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First molecular detection and characterization of *Citrivirus citri* (citrus leaf blotch virus) in citrus in Turkiye

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Summary. *Citrivirus citri* (*Betaflexiviridae*) (citrus leaf blotch virus; CLBV), is a positive-sense, single-stranded RNA virus, that has been associated with bud union disorders and chlorotic leaf blotching in citrus. Although this virus has been reported from several citrus-producing regions, its occurrence in Turkiye has not been molecularly confirmed. In this study, 143 samples of satsuma mandarin (*Citrus unshiu* Marc.) and Nagami kumquat (*Fortunella margarita* (Lour.) Swingle) collected from the Çukurova region on the Mediterranean coast of Turkiye between 2019 and 2024 were assessed using RT-PCR, with representative samples confirmed by RT-qPCR and sequencing. CLBV infections were detected in 37% of kumquat and 21% of and satsuma mandarin samples. Positive samples included symptomatic and asymptomatic trees, indicating that visual inspection alone is insufficient for detection of CLBV infections. Coat protein gene sequences showed high similarity among most isolates from Turkiye, but one isolate was closely related to an isolate from China. The RdRp-based phylogenetic analyses revealed greater genetic diversity among isolates from Turkiye than was observed in the coat protein (CP)-based phylogenetic analyses. Transmission electron microscopy revealed flexuous filamentous particles in CLBV-infected kumquat and experimentally inoculated *Nicotiana benthamiana* plants, that were consistent with members of the *Betaflexiviridae*. These results provide the first molecular evidence of CLBV in citrus from Turkiye, and support the inclusion of CLBV screening in routine citrus certification and indexing programmes.

Keywords. Citrus, bud union disorders, CLBV, genetic diversity.

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INTRODUCTION

Citrus cultivation is a major component of agricultural production in the Mediterranean basin, where grafting onto selected rootstocks is widely used to optimize fruit yields, quality, and adaptation to local soil conditions. However, bud union disorders remain significant constraints in citrus production, often leading to reduced tree vigour and long-term productivity losses. Although incompatibility reactions are often genetically determined and associated with specific scion-rootstock combinations, graft-transmissible

pathogens may also contribute to bud union abnormalities (Schneider, 1978; Garnsey *et al*., 2005).

Several graft-transmissible agents, including viruses and viroids, have been reported in citrus (Roistacher, 1991; Moreno *et al*., 2008). Among these, citrus leaf blotch virus (CLBV) has been associated with bud union disorders in particular scion–rootstock combinations (Navarro *et al*., 1984; Galipienso *et al*., 2000; Vives *et al*., 2001). CLBV, the type species of the *Citrivirus* (*Betaflexiviridae*), is a positive-sense single-stranded RNA virus encoding replication, movement, and coat proteins (Vives *et al*., 2001). This virus was originally identified in kumquat grafted onto Troyer citrange in Corsica, where it was linked to bud union crease symptoms (Navarro *et al*., 1984). Symptom expression in infected plants may include vein clearing and chlorotic blotching of the leaves, and stem pitting, which is strongly influenced by scion–rootstock interactions (Galipienso *et al*., 2000).

Beyond citrus, CLBV has also been detected in several woody hosts, including apple, cherry, kiwifruit, and ornamentals, indicating a broad host range (Wang *et al*., 2016; Zhu *et al*., 2016; Kim *et al*., 2023; Huang *et al*., 2024). The virus is efficiently transmitted via grafting, and has been shown to be seed-transmissible in several citrus hosts, including Troyer citrange, Nagami kumquat, and sour orange, increasing the risk of dissemination through propagation material (Guerri *et al*., 2004). Experimental mechanical transmission has also been demonstrated in herbaceous hosts, including *Nicotiana* spp. (Vives *et al*., 2008; Guardo *et al*., 2009). No evidence of vector-mediated transmission has been reported (Hernández-Rodríguez *et al*., 2016). Therefore, CLBV is considered relevant in certification and quarantine frameworks, and is listed as a regulated non-quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO, 2024).

Turkiye is a major citrus-producing country in the Mediterranean basin, and the Çukurova region is a major citrus production region in this country. Sour orange (*Citrus aurantium*) is the predominant rootstock because of its adaptation to calcareous soils, despite its known susceptibility to several pathogens. The diversification in rootstock use in the region highlights the importance of latent virus infections such as CLBV, which may remain asymptomatic or become associated with bud union disorders, depending on the specific scion-rootstock combination (Tuzcu *et al*., 1998).

