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Uncovering the Virome of Sweet Cherry Using VANA-Based High-Throughput Sequencing and RT-PCR in Türkiye

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Abstract

In the last decade, high-throughput sequencing approaches have been increasingly applied in plant virology. In this study, virion-associated nucleic acid (VANA)-based high-throughput sequencing (HTS) was performed on a pooled sample of 29 sweet cherries (*Prunus avium* L.) exhibiting virus-like symptoms in Osmaniye Province, Türkiye. Based on viruses detected by VANA-based sequencing, 259 samples collected from major cherry-growing provinces were tested by virus-specific RT-PCR analyses to confirm the presence and prevalence of these viruses. HTS and RT-PCR analyses confirmed the presence of prune dwarf virus (*Ilarvirus PDV*, PDV) cherry green ring mottle virus (*Robigovirus viridiavii*, CGRMV), little cherry virus 1 (*Velarivirus nanoavii*, LChV-1), cherry necrotic rusty mottle virus (*Robigovirus necroavii*, CNRMV), cherry latent virus 1 (CLV-1) and cherry virus Turkey (CVTR). Among tested samples, CNRMV and CLV-1 were first report for the country and most frequently detected viruses were PDV and CGRMV. The results demonstrate that VANA-based high-throughput sequencing, followed by targeted RT-PCR confirmation, is an effective approach for the rapid and reliable detection of known and novel viruses in cherries and provides valuable data for virological surveys and certification programs.

Key words: *Prunus avium*, novel viruses, HTS, prevalence, RT-PCR

Introduction

In terms of economic value and genetic diversity, Türkiye is one of the leading countries in cherry (*Prunus avium* L.) cultivation. According to FAO data (2023), Türkiye ranks first in global cherry

production with 736791 tons, placing it in a strategic position within the global supply chain. However, such intensive production also increases the risk of spreading viral agents and is one of the most important factors threatening the sustainability of cherry production. Sweet cherry is affected by more than 35 virus and virus-like diseases, many of which can significantly reduce vegetative growth, tree vigor, and fruit quality leading to substantial economic losses (Reinhold and Pscheidt, 2023). Among these viruses prunus necrotic ring spot virus (*Ilarvirus* PNRSV, PNRSV), prune dwarf virus (*Ilarvirus* PDV, PDV) (Çağlayan et al., 2008), little cherry virus 1 (*Velarivirus nanoavii*, LChV-1) (Serçe et al., 2011), cherry green ring mottle virus (*Robigovirus viridiavii*, CGRMV) (Sipahioğlu et al. 2008), plum bark necrosis stem pitting associated virus (*Ampelovirus pruni*, PBNSPaV) (Sipahioğlu et al. 2011) and cherry virus A (*Capillovirus alphavii*, CVA) (Nursal et al., 2026) have previously been reported in cherries in Türkiye. PDV and PNRSV are generally cause mild symptoms but widespread due to their ability to spread by pollen. Some viruses, like CNRMV, cause angular chlorotic spots on leaves and necrotic and rusty speckling in infected sweet cherry trees, resulting in reduced development, yield loss, and early tree mortality. Although CGRMV is primarily latent, it can cause symptoms such as leaf necrosis, curling, and fruit necrosis in susceptible varieties, making the use of disease-free propagation material crucial for its spread due to the mechanical transmission and the absence of a known vector (Jelkmann et al., 2011).

The high resolution provided by the HTS method has enabled the discovery of many new viruses in cherries in recent years. Studies conducted in the United States have identified Prunus virus F (PrVF) in U.S.A (Villamor et al., 2017), cherry virus F (CVF) in Greece and the Czech Republic (Koloniuk et al., 2018), cherry virus Trakia (CVT) in Bulgaria (Milusheva et al., 2019), cherry robigovirus 5 (CRV-5) from Georgia (Wu et al., 2019), cherry virus Turkey (CVTR) in Türkiye and Greece (Caglayan et al., 2019; Katsiani et al., 2021), cherry latent virus 1 (CLV-1) on cherry accession imported into the USA from the Republic of Georgia (Brewer et al., 2020), and cherry virus T (ChVT) in the Czech Republic (Marais et al., 2020). Additionally, the detection of a new virus belonging to the genus *Ilarvirus* infecting sweet cherry in Northern Greece has once again demonstrated the effectiveness of HTS in determining intraspecific and interspecific viral diversity (Orfanidou et al., 2021). Recently, high-throughput sequencing (HTS) methods in the field of plant virology have brought about revolutionary advancements in the rapid, comprehensive, and reliable detection of viruses (Massart et al., 2014). Thanks to HTS technology, both known and novel viral agents can be detected from a single sample, and complete genome sequencing can be performed rapidly and with high precision (Maliogka et al., 2018). These approaches utilize various types of nucleic acids, including total RNA, virion-associated nucleic acids (VANA), double-stranded RNA (dsRNA), and small interfering RNAs (siRNA) (Roossinck et al., 2015). Specifically, the

VANA method enables the enrichment of viral genomes free of host-derived nucleic acids, allowing for the simultaneous detection of both DNA and RNA viruses. Comparative studies have shown that the VANA approach produces a higher genome coverage rate and longer contigs compared to siRNA-based methods (Candresse et al., 2014). In this context, HTS-based metagenomic approaches not only facilitate the identification of plant pathogens but also stand out as a critical tool for exploring viral diversity in natural ecosystems (Moubset et al., 2022). Beside several cherry viruses, including PDV, PNRSV, ACLSV, ApMV, CLRV, CGRM, LChV-1, and CVA, have previously been reported in Türkiye using molecular and serological methods (Çağlayan et al., 2008; Sajid and Elci, 2019), HTS analyses performed using an RNA-seq approach in cherries have identified only CVTR to date. Owing to its broad genome coverage, ability to generate long contigs, and capacity to simultaneously identify multiple viral agents through HTS, this study aimed to evaluate the effectiveness of the VANA approach in detecting viruses in *P. avium* in Türkiye and to confirm the presence of the identified viruses by RT-PCR. The findings aim to establish a significant scientific resource for monitoring and managing cherry viral diseases, highlighting the contribution of the VANA method to sensitive and comprehensive virus detection in plant virology.

