





Research Article

**Genetic variability of *Grapevine Leafroll-Associated Virus-1,2,3,4* Infecting Vineyards in Tokat Province of Turkey\*\***

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**Keywords:**

Grapevine leafroll disease, *Vitis vinifera*, virus, RT-PCR, Tokat

**Abstract.** Grapevine leafroll disease (GLRD) is one of the most important viral diseases of grapevine worldwide. The aim of this study was to investigate the prevalence of *Grapevine leafroll associated virus-1* (GLRaV-1), *Grapevine leafroll-associated virus-2* (GLRaV-2), *Grapevine leafroll-associated virus-3* (GLRaV-3), *Grapevine leafroll-associated virus 4* (GLRaV-4), and *Grapevine leafroll-associated virus-9* (GLRaV-9) in Tokat province of Turkey. In 2018, leaf samples and one-year-old shoots of plants, showing virus-like symptoms, were collected from different vineyards in Central, Erbaa, Niksar, and Pazar districts of Tokat province. Totally 197 samples were obtained and tested molecularly using two-step polymerase chain reaction (PCR) by using virus-specific primers for GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4 and GLRaV-9. At least 106 (53.80%) samples were found to be infected showed single or multiple infections. The 103 (52.28%) samples resulted in positive with GLRaV-1 while the infection rates were %5.18 for GLRaV-3 with 10 samples, %2.53 for GLRaV-4 with 5 samples, %1.03 for GLRaV-2 and GLRaV-9 with 2 samples, respectively. The amplified PCR products of GLRaV-1, GLRaV-2 and GLRaV-3 were sequenced, aligned with MEGAX, and analyzed in BLAST for multiple comparisons of other isolates in the GenBank. Sequence analysis of viruses showed 94-96 %, 96-99 % and, 99-100 % nucleotide identity with references isolates in the GenBank for GLRaV-1, GLRaV-2, and UO3-3 isolate of GLRaV-3 respectively.

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**Tokat ili Bağlarda *Grapevine leafroll-associated virus -1,2,3,4*'ün yaygınlığı ve Genetik Değişkenliği**

**Anahtar kelimeler:**

Asma yaprak kıvrıcıklığı hastalığı, *Vitis vinifera*, virus, RT-PCR, Tokat

**Özet.** Asma yaprak kıvrıcıklığı hastalığı (GLRD), dünya çapında asmanın en önemli viral hastalıklarından biridir. Bu çalışma, *Grapevine leafroll associated virus -1* (GLRaV-1), *Grapevine leafroll associated virus -2* (GLRaV-2), *Grapevine leafroll associated virus -3* (GLRaV-3), *Grapevine leafroll-associated virus 4* (GLRaV-4), ve *Grapevine leafroll-associated virus-9* (GLRaV-9) etmenlerinin yaygınlığının araştırılması amacıyla yapılmıştır. Bunun için Tokat ili Merkez, Erbaa, Niksar ve Pazar ilçelerindeki farklı bağ alanlarından virüs benzeri belirti gösteren asmalardan yaprak örnekleri ve bir yıllık bitki sürgünleri toplanmıştır. Surveyde elde edilen toplam 197 örnek GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4 ve GLRaV-9 etmenlerine özgü primerler ile iki aşamalı polimeraz zincir reaksiyonu (PCR) kullanılarak moleküler olarak test edilmiştir. 197 örnekten 106'sı (%53.80) bu virüslerle tekli veya çoklu enfeksiyon tespit edilmiştir. 103 (%52,28) örnek GLRaV-1 ile pozitif sonuçlanırken, enfeksiyon oranları 10 örnekle GLRaV-3 için %5,18, GLRaV-4 için 5 örnekle %2,53, GLRaV-2 ve GLRaV-9 için %1,03 olarak belirlenmiştir. GLRaV-1, GLRaV-2 ve GLRaV-3'ün pozitif PCR ürünleri çift yönlü sekans analizinden sonra MEGAX ile GenBank'taki referans diğer izolatlarla karşılaştırılması yapılmıştır. GLRaV-1 izolatlarının sekans analizine göre GenBank'taki referans izolatlarla %94-96, GLRaV-2 izolatları %96-99 ve GLRaV-3'ün UO3-3 izolatı %99-100 nükleotid benzerliği göstermiştir.

