russia	Hop stunt viroid	Grapevine	ON669243	95.68	Shvets et al. (2022b)
Russia	Hop stunt viroid	Grapevine	OL799308	95.68	Navrotskaya et al. (2021)
Russia	Hop stunt viroid	Grapevine	OP885293	92.79	Shvets et al. (2022a)
Slovakia	Hop stunt viroid	Grapevine	OP918904	95.68	Alaxin et al. (2023)
South Korea	Hop stunt viroid	Plum	KM052627	90.39	Cho et al. (2014) unpublished
Spain	Hop stunt viroid	Grapevine	KJ466332	92.73	Fiore et al. (2016)
Spain	Hop stunt viroid	Apricot	Y09344	91.39	Kofalvi et al. (1997)
Spain	Hop stunt viroid	Almond	AJ011813	90.69	Canizares et al. (1999)
Spain	Hop stunt viroid	Citrus	AF213503	98.00	Palacio-Bielsa (2004)
Tunisia	Hop stunt viroid	Pistachio	KC771547	90.06	Elleuch et. al. (2013)
Türkiye	Hop stunt viroid	Citrus	MZ995256	100.00	Gök and Önelge (2018)
Türkiye	Hop stunt viroid	Medlar	OR257530	–	this study
Türkiye	Hop stunt viroid	Medlar	OR257531	–	this study
Türkiye	Peach latent mosaic viroid	Persimmon	MZ289070	49.52	Oksal et al. (2021)

group for better branching. Using the GP program, secondary structure predictions of the new variants were conducted at 20 ℃, following the methodology described by Andronescu et al. (2007).

RESULTS

Survey and Sample Collection. Even though the samples were collected and tested in the spring of 2021, the trees were monitored for symptoms throughout the year. During the vegetation season (spring and summer months), trees were checked every two weeks. During the dormant phase (autumn and winter months), symptoms of the viroid were controlled monthly, with no evident symptoms or observable signs on flowers, leaves, fruits, or trees in general. The sampled trees looked relatively healthy, with no signs of damage. The symptoms looked for were typical HSVd symptoms on infected trees, such as early blooming and deformed flowers during the flowering period, crinkling, blistering, mosaic symptoms, yellow spots, leaf curling on leaves, fruit disorder and colour changes on the fruits, and stunting,

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short internodes, gum impregnation of the bark, and overall retarded maturation of the tree.

RT-PCR detection. Out of twenty medlar tree samples collected and tested, HSVd was detected in five. Two randomly selected HSVd medlar variants were subjected to sequencing and deposited in the gene bank with the accession numbers OR257530 and OR257531. The presence of HSVd in these samples was verified using RT-PCR assays and sequencing. Agarose gel electrophoresis was performed to assess PCR amplicons, revealing characteristic bands specific to HSVd in the infected samples (approximately 300 bp) on the gel. No amplicon was observed in the negative control, where DNase-free water was used as the template.

Phylogenetic analyses and prediction of the most stable secondary structure. The BLAST analyses of two complete HSVd revealed that the medlar variants share 89.7–100% identity with 27 available HSVd sequences infecting different crops worldwide (Table 1). Both medlar HSVd Malatya isolates showed 100% similarity with the HSVd citrus reference strain (NC_001351).

Five major clusters were observed in the phylogenetic dendrogram of HSVd (Figure 3). The two HSVd variants were shown to be genetically connected in the citrus group (in pink colour). Other isolates compared with the variants generated in this study were in the plum group (in red colour), hop group (in yellow colour) plum citrus group (in blue colour) and plum hop group (in brown colour).

Analysing the alignment of multiple sequences and conducting phylogenetic analyses revealed that identified HSVd variants clustered with citrus Türkiye (MZ995256), citrus Italy (KC584022), citrus Iran (GQ260203) and citrus Japan (X06719) isolates with 100% similarity rate and citrus China (FJ716172) and citrus Spain (AF213503) isolates with 99.5% and 98.0% similarity rates, respectively.