Methods

Plant materials

Owing to the presence of virus-like symptoms, 29 cherry leaf samples (cv. Ziraat0900) (OK1–OK29) were collected in September 2019 from orchards with ancient cherry trees in the Bahçe district of Osmaniye province, and these samples were subsequently used as material for Illumina sequencing analyses based on the VANA method. For the survey studies, a total of 259 cherry samples (Supplementary file 1) were collected in 2019 from İzmir, Osmaniye, Bursa, Konya, Antalya, and Niğde provinces, thereby enabling a comprehensive assessment of viruses detected through HTS (Fig. 1).



Fig. 1. The provinces in Türkiye where the surveys for cherry viruses were conducted are marked by black circles.

Virus purification by the VANA protocol and library preparation

Virus particles were purified from pooled samples derived from 29 different cherry trees (21 symptomatic and 8 asymptomatic) (OK1-OK29) using the VANA method described by Maclot et al.(2021). Briefly, 60 g of leaves were ground into a fine powder in liquid nitrogen and homogenized in a mixer containing 500 ml of extraction buffer for 20 minutes. Then the mixture was passed through a double-layered cheesecloth and centrifuged at 4,000 rpm for 20 minutes at 4°C. The supernatant (80 ml) was collected, and 5 ml of 20% sucrose solution and 0.1 M Tris buffer (pH=8.2) were added. After the second centrifugation (40000 rpm for 90 minutes), the resulting pellet was incubated overnight in 200 µL of HBSS buffer.

Subsequently, non-viral nucleic acids were eliminated by DNase and RNase treatment at 37°C for 1.5 hours. Viral RNA and DNA were then isolated from 200 µL of the preparation using the PureLink™ Viral RNA/DNA Mini Kit (ThermoFischer Scientific, country). The extracted RNAs were converted to cDNA using Superscript III reverse transcriptase (ThermoFisher Scientific/Invitrogen), the cDNAs were further purified with the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France), and a complementary strand was synthesized using the Klenow fragment of DNA polymerase I. Finally, PCR amplification was performed by adding barcoded dodecamers and corresponding MID primers during reverse transcription and PCR, respectively.

Preparation of samples, library construction, and RNA-Seq analysis

A total of 29 cherry samples collected from the Bahçe district of Osmaniye province were firstly tested for widely known viruses such as PPV, ApMV, ACLSV, PNRSV, and PDV using RT-PCR analysis. Based on RT-PCR analysis, only PDV was detected in 11 samples (Supplementary file 1). All 29 samples were mixed, and a library was prepared and sequencing was performed on Nextseq 500 sequencing platform using 2×150 nt paired-end reads. The graphical flowchart of the symptoms, VANA-HTS workflow, and downstream data analysis is shown in figure 2.