\*\* This study is a part of a master's thesis of Kübra Sarıtaş

## INTRODUCTION

Turkey is located within the most suitable climate zone of the world for viticulture. Thus, the country has a quite old history of viticulture. According to 2017 statistics, the world vineyard area was 6 931 353 ha with an annual grape production of 74 276 583 tons (FAO, 2017). According to 2017 FAOSTAT data, Spain has the greatest vineyard area and China is the greatest grape producer in the world. Among the viticulture practicing countries, Turkey has 5th place in vineyard area and 6th place in grape production (FAO, 2017). The quantity of production; 1 945 262 tons were table grapes, 1 524 091 tons were for dried grapes and 463 647 tons were for wine production. Turkey also is the world's largest producer for dried grapes constituting 28% of global production in 2014 (FAO, 2016).

Viticulture is an important agricultural activity in Turkey both for fresh consumption and wine production. While some of the annual fresh production is consumed as table grapes, some others are consumed either seedless or seeded raisins. Grape export constitutes a significant revenue for the country's economy. Besides grape, the leaf is another product obtained from vineyards. The demand for fresh and brined grape leaves is always increasing. Most of the vineyards in the province were established with this grape cultivar. More than 80 viruses and five viroids have been identified in *Vitis* spp., making grapevine the most virus-infected agricultural commodity known to date (Martelli, 2017). Grapevine leafroll disease (GLRD) is the most widespread and economically destructive disease among the grapevine virus diseases. GLRD are represented by the species GLRaV-1, -2, -3 and -4 and recently, GLRaV-5, -6, -7, -9, GLRaVPr, GLRaV-De, and GLRaV-Car were recognized as strains of GLRaV-4 (Martelli *et al.*, 2012; Martelli, 2014). All these viruses belong to the genus *Ampelovirus* except for GLRaV-2, which is in the genus *Closterovirus*, and GLRaV-7, which is in the tentative genus *Velarivirus* (Al Rwahnih *et al.*, 2011).

Several studies have been conducted to determine the problems caused by GLRaV's in Turkish vineyards production areas. Gursoy (1991) reported that GLRaV-1 and GLRaV-3 were common in vineyards of Tekirdağ, Yalova and Manisa provinces; Buzkan *et al.* (2012) conducted a survey in Turkish vineyards and noted that GLRaV-1 and GLRaV-3 were generally found in the vineyard areas of the Eastern Mediterranean (Kahramanmaraş, Mersin, Hatay) and Southeast Anatolia (Adıyaman, Gaziantep, Kilis, Diyarbakır and Şanlıurfa) regions of Turkey. Kaya and Erilmez (2014) reported the existence of GLRaV-1, -2, -3, -4 and -9 in vineyards in İzmir, Manisa, Çanakkale and Denizli provinces; Ince and Yavuz (2015) found GLRaV-1, -2, -3, -4, -6 and -9 in Eastern Mediterranean Region by serologically. Onder (2016) identified GLRaV-9, GLRaV-Pr and GLRaV-De viruses for the first time in İzmir, Aydın, Denizli, Manisa and Uşak provinces. Omeroğlu (2016) identified GLRaV-1, -2, -3 and -5 in Tekirdağ province, Sarköy and Marmara Ereğlisi districts. Yakar (2017) showed the presence of GLRaV-2 in the Eastern Mediterranean and Southeastern Anatolia regions.

Tokat province stands out especially with the production of pickled leaves. Karadeniz *et al.* (2018) conducted some serological tests in Tokat province and detected GLRaV-1 in the survey area. The main goal of this study, to understand the molecular structure of other GLRaV's by sequencing and comparing the related data with reference sequences from Genbank. This study was conducted to identify important and emerging grapevine viruses (GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4 and GLRaV-9) through molecular methods in important grapevine growing fields of Tokat province. This study will provide a scope for further research in grapevine infecting viruses based molecular studies.

## MATERIAL AND METHOD

### **Plant Material and RNA Extraction**

Grapevine leaves and annual shoot samples were collected from plants showing virus infection symptoms in the grapevine from the Centre, Erbaa, Niksar and Pazar districts of Tokat during the autumn in 2018 growing period. In total 197 samples, cvs Sultani, Narince, which is the local variety and cv Syrah, were collected and stored at 20 °C until use. Total nucleic acids were extracted from each sample using the main veins of the leaf, petiole, stalks and phloem scrapings of annual shoots. RNA isolation was made according to Li *et al.* (2008)'s protocol with some addititioan modifications.

