

Genetic diversity and population structure of tomato brown rugose fruit virus (ToBRFV) variants from Antalya province, Turkey

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Abstract

In this study, bioinformatic analyses were carried out according to the fully coded CP and MP gene regions of the agent, using six novel tomato brown rugose fruit virus (ToBRFV) variants obtained from the production greenhouses in Antalya, where the infection was first detected in Turkey and global variants. Molecular evolutionary analyses using both CP and MP gene regions showed that all variants were distributed in three major clades. Population dynamics studies for both gene regions have shown that there was very low nucleotide diversity and haplotype diversity. The low haplotype diversity for the CP and MP genes indicated almost no recombination status. A strong negative selection was determined for CP and MP gene regions, $dN/dS=0.0877$ and $dN/dS=0.2104$, respectively. Neutrality test results revealed that ToBRFV populations are in an expansion phase. Pairwise comparisons were performed between populations separated in the geographic hierarchy as American, European, and Asian variants, and the findings showed intense gene flow and high genetic similarity ($F_{ST}<0,33$ and migration rate >1). The results of this study reveal the recent population structure of the virus and suggest that necessary precautions should be taken in the international seed trade against contaminated seeds.

Keywords: genetic diversity; pepper; ToBRFV; tomato; Turkey

Introduction

Tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annuum* L.), which are members of the Solanaceae family, are vegetables that are grown around the world and have a very high supply in the market. The annual tomato and pepper production amount reaches about 16 million tons in Turkey (Turkstat, 2022). A large part of this production volume is realized in the regions located in the western and southern parts of Turkey, which are dominated by the Mediterranean climate zone (TurkStat, 2022). In the production of these vegetables, particularly Antalya province has an important place in terms of exports and significantly contributes to the regional economy in terms of agriculture.

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Tomatoes and peppers have an important part in human nutrition, and in addition to providing countries with positive economic returns, their products are damaged by a variety of phytopathogens that may have an adverse effect on the quality and yield in growing conditions such as greenhouses and open fields. Furthermore, the damage and yield loss resulting from this may reach 100% because there is no direct control method for viruses. In Turkey, infections caused by tomato spotted wilt virus (TSWV), cucumber mosaic virus (CMV), southern tomato virus (STV), Pepper mild mottle virus (PMMoV), and tobacco mild green mosaic virus (TMGMV) have been reported in pepper and tomato cultivation areas (Çağlar *et al.*, 2013; Randa-Zelyüt *et al.*, 2023; Karanfil, 2021). However, ToBRFV was first detected in tomato-growing areas in Antalya province of the Mediterranean region of Turkey in 2019 (Fidan *et al.*, 2019). Later, Tomato brown rugose fruit virus (ToBRFV) infections were also reported in the Black Sea and Central Anatolia regions of the country (Çelik *et al.*, 2022).

ToBRFV is a member of the genus Tobamovirus, which has only a single genomic RNA in contrast to other genus members of the Virgaviridae family (King *et al.*, 2012). The single-stranded positive-sense (+ss RNA) genome of ToBRFV is 6392 nt in length and has four open reading frames: a movement protein (MP) (ORF2), a coat protein (CP) (ORF3), 183 kDa and 126 kDa (ORF1a and ORF1b) replication proteins, and 5' and 3' untranslated regions (Salem *et al.*, 2016; Luria *et al.*, 2017). The agent, which has typical genome organization of tobamovirus features, is a newly identified virus that has recently infected peppers and tomatoes (Salem *et al.*, 2016; Luria *et al.*, 2017). A recent study showed that the MP gene of ToBRFV plays a critical role in overcoming *Tm-2*²-mediated resistance in transgenic *Nicotiana benthamiana* plants and tomato plants and is a virulence determinant (Yan *et al.*, 2021).

ToBRFV infections have been reported in many countries in different parts of the world: Turkey (Fidan *et al.*, 2019), Italy (Panno *et al.*, 2019), Syria (Hasan *et al.*, 2022), Israel (Luria *et al.*, 2017) Malta, Hungary, France, Poland, Belgium, Norway, Bulgaria, Slovenia, Estonia, Portugal, Austria, Czech Republic, Cyprus (Eppo, 2023), Spain (Alfaro-Fernández *et al.*, 2021), Egypt (Amer and Mahmoud, 2020), Greece (Beris *et al.*, 2020), Mexico (Camacho-Beltrán *et al.* 2019), China (Yan *et al.*, 2019), Netherlands (van de Vossenberg *et al.*, 2020), Germany (Menzel *et al.*, 2019), the United States of America (California) (Ling *et al.*, 2019) (Florida) (Dey *et al.*, 2021), the United Kingdom (Skelton *et al.*, 2019). Symptoms induced by ToBRFV include mild to severe leaf mosaic, deformation in sepals, necrosis on young leaves, and puckering, while fruits exhibit marbling and brown rugose (Salem *et al.*, 2016; Luria *et al.*, 2017; Davino *et al.*, 2020). ToBRFV is efficiently transmitted mainly by mechanical contact, but it may also spread widely via contaminated seeds or fruits, just like other tobamoviruses (Salem *et al.*, 2016; Luria *et al.*, 2017). Because of its stable particle structure, the agent can also persist in agricultural tool equipment, on various surfaces of post-harvest greenhouses, in the soil, and be spread by bumblebee species like *Bumblebee terrestris* (Wilstermann and Ziebell, 2019; Levitzky *et al.*, 2019).

Nucleotide sequences of ToBRFV variants documented from different countries have been reported to be closely related genetically to each other, strongly supporting the hypothesis that the virus evolved from a unique common ancestor (Oladokun *et al.*, 2019). Similarly, very low genetic variation has been reported in TMGMV, one of the important species of tobamoviruses (Karanfil *et al.*, 2023). Furthermore, studies to reveal the genetic diversity and population structure of ToBRFV are very limited (Çelik *et al.*, 2022; Abrahamian *et al.*, 2022). In addition, many of the sequenced ToBRFV genomes were obtained from the Netherlands (van de Vossenberg *et al.*, 2020). On the other hand, genetic diversity studies of tobamoviruses, an ancient genus thought to have evolved with their angiosperm hosts thought to be 120-140 million years old, have greatly contributed to the development of all areas of virology and evolutionary biology, including the evolution of viruses (Fraile and Garcia-Arenal, 1990; Gibbs, 1999). The objective of this study is to understand how population structure is formed and the relationship between haplotypes using the complete encoded MP and CP gene regions of the variants obtained from tomato and pepper greenhouses in the Mediterranean region of Turkey as well as the global isolates provided by GenBank.

Materials and Methods

Sample collection and molecular assays

Field studies were carried out during the vegetative period of the plants in pepper and tomato greenhouses in Antalya province in the Mediterranean Region of Turkey in 2022. A total of 86 samples were collected from symptomatic and asymptomatic plants including 53 tomatoes and 33 pepper. Among these samples, 12 tomato samples and 10 pepper samples were asymptomatic. After that, the samples were stored at -20 °C until they were used in molecular studies.

Total RNA was isolated to extract viral RNA using the silica-based method described by Foissac *et al.* (2001). Synthesis studies were performed according to the protocol recommended by the manufacturer (Takara, Japan) using a random hexamer primer to obtain cDNAs from the samples. All cDNAs were screened by PCR to detect viral infection and amplify complete CP and MP genes of ToBRFV. Forward and reverse primer pairs (F- 5'-GACTTACGTCGCCGATTCAGA-3' and R- 5'-CGTGTGTTTTGCAGACACAATC-3' for the CP gene region, and F- 5'-GATGGCTCTTGTTAAGGGTAAA-3' and R- 5'-CCCATGCTGATGACAAAAAC-3' for the MP gene region) were designed using Snap Gene software (version 4.1.3). Furthermore, The PEPQRO variant from Mexico with GenBank accession number OQ427353 was used in primers designed to obtain the entire MP and CP gene regions.

Cloning, sequencing, and phylogenetic inferences

For molecular characterization, clear PCR-positive bands, 4 from tomatoes and 2 from peppers were selected for prokaryotic cloning, purified from the gel (GeneJET Gel Extraction Kit, Thermo Scientific, USA), inserted directly into the T-A cloning system (Promega, USA), and transformed into *E. coli* (JM 109 strain) electrophoretically. Purified recombinant plasmids from bacteria-containing inserts (GeneJET Plasmid Miniprep Kit, Thermo Scientific, USA) were sequenced by the Sanger sequencing method (BMLabosis BM Lab, Ankara, Turkey).

The nucleotide sequences of the MP and CP gene regions of the ToBRFV variants were obtained from sequencing, and the raw data were edited and matched using the software BioEdit version 7. 2. 5. (Hall, 1999). Sequence comparison using BlastN was also performed to ascertain the nucleotide similarity of the data. After that, the GenBank was loaded with all the sequence data, and the accession numbers for the MP and CP gene regions of ToBRFV were given.

Phylogenetic relationships were deduced based on the complete CP and MP gene regions of ToBRFV variants identified in the present study and global variants accessible in GenBank. The Nt sequences of the CP and MP genes were aligned using ClustalW in the MEGA 11 software (Tamura *et al.*, 2021). The unrooted phylogenetic trees were constructed utilizing the Neighbour-Joining (NJ) statistical method according to the Tamura-3 parameter model (Tamura, 1992) with uniform rates of partial deletion for the complete CP and MP gene regions of ToBRFV.

Haplotype network analyses

At the geographic level, haplotype networks were generated to represent the genetic variation of aligned sequences for each of the CP (N= 229) and MP (N=210) genes of ToBRFV. For the analysis, the countries according to the geographies from which the ToBRFV variants were obtained; It is classified as Asia populations (Jordan, Iran, Egypt, Palestine, Israel, China, Turkey), Europe (Netherlands, Germany, United Kingdom, Italy, Switzerland, Belgium, France, Czech Republic, Greece), and America (Canada, Peru, Mexico, U.S.A). Haplotype data files of the complete MP and CP genes of ToBRFV were implemented and calculated in the DnaSP V6.12.03 software (Rozas *et al.*, 2017). The haplotype networks were formed using the Median

Joining (MJ) algorithm (Bandelt *et al.*, 1999) and mapped using PopART software (<http://popart.otago.ac.nz>) (Leigh and Bryanti, 2015).

Genetic parameters, gene flow, and differences among geographic populations analyses

The number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π) analyses were performed based on complete MP and CP genes using DnaSP v.6.12.03 (Rozas *et al.*, 2017) software to determine genetic variations among geographic populations. At the geographical level $dN/dS = \omega$ ratio was also calculated to estimate selection pressure on MP and CP genes in the same software. However, if this ratio was less than 1, equal to 1, and greater than 1, the gene region was considered as negative selection, neutral and positive selection, respectively. Fu and Li D^* and F^* (Fu and Li, 1993), and Tajima's D (Tajima, 1989) test statistics were calculated to detect natural selections using the CP and MP nt sequences.

To evaluate genetic differentiation and gene flow between the geographic populations of ToBRFV according to MP and CP gene regions were used independent test statistics: KST^* (values near zero demonstrate that there is no population differentiation), Z^* , (lower values indicate less genetic differentiation between among populations), S_{nn} -the nearest neighbor statistic (value near to 1 show between populations are differentiated), the fixation index (F_{st}) (value is zero shows that there is no genetic differentiation between populations, whilst the value of 1 shows that there is a complete differentiation between populations) and the number of effective migrants (Nm) (Hudson *et al.*, 1992; Hudson, 2000; Wright, 1965). Generally, the following approach has been used to assess differences in virus populations: If $F_{st} > 0.33$ or $Nm < 1$, this shows restricted gene flow, but if $F_{st} < 0.33$ or $Nm > 1$, this suggests substantial gene flow between populations (Lu *et al.*, 2021; Randa-Zelyüt *et al.*, 2023).

Results

Survey observations and molecular detection of ToBRFV infection

During the 2022 crop growing season, a total of 86 plant samples, including 53 tomatoes and 33 peppers, were collected from plastic and glass greenhouses in the Mediterranean Region of Turkey. During the survey studies, discoloration, deformations, necrotic brown spots, non-severe yellow spots, and necrotic spots were observed on the leaves, especially on the fruits of symptomatic plants (Figure 1). Molecular studies revealed that 12 peppers and 22 tomatoes from these plants were infected with ToBRFV. However, negative results were obtained from 12 tomato and 10 pepper plants that did not show any symptoms. Thus, the gene-specific primers designed in this study successfully amplified the entire CP and MP gene regions of ToBRFV. Consequently, fragments of about 859 and 559 bp were obtained from the amplification assays, covering the complete MP and CP gene regions, respectively.

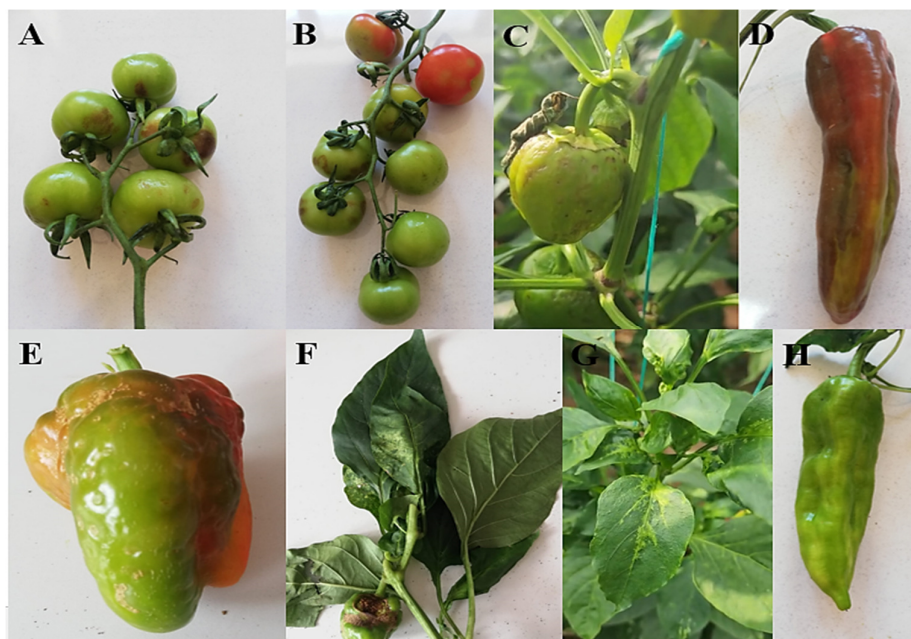


Figure 1. Symptoms triggered on fruit and leaves of ToBRFV-infected tomato and pepper plants (A, B, C) Necrosis, dark spot, and rugosity symptoms in tomato and pepper fruit; (D, E) discoloured and deformed pepper fruit; (F and G) Yellow spot and malformed leaves in pepper; (H) deformed pepper fruit

Sequence processing and molecular evolution analyses

Six samples were selected from different greenhouses where ToBRFV infection was detected. The nt sequences containing the complete CP and MP gene regions of novel Turkey ToBRFV variants were obtained from sequencing processing. These variants have been uploaded to GenBank under access numbers OR393421-OR393432.

Phylogenetic analyses were conducted based on the complete CP (N= 229) and MP (N=210) nt datasets and using the neighbor-joining (NJ) methods performed in MEGA11. For both gene regions, three major clades emerged as group I/II/III. However, most of the variants for both gene regions are clustered in two main groups (Figure 2). For the MP gene, a total of 210 variants were divided into major clades I, II, and III with 151, 51, and 8 variants, respectively (Figure 2a). Furthermore, only two variants from France of the total 229 CP gene variants formed group III (access no MW284988-22006291-L and access no MW284987-22006291-H), but the other 176 and 51 variants were distributed in main group I and group II, respectively (Figure 2b). The order of the variants was given in the clockwise order of the phylogenetic tree Supp. Tables 1 and 2.

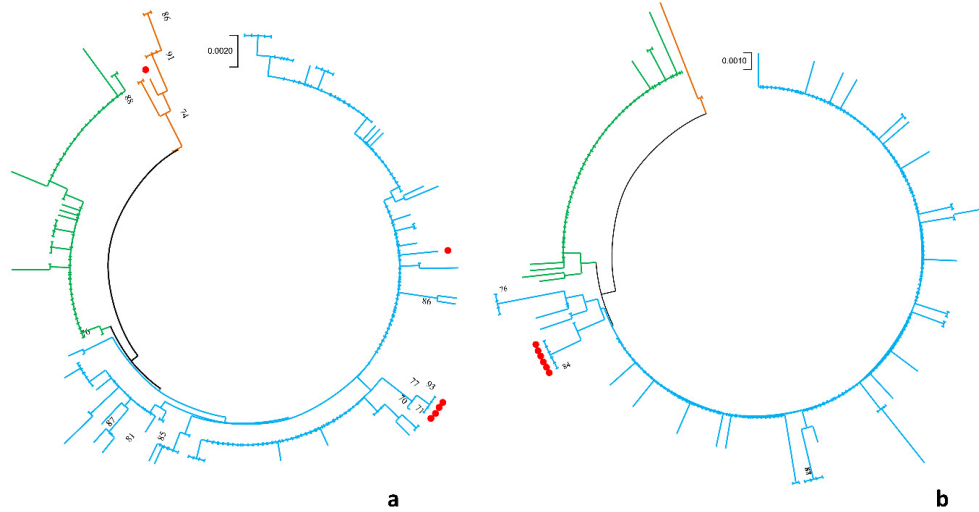


Figure 2. Phylogenetic analyses were conducted based on the complete (a) MP (N=210) and (b) CP (N=229) and nt datasets and using the neighbor-joining (NJ) methods performed in MEGA11. Red circles indicate variants from this study. Blue, green, and orange nodes represent groups I, II, and III, respectively. The order of the variants was given in the clockwise order of the phylogenetic tree Supp. Tables 1 and 2.

Haplotype diversity of ToBRFV variants

To analyze and visualize the MP and CP gene domains of ToBRFV variants at the geographical population level (Europe, Asia, and America populations) the haplotype network structure was constructed (Figure 3). A total of 66 haplotypes were found from 210 MP gene global variants and six novel MP Turkey variants had 3 haplotypes (Figure 3a). Furthermore, Hap_10, Hap_15, and Hap_16 showed the highest prevalence and were geographically found in the Europe variants. Also, Hap_22 demonstrated the greatest prevalence and was geographically found in Europe, Asia, and America (Figure 3a). The haplotype distribution analysis for the CP gene domain demonstrated that one haplotype was found in six novel Turkey variants and 42 haplotypes for the other global variants (Figure 3b). Especially, Hap_1 and Hap_2 had the greatest prevalence among all CP haplotypes. Moreover, the Hap_1 haplotype consisted of 43 variants from Europe alone, while the Hap_2 haplotype consisted of 127 variants from Europe, Asia, and the Americas (Figure 3b).

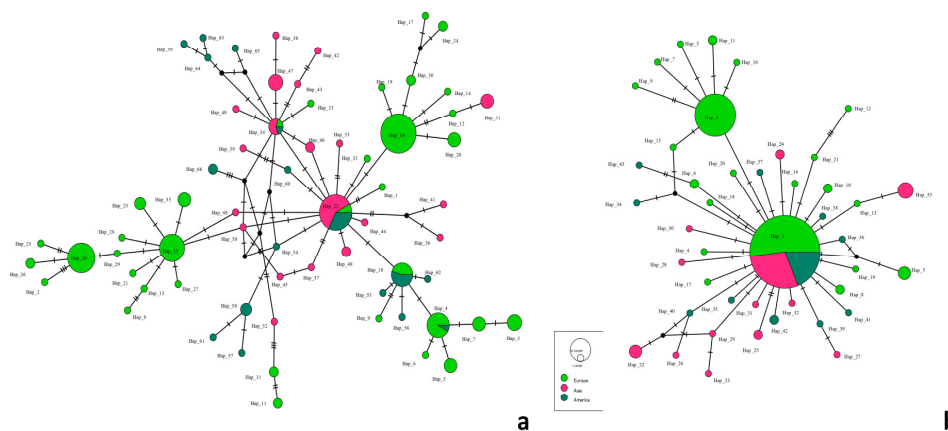


Figure 3. Network analysis of ToBRFV haplotypes. The colour of each node represents different geographical populations and allele types. Green: European, Pink: Asian, Dark green: American populations **a.** Indicates haplotypes of the MP gene **b.** Indicates haplotypes of the CP gene

Population genetic diversity and neutrality test results

Geographic populations of ToBRFV variants were used to evaluate genetic parameters to better comprehend the evolution of the CP and MP gene domains. The mean nucleotide diversities (π) for all populations of the MP and CP gene domains were 0,00474 and 0,00232, respectively (Table 1). Furthermore, the nucleotide diversity (π) values for the MP gene sequences varied from 0,00166 to 0,00273, and the haplotype diversity (Hd) values from 0,873 to 0,923 in geographic populations. On the other hand, the nucleotide diversity (π) values for the CP gene sequences ranged from 0,00166 to 0,00273, and the haplotype diversity (Hd) values from 0.534 to 0.662 in geographic populations (Table 1).

