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Research Article

Molecular Characterization of Partial RdRp Genes of *Tomato Ringspot Virus* Isolates from Turkey

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Abstract

Tomato ringspot virus (ToRSV) is a virus having isometric structure and single helix positive sense and is formed by a combination of two genomic RNA (RNA-1 and RNA-2) in the *Secoviridae* family from the Nepovirus group, and it causes economically devastating diseases in a wide range of hosts. In this study, 300 leaf samples from tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), cucumber (*Cucumis sativus*) and grapevine (*Vitis vinifera*) plants exhibiting virus and virus-like symptoms were collected from three different districts of Hakkari province between September 2014 and July 2015. The samples were tested for ToRSV infection on DNA fragments of 411 bp in size by Reverse Transcription Polymerase Chain Reaction (RT-PCR), using specific primers for the RNA dependent RNA polymerase (RdRp) gene. Seven out of 300 leaf samples (2.3%) were found to be infected with ToRSV. The virus was detected in tomato, pepper and cucumber samples, but its presence was not so evident in grapevine plants. The DNA sequences were submitted to GenBank (Accession numbers; KT728407, KT728408, KT728409 and KT728410). Our ToRSV isolates were compared with the ones available in GenBank, and the analysis of four ToRSV isolate RdRp gene sequences revealed that the nucleotide and amino acid homology were 83-100% and 94-100%, respectively. Phylogenetic analysis suggested that Turkish ToRSV isolates and currently available ToRSV isolates in GenBank were divided into three distinct groups. This analysis provides, to the best of our knowledge, the first report on the partial RdRp sequence of ToRSV in Turkey.

Keywords: Tomato ringspot virus, Partial RNA dependent RNA polymerase gene, Reverse Transcription Polymerase Chain Reaction (RT-PCR), Sequence analysis.

Türkiye'den *Domates Halkalı Leke Virüsü* İzolatlarının Kısmi RdRp Genlerinin Moleküler Karakterizasyonu

Öz

Domates halkalı leke virüsü (*Tomato ringspot virus*, ToRSV); Nepovirüs grubundan *Secoviridae* familyasında yer alan, iki genomik RNA (RNA-1 ve RNA-2) kombinasyonu ile oluşan izometrik yapıda ve tek sarmal pozitif duyarlı bir virüs olup, çeşitli konukçularda ekonomik olarak yıkıcı etkilere neden olmaktadır. Bu çalışmada, Eylül 2014 - Temmuz 2015 tarihleri arasında Hakkari ilinin üç farklı ilçesinden virus ve virüs benzeri belirtiler gösteren domates (*Solanum lycopersicum*), biber (*Capsicum annuum*), hıyar (*Cucumis sativus*) ve asma (*Vitis vinifera*) bitkilerinden 300 yaprak örneği toplanmıştır. Örnekler, RNA'ya bağlı RNA polimeraz (RdRp) geni için spesifik primerler kullanılarak Ters Transkripsiyon Polimeraz Zincir Reaksiyonu (RT-PCR) ile 411 bç boyutundaki DNA fragmanlarında ToRSV enfeksiyonu için test edilmiştir. 300 yaprak örneğinden yedisinin (%2.3) ToRSV ile enfekte olduğu tespit edilmiştir. ToRSV, domates, biber ve hıyar örneklerinde tespit edilirken asma örneklerinde tespit edilmemiştir. Bu çalışma sonucunda elde edilen DNA dizileri GenBank'a yüklenmiştir (Erişim numaraları; KT728407, KT728408, KT728409 ve KT728410). Ülkemiz ToRSV izolatları GenBank'ta bulunan ToRSV izolatları ile karşılaştırılmış ve dört ToRSV izolatı RdRp gen sekansının analizi ile nükleotid ve amino asit benzerliğinin sırasıyla %83-100 ve %94-100 olduğu tespit edilmiştir. Filogenetik analiz, ülkemiz ve GenBank'ta mevcut bulunan ToRSV izolatlarının üç farklı gruba ayrıldığını göstermiştir. Yapılan bu çalışma, bilgimiz dahilinde, Türkiye'deki ToRSV'nin kısmi RdRp dizisinin ilk raporudur.

Anahtar Kelimeler: Domates halkalı leke virüsü, Kısmi RNA bağımlı RNA polimeraz geni, Ters Transkripsiyon Polimeraz Zincir Reaksiyonu (RT-PCR), Dizi analizi.

