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Investigation of Viral Agents in Walnut (*Juglans* spp.) Trees by High Throughput Sequencing from Niğde province, Türkiye

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ABSTRACT
In October 2023, virus-like symptomatic walnut trees in Niğde province were investigated using high-throughput sequencing (HTS) to investigate viral infections. Double-stranded RNA (dsRNA) extraction was conducted, followed by conversion of dsRNA into cDNA. PCR was then performed using barcoding with MID primers for metagenomic analyses. The PCR products from walnut samples B13, B22, B23, and B21 + B5 were sequenced using the Illumina NovaSeq 6000 sequencing platform. The sequencing data underwent bioinformatics analysis using Geneious Prime and CLC Genomic Workbench. In this comparison, more contigs were constructed in CLC Genomic Workbench, resulting in more precise outcomes with BLASTx analysis. According to the map, to reference analyses of B21+B5 HTS data, 29,574 reads matched with cherry leaf roll virus RNA1 (CLRV) RefSeq NC_015414, and 405 reads matched with CLRV RNA2 RefSeq NC_015415. However, 4 reads matched with cucumber mosaic virus (CMV) RefSeq NC_001440 from B23 HTS data. To confirm the presence of these viruses, virus-specific primers were used to test the total RNA extracted from walnuts. CLRV was detected in sample B21 but not in B5. CMV was not detected in sample B23. Improving nucleic acid extraction methods is recommended to enhance the detection of viral agents using high-throughput sequencing.

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Niğde İli Ceviz (*Juglans* spp.) Ağaçlarındaki Viral Etmenlerin Yüksek Kapasiteli Dizileme ile Araştırılması

ÖZET
Niğde ilinde, Ekim 2023'te, virüs benzeri simptomlar gösteren ceviz ağaçları, viral enfeksiyonları araştırmak için yüksek kapasiteli dizileme kullanılarak analiz edilmiştir. Çift sarmallı RNA (dsRNA) ekstraksiyonu yapılmış, ardından dsRNA'lar cDNA'ya dönüştürülmüştür. Daha sonra metagenomik analizler için MID primerleri ile barkodlama kullanılarak PCR gerçekleştirilmiştir. B13, B22, B23 ve B21+B5 ceviz örneklerinin PCR ürünleri Illumina NovaSeq 6000 dizileme platformu kullanılarak dizilenmiştir. Dizileme verisi Geneious Prime ve CLC Workbench kullanılarak biyoinformatik analize tabi tutulmuştur. Bu karşılaştırmada, CLC Workbench'te daha fazla contig oluşturulmuş ve BLASTx analizi ile daha doğru sonuçlar elde edilmiştir. B21+B5 HTS verisinin referansa göre haritalama-analizlerine göre, 29.574 okuma cherry leaf roll virüsü RNA1 (CLRV) RefSeq NC_015414 ile ve 405 okuma CLRV RNA2 RefSeq NC_015415 ile eşleşmiştir. Ancak, B23 HTS verisindeki 4 okuma cucumber mosaic virus (CMV) RefSeq NC_001440 ile eşleşmiştir. Bu virüslerin varlığını doğrulamak amacıyla, cevizlerden ekstrakte edilen total RNA'yı test etmek için virüse özgü primerler kullanılmıştır. CLRV, B21 örneğinde tespit edilmiş ancak B5'te tespit edilmemiştir. CMV, B23 örneğinde tespit edilmemiştir. Yüksek kapasiteli dizileme kullanılarak viral ajanların tespitini arttırmak için nükleik asit ekstraksiyon yöntemlerinin iyileştirilmesi önerilmektedir.

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INTRODUCTION

Walnut (*Juglans regia L.*) belongs to the *Juglandaceae* family and is considered one of the highest-quality nuts in temperate regions, particularly Central Asia. Walnuts are used in various culinary applications and offer numerous nutritional benefits, including minerals, antioxidants, and vitamins that support heart and brain health (Sütyemez, 2008). Walnuts are widely distributed globally and can be cultivated in many regions, including all parts of Türkiye (Budak, 2010). In Türkiye, 360,000 tons of walnuts were produced, with significant contributions from the provinces of Kahramanmaraş, Bursa, and Bilecik (TUIK, 2023). It is reported that Türkiye will achieve self-sufficiency in walnuts from 2020 to 2045 (Güvenç and Purlu, 2022).