### **Molecular Studies**

#### **Complementary DNA (cDNA) Synthesis and PCR**

Complementary DNA (cDNA) synthesis was performed in a 10 µl total volume and used as a template for PCR analysis. The 5 µl of extracted RNA was used as a template and added to a PCR tube and incubated 5 min at 65°C.

Then the reaction mix containing 1  $\mu$ l 10X reaction buffer (Wizscript), 0.5  $\mu$ l 20X dNTP (2.5mM), 1  $\mu$ l random hexamer primer, 0.5  $\mu$ l reverse transcriptase enzyme (Wizscript), 0.25  $\mu$ l RNase inhibitor, 1.75 of nuclease-free water added to the tube. RT reaction was performed with a 10 min incubation at 25 °C, followed by at 37 °C for two h and 85 °C for 5 min.

The PCR was performed with 2.5  $\mu$ l of cDNA, 0.2  $\mu$ l of 25mM dNTPs, 2  $\mu$ l of 25 mM MgCl<sub>2</sub>, 5  $\mu$ l of 5x green reaction buffer and 0.5  $\mu$ l of 10  $\mu$ M of each specific primers (Table 1) adding with 0.25  $\mu$ l of 5 units  $\mu$ L<sup>-1</sup> Taq DNA polymerase (GoTaq® DNA Polymerase, Promega Corp., USA) and 16.5  $\mu$ l dH<sub>2</sub>O. PCR amplifications were conducted according to references in Table 1. The PCR products were analyzed by electrophoresis in 1.2 % agarose gel containing 10 mg/ml ethidium bromide and visualized under UV light.

**Table 1.** The primers used in this study.

Çizelge 1. Çalışmada kullanılan primerler.

Primer	Primer sequence	Long	References
GLRaV 1-F	CGACCCCTTTATTGTTTGAGTATG	401 bp	Martin <i>et al.</i> , 2005
GLRaV 1-R	GTTACGGCCCTTTGTTTATTATGG		
GLRaV 2-F	ATAATTCGGCGTACATCCCCACTT	332 bp	Martin <i>et al.</i> , 2005
GLRaV 2-R	GCCCTCCGCGCAACTAATGACAG		
GLRaV 3- LR3-8504V	ATGGCATTGAACTGAAATT	940 bp	Ling <i>et al.</i> , 2004
GLRaV 3- LR3-9445C	CTACTTCTTTTGCAATAGTT		
GLRaV 4up	CCAAGTGTCTGGGTATAAGGAAT	243 bp	Maliogka <i>et al.</i> , 2008
GLRaV 4do	CCCAGACACCGGTCTATACTIA		
GLRaV 9-F	CGGCATAAGAAAAGATGGCAC	393 bp	Alkowni <i>et al.</i> , 2004
GLRaV 9-R	TCATTCACTGCTTTGAAC		

### Sequencing and Phylogenetic Analysis

The PCR products were sequenced in two directions (forward and reverse) with Sanger technology. The obtained nucleotide sequences were analyzed by the program Molecular Evolutionary Genetics Analysis using neighbor-joining method (NJ methods) (software MEGAX (Kumar *et al.*, 2018). The Bootstrap analysis was performed with 1000 replications.

## RESULTS AND DISCUSSION

### Symptoms

A total of 197 vine samples were collected from Tokat Central, Erbaa, Niksar and Pazar towns in 2018. Symptoms on collected samples included; mosaic, yellowing, green veins-yellowing in interveinal, chlorosis in veins-green color formation in interveinal, color desaturation in veins, vein banding, shape disorders, reddening, necrotic spots, brown spots, mottling, swells, leaf curling inside, increase in leaf teeth, fan-like leaf formation, decrease in a number of clusters and cluster size, decrease in fruit set, irregular (small-large) berry formation, irregular coloration, recess in development and general dwarfness (Figure 1).



**Figure 1.** a) Mosaic, irregular (small-large) berry formation, chlorosis in veins-green color formation in interveinal, reddening. Şekil 1. a) Mozaik b) İrili ufaklı tane oluşumu c) Damar aralarında sararma d) Kırmızılaşıma.

