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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

First Report of Natural Infection of Watermelon Mosaic Virus (WMV) Infecting Bottle Gourd and Snake Melon

Su Kabağı ve Acur'da Doğal Enfeksiyona Neden Olan Karpuz Mozaik Virüsü (WMV)'nün İlk Raporu

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Abstract

Cucurbitaceous crops, one of the main crops of agriculture, are sensitive to many plant viruses. In August 2019, virus-like symptoms were observed on some cucurbit plants grown in private home gardens in Antalya and Denizli provinces (Turkey). A total of 53 leaf samples were sampled from plants with the most symptoms (melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* L.), bottle gourd (*Lagenaria siceraria* (Molina) Standl.), and snake melon (*Cucumis melo* var. *flexuosus*) and tested by Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) against possible watermelon mosaic potyvirus (WMV) infection. The coat protein gene (CP) specific primer sets amplified a gene product of nearly 820 bp fragment from symptomatic plants. WMV infections were detected in 31 individual cucurbit plants, including 11 melons, 8 watermelons, 7 snake melons and 5 bottle gourds. The presence of viral infection was found only in ornamental squash plants in Antalya province and in all cucurbits sampled in Denizli province. To better comprehend the molecular characteristics of virus isolates, the amplified viral DNA fragments were cloned in a proper prokaryotic plasmid, sequenced by Next Generation Sequencing (NGS) and recorded to GenBank. Bioinformatic analyses using the Basic Local Alignment Search Tool (BLAST) showed that the identified CP gene sequences exhibited significant nucleotide homogeneity, supported by a high nucleotide similarity index with that of other isolates around the world. In addition, Turkish isolates isolated from Antalya and Denizli regions showed approximately 94% nucleotide similarity among themselves. For phylogenetic inference, WMV sequences were subjected to multiple alignments with isolates from different geographic origins of the same viruses. Molecular phylogeny showed that all WMV isolates are closely related to other world WMV isolates at variable rates. WMV is wide host range viruses in cucurbit crops, however, this work is the first scientific report of WMV isolates detected in bottle gourd and snake melon from the South and West Regions of Turkey all over the world.

Keywords: WMV, RT-PCR, Molecular Phylogeny, Bottle gourd, Snake melon

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Öz

Tarımın ana ürünlerinden biri olan kabakgiller, birçok bitki virüsüne karşı hassastır. Ağustos 2019'da Antalya ve Denizli ilinde (Türkiye) müstakil ev bahçelerinde yetiştirilen bazı kabakgil bitkilerinde virüs tipi semptomlar gözlenmiştir. Çoğu belirtili olan bitkilerden (kavun (*Cucumis melo* L.), karpuz (*Citrullus lanatus* L.), su kabağı (*Lagenaria siceraria* (Molina) Standl.) ve acur (*Cucumis melo* var. *flexuosus*) toplam 53 yaprak örneği toplanmış ve olası watermelon mosaic virus (WMV) enfeksiyonuna karşı Revers-Transkriptaz Polimeraz Zincir Reaksiyonu (RT-PCR) ile test edilmiştir. Kılıf protein genine (CP) özel primer setleri, simptomatik bitkilerden yaklaşık 820 bp'lik bir gen ürününü amplifiye etmiştir. WMV enfeksiyonları, 11 kavun, 8 karpuz, 7 acur ve 5 su kabağı dahil olmak üzere 31 farklı kabakgil bitkisinde tespit edilmiştir. Antalya ilinde sadece su kabağı bitkilerinde, Denizli'den toplanan tüm kabakgil örneklerinde viral enfeksiyon varlığı saptanmıştır. Virüs izolatlarının moleküler özelliklerinin daha iyi anlaşılması için amplifiye edilmiş viral DNA fragmanları uygun bir prokaryotik plazmide klonlanmış, Yeni Nesil Dizileme (NGS) ile dizilenmiş ve gen bankasına kaydı yapılmıştır. Basic Local Alignment Search Tool (BLAST) ile gerçekleştirilen biyoinformatik analizler, belirlenen WMV-CP gen dizilerinin dünyadaki diğer izolatlarınkiyle yüksek bir nükleotid benzerlik indeksi ile desteklenerek önemli bir nükleotit homojenitesi sergilediğini göstermiştir. Ayrıca, Antalya ve Denizli illerinden izole edilen Türk izolatları kendi aralarında yaklaşık %94 oranında nükleotit benzerliği göstermiştir. Filogenetik çıkarımlar için, WMV dizileri, aynı virüslerin farklı coğrafik kökenlerinden gelen 30 izolatla çoklu hizalamaya tabi tutulmuştur. Moleküler filogeni ise tüm WMV izolatlarının değişen oranlarda diğer dünya WMV izolatları ile yakından ilişkili olduğunu göstermiştir. WMV, kabakgil bitkilerinde geniş konukçu dizisine sahip virüsler arasında yer almakta ve bununla birlikte bu çalışma, Türkiye'nin Güney ve Batı Bölgeleri'nde su kabağında ve acurda belirlenen WMV izolatlarının tüm dünyadaki ilk bilimsel raporudur.

Anahtar Kelimeler: WMV, RT-PCR, Moleküler filogeni, Su kabağı, Acur

1. Introduction

Cucurbits are one of the important plant groups that support humans for their consumable products, fiber sources and other purposes. Family Cucurbitaceae contains nearly 1000 species from five subfamilies, including watermelon, melon, bowler, cucumber, fig-leaf gourd, porongo, winter squash, and pumpkin (Bisognin, 2002). Among these, snake melon (*Cucumis melo* var. *flexuosus*), which is considered to be native to Anatolia, Iran, Afghanistan, and Southwest Asia, is an open field vegetable consumed in the immature stage for table and pickle, with a production of around 22 000 tons in Turkey (Vural et al., 2000). It is a source of vitamins and minerals (potassium, phosphorus, magnesium, vitamin A) as well as functional bioactive compounds (secondary metabolites, polyphenols) that serve as curatives for human health (Ilahy et al., 2019). Originating from Africa, bottle gourd (*L. siceraria*) is a climbing perennial cucurbit vegetable grown in tropical regions, with 24 different shaped varieties (Stephens, 1994; Awala et al., 2019). Besides its decorative use, its fruit is widely used for medicinal purposes thanks to its different natural biological ingredients (Saeed et al., 2022).

Cucurbits are invaded by many pathogens, including viruses, bacteria, and fungi (Horuz and Aysan, 2018). Cucurbit viruses are the largest production-limiting pathogens causing product losses by up to 100% on sensitive varieties under the appropriate conditions (Coutts et al., 2011)). More than 50 viruses encompassing zucchini yellow mosaic virus (ZYMV), cucumber mosaic virus (CMV), squash mosaic virus (SqMV), and WMV, are the main hosts of cucurbits (Karanfil and Korkmaz, 2020). WMV, one of the first described pathogens of cucurbit mosaic infections, was first isolated in watermelon (*Citrullus lanatus*) Rio Grande Valley (Webb and Scoot, 1965). WMV, a member of the Potyvirus genus, is infectious to more than 170 plant species, including cucurbits and weeds, and causing rapid and severe outbreaks in cucurbit-grown fields worldwide. Severe WMV infection reduces the quality of cucurbit crops, rendering them unmarketable, resulting in crop losses of up to 100%, especially in early-season infections (Fletcher et al., 2000; Katis et al., 2006; Coutts et al., 2011). An insect-transmitting pathogen is transmitted by aphids in a non-persistent style (Brunt et al., 1996). Depending on the host and the time of infection, the virus causes mosaic, dark green blistering, mottling, deformity on leaves and knobbly, discoloration, and distortion in fruit (Delmiglio and Pearson, 2006). DNA-based molecular methods and protein-based serological methods were commonly employed to accurately diagnose the associated pathogen (Al-Ani et al., 2011; Khalifa et al., 2015; Kızmaz et al., 2016).

The occurrence of WMV has been reported almost worldwide as a prevalent virulent pathogen of cucurbit plants, chiefly in Mediterranean countries (Akbar et al., 2015; Chatzivassiliou et al., 2016; Niu et al., 2017; Qiu et al., 2018). In Turkey, WMV infection has mostly been reported in cucurbits such as cucumber, melon and watermelon (Sevik and Arli-Sokmen, 2003; Yeşil, 2018; 2019; 2020). However, no attempt has been made to detect WMV infection in bottle gourd and snake melon so far. In this study, we attempted to determine the causal viral pathogens in cucurbit showing viral-suspicion cultivated in Denizli and Antalya Provinces of Turkey. We analyzed their respective gene sequences and phylogenetic relationships.

2. Materials and Methods

2.1. Sample collection and virus source

In 2019, cucurbit leaves samples showing virus-like symptoms were observed in Denizli and Antalya provinces (Turkey). Samples were bulked from mostly suspicious plants in a home garden. The number of samples collected is listed in *Table 1*. Samples were transported to the laboratory by placing them in a styropor box containing an ice pack immediately and maintained at -80 ºC until analyzed.

Table 1. Number of cucurbit plants collected from the South and West region of Turkey

District	Number of samples				Total
	Melon	Watermelon	Snake melon	Bottle gourd	
Denizli	**15**	**10**	**10**	**5**	**40**
Antalya	**-**	**9**	**-**	**4**	**13**
Total	**15**	**19**	**10**	**9**	**53**

2.2. Total RNA extraction

The RNA extraction was accomplished by the silica-capture method with a minor difference according to Foissac et al. (2001). Frozen cucurbit tissues (100 mg) were grounded in grounding buffer added 1 µl of β-

mercaptoethanol and transferred to 1.5 ml microfuge tubes. The homogenates added 100 µl sarkosyl (10%) were incubated for 10 min at 70 °C and then on ice for 5 min and centrifuged for 10 min at 13.000 rpm. 300 µl of the liquid phase were carefully was poured into a new tube consisting of 150 µl EtOH, 300 µl NAI (6M), and 25 µl resuspended silica. The solution was vortexed thoroughly to attain a homogeneous suspension before incubation in intermittent shaking for 10 min. The tubes were centrifuged for 1 min at 6000 rpm. The upper phase was removed and the pellet was dissolved with 500 ml wash buffer (repeat 2 wash steps). The pellet containing RNAs was resuspended with 100 µl of RNA-free water. The tubes were centrifuged for 10 min at 13000 rpm and supernatant consisting of total RNAs was preserved at-80°C until the cDNA and RT-PCR process.

2.3. Amplifications of Coat Protein Gene (CP)

RNA-enriched solutions were used as a template for cDNA synthesis. The cDNA synthesis was fulfilled using a reverse primer with 2 µl of extracted RNA following the instruction from the RevertAid First Strand cDNA kit (Vilnius, Thermo-Fermentas, Lithuania). For WMV-specific detection, CP-specific primer pairs generating 822 bp amplicons were utilized based on the reference publications (Sharifi et al., 2008). A reverse transcription-polymerase chain reaction (RT-PCR) method was set up to detect the occurrence of each viral agent in infected cucurbit tissues. The 2 µl of synthesized cDNAs were submitted to PCR assays in a final volume of 25 µl containing 18.3 µl of nuclease-free water, 2.5 µl of 10× reaction buffer, 0.5 µl of dNTPs (20 mM), 1.5 µl of $MgCl_2$ (25 mM), 0.5 µL of each primer (100 pmol), 0.2 µl of Taq DNA polymerase. The primers and temperature cycles used in the PCR reaction are listed in *Table 2*.

Table 2. Primers and termocycling program used in PCR tests to detect watermelon mosaic potyvirus infections

	Forward primer	Reverse primer	Cycling program
WMV	5'-ATTCACGTCCCTTGCAGTGTG-3'	5'-GAATCAGTGTCTCTGCAATCAGG-3'	3m 94°C **1m 94°C** **1m 60°C** **1m 72°C** } **35 cycles** 10m 72°C

2.4. Molecular cloning and nucleotide sequencing

All PCR-amplified fragments are gel-purified using a gel extraction kit (Thermo Scientific) and introduced into a prokaryotic cloning vector (pGEM T-Easy vector system, Promega) using standard cloning techniques with some modifications, and afterward transferred separately in *E. coli* (JM109 strain). At least one independent isolate of each sample was grown in LB medium containing ampicillin (1%). The recombinant plasmids consisting of viral CP gene were purified from bacterial solution and sequenced by next-generation sequencing (Sentebiolab/Ankara/Turkey).

2.5. Phylogenetic inference and Multiple alignment

To determine the sequence similarity of the viral CP genes, the unpublished nucleotide sequences of cucurbit isolates were compared with the viral nucleotide sequences stored in NCBI (nucleotide BLAST, BLASTn). The definitively diagnosed viral sequences were recorded in GenBank. Phylogenetic relationship, multiple alignments, and nucleotide analysis of sequences detected were achieved using CLC Main Workbench (version 6.7.1), Sequence Demarcation Tool (Version 1.2), and Mega X program (Kumar et al., 2018). The evolutionary relationship was presumed using the Neighbor-Joining method and robustness was calculated by 1000 bootstraps search. Soybean mosaic virus isolate (FJ376388) was assigned as an outgroup virus isolate.

3. Results and Discussion

3.1. Viral detection

In the inspection of cucurbit plants carried out in Antalya and Denizli provinces, virus-like symptoms were observed in melon, watermelon, snake melon, and bottle gourd (*Figure 1*). WMV-related symptoms of cucurbit leaves were summarized in *Table 4*. The symptoms produced by plant viruses can be explained by a close relationship between the virus and the host. Plant viruses have developed various strategies that have a suppressive effect on plant physiology (Krajcsi and Wold, 1998). Although they have few genes compared to their hosts, they have the potential to turn the plant cell's reproductive mechanism in their favor by stopping the synthesis of various

macromolecules to capture the host cells. After viral infection, various symptoms occur, which are manifestations of the disease because of abnormal metabolic or morphological changes occurring in the host (Martelli and Russo, 1985).

Figure 1. Some signs induced by watermelon mosaic potyvirus infection in cucurbit specimens collected from surveyed areas. A-B, C, E-F, and G-H represent the snake melon, bottle gourd, watermelon, and melon samples from Denizli province, respectively, while D is the bottle gourd sample from Antalya province.

Gathered samples were tested by RT-PCR against the WMV infections. DNA fragments equivalent to 820 bp were obtained on agarose gel, corresponding to the CP gene of the causative agent in diseased cucurbits (*Figure 2*). RT-PCR assays amplified ~820 bp products in 31 out of 53 symptomatic and asymptomatic cucurbit samples. WMV amplicons were detected in 30 samples from Denizli and 1 from Antalya. No DNA band is amplified from negative control. Plant-based infection-related information based on RT-PCR tests is presented in *Table 3*.

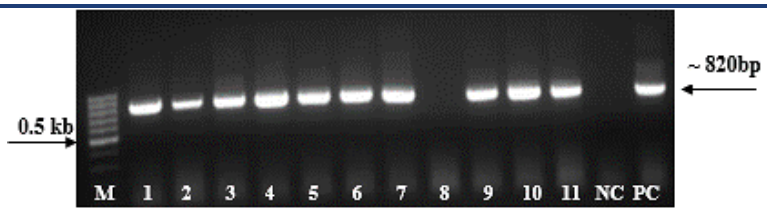

Figure 2. DNA fragments of watermelon mosaic potyvirus amplified using RT-PCR assay in infected cucurbits

WMV is a prevalent biotroph pathogen for cultivated plants and weeds (Mahgoub et al., 1997; Kheder et al., 2017). Common symptoms of cucurbit-related WMV infections were well-documented in many agroecosystems worldwide. Similar symptoms as in this study have also been reported in New Zealand from buttercup squash (Fletcher et al., 2000), watermelon in Saudi Arabia (Santosa et al., 2018), cucurbitaceous vegetables in the Czech Republic (Svoboda and Hale, 2011), cucurbit crops in USA and southern United States (Fernandes et al., 1991; Ali et al, 2012), watermelons and pumpkins in Uganda (Masika et al., 2017).

In this study, WMV infection was not detected in all cucurbits collected, probably due to different viral agents. However, WMV was detected in all symptomatic cucurbit species (melon, watermelon, snake melon, and bottle gourd) within the study. Although there have been many previous reports of WMV in melons and watermelons, according to the literature review, there are no reports of the natural presence of WMV in snake melons and bottle gourds. Based on our online surveys, the only record is that Cucumber green mottle mosaic virus has been determined in bottle gourd plants in Saudi Arabia biologically and molecularly (Amer, 2015). However, several cucurbit plant-associated WMV isolates, including snake melon and bottle gourd (Acc. no: AB127934 and AB218280), have been characterized experimentally (mechanical inoculation) in Pakistan in host range tests (Ali et al., 2006). In addition, in Argentina, WMV partial polyprotein gene sequences (825 bp) from bottle gourd have been directly submitted in the GenBank public database (MN006915.1 and MN006914.1), not published in any journal. Therefore, the above reports confirmed that natural WMV infections of snake melon and bottle gourd plants in this study are the first published report globally.

The presence of WMV infections in Turkey was well-determined using various methodologies, such as serological, molecular, and biological indexing in different localizations and plant sources. Randa-Zelyüt et al. (2022) reported natural infection of WMV in wild carrot (*Daucus carota*) in Çanakkale province of Turkey. It was also determined for the first time that WMV-2 naturally infects *S. angulatus* in the Black Sea Region of Turkey (Korkmaz et al., 2016). WMV infection of cucurbit plants has been extensively studied in distinct localities of Turkey. In Uşak province, viral agents were confirmed in 106 of 175 cucurbit samples from 5 species in cucurbit growing areas using DAS-ELISA and biological indexing. Following this study, signs such as yellowing, vein banding, blistering, curling, asymmetry in the leaves and stunting were observed in field observations (Dikici and Tarla, 2020). In Tekirdağ, Edirne, and Kırklareli provinces, 502 melon and watermelon samples were tested against 7 viruses, including WMV. Overall, the associated pathogen was confirmed in all survey areas (Köklü and Yilmaz, 2006). In Samsun province, WMV infection has been reported on cucumber, melon, pumpkin, squash and watermelon plants using serological methods by Sevik and Arli-Sokmen, (2003). In Turkey, on the other hand, WMV has been detected in different agricultural areas of Turkey (Konya, Karaman and Aksaray) (Yeşil, 2013; Yeşil and Ertunç, 2012; 2013), Diyarbakır and Mardin (Kızmaz et al., 2016; Korkmaz et al., 2021), Tokat (Korkmaz et al., 2018), Ankara and Antalya (Topkaya et al., 2019), Adana (Kamberoğlu et al., 2015), Van (Usta et al., 2018), Eastern Mediterranean Region (Adana, Osmaniye, Mersin) (Kamberoğlu and Keçe, 2016) and Bingöl (Güller and Usta, 2020). WMV infecting cucurbits is also associated with seeds of squash plants (*Cucurbita pepo*). In Turkey, WMV infection in squash seeds has been reported in Aksaray, Yozgat and Nevşehir provinces of Turkey (Yeşil, 2018; 2019; 2020).

Table 3. Watermelon mosaic potyvirus infection based on plant samples and numbers according to the provinces

District	TS				IS				HS			
	M	**WM**	**SM**	**BG**	**M**	**WM**	**SM**	**BG**	**M**	**WM**	**SM**	**BG**
Denizli	15	10	10	5	11	8	7	4	4	2	3	1
Antalya	-	9	-	4	-	-	-	1	-	9	-	3
Total	**15**	**19**	**10**	**9**	**11**	**8**	**7**	**5**	**4**	**11**	**3**	**4**
	53				**31**				**22**			

TS: Tested Samples, IS: Infected Samples, HS: Healthy Samples, M; melon, WM: watermelon, S: Snake melon, BG: Bottle gourd

3.2. Sequencing, Multiple Alignment, and Phylogenetic Relationship

The CP-DNA bands of some positive isolates (1 of bottle gourd, 2 of melon, 1 of watermelon, and 1 of snake melon from Denizli province, and 1 of bottle gourd from Antalya province) were successfully cloned and sequenced. The CP gene sequences of causative agents were further analyzed using BLAST analysis at the nucleotide level. Nucleotide BLAST analysis showed that sequences exhibited substantial nucleotide consensus with that of other isolates in the world, displaying a high nucleotide similarity score. After sequence validation, all six WMV sequences, 5 from Denizli and 1 from Antalya, were archived in the GenBank database under the accession numbers submitted in *Table 4*.

Table 4. Symptoms and distribution of watermelon mosaic potyvirus isolates related to various cucurbit crops in Turkey along with Genbank accession numbers

Cucurbit species	District	Isolate name	Acc. No	Symptoms
Melon	Denizli	Denizli 8	OM988079	mosaic, mottle, malformation, filiformis
Melon	Denizli	Denizli 32	OM988082	
Watermelon	Denizli	Denizli 16	OM988080	mosaic, mottle, little leaf, flecking
Snake melon	Denizli	Denizli 24	OM988081	mosaic, mottle, rosetting, bumps in fruit
Bottle gourd	Denizli	Denizli 5	OM988078	vein banding, crumpled leaves, mosaic in fruit
Bottle gourd	Antalya	Antalya 40	ON010742	

Multiple alignment analyses using 30 verified sequences showed that Denizli-WMV isolates have high nucleotide similarity among themselves (OM988078, 79, 80, 81, and 82), but not with Antalya isolate (ON010742). Approximately 94% nucleotide similarity was determined amongst Turkish isolates from Antalya and Denizli districts (*Figure 3*). In a comparison of the nucleotide sequences using the sequence demarcation tool, nucleotide variations were on the order of 3.28%, corresponding to 27 nucleotides, among isolates from different localities in this study. This data showed the genetic diversity of RNA-structured viruses in cucurbit plants.

3.3. Phylogenetic Relationship of Identified Isolates

The phylogenetic inference of WMV sequences was investigated along with 30 related sequences from distinct hosts and ecological origins. Consistent with the nucleotide similarity index, the phylogenetic dendrogram divided all WMV isolates into two dominant groups, possibly resulting from the presence of two distinct developmental pathways. The phylogenetic tree clustered all Denizli-WMV isolates in the same group, but not the Antalya isolate, probably because of similar genetic traits originating from the same region, the Turkish-Antalya isolate showed a close phylogenetic affinity with the isolates from France, Turkey, and China (*Figure 4*).

Different host populations of WMV have the potential to cause epidemics in agroecological areas worldwide. Therefore, it is essential to eliminate or control viruses from commercial areas growing cucurbit crops. Systematic monitoring and early detection of viral disease agents, together with ecological and quantitative epidemiological approaches, may facilitate their control (Jeger, 2020; McLeish et al., 2020). Different control approaches, such as prophylactic measures, cross-protection, and resistant cultivars, have been adopted for general plant viral diseases. In particular, prophylactic measures preventing or limiting the contact of virus-infected aphids and cucurbitaceous plants are important in protection against plant viruses. Considering that WMV is carried by over 35 species of aphids, insecticidal measures come to the fore (Lecoq and Desbiez, 2008). In addition, weed removal near planting areas and crop rotation in the same area can reduce the seasonal virus population. Plastic mulching also has a repellent effect on aphids and can significantly delay the viral spread.

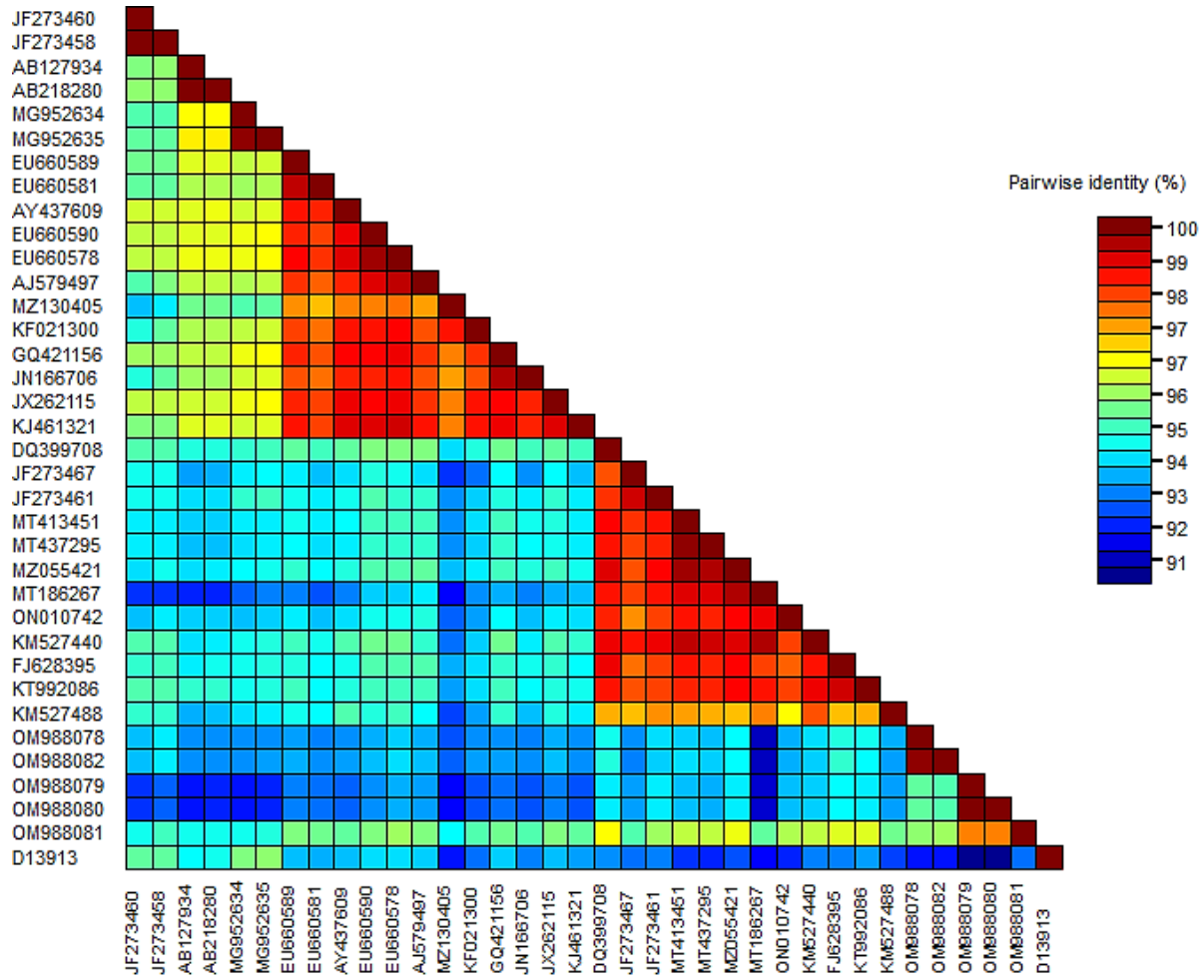

Figure 3. Nucleotide similarity rates of watermelon mosaic potyvirus sequences detected in diseased cucurbits worldwide, along with Antalya and Denizli isolates. The isolates belong to; France (JF273460, JF273461, JF273467, JF273458, EU660589, EU660581, AY437609, EU660578), Pakistan (AB127934, AB218280), Turkey (MG952634, MG952635, MZ130405, KF021300, MT413451, MT437295, MZ055421, MT186267), Italy (EU660590), Spain (AJ579497), Iran (GQ421156, JN166706), Serbia (JX262115), Ukraine (KJ461321), China (DQ399708, KM527440, KM527488), Poland (FJ628395), South Korea (KT992086) and, USA (D13913).

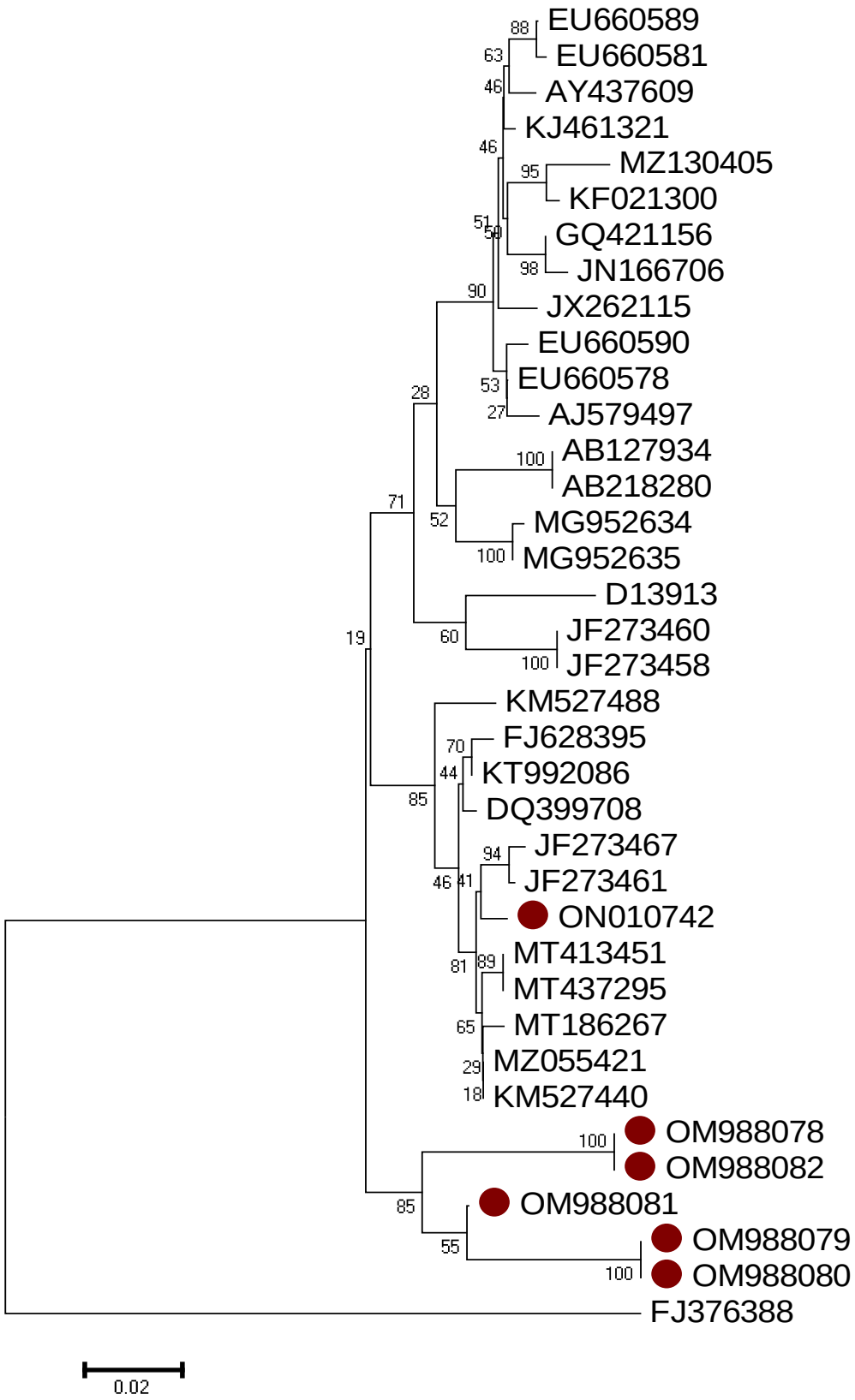

Figure 4. Phylogenetic dendrogram of watermelon mosaic potyvirus isolates. Soybean mosaic virus (FJ376388) was assigned as an outgroup. Bootstrap scores are demonstrated on each branch. The isolates from this study are marked with a red circle.

4. Conclusions

Turkish WMV isolates denominated Denizli 8, Denizli 32, Denizli 16, Denizli 24, Denizli 5 and Antalya 40 were obtained from symptomatic melon, watermelon, snake melon and bottle gourd plants. Two molecular phylogroups emerged in the phylogenetic relationships of the detected WMV isolates. In the dendrogram, the Denizli isolates formed an independent group, while the Antalya isolate showed a genetic affinity with isolates from different hosts from Asia and Europe. This is the first molecular detection and molecular characterization of WMV isolates from snake melon and bottle gourd in Turkey and the world.

Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

Concept: Korkmaz, G., Dermirel, S.; Design: Dermirel, S., Usta, M.; Data Collection or Processing: Usta, M., Güller, A.; Literature Search: Güller, A., Korkmaz, G.; Writing, Review and Editing: Güller, A., Usta, M.

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