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Molecular detection and characterization of viruses infecting greenhouse-grown tomatoes in Albania

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Summary. During the 2023–2024 growing season, a total of 45 tomato greenhouses were visited across five major production regions of Albania (Berat, Lushnje, Fier, Tirana, and Shkodra). A total of 196 greenhouse-grown tomato leaf samples, representing 31 varieties, were collected from plants showing virus-like symptoms. All samples were tested by RT-PCR and qPCR assays for the possible presence of significant tomato-infecting viruses and viroids, including alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), tomato brown rugose fruit virus (ToBRFV), tomato yellow leaf curl virus (TYLCV), tomato chlorosis virus (ToCV), tomato infectious chlorosis virus (TICV), tomato mottle mosaic virus (ToMMV), tomato spotted wilt virus (TSWV), tomato mild mottle virus (ToMMoV), tobacco mosaic virus (TMV), pepino mosaic virus (PepMV), potato viruses X and Y (PVX, PVY), potato leafroll virus (PLRV), tomato apical stunt viroid (TASVd), and potato spindle tuber viroid (PSTVd). In addition, Next-generation sequencing (NGS) using *Illumina* and *MinION* nanopore technologies were performed to characterize the complete genome of two Albanian ToBRFV isolates. RT-PCR and qPCR showed that ToCV, ToBRFV, AMV, and CMV were present in 25, 9.1, 4.1, and 4.1% of the samples, respectively, whereas all remaining viruses and viroids were absent. *Illumina* and *MinION* sequencing unveiled the complete genome sequences of ToBRFV (6,381 nucleotides) and of two additional viruses, i.e., Southern tomato virus, STV (3,437 nts) and tobacco vein clearing virus, TVCV (7,596 nts), both of which were not included in our initial screening. The two latter viruses were afterward diagnosed by PCR and found to be present in 2 and 3% of the tested samples, respectively. ToCV was most prevalent in Lushnje and Fier regions, while ToBRFV in Berat and Fier. The phylogenetic analyses predominantly clustered together the different Albanian viruses isolate, suggesting a local or regional origin. This study reports for the first time the presence of STV and TVCV in Albania and highlights the emergence of ToCV and ToBRFV in the country.

Keywords. RT-PCR, qPCR, next-generation sequencing, phylogenetic analysis, emerging viruses.

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INTRODUCTION

Tomato production in Albania has shown significant growth in recent years, with a total cultivated area reaching 6,663 hectares (FAO, 2022). Albania harbored ancient local tomato varieties, known for their potential to enhance agricultural diversity and emphasize rural economic prospects. Thanks to the dedicated efforts of Albanian farmers, the Plant Genetic Resources Institute (Agriculture University of Tirana) now houses 67 autochthon cultivated and inherited tomato varieties, reflecting the Albanian wealth agricultural heritage. In Albania, vegetable cultivation spans a total of 42,994 hectares (Dhuli, 2022), with 6,693 hectares dedicated to tomato farming, including 2,100 hectares for greenhouse-grown tomatoes. The country's annual vegetable production amounts to 1.3 million tons, of which tomatoes account for approximately 300,000 tons. In 2022, Albania exported an average of 29,760 tons of tomatoes, valued at approximately 26.2 million USD. The country's tomato cultivation is concentrated in five main regions, with Fier being the most productive. It accounts for 24.8% of the total cultivated area and 37.3% of total production, followed by Berat, which represents 14.7% of the cultivation area and 17.7% of production. Shkodra ranks third, with around 5.5% of both the cultivated area and total production (Dhuli, 2022).

In Albania, several viruses have been reported to infect tomatoes, causing various symptoms that impact both production and quality. Notably, tomato brown rugose fruit virus (ToBRFV), first detected in 2022, has emerged as a major concern due to its severe symptoms, including fruit deformation and plant stunting on different cultivars (Orfanidou *et al*., 2022a). The alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), tomato spotted wilt virus (TSWV) and potato virus Y (PVY), all identified for the first time in 2005 (Finetti-Sialer *et al*., 2005), together with tomato chlorosis virus (ToCV) in 2022 (Orfanidou *et al*., 2022b), were associated with different symptoms, i.e., yellowing, leaf curl, and yield reduction in the country. These viruses, together with many others uninvestigated previously in the country are of a significant concern for tomato farmers, particularly in greenhouse systems where conditions may favor the spread of viral diseases.

