J Genet Resour 2026; 12(1): 84-93 *Homepage: http://sc.journals.umz.ac.ir/*

RESEARCH ARTICLE *DOI: 10.22080/jgr.2026.31520.1461*

Evolutionary Analysis of Tomato Brown Rugose Fruit Virus Reveals Purifying Selection on the Coat Protein Gene

Mahsa Mostafavi, Sahar Kordbacheh, Parastoo Pouraziz and Davoud Koolivand*

Department of Plant Protection, Faculty of Agriculture, University of Zanjan, Zanjan, Iran

ARTICLE INFO

Article history:
Received 27 November 2025
Accepted 29 December 2025
Available 22 January 2026

Keywords:
Coat protein
Genetic diversity
Phylogenetic analysis
Tobamovirus
Tomato brown rugose fruit virus

***Corresponding authors:**
✉ D. Koolivand
koolivand@znu.ac.ir

p-ISSN 2423-4257
e-ISSN 2588-2589

ABSTRACT

Tomato (*Solanum lycopersicum*) is one of the world's most economically important vegetable crops, yet its productivity is increasingly threatened by viral pathogens that reduce yield and fruit quality. Among emerging viruses, tomato brown rugose fruit virus (ToBRFV), a member of the genus *Tobamovirus*, has become a global concern due to its rapid mechanical and seed transmission and its capacity to infect both greenhouse and open-field tomato production systems. This study aimed to characterize the genetic diversity and evolutionary dynamics of ToBRFV populations in Iran using complete coat protein (CP) gene sequences. Twenty-eight symptomatic tomato samples were collected from greenhouses located in Bam and Qanad, Kerman Province, of which five were confirmed as ToBRFV-positive via PCR targeting a 620 bp fragment of the CP gene. Phylogenetic analysis was performed using the Maximum Likelihood method in MEGA (Version 12.1), whereas population genetic parameters and neutrality tests (Tajima's D; Fu and Li's D and F) were estimated using DnaSP. Recombination events were assessed using multiple algorithms implemented in RDP4 (Version 4.8). The Iranian isolates exhibited high haplotype diversity (Hd= 0.9) but low nucleotide diversity (π= 0.01375), indicating substantial haplotypic variation despite overall sequence conservation. A dN/dS ratio of 0.598 indicated that the CP gene is under purifying selection, and no statistically supported recombination events were detected. These findings demonstrate that ToBRFV populations in Iran maintain considerable genetic diversity at the haplotype level while preserving evolutionary stability in the CP gene, reflecting its functional constraints and importance in viral fitness.

Please cite this paper as: Mostafavi, M., Kordbacheh, S., Pouraziz, P., & Koolivand, D. (2026). Evolutionary analysis of tomato brown rugose fruit virus reveals purifying selection on the coat protein gene. *Journal of Genetic Resources*, 12(1), 84-93. doi: 10.22080/jgr.2026.31520.1461

Introduction

Tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) are widely cultivated solanaceous crops of major agronomic and economic importance worldwide (Baenas *et al.*, 2019. Like many other cultivated plants, these crops are susceptible to numerous pests and pathogens. Among these, viral diseases represent a major constraint to production, leading to reduced yields and deterioration of fruit quality and marketability, ultimately resulting in substantial economic losses (Hanssen and Lapidot, 2012; Jones and Naidu, 2019). Among plant viruses, members of the genus *Tobamovirus* represent a major threat to crop production, as this group includes several destructive pathogens such as Tobacco mosaic virus/TMV, Tomato mosaic virus/ToMV, Tomato mild mottle virus/ToMMV, Pepper mild mottle virus/PMMoV, and Cucumber green mottle mosaic virus/CGMMV (Zhang *et al.*, 2022). In addition to these well-established viruses, newly emerging viral diseases are increasingly posing serious challenges to global crop production and plant

health management (Çelik *et al*., 2022; Oladokun *et al*., 2019).

Recently, the emerging Tomato brown rugose fruit virus (ToBRFV) has been identified infecting tomato plants. ToBRFV is a highly destructive pathogen that has rapidly become a global threat to tomato and pepper production (Salem *et al*., 2016; Zhang *et al*., 2022).