Despite its reported distribution in Mediterranean regions, including Spain, France (Corsica), and Italy (Galipienso *et al*., 2001; Guardo *et al*., 2007; Navarro *et al*., 1984). CLBV has not been systematically investigated or molecularly confirmed in Turkiye. CLBV has also been reported from other citrus-producing regions, including Japan, Australia, New Zealand, Cuba, South Korea, and China (Vives *et al*., 2002; Guardo *et al*., 2007; Harper *et al*., 2008; Hernández-Rodríguez *et al*., 2016; Cao *et al*., 2017; Park *et al*., 2019). This lack of pathogen identification is important, because CLBV can occur without obvious symptoms and may be disseminated through infected propagation material.

The objectives of the present study were: 1) to determine whether CLBV is present in citrus orchards in the Çukurova region; 2) to estimate CLBV incidence levels in satsuma mandarin and Nagami kumquat trees; 3) to document associated field symptoms; 4) to confirm virus identity using RT-PCR and sequencing; and 5) to evaluate the genetic relationships of selected isolates from Turkiye within an international context.

MATERIALS AND METHODS

Survey design and sample collection

Field surveys were carried out between spring 2019 and summer 2024, during active vegetative growth periods, in commercial orchards and nurseries located in the provinces of Adana, Mersin, and Hatay within the Çukurova region of Turkiye. A combined targeted and random sampling strategy was employed to capture symptomatic and asymptomatic virus infections.

Targeted sampling focused on trees exhibiting symptoms potentially associated with graft-transmissible pathogens, including bud union disorders (e.g., creasing and swelling above graft unions), chlorotic leaf blotching, stunting, and general decline. In addition, asymptomatic trees within the same orchard blocks were randomly selected to assess for latent virus infections. The survey focused on kumquat and Satsuma mandarin trees, based on field observations and reports of bud-union disorders in these hosts in the study region. In total, 143 samples were collected, including 38 from satsuma mandarin and 105 from Nagami kumquat plants. Geographic coordinates of each sampling site were recorded using a handheld GPS device.

Molecular detection and sequencing

Total nucleic acid (TNA) was extracted from approx. 300 mg of leaf midrib and petiole tissue from each field sample, using a CTAB-based protocol modified from that of Li *et al*. (2008). Specifically, compared with the original protocol, the amount of sample tissue was increased from 100 to 300 mg, the volume of CTAB

Table 1. Primers used for the detection of Citrus leaf blotch virus (CLBV).

Primer	Sequence (5'–3')	Target gene	Product size (bp)	Reference
KU-27	GATGCAAGCCAGGATGAATAC	RdRp (ORF1)	456	Vives *et al.,* 2002
KU-15	CAGACACTCCAAGACCTTTCC	RdRp (ORF1)	456	Vives *et al.*, 2002
KU-18	TTAAGATTACAGACACGAAGG	CP (ORF3)	438	Vives *et al.*, 2002
KU-19	CTGTTTTTGAATTTTGCTCG	CP (ORF3)	438	Vives *et al.,* 2002

extraction buffer was increased from 1 to approx. 2 mL, and the incubation time at 65 °C was extended from 15 to 60 min. RNA concentrations and purity were determined using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific). Extracted nucleic acids were stored at −80°C until further analyses.

CLBV detection was carried out using SuperScript™ One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen), with the two previously reported primer pairs KU-27/KU-15 and KU-18/KU-19 (Vives *et al.,* 2002) (Table 1). The KU-27/KU-15 primer pair amplifies a 456 bp fragment of the RNA-dependent RNA polymerase (RdRp) gene (ORF1), and the KU-18/KU-19 pair amplifies 438 bp fragments of the coat protein (CP) gene (ORF3). Other citrus viruses and viroids associated with bud union disorders were excluded from the PCR tests carried out on the collected samples.