This study extensively investigates for the first time the presence and prevalence of some important tomato viruses and viroids, possibly infecting greenhouses-grown tomato plants in the country, i.e., ToCV, alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), tomato brown rugose fruit virus (ToBRFV), tomato yellow leaf curl virus (TYLCV), tomato infectious chlorosis virus (TICV), tomato mottle mosaic virus (ToMMV), tomato spotted wilt virus (TSWV), tomato mild mottle virus (TMMoV), tobacco mosaic virus (TMV), pepino mosaic virus (PepMV), potato viruses X and Y (PVX, PVY), potato leafroll virus (PLRV), tomato apical stunt viroid (TASVd), and potato spindle tuber viroid (PSTVd); for which the results are hereafter reported.

MATERIALS AND METHODS

Source and location of plant material

During the 2023-2024 growing season, a total of 45 tomato greenhouses across five regions, i.e., Berat (5), Lushnje (25), Fier (13), Tirana (1), and Shkodra (1), were visited. These regions are known for their significant tomato production, accounting for 70% of the national output. A total of 196 tomato leaf samples, representing 31 varieties (11 of which were local; Supplementary Table 1), were collected from plants exhibiting virus-like symptoms, such as yellowing, stunting, mottling, chlorosis, necrosis, deformed and brown fruits. The samples were placed in labeled plastic bags with wet filter paper and were transported in a cool box for laboratory processing.

Extraction of total nucleic acids (DNA and RNA)

Two protocols were adopted for extracting the total nucleic acids (TNA). The total RNAs were extracted from 0.1 g of leaf veins, homogenized in 1 mL grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc pH 5.0 and 2.5% w/v PVP-40), and purified using Silica particles, according to Foissac *et al*. (2001). The total DNAs were extracted from 1 g of leaf tissues, following the CTAB protocol (Doyle, 1991). The TNA quality was evaluated by NanoDrop™ One/OneC Microvolume UV–Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, United States) and electrophoresis in 1.2% agarose gel of 1X TBE buffer (Tris-Borate-EDTA). The purified TNAs were then stored at -20°C until processing.

Complementary DNA synthesis (cDNA), PCR and qPCR assays

Total RNA (0.5 µg) was reverse-transcribed into cDNA using 1 µL of random hexamer primers (0.5 mg/

Table 1. List of primers used in RT-PCR and qPCR for detecting tomato viruses and viroids, and for complete genome PCR amplification and Sanger sequencing of ToBRFV, STV and TVCV. F and R: forward and reverse primers, respectively.