Figure 4 depicts the virtual gel of the HSVd isolates collected in this study and additional HSVd isolates created with GP from diverse crops world-

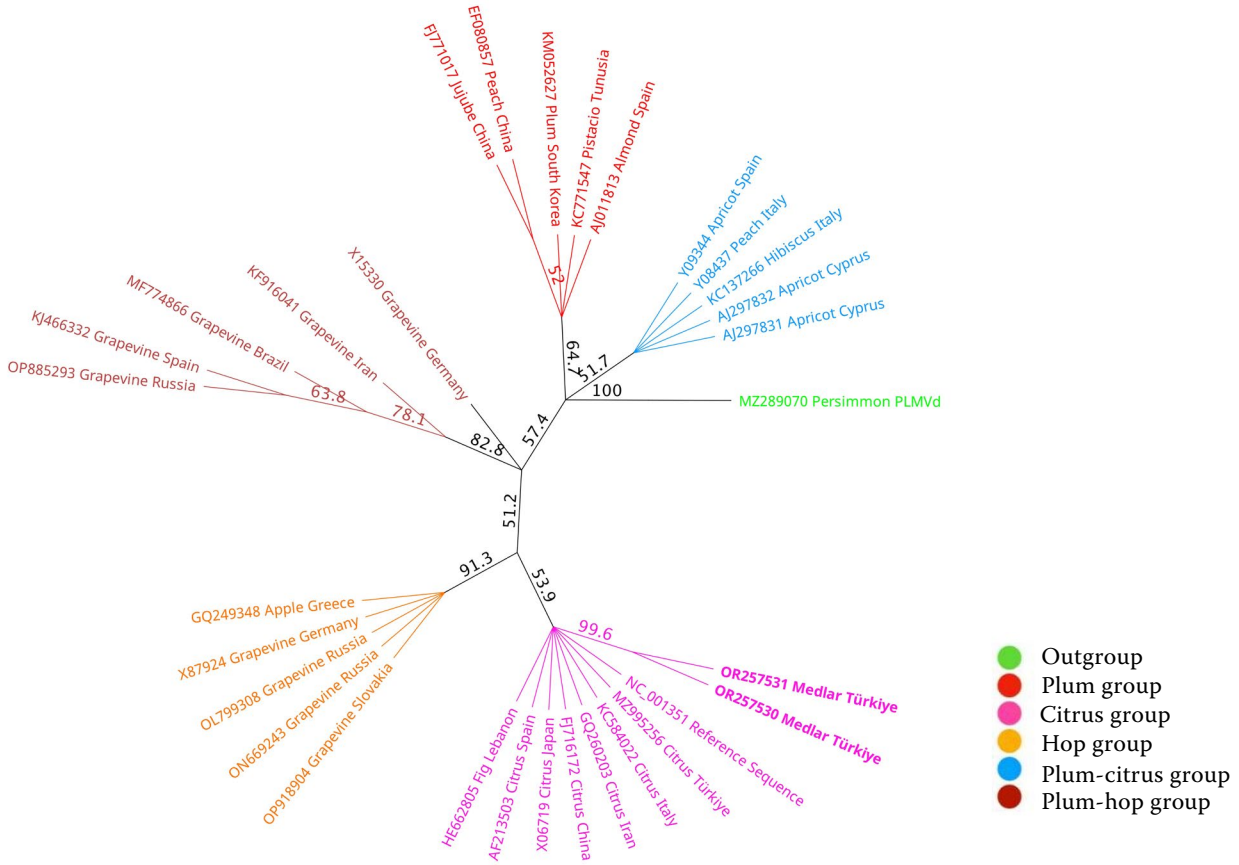

Figure 3. Phylogenetic relationships of Turkish Hop stunt viroid (HSVd) isolates of the medlar. The variants generated in this study were compared to the reference HSVd isolate, and 27 similar genetically related species were reconstructed from a full-length viroid genome. A PLMVd isolate from Türkiye (in green colour) was included as an outgroup