However, walnut trees are susceptible to fungal, bacterial, viral, and virus-like diseases. Notably, the cherry leaf roll virus (CLRV) (*Nepovirus avii*) and plum pox virus (PPV) (*Potyvirus plumpoxi*) have been identified in walnuts (Savino et al., 1977; Baumgartnerova, 1996). CLRV was reported in walnuts in the Lake Van Basin in Türkiye (Ozturk et al., 2008; Siphaioglu et al., 2011; Yegül and Baloğlu, 2019).

Plant pathogenic agents can be identified through high-throughput sequencing (HTS) technologies like Illumina sequencing combined with bioinformatics analysis. In the Illumina sequencing, a little slide is flooded with fluorescently labeled nucleotides and DNA polymerase. Also, there is one terminator for stopping the length read. When the terminator sees the fluorescently labeled nucleotides, it stops and reads the read lengths. In this working principle, a slide is used to detect the lengths. It read an average of 50-300 bp (Sharma et al., 2022).

HTS can identify all DNA and RNA viruses in a sample through a single analysis when appropriate methodology is used. This advanced technique provides an in-depth and comprehensive assessment of a plant's viral health status, allowing for a thorough understanding of any potential viral threats that may affect its overall phytosanitary condition (Kreuze et al., 2009; Moubset et al., 2022). Furthermore, the acquired sequence data can be leveraged for multiple applications, including elucidating the population structuring, ecological interactions, and evolutionary dynamics of viruses. It also aids in differentiating variants with varying impacts on disease etiology.

HTS offers flexibility in its implementation, allowing for individual, bulked, or mixed samples (Maree et al., 2018). A range of nucleic acid populations can be utilized for HTS. The primary methods have focused on double-stranded RNA (dsRNA), virus-derived small interfering RNA (siRNA), virion-associated nucleic acids (VANA), total RNA, whether or not it has undergone rRNA depletion, and polyadenylated RNA. The advantages and disadvantages of each approach for virus discovery and etiology research can vary significantly (Ma et al., 2019). Regardless of the assessment criteria, the dsRNA-based method showed a greater diversity of RNA viruses than the VANA method. Dissimilarity analyses revealed that both methods were reproducible but not necessarily convergent (Ma et al., 2019).

Although there has been no record of virus identification in walnuts by HTS, there are many examples from other perennial crops like grapevine, mulberry, and pomegranate (Maliogka et al., 2015; Caglayan et al., 2020; Gürcan et al., 2021).

In October 2023, walnut trees exhibiting virus-like symptoms were observed in Niğde province. These symptoms included line patterns, yellow discoloration between the lateral veins of the leaves, dark green patches on the leaf surface, bright yellow veins, black veins, and narrow leaves. We collected walnut leaf samples that exhibited these symptoms to explore the connection between the observed symptoms and potential viral agents. The samples were then subjected to dsRNA extraction, followed by Illumina sequencing and subsequent bioinformatics analysis.

MATERIAL and METHOD

Plant Materials

In October 2023, shoot samples were collected from five walnut trees (B5, B13, B21, B22, B23) showing virus-like disease symptoms in Ulukışla and central Niğde province. Two of these samples (B5 + B21) were analysed as a bulk sample randomly due to the sequencing quota, while the remaining samples were analysed individually. The samples were preserved using liquid nitrogen and stored at -80°C until analysis.

Double-strand RNA Isolation and Viral Nucleic Acid Enrichment

Double-stranded RNAs (dsRNA) were purified from each sample using two rounds of CF11 cellulose chromatography, following the protocol established by Marais et al. (2018). The undiluted dsRNA samples were then subjected to cDNA synthesis with Dodeca primers, followed by PCR amplification employing MID primers as described by François et al. (2018). For the cDNA amplification with the Dodeca primer, the preparation for each sample included 0.6 µL of nuclease-free water, 0.4 µL of Dodeca primer (as shown in Table 1), and 9.5 µL of dsRNA, resulting in a total volume of 10.5 µL. Subsequently, the mixture underwent a short-term denaturation at 95°C for 5 minutes.