Since its first official report in 2014, the virus has spread to multiple continents, affecting both open-field and greenhouse cultivation systems (Zhang *et al*., 2022). Infected plants exhibit a range of symptoms, including mosaic patterns, leaf narrowing, mild to severe chlorotic mosaic, deformation of young leaves, and necrosis (van de Vossenberg *et al*., 2020). In fruits, symptoms include discoloration of immature fruits, marbling, deformation, and necrotic lesions. These infections can lead to significant economic losses by reducing fruit quality, yield, and market value (Lanfermeijer *et al*., 2004). The rapid international spread of ToBRFV has raised serious concerns regarding agricultural biosecurity, trade regulations, and global food security. ToBRFV belongs to the genus *Tobamovirus*, members of which are primarily transmitted through mechanical means (Lanfermeijer *et al*., 2004). In addition, the virus can be transmitted through seeds, making it particularly difficult to control in intensive production systems (Davino *et al*., 2020; Salem *et al*., 2022). The ToBRFV genome consists of a single-stranded, positive-sense RNA of approximately 6.4 kb in length (Lanfermeijer *et al*., 2004). The typical tobamovirus genome contains four open reading frames (ORF1-ORF4). ORF1 and ORF2 are translated directly from the genomic RNA, whereas ORF3 and ORF4 are expressed from subgenomic RNAs (Ishibashi and Ishikawa, 2016; Knapp and Lewandowski, 2001). ORF3 encodes the movement protein (MP), which facilitates cell-to-cell movement of the virus, while ORF4 encodes the coat protein (CP) (Hak and Spiegelman, 2021; Yan *et al*., 2021).

Given its rapid global spread, integrated disease surveillance and development of resistant cultivars have become essential strategies for ToBRFV management. In Iran, ToBRFV was first reported from a greenhouse in Isfahan and Markazi by Ghorbani *et al*. (2021) and (Esmaeilzadeh *et al.* 2021, 2022). However, no study has yet investigated the possibility of intra-host genetic variation of ToBRFV among different organs of the same infected plant. Therefore, the present study was designed not only to characterize the phylogenetic relationships of Iranian ToBRFV isolates but also to investigate whether isolates obtained from different symptomatic organs of naturally infected tomato plants, including leaves, stems, fruits, and calyces, exhibit any tissue-associated genetic variation within the CP gene region. In addition, the possible relationship between symptom severity and phylogenetic clustering was comparatively evaluated to provide further insight into the evolutionary behavior of ToBRFV populations in Iran.

Materials and Methods

Sampling and virus detection

In late summer 2025, tomato crops in 20 greenhouses (around 50 hectares) located in Bam, Qanad, Kerman Province, were surveyed, and sampling was conducted based on characteristic symptoms associated with ToBRFV infection. A total of 28 symptomatic tomato plants showing mosaic patterns, leaf narrowing, chlorosis, deformation of young leaves, and necrosis were collected. To ensure comprehensive detection of the virus within each plant, samples were taken from multiple tissues, including leaves, stems, fruits, and sepals.

Total RNA was extracted from symptomatic tissues using the CTAB method (Gambino *et al*., 2008). Complementary DNA (cDNA) was synthesized from the extracted RNA using a commercial reverse transcription kit with random hexamer primers, following the manufacturer's instructions. Detection of ToBRFV was performed *via* polymerase chain reaction (PCR) using primers specific to the complete CP gene region (Forward: 5'CACAATCGCAACTCCAT CGC; Reverse: 5'GTGCCTACGGATGTGTAT GA), which amplify a ~620 bp fragment. PCR amplification was conducted under the following thermal cycling conditions: an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 90 s; followed by a final extension at 72°C for 10 min.

Phylogenetic analysis

To investigate the phylogenetic relationships between the ToBRFV isolates detected in Iran and those previously reported worldwide, complete CP gene sequences were aligned using MEGA version 12.1 (Tamura *et al*., 2021) with the Clustal W program (Larkin *et al*., 2007). Phylogenetic trees were constructed using the Maximum Likelihood (ML) method based on the Tamura 3-parameter model (Tamura, 1992), and branch support was assessed with 1,000 bootstrap replicates. Tree topology was evaluated for clustering patterns and evolutionary relationships. To ensure reliable rooting and minimize long-branch attraction, three closely related tobamoviruses were included as outgroups (Olmstead, 1996).

Split network analysis

To complement the phylogenetic tree and examine potential conflicting phylogenetic signals, a split network analysis was performed using SplitsTree v4.17.1. The aligned CP gene sequences in FASTA format were imported into the software, and a phylogenetic network was constructed using the Neighbor-Net algorithm. This approach enabled visualization of alternative evolutionary relationships and potential reticulation among ToBRFV isolates.

Population genetic parameters

Genetic diversity and population structure of the analyzed ToBRFV isolates were assessed using DnaSP v6.10.01 (Rozas *et al*., 2017). Several population genetic parameters were calculated, including nucleotide diversity (π), number of haplotypes (h), haplotype diversity (Hd), and number of polymorphic sites (S). Neutrality tests, including Tajima's D (Tajima, 1989) and Fu and Li's D and F statistics (Fu and Li, 1993), were performed to evaluate whether the viral population deviates from neutral evolution, which may indicate the influence of natural selection or demographic processes such as population expansion or bottlenecks.

