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Genetic Diversity of *Cucumber mosaic virus* in Cucumber Plants Grown in Diyarbakır Province

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Abstract: The cucumber plant (*Cucumis sativus* L) is an important cultivated plant produced worldwide. *Cucumber mosaic virus* (CMV), one of the common viral diseases, causes economic losses by reducing the yield and quality of the cucumber plant. In the observations performed in Diyarbakır in September 2021, cucumber plants showing virus-like symptoms such as mosaic, irregular yellowish spots, and deformity on the leaves were observed. Symptomatic and non-symptomatic samples were collected and subjected to a reverse transcription polymerase chain reaction (RT-PCR) using CMV-specific primers, and the produced DNA bands were visualized on an agarose gel. CMV infection was detected in seven of the 15 samples. Bacterial cloning and sequencing of a randomly selected specimen determined that the CMV partial coat protein gene was 593 bp long and was registered in the NCBI database with the accession number MW962979.1. According to the phylogenetic tree performed with different isolates of CMV, Diyarbakir CMV isolate clustered with CMV isolates from Australia, Israel, Spain, Hungary, Japan, and Korea forming Subgroup IA. The presence of CMV and group/subgroup diagnosis in cucumber plants grown in the Diyarbakır region were confirmed molecularly for the first time by this study.

Diyarbakır İlinde Yetiştirilen Hıyar Bitkilerinde Hıyar Mozayik Virüsü'nün Genetik Çeşitliliği

Anahtar Kelimeler
Kılıf proteini, RT-PCR, Klonlama

Öz: Hıyar bitkisi (*Cucumis sativus* L.) dünya çapında üretilen önemli bir kültür bitkisidir. Yaygın viral hastalıklarından biri olan Hıyar mozayik virüsü (*Cucumber mosaic virus*, CMV) hıyar bitkisinde verim ile birlikte kaliteyi düşürerek ekonomik kayıplara neden olmaktadır. Diyarbakır ilinde 2021 yılı Eylül ayında yapılan gözlemlerde, yapraklarda mozaik, düzensiz sarımsı lekeler ve deformite gibi virüs benzeri simptomlar gösteren hıyar bitkileri gözlenmiştir. Simptom gösteren ve göstermeyen örnekler toplanarak CMV spesifik primerler kullanılarak ters transkriptaz polimeraz zincir reaksiyonu (RT-PZR)'na tabi tutulmuş ve üretilen DNA bantları agaroz jelde görüntülenmiştir. CMV infeksiyonu 15 örnekten yedisinde belirlenmiştir. Rastgele seçilen bir örneğin bakteriyel klonlanması ve dizilenmesi sonucunda, CMV kısmi kılıf proteni geninin 593 bp uzunluğunda olduğu belirlenmiş ve NCBI veri tabanına MW962979.1 erişim numarası ile kaydedilmiştir. CMV'nin farklı izolatlarıyla gerçekleştirilen filogenetik ağaca göre, Diyarbakır CMV izolatı Subgrup IA grubunu oluşturan Avustralya, İsrail, İspanya, Macaristan, Japonya ve Kore'ye ait CMV izolatları ile kümelenmiştir. Diyarbakır bölgesinde yetiştirilen hıyar bitkilerinde CMV'nin varlığı ve grup/subgrup teşhisi ilk defa bu çalışma ile doğrulanmıştır.

1. INTRODUCTION

Cucumber mosaic virus (CMV), responsible for significant agricultural losses in many cultivated plants worldwide, is probably one of the viruses with the largest host range among plant viruses. CMV firstly described in 1916 by Doolittle in Michigan and by Jagger in New York is a disease of cucurbits. CMV has a high degree of diversity and a large number of isolates due to differing biological and molecular properties [1, 2]. CMV, a type of member of the Cucumovirus genus, has a wide host range with 1241 species in 101 plant families consist of monocotyledon and dicotyledonous plants. In addition, the virus's host range includes a large number of wild species that are important for its year-round survival, as well as plants from all kinds of cultures such as food and feed products, ornamental plants [3].

CMV virions are icosahedral particles having a diameter of 29 nm and include 18% RNA and a single capsid protein (CP) with 180 subunits. CMV genome having single-stranded positive-sense RNA consists of three pieces named as RNA1, RNA2 and RNA3 in decreasing order of size. RNA1, which is monocistronic, encodes protein 1a. This fragment includes an admitted methyltransferase domain at its N-terminal portion and also a helicase domain at its C-terminal portion. RNA2 encodes the huge 2a protein containing the GDD motif typical responsible for an RNA-dependent RNA polymerase (RdRp), as well as the small 2b protein expressed from an open reading frame 2b (ORF2b). 2b protein is a suppressor of RNA silencing. RNA3 encodes the coat (CP) and the movement proteins (MP) has a bicistronic structure. The first ORF of each bicistronic RNA is expressed from genomic RNA, but the second ORFs are expressed from RNA4 and RNA4A [4]. CMV divided into two groups as group I and II according to their serological relationships and genetic diversity by Palukaitis et al. [5]. CMV causes typical mosaic symptoms on melon and cucumber leaves, stunting and reduced fruit yield. Symptoms on fruits are usually seen in the form of spots or mosaics. Adult plants of some cucumber cultivars may show rapid and complete wilting a few days after CMV infection. In pumpkin, the symptoms of CMV are very severe, including mosaic, yellow spots and leaf rot. Infected plants often deform fruit, drastically reducing fruit retention, or even stopping [7]. The existence of CMV has been reported in many countries in the world and has been detected in many regions and plants in Türkiye [8, 9, 10, 11, 12].

Recently, the research in the world shown that CMV subgroup I divided into two subgroups (Ia and Ib) with the analysis of non-protein coding regions at the 5' end and the CP gene [6]. Previous studies have shown the presence of CMV infections in various hosts in Turkey, as described by several researchers [29, 32, 36, 37, 38]. A few studies have shown that subgroup IA isolates are frequently observed in molecular investigations carried with CMV [34, 39, 40]. Moreover, Group II and subgroup IB have been seen in Turkey in recent years [33]. Although CMV infection has been reported in cucumber growing regions in our country, the genetic diversity of these isolates remains unknown.

Turkey stands out as one of the leading nations worldwide in the production of vegetables from the *Cucurbitaceae* family, with Diyarbakır playing a significant role in this contribution [31]. Cucurbits are susceptible to a wide variety of viral infections, which may result in considerable productivity reductions in these crops [18]. The objective of this study is to identify CMV in the cucumber plant and to investigate its genetic diversity in the cucumber growing areas of Diyarbakır, which is located in the Eastern Anatolia region of Türkiye. It has also been investigated the phylogenetic relationships of Diyarbakır isolate using other worldwide CMV isolates from the gene bank for determine the genetic diversity.

2. MATERIAL AND METHOD

2.1. Virus Isolates and Total RNA Extraction

In 2021, fresh leaves were collected from 5 CMV-suspicious and 10 healthy-looking plants out of a total of 15 plants in the five fields where cucumbers were grown Çınar town of Diyarbakır. Total RNA isolation was performed by using method described by Foissac et al. [13]. The RNA samples obtained were stored at -80°C for testing.

2.2. cDNA Synthesis and PCR

The cDNA synthesis was performed by using RNAs obtained from the previous step. Gene-specific oligonucleotide designed for CP was used in cDNA synthesis. cDNA synthesis was carried out in two steps. In the first step, 12 µl of the reaction mixture (2 µl RNA, 1 µl dNTP, 8 µl RNase-free water and 1 µl reverse primer were incubated at 65°C for 5 minutes and then was kept on ice for 5 minutes. In the second step, a mixture of components of 1 µl RNase-free water, 4 µl 5X RT buffer, 1 µl reverse transcriptase and 2 µl 0.1 M DTT was incubated at 42°C for 45 minutes and then at 70°C for 15 minutes, respectively. cDNAs in a total volume of 20 µl were stored at -80°C until use. Specific forward and reverse primers were used to obtain the coat protein gene of CMV (Table 1). 25 µl RT-PCR reaction volume contains 3 µl cDNA, 15.6 µl nuclease-free water, 0.5 µl dNTP, 0.4 µl DreamTaq DNA polymerase (Thermo Scientific), 1.5 µl $MgCl_2$, 2.5 µl 10X Taq buffer, 0.5 µl forward and reverse primer. RT-PCR reactions were performed with the following conditions: initial denaturation 1 cycle at 94°C for 2 min, 36 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 and final extension of 1 cycle at 72°C for10 min. The standard marker (1 kb, Thermo Scientific) (5 µl) and obtained reaction products (15 µl) were run on an agarose gel (1.5%) including 1×TAE Buffer and EtBr (1%) for 45 minutes at 85 volts and visualized under UV light. In order to strengthen the accuracy of PCR tests, CMV [14] isolate obtained from previous studies was used as a positive control and asymptomatic cucumber samples were used as negative controls.

Table 1: Primer information that is used for the CMV CP gene

Primer name	Primer sequence	Position	Product size (bp)	Ref.
Forward primer	5'GCCACCAAAAATAGACCG3'	1484–1502		[35]
Reverse primer	5'ATTCGCTGGCGTGGATTTCT3'	2057–2076	593	[35]

2.3. Sequencing analysis, BLAST and Phylogenetic construction

RT-PCR amplified DNA was purified with the Gene Jet " (Cat. No. K0691, Thermo) in accordance with the manufacturer's instructions. Purified products were cloned into the prokaryotic cloning vector (pGEM T-Easy, Promega) and transferred to *E. coli* by electro transformation method. Recombinant plasmids including CMV coat protein in bacteria were purified with Miniprep Kit (Thermo GeneJet Plasmid, Cat. No. K0503,) and DNA fragment of CMV coat protein sequenced by next generation sequencing (NGS) (Sentebiolab/Ankara/Türkiye) [41]. The resulting sequence has been registered in the NCBI database.

A phylogenetic tree was generated with the CMV coat protein sequence belong to Diyarbakir isolate, 25 different sequences from different hosts and countries (including CMV IA, CMV IB and CMV II group) on the NCBI website. Pairwise identity of CMV isolates used in the present study calculated with Sequence Demarcation Tool Version 1.2 (SDTv1.2). The phylogenetic dendogram was created with the Neighbor-Joining method (NJM) using CLC Main Workbench 6.7.1 software with 100 replications. Tomato aspermia virus, accession number EF153735, was assigned as outgroup in order to promote better branching of the tree.

3. RESULTS

As a result of the field studies carried out in the province of Diyarbakır in the Eastern Anatolia region, symptoms caused by viruses and virus-like factors such as mosaic and blistering, yellowish spots and abnormal leaves were observed on the leaves from the cucumber production areas. The total of 15 cucumber leaves in this study were collected and RT-PCR tests applied to the collected samples yielded DNA bands of approximately 593 bp in 7 samples, confirming the presence of CMV (Figure 1). According to the PCR test results, the percentage of infection was calculated as 46.6.

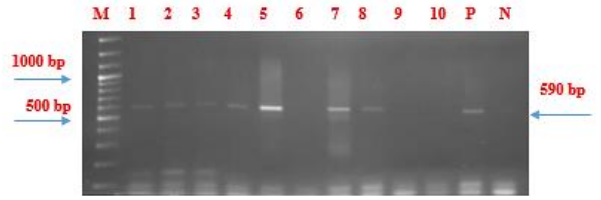

Figure 1. Agarose gel electrophoresis image of RT-PCR products obtained using CP specific primers for cucumber samples collected from Diyarbakır province. M: Marker, P: Positive control, N: Negative control

After the RT-PCR result, bacterial cloning of a CMV isolate from 7 samples that gave positive results among the plants tested in Diyarbakır province was performed and its nucleotide sequence was revealed. The resulting partial coat protein nucleotide sequence was named Diyarbakir D4 and registered in the gene bank with the accession number MW962979.1 as shown in figure 2.

BLAST analysis was performed to determine the nucleotide similarity ratio of the obtained sequence from the current study. According to the comparison based on other CMV isolates worldwide, the similarity rate of Diyarbakır D4-CMV isolate was found to be between 96.63% and 99.49% at the nucleotide level (Figure 3). Based on the sequence of 593 nucleotides, we also investigated the phylogenetic relationships of Diyarbakır D4-CMV isolate with other isolates and to which group it belongs. While constructing the tree, 25 different DNA sequences selected from Group I (A and B) and Group II from different gene sources were used (Table 2).

GCCACCAAAAATAGACCGTGGGTCTTATTACGGTAAAAGGTTGTTACTACCTGATTCAGTCACGGA
ATATGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTGCCGAAATTTGATTCTACCG
TGTGGGTGACAGTCCGTAAAGTTCCTGCCTCCTCGGACTTATCCGTTGCCGCCATTTCTGCTATGTTT
GCGGACGGAGCCTCACCGGTACTGGTTTATCAGTATGCCGCATCTGGAGTCCAAGCCAACAACAA
ACTGTTGTATGATCTTTCGGCGATGCGCGCTGATATAGGTGACATGAGAAAGTACGCCGTCCTCGT
GTATTCAAAAGACGATGCGCTCGAGACGGACGAGCTAGTACTTCATGTTGACATCGAGCACCAAC
GCATTCCCACGTCTGGAGTGCTCCCAGTCTGATTCCGTGTTCCCAGAATCCTCCCTCCGATCTCTGT
GGCGGGAGCTGAGTTGGCAGTTCTGCTATAAACTGTCTGAAGTCACTAAACGTTTTTTACGGTGAA
CGGGTTGTCCATCCAGCTTACGGCTAAAATGGTCAGTCGTGGAGAAATCCACGCCAGCAGAT

Figure 2. Partial coat protein nucleotide sequence (593 bp) obtained by cloning of CMV isolate (MW962979.1) obtained from Diyarbakir province

Table 2. Gene bank information relating to the genes used in the investigation of phylogenetic relationships of Diyarbakır D4 isolate

No	Country	Accession number	Plant source	Gene source	Virus types noted in NCBI
1	Australia	U22821	-	Complete	Group I and II
2	Israel	U66094	*Cucurbita pepo*	Complete	-
3	Spain	AM183119	Tomatoes	Complete	Group IB
4	Hungary	AJ517802	*Raphanus sativus*	Genomic RNA	-
5	Japan	D28487	*Licopersicon esculentum*	Complete	Subgroup I
6	Korea	L36251	-	Complete	-
7	Korea	AF013291	-	Complete	-
8	India	AF281864	*Datura innoxia*	Complete	-
9	Thailand	AJ810259	Chili pepper	CP	-
10	China	KJ746022	*Nicotiana tabacum*	CP	-
11	Türkiye	KY474380	Kidney bean	CP	-
12	Türkiye	MW962980	*Cucumis melo*	CP	-
13	Türkiye	MT361015	*Cucumis melo*	CP	-
14	Türkiye	MT361015	*Cucumis melo*	CP	-
15	Japan	AB042294	-	Complete	-
16	USA	U31220	Musa	Complete	-
17	China	AF268598	Banana	Complete	-
18	-	M21464	-	Complete	-
19	India	AJ585086	Lilium	Genomic RNA	-
20	USA	AF127976	-	Complete	-

21	India	HE583224	*Nicotiana glutinosa*	Genomic RNA	-
22	-	L15336	-	Complete	-
23	Japan	AB006813	-	Complete	-
24	Netherlands	AJ304397	Alstroemeria	CP	-

CP: Coat protein

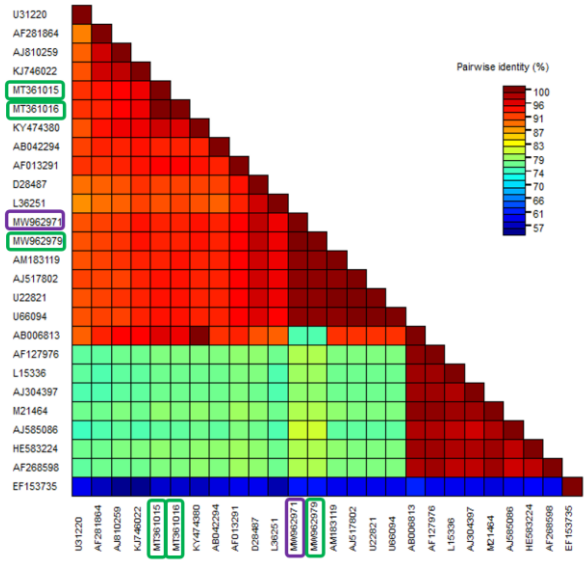

Figure 3. Similarity matrix created by using nucleotide sequences of CP genes of Diyarbakır D4 isolate and world CMV isolates

Based on sequence similarity ratios, the results are consistent with the phylogenetic tree constructed with the same sequences. According to the phylogenetic tree formed by the neighbor-joining method, 3 groups were formed, namely Group I (A and B) and Group II. According to the phylogeny, it was determined that the CMV D4 isolate clustered with the isolates of Subgroup IA (Figure 4).

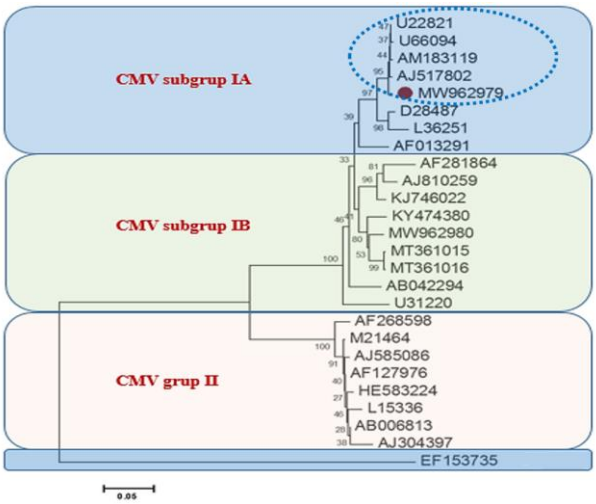

Figure 4. Phylogenetic dendrogram of CP gene sequences of CMV isolates. Diyarbakır D4-CMV isolate is shown in red (MW962979)

CMV isolates from Australia, Israel, Spain, Hungary, Japan and Korea were also included in the cluster (Subgroup IA) in which Diyarbakır isolate accession number MW962979 is located. In addition, as result of the isolate obtained from the cucumber showed a closer phylogenetic relationship with the CMV isolates isolated from Australia, Israel, Spain and Hungary. Different clustering of Japanese and Korean isolates with other isolates in subgroup IA by tree supports this information. On the other hand, although they share almost the same geography, the Diyarbakır cucumber isolate and the melon plant isolates with accession numbers MW962980, MT361015 and MT361015, also reported from Türkiye, were in different groups. On the other hand, although they share almost the same geography, the Diyarbakır cucumber isolate and the melon plant isolates (MW962980, MT361015 and MT361015) reported from Türkiye, clustered in different groups. Also, it can be concluded that plant species as based on Table 2 is not important in phylogenetically separating CMV groups.

4. DISCUSSION AND CONCLUSION

Cucurbits are an important host of many viral pathogens. The viruses can cause %50 to 100% damage depending on the plant species, vector density, virus strain and environmental conditions. Mixed infection of these viruses with other viruses has the potential to cause epidemics in vegetable growing areas around the world [15, 16]. CMV is prevalent in mostly all continents, including Europe, Asia, North America, Africa, and Australia [17]. CMV is also widespread in agriculture in Türkiye and constitutes a wide viral study area. The presence of CMV has been reported in many cultivated and wild plants such as tobacco, beans, spinach, peppers, tomatoes, cucumbers olives and *Polygala myrtifolia* [18, 19, 20, 21, 22, 23, 24]. Virus and virus-like symptom symptoms, including mosaic and blistering, yellowish spots, and abnormal leaves, were noted on cucumber leaves harvested from in this study. These symptoms are in agreement with those reported in other studies in the literature [25, 26].

CMV has been reported nationally in different geographies and in different hosts in Türkiye. The 113 tomato leaves in total with virus and virus-like symptoms from production areas in the Marmara region were tested with the DAS-ELISA test (Double-antibody sandwich enzyme linked immuno sorbent assay) by Karanfil [27]. The infection rate was founded 30.08% in the samples collected. In a similar study conducted in the same region (Çanakkale, Bursa and Bilecik), the presence of CMV with the DAS-ELISA test was determined in 67 of 77 samples belong to different plant species and the infection rate was reported as 87% [22]. A research conducted in the Western Mediterranean area revealed that 53 out of 138 tomato samples, accounting for 38.40% of the total, were found to be infected with CMV [28].

In the surveys carried out in Bingöl province in 2019, melon leaf samples with mosaic patterns in different shades of green and vein bands and leaf deformations were collected and analyzed with PCR to detect the presence of CMV and Watermelon mosaic virus (WMV). As a result of RT-PCR performed using primer sets specific to the coat protein (CP) gene, it was determined that the partial coat protein gene length contained 657 bp [29]. Furthermore, two separate research have shown the existence of CMV isolate in the region of Diyarbakır. [30,

31]. In the study carried out by Öztürk [30] in the fields of watermelon cultivation, leaf curling, chlorotic mottling, mosaic formations, thinning and fruit deformations were detected in plants and tested with DAS-ELISA tests against various cucurbit viral agents. In the study, 53 of 60 leaf samples infected with one or more viruses. While Zucchini yellow mosaic virus (ZYMV) had the most infection, the lowest infection rate was found in CMV. In the other study carried out in Diyarbakır, a total of 547 samples, including 34 cucumbers, 176 zucchini, 142 watermelons and 195 melons, were collected and tested by applying the DAS-ELISA method against 7 cucurbit viruses. As a result of the analysis, CMV pathogen was reported with an infection rate of 18.28% in 100 of 547 samples [31]. The result of previous studies regarding molecular characterization of CMV illustrated that CMV isolates of Türkiye are generally 80-100% similar to the world isolates [32, 33]. In this regard, the results provided from this study are consistent with those from previous studies.

Geographical conditions have been suggested as a potential influence on phylogenetic groupings. However, it has been shown that the geographical origins of CMV do not have a significant impact on its phylogenetic grouping. It has been suggested that isolates from the immediate region are distributed in different phylogenetic groups [34]. In spite of the fact that a great number of research on CMV have been conducted in our country, the fact that the majority of these studies were conducted using the ELISA test results in uncertainty about the differentiated groups and subgroups of CMV isolates. The subgroup IA CMV isolates are present in different plants in Türkiye [27, 33]. Moreover, the availability of subgroup IB and II isolates has been stated in different studies.

In the present study, the phylogenetic relationships of CMV and its infection in cucumber plants obtained from Diyarbakır province were revealed. Moreover, this study is the first report in Diyarbakır province where this isolate is included in Subgroup IA. In addition, the registration of the CP partial gene sequence of the cucumber CMV isolate to GenBank for the first time is among the original outputs of the study. Although the infection rate was calculated as 46.6 according to the collected samples, it is recommended to test the samples with the PCR test, which is more sensitive than the DAS-ELISA test by using more samples for the current rate. In addition to the partial coat protein, the complete gene of coat protein of belong to CMV Diyarbakir D4 isolate should be amplified and molecular characterization should be made.

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