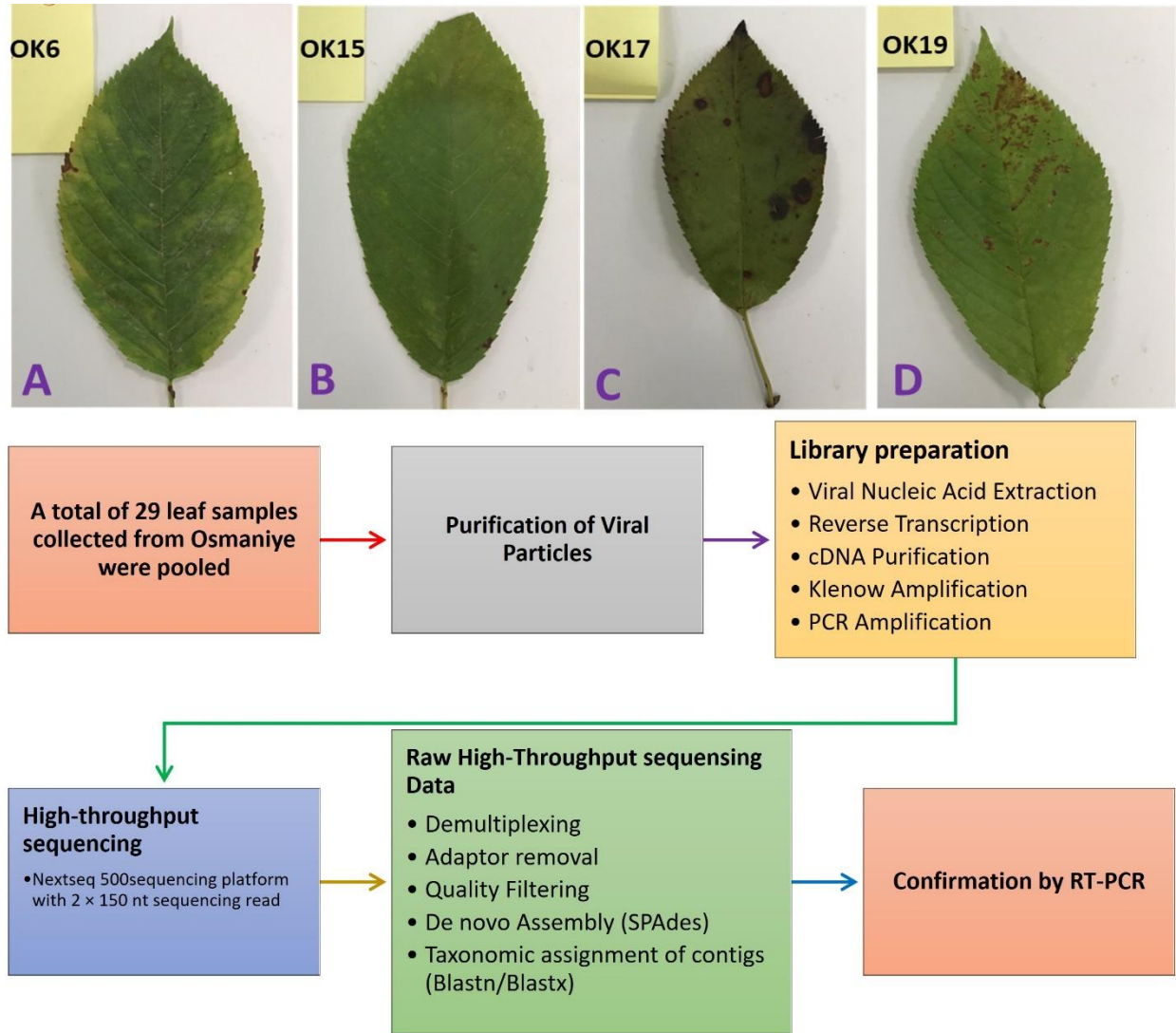


Fig. 2. Schematic representation of the experimental workflow for high-throughput sequencing (HTS) analysis followed by RT-PCR of 29 cherry samples (OK1–OK29) collected from Bahçe district, Osmaniye province-Türkiye.

Virus screening by RT-PCR

For the screening of the viruses detected in HTS analyses, 259 cherry samples (cultivars ‘Regina,’ ‘Early Lory,’ ‘Stark’s Gold,’ ‘North Wonder’, and ‘Prime Giant’) showing virus-like symptoms

and as well as symptomless ones were collected from major cherry-growing areas in Türkiye (Supplementary file 1). Total RNAs were extracted using an optimized method to obtain high-quality RNA developed by Morante-Carriel et al.(2014). cDNA was synthesized from five µl of total RNA and one µl of random hexamer primer using (Thermo Fisher Scientific, USA). The mixture was incubated at 94°C for 5 minutes and then on ice for 5 minutes. Later, 4 µl of 5× RT buffer, 2 µl of ddH₂O, 0.5 µl of dNTP (10 mM), and 1 µl of reverse transcriptase enzyme (Thermo Fisher Scientific, USA) were added and incubated at 42°C for 1 hour, followed by 72°C for 10 minutes. The PCR ingredients for CNRMV and CGRMV viruses were as follows: 17.3 µl ddH₂O, 2.5 µl 10× buffer, 0.5 µl dNTP (10 mM), 1.5 µl MgCl₂ (25 mM), 0.5 µl primer pair (10 pmol/µl each), and 0.2 µl Taq-DNA polymerase. The thermal cycling was one cycle at 94°C for 3 minutes; 35 cycles at 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 minute; and one cycle at 72°C for 10 minutes (Table 1).

Table 1. Sequences of detection primers used for identifying known cherry viruses, their amplicon sizes, annealing temperatures, and amplified gene regions.

Primer name	Virus name	Primer Sequence (5'-3')	Product Size (bp)	Annealing Temperature (°C)	Target Gene Region	Reference
CNRMV-F	CNRMV	CTTCAAGAARGAGYGAT ATTG	658	55	CP	Villamor and Eastwell, 2013
CGRMV-F	CGRMV	CTAAGTCCTCCAACCCTC A	685	60	CP	
NGRRM-TL CPR (reverse)	CGMRV/CNRMV	ATNGGTTGAATTTGGCC AGT				
CVTR 7306F	CVTR	CCTTACGCGCATGGCTTA GGTT	828	60	CP	Caglayan et al., 2019
8134R	CVTR	CTGATTGATGGCCTTGAT GGG				
1LC-12776F	LChV1	TCAAGAAAAGTTCTGG TGTGC	449	55	CP	Glasve et al., 2015
1LC-13223R		CGAGCTAGACGTATCA GTATC				

PD3-331F	PDV	AGTTTCCGCTGAAGATT GG	172	54	CP	Parakh ve et al .,1995
PD3-1086R		ACAGACTCGGCTTCCTT GA				
8F	CLV-1	GAGTGATGGCAGCAATC TTG	585	56	CP	This study
8R		ATCTGCCAGAAAAGAGC CG				

Sequence analysis, genetic variability, phylogenetic and recombination analysis

The nucleotide and protein sequences were aligned using Clustal W (1.6) embedded in MEGA 11 (Tamura et al., 2021). The phylogenetic trees were constructed using MEGA 11 with the neighbor-joining method, employing the most appropriate model and bootstrapping with 1000 replicates. Genetic distance characteristics (pairwise distances, overall distances, within-groups mean distances, between-groups mean distances, and net between-group mean distances) were estimated by MEGA11 using the best-fit model (Tamura, 1992). All positions with less than 95% site coverage were eliminated. Recombination analyses of all robigoviruses with CNRMV and Turkish isolates were performed with the RDP 5 software with a P-value of 0.05 using the following programs: RDP (Martin et al., 2005b), GENECONV (Sawyer, 1989), Bootscan (Martin et al., 2005a), MaxChi (Smith, 1992), Chimaera (Posada and Crandall, 2001), and SiScan (Gibbs et al., 2000).

Results

Identification of cherry viruses by VANA based HTS analyses

A total of 386,742 reads were obtained from HTS, which were analyzed using Geneious Prime 2019. 2.1 software (<http://www.geneious.com>). After quality control, trimming for low quality and short reads was performed using BBduk, and duplicates were removed, resulting in 154,008 high-quality reads. Subsequently, contigs ranging in length from 101 to 7300 nt were generated using the SPAdes assembler. The contigs were filtered by BLASTn/x analyses, and various plant viruses were detected. These viruses included CGRMV, CNRMV, CVT, CLV-1, CVA, PDV, apple chlorotic leaf spot virus (*Trichovirus mali*, ACLSV), LChV-1 and PrVF (Table 2).

Table 2. Number of contings, coverage, identity percentages of viral contigs obtained from HTS analysis of a mixture of 29 cherry samples collected from Osmaniye province compared with the corresponding reference sequences in GenBank and confirmation of the results by RT-PCR.