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1. Introduction

Plants are exposed to a multitude of exogenous stimuli that include attacks by pathogens and herbivores and changes in environmental conditions (Jovel et al., 2011). Plant viruses are an important group of pathogens leading to several diseases in agricultural products around the world. Plant virus diseases cause significant economic losses by lowering the efficiency and degrading the quality of the yield (Matthews, 1992). Viral diseases are often asymptomatic and do not pose a risk; however, they might cause serious problems such as severe damage and even death when the virus is transmitted to a susceptible host (Mathews, 2010). ToRSV (*Tomato ringspot virus*) has (genus Nepovirus, subgroup C, family *Secoviridae*) a bipartite single-stranded positive-sense RNA genome encapsidated in icosahedral particles (Stace-Smith, 1996; Sanfaçon et al., 2006, 2009; Walker et al., 2015). Both RNA1 and RNA2 are translated into polyproteins that are cleaved at specific sites by an RNA1-encoded proteinase to yield several functional proteins. RNA1 encodes two proteins of unknown function (X1 and X2), a putative nucleotide triphosphate binding (NTB) helicase, a genome-linked viral protein (VPg), a protease (Pro) and a putative RNA dependent RNA polymerase (Pol) (Rott et al., 1995; Li et al., 2011). In nature, ToRSV is transmitted by mechanical ways, seeds, transplant, pollen, vegetative organs and different species of *Xiphinema* nematode (Braun and Keplinger, 1973; Bitterlin et al., 1987; Brown et al., 1994; Pinkerton et al., 2008).

Genetic variation and the phylogenetic analysis of whole genomes, genome segments, and complete and partial sequences of specific genes including coat protein (CP), Pro, VPg and Pol of ToRSV isolates have been studied to understand their genetic diversity. Due to the availability of more sequences from diverse geographical regions, RdRp gene was also used for RT-PCR studies of ToRSV isolates (Wang and Sanfaçon, 2000; Wei and Clover, 2008; Fuchs, 2010; Farmahini et al., 2014). ToRSV isolates infect herbaceous hosts with varying degrees of virulence (Bitterlin et al., 1987; Wang and Sanfaçon, 2000; Li et al., 2011). Previous studies have shown the presence of ToRSV in some deciduous fruit trees, rose, almond, grapevine, apple, soybean, tomato and eggplant in Iran, Jordan, Lebanon, Alabama and Latvia (Abou Ghanem-Sabanadzovic et al., 2003; Pourrahim et al., 2004; Golnaraghi et al., 2004; Massumi et al., 2009; Al-Nsour et al., 2010; Coneva et al., 2010; Moini, 2010; Moini et al., 2010; Sattary et al., 2011; Safaizadeh and Saidi, 2011; Sokhansanj et al., 2012; Gospodaryk et al. 2013; Farmahini et al., 2014). The detection and molecular characterization of ToRSV isolates is significant for understanding the epidemiology of the virus and management of the diseases caused by this virus.

Turkey is the gene center of many important vegetable species, and the vegetable sector is an important branch of agriculture (Turhan and Korkmaz, 2006). The presence of ToRSV in Turkey was reported in the literature (Fidan, 1995; Sertkaya, 2010; Yeşilçöllü et al., 2011; Sertkaya et al. 2013). Since no information was available on virus diseases of vegetables and grapevines in Hakkari, the samples used in this study were collected from different areas and tested using RT-PCR, a technique which successfully confirms the presence of both isolates in the leaves of infected tomato, pepper and cucumber plants.

2. Material and Method

2.1. Field Surveys

In the early autumn of 2014 and the summer of 2015, a total of 300 leaf samples of tomato, pepper, cucumber and grapevine plants (75 samples from each host) were collected from Çukurca, Şemdinli and Center districts of Hakkari province (Figure 1). The samples showing suspicious viral symptoms such as necrosis, chlorosis and ring stains on leaves were transported to the laboratory in cool conditions and were stored at 4°C (Figure 2).

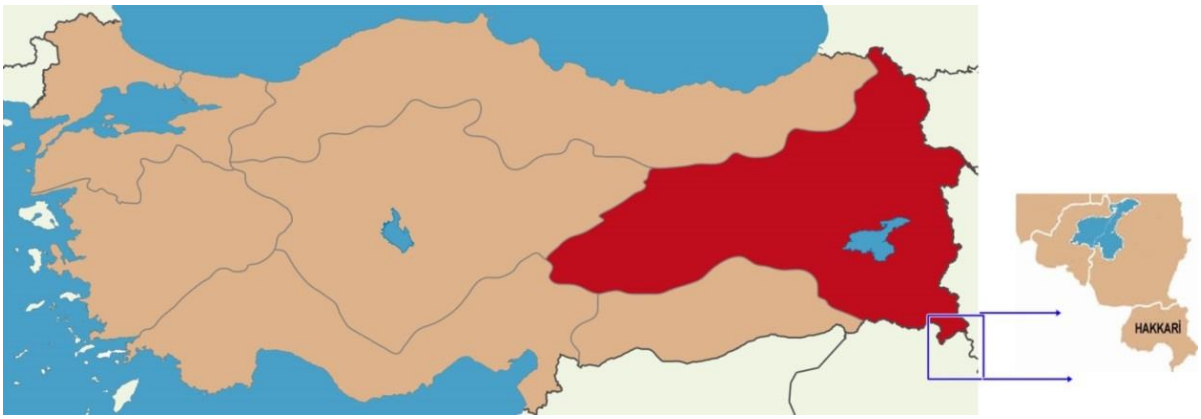

Figure 1. Survey location on the map of Turkey

Figure 2. Symptomatic plants collected in the field survey from Hakkari province: a) cucumber, b) pepper, c) grapevine, d) tomato