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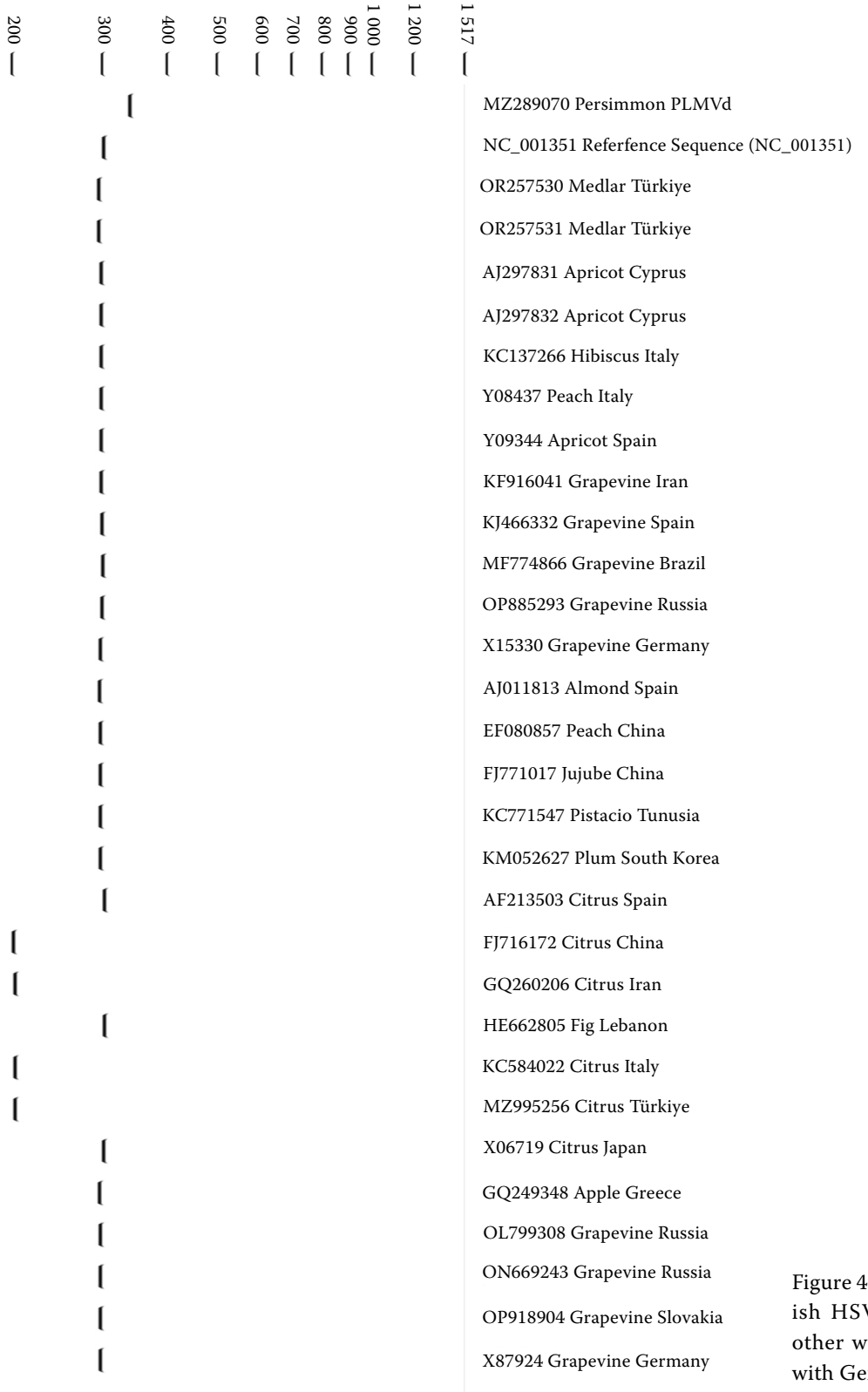

Figure 4. The virtual gel of Turkish HSVd medlar isolate and other world isolates generated with Geneious Prime (GP)

wide. At less than 300 kb, several isolates with lower nucleotide counts were evident on the gel.