Virus	Primer sequence (5´ - 3´)	PCR amplicon (bp)	qPCR amplicon (bp)	Reference
Tomato brown rugose fruit virus (ToBRFV)	F-GTAAGGCTTGCAAAATTTCGTTCG R-CTTTGGTTTTTGTCTGGTTTCGG		79	(Panno *et al*., 2019)
	F-GAAGTCCCGATGTCTGTAAGG R-GTGCCTACGGATGTGTATGA	842		(Ling *et al*., 2019)
Pepino mosaic virus (PepMV)	F-ACTCCTAGAGCTGACCTCAC R-TCTCCAGCAACAGGTTGGTA		107	(Ling, 2007)
Tomato yellow leaf curl virus (TYLCV)	F-TGTTGTAAGGGCCCGTGACT R-GACGGGCGTGGAAATGATTA		62	(Papayiannis *et al*., 2010)
Tomato spotted wilt virus (TSWV)	F-GCTTGTTGAGGAAACTGGGAATT R-AGCCTCACAGACTTTGCATCATC		150	(Roberts *et al*., 2000)
Tomato chlorosis virus (ToCV)	F-TCTCGAACCTGCTTATGAAAAGAAA R-ATGCAAGTTGGTTAACGTTGTACAGT		80	(Lozano *et al*., 2006)
	F-AAGAGGGTGTCAGCAACAGG R-TGGGTTCTGAGGTTGAGAGT	760		(Hirota *et al*., 2010)
Tomato infectious chlorosis virus (TICV)	F-AAAGCGGGACATTTTTTATCATATG R-TGTTTCCAGACTAGATCGCATGAAT		89	(Vaira *et al*., 2002)
Tomato mosaic virus (ToMV)	F-TTGCCGTGGTGGTGTGAGT R-GACCCCAGTGTGGCTTCGT		72	(Boben *et al*., 2007)
Alfalfa mosaic virus (AMV)	F-TCGTCACGTCATCAGTGAGAC R-CCATCATGAGTTCTTCACAAAAG	351		(Xu and Nie, 2006)
	F-GTTGATGCTGCTGCTGCTG R-GCTGCTGCTGCTGCTGCTG		100	(Trucco *et al*., 2022)
Cucumber mosaic virus (CMV)	F-CTTTCGCGACTTAATAAGACGTT R-CACAGTAGAATCAAATTTCGGCA	230		(Srivastava *et al*., 2019)
	F-ATCCGGAGTTTTCGATTA R-GCATCATCATATATTCCAATTC		96	(Xinying *et al*., 2022)
Tomato mottle mosaic virus (ToMMV)	F-CTGGAGAAGACTGGGTCTAG R-TTCGGTAAGTTCAATGGGACCT		50	(Fowkes *et al*., 2022)
Tomato apical stunt viroid (ToASVd)	F-GGG ATC CCC GGG GAA AC R-AGCTTCAGTTGTATCCACCGGGT		196	(Verhoeven *et al*., 2012)
Tomato mild mottle virus (ToMMoV)	F-CGACCCTGTAGAATTAATAAATATT R-CACTCTGCGAGTGGCATCCAAT		289	(Sui *et al*., 2017)
Potato virus Y (PVY)	F-CCA ATC GTT GAGAATGCAAAAC R-ATA TACGCTTCTGCAACATCTGAC A		74	(Cárdenas *et al*., 2017)
Potato virus X (PVX)	F-AAGCCTGAGCACAAATTCGC R-GCTTCAGACGGTGCCG		101	(Agindotan *et al*., 2007)
Tobacco mosaic virus (TMV)	F-ATTAGACCCGCTAGTCACAGCAC R-GTGGGGT TCGCCTGATTTT		83	(Yang *et al*., 2012)
Tomato brown rugose fruit virus (ToBRFV)	1F-CAACTACAATACTTAACAAC 1R- CTCCTTCATGTAGACCTCTC	956		This study
	2F- AATACGTGTGCAAAACTTAC 2R- CATGGATGGTTCTTCAACAT	1097		
	3F- AGGGTGCACTAGTGGTTACT 3R- TGTACACATGACATGTCCTT	1079		
	4F- AGCCGACATTACCCACTATC 4R- GCTTTAATCATATGCCTGTA	1005		
	5F- GCTTGCAGATTTTGATTTTG 5R- ACACTCTTAACAGGTGTGAA	1081		
	6F- GGTAAAGTCAATATTAATGA 6R- AAATTGCCGTTGAACGGTTG	935		
	7F- TGGGCCGACCCTATAGAATT 7R- CGCCCCTACCGGGGGTTCCG	628		

(Continued)

Table 1. (Continued).