For one-step RT-PCR, each 25 µL reaction mixture contained 1× reaction buffer, 3 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 µM of each primer, 1 µL of TNA, 4 U RNaseOUT™, 20 U SuperScript™ II reverse transcriptase, and 1 U Taq DNA polymerase. Thermal cycling conditions consisted of reverse transcription at 42 °C for 45 min, followed by initial denaturation at 94 °C for 2 min; 40 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C (RdRp) or 45 °C (CP) for 20 s, and extension at 72 °C for 30 s; then a final extension at 72 °C for 5 min. Amplification products were analyzed by electrophoresis on 2% agarose gels, that were stained with ethidium bromide and visualized using a UV-transilluminator.

To validate RT-PCR results, SYBR Green-based reverse transcription quantitative PCR (RT-qPCR) assays targeting the coat protein (CP) gene were carried out, using the KU-18/KU-19 primer pair. Reverse transcription was performed before quantitative PCR amplification, and reactions were carried out using standard SYBR Green chemistry on a LightCycler® 480 system (Roche Diagnostics GmbH). For interpretation of the qPCR results, Ct values were classified as low ($Ct < 20$), medium ($20 \leq Ct < 23$), or high ($Ct \geq 23$).

Selected RT-PCR-positive samples were subsequently subjected to sequencing of the CP and RdRp gene regions amplified using, respectively, the KU-18/KU-19 and KU-27/KU-15 primer pairs. PCR products were purified and sequenced bidirectionally by a commercial service provider (Telomer).

Phylogenetic analyses of viruses

Raw sequence data were edited and assembled into consensus sequences using MEGA11 (Tamura *et al.,* 2021). Pairwise nucleotide similarities were calculated using the Sequence Demarcation Tool (SDT v1.2) (Muhire *et al.,* 2014). Reference sequences representing global CLBV diversity were retrieved from GenBank. Multiple sequence alignments were carried out using the MUSCLE algorithm implemented in MEGA11. Phylogenetic trees based on the nucleotide sequences of the CP and RdRp genes were constructed using the Neighbor-Joining method with the Tamura–Nei substitution model. The robustness of the inferred trees was evaluated by bootstrap analyses with 1000 replicates. Apple stem grooving virus (ASGV; GenBank accession no. AF522459) was used as an outgroup.

Transmission electron microscopy (TEM)

Leaf dip preparations from CLBV-infected kumquat and experimentally inoculated *Nicotiana benthamiana* plants were prepared in 0.1 M phosphate buffer (pH 7.2). Samples were negatively stained with 2% (w/v) uranyl acetate, and were then examined using a JEOL JEM-1400 Flash transmission electron microscope.

RESULTS

Field symptoms

Field observations revealed distinct differences in symptom expression between citrus hosts and scion–rootstock combinations. The sampled Satsuma mandarin trees were more than 10 years old, and were grafted onto sour orange (*Citrus aurantium*), trifoliate orange (*Poncirus trifoliata*), or their hybrids. In satsuma man-

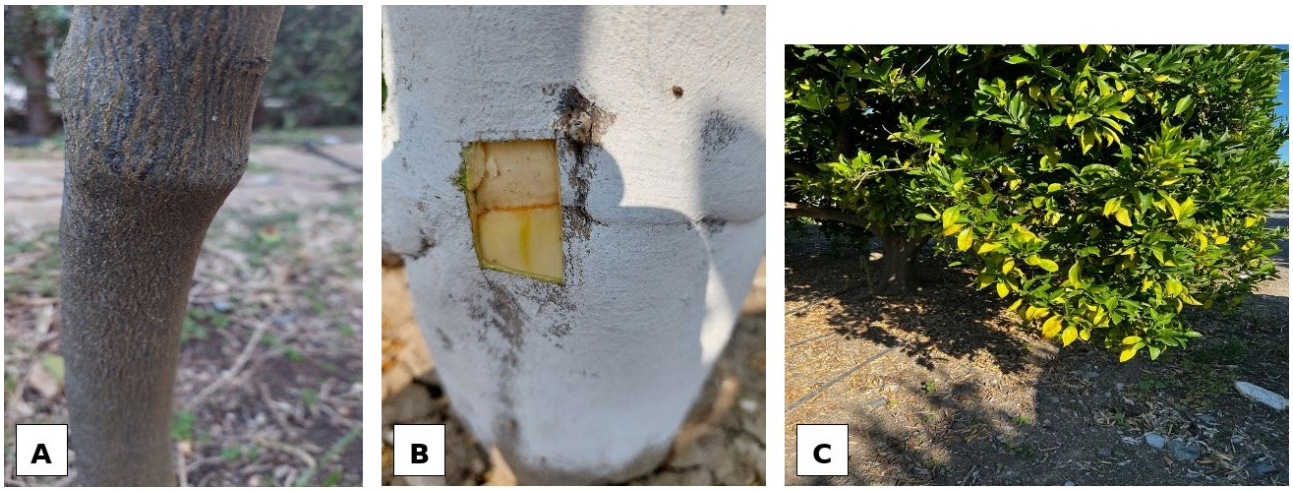


Figure 1. Field symptoms associated with CLBV in satsuma mandarin grafted onto sour orange rootstock. (A) Swelling above a bud union. (B) Discolouration and tissue alteration at a graft interface. (C) General chlorosis and decline of a citrus tree.



Figure 2. Field symptoms associated with CLBV in Nagami kumquat. (A) General decline and chlorosis. (B) Chlorotic blotching on leaves. (C) Bud union abnormalities and tissue alterations.

darin grafted onto sour orange rootstock, pronounced swellings above the bud unions were frequently observed, often each accompanied by a distinct crease or brown line at the interface (Figure 1). Affected trees showed mild dieback, shortened internodes, and general chlorosis.

The sampled Nagami kumquat trees were 6 years or older, and were generally grafted onto trifoliate orange (*Poncirus trifoliata*) and its hybrids, as well as on sour orange (*Citrus aurantium*). In contrast, Nagami kumquat grafted onto sour orange or Carrizo citrange rootstocks typically exhibited sharp bud union creases without noticeable overgrowths. These trees frequently displayed severe chlorotic leaf blotching (Figure 2), accompanied by pronounced stunting.

Virus detection, prevalence, and qPCR validation

RT-PCR analyses detected citrus leaf blotch virus (CLBV) in the two citrus hosts, with a greater proportion of positives in kumquat than in satsuma mandarin (Table 2). Overall, CLBV was detected in 47 of 143 samples (32.9%). Because sampling included targeted and random collections, this incidence proportion should be interpreted as an estimate of the surveyed material, rather than as an unbiased regional incidence estimate.

RT-qPCR analysis was performed on 53 samples, including the 47 RT-PCR-positive samples and six RT-PCR-negative samples used as controls. RT-qPCR confirmed all RT-PCR-positive samples, with Ct values ranging from 20 to 27. No amplification was detected in the RT-PCR-negative control samples.

Table 2. Prevalence of CLBV in citrus samples collected from the Çukurova region of Turkiye.

Host species	Province	Symptom status	RT-PCR positive	RT-PCR negative	Infection rate (%)
		Symptomatic / Asymptomatic	Symptomatic / Asymptomatic		
Nagami kumquat	Mersin	41 / 9	18 / 3	29	42.0
Nagami kumquat	Adana	34 / 10	12 / 2	30	31.8
Nagami kumquat	Hatay	11 / 0	4 / 0	7	36.4
Nagami total	—	86 / 19	34 / 5	66	37.1
Satsuma mandarin	Mersin	6 / 3	1 / 0	8	11.1
Satsuma mandarin	Adana	14 / 3	3 / 1	13	23.5
Satsuma mandarin	Hatay	7 / 5	3 / 0	9	25.0
Satsuma total	—	27 / 11	7 / 1	30	21.1
Overall total	—	113 / 30	41 / 6	96	32.9

Table 3. Details for selected CLBV isolates from Turkiye used for sequence and phylogenetic analyses.

Isolate	Location	Host	Symptom	CP accession	RdRp accession
CLBV1	Mersin	Nagami kumquat	Symptomatic	OQ718432	PP726108
CLBV2	Adana	Nagami kumquat	Symptomatic	OQ718433	PP726107
CLBV3	Mersin	Satsuma mandarin	Symptomatic	OQ718434	PQ818280
CLBV4	Mersin	Nagami kumquat	Asymptomatic	PQ818276	PQ818283
CLBV5	Mersin	Nagami kumquat	Symptomatic	PQ818277	PQ818284
CLBV6	Adana	Satsuma mandarin	Asymptomatic	PQ034506	PQ818285
CLBV7	Hatay (Erzin)	Satsuma mandarin	Symptomatic	PQ818278	PQ818286
CLBV8	Mersin	Nagami kumquat	Symptomatic	PQ818279	PQ818287
CLBV9	Hatay (Erzin)	Nagami kumquat	Symptomatic	PQ818280	PQ818288
CLBV10	Hatay (Erzin)	Satsuma mandarin	Symptomatic	PQ818281	PQ818289