Table 1. Primer sequences used for cDNA and PCR amplifications of walnut dsRNAs
Çizelge 1. Ceviz dsRNA'larının cDNA ve PCR amplifikasyonları için kullanılan primer dizileri

Primer	Base Sequence (5'- 3')	Walnut Sample Name
LDF_007_Dodeca	CGTGGAGACTCTGGNNNNNNNNNNT	B23
LDF_011_Dodeca	ACGCCATCACACGGNNNNNNNNNNT	B13
LDF_078_Dodeca	GTGACCGACACCGTNNNNNNNNNNT	B5+B21
LDF_084_Dodeca	TACGACCGCTGCACNNNNNNNNNNT	B22
Tag156_4_LDF_007	AAGGTAGAAGCGTGGAGACTCTGG	B23
Tag34_4_LDF_011	AATACTGTGGACGCCATCACACGG	B13
Tag590_4_LDF_078	GCAAGATGTAGTGACCGACACCGT	B5+B21
Tag684_4_LDF_084	GGCATATACCTACGACCGCTGCAC	B22

The total volume of the reaction mix for cDNA amplification was 9.5 µL, which included the following components: 2 µL of dNTP (10 mM), 2 µL of DTT (100 mM), 0.5 µL of RNase OUT (20 U/µL), 4 µL of 5X Reverse Transcriptase Buffer, and 1 µL of SuperScript Reverse Transcriptase (200 U/µL). These were combined in each tube containing Dodeca primers and dsRNA to achieve a final cDNA volume of 20 µL. The cDNA synthesis procedure involved 10 minutes at 25°C, 1 hour at 42°C, and 5 seconds at 70 °C.

The PCR reaction mix for enriching viral nucleic acids consisted of the following components: 33.50 µL of water, 5 µL of 10X Buffer, 1.25 µL of dNTP, 5 µL of Tag (MID) Primer (refer to Table 1), 0.25 µL of Dream Taq polymerase enzyme, and 5 µL of cDNA sample, totaling 50 µL. The amplification conditions were set as follows: 1 minute at 94 °C, 1 minute at 65 °C, and 45 seconds at 72 °C, followed by 40 cycles of 1 second at 94 °C, 1 second at 45 °C, and 5 minutes at 72 °C. The process concluded with a final extension of 5 minutes at 72 °C and 5 minutes at 37 °C. To visualize the PCR products, 10 µL of the reaction was loaded onto a 1.5% agarose gel for electrophoresis.

Sequencing

The PCR products obtained were sent to Genoks (Ankara, Türkiye). Library construction was carried out using the Illumina DNA Prep Kit, previously known as Nextera XT. We requested sequencing for each sample at a depth of 5 M, with a read length of 2 x 150 bp. The sequencing platform used was the Illumina Novaseq 6000.

Bioinformatics Analysis

After performing quality analyses on the FASTQ data provided by the company, we conducted a set of paired, trimming, and merging analyses using the reads. Trimming was performed using the BBDuck Adapter/Quality Trimming Version 38.37 for Geneious Prime and in CLC Genomic Workbench with the default parameters. *De novo* assembly was performed with default parameters of the SPAdes assembler in Geneious Prime 2024.0.5, while CLC's assembly system in CLC Genomic Workbench 11.0.1. Subsequently, BLASTn analyses were carried out against a custom viral database (retrieved on 19 May 2024) using the resulting contigs. BLASTx analysis was performed against NCBI nucleotide data only using contig data by CLC Genomic Workbench. After the blast analyses, map-to-reference analyses were conducted using the detected viruses as references with both software tools. The BLASTn and BLASTx analyses used contigs from de novo assembly, while map-to-reference analyses utilized read pairs obtained after trimming.

Confirmatory Analyses for Detected Viruses

To validate the viruses detected in Illumina sequencing data, total RNA extraction, cDNA synthesis, and virus PCR tests were performed on the samples using virus-specific primers.

Total RNA extraction was performed with modifications based on the protocol by MacKenzie et al. (1997). After cDNA synthesis, the total RNA extracted from the samples was subjected to PCR using virus-specific primers. The analysis included CLRV and cucumber mosaic virus (CMV) (*Cucumovirus CMV*), which were tested with virus-specific primers (Werner et al., 1997; Grieco et al., 2000) (Table 2).