2.2. Preparation of Primers and RNA Extraction

The primer pairs were synthesized to amplify about 411 base pairs designed to protect the regions of the gene RdRp (Table 1). RNA extraction from the leaf samples of tomato, pepper, cucumber and grapevine plants were performed using the RNeasy Plant Mini Kit from Qiagen GmbH (Hilden, Germany) according to the manufacturer's protocol.

Table 1. Primer information used in RT-PCR analyses

Primer		Sequence	Reference
ToRSV-RdRp (Forward primer)	F	5'-GAAGAGCTAGAGCCTCAACCAGG-3'	Sokhansanj and Rakhshandehroo, 2012
ToRSV-RdRp (Reverse primer)	R	5'-AGTCTCAACTTAACATACCAC-3'	

2.3. RT-PCR

The partial RdRp gene of ToRSV was amplified by using a two-step RT-PCR method. A one-step RT-PCR was performed using Qiagen OneStep RT-PCR Kit. As a positive control, the plant sample (the original host: *Pelargonium* sp) obtained from Leibniz-Institut DSMZ German Collection of Microorganisms and Cell Cultures (Germany) was used. The total RT-PCR reaction volume was 25 µl and consisted of 1 µl of primers F and R (0.6 µM), 11 µl RNase-free water, 5 µl of 5X Qiagen OneStep RT-PCR buffer, 1 µl dNTPs (400 µM), 1µl reverse transcriptase and 5 µl of RNA isolated from the leaf samples. PCR conditions were conducted in two phases. In the first phase, reverse transcription cDNA (complementary DNA) was synthesized at 50°C for 30 minutes. In the second phase, HotStarTaq DNA polymerase was activated at 95°C for 15 min during the heating stage. The next step was carried out according to the PCR procedure. First, a gradient PCR with an annealing temperature ranging from 57 to 67.9°C was conducted to determine the optimum temperatures for the primer set by using the positive control sample. The obtained results demonstrated that 67.5°C was the optimum binding temperature for the primer set used for the amplification of RdRp gene. The temperature was raised to 94°C for 1 min to denature the template, followed by a 67.5°C anneal for 30 s and a 72°C extension for 1 min. Normally, 40 cycles were performed, followed by a 10 min extension at 72°C and then lowering it to a holding temperature of 4°C. The obtained PCR products with 100 bp markers of DNA were seperated using a 1.5% agarose gel electrophoresis imaging under ultraviolet light by using a Transilluminator (White/2UV) (UVP, USA).

2.4. Purification of PCR Products

The RT-PCR products were purified using a High Pure PCR Purification Kit (Roche, Germany) according to the instructions specified by the manufacturer.

2.5. Sequencing and Sequence Comparison

The nucleotide sequences of RdRp gene were determined by sequencing them with an automated DNA sequencer (Applied Biosystems) directly sequenced at the Refgen (Ankara, Turkey). The resulting DNA sequences were transferred to the Vector NTI DNA sequence analysis program. The sequence analysis was performed with Vector NTI AdvanceTM software (Invitrogen, Carlsbad, CA, USA). In order to identify their similarities, the nucleotide and amino acid sequences in the RdRp genes of ToRSV Turkish isolates were compared with each other by doing multiple sequence comparisons with Align X program. The ToRSV nucleotide and amino acid sequences were compared with the sequences from the ToRSV isolates available in the GenBank database (Table 2).