Phylogenetic and genetic diversity analyses

BLASTn analysis of CP gene sequences showed that nine isolates from Turkiye shared close nucleotide similarity (98 to 100%) among themselves and more than 96% similarity with previously reported global isolates. The characteristics of the selected CLBV isolates used for sequence and phylogenetic analyses are summarized in Table 3.

Pairwise nucleotide similarity analyses of the CP sequences were consistent with the BLASTn results and the clustering pattern observed in the CP phylogeny (Figure 3; Supplementary Figure S1). One isolate, CLBV6 (PQ034506), obtained from asymptomatic Satsuma mandarin in Adana, had a distinct profile, with 99.53% nucleotide similarity with an isolate MW366992 from China. Phylogenetic analyses based on CP gene sequences showed that isolates from Turkiye clustered closely together, forming a well-defined group distinct from most international isolates (Figure 3). In contrast, isolate CLBV6 grouped with isolates from China. CLBV has previously been reported in China (Cao *et al*., 2017).

Analyses of the RdRp gene showed a more heterogeneous pattern than for the CP gene, with isolates from Turkiye distributed across multiple clusters (Figure 4). Some isolates were closely related to isolates from East Asia, including those from China and South Korea, whereas others formed separate groupings. CLBV has previously been reported in China and South Korea (Cao *et al*., 2017; Park *et al*., 2019). Pairwise nucleotide identity analyses of the RdRp sequences were consistent with the clustering pattern observed in the RdRp phylogeny (Figure 4; Supplementary Figure S2).

Electron microscopy

Transmission electron microscopy of leaf dip preparations from CLBV-infected Nagami kumquat and experimentally inoculated *Nicotiana benthamiana* plants showed flexuous filamentous particles, approx. 800 to 900 nm in length, and characteristic of *Betaflexiviridae* (Figure 5).