Table 2. Specific primers used for virus tests of B5, B21, and B23 walnut samples
Çizelge 2. B5, B21 ve B23 ceviz numunelerinin virüs testleri için kullanılan spesifik primerler

Virus Scientific Name	Nucleotide Sequence (5'-3')	Reference
Nepovirus avii (CLRV)	F: TGGCGACCGTGTAACGGCA R: GTCGGAAAGATTACGTAAAAGG	Werner et al., 1997
Cucumovirus CMV (CMV)	F: CCATCACCTTAGCTTCCATGT R: TAACCTCCCAGTTCTCACCGT	Grieco et al., 2000

For cDNA synthesis, a mixture was prepared containing 4 µL of nuclease-free water, 1 µL of random hexamer, 4 µL of RNA, and 1 µL of dNTP (10 mM), making a total volume of 10 µL. This mixture was subjected to short-term denaturation at 65 °C for 5 minutes. Following denaturation, a second mixture was added to each sample, consisting of 3.2 µL of nuclease-free water, 0.5 µL of DTT (100 mM), 0.3 µL of RNase OUT (20 u/µL), 5 µL of 5X RT Buffer, and 1 µL of MML-V Reverse Transcriptase, bringing the final volume to 20 µL. The cDNA synthesis procedure included 10 minutes at 25 °C, 45 minutes at 42 °C, and 10 minutes at 70 °C.

PCR amplification was conducted using a mixture of 6.4 µL of nuclease-free water, 10 µL of 2X Master Mix, 0.8 µL of forward primer (10 µM), 0.8 µL of reverse primer (10 µM), and 2 µL of the cDNA sample. The amplification program comprised an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of 45 seconds at 95 °C, 45 seconds at 60 °C (for CMV) or 50 °C (for CLRV), and 45 seconds at 72 °C, concluding with a final extension at 72 °C for 10 minutes.

Each PCR product was visualized using 1.2% agarose gel electrophoresis. The gels were stained with ethidium bromide and visualized using a gel imaging system.

RESULTS and DISCUSSION

In walnut samples, abnormal leaf development was observed. Yellow discolorations along the lateral veins, bright yellow lines following the veins, dark green areas between the veins, and blackening of the veins were also monitored (Figure 1).

Figure 1. Symptoms observed in walnut trees: A) Sample B5, abnormal leaf development; B) Sample B13, blackening of the main veins on the leaves; C) Sample B21, yellow discoloration between the lateral veins of the leaves; D) Sample B22, bright yellow blotches along the main and lateral veins of the leaves; E) Sample B23, dark and light green blotches on the leaves.

Şekil 1. Ceviz ağaçlarında görülen belirtiler: A) Örnek B5, anormal yaprak gelişimi; B) Örnek B13, yapraklardakianadamarların kararması; C) Örnek B21, yaprakların yan damarları arasında sarı renk değişikliği; D) Örnek B22, yaprakların ana ve yan damarları boyunca parlak sarı lekeler; E) Örnek B23, yapraklarda koyu ve açık yeşil lekeler.

The dsRNAs randomly amplified using Dodeca and Tag (MID) primers exhibited multiple bands and smears (Figure 2). The dsRNA extraction protocol eliminated DNA by applying DNase I, as the target genomes are RNAs in this method.

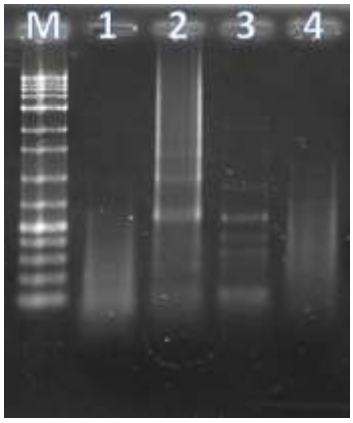

Figure 1. dsRNA products on agarose gel amplified using Dodeca and Tag (MID) primers. M: Marker; 1: B13, 2: B21+B5, 3: B22, 4: B23 walnut samples.

Şekil 2. Dodeca ve Tag primerleri kullanılarak çoğaltılmış agaroz jel üzerindeki dsRNA ürünleri. M: Markör; 1: B13, 2: B21+B5, 3: B22, 4: B23 ceviz örnekleri.