### RT-PCR Results

A total of 197 vine samples were subjected to RT-PCR analysis with a virus or strain-specific primers for diagnosis of GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4 viruses and GLRaV-9 strain. According to RT-PCR results, virus infection was identified in 106 samples of 197 samples. Of the total samples, 103 (52.28%) were identified as GLRaV-1, 10 (5.18%) with GLRaV-3, 5 (2.53%) with GLRaV-4, 2 (1.03%) with GLRaV-2 and 2 (1.01%) with GLRaV-9 strain infected (Table 2). GLRaV-1 was the most common virus in samples collected from Central and the other parts of Tokat province and it was followed by GLRaV-3. The least infecting rates showed viruses were identified as GLRaV-2 and GLRaV-9 strain.

**Table 2.** Town-based distribution of identified viruses.

Çizelge 2. Tespit edilen virüslerin ilçelere göre dağılımı.

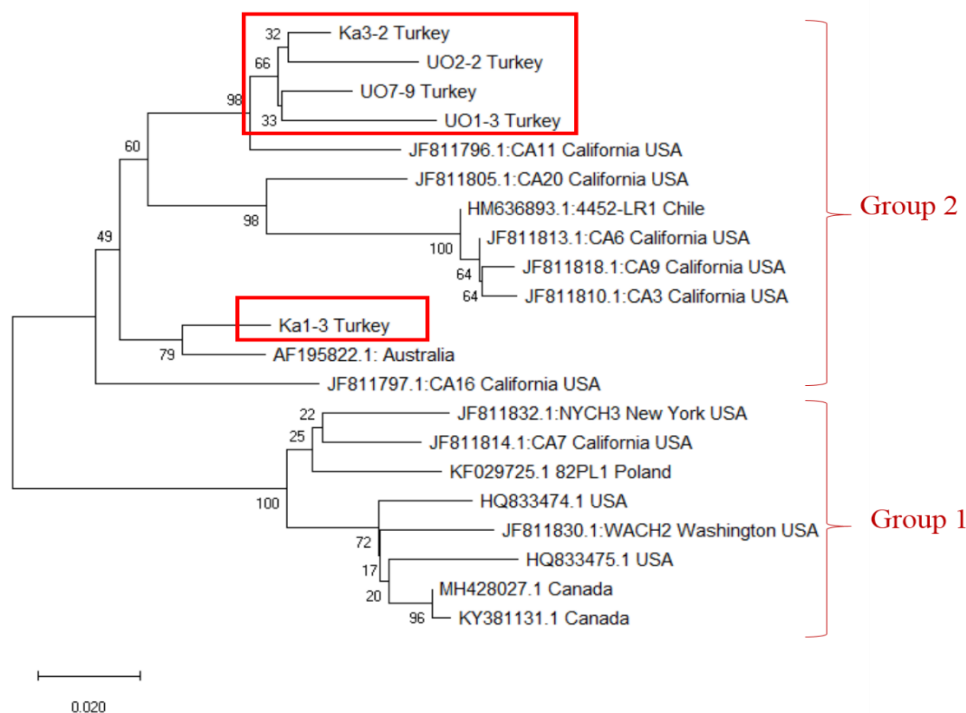
Town	Number of tested sample	Number of Infected Plants	GLRaV-1	GLRaV-2	GLRaV-3	GLRaV-4	GLRaV-9 strain
Central	47	31	30	–	1	1	–
Erbaa	48	17	17	–	–	–	–
Niksar	21	14	14	1	–	–	–
Pazar	81	44	42	1	9	4	2
Total	197	106	103	2	10	5	2

The symptoms of viruses diagnosed with RT-PCR tests were compared and symptoms observed in GLRaV-1-infected plants included mosaic, vein banding, and reddening, symptoms in GLRaV-3-infected plants included reddening, the symptoms in infected with mixed GLRaV-1 and GLRaV-2 included reddening in leaves.

### Phylogenetic Analysis

Sequence information of five isolates infected with GLRaV-1, one isolate infected with GLRaV-2 and one isolate infected with GLRaV-3 were used for phylogenetic analysis. At the end of sequencing analysis, five samples of GLRaV-1 and one GLRaV-2 sample yielded bilateral outcome and one sample of GLRaV-3 yielded reverse outcome and tests were done with these data.

Among RT-PCR positive resulted samples by using specific primers for coat protein duplicate 2 (CPd2) for GLRaV-1, five isolates (UO1-3, UO2-2, UO7-9, Ka3-2, Ka1-3) were selected and sequenced. Sequence data was deposited in the Genbank under the accession numbers MN871916-MN871920. According to phylogenetic analysis, Ka1-3 isolate exhibited similar branching with Australian isolate (AF195822) and the other four isolates showed close similarity with Chilean isolate and generated a separate branching (Figure 2). Five Turkey isolates were grouped into Group 2 with a reference isolate AF195822.

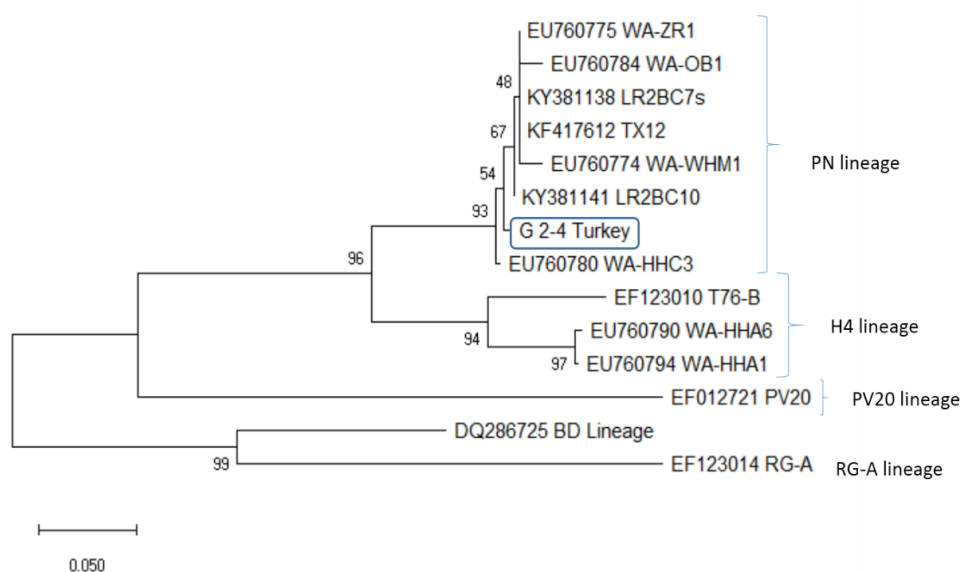


**Figure 2.** Phylogenetic tree obtained with GLRaV-1. The sequences obtained in this study are shown in the red boxes.

Şekil 2. GLRaV-1 ile elde edilen filogenetik ağaç. Bu çalışmada elde edilen izolatlar kırmızı kare ile işaretlenmiştir.

For the CPd2 analysis, 5 isolates from Turkey and reference isolates from GenBank were used. According to phylogenetic analyses of 5 CPd2 gene sequences of GLRaV-1, two main groups were obtained (Figure 2) as before reported by Alabi *et al.* (2011) and Elçi (2019) and the Turkish isolates were clustered into Group 2 and clustered different cluster in this group. California isolates from the USA (CA11) were found highly similar to Turkish isolates with high bootstrap values. The GenBank sequences obtained from Poland, some isolates from California, Washington and Canada were clustered into Group 1 but none of the Turkish isolates were clustered there (Figure 2).

The 327 bp-long nucleotide obtained with heat shock protein (hsp70h gene region) of GLRaV-2 were compared with the isolates recorded in the GenBank (Figure 3). The G2-4 GLRaV-2 isolate showed 98-99% similarity with the isolates from Canada (KY381141), USA (EU760780), and China and was placed over the same branch in the phylogenetic tree. So far, for GLRaV-2, six variants were reported by researchers, the variant 'Pinot Noir (PN)', 93/955, H4, BD, RG and PV20 (Beuve *et al.*, 2007; Bertazzon *et al.*, 2010). The groups PN and H4 are more common groups. The RG group contains isolates provided from the variety Red Globe obtained in California. The PV20, BD and 93/955 groups were less represented up to now. In our study two GLRaV-2 isolates founded in the surveying area and one isolate (G2-4) was sequenced. According to sequence and phylogenetic analysis results, G2-4 isolates grouped with PN isolates. So far, the PN variant was reported to be most widespread worldwide and associated with leafroll symptoms and graft incompatibility on grapevines. More studies are required to understand the presence of other genetic variants of GLRaV-2 in Tokat vineyards.