Virus name	Genus name	Number of Contings	Coverage of reference sequence (%)	Nucleotide Identity (%)	GenBank Accession number	RT-PCR
CNRMV	<i>Robigovirus</i>	16	98%	98,39%	KF030832	+
CGRMV	<i>Robigovirus</i>	2	99%	97,27%	LC522992	+
CVT	<i>Picornavirales</i>	1	86%	94,70%	NC040561	Not tested
CLV-1	<i>Trichovirus</i>	3	99%	81,08%	MK770441	+
CVA	<i>Capillovirus</i>	10	27,8%	75,1%	KY510918	-
CVTR	<i>Robigovirus</i>	80	100	60,7%	MK600387	+
PDV RNA1	<i>Ilarvirus</i>	11	60.5%	76.0%	KU215408	+
PDV RNA2	<i>Ilarvirus</i>	6	35.9%	72.3%	MZ220868	+
PDV RNA 3	<i>Ilarvirus</i>	9	62,1%	67,2%	KU215404	+
ACLSV	<i>Trichovirus</i>	5	13.8%	73.9%	MK368735	-
LChV-1	<i>Velarivirus</i>	108	89.1%	67.5 %	KX192366	+
PrVF RNA1	<i>Fabavirus</i>	10	28.8%	87.7%	OR545804	Not tested
PrVF RNA2	<i>Fabavirus</i>	3	14.4%	75.5%	OR545805	Not tested

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174 To confirm the presence of viruses detected by HTS analysis in 29 cherry samples collected from

175 Osmaniye Province, each sample was individually tested for CNRMV, CGRMV, CLV-1, CVA,

176 CVTR, PDV, ACLSV, and LChV-1 using RT-PCR. The expected amplifications were obtained

177 in 11 cherry samples for PDV (37.9%), 10 samples for CGRMV (34.4%), seven samples for CLV-

178 1 (24.1%), four samples for LChV-1 (13.7%), two samples for CVTR (6.8%) and one sample for

179 CNRMV (3.4%) (Fig 3, Supplementary file 1). The samples tested for the viruses were also

180 confirmed by Sanger sequencing and the sequences were submitted to the NCBI database

181 (Supplementary file 1). Among 29 samples included in the VANA-HTS analysis, a total of 9

182 samples were found to be mixed infected. Of these, 6 were symptomatic and 3 were asymptomatic.

183 Mixed infections in symptomatic samples were detected as PDV+CGRMV in 3 samples (OK4,

184 OK14, OK16), PDV+LChV-1 in one sample (OK2), PDV+CGRMV+LChV-1 in one sample

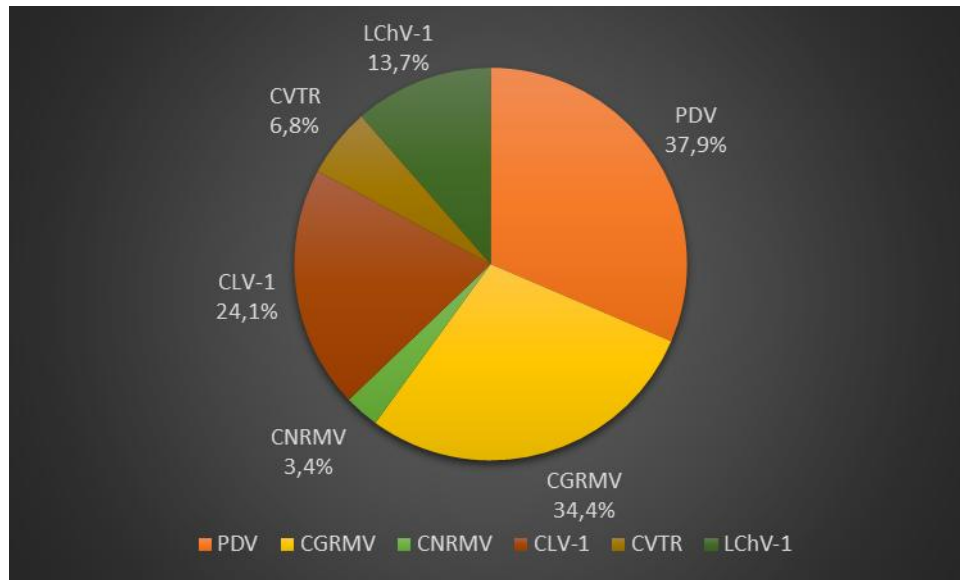
185 (OK7), and PDV+CVTR in one sample (OK13). In asymptomatic samples, mixed infections were

186 identified as CGRMV+CLV-1 in one sample (OK15), CGRMV+CNRMV+LChV-1 in one sample

187 (OK18), and CLV-1+CVTR+LChV-1 in one sample (OK19). Both CNRMV and CLV-1 were

188 detected in Türkiye for the first time through HTS analysis and results were confirmed by RT-

189 PCR.



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Fig. 3. Prevalence of viruses detected by HTS analysis in 29 cherry samples collected from Osmaniye province using RT-PCR analysis.

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Sequence identity analyses of CVTR and LChV-1 isolates were evaluated based on nucleotide and amino acid data. The CVTR OK19 isolate showed 91.4% nucleotide identity with the Greek isolate, while the highest nucleotide identity (97.9%) was detected with Turkish Bursa isolates (MH986197 and MH986198), and the lowest identity (87.4%) was observed with the MH986213 isolate; amino acid identities ranged from 78.8% to 93.6%. In the case of LChV-1, the highest nucleotide identity (98.3%) was observed between PX103079 (OK7) and the Spanish isolate (KX192367), while the lowest identity (80.7%) was detected between PX103081 (OK19) and the Italian isolate (EU716000); among Turkish isolates, nucleotide identity ranged between 82.7% (PX103080–PX103081) and 95.3% (PX103078–PX103079), whereas amino acid identity was 100% between PX103081 (OK19) and HG792420 (Greek isolate) (Fig 4).