The dN/dS ratios for the MP and CP gene domains of ToBRFV were less than 1, indicating that both gene regions were under strong purifying (negative) pressure. However, these values revealed that the CP gene ($\omega=0,0877-0,2321$) was under more negative selection pressure than the MP gene ($\omega=0,1889-0,4275$). Moreover, in the analysis of the MP, CP, and geographic populations, neutrality tests (Tajima's D and Fu and Li's D^* & F^* tests) using three parameters consistently produced negative values for all tested variants. On the other hand, statistically insignificant negative values were obtained for both gene regions for Asian population variants, while similar results were obtained for the MP gene region of the American variants. Moreover, the neutrality test results were statistically significant for other geographic populations and for all variants constituting the gene clusters (Table 1).

To assess genetic differentiation and migration between three geographic populations consisting of Europe, Asia, and America, independent statistical tests based on permutation with 1000 replicates, K_{st}^* , Z^* , S_{nn} , F_{st} , and Nm (migration rate) were used (Table 2). However, the absolute value of F_{st} among geographic populations was less than 0,33 and the absolute migration rate was greater than 1. These results indicate a high gene flow among geographic population clusters of European, Asian, and American variants according to CP and MP gene domains. All pairwise comparisons for both gene regions showed significant and non-significant p values and relatively low values of K_{st}^* and Z^* metrics. Especially, the S_{nn} metric, which gave results less than 0, revealed that there was no significant genetic difference between geographic populations (Table 2).

Table 1. Genetic diversity parameters of ToBRFV variants and Neutrality test results

Gene region	Population	N	π	H	Hd	Fu and Li's D^*	Fu and Li's F^*	Tajima's D^*	dN/dS
CP	All	229	0,00232	43	0,658	-5,91497**	-5,31115**	-2,50838***	0,0877
	Europe	135	0,00202	21	0,662	-4,86077**	-4,59118**	-2,23107*	0,0626
	Asia	59	0,00273	13	0,597	-1,78538 ns	-2,11300 ns	-1,77628 ns	0,1180
	America	35	0,00166	11	0,534	-4,04474**	-4,13428**	-2,39292**	0,2321
MP	All	210	0,00474	66	0,946	-3,81419**	-3,65836**	-2,14259**	0,2104
	Europe	146	0,00509	43	0,923	-3,02492*	-3,00874**	-1,81161*	0,1889
	America	33	0,00355	17	0,900	-1,75609 ns	-1,98947 ns	-1,54755 ns	0,4275
	Asia	45	0,00350	20	0,873	-2,31818 ns	-2,62105 ns	-1,99934*	0,2538

Statistical significance: **, P < 0.02 ***, P < 0.001, **, P < 0.01 *, P < 0.05 Not significant: P > 0.10, 0.10 > P > 0.05

Table 2. Gene flow, genetic differences and migration rate results among geographic populations

Gene region	Populations	Kst*	Z*	S _{nn}	F _{ST}	Nm
MP	Europe (n=146)/Asia (n=45)	0,03847 (0,0000***)	8,72702 (0,0000***)	0,91020 (0,0000***)	0,10339	2,17
	Europe (n=146)/America (n=33)	0,02914 (0,0000***)	8,62833 (0,0000***)	0,79110 (0,0000***)	0,10935	2,04
	America (n=33)/Asia (n=45)	0,03185 (0,0000***)	6,98688 (0,0000***)	0,76629 (0,0000***)	0,06756	3,45
	Populations					
CP	Europe (n=135)/Asia (n=59)	0,05429 (0,0000***)	8,90529 (0,0000***)	0,71510 (0,0000***)	0,13383	1,62
	Europe (n=135)/America (n=35)	0,04026 (0,0000***)	8,65649 (0,0000***)	0,73766 (0,0000***)	0,13429	1,61
	Asia (n=59)/America (n=35)	0,00954 (0,0520 ns)	7,51047 (0,0880 ns)	0,58476 (0,0060**)	0,03377	7,15

Europe population: Netherlands, Greece, Switzerland, UK, Belgium, Germany, Italy, France, Czech Republic

Asia population: Israel, Turkey China, Egypt, Jordan, Palestine, Cyprus, Iran, and America population: Mexico, USA, Peru, Canada

Discussion

Several studies have revealed that members of the Solanaceae family are infected by numerous viral phytopathogens, which significantly reduce crop quality and yield (Hanssen *et al.*, 2010; Hančinský *et al.*, 2020). Of all viruses, tobamoviruses have been extensively studied. However, there is still much to discover about the severity of the infections they cause, the molecular mechanisms within their genomes, their interactions with other viruses, and their population genetic structures (Ilyas *et al.*, 2022; Karanfil *et al.*, 2023). ToBRFV, which has emerged with its destructive effect recently and succeeded in infecting tomato varieties with high resistance genes, has entered the field of phytopathology as an unusual tobamovirus. The agent is being reported in more and more countries every day, and the number of studies on it is also increasing. In this study, the population genetic structure and haplotype distributions formed recently by the viral agent were investigated by using the fully encoded CP and MP gene regions of variants obtained from the region where ToBRFV was first reported from Turkey and other global variants obtained from GenBank.

The severity and symptoms of infections caused by tobamoviruses are closely related to the host plant, species of the agent, and environmental conditions. Thus, it has been noted that symptoms induced by ToBRFV differ based on factors such as photoperiod, plant growth stage, plant age, and temperature (Caruso *et al.*, 2022). More specifically, like tomato plants, it has been reported that pepper plants often develop symptoms such as mosaic, mottling, yellowing, and necrotic lesions on their leaves, while tiny yellow to brown rugose spots and necrotic blotches appear on their fruits (Salem *et al.*, 2020; Fidan *et al.*, 2021). Similar to previous studies, deformations, and necrotic or brown spots were observed on the fruits of ToBRFV-infected tomato and pepper plants grown in greenhouse areas, while non-severe yellow spots and necrotic spots were seen on the leaves (Figure 1). This result indicates that ToBRFV, which has a very stable particle structure, may pose a potential risk for regional greenhouse cultivation.

Negative selection accelerates the rate at which harmful gene mutations are eliminated and a stable population genetic structure forms, whereas natural selection is another fundamental evolutionary mechanism and driving force behind virus population variation (Pérez *et al.*, 2008). The dN/dS ratio values obtained in this study indicated that the MP and CP genes were under strong purifying selection. Similar findings were also reported in the ToBRFV population genetics studies by Abrahamian *et al.* (2022) and Çelik *et al.* (2022). However, in a study by Hak and Spiegelman (2021), it was reported that the MP gene of ToBRFV is effective

in breaking resistance in plants containing the *Tm-2²* gene. On the other hand, in a study by Yan *et al.* (2021), when they removed the seven amino acids in the center of the MP gene, which is effective in breaking the *Tm-2²* resistance of ToBRFV, the virus lost its pathogenicity. Thus, MP has been reported to be under negative selection pressure, as any mutation at critical amino acid positions is destructive to the virus (Abrahamian *et al.*, 2022). In addition, when the nucleotide diversity values of the CP and MP gene regions obtained from this study were compared, it was determined that MP variants were higher than CP variants. In conclusion, these findings may indicate that the stable particle structure of ToBRFV plays an effective role in both gene regions for host and environmental adaptation, eliminating deleterious mutations for survival success.

The study of plant virus population genetics provides crucial information about their origins, transmission patterns, and adaptation to changing environments. Thus, in this study, demographic analyses were conducted to elucidate these processes of ToBRFV. Haplotype diversity and nucleotide diversity were computed among the clusters formed from the variants according to their geographic status to better understand the expansion status of the agent population using the fully encoded gene sequences of both CP and MP regions. The values from the genetic diversity analyses have been shown in Table 1, and the findings revealed that the MP gene region had extremely low nt diversity (π) (0,00350-0,00509) and high haplotype diversity (Hd) (0,873-0,923) according to geographic groups. In conclusion, considering the state of the MP gene, it may be possible to conclude that there are several genetically similar haplotypes and that the population has recently gone through an expansion phase. Therefore, despite the highest haplotype diversity, the lowest nt diversity values indicate relatively minor variation between haplotypes (Karanfil *et al.*, 2023). Furthermore, both low nt diversity (π) (0,00166- 0,00273) and very low haplotype diversity (Hd) (0,534- 0,662) values were obtained for the CP gene. This haplotype diversity value, which is quite low, may indicate that the recombination situation is almost non-existent. In fact, no recombination evidence was found in the analyses performed by Çelik *et al.* (2022) according to the ORF4/CP gene regions of 185 ToBRFV variants. Also, for geographic populations, neutrality tests (Tajima's D , Fu and Li F^* & D^*) were used to assess the selective neutrality of nt variability of gene regions and determine the rate of growth of ToBRFV populations based on both MP and CP gene regions. For the MP gene, the American and Asian populations gave negative results that were not statistically significant, while only the Asian populations for the CP gene. However, statistically significant negative results were obtained for the other populations and all variants. Similar findings were reported by Çelik *et al.* (2022). This finding could suggest a connection between the structural and functional MP and CP gene regions and the recent expansion of ToBRFV populations and their positive selection relationships.

Phylogenetic studies have been carried out recently to understand the molecular evolutionary relationships of ToBRFV. Herein, phylogenetic analyses were carried out with variants currently available in GenBank, including six novel CP (N=229) and MP (N=210) variants obtained from the region where ToBRFV infection was first detected in Turkey. The variants for both gene regions in the trees were distributed in three main branches as clades I/II/III. However, the main groups that emerged did not specifically reflect any geographic situation. The division of ToBRFV into three main clades has been similarly reported in studies by Çelik *et al.* (2022) and Abrahamian *et al.* (2022). On the other hand, the variants obtained from this study were clustered in the main clade I, which constitutes the largest branch for the CP gene, while these variants were clustered in major clades I and III for the MP gene. Interestingly, a single MP variant (Trky-52) from infected tomatoes clustered with well-known Peruvian variants such as S17 (OM892676) and S19 (OM892678).

In our study, F_{st} and migration rate values were evaluated together with K_{st}^* , Z^* , and S_{nn} test statistics to determine gene flows and genetic differences between geographic populations. Thus, it was calculated that the statistical values of K_{st}^* , Z^* , and S_{nn} obtained based on the CP and MP genes of ToBRFV were highly correlated with each other, and these values were quite low among European, American, and Asian populations. To confirm these findings, the fixation index (F_{st}) and migration rate (Nm) values were evaluated together, and

the results for both gene regions indicated that there was an intense and continuous gene flow and that the populations were very closely related to each other. Furthermore, in a study by Davino *et al.*, it was determined that ToBRFV particles were effectively localized in seeds, and the presence of the agent was reported in seeds imported from other countries in the Netherlands (HortiDaily, 2020). Our findings from these statistical tests indicate that the virus is effectively carried by seed. In addition, haplotype network maps were also obtained to evaluate the relationship of seed transmission with haplotypes of CP and MP variants and present the results. For the MP gene, Hap_22 included variants from the American, European, and Asian populations, while Hap_15, Hap_16, and Hap_10 cumulatively consisted of only European variants. In addition, 66 haplotypes were obtained from 210 variants of the MP gene (Figure 3a). Strikingly, in the haplotype map obtained from the CP gene region, Hap_1 included 127 out of a total of 229 variants from the American, European, and Asian populations (Figure 3b). Hap_22 included 43 variants that were only reported from Europe. Consequently, findings from haplotype network analyses showed consistent results with gene flow and genetic variation analyses.

Conclusions

The findings from this study showed bioinformatics analyses that the population of ToBRFV is going through an expansion phase and that contaminated seeds can be strongly effective in its global spread. However, haplotype analyses revealed that the MP gene behaves more dynamically than the Cp gene and may indeed play an important role in overcoming plant resistance genes. In future studies, there is a need to report more variants and examine other replication genes in terms of bioinformatics to obtain more genetic information.

Authors' Contributions

Conceptualization, Software; Supervision; Validation; Visualization; Writing - original draft: FRZ. First-writing draft: AG; Field surveys, primer design, laboratory experiments: AG and MU. All authors contributed to the writing of the manuscript. All authors have read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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