Virus	Primer sequence (5′ - 3′)	PCR amplicon (bp)	qPCR amplicon (bp)	Reference
Tobacco vein clearing virus (TVCV)	1F-ATATGTATGATTTATATGAT 1R-CATTTCCGTCTATTTCATTT	1009		This study
	2F-GATAAAGAGCTAGAATTAAC 2R-GTTGTACATCCAAATTGTGG	1061		
	3F-ATGGCACCACAATTTGGATG 3R-GCTTCCTGGTGCTAGTTCTA	989		
	4F-CTATAGAAAAAAGCGTAATT 4R-GGCATTCCTAATAACATATC	1120		
	5F-TGGTAGTAACAGGATTTAAT 5R-GTATTCCTAGAAATTCTATC	1080		
	6F-ATTCAGGTATAAGTTTAAGT 6R-TAGATTGTCCTGCTGTAGAT	1000		
	7F-GCATGACTATAAACATGCGG 7R-AGTCTATTCCAGTTGTACTC	1093		
	8F-GACTTGATGACCAGATACTG 8R-CATAATAAAGtTCATCTTGT	1005		
Southern tomato virus (STV)	1F- GATAAATTTAGTAAGCTACC 1R- CTGGAGCTCATCCTTCACAT	935		This study
	2F- AGCCTACTAGGAAGCAAGTC 2R- CCCACCCTTGCAGCAAAATA	905		
	3F- CTGATGGAGGATATCTACTG 3R- TTCTCCAAGTTCTCGTGAGA	1010		
	4F- CATAGGTGGGAGTACAGGTT 4R- GAAGACGCGCTACTCTAATA	878		

mL) and the M-MLV reverse transcriptase enzyme, following the manufacturer's instructions (Thermo Fisher). All samples were initially screened using qPCR assay, only positive samples were further tested by RT-PCR and confirmed via Sanger sequencing. Both PCR and qPCR were performed on the cDNA using a comprehensive set of sense and antisense primers designed to target all tomato-infecting viruses and viroids examined in this study (Table 1). PCR and qPCR primers, conditions, and cycles were applied according to each virus and viroid and relative reference (Table 1). PCR reactions, conducted for further sequencing, were carried out in a final volume of 25 µL, of which 2.5 µL of cDNA or total DNA were used as a template. Cycling conditions included 30 sec of denaturation at 95°C, followed by 40 cycles of PCR amplification at 95°C for 50 sec, annealing at 50–58°C (according to each virus and viroid primer pair) for 30 sec and an elongation at 72°C for 40 sec. qPCR based on iTaq Universal SYBR Green Supermix [2x concentrated, ready-to-use master reaction mix, optimized for dye-based quantitative PCR (qPCR)] was carried out for viruses and viroids detection (Table 1), using Rotor- Gene Q and CFX96 BioRad thermocyclers (Milan, Italy). Specific primer sets were designed based on NGS generated sequences for ToBRFV as well as Southern tomato virus (STV) and tobacco vein clearing virus (TVCV), both initially not included in our investigation (Table 1).

Cloning, sequencing and computer-assisted analysis

All the PCR amplicons obtained from different viruses were ligated into the StrataCloneTM PCR cloning vector pSC-A, subsequently cloned into *Escherichia coli* DH5 cells, and automatically sequenced (Eurofins Genomics, Koln, Germany). Nucleotide (nt) and amino acid (aa) sequences were analyzed with the assistance of Geneious Prime 2024.0.5 (San Diego, CA, USA). Search for nt and aa identities in the GenBank was carried out using BLASTX and BLASTP tools (http://www.ncbi.nlm.nih.gov/) (Altschul *et al*., 1990). Sequence alignment and tentative phylogenetic trees were performed using "Clustal Omega" and "Maximum Likelihood" packages included in Geneious Prime 2024.0.5.

Next-generation sequencing using Illumina and MinION nanopore technologies

Next-generation sequencing (NGS) was performed on two ToBRFV-infected samples using both *Illumina* and *MinION* nanopore technologies. This analysis aimed to determine the complete genome sequences of two Albanian isolates from the highly affected regions of Fier and Lushnje and to assess their phylogenetic relationships with homologous sequences reported in the GenBank. Accordingly, the total RNA was ribo-depleted, fragmented, and double-stranded (ds) cDNA was synthesized using random hexamers and used as input in the end-prep step in the direct cDNA sequencing kit (SQK-DCS109). The prepared library was loaded onto a *MinION* flow cell (FLO-MIN114), and sequencing was conducted using ONT *MinION* sequencing device with Flongle flow cells, generating real-time data. Raw reads were base-called, quality-controlled, and aligned to a reference genome. *Illumina* sequencing was conducted on the reverse-transcribed templates that were sequenced in a run of 2 × 150 bp paired-end mode (Eurofins Genomics, Germany). The reads were trimmed, error corrected and normalized using BBDuck package in Geneious Prime 2024.0. The Tadpole tool with different k-mers was used for *de novo* assembly of all the previously filtered reads into larger contigs. BLASTX (e-value with cut-off from 10^{-6} to 10^{-2}) and BLASTP were used for sequence homology screening of the assembled contigs.