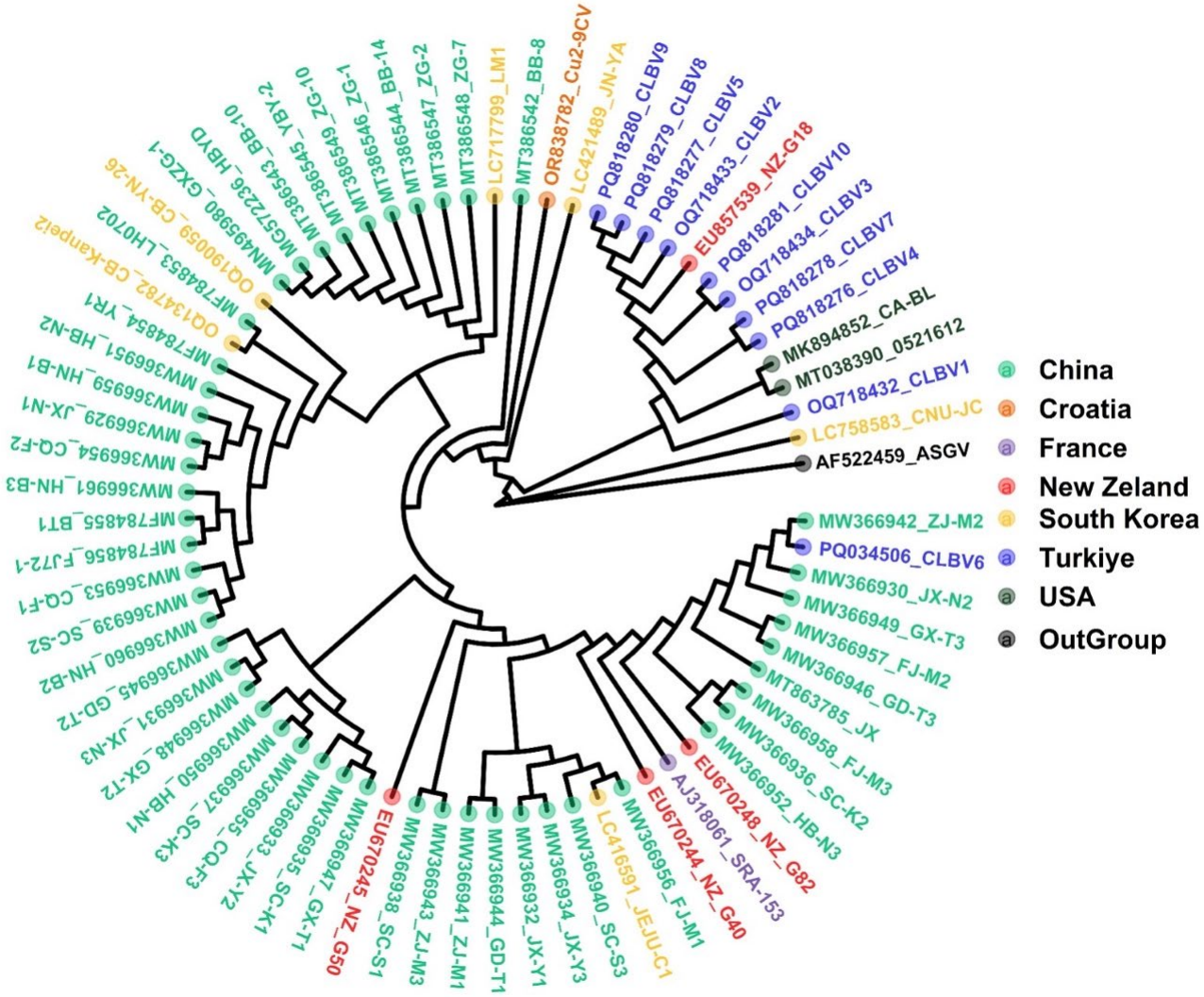


Figure 3. Phylogenetic tree based on the coat protein (CP) gene of citrus leaf blotch virus (CLBV) isolates. The tree was constructed using the Neighbor-Joining method with the Tamura–Nei model. Bootstrap values (1000 replicates) are shown at branch nodes. Isolates from Turkiye identified in the present study are included in the analysis. Apple stem grooving virus (ASGV) was used as the outgroup. Accession numbers of sequences are indicated next to each isolate. Different colours indicate the countries of origin of the sequences.

DISCUSSION

The detections of CLBV in Nagami kumquat and satsuma mandarin plants confirms that the virus is present in citrus in the Çukurova region of Turkiye. The greater proportion of CLBV positives in kumquat than in satsuma mandarin may reflect host effects, differences in propagation history, or the sampling design, which included targeted and random collections. Therefore, the prevalence values reported here should be interpreted as estimates for the surveyed material, rather than as region-wide CLBV incidences.

The detections of CLBV in asymptomatic plants are particularly important for citrus certification, as these are not currently part of the phytosanitary screening process. Visual symptoms varied between hosts and scion-rootstock combinations, with satsuma mandarin showing swelling and discolouration near the affected tree bud unions, whereas Nagami kumquat more often showed sharp bud union creases, chlorotic leaf blotching, and stunting. These observations are consistent with previous reports that CLBV-associated symptoms depend on host genotype and graft compatibility (Navarro et al., 1984; Galipienso et al., 2000). Because infected plants may remain symptomless, field inspections alone are unlikely to detect all infected propagation material.