Read numbers were changed according to the samples, around 11.7-21.2M after sequencing (Table 3). Trimming in the CLC Genomic Workbench led to removing reads from 107.000 to 337.000 (Table 4). A quality score below 0.05 for CLC Genomic Workbench and sequence reads quality below Q10 for Geneious Prime was trimmed. The purpose of trimming is to eliminate adapter sequences and low-quality reads, enhancing the accuracy of results by removing low-quality data.

Table 3. Illumina data obtained after sequencing

Çizelge 3. Sekanslamadan sonra elde edilen Illumina verisi

Sample Name	Number of Reads for R1	Number of Reads for R2	Total Number of Reads (R1+R2)	Range of Sequence Length
B13	7,397,993	7,397,993	14,795,986	35-151
B22	9,514,828	9,514,828	19,029,656	35-151
B23	10,624,402	10,624,402	21,248,804	35-151
B21+B5	5,871,496	5,871,496	11,742,992	35-151

Table 4. Number of reads after trimming in the CLC Genomic Workbench and merging in Geneious Prime

Çizelge 4. CLC Genomic Workbench'te kırpma ve Geneious Prime'da birleştirme sonrasında okuma miktarı

Sample Name	Number of Total Reads	CLC Genomic Workbench	Geneious Prime	
		Number of Reads After Trimming	Number of Merged Reads	Number of Unmerged Reads
B13	14,795,986	14,678,130	4,752,456	2,194,808
B22	19,029,656	18,692,547	5,303,888	3,146,332
B23	21,248,804	21,131393	6,876,590	3,393,604
B21+B5	11,742,992	11,624,878	3,701,881	1,245,290

Merged read amounts were changed around 3.7-6.9M, unmerged reads were around 1.3-3.4M according to samples in Geneious Prime (Table 4). When the paired reads are aligned and combined, they are called "merged" and the others are called “unmerged”. Both types of files (merged and unmerged) were selected for the *de novo* assembly stage.

Comparison of Contigs After Assembly with Geneious Prime and CLC Genomic Workbench

For the B13 sample, the total number of contigs was 908 in CLC Genomic Workbench, while Geneious Prime showed only 152 contigs. In the case of the B21+B5 sample, CLC Genomic Workbench produced 3.566 contigs compared to just 69 in Geneious Prime. For the B22 sample, CLC Genomic Workbench recorded 964 contigs, whereas Geneious Prime had 73. Lastly, for the B23 sample, CLC Genomic Workbench yielded 1.910 contigs, while Geneious Prime resulted in 210. When comparing the two bioinformatics software, it is evident that despite N50

values and length of the produced contigs being higher for Geneious Prime, CLC Genomic Workbench is more effective for generating of fold higher number of contigs than Geneious Prime. In addition to this, minimum contig lengths were found to be higher in the CLC Genomic workbench than in Geneious Prime. As shown in Table 5, since N50 values were lower, CLC Genomic Workbench consistently produced more contigs than Geneious Prime. Means that the number of bases in all contigs longer than N50 will be close to the number of bases in all contigs shorter than N50. A higher number of contigs enhances the likelihood of detecting viral genomes in blast analyses (Table 5).

Table 5. *De novo* assembly results were derived from different assemblers
Çizelge 5. Farklı montajcılar kullanılarak elde edilen De novo montaj sonuçları

Sample Name	*De novo* Assembly Tools	Min. Contig Length	Max. Contig Length	Total Numbers of Contig	N50 value
B13	CLC	100	844	908	224
	Geneious (SPAdes)	74	1031	152	656
B21+B5	CLC	100	1258	3566	295
	Geneious (SPAdes)	73	2001	69	806
B22	CLC	100	2802	964	246
	Geneious (SPAdes)	81	2806	73	1038
B23	CLC	100	3018	1910	232
	Geneious (SPAdes)	74	3136	212	899

BLASTn Analysis of Contigs Using Geneious and CLC Genomic Workbench

A custom viral database of the NCBI virus RefSeq was created for both software programs to conduct blast analyses. BLASTn analysis was performed using contigs obtained from four samples (B13, B22, B23, and B21 + B5). As shown in Table 6, most contigs exhibited short-length sequence hits (less than 50 nt) to viruses in the database, and many contigs did not match any entries in the database.