Using the GP program, secondary structure predictions of the new variants were conducted at 20 °C, following the methodology described by Andronescu et al. (2007). The Turkish Malatya HSVd isolates exhibited a highly stable secondary structure resembling the HSVd reference sequence,

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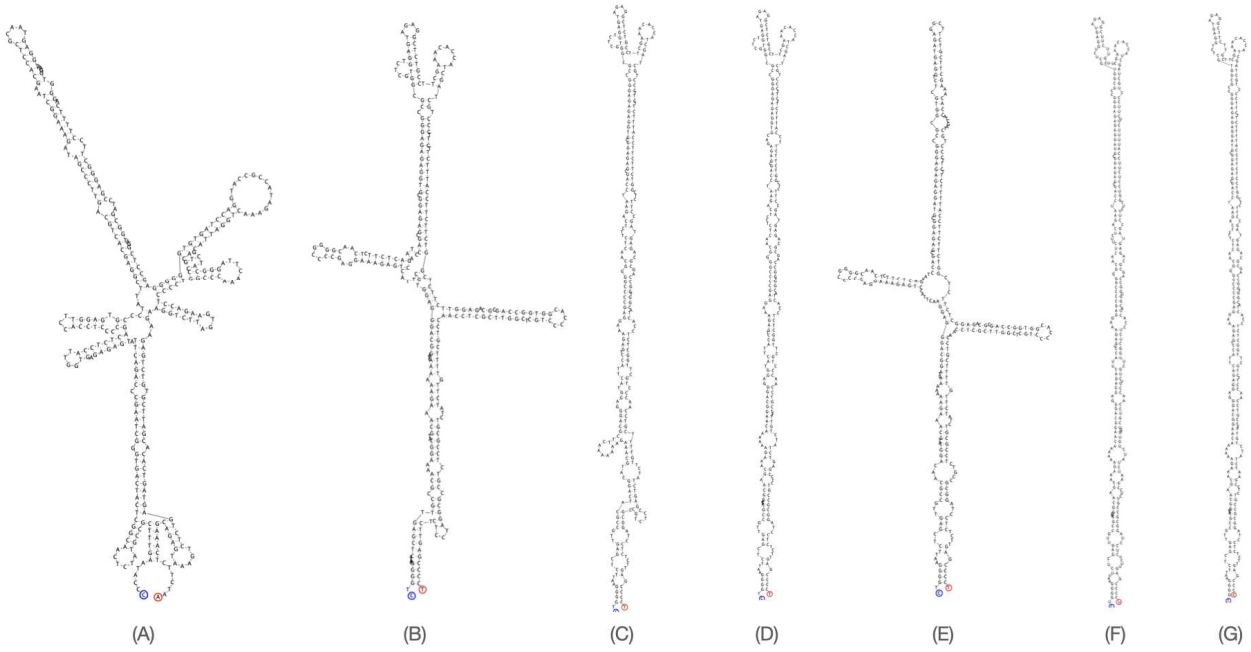

Figure 5. The most stable secondary structures of PLMVd, five different HSVd groups, and the medlar HSVd Turkish isolates were generated with Geneious Prime (GP)

(A) PLMVd (access. No. MZ289070 persimmon, Türkiye, outer group); (B) plum group (access. No. AJ011813, almond, Spain); (C) plum hop group (access. No. X15330, grapevine, Germany); (D) hop group (access. No. X87924, grapevine Germany); (E) plum citrus group (access. No. Y08437, peach, Italy); (F) citrus group (Reference Sequence access. No. NC_001351); and (G) HSVd medlar isolate (access. No. OR257530)

with a 100% nucleotide identity. The HSVd citrus strain displayed a rod-like shape as its most stable secondary structure, consisting of two distinct domains: a CCR and a conserved hairpin at the terminus. Typically, secondary structures undergo alterations when there are mutations in the nucleotide sequence of the isolates. The five groups of HSVd variants demonstrated diverse secondary structures, as depicted in Figure 5.

DISCUSSION

Reverse transcription polymerase chain reaction (RT-PCR), S-Page, and dot-blot hybridisation can all be used to detect viroids. Maddahian et al. (2019) amplified, cloned, sequenced, and compared 11 isolates of pistachio HSVd to those in GenBank. They detected HSVd infection for the first time in Iran and discovered that five isolates were from the hop group, two were from the citrus group, and the remaining five were from the recombinant isolates group (Ph/cit3). Guo et al. (2008) reported the presence of HSVd in hop using dot blot hybridisation for the first time. They also amplified three samples by PCR and sequenced and proved that the HSVd samples belong to the hop group. Elbeaino et al. (2012) tested 90 fig samples for HSVd by PCR and detected HSVd in 13.3% of the samples, forming a distinct clade known as the M-group in phylogenetic analyses. Gorsane et al. (2010) employed dot-blot hybridisation, S-Page, and reverse transcription polymerase chain reaction (RT-PCR) to identify HSVd in pomegranate. Through sequence and phylogenetic analysis, it was revealed that HSVd variations in Tunisian pomegranates could be categorised into two groups: citrus-type and recombinant citrus-plum-type. RT-PCR method was preferred to detect the HSVd infection in this study, and bidirectionally sequenced nucleotides were subjected to phylogenetic analysis to detect the group and molecular characterisation of the variants.