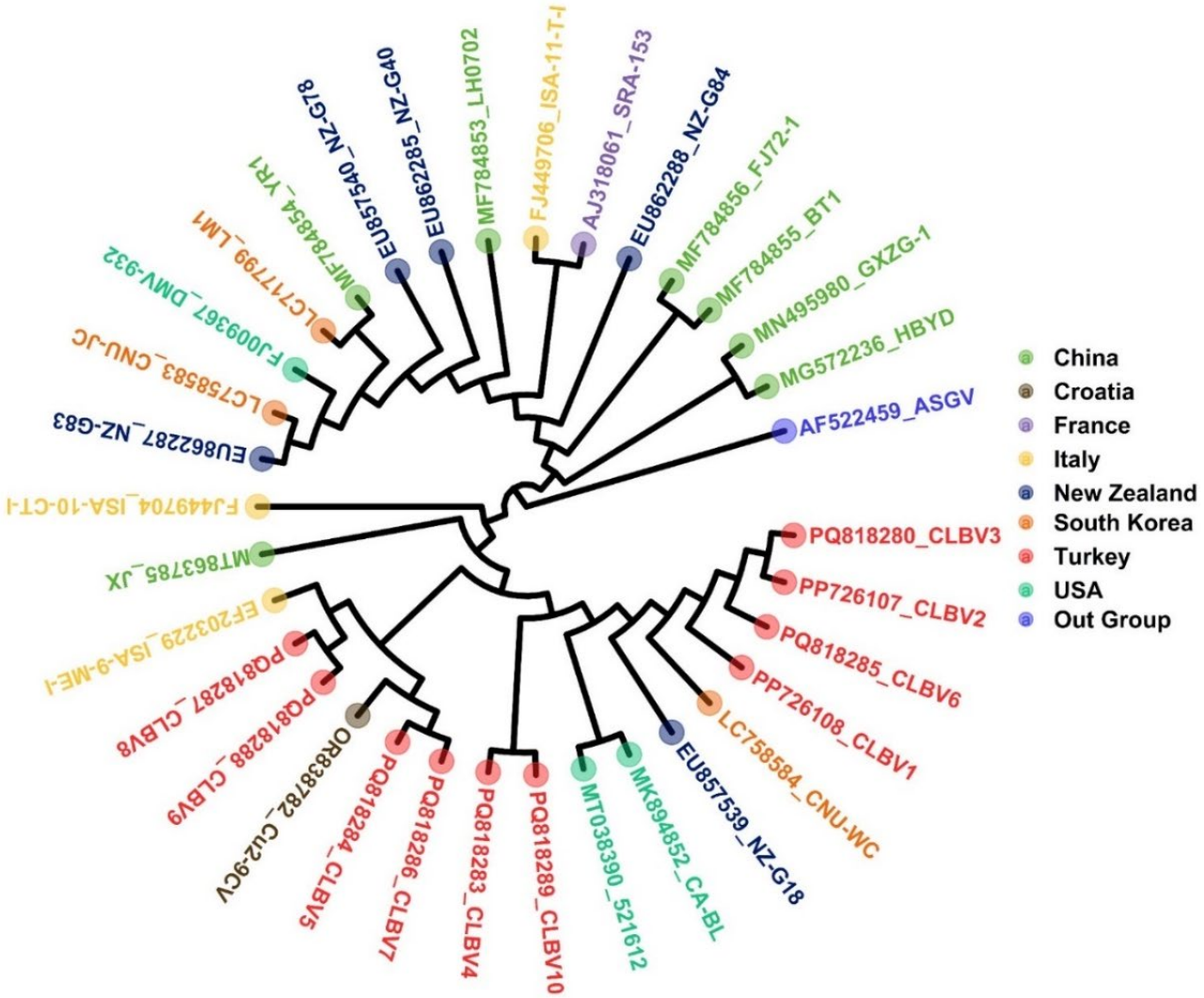


Figure 4. Phylogenetic tree based on the RNA-dependent RNA polymerase (RdRp) gene of isolates of citrus leaf blotch virus (CLBV). The tree was constructed using the Neighbor-Joining method with the Tamura–Nei model. Bootstrap values (1000 replicates) are shown at branch nodes. Isolates from Turkiye identified in the present study are distributed across multiple clusters. Apple stem grooving virus (ASGV) was used as the outgroup. Accession numbers of sequences are indicated next to each isolate. Different colours indicate the countries of origin of the sequences.

Most of the CLBV isolates from Turkiye were very similar in their CP regions, and formed a closely related group. This indicates either limited sequence divergence or dissemination through related propagation material. However, isolate CLBV6 grouped with an isolate from China, indicating that the Turkish population may not derive from one source. Because only partial genomic regions were analyzed, this interpretation should be considered preliminary. Whole-genome sequencing, and broader sampling than was carried out in the present study, would be required to determine whether distinct introductions, recombinations, or local diversification, explain the observed isolate characterizations.

The RdRp analysis showed greater sequence variability than the CP analysis, with Turkish isolates distributed across multiple clusters. This difference between genomic regions suggests that CP-based analyses alone may underestimate diversity within the sampled CLBV population. Similar patterns of greater variability in the RdRp region than in the CP region have been reported previously (Hernández-Rodríguez *et al*., 2016), supporting assessment of multiple genomic regions when evaluating CLBV diversity and relationships among isolates.