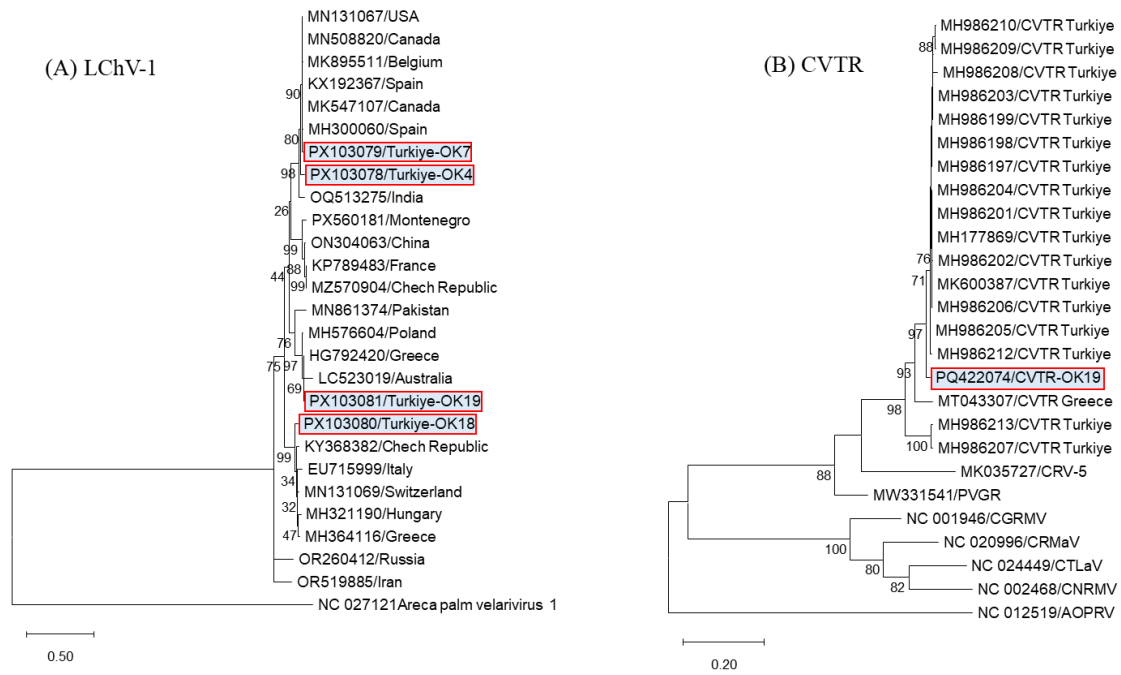


Fig. 4. Phylogenetic trees based on partial coat protein gene sequences of (A) LChV-1 and, (B) CVTR, isolates. The trees were constructed using the Neighbor-Joining method in MEGA 11 with 1000 bootstrap replicates. Bootstrap support values (%) are indicated at the nodes. Isolates obtained in this study are labeled with sample IDs (OK numbers) and corresponding accession numbers and are highlighted in the trees. Scale bars represent the number of nucleotide substitutions per site.

Detection of cherry-infecting viruses in large scale field survey

Symptoms observed in the collected samples of different cherry varieties included leaf discoloration, severe mosaic patterns, chlorotic and necrotic spots, interveinal yellowing, and browning (Fig. 5).

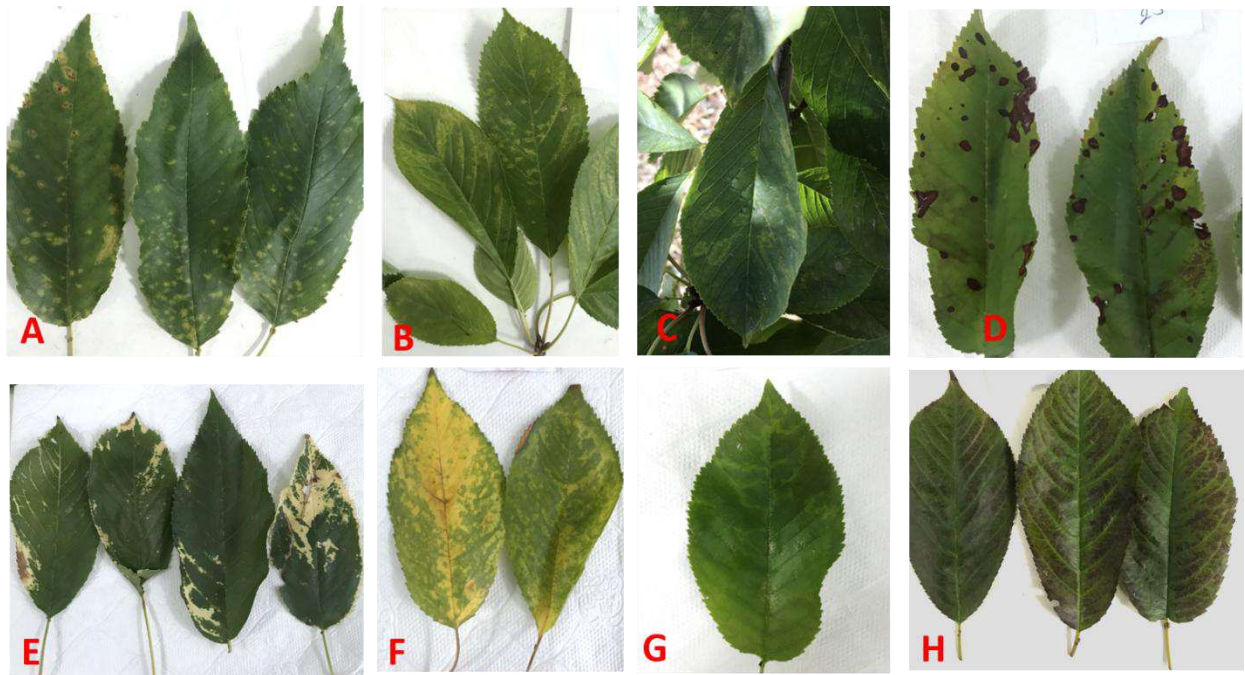


Fig. 5. Various symptoms were observed in samples collected from the provinces of İzmir, Osmaniye, Bursa, Konya, Antalya, and Niğde. A: chlorotic and necrotic spots; B: vein clearing and chlorotic spot C: mosaic and ring spots D; dark brown necrotic spots; E: discoloration; F: vein yellowing and severe mosaic; G: chlorotic ring; H: interveinal browning.