RESULTS

PCR and qPCR detection of tomato viruses and viroids

PCR and qPCR assays conducted on 196 tomato samples identified the presence of only four viruses, i.e., ToCV, ToBRFV, AMV, and CMV, among those tested, while no viroids were detected. A total of 87 tomato plants were found to be infected, representing an overall infection rate of 44.4% (Table 2). The Fier region showed the highest infection rate at 47.2%, with 26 PCR-positive samples out of 55 tested, followed by Lushnje with a rate of 43.5%, based on 44 infected samples out of 101 collected. The analysis revealed that ToCV and ToBRFV were the most prevalent viruses, with ToCV being dominant in the Lushnje and Fier regions and ToBRFV more prevalent in Berat and Fier (Table 2). Among the infected samples, six plants exhibited double infections, while 81 had single infections.

Symptoms in greenhouses

The symptoms found associated with ToBRFV in greenhouses-tomato infected plants were varied and ranged from light to severe. Affected leaves exhibited moderate to severe mosaic patterns, accompanied by dark green wrinkling, blistering, narrowing, deformation, and necrotic spots (Figure 1a). On the fruit, irregular brown necrotic lesions, deformities, and yellowing spots were commonly observed, making the tomatoes unsuitable for market (Figure 1b). Plants infected with ToCV had the same type of symptoms, characterized by vein clearing, yellowing, crinkle and deformed leaves (Figure 1c). Light mottling and vein clearing symptoms were observed on TVCV-infected tomato plants (Figure 1d). Symptoms of both viruses were prevalent across numerous tomato plants in various locations. However, no symptoms suggestive of viral infection were observed and associated with AMV-, CMV-, and STV-infected plants.

Illumina and MinION nanopore sequencing

The NGS runs using *Illumina* and *MinION* platforms were performed on total RNA extracted from two ToBRFV-infected samples (Alb-L1 and Alb-F5). The *Illumina* runs yielded approximately 6.2 million paired-

Table 2. Detection and incidence analysis of tomato viruses in samples from five regions of Albania.

Region	Tomato samples	Infected samples	Infection rate %	ToCV	ToBRFV	AMV	CMV	STV	TVCV
Berat	32	12	37.5	3	6	1	0	2	1
Lushnje	101	44	43.5	26	4	3	8	2	4
Fier	55	26	47.2	18	6	3	0	0	1
Tirana	5	2	40	0	2	0	0	0	0
Shkodra	3	3	100	2	0	1	0	0	0
Total	196	87	44.4	49 (25%)	18 (9.1%)	8 (4.1%)	8 (4.1%)	4 (2%)	6 (3.1%)

Figure 1. Symptoms of viral infections in tomato plants: ToBRFV causes visible damage on both leaves and fruits (a, b), whereas ToCV (c) and TVCV (d) primarily affect the leaves. White arrows highlight symptom locations.

end reads per sample, while *MinION* produced approximately 0.71 million reads per sample. After quality filtering, these numbers were reduced to approximately 3.7 and 3.6 million high-quality reads for *Illumina* and 0.49 and 0.47 million high-quality reads for *MinION*. All high-quality reads were used for de novo assembly, generating around 12,334 and 11,259 contigs from *Illumina* and 3,108 and 3,548 contigs from *MinION,* with contig lengths ranging from 150 to 4,720 nucleotides. The alignment of high-quality *Illumina* and *MinION* reads to reference ToBRFV sequences from GenBank (Table 4) revealed that *Illumina* sequencing provided higher resolution for genome assembly, which is critical for accurate virus characterization. It successfully uncovered the complete genome sequences of ToBRFV in both samples, as well as the full genome sequences of STV and TVCV, which were not initially included in our screening (Table 3). The complete genome sequences of both ToBRFV isolates obtained in this study were identical, spanning 6,381 nts. They shared 99.8% identity with the Israeli isolate "6166394_2" (GenBank accession number OM515237). One representative sequence for ToBRFV (Alb-L1 isolate) has been deposited in GenBank under the accession number PQ643185.

Similarly, *Illumina* outperformed *MinION* in generating and fully covering the nucleotide sequences of the STV and TVCV genomes, each uniquely identified in two distinct samples using these platforms (Table 3). The complete genome sequences of TVCV and STV were 7,596 and 3,437 nts in length, respectively, sharing 95.3% identity with the Iraqi isolate Iraq-1 (accession number ON684329) and 99.8% identity with the German isolate JKI ID1904214/15 (accession number MK948545). The accuracy of the full genome sequences of ToBRFV, TVCV, and STV was verified by Sanger-sequencing of PCR-amplified amplicons using specific primers designed from the NGS-generated sequences (Table 1).

Sequence and phylogenetic analyses

The sequencing analysis of PCR amplicons generated distinct sequence types across different virus isolates from tested samples. Sequence types were obtained from 6 out of 8 samples for AMV, 4 out of 8 for CMV, 10 out of 18 for ToBRFV, 13 out of 49 for ToCV, 1 out

Table 3. Genomics features provided by *Illumina* and *MinION* sequencing. Alb-L1: sample from Lushnje region: Alb-F5:sample from Fier region.

Virus	Method	Sample	Reads	Coverage %	Genome sequence length (nts)	GenBank Accession number
ToBRFV	*Illumina*	Alb-L1	298,175	100	6,381	PQ643185
		Alb-F5	287,741			
	MinION	Alb-L1	110,112	98.9	6,310	
		Alb-F5	109,187	99.3	6,336	
TVCV	*Illumina*	Alb-L1	250,149	100	7,596	PQ643187
	MinION	Alb-L1	95,545	97.2	7,383	
STV	*Illumina*	Alb-F5	210,580	100	3,437	PQ643186
	MinION	Alb-F5	80,633	97.7	3,357	

Table 4. List of virus isolates sequenced in this study and their identity levels with homologue in the GenBank

Virus	Accession No. deposited in GenBank	Intraspecies identity of Albanian isolates (%)	Reference isolate (Accession No.)	Origin	Highest identity with homologue in GenBank (%)
AMV	PQ613741- PQ613746	95–98	Tec1 (FR715042)	Spain	98.7
CMV	PQ643215- PQ643218	96–99	SKO20ST1 (OL472046)	Slovenia	99.5
ToBRFV	PQ643188- PQ643197	98–100	Gr2 (OQ190155)	Greece	100
ToCV	PQ643201-PQ6432013	96–99	DSMZ PV-1242 (ON398513)	Greece	99.3
TVCV	PQ643187	100	Iraq-1 (NO684329)	Iraq	95.3
STV	PQ643186	100	Mexico-1 (NC_011591)	Mexico	98.7

of 6 for TVCV and 1 out of 4 for STV. The resulting clones exhibited varying degrees of sequence identity both among themselves and with reported isolates in the GenBank (Table 4).

The phylogenetic analyses of ToCV and ToBRFV, identified as the two most significant viruses in our study and among the most prevalent in Albania, revealed notable genetic similarities with isolates from various countries. Notably, ToCV isolates from Albania formed a distinct clade, indicating a high level of sequence conservation among strains within the country. These isolates were closely related to those from Greece (Gr-535, Acc. No. EU284744; DSMZ-PV-1242, Acc. No. ON398513) but more distantly related to isolates from China and Korea (Figure 2).

The phylogenetic allocation of ToBRFV isolates from Albania showed a pattern somewhat analogous to that of ToCV. The Albanian isolates displayed genetic proximity to one another but were distributed across two main clades. One clade included some of the Albanian isolates alongside isolates from Greece (Gr2, Acc. No. OQ190155) and Israel (41106995, Acc. No. OM515266), forming two closely related clusters. The second clade grouped other Albanian isolates into two clusters, along with isolates from Cyprus (DSMZ-PV-1300, Acc. No. OL311702), the United Kingdom (ToBRFV.21930919, Acc. No. MN182533), Belgium (GBVC-01, Acc. No. MZ945 419), and the Netherlands (39941430A, Acc. No. MN882046) (Fig. 2). Overall, the Albanian ToCV and ToBRFV isolates showed genetic clustering with isolates from geographically neighboring regions and countries such as Greece and Israel, suggesting the potential circulation of closely related and genetically homogeneous strains across these areas.

DISCUSSION AND CONCLUSION

This study provides an in-depth analysis of viruses affecting greenhouse-grown tomatoes in Albania, highlighting several significant findings. RT-PCR and qPCR assays performed on greenhouse-grown tomato plants from five Albanian tomato growing regions detected six viruses, i.e., AMV, CMV, STV, TVCV, ToCV, and ToBRFV. Both methods provided consistent results, with PCR amplicons enabling molecular analyses. Among the viruses tested, ToCV and ToBRFV emerged as the most prevalent, accounting for 25 and 9.1% of the infected samples, respectively. ToCV showed higher prevalence in Lushnje and Fier regions, whereas ToBRFV was dominant in Berat and Fier. Notably, STV and TVCV were identified in Albanian greenhouses for the first time, using *Illumina* and *MinION* sequencing. This study reports for the first time the complete genome sequences of ToBRFV (6,381 nts), STV (3,437 nts), and TVCV (7,596 nts) Albanian isolates. The prevalence of the two latter, although low, indicates the need for further monitoring and study of their epidemiological impact in the country. Notably, ToBRFV earlier reported in a few samples from the Fier and Berat regions (Orfanidou *et al*., 2022a), has now been detected in two additional regions, Lushnje and Tirana, suggesting its potential spread. Similarly, ToCV, which was detected in 10 out of 15 tested tomato plants from the Fier and Berat regions (Orfanidou *et al*., 2022b), was found in 49 out of 196 samples in our survey, indicating a higher infection rate of 25%.

Illumina provided 100% genome coverage for ToBRFV, STV, and TVCV, while *MinION* achieved slightly lower coverage (97.2-99.3%) and shorter sequences for STV and TVCV. Therefore, *Illumina's* higher accuracy makes it preferable for detailed viral characterization, whereas *MinION* 's could be more valuable for diagnostics when it deals with accuracy/speed efficiency ratio.

The phylogenetic analyses showed that the Albanian isolates of ToCV and ToBRFV are closely related to those from neighboring regions, such as Greece, suggesting regional movement of these pathogens. An analogous phylogenetic profile for AMV and CMV isolates had also emerged (data not shown).

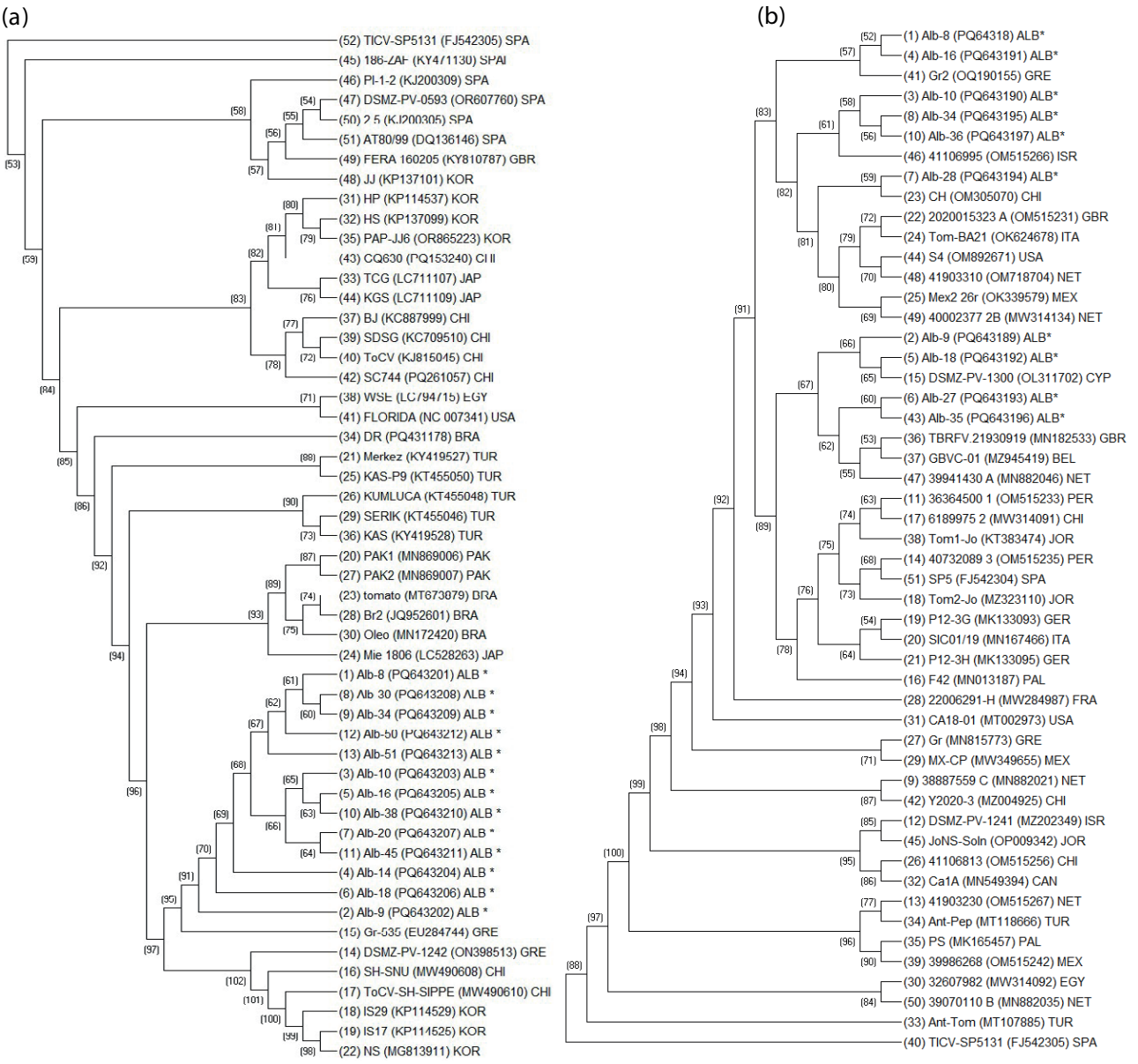

Figure 2. Phylogenetic trees constructed using nucleotide sequences of PCR amplicons of tomato chlorosis virus (a) and tomato brown rugose fruit virus (b) isolates from Albania (indicated with asterisks), along with sequences retrieved from GenBank for both viruses. Tomato infectious chlorosis was used as the outgroup species. Accession numbers of the sequences used are provided in brackets. The origins of the isolates are abbreviated as follows: Spain (SPA), United Kingdom (GBR), Korea (KOR), China (CHI), Japan (JAP), Egypt (EGY), United States of America (USA), Brazil (BRA), Turkey (TUR), Pakistan (PAK), Palestine (PAL), Mexico (MEX), Greece (GRE), Italy (ITA), France (FRA), Peru (PER), Germany (GER), Canada (CAN). Bootstrap values are indicated at branch nodes.

This study can be considered as a step toward sustainable tomato production in Albania, addressing emerging viral challenges while emphasizing the value of modern diagnostic technologies in agricultural research, such as NGS. Effective control measures, including managing insect vectors, using resistant varieties, ensuring virus-free seeds, and implementing strict sanitation practices, are essential to limit the spread of these viruses. Overall, the growing prevalence of viruses such as ToBRFV and ToCV, and the finding of new viruses never reported before in the country, underscores the need for improved disease management strategies in Albanian tomato production.

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