Typically, viroids can remain inactive in woody plants for periods. While symptoms may not manifest until the tree starts producing fruits, apple viroids and grapevines can remain asymptomatic indefinitely, even when infected with HSVd (Kawa-

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guchi-Ito et al. 2009). HSVd was detected exclusively on trees with discoloured areas on the fruit skins, according to Ragozzino et al. (2002), and it was not found in any symptomless samples. For the first time, Balsak (2017) has detected HSVd in pistachio trees in Kahraman Maraş province of Türkiye. No apparent symptoms were observed in the examined 50 plants. Blast analysis indicated that the HSVd isolates from Turkish pistachios exhibited a 99% nucleotide similarity with a Japanese HSVd isolate. Marquez-Molins et al. (2021) described the variety of HSVd isolates across its host range using a low-fidelity replication of RNA polymerase II, which is compelled to employ viroid RNAs as templates. Gazel et al. (2008) detected HSVd in Türkiye from naturally infected apricot, plum and peach trees. They sequenced eleven isolates and identified five novel sequence variants, ranging in length from 296 to 297 nucleotides, comparable to previously described HSVd isolates. Phylogenetic analysis revealed that one apricot isolate belonged to the recombinant P-H/cit3 group, while the others belonged to the hop group, indicating molecular variability among HSVd isolates. Geographical origin appeared more influential than host specificity in the sequence variability. Zhang et al. (2009) detected HSVd in 1.8% of tested jujube trees in China, with the isolates sharing 92.6–92.8% homology with the first known HSVd sequence and categorised in the plum subgroup. Amari et al. (2001) identified 16 novel HSVd sequence variations from Mediterranean countries (Türkiye, Morocco, Greece and Cyprus) where the viroid was not previously recorded, highlighting the prevalence of sequence variations in minor recombinant subgroups. According to phylogenetic analyses, the two unique nucleotide sequences discovered in this study are 300 nt in length and belong to the citrus group.

Hagemann et al. (2023) stated that HSVd was detected in one-third of the German samples and two-thirds of the Slovenian samples, primarily in lemons, grapefruit, and oranges. Furthermore, two-thirds of all grapes tested in Germany contained HSVd. It is stated that HSVd is latent in grapes but poses a high risk to neighbouring hop gardens due to the possibility of transmission. Because it is still unknown how the original infection occurred in Malatya and how HSVd can be transmitted by mechanical inoculation and contaminated equipment, producers should be warned and raise awareness about this issue. Malatya province has no commercial hop production, but other potential hosts such as apricots, grapevines, peaches, and plums are commonly grown. However, citrus cannot be produced owing to climatic circumstances.

CONCLUSION

In the spring of 2021, medlar trees in an orchard in the Malatya region of Türkiye were surveyed for the presence of HSVd, which has the broadest host range among the viroidsLeaf and flower samples were collected from 20 medlar trees and examined by RT-PCR. Hsvd was found in five of the samples, with unique sequence variants. Two of the five HSVd variations were randomly chosen and entered into GenBank under the accession codes OR257530 and OR257531. Both Turkish HSVd isolates have genomes of 300 nucleotides. The complete genome sequence of these variations was compared to isolates from throughout the world in GenBank. The nucleotide sequences of HSVd isolates were found to be 89.7–100% similar to HSVd isolates found in other crops around the world and were found to be in the citrus group of HSVd. This is the first report of medlar as a new natural host for HSVd. This study highlights the ubiquity and genetic variability of HSVd, a viroid that infects numerous crops cultivated worldwide, by identifying medlar as a distinctive natural host.

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