Due to large scale of surveys for PDV, CVTR and LChV-1 were already conducted in Türkiye (Caglayan et al.,2019; Santosa et al.,2023; Ulubas Serce.,2011), in frame of this study the prevalence of CNRM, CGRMV and CLV-1 was investigated by RT-PCR. Among 259 samples, 27 were found to be infected with CGRM, 54 were found with CLV-1 while 10 were found to be infected with CNRMV. Based on the RT-PCR analyses, mixed infections were identified in seven samples, including four samples with CGRMV + CLV-1, two samples with CNRMV + CLV-1, and one sample with CGRMV + CNRMV. The highest infection rate of CGRMV was found in Osmaniye province (14.9%), followed by Bursa (12.5%), Konya (11.11%), İzmir (10%), and Antalya (3.9%). Regarding CNRMV, the highest infection rate was recorded in Antalya (11.76%), while İzmir (7.5%) and Niğde (7.4%) exhibited comparable infection levels, and no infections were observed in Bursa and Konya. For CLV-1, the highest infection rate was observed in Konya (29.4%), followed by Niğde (25.9%), Bursa (21.3%), Antalya (17.6%), Osmaniye (16.4%), and İzmir (17.5%). (Fig. 6).

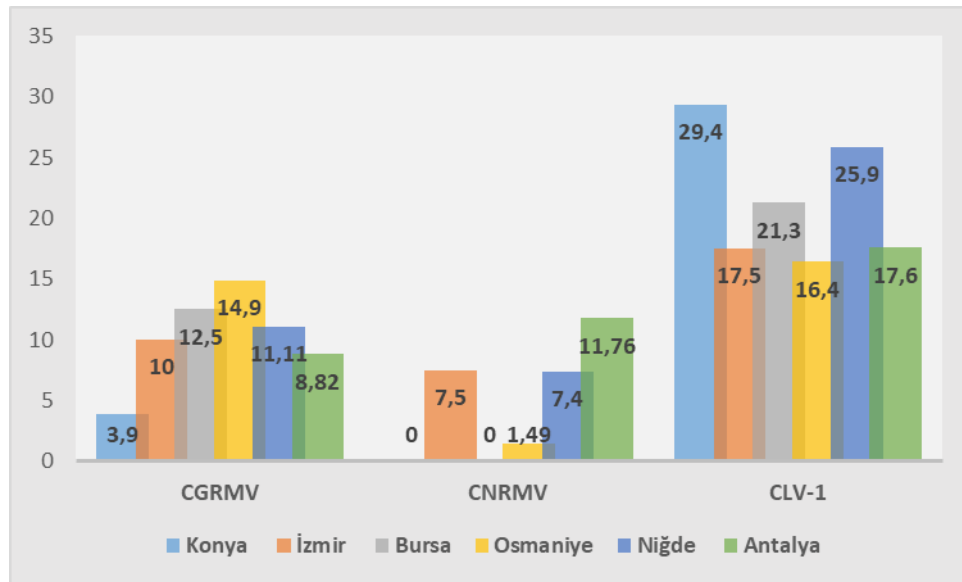


Fig. 6. Prevalence of cherry green ring mottle virus (CGRMV) and cherry necrotic rusty mottle virus (CNRMV) and Cherry latent virus 1 (CLV-1) infections in cherry samples collected from different provinces of Turkey, based on RT-PCR results.

Genetic diversity and phylogenetic analysis

A total of 26 CGRMV isolates were sequenced and submitted to GenBank (Supplementary file 1). The highest nucleotide identity was found to be 99% between Canada Cherry and Osmaniye OK18 isolates (MZ148104 and OR820900). In contrast, the lowest identity was recorded as 77.7% between the Mexico plum isolate (KY774448) and the Antalya Ank21 (OR820882) and Bursa (OR820876) cherry isolates. Turkish isolates showed the highest nucleotide identity of 99% among Osmaniye samples (OR820899/OK17 and OR820898/OK16), while the lowest nucleotide identity was found to be 94.4% between the İzmir (OR820890/I257) and Niğde (OR820893/NK107) isolates (Figure 7). Furthermore, the Niğde isolate (OR820893/NK107) and a USA cherry isolate (AB863734) were identical at the amino acid (a.a.) level. At the same time, the lowest identity (96.9%) was found between Mexican plum (KY774448) and all Turkish isolates. The Turkish isolates from Antalya, Bursa, Niğde, and Konya, as well as Osmaniye, were identical at the a.a. level. In contrast, the lowest nucleotide identity was 99.6% between İzmir samples and other isolates.

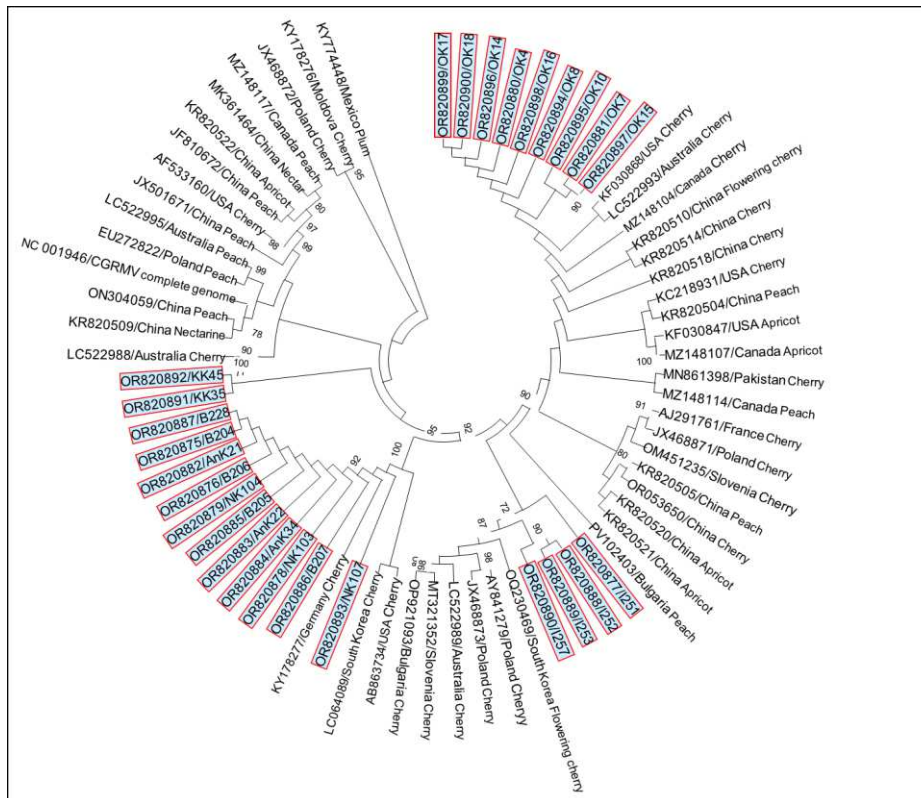


Fig. 7. Phylogenetic tree based on the nucleotide sequences of CGRMV isolates obtained in this study from Turkey (highlighted in blue box) and other countries registered in GenBank. Phylogenetic analysis was performed using MEGA 11 with Neighbor-Joining (NJ) method software. Bootstrap values on the branches are shown as percentages (%).

Sanger sequencing was performed on the five CNRMV positive samples, and the sequences were submitted to GenBank (Fig 8). The highest nucleotide identity as 99.7% was observed between an Izmir isolate (I281-OR820871) and the Polish peach isolate (MH681791). Comparison with isolates in the GenBank database revealed the lowest nucleotide identity as 84.1% was between the AnK13 (OR820870) Antalya and I281 Izmir (OR820871) isolates and a Chinese nectarine isolate (MK361472 China). Turkish-Niğde isolates showed 100% nucleotide identity (OR820872/NK110 and OR820873/NK113), whereas the lowest nucleotide identity (92.2%) was observed between the Antalya isolate OR820870/AnK13 and the Osmaniye isolate OR820874/OK18. When the Turkish isolates were compared among themselves, 100% amino acid identity was determined among the Antalya isolate OR820870, the Niğde isolates OR820872 and OR820873, and the İzmir isolate OR820871, indicating complete conservation at the amino acid level. In contrast, the Osmaniye isolate OK18 (OR820874) exhibited a slightly lower similarity, sharing 99.3% amino acid identity with the remaining Turkish isolates.

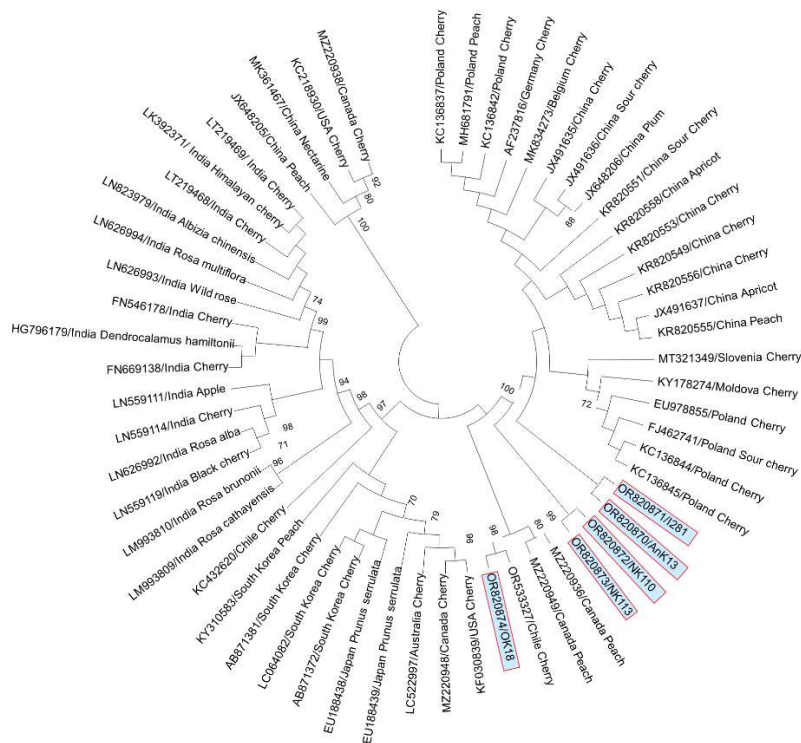


Fig. 8. Phylogenetic tree based on the nucleotide sequences of Turkish CNRMV isolates (highlighted in the blue box) and other isolates registered in GenBank. Phylogenetic analysis was performed using the Neighbor-Joining (NJ) method in MEGA 11 software. The bootstrap values on the branches are shown as percentages (%).

When the nucleotide and amino acid identities of CLV-1 isolates were evaluated, 100% nucleotide identity was observed between PX564504 (OK22, Osmaniye) and PZ244700 (OK60, Osmaniye). The lowest nucleotide identity was detected between PZ244700 (OK60, Osmaniye) and PZ244683 (AnK11, Ankara). In comparisons with global isolates, the highest nucleotide identity was found between the Greek isolate (OP750486) and PZ244680 (AnK33, Ankara) with 95.8%, whereas the lowest identity was observed between PZ244684 (B188, Bursa) and the GenBank reference NC076710 (Georgia isolate). At the amino acid level, the highest identity was 100% between PX564504 (OK22, Osmaniye) and PZ244700 (OK60, Osmaniye), while the lowest identity was 99% between PZ244683 (AnK11, Ankara) and PZ244697 (NK107, Niğde). In comparison with global isolates, the highest amino acid identity was 98.5% between PZ244682 (AnK26, Ankara) and the Greek isolate (OP750485), whereas the lowest identity was 94.9% between PX564506 (OK27, Osmaniye) and the GenBank reference NC076710 (Georgia isolate) (Fig 9). According to the species demarcation criteria established by the ICTV for the Trichovirus genus, viruses are considered distinct species when they share less than approximately 72% nucleotide or 80% amino acid identity in the CP or Rep genes; therefore, the identity values obtained in this study clearly

the most closely related to Slovenian isolates (0.0038 ± 0.00), supporting a European origin or a shared epidemiological history for Turkish CNRMV isolates.

Recombination analysis

Recombination analyses were performed on the partial CP gene sequences of all robigovirus isolates using RDP5 software (Martin et al., 2021), resulting in the identification of two putative recombination events. In the first event, a potential recombination region was detected in the KF356396 accession (CRMV isolate 8804) by the SiScan, MaxChi, Chimaera, and GENECONV algorithms, with the OK4 isolate identified as the putative major parent and the MZ221022 accession (CRMV isolate 13TF134) as the minor parent. In the second event, a recombination region was identified within the CP gene of the I251 isolate, where MZ148103 was inferred as the major parent and MZ148112 as the minor parent; this event was supported by the BootScan, MaxChi, Chimaera, and SiScan algorithms ($p < 0.01$).

Discussion

The objective of this study was to thoroughly identify viral agents in cherry trees in Türkiye by employing the enhanced sensitivity offered by VANA-based HTS method. In frame of this study, in addition to CGRMV, LChV-1, CVA and CVTR, which were already identified in previous studies, viruses such as CLV-1, CNRMV and PrVF are reported for the first time in the country. The results align with Massart et al. (2014) and Maliogka et al. (2018), who characterize HTS as an "exploratory diagnostic tool" in plant virology. Furthermore, the results presented by Candresse et al. (2014) regarding sugarcane samples illustrate the superior efficiency of the VANA method in terms of long contig generation and genome coverage.

Confirmation of HTS results with RT-PCR analyses definitively revealed the presence of CGRMV, CNRMV, and CLV-1 in Türkiye. Although the presence of CGRMV in cherries was already reported in the Eastern Anatolia Region in 2008 (Sipahioğlu et al., 2008), CNRMV and CLV-1 are the first report for the country. Previous study on CGRMV showed quite high infection rate as 33% in Eastern Anatolia while CGRMV infection rates were found much lower in the Mediterranean Region as 14.9% in Osmaniye and 11.76% in Antalya provinces in this study. It might be due to less amount of tested samples (34) in previous study or linked to a range of variables, including the sources of breeding material, host genotypes, and regional climate. In addition, the high prevalence of CLV-1 (20.8%, 54/259) highlights its widespread distribution across surveyed regions and suggests a potentially significant epidemiological role in Turkish cherry production systems. It is thought that most of these viruses are spread through grafting material, as there are no known alternative means of transmission. This evidence highlights the

importance of using certified, disease-free seedlings. Phylogenetic analyses showed that CGRMV and CNRMV isolates obtained from Türkiye have high genetic identity to international isolates (94–100%). The close relationship between CGRMV and CNRMV isolates, as well as those from Canada and China, suggests that these viruses can be spread through the movement of international trade goods. The Turkish isolates had amino acid levels that were almost 100%. Within the *Betaflexiviridae* family, species demarcation in the *Robigovirus* genus is primarily based on approximately 72% nucleotide or 80% amino acid identity in the Rep and CP genes. Accordingly, the CVTR isolates can be considered to belong to the same species, although the observed amino acid variability indicates a certain level of genetic diversity within the population. In contrast, species demarcation in the *Velarivirus* genus, to which LChV-1 belongs, is mainly defined by more than 25% amino acid divergence in key proteins such as RdRp, CP, and HSP70h. In the present study, despite the high conservation at the amino acid level, the relatively low nucleotide identity (80.7%) indicates notable genetic divergence, which is better interpreted as intraspecies variation rather than evidence of a distinct species.

Recombination studies have demonstrated the existence of multiple recombination regions within the CGRMV genome. These findings suggest that recombination may play a significant role in preserving viral genetic diversity. Subsequent research should examine the effects on virulence, host compatibility, and environmental tolerance.

This study has demonstrated the potential for seemingly latent robigoviruses to spread quietly in cherry-producing regions of Turkey. Regular RT-PCR or HTS-based monitoring programs are necessary because infections can only be detected at the molecular level, often due to the absence of visible symptoms. Turkey is the world's largest producer of cherries (FAO, 2023), so these mild infections could be harmful for exports. Therefore, it is essential to use disease-free propagation material in the cherry production chain, conduct regular virological tests, and establish national certification systems.

To sum up, this study reports for the first time the presence of CNRMV and CLV-1 in Türkiye, confirmed by both HTS and RT-PCR analyses, while the presence of PrVF was detected only by HTS analysis, indicating the need for further studies. The subsequent detection of CGRMV in different locations in Türkiye indicates that the virus remains present there together with other robigoviruses like CNRMV and CVTR. The large amount of data from HTS has helped demonstrate the accuracy of viral diversity in cherries. Recombination analyses have demonstrated the flexibility of the genetics in this group of viruses. The results obtained provide a crucial foundation for enhancing the ability to conduct regular virological tests, developing strategies for virological monitoring, and establishing a sustainable cherry production system in Türkiye.

Author contribution statement

BT, KÇ, VR, JSR, Investigation, Formal analysis, Data curation, Methodology; BT, KÇ, VR Writing-original draft; KÇ, JSR, Conceptualization, Supervision, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data availability The nucleotide sequences generated in this study have been deposited in GenBank (NCBI) under accession numbers OR820870–OR820900, PQ422074, PX103078–PX103081, PX564504–PX564506 and PZ244680–PZ244700. All other data supporting the findings of this study are available within the article and its Supplementary Information files.

Ethics approval and consent to participate

All plant samples were collected with permission from the landowners of the sampling sites, and no additional ethical approval was required.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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