Table 2. List of ToRSV isolates used in this study

Accession number	Isolate	Host	Origin	Size (bp)	Reference
AF135407	*Grape Yellow Wein* (GYV)	grape	USA	2958	Wang and Sanfacon, 2000
AF135408	*Peach Yellow Bud Mosaic*; PYB-1	peach	USA	2958	Wang and Sanfacon, 2000
AF135409	Raspberry; Rasp-1	raspberry	USA	2958	Wang and Sanfacon, 2000
AF135410	T392	-	USA	2958	Wang and Sanfacon, 2000
DQ641947	raspberry	raspberry	Canada	2133	unpublished
GQ141525	*Tomato ringspot virus* strain 19-7	blueberry cv. Bluecrop	USA	585	Fuchs, 2010
GQ141526	*Tomato ringspot virus* strain 4-5	blueberry cv. Patriot	USA	585	Fuchs, 2010
GQ141527	*Tomato ringspot virus* strain 5-11	blueberry cv. Patriot	USA	585	Fuchs, 2010
GQ141528	*Tomato ringspot virus* strain 11-12	blueberry cv. Patriot	USA	585	Fuchs, 2010
JQ972695	ToRS-Teh	pepper	IRAN	373	Sokhansanj et al., 2012
KM083890	13C280	Prunus sp	USA	8209	Walker et.al., 2015
KM083892	GYV-2014	grapevine	USA	8358	Walker et.al., 2015
KM083894	Rasp1-2014	raspberry	USA	8224	Walker et.al., 2015
KT728407	TR/HAKKARI/2014/DN1	tomato	Turkey	282	this study
KT728408	TR/HAKKARI/2014/DF5	tomato	Turkey	282	this study
KT728409	TR/HAKKARI/2014/BR9	pepper	Turkey	282	this study
KT728410	TR/HAKKARI/2014/BZ127	pepper	Turkey	282	this study
KR911669	Rasp-CL	raspberry	Chile	8209	Rivera et. al., 2016
L19655	-	raspberry	Canada	8214	Rott et. al., 1995
MF176958	OG1	grapevine	USA	5987	Yao et al., 2018
MH427294	ToRSV-RNA1-WA	*Apis mellifera*	Australia	3978	Roberts et al., 2018

2.6. Phylogenetic Analysis

Phylogenetic analysis was performed to determine the relationship between ToRSV Turkish isolates and related ToRSV isolates in the GenBank database. The partial sequence of the RdRp genes of ToRSV isolates was compared using Mega (version X) program (Kumar et al., 2018). By means of utilizing the phylogenetic analysis data, a phylogenetic tree was created using the neighboring joining method which is applied to Kiamura two parameter algorithm. 100 repetitive bootstrap analyses were performed in order to statistically determine the accuracy of the generated parentage (Kimura, 1980).

3. Results and Discussion

3.1. Molecular Detection

RT-PCR analysis of 300 samples collected from the field surveys revealed that seven samples were infected with ToRSV. A fragment of 411 bp of the RdRp gene was amplified from the infected leaves of tomato, pepper, and cucumber plants (Figure 3). According to RT-PCR results, ToRSV was detected in three tomatoes and two peppers collected from Çukurca district and one cucumber and one pepper collected from the Center district of Hakkari province. None of the grapevine plants collected from Çukurca, Şemdinli, and the Center districts of Hakkari were found to be infected with ToRSV. 2.3% a total of the collected samples were found ToRSV infected in Hakkari. The highest ToRSV incidence rate was observed in Çukurca (6.84%), while the lowest incidence rate was seen in the Center (1.28%). ToRSV wasn't detected in the samples collected from Şemdinli (Table 3). The results showed that although ToRSV is still not very common and not wide-spread in all districts of Hakkari.

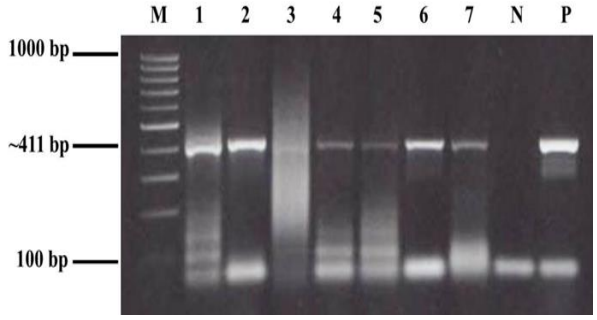

Figure 3. Amplification of the partial RdRp gene of ToRSV from tomato and pepper samples by using RT-PCR. Lane M-100 bp DNA marker, Line 1(tomato /Çukurca), 2 (tomato/Çukurca), 3 (tomato/Çukurca), 4 (pepper/Çukurca), 5 (pepper/Çukurca), 6 (cucumber/Center), 7 (pepper/Center), N-Negative control; water, P-Positive control

Table 3. Infection rates and collected/infected plant samples from Hakkari province for RT-PCR

District	Collected Samples / Infected Samples				Infection rate (%)
	Tomato	Pepper	Cucumber	Grapevine	
Center	39/0	43/1	44/1	30/0	1.28
Çukurca	28/3	18/2	19/0	8/0	6.84
Şemdinli	8/0	14/0	12/0	37/0	0
Total	75/3	75/3	75/1	75/0	2.33

3.2. Sequencing and Phylogeny

The partial RdRp gene sequence of the ToRSV Turkish isolates was deposited in GenBank under the accession numbers KT728407, KT728408, KT728409, and KT728410. The ToRSV isolates obtained from tomatoes were named TR/HAKKARI/2014/DN1 and TR/HAKKARI/2014/DF5, and the isolates obtained from the peppers were named TR/HAKKARI/2014/BR9 and TR/HAKKARI/2014/BZ127.