For the B13 sample, Geneious Prime identified hits from 13 viruses, while CLC Genomic Workbench detected hits from 16 viruses. In the B22 sample, Geneious Prime found hits from four viruses, whereas CLC Genomic Workbench identified hits from 13 viruses. For the B23 sample, Geneious Prime matched hits from five viruses, while CLC Genomic Workbench found hits from 42 viruses. Finally, in the B21+B5 sample, Geneious Prime recorded hits from four viruses, compared to CLC Genomic Workbench, which detected hits from 33 viruses.

Table 6. Number of plant viruses revealed via BLASTn analyses in Geneious Prime and CLC Genomic Workbench
Çizelge 6. Geneious Prime ve CLC Genomic Workbench'te BLASTn analizi ile tespit edilen edilen bitki virüslerinin sayısı

Sample Name	A number of plant viruses	
	Geneious Prime	CLC Genomic Workbench
B13	13	16
B21+B5	4	13
B22	5	42
B23	4	33

BLASTx Analysis of Contigs Using CLC Genomic Workbench

BLASTx was performed against the NCBI database using the contigs from four samples: B13, B22, B23, and B21 + B5. BLASTx is useful for identifying viral protein motifs, which allows for the discovery of unknown viral agents. This method is particularly effective for detecting newly discovered viruses, as it targets proteins.

For the B13 sample, the contigs were matched to three viruses using CLC Genomic Workbench. In the case of the B23 sample, contigs were matched to one virus, while for the B21+B5 sample, the contigs were matched to two viruses (Table 7).

BLASTx analysis against NCBI was more accurate than BLASTn against the custom viral database in detecting viruses, as indicated by resulting read matches in the map-to-reference analysis.

Genome Mapping to Reference Sequences in Geneious Prime

Blueberry scorch virus, cereal yellow dwarf virus, orchid fleck virus genomic RNA, pedilanthus leaf curl virus, pepper chlorotic spot virus, potato virus Y, raspberry leaf blotch virus, red clover associated virus, spinach

amalgavirus, tomato chlorotic leaf distortion viruses were selected as reference for sample B13 to perform map to reference analysis. High Plains wheat mosaic virus, longan witches broom-associated virus, rice yellow mottle virus, and turnip vein-clearing virus were selected as references for sample B22 to perform map-to-reference analysis. CLRV RNA1, High Plains wheat mosaic virus, sunn-hemp mosaic virus, and tomato leaf curl Cebu virus were selected as references for sample B21+B5 to perform map-to-reference analysis. Only CLRV RNA 1 had a high match with the B21+B5 contigs with Geneious Prime. CLRV RNA 1 had 27.112 reads matched with the B21+B5 sample in BLASTn with Geneious Prime.

Table 7. BLASTx results from CLC Genomic Workbench for walnut samples (B13, B22, B23, B21+B5)
Çizelge 7. Ceviz numuneleri için CLC Genomic Workbench'ten elde edilen BLASTx sonuçları (B13, B22, B23, B21+B5)

Accession Number	Description (CLC Genomic Workbench B13)	Hits for Total Contig Number	Min-max read length (nt)
NP_619665	coat protein [Grapevine virus A]	1	28-28
NP_597746	Replicase [Tobacco mosaic virus]	1	32-32
YP_008492928	RNA-dependent RNA polymerase [Tomato mottle mosaic virus]	1	27-27
Accession Number	**Description (CLC Genomic Workbench B23)**	**Hits for Total Contig Number**	**Min-max read length (nt)**
NP_040777	capsid protein [Cucumber mosaic virus]	1	54-54
Accession Number	**Description (CLC Genomic Workbench B21+B5)**	**Hits for Total Contig Number**	**Min-max read length (nt)**
YP_004382746	polyprotein 1 [CLRV]	3	71-418
YP_004382747	polyprotein 2 [CLRV]	2	29-50

Genome Mapping to Reference Sequences in CLC Genomic Workbench

According to BLASTn results, bean necrotic mosaic virus, chicory yellow mottle virus satellite, eