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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Genetic Diversity of Tobacco Mosaic Virus (TMV) Isolates from Tobacco Growing Fields of Western Anatolia, Türkiye

Batı Anadolu Bölgesi Tütün Üretim Alanlarından Elde Edilen Tütün Mozaik Virüsü (TMV) İzolatlarının Genetik Çeşitliliği

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Abstract

Tobacco mosaic virus (TMV) is an important plant virus in agriculture. It is the first evidence of the existence of viruses in history. Studies on the genetic diversity of the CP gene of TMV, which plays a leading role in host interaction, are limited both in our country and worldwide. Genetic diversity analyses were conducted on ten isolates of the full CP gene region of TMV obtained from the most intensive tobacco cultivation areas in Türkiye, and compared with global isolates. TMV infection was detected in 32 out of 300 plants collected from the Aegean and Marmara regions (Çanakkale, Balıkesir, İzmir, Manisa, Uşak, Aydın and Denizli) between 2019 and 2020 using conventional molecular techniques. To genetically characterize the virus, 10 samples were selected from each region, and the complete CP gene region sequences were determined. The aligned CP gene region sequences of TMV from Türkiye and its global isolates exhibited nucleotide homology ratios ranging from 87.7% to 100%, with amino acid ratios ranging from 88.7% to 100%. The Türkiye isolates displayed similarity rates of 98.5% to 100% at the nucleotide level and 98.7% and 100% at the amino acid level. In phylogenetic analysis, the 196 known isolates of TMV registered in GenBank, belonging to the CP gene region, were divided into two main clades (I and II) and two subclades (Ia and Ib). Türkiye isolates were clustered in the major branch with the main clade I and subclade Ia isolates. Therefore, genetic analyses were performed on the CP gene region isolates obtained from different parts of the world and a wide range of hosts, including the isolates obtained from Türkiye. The results showed high genetic stability, similar to many tobamoviruses.

Keywords: TMV, Tobacco, RT-PCR, Phylogenetic analyses, Genetic diversity

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Öz

Tütün mozaik virüsü (TMV), tarımda yaygın olarak görülen önemli bir bitki virüsüdür. Tarihte virüslerin varlığının ilk kanıtıdır. Konakçı etkileşiminde öncü rol oynayan TMV' nin CP geninin genetik çeşitliliğine ilişkin çalışmalar hem ülkemizde hem de dünyada sınırlıdır. Bu çalışma kapsamında, ülkemizin en yoğun tütün üretimi yapılan bölgelerinden elde edilen 10 TMV izolatı ile gen bankasında bulunan küresel varyantların genetik çeşitlilik analizleri yapılması gerçekleştirilmiştir. Ege ve Marmara bölgelerinden (Çanakkale, Balıkesir, İzmir, Manisa, Uşak, Aydın and Denizli) 2019-2020 yılları arasında toplanan 300 bitkiden 32'sinde konvansiyonel moleküler teknikler kullanılarak TMV enfeksiyonu tespit edilmiştir. Genetik karakterizasyon için her bölgeyi temsil edecek şekilde 10 TMV izolatı seçilerek CP gen bölgesine ait nükleotid dizilimlerinin tamamı elde edilmiştir. Türk ve global TMV izolatlarının CP gen bölgesi dizilerinin çoklu dizi analizleri sonucunda nükleotid benzerlik oranları %87.7 ile 100 arasında, amino asit benzerlik oranlarının ise %88.7 ile 100 arasında değişen değerler gösterdiği belirlenmiştir. Türk izolatlarının kendi içlerinde ise nükleotit düzeyinde %98.5 ile 100, amino asit düzeyinde ise %98.7 ile 100 arasında benzerlik oranları gösterdiği görülmüştür. Gen bankasında kayıtlı tüm bilinen TMV izolatlarının (n=196) CP gen bölgesine göre gerçekleştirilen filogenetik analizleri sonucunda iki ana gruba (I ve II) ve bunlardan da birincisinin de iki alt gruba (Ia ve Ib) ayrıldığı belirlenmiştir. Türk izolatlarının Ia alt grubu içinde I ana grubuna ait olduğu görülmüştür. Bu analizler ile dünyanın farklı coğrafyalarından ve geniş bir konukçu yelpazesinden elde edilen izolatların yanı sıra Türkiye'den elde edilen izolatların da CP gen bölgesine göre genetik analizleri gerçekleştirilmiştir. Gerçekleştirilen bu çalışmaların sonucunda; birçok tobamovirus'larda olduğu gibi TMV'nin de yüksek genetik kararlılık gösterdiği belirlenmiştir.

Anahtar Kelimeler: TMV, Tütün, RT-PCR, Filogenetik analiz, Genetik çeşitlilik

1. Introduction

Türkiye has a long-standing tradition of cultivating tobacco (*Nicotiana tabacum*), a crop that rapidly spread in the New World and has been one of the significant agricultural products produced for many years (Gül et al., 2009). The tobacco, which has adapted significantly to temperate climate conditions, is primarily cultivated in the Marmara and Aegean regions of Türkiye, accounting for approximately 90% of its production (TURKSTAT, 2023). Globally, recognized as Turkish or Oriental tobacco in the world market, due to its distinctive aroma.

Tobacco plants are susceptible to various phytopathogens, including viruses, which can negatively impact the quality and quantity of the plant's leaves. It is important to note that viruses cannot be effectively controlled through direct means. The global spread of both established and newly developed tobamoviruses has significantly affected the production of various crops (Smith and Dombrovsky, 2020). Among these tobamoviruses, tomato brown rugose fruit tobamovirus (ToBRFV) has recently caused a significant reduction in the production of Solanaceae plants (Luria et al., 2017). On the other hand, infections caused by the tobacco mosaic tobamovirus (TMV) pathogen, one of the oldest known viruses and a pioneer in the field of virology, continue to be common in growing areas (Gibbs, 1999).

The genome of TMV is a single-stranded positive-polarity RNA molecule, termed (+) ssRNA, and has a compact size of 6.4 kilobases (kb) in length (Goelet, 1982). One feature that distinguishes TMV within the *Virgaviridae* family is its undivided genome structure, a characteristic not shared by other genera in the family (King et al., 2012). Virions are typically about 300–310 nm in length and 18 nm in diameter (King et al., 2012). Studies of TMV strains have revealed a complex genome consisting of at least three non-structural proteins (P183, P126 replication proteins and the 30 kD MP movement protein), a 54 kD protein of unknown function and the coat protein (17.6 kD-CP coat protein) (Okada, 1999; Zaitlin, 1999). These genomic features play a major role in the life cycle of TMV and its interactions with host plants and are the subject of ongoing virology research.

TMV is transmitted mechanically through contaminated tools or hands, and it also spreads systemically through plant vascular tissues, causing widespread infection (Sacristán et al., 2011). The virus's stable particle structures are widely believed to be the reason for the difficulty in controlling TMV in tobacco cultivation fields (Alonso et al., 2013). On the other hand, research has shown that both tobacco mild green mosaic virus (TMGMV) and TMV exhibit limited genetic diversity. This suggests that the viruses can infect new hosts without undergoing significant adaptive evolution, regardless of their habitat or host plant taxonomy (Zamfir et al., 2023). However, there is currently no comprehensive study on the genetic diversity of TMV in our country. This study aimed to determine the incidence of TMV in Western Anatolia, which is one of Türkiye's most intensive tobacco cultivation areas. Additionally, we conducted a molecular evolutionary characterization of its structural gene, the coat protein (CP gene).

2. Materials and Methods

2.1. Virus sources

Field studies were carried out within the scope of the TUBITAK project (119O625), which aims to determine the genetic diversity of TMV. The studies were carried out in tobacco production areas in Türkiye's Marmara and Aegean regions between 2019 and 2020. A total of 7 provinces were in the study: Çanakkale, Balıkesir, İzmir, Manisa, Uşak, Aydın, and Denizli. Samples were taken from plants showing virus and virus-like symptoms in randomly selected sampling areas.

2.2. Molecular assay

TMV was identified at the species level using reverse transcription polymerase chain reaction (RT-PCR) methods. The CTAB method with minor modifications, as previously detailed by Li et al. (2008), was used to extract total nucleic acid (TNA) from plant leaves. the cDNA Synthesis Kit (Takara, Japan) was used to generate complementary DNA (cDNA) libraries from the extracted TNA, along with a random hexamer primer (5′–NNNNNN–3′). The cDNA libraries were used in amplification procedures with the 2X Emerald PCR Master Mix (Takara, Japan) and gene-specific primer pairs Forward (5'-AGTGATGTCCGTAAAGGGA-3') and Reverse (5'-CGTTATCGTACGYACCACG-3') (Karanfil et al., 2023). The outcomes were validated through agarose gel electrophoresis stained with 1.5% Ethidium Bromide (EtBr) and visualized using a UV imager.

2.3. Sequencing studies and phylogenetic inference

To determine the nucleotide sequences of TMV isolates within the CP gene regions, we employed the PCR products resulting from the amplification studies. These products were cloned using the T-A cloning kit from Promega (USA) and underwent bidirectional sequencing through the Sanger sequencing method using the M13F-R universal primer pair for each gene region. The raw sequence data obtained were subsequently assembled using the CLC Main Workbench V.20.3 software. To classify the complete CP gene region of TMV objectively, we determined genetic similarity ratios between sequence pairs using the Sequence Demarcation Tool Version 1.2 (SDTv1.2) software (Muhire et al., 2014).

Phylogenetic relationships were determined by analysing the complete CP gene region of ten TMV isolates identified in this study, as well as 196 global isolates available in GenBank. The nucleotide sequences of the CP gene were aligned using the ClustalW algorithm in MEGA 11 software (Tamura et al., 2021). The phylogenetic trees were constructed using the Neighbour-Joining (NJ) statistical method, following the Tamura-3 parameter model (Tamura, 1992). Uniform rates of complete deletion were applied to the entire CP gene region of TMV. To increase the reliability of the main nodes, 1000 bootstrap tests supported the analyses. Tobamoviruses, including pepper mild mottle virus (PMMoV; access no AF1037777, South Korea), tomato mosaic virus (TomV; access no MZ323245, China), and tobacco mild green mottle virus (TMGMV; access no MH636301, China), were used as outgroups. The phylogenetic outcomes were also represented graphically using the Interactive Tree of Life (iTOL) v5 online software (Letunic and Bork, 2021).

3. Results and Discussion

3.1. Symptomatology and disease prevalence

During the field research, 300 plant samples displaying typical virus and virus-like symptoms were collected from the primary tobacco-producing areas in Western Anatolia, namely the Marmara and Aegean regions. Almost all the collected samples exhibited various degrees of mosaics, including mild and severe symptoms (*Figure 1*). In addition to the mosaic symptoms, a small number of plants also displayed stunting symptoms. Molecular testing using species-specific primers detected TMV infection in 32 out of 300 tobacco plants (10.66% infection rate) (*Table 1*). Additionally, as a result of amplification studies, fragments of 703 bp were obtained for the fully encoded CP gene region of TMV (*Figure 2*).

Figure 1. TMV-infected tobacco plant exhibiting mosaic symptoms of varying severity

Table 1. The number of tobacco mosaic virus isolates in the collected samples.

Region	Province	No of TMV isolates	No of collected samples
Marmara	Çanakkale	8	20
	Balıkesir	13	38
Aegean	İzmir	-	35
	Manisa	1	64
	Uşak	9	41
	Aydın	-	40
	Denizli	1	62
Total		32	300

Figure 2. Complete encoded CP gene region of TMV with a size of 703 bp

Research on tobamoviral genetic variability has enhanced our understanding of genetic diversity in viruses, elucidating the mechanisms responsible for its generation (Fraile and García-Arenal, 2018). Furthermore, it has provided insights into the factors influencing viral population dynamics (Karanfil et al., 2023). Thus, this study sheds light on the genetic diversity of the CP gene region, which plays a crucial role in the complex interaction between host and virus.

Symptoms of tobamovirus infections can vary depending on the virus species, the host plant, and environmental conditions. Infected plants typically exhibit leaf deformation, mosaic patterns, and mottled appearances on their leaves (Melcher et al., 2021). The severity of these symptoms can range from mild to severe, depending on the tobamovirus and its impact on the leaves or fruits of the host plant (Fraile et al., 1997). The existence of TMV and its symptoms have been known in our country since 1969 (Erdiller, 1969). TMV has been known to cause significant losses in yield and quality, particularly in economically valuable crops such as eggplant, pepper, tobacco, and tomatoes (Erdiller, 1969; Erkan and Yorgancı, 1988; Çulal-Kılıç et al., 2017). Infections caused by the agent in these plants often result in symptoms such as mosaic patterns, blistering and deformation of leaves, as well as reduced fruit size and mottling. However, recent advancements have shown that these symptoms are not exclusively caused by TMV. It has been demonstrated that both tobamoviruses and members of other genera can induce similar symptoms. Recent studies conducted in Türkiye have reported that tobamoviruses frequently cause single or multiple infections, often co-occurring with viruses from the same genus (Karanfil et al., 2023; Balsak et al., 2022). These infections are typically documented as symptoms of leaf yellowing, deformations, and mosaics on tobacco and pepper (Karanfil et al., 2023; Balsak et al., 2022). Randa-Zelyüt et al. (2022) reported the first description of 16Sr XII-A (stolbur group) phytoplasma infections causing abnormalities in tobacco leaves in Türkiye. The infected tobacco isolates used in this study were described in the study on TMGMV by Karanfil et al. (2023). Tobacco plants infected with TMV or multiple infections exhibited moderately mosaic symptoms. Therefore, it is hypothesized that the prevalence of TMV infections in our country is quite low, and the symptoms it causes are not severe. This hypothesis is supported by findings from a study that examined the prevalence and severity of TMV infections in cucurbits (Karanfil, 2022).

3.2. Molecular evolutionary inferences and sequence similarities

Molecular evolutionary analysis indicated that the TMV isolates (n=206) were grouped into two main clades (I and II) at the CP gene level, with strong bootstrap support (≥90). Furthermore, clade I was divided into two subgroups, I-a and I-b. The I-a subclade consisted of isolates from Indonesia, Finland, the United Kingdom,

Thailand, Spain, Germany, Canada, Italy, the United States, Vietnam, South Korea, Japan, India, Brazil, and China, making it the largest subclade (n=194). All 10 novel TMV isolates obtained from this study, along with 3 isolates from the eastern region of Türkiye, were positioned within subclade I-a (n=194). Subclade I-b (n=6) encompassed six isolates originating from Germany and South Africa. The main clade II (n=6) comprised six TMV CP gene isolates, which originated from Germany, Russia, China, the United States, Taiwan, and Mexico (*Figure 3*).

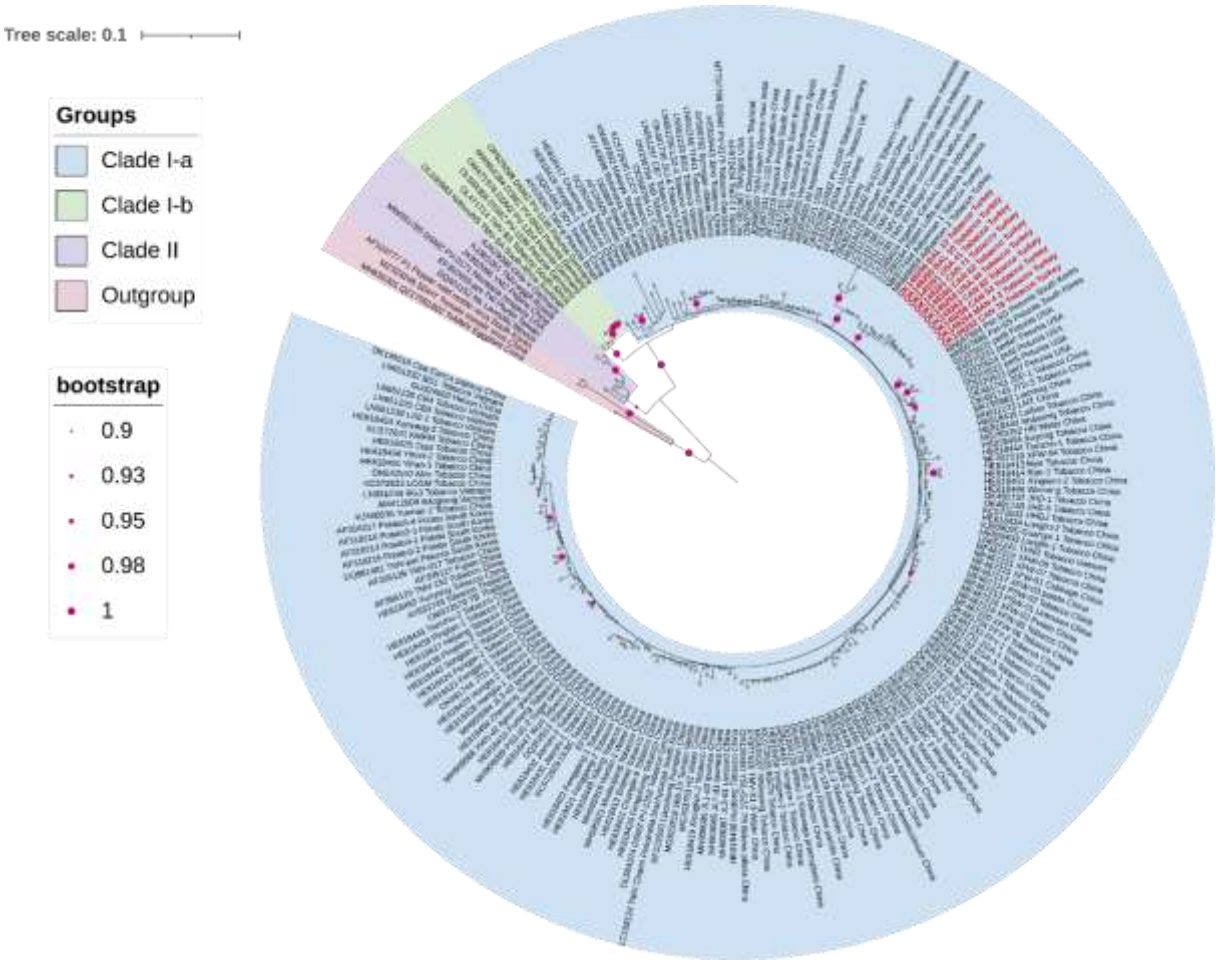

Figure 3. Phylogenetic relationship of tobacco mosaic virus isolates

The nucleotide sequence data for the entire CP gene region of ten randomly selected TMV-infected samples from both the Marmara and Aegean regions have been submitted to GenBank with accession numbers OK149254-63. The nucleotide similarity rates among the ten novel Turkish isolates obtained in this investigation ranged from 98.5% to 100% for the CP gene of TMV (*Figure 4*). The amino acid similarity rates among the ten novel Turkish isolates ranged from 98.7 to 100 % (*Figure 5*). Subgroup I-a isolates had nucleotide similarity ranging from 99.8% to 87.9% among themselves, while subgroup I-b isolates had nucleotide similarity ratios ranging from 99.2% to 99%. Main clade II isolates had a similarity ratio between 95.4% and 96% nucleotide among themselves. The nucleotide sequence similarity ranged from 87.7% to 100% among global isolates. Furthermore, the homogeneity of amino acid ranged from 88.7% to 100%. These results indicate that there was no significant variation among the isolates.

RNA genomes encode the RNA-dependent RNA polymerase (RdRp) enzyme for the replicating of many plant RNA viruses. Due to the lack of proofreading activity in this process, a high rate of nucleotide sequence errors occurs (Drake and Holland, 1999). Mutation rates were first reported for tobacco mosaic virus and ranging from 10^{-3} to 10^{-6} nucleotide substitutions per site per round of replication in viruses that infect animals or bacteria (Malpica et al., 2002; Sanjuán et al., 2009; Sanjuán et al., 2010). Our study revealed that TMV isolates clustered in Clade II had more mutations in the CP gene and showed 86% nucleotide homogeneity with isolates clustered in other branches. On the other hand, isolates that form subsets of the main clade I exhibit nucleotide homogeneity

of over 88%. These findings, obtained for the CP gene of TMV, support the hypothesis that some RNA viruses exhibit less genetic variation (Nichol, 1996). Studies have reported genetic stability in some RNA virus populations, including turnip yellow mosaic virus (TYMV), tobacco mild green mosaic virus (TMGMV), and tomato brown rugose fruit virus (ToBRFV) (Skotnicki et al., 1993; Karanfil et al., 2023; Güller et al., 2023).

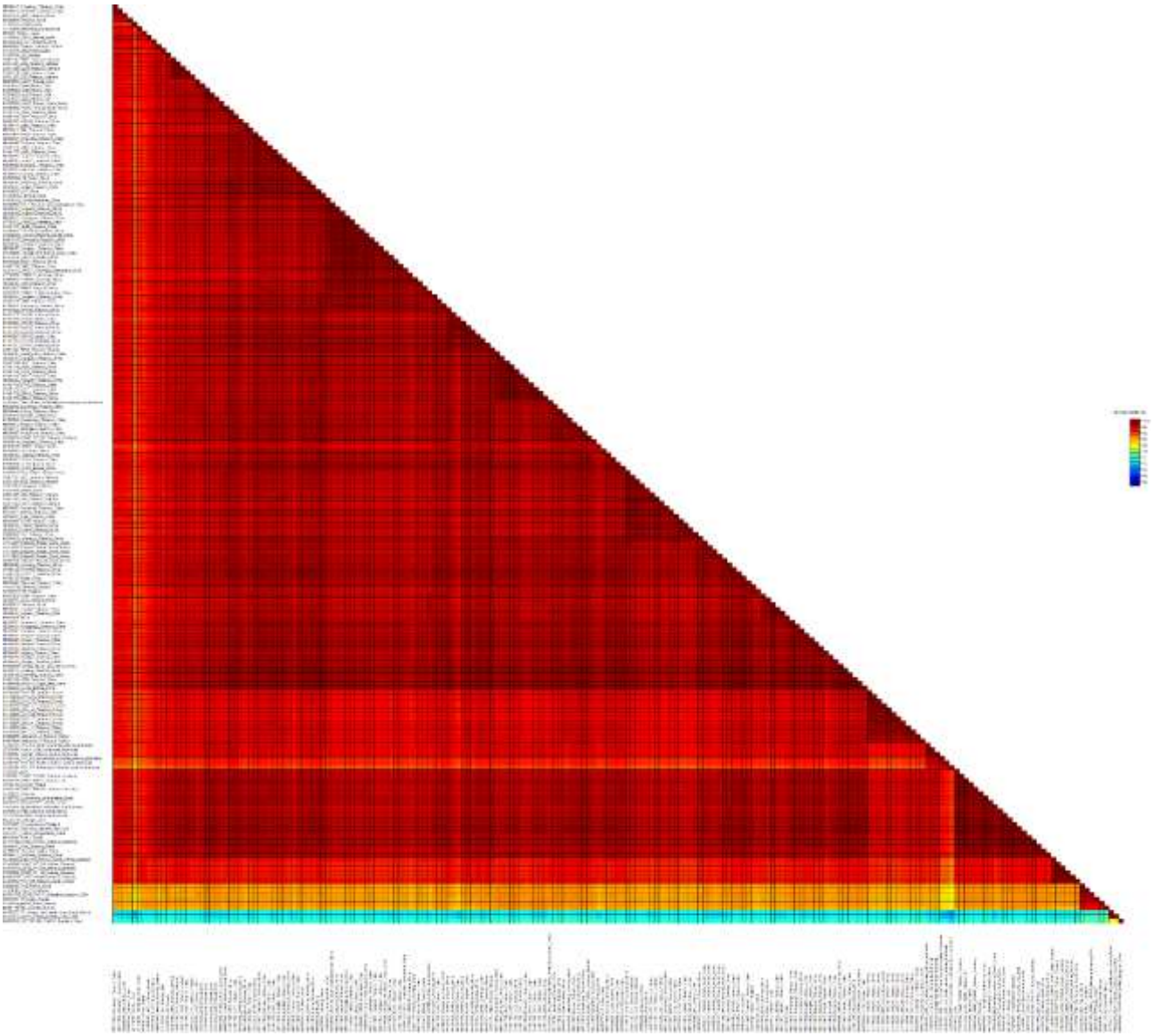

Figure 4. Similarity rates of tobacco mosaic virus isolates based on nucleotide sequences of the coat protein gene region

Phylogenetic analysis of the fully encoded CP gene region of TMV revealed that it is divided into two main groups: Clade I and II. The main group of Clade I is further divided into two subgroups (Ia and Ib). Previous studies have reported that TMV diverged into three branches (Alishiri et al., 2013; Kimaru et al., 2020). Molecular evolutionary analyses (n=206) were conducted using available isolates in GenBank to provide a detailed molecular characterization of both the main and subgroup branches. The main branches emerging in the phylogenetic trees did not reflect any geographical origin or host species from which the isolates were obtained (Alishiri et al., 2013). The low genetic diversity supports this, as does the determination that TMV isolates from different host species have the same dominant genotype. This suggests that host species do not contribute to the differentiation of the virus population, as observed in other plant viruses (Garcia-Arenal et al., 2001).

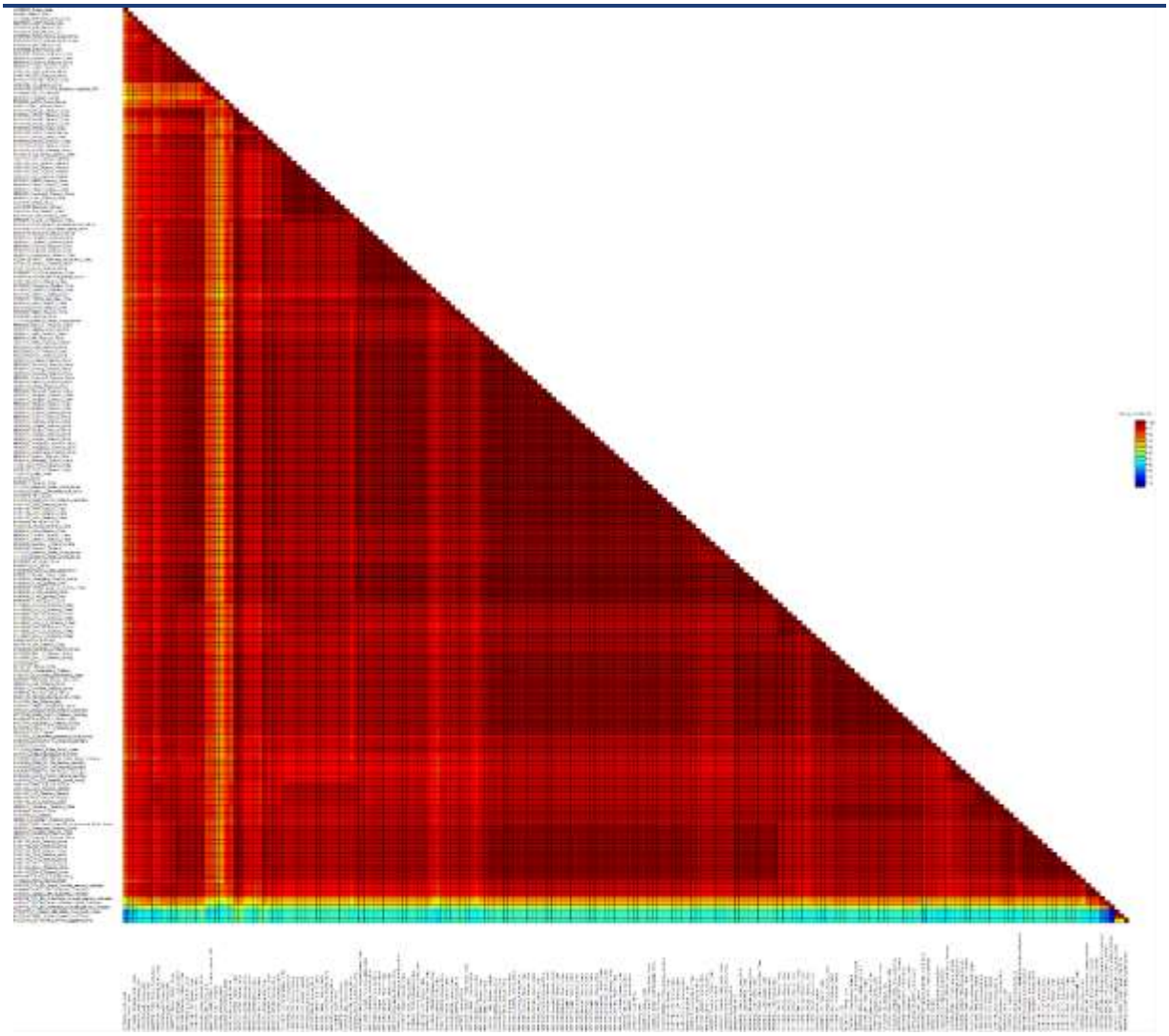

Figure 5. Similarity rates of tobacco mosaic virus isolates based on amino acid sequences of the coat protein gene region

4. Conclusions

The study comprehensively analyzed the genetic diversity of the complete CP gene of TMV, which plays a crucial role in host interaction. The results indicate that TMV isolates obtained from Türkiye do not exhibit a high level of diversity and global isolates also demonstrate genetic stability.

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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: Randa-Zelyüt, F.; Design: Randa-Zelyüt, F., Karanfil, A.; Data Collection or Processing: Karanfil, A.; Statistical Analyses: Randa-Zelyüt, F.; Literature Search: Randa-Zelyüt, F., Karanfil, A.; Writing, Review and Editing: Randa-Zelyüt, F., Karanfil, A.; Korkmaz, S.

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