The genetic diversity of ToRSV Turkish isolates and international isolates was determined through the analysis of the RdRp gene, the sequences of which were available in the GenBank database. Due to the different size of the reference gene sequences, all sequence data were evaluated at 282 bp of equal size. The sequence of partial RdRp gene and the full-length of RNA1 of ToRSV isolates from several countries and available in the GenBank database were found to be 16 (12 from the USA, 1 from Chile, 2 from Canada and 1 from Australia. The RNA1 full-length sequence is currently available for eight isolates of ToRSV (Accession nos; AF135407, AF135408, AF135409, AF135410, KM083890, KM083892, KM083894, L19655, KR911669, MH427294, MF176958, DQ641947) and the partial sequence information of the RdRp gene is available for four isolates of ToRSV (Accession nos; GQ141525, GQ141526, GQ141527, GQ141528). The geographical origin, isolate, host, and accession numbers of comparable ToRSV isolates from around the world are given in Table 2**.**

The RdRp gene sequences of four ToRSV Turkish isolates were highly conserved with 98-100% and 96-100% sequence identity at the nucleotide and amino acid level, respectively (Table 4, 5). A low variation (up to 4%) was found between the four Turkish isolates. The sequences of 16 international isolates, including four Turkish isolates, showed 83-100% identity in the nucleotide sequence of the partial RdRp gene and the full-length of RNA1. Thus, the RdRp gene of ToRSV isolates had a maximum of 17% sequence difference. The obtained results showed that the ToRSV strains from the USA (Accession nos; AF135409, AF135410, KM083890), Australia (Accession no; MH427294) and Canada (Accession no; L19655) had the highest similarity with ToRSV-TR/HAKKARI/2014/DN1 isolate (Accession no; KT728407) (96-100%), ToRSV-TR/HAKKARI/2014/DF5 isolate (Accession no; KT728408) (96−100%), ToRSV-TR/HAKKARI/2014/BR9 isolate (Accession no; KT728409) (94-98%) and ToRSV-TR/HAKKARI/2014/BZ127 (Accession no; KT728410) (96-99%), while the ToRSV isolates from the USA (Accession nos; AF135407, GQ141525, GQ141526, GQ141526, GQ141527, GQ141528, KM083892, KM083894, KR911666, MF176958) and Canada (Accession no; DQ641947) had the lowest similarity with Turkish isolates of ToRSV (86-90%) (Table 4).

Table 4. Nucleotide identity percentage of the RdRp gene sequences of ToRSV isolates

Identity (%) nucleotid

GenBank accession no	KM083892	DQ641947	KM083894	GQ141527	GQ141528	KR911669	GQ141525	GQ141526	MF176958	AF135409	L19655	AF135410	KM083890	MH427294	AF135408	KT728407	KT728408	KT728409	KT728410
AF135407	100	83	83	84	84	84	84	86	86	89	89	89	87	88	88	88	88	86	88
KM083892		83	83	84	84	84	84	86	86	89	89	89	87	88	88	88	88	86	88
DQ641947			100	98	98	98	97	94	90	89	89	89	88	89	89	89	89	88	89
KM083894				98	98	98	97	93	90	88	88	89	88	88	88	88	89	87	89
GQ141527					100	99	96	94	91	90	90	90	89	90	90	90	90	89	90
GQ141528						100	97	93	90	89	89	90	89	89	89	89	90	88	90
KR911669							97	94	91	89	89	89	89	89	89	89	89	88	89
GQ141525								93	91	90	90	91	90	90	90	90	90	89	90
GQ141526									91	90	90	90	90	89	90	90	89	88	89
MF176958										89	89	89	88	89	90	90	90	89	90
AF135409											100	100	98	98	98	98	97	96	97
L19655												100	98	98	98	98	97	96	97
AF135410													98	98	98	98	98	96	98
KM083890														97	96	96	96	94	96
MH427294															97	97	97	95	97
AF135408																100	100	98	99
KT728407																	100	98	99
KT728408																		98	100
KT728409																			98

94 amino acid sequences of the RdRp genes of Turkish isolates were compared with ToRSV isolates in GenBank with results ranging from 94-100%. The obtained results demonstrated that the ToRSV strains from the USA (Accession nos; AF135407, AF135408, AF135409, AF135410, GQ141525, GQ141526, GQ141527, GQ141528, KM083890, KM083892, KM083894), Canada (Accession nos; L19655, DQ641947), Chile (Accession no; KR911666) and Australia (Accession no; MH176958) had the highest similarity with ToRSV-TR/HAKKARI/2014/DN1 isolate (97-100%) and ToRSV-TR/HAKKARI/2014/DF5 (97-100%) isolate, while ToRSV-TR/HAKKARI/2014/BR9 isolate (94-97%) and ToRSV-

TR/HAKKARI/2014/BZ127 (96-99%) isolate had the lowest similarity with Turkish ToRSV isolates (Table 5).

Table 5. Amino acid identity percentage of the RdRp gene sequences of ToRSV isolates

Identity (%) aminoacid

GenBank accession no	KM083892	AF135410	L19655	AF135408	AF135409	DQ641947	GQ141525	GQ141526	GQ141527	GQ141528	KM083890	KM083894	KR911669	KT728407	KT728408	KT728409	KT728410	MF176958	MH427294
AF135407	100	97	97	97	97	97	96	97	97	97	97	97	97	97	97	94	96	96	97
KM083892		97	97	97	97	97	96	97	97	97	97	97	97	97	97	94	96	96	97
AF135410			100	100	100	100	99	100	100	100	100	100	100	100	100	97	99	99	100
L19655				100	100	100	99	100	100	100	100	100	100	100	100	97	99	99	100
AF135408					100	100	99	100	100	100	100	100	100	100	100	97	99	99	100
AF135409						100	99	100	100	100	100	100	100	100	100	97	99	99	100
DQ641947							99	100	100	100	100	100	100	100	100	97	99	99	100
GQ141525								99	99	99	99	99	99	99	99	96	98	98	99
GQ141526									100	100	100	100	100	100	100	97	99	99	100
GQ141527										100	100	100	100	100	100	97	99	99	100
GQ141528											100	100	100	100	100	97	99	99	100
KM083890												100	100	100	100	97	99	99	100
KM083894													100	100	100	97	99	99	100
KR911669														100	100	97	99	99	100
KT728407															100	97	99	99	100
KT728408																97	99	99	100
KT728409																	96	96	97
KT728410																		98	99
MF176958																			99

Phylogenetic analyses are useful for determining the genetic relationships among different isolates. The use of greater numbers and more diverse isolates improve the reliability of phylogenetic trees constructed for identification and classification of isolates. Phylogenetic analysis using the maximum likelihood analysis yielded three major phylogenetic clusters (I, II, III) of ToRSV isolates shown in Figure 4, some of them being composed of a large majority of isolates (Group I).

According to the phylogenetic tree that was formed based on the partial RdRp gene sequence of ToRSV, the first phylogenetic group (I) contains isolates from the USA, Australia and Turkey. Phylogenetic analyses inferred a clustering of Turkish isolates into a single group. A phylogenetic tree based on RdRp gene sequences revealed that Turkish isolates in this study belonged to Group Ia, clustering with the isolates from the USA. All ToRSV isolates represented phylogenetic groups I, II and III originating with raspberry, grapevine, prune, peach, blueberry and bee isolates from the USA, Canada, Chile and Australia.Phylogenetic analysis based on the nucleotide sequence of the RdRp gene showed that Turkish isolates and other known ToRSV strains might be divided into three distinct groups. A moderate level of genetic variation in the RdRp gene was observed among these groups on the basis of their geographical and host origins. Variations up to 4% in the RdRp gene nucleotide sequence of bases differentiated at least three distinct subgroups of ToRSV strains or isolates. The second and third phylogenetic groups (II and III) contain isolates from the USA, Canada and Chile. Turkish ToRSV isolates may be considered as separate strains from the other isolates in these groups. ToRSV isolates from the generated parental strain are thought to be genetically similar to the RdRp gene and may be a genetic variation, especially of the isolates from the USA. The analysis placed Turkish isolates in phylogenetic Group I, which mainly harbors raspberry, *Apis mellifera* and prune isolates. It is noteworthy that the host of the Australian isolate is not a plant, it is honey bee, and is evaluated in the same group for phylogenetic analysis. The analysis of the phylogenetic relationships of Turkish isolates with other ToRSV isolates showed a closer relationship with *Peach Yellow Bud Mosaic* PYB-1 isolate (Accession no AF135408).

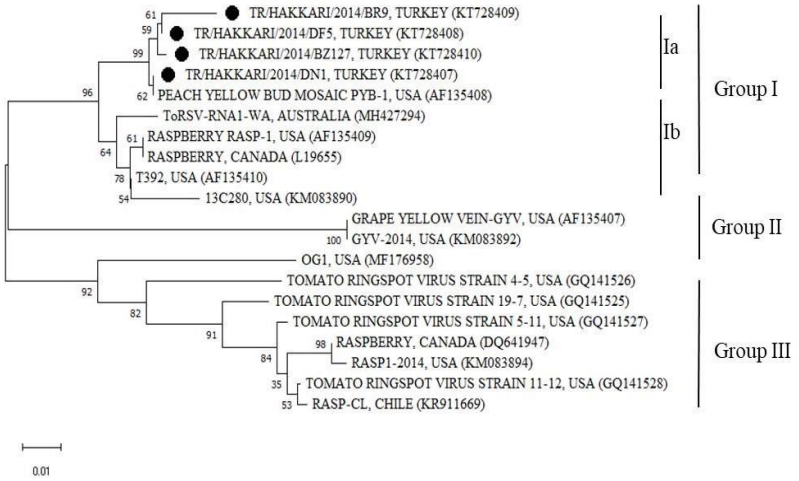

Figure 4. Phylogenetic tree based on the nucleotide sequence of a RdRp gene of the ToRSV. All data obtained from the GenBank nucleotide database were indicated by accession numbers. Data analysis and tree construction were performed using the MEGA (Version X) program.

In this study, ToRSV was detected in tomato samples and pepper plants from Hakkari province. Azeri (1994) was detected presence of ToRSV in the samples of stone fruit trees tested by ELISA in Aegean Region and later ToRSV was determined on tomato, pepper, cucumber and eggplant, blackberry, grape, strawberry in Turkey (Fidan, 1995; Sertkaya, 2010; Yeşilçöllü et. al., 2011; Sertkaya et. al., 2013). ToRSV was detected in wild blackberry (*Rubus fruticosus* L., Rosaceae) some stunted plants growing in the border of stone-fruit orchards in Hatay province in Eastern Mediterranean Region by Sertkaya, 2010. The results of biological indexing were also confirmed by serological assays (ELISA). This work represents the first report of ToRSV in wild blackberry (*R. fruticosus*) in Turkey. Yeşilçöllü et al. (2011) were determined to incidence of strawberry viruses in the important growing areas of Aegean Region, the total of 221 plant samples were randomly collected from strawberry plantations with virus like symptoms. ToRSV in 7 samples were found to be present by RT-PCR. Sertkaya et al. (2013) were investigated *Raspberry ringspot virus* (RpRSV), *Grapevine fan leaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Strawberry latent ring spot virus* (SLRV) *Tomato black ring virus* (TBRV), ToRSV and *Tobacco ringspot virus* (TRSV) in the Hatay Province vineyards by DAS-ELISA. Mixed infection with 4 samples GFLV + ArMV and 2 samples GFLV + ToRSV were determined. ToRSV has not been detected intensively in the studies in our country, but there is not much molecular determination.

Only 7 of the 300 samples collected in this study were identified as ToRSV infected. Since samples showing symptom of virus are collected, it can be thought that samples without ToRSV may be infected with different viruses. 2.3% a total of the samples were found ToRSV infected. A total of 104 symptomatic eggplant leaf samples were collected in fields in the province of Tehran (Iran) by Sokhansanj et al. (2012) and analyzed by DAS-ELISA and dot-blot assay (DIBA) for the presence of ToRSV using specific polyclonal antibodies (Agdia, USA). Results showed the presence of ToRSV in 23% of the samples. Field surveys were conducted to asses the incidence of ToRSV infection in stone fruit trees in Jordan by Al-Nsour et al. (2010). A total of 2546 samples collected from commercial orchards, a mother block, nurseries and a varietal collection were tested for ToRSV infection by DAS-ELISA. Results showed that 16% of the tested samples were infected with the virus.

It was found that the country which mostly introduced the disease to Turkey was Iran. We conceived that the geographical position of Hakkari could be the reason for this situation. The studies conducted in Iran by using ELISA, DAS-ELISA, Dot Blot Hybridization and RT-PCR reported that ToRSV was detected in 20% of apple, walnut, rose, almond, tomato, tabasco, pepper, and eggplant plants (Moini, 2010; Moini et al., 2010; Safaeizadeh and Saidi, 2011; Sattary et al., 2011; Sokhansanj and Rakhshandehroo, 2012; Sokhansanj et al., 2012). Here, it was presumed that the host differences in the study may affect the results. As the overall results obtained in this study revealed, the occurrence of ToRSV in Iran was relatively high compared to Hakkari.

Wang and Sanfaçon (2000) showed that direct sequenced PCR products could be used to make phylogenetic inferences. The sequence data obtained in this study were obtained directly from the RT-PCR products. In the present study, full-length and shorter sequences of the RdRp gene available in GenBank and obtained from ToRSV Turkish isolates were used to determine the RdRp based genetic diversity and phylogenetic classification of ToRSV isolates from around the world. It would be interesting to further characterize the genetic variability of different ToRSV isolates using partial RdRp gene sequences to advance our knowledge about the diversity of these viruses. Sequence analysis of nucleotides and amino acids showed that ToRSV isolates were closely related to each other, and there were only slight differences in both nucleotide and amino acid sequences in their RdRp gene. The little genetic variation among them probably indicates that there is only one strain of Turkish isolate. The resulting nucleotide sequence T32 isolate (Accession no; AF135410) had the highest identity (98%) of the RdRp gene in ToRSV isolates from blueberry and raspberry samples (Accession nos; MF176958, AF135409) and had the lowest identity (86%) in the ToRSV isolate from grapevine (Accession no; AF135407). ToRSV was detected in the symptomatic 112 pear leaf samples from all surveyed provinces with infection ranging from 32.4% up to 35.0% by Farmahini et al. (2014). DNA fragments of 580 bp in size were RT-PCR amplified using specific primers designed according to RdRp genes of ToRSV, which indicated 96% highest identities with available sequences of ToRSV isolates in GenBank. Fuchs, (2010) showed that plantings of highbush blueberry cultivars

'Patriot' and 'Bluecrop' showing virus-like symptoms and decline in vigor in New York were surveyed for the occurrence of viruses. ToRSV was identified in leaf samples by DAS-ELISA. Their presence was confirmed by RT-PCR with amplification of 320 bp of the RdRp genes. Comparative sequence analysis of New York isolates indicated moderate (80.7%) to high (90.8%) nucleotide sequence identities with other ToRSV. Sokhansanj and Rakhshandehroo (2012) were detected presence of ToRSV on chili pepper (Capsicum frutescens) in Iran. The sequences (Accession no. JQ972695) obtained by cloning was compared and found 91-94% similarity with blueberry cv. Patriot (Accession no. GQ141528) and blueberry cv. Bluecrop (Accession No. GQ141525).

The difference in host types is remarkable in phylogenetic analysis. There were no tomato and pepper plants as hosts in the isolates recorded in the GenBank database. TR/HAKKARI/2014/DN1 and TR/HAKKARI/2014/DF5 isolates were obtained from tomatoes and showed 100% similarity to nucleotide and amino acid sequences. In this group, ToRSV-TR/HAKKARI/2014/DN1 isolate was slightly different from the other three ToRSV Turkish isolates. It was observed that the host varieties did not cause any genetic variation. This may be due to the fact that geographically close isolates belong to the same group and the isolates from our country show similarities to these isolates, which might stem from the importation of plant products from these countries. The analysis placed blueberry, raspberry and grapevine ToRSV isolates in phylogenetic Group II and all other grapevine isolates in phylogenetic Group III. The results obtained from host range and phylogenetic analyses revealed that Turkish isolates were close to each other. Little genetic variation was found among them, which probably indicates more similarity with ToRSV isolates from the USA. Despite the fact that the RdRp gene and gene products of all analyzed ToRSV isolates were very similar, it was clear that the sequence of the ToRSV isolates from the USA more closely matched with the sequence of Turkish isolates based on the alignment of nucleotide and amino acid sequences and the phylogenetic analysis of nucleotide sequences of ToRSV isolates. The results obtained from the phylogenetic analysis of Turkish isolates detected in different plant varieties from the same districts are significant. It is believed that the Turkish isolates being genetically similar may be a genetic variation relative to the host and phylogenetically closer to each other.

ToRSV is transmitted by different species of *Xiphinema* nematode (Brown et al., 1994; Pinkerton et al., 2008). The presence of ToRSV in this region of Turkey has been attributed to the possibility of importing vectors and vegetables from other countries. Plant viruses are generally carried by vectors and for ToRSV, this is the nematode vector *Xiphinema americanum* sensu lato. Elekçioğlu et al. (1994) identified species belonging to the *Xiphinema* genus in the Eastern Mediterranean region. In the light of our findings, it's possible to talk about the presence of this nematode vector *Xiphinema* in Hakkari despite the fact that its presence in Hakkari hasn't been reported so far and that its natural spread is considered to be limited.

4. Conclusions and Recommendations

It was noted that the use of pesticide and domestic seeds was not common in the fields where fieldwork was conducted in Hakkari. The presented data together with the other available sequences provide useful information concerning ToRSV isolates found in Turkey. These findings may be particularly relevant in the light of the recent reports on the presence of ToRSV in vegetables in Turkey. The given data indicates a growing distribution of the ToRSV infections worldwide. The RdRp gene is also highly conserved in various hosts. However, a significant level of variability was not found within the same genomic stretch between the four Turkish virus isolates, which means more extensive molecular investigation is required. In this report, four distinct isolates of ToRSV were described. To the best of our knowledge, this is the first report on the partial genome sequences of ToRSV isolates from Turkey.

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