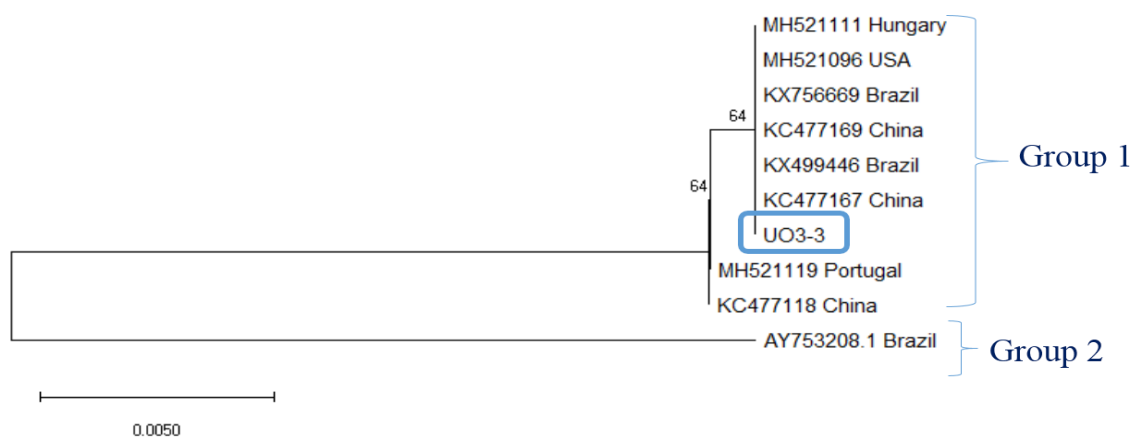


**Figure 3:** Phylogenetic tree for G2-4 isolate infected with GLRaV-2. The sequences obtained in this study are shown in the blue box.

Şekil 3. GLRaV-2 ile enfekteli G2-4 izolatına ait filogenetik ağaç. Bu çalışmada elde edilen izolatlar mavi kare ile işaretlenmiştir.

The 505 bp-long nucleotides belong to coat protein region of GLRaV-3 was compared with the isolates recorded in the GenBank (Figure 4) and UO3-3 isolate showed 100% similarity with the isolates from China, Brazil, Hungary, and the USA and placed over the same branch in the phylogenetic tree. The isolate exhibited 99% identity with two isolates from Portugal and China and, a separate branching in the phylogenetic tree. When compared the nucleotide sequence of UO3-3, only one base was different from references isolates. However, such one base difference did not generate a difference in the protein. The CP sequences of UO3-3 revealed restricted nucleotide and amino acid divergences among reference isolates, suggesting low variability among the analyzed isolates. The nucleotide sequence of Turkish isolates of UO3-3 GLRaV clustered together isolates from Hungary, USA and Brazil (Figure 3), in group I as defined by Maree *et al.* (2015) and Moura *et al.* (2018) and showed nucleotide and amino acid identities 100 %.





**Figure 4.** Phylogenetic tree for UO3-3 isolate infected with GLRaV-3. The sequences obtained in this study are shown in the blue box.

Şekil 4. GLRaV-3 ile enfekteli UO3-3 izolatına ait filogenetik ağaç. Bu çalışmada elde edilen izolatlar mavi kare ile işaretlenmiştir.

## DISCUSSION AND CONCLUSION

Viral diseases influence culture plants at different levels and cause yield losses. Such a loose continues every year in perennial species like grapevines and can emerge even 10-20 years after planting. Perennial plant species can stay infected throughout the entire lifetime and serve as a source of inoculum. Yield loses in virus-infected trees may vary from season to season. Non-yielding trees with severe infections or in cases with the fast spread of the disease, trees may be cut down. Besides instant loses, continuous economic losses may also be generated until the newly planted trees reach to the full-yield stage.

Viruses result in serious yield losses in cultural plants. Contrary to the other pathogens in plants, there are not any direct control methods for viruses. Since the majority of the methods used in control of viral diseases aim at protection, there is a need for reliable and precise methods or techniques for the diagnosis of viruses. Besides serological methods, molecular methods are also employed in the diagnosis of viral diseases. Since these methods are versatile methods, they yield reliable outcomes (Erkan *et al.*, 2011). Viruses and viroids result in a recess in growth and development, shape disorders, color changes in leaves and yield losses. The most significant viral disease agents of grapevines result in short internodes and leaf curling (Martelli, 2014). GLRaVs generally result in curling, irregular fruit set, general dwarfness and recessed growth in grapevines. They also result in 10-70% yield losses and reduce fruit sugar content. These viruses reduce grafting performance and plant rooting ability and reported in Turkish vineyards (Turkmen, 2014; Onder *et al.*, 2016).

To date, there are several serological studies on GLRaV-1 incidence in grapevine growing areas of Turkey. Ince and Yavuz (2015) diagnosed GLRaV-1 in Eastern Anatolia region through DAS-ELISA tests; Omeroglu (2016) diagnosed GLRaV-1 and GLRaV-3 as the most common viruses in Central, Sarkoy and Marmara Ereglisi towns of Tekirdag province. The other studies conducted for the diagnosis of GLRaV agents revealed that GLRaV-1 and GLRaV-3 were the most common leaf curling agents in grapevines. In the present study, GLRaV-1 and GLRaV-3 were also diagnosed as the most common viral disease agents in vineyards of Tokat province. Turkmen (2014) conducted DAS-ELISA tests in vineyards of Ankara, Manisa, Izmir, Denizli, Nevsehir, Elazığ, Canakkale, Edirne, Tekirdag and Kırklareli provinces and reported GLRaV 4-9 viruses as the most common ones, but GLRaV-4 and GLRaV-9 strain were not common in Tokat province. In another study conducted in Tokat province, Karadeniz *et al.* (2018) used a serological method of DAS-ELISA and diagnosed GLRaV-1 (9%) as the most common disease agent.

In the present study, the molecular method of RT-PCR was used, and GLRaV-1 was diagnosed in more than half of the samples (52%) collected from different districts of Tokat province.. It was observed that GLRaV-3 was also common in vineyards of Tokat province. With this study, GLRaV-2, GLRaV-3 and GLRaV-9 strain viral disease agents were reported for the first time in Tokat province and GLRaV-2 and GLRaV-9 strain were molecularly reported for the first time in Turkey. In this study, GLRaV-2 and GLRaV-9 strain were the least common disease agents.

Literature reviews revealed that this study was the first one conducted at a molecular level for detection of vine viruses in Tokat province. Within the scope of this study, the existence of GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4 and GLRaV-9 strain viral disease agents was investigated in vineyards of Tokat Central, Erbaa, Niksar and Pazar towns. Present tests revealed that 106 (53.80%) of 197 samples had one or more the investigated disease agents. GLRaV-1 was diagnosed as the most common virus in vineyards of Tokat province and it was followed by GLRaV-3. Among the mixed infections, GLRaV-1 + GLRaV-3 was the most common one. In this study, a high frequency of GLRaV-1 in grapevines was detected with RT-PCR and representative isolates were sequenced. Taken together with the nucleotide comparisons, phylogenetic analysis grouped Turkish isolates into Group 2 as before reported by Alabi *et al.*, (2011). Similarly, Elçi (2019) reported that some Turkey isolates belong to Group 2. According to these results, there is only Group 2 among the Turkish isolates. In previous studies reported that the most divergent region was CPd2 while the lowest region was HSP70h among the GLRaV-1 genes and GLRaV-1 genes have a high genetic variation and the CPd2 gene is the most variable gene (Alabi *et al.*, 2011; Elçi, 2019).

As it was in the world and different regions of Turkey, GLRAV-1 was diagnosed at high levels in vineyards of Tokat province. The other GLRaV agents (GLRAV-3 GLRAV-2, 4 and 9 strain) were also diagnosed in vineyards of Tokat province. Existence of these disease agents even at quite low levels threatens the viticulture of the province. These disease agents are transmitted through different vectors, but the primary transmission material is scions taken from infected plants. In this sense, it is recommended that plant material to be used in grafting and the samplings to be used in vineyard establishments should definitely be composed of registered materials. The present survey revealed for the first time the occurrence of GLRaV-2, and -4 strain 9 on grapevines of Turkey. In conclusion, viral diseases are among the most important problems and cause serious yield losses in viticulture.. Mostly preventive measures are taken against viral diseases and infections. Today, there aren't any control methods except for eradication and cultural measures against viruses. Therefore, early and reliable diagnosis of viruses with the use of modern techniques is a quite significant issue in the control of viral diseases (Onder *et al.*, 2016). Early diagnosis of viral agents in a short time will allow producer to prevent infection and spread of these agents and thus will contribute to the prevention of resultant losses.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DECLARATION OF AUTHOR CONTRIBUTION

KS: Survey and laboratory studies

ŞT: Planning the study, evaluation of analyzes, design of the article and writing of the article

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