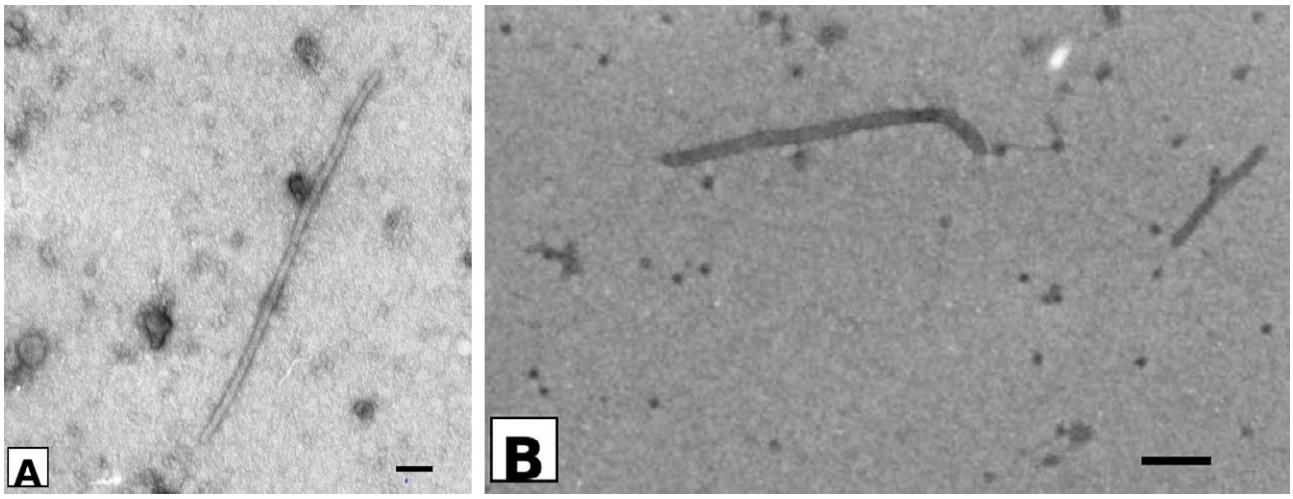


Figure 5. Transmission electron microscopy (TEM) images of flexuous filamentous virus particles in leaf dip preparations from (A) Nagami kumquat, and (B) *Nicotiana benthamiana*. The particles are consistent with those of *Betaflexiviridae*. Scale bars = 100 nm.

Transmission electron microscopy provided additional support for the molecular results by revealing flexuous filamentous particles consistent with *Betaflexiviridae*, as previously described for CLBV (Vives *et al*., 2001). TEM does not by itself identify the virus species, but the particle morphology detected in the present study was consistent with the RT-PCR, sequencing, and phylogenetic results.

From a phytosanitary perspective, detection of CLBV in symptomatic and asymptomatic plants indicates risk of spread of the virus through infected propagation material. Because CLBV is graft-transmissible and has also been reported to be seed-transmissible, symptom-based certification is insufficient. The present study results support the inclusion of CLBV in molecular indexing and routine phytosanitary monitoring programmes for citrus in Turkiye.

Taken together, the results from the present study indicate that CLBV has likely been overlooked in citrus surveys in Turkiye. Broader surveys, inclusion of additional citrus hosts and rootstocks, and whole-genome sequencing, would help clarify the distribution, genetic structure, and epidemiological significance of CLBV in Turkish citrus production.

CONCLUSIONS

This study has confirmed the presence of Citrus leaf blotch virus (CLBV) in citrus plants in the Çukurova region of Turkiye. The virus was detected in Nagami kumquat and satsuma mandarin, including trees without obvious symptoms, indicating that field inspections alone are not reliable for identifying plants infected by this virus.

Molecular analyses revealed limited CP gene diversity among most CLBV isolates from Turkiye, but greater variation in the RdRp region, suggesting that additional sampling and whole-genome analyses are warranted. One isolate showed close similarity to an isolate from China, which may indicate a separate introduction or movement of infected plant material.

These findings support the inclusion of CLBV in molecular indexing and certification programmes for citrus propagation material in Turkiye, and provide a basis for future studies on distribution and epidemiology of this virus.

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