Selective pressure acting on the CP gene was further assessed by estimating the ratio of nonsynonymous to synonymous substitutions (dN/dS, ω). A ratio of $\omega < 1$ indicates purifying (negative) selection, $\omega = 1$ indicates neutral evolution, and $\omega > 1$ suggests positive (diversifying) selection (Rozas *et al*., 2017). To evaluate genetic differentiation between phylogenetic clades, additional statistical tests, including KST*, Z*, Snn (Hudson, 2000), and FST, were performed. The statistical significance of these estimates was determined using permutation tests implemented in DnaSP.

Recombination

The potential occurrence of recombination events within the complete CP gene sequences of the ToBRFV isolates was assessed using RDP4 software (Ver. 4.8). Multiple detection algorithms were employed, including RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, and 3Seq, to ensure robust identification of recombination signals. Recombination events were considered credible only when supported by at least three independent methods and associated with a p-value< 0.05.

Results and Discussion

Sampling and virus detection

According to the samples studied in the city of Bam and Qanad, Kerman Province, the results showed that five samples tested positive for the virus. Negative control samples included in the assay showed no amplification, confirming the reliability of the results and indicating the absence of laboratory contamination (Fig.1).

Phylogenetic analysis

Phylogenetic relationships among the ToBRFV isolates obtained in this study and previously reported isolates were investigated using the complete sequence of the CP gene. A phylogenetic tree was constructed using the Maximum Likelihood method with the best-fit nucleotide substitution model implemented in MEGA 12.1 (Fig. 2). To properly root the tree and infer evolutionary direction, three tobamovirus sequences (NC_001367.1, AF332868.1, and JX534224) were included as outgroups.

The inclusion of these outgroup sequences provided a stable reference point for the phylogenetic reconstruction and confirmed that the analyzed ToBRFV isolates share a relatively recent common ancestor. The resulting phylogenetic topology revealed that the isolates grouped into several well-supported evolutionary lineages, indicating a structured but relatively

limited level of genetic diversity within the virus population. Notably, the phylogenetic tree clearly separated the analyzed sequences into four major clades (Fig. 2). This clustering pattern suggests that, despite the overall genetic similarity characteristic of ToBRFV populations worldwide, distinct evolutionary lineages are circulating. The distribution of isolates across these clades reflects ongoing microevolution within the CP gene, likely driven by mutation and local adaptation while maintaining a generally conserved genomic structure typical of tobamoviruses.

Fig. 1. The most common symptoms observed in the greenhouse, indicative of ToBRFV infection, were detected in both leaves and fruits

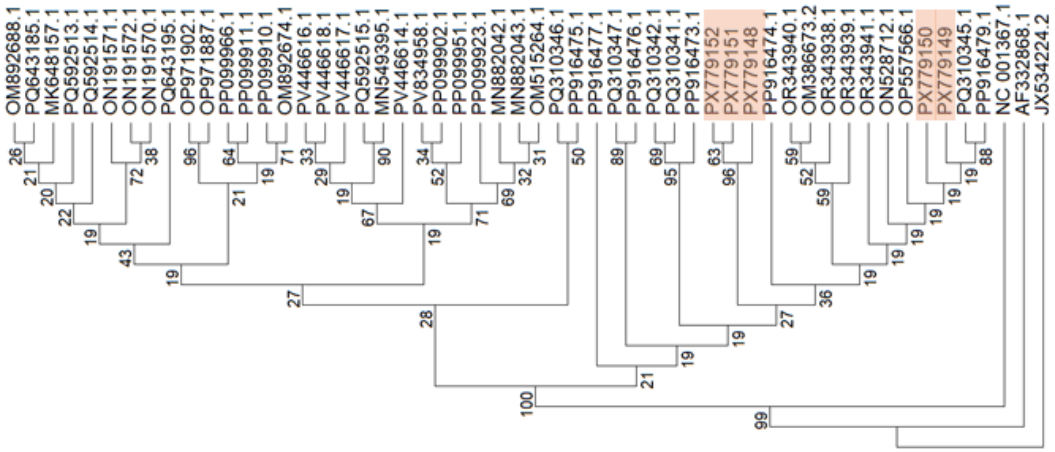

Fig. 2. Phylogenetic analysis of ToBRFV isolates based on the nucleotide sequence of the CP gene. Determination of the strains of the detected isolates based on the sequences of different strains in MEGA (Ver. 12.1) software using the Maximum Likelihood (ML) methods and the Tamura 3-parameter model.

Phylogenetic relationships and evolutionary insights

Phylogenetic analysis of the complete CP gene sequences revealed distinct evolutionary groupings among the global ToBRFV isolates. The resulting tree topology demonstrated the close genetic affiliation of sequences, clustering isolates from various geographical locations, including Iran, the Netherlands, Canada, the USA, Turkey, Albania, Jordan, Peru, and Mexico. This finding underscores the extensive global dissemination and interconnectedness of the virus population.

A key observation was the short branch lengths observed at the terminal ends of the tree, which signifies a low overall genetic distance among the contemporary isolates and suggests a relatively slow rate of evolutionary divergence within the highly conserved CP gene region. Consistent with previous studies, the newly identified Iranian isolates clustered tightly with previously reported Iranian sequences, confirming their provenance within the established national pool.

Phenotypically, all local Iranian isolates induced characteristic ToBRFV symptoms, including mosaic patterns, leaf deformation, and severe fruit rugosity. Symptom severity exhibited a range from mild to severe across the field samples; however, based on the drawn phylogenetic tree, no significant correlation was observed between symptom severity and the placement of isolates in phylogenetic clades, and further tests should be performed.

Collectively, the phylogenetic tree confirms that the newly identified Iranian isolates are integral components of a relatively homogeneous global ToBRFV population. Simultaneously, their specific clustering provides a distinct genetic signature that is crucial for future epidemiological tracking, outbreak source identification, and ongoing evolutionary studies of this economically devastating virus.

Network Analysis of Phylogenetic Relationships

To further explore potential complexities such as phylogenetic uncertainty or potential recombination signals masked by a strict tree structure, a phylogenetic network was constructed. The evolutionary relationships among the ToBRFV isolates were visualized using the Neighbor-Net algorithm implemented in SplitsTree4 software, based on the complete CP gene sequence data (Fig. 3).

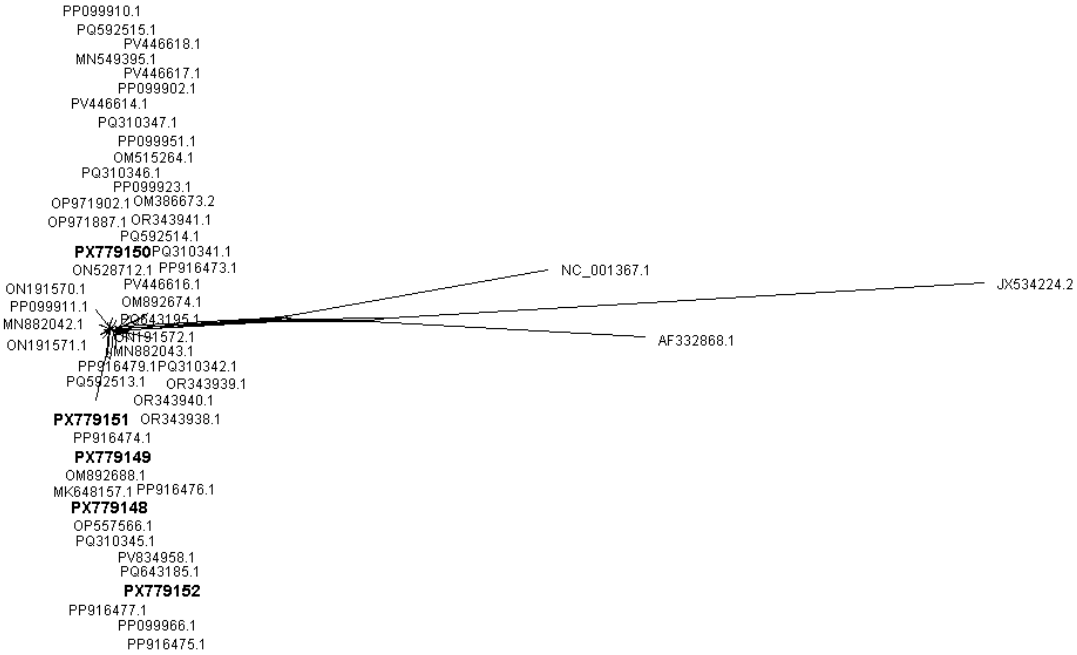

Fig. 3. Splits network analysis of ToBRFV CP gene sequences. This network was reconstructed using SplitsTree software to investigate the evolutionary relationships and complex genetic structure between isolates.

Network analysis revealed that the majority of ToBRFV isolates formed a compact cluster characterized by short branch lengths, indicating low genetic diversity and a high degree of evolutionary relatedness at the global level. The Iranian isolates were positioned within this central cluster and did not exhibit a distinct geographic structure, suggesting extensive gene flow and rapid dissemination of the virus across different regions.

In contrast, sequences of related tobamoviruses, including *Tomato mosaic virus* (ToMV), *Tobacco mosaic virus* (TMV), and *Tobacco mild green mosaic virus* (TMGMV), were clearly separated from the main ToBRFV cluster and appeared as distinct outgroup branches. This clear separation further supports the correct taxonomic identification of the analyzed isolates as ToBRFV.

Population genetic parameters

Population genetic analysis performed using DnaSP revealed considerable haplotypic variation among ToBRFV isolates, including the five newly identified Iranian samples. The overall haplotype diversity was high (Hd= 0.9), indicating the presence of multiple distinct haplotypes within the analyzed population despite the generally conserved nature of the CP gene.

Further analysis of the major phylogenetic groups showed that the three investigated clades (Clade I, Clade II, and Clade IV) also exhibited high haplotype diversity (Table 1). This pattern suggests that although the global ToBRFV population displays relatively low nucleotide divergence, it still maintains notable genetic variability at the haplotype level, reflecting ongoing microevolution within circulating virus populations.

Table 1. Data on population parameters of different ToBRFV populations based on the CP gene.

Population	*N*	*h*	*Hd*	*S*	η	*k*	*π*	dS	dN	ω
Iran (new isolate)	5	4	0.900	14	14	6.600	0.01375	0.02004	0.01198	0.59780
Clade I	28	11	0.918	13	14	2.799	0.00583	0.00845	0.00510	0.60355
Clade II	2	2	1.000	1	1	1.000	0.00208	0.00000	0.00272	-
Clade III	1	-	-	-	-	-	-	-	-	-
Clade IV	20	15	0.942	37	39	6.142	0.01285	0.01830	0.01147	0.62677

N= Number of sequence; *h*= Number of haplotypes; *Hd*= Haplotype diversity; *S*= Polymorphic sites; η= Total number of nucleotide; *k*= Average number of nucleotide difference; *π*= Nucleotide ersity; dS= Synonymous substitution Rate; dN= Non synonymous substitution Rate; ω= dN/dS ratio.

Although the number of polymorphic sites (S= 14) and the total number of nucleotide differences (η= 14) indicate measurable genetic variation among the analyzed isolates, the overall nucleotide diversity was relatively low (π= 0.01375). This low π value reflects a high level of nucleotide similarity among the Iranian ToBRFV samples, consistent with the conserved nature of the CP gene. The ratio of nonsynonymous to synonymous substitutions (ω= 0.598) further supports this observation, indicating that the Iranian isolates are under purifying (negative) selection, which restricts amino acid-altering mutations and preserves essential CP protein function.

At the global scale, clade-level analyses revealed a similar pattern. Clade I, which included 28 isolates, showed high haplotype diversity despite overall sequence conservation. Clade II (two isolates), Clade III (one isolate), and Clade IV (20 isolates containing 15 haplotypes; Hd = 0.942, π= 0.01285) also exhibited substantial haplotype variability. All clades were characterized by signatures of negative selection, reflecting evolutionary pressure to maintain CP protein integrity across diverse geographic regions.

Overall, the results demonstrate that ToBRFV populations are highly diverse at the haplotype level, yet exhibit low nucleotide diversity and are consistently shaped by strong purifying selection. This evolutionary pattern suggests that while the virus circulates widely and undergoes frequent mutation, functional constraints on the coat protein maintain a stable genomic structure. Such findings have important implications for epidemiological tracing, global spread monitoring, and the development of resistant cultivars, as the conserved CP region represents a stable molecular target.

The observed combination of low nucleotide diversity and strong purifying selection is consistent with previous global reports on ToBRFV population structure (Fougere *et al*., 2025), supporting the hypothesis of rapid worldwide dissemination accompanied by functional constraints on the CP gene (Abrahamian *et al*., 2022). Neutrality tests, including Tajima's D and Fu and Li's D* and F* statistics, were performed to further assess the evolutionary dynamics of the ToBRFV population (Table 2).

Neutrality tests were conducted to further evaluate the evolutionary dynamics of the ToBRFV population. In the newly identified Iranian isolates, Tajima's D as well as Fu and Li's D* and F* statistics showed negative but statistically non-significant values (p-value> 0.10), indicating no significant deviation from the neutral model of evolution. This pattern suggests that the virus population in Iran is currently close to evolutionary equilibrium, with no clear evidence of recent population expansion or strong directional selection (Abrahamian *et al*., 2022). A similar but statistically non-significant pattern was observed in Clade I, consistent with demographic stability**.** In Clade IV, neutrality statistics were more strongly negative; however, they still did not reach statistical significance. This trend may reflect a weak tendency toward the accumulation of rare mutations or a recent, mild population expansion, although the signal remains insufficient to draw firm evolutionary conclusions. Interpretation for Clades II and III is limited due to small sample sizes, preventing meaningful statistical inference.

Table 2. Genetic differentiation estimates for populations of the ToBRFV CP gene.

Population	Fu and Li's D*	Fu and Li's F*	Tajima's D
Iran (new isolate)	-0.13015, P> 0.10(ns)	-0.13846, P> 0.10(ns)	-0.13015, P> 0.10(ns)
Clade I	0.70396, P> 0.10(ns)	0.29684, P> 0.10(ns)	-0.75166, P> 0.10(ns)
Clade II	-	-	-
Clade III	-	-	-
Clade IV	-1.67242, P> 0.10(ns)	-1.97745, P> 0.10(ns)	-1.75578, 0.10 > P > 0.05(ns)

Overall, these neutrality test results align with the broader population genetic patterns observed in this study: high haplotype diversity coupled with low nucleotide diversity, and a dN/dS ratio below one, collectively indicating that purifying (negative) selection is the dominant evolutionary force maintaining the structural and functional stability of the CP gene. Combined with the absence of credible recombination events, the neutrality patterns suggest that ToBRFV evolution in both Iranian and global populations is primarily driven by limited point mutations and strong functional constraints, rather than by recombination, rapid diversification, or positive selection (Abrahamian *et al*., 2022). Such evolutionary behavior is characteristic of RNA viruses with essential structural proteins, where genome stability must be maintained despite ongoing mutation, allowing only gradual, functionally tolerated adaptations (Abrahamian *et al*., 2022; Elena and Sanjuán, 2005; Panno *et al*., 2020).

To evaluate genetic differentiation and population subdivision among the ToBRFV clades, several sequence-based statistics were calculated using DnaSP, including Ks*, Kst*, Z*, the nearest-neighbor statistic (Snn), and the fixation index (FST) (Table 3). These indices provide complementary measures of genetic structuring, enabling assessment of the degree of separation between clades and the extent of gene flow occurring among them. By comparing these parameters across the major phylogenetic groups, it is possible to identify whether the observed clades represent distinct evolutionary units or are interconnected through frequent genetic exchange within a broadly mixed global population.

Table 3. Neutrality tests of ToBRFV isolates based on phylogroups.

Comparison	$^{\alpha}K_S$*	$^{\alpha}K_{ST}$*	*p*- Value	$^{\alpha}Z$*	*p*- Value	S_{nn}	*p*- Value	$^{\beta}F_{ST}$
Clade I (n=2^8)/clade II (n=2)	1.24011	0.01273	0.1730 ns	5.09024	0.1500 ns	1.00000	0.0100 *	0.37795
Clade I (n=2^8)/clade III (n=1)	-	-	-	-	-	-	-	-
Clade I(n=2^8)/clade IV (n=20)	1.44547	0.09355	0.0000 ***	5.79798	0.0000 ***	1.00000	0.0000 ***	0.20725
Clade II (n=2)/clade III (n=1)	-	-	-	-	-	-	-	-
Clade II (n=2)/clade IV (n=20)	1.74210	0.5210 ns	0.6390 ns	4.46740	0.4620 ns	1.00000	0.0150 *	0.31326
Clade III (n=1)/clade IV (n=20)	-	-	-	-	-	-	-	-

The results indicated that the highest level of genetic differentiation occurred between Clade I and Clade IV. In this comparison, the KST*, Z*, and Snn statistics were all highly significant (P< 0.001), and the Snn value of 1.000 suggested complete sequence separation between these two

clades. The βFST value (0.20725) further supports moderate to high genetic differentiation, indicating restricted gene flow between these populations.

In contrast, comparisons between Clade I and Clade II, as well as between Clade II and Clade IV, revealed weaker and mostly non-significant genetic differentiation. This pattern is likely influenced by the small sample size available for Clade II, which limits the statistical power of the analysis. Similarly, the absence of sufficient data for Clade III prevented reliable statistical inference for this group. Overall, these findings are consistent with the broader genetic diversity patterns observed in this study, characterized by high haplotype diversity, low nucleotide diversity, and a dN/dS ratio below one. Together, these indicators suggest a structured viral population evolving under stable purifying (negative) selection. The observed differentiation between certain clades is therefore likely associated with geographic separation and independent dissemination routes of the virus. At the same time, the absence of clear recombination signals indicates that the evolution of these populations has largely occurred through the gradual accumulation of point mutations rather than through recombination-driven diversification (Abrahamian *et al*., 2022).

Genetic recombination among the examined isolates was assessed using RDP4 software (Ver. 4.8). Although one putative recombination event was initially detected, it was supported by only a single detection algorithm. Because our analysis required confirmation by at least three independent methods to be considered reliable, no credible recombination event was identified. This result indicates that the Iranian isolates show no significant evidence of recombination within the CP gene (Esmaeilzadeh *et al*., 2023a; 2023b) and that their genetic variation is primarily attributable to limited point mutations.

Overall, these findings demonstrate that, despite the presence of considerable haplotypic diversity at both regional and global scales, the ToBRFV CP gene remains highly conserved. Such a pattern is characteristic of RNA viruses encoding essential structural proteins, where strong functional constraints maintain sequence stability. The absence of recombination also suggests that ToBRFV evolution is driven mainly by gradual, mutation-based diversification rather than by major genomic rearrangements. This emphasizes the importance of ongoing genetic surveillance to detect possible future shifts in pathogenicity, geographic dispersal patterns, or the emergence of variants with implications for resistance breeding and disease management (Abrahamian *et al*., 2022; Ishibashi and Ishikawa, 2016).

Conclusion

In this study, extensive areas were not sampled; however, genetic analysis of the complete CP gene in the examined isolates from a part of Bam and Qanad Kerman Province revealed high haplotype diversity despite relatively low overall nucleotide diversity, indicating the presence of limited but structured genetic diversity within the circulating viral population. Similar patterns of restricted sequence divergence have been reported in other regions, suggesting that viral evolution may be constrained by the functional requirements of the CP gene. Phylogenetic analysis showed that the Iranian isolates are closely related to previously reported global strains. In addition, neutrality test results were negative but not statistically significant, supporting the hypothesis that the CP gene region is evolving under relative evolutionary stability rather than undergoing rapid demographic expansion or strong directional selection. The absence of recombination is consistent with the evolutionary characteristics of many plant RNA viruses, in which the gradual accumulation of mutations plays a major role in shaping genetic diversity. Overall, the molecular evolutionary analyses suggest that the CP gene of ToBRFV circulating in Iran remains relatively conserved and is predominantly subject to purifying selection. This evolutionary stability may have practical implications for virus detection strategies and for long-term epidemiological monitoring of ToBRFV populations.

Conflict of interests

The authors declare no conflict of interest.

References

Abrahamian, P., Cai, W., Nunziata, S. O., Ling, K.-S., Jaiswal, N., Mavrodieva, V. A., Rivera, Y., & Nakhla, M. K. (2022). Comparative

analysis of tomato brown rugose fruit virus isolates shows limited genetic diversity. *Viruses*, 14(12), 2816. https://www.mdpi.com/1999-4915/14/12/2816

Baenas, N., Belović, M., Ilic, N., Moreno, D. A., & García-Viguera, C. (2019). Industrial use of pepper (*Capsicum annum* L.) derived products: Technological benefits and biological advantages. *Food Chemstry*, 274, 872-885. https://doi.org/10.1016/j.foodchem.2018.09.047

Çelik, A., Coşkan, S., Morca, A. F., Santosa, A. I., & Koolivand, D. (2022). Insight into population structure and evolutionary analysis of the emerging tomato brown rugose fruit virus. *Plants*, 11(23), 3279. https://doi.org/10.3390/plants11233279

Davino, S., Caruso, A. G., Bertacca, S., Barone, S., & Panno, S. (2020). Tomato brown rugose fruit virus: Seed transmission rate and efficacy of different seed disinfection treatments. *Plants*, 9(11), 1615. https://doi.org/10.3390/plants9111615

Elena, S. F., & Sanjuán, R. (2005). Adaptive value of high mutation rates of RNA viruses: Separating causes from consequences. *Journal of Virology*, 79(18), 11555-11558. https://doi.org/10.1128/JVI.79.18.11555-11558.2005

Esmaeilzadeh, F., Koolivand, D. (2021). Occurrence of tomato brown rugose fruit virus in tomato in Iran. *Journal of Plant Pathology*, 104(3) 457. https://doi.org/10.1007/s42161-021-01009-7

Esmaeilzadeh, F., Koolivand, D. (2022). First report of tomato brown rugose fruit virus infecting bell pepper in Iran. *Journal of Plant Pathology*, 104(2) 893. https://doi.org/10.1007/s42161-022-01094-2

Esmaeilzadeh, F., Santosa, A. I., Çelik, A., & Koolivand, D. (2023a). Revealing an Iranian isolate of tomato brown rugose fruit virus: Complete genome analysis and mechanical transmission. *Microorganisms*, 11(10), 2434. https://doi.org/10.3390/microorganisms11102434

Esmaeilzadeh, F., & Koolivand, D. (2023b). Co-infection of tomato brown rugose fruit virus and tomato spotted wilt virus in pepper from Iran. *Journal of Plant Pathology*, 105(4), 1129–1134. https://doi.org/10.1007/s42161-023-01436-8

Fougere, G. C., Xu, D., Gaiero, J. R., McCreary, C., Marchand, G., Despres, C., Wang, A., Fall, M. L., & Griffiths, J. S. (2025). Genomic diversity of tomato brown rugose fruit virus in Canadian greenhouse production systems. *Viruses*, 17(5), 696. https://doi.org/10.3390/v17050696

Fu, Y.-X., & Li, W.-H. (1993). Statistical tests of neutrality of mutations. *Genetics*, 133(3), 693-709. https://doi.org/10.1093/genetics/133.3.693

Gambino, G., Perrone, I., & Gribaudo, I. (2008). A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. *Phytochemical Analysis*, 19(6), 520-525. https://doi.org/10.1002/pca.1078

Ghorbani, A., Rostami, M., Seifi, S., & Izadpanah, K. (2021). First report of tomato brown rugose fruit virus in greenhouse tomato in Iran. *New Disease Reports*, 44, e12040. https://doi.org/10.1002/ndr2.12040

Hak, H., & Spiegelman, Z. (2021). The tomato brown rugose fruit virus movement protein overcomes Tm-2² resistance in tomato while attenuating viral transport. *Molecular Plant-Microbe Interactions*, 34(9), 1024-1032. https://doi.org/10.1094/MPMI-01-21-0023-R

Hanssen, I. M., & Lapidot, M. (2012). Major tomato viruses in the Mediterranean basin. *Advances in Virus Research*, 84, 31-66. https://doi.org/10.1016/B978-0-12-394314-9.00002-6

Hudson, R. R. (2000). A new statistic for detecting genetic differentiation. *Genetics*, 155(4), 2011-2014. https://doi.org/10.1093/genetics/155.4.2011

Ishibashi, K., & Ishikawa, M. (2016). Replication of tobamovirus RNA. *Annual Review of Phytopathology*, 54, 55-78. https://doi.org/10.1146/annurev-phyto-080615-100217

Jones, R. A. C., & Naidu, R. A. (2019). Global dimensions of plant virus diseases: Current status and future perspectives. *Annual Review of Virology*, 6(1), 387-409. https://doi.org/10.1146/annurev-virology-092818-015606

Knapp, E., & Lewandowski, D. J. (2001). Tobacco mosaic virus, not just a single component virus anymore. *Molecular Plant Pathology*, 2(3), 117-123. https://doi.org/10.1046/j.1364-3703.2001.00064.x

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., & Lopez, R. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, 23(21), 2947-2948. https://doi.org/10.1093/bioinformatics/btm404

Lanfermeijer, F. C., Jiang, G., Ferwerda, M. A., Dijkhuis, J., de Haan, P., Yang, R., & Hille, J. (2004). The durable resistance gene Tm-22 from tomato confers resistance against ToMV in tobacco and preserves its viral specificity. *Plant Science*, 167(4), 687-692. https://doi.org/10.1016/j.plantsci.2004.04.027

Oladokun, J., Halabi, M., Barua, P., & Nath, P. (2019). Tomato brown rugose fruit disease: Current distribution, knowledge and future prospects. *Plant Pathology*, 68(9), 1579-1586. https://doi.org/10.1111/ppa.13062

Olmstead, R. G. (1996). Molecular systematics (2nd ed.), edited by David M. Hillis, Craig Moritz, and Barbara K. Mable. *Systematic Biology*, 45(4), 607-608. https://doi.org/10.1093/sysbio/45.4.607

Panno, S., Caruso, A. G., Barone, S., Lo Bosco, G., Rangel, E. A., & Davino, S. (2020). Spread of tomato brown rugose fruit virus in Sicily and evaluation of the spatiotemporal dispersion in experimental conditions. *Agronomy*, 10(6), 834. https://doi.org/10.3390/agronomy10060834

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution*, 34(12), 3299-3302. https://doi.org/10.1093/molbev/msx248

Salem, N., Mansour, A., Ciuffo, M., Falk, B. W., & Turina, M. (2016). A new tobamovirus infecting tomato crops in Jordan. *Archives of Virology*, 161(2), 503-506. https://doi.org/10.1007/s00705-015-2677-7

Salem, N. M., Sulaiman, A., Samarah, N., Turina, M., & Vallino, M. (2022). Localization and mechanical transmission of tomato brown rugose fruit virus in tomato seeds. *Plant Disease*, 106(1), 275-281. https://doi.org/10.1094/PDIS-11-20-2413-RE

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123(3), 585-595. https://doi.org/10.1093/genetics/123.3.585

Tamura, K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution*, 9(4), 678-687. https://doi.org/10.1093/oxfordjournals.molbev.a040752

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*, 38(7), 3022-3027. https://doi.org/10.1093/molbev/msab120

van de Vossenberg, B., Visser, M., Bruinsma, M., Koenraadt, H. M. S., Westenberg, M., & Botermans, M. (2020). Real-time tracking of tomato brown rugose fruit virus (ToBRFV) outbreaks in the Netherlands using Nextstrain. *PLoS One*, 15(10), e0234671. https://doi.org/10.1371/journal.pone.0234671

Yan, Z. Y., Ma, H. Y., Wang, L., Tettey, C., Zhao, M. S., Geng, C., Tian, Y. P., & Li, X. D. (2021). Identification of genetic determinants of tomato brown rugose fruit virus that enable infection of plants harbouring the Tm-2^2 resistance gene. *Molecular Plant Pathology*, 22(11), 1347-1357. https://doi.org/10.1111/mpp.13115

Zhang, S., Griffiths, J. S., Marchand, G., Bernards, M. A., & Wang, A. (2022). Tomato brown rugose fruit virus: An emerging and rapidly spreading plant RNA virus that threatens tomato production worldwide. *Molecular Plant Pathology*, 23(9), 1262-1277. https://doi.org/10.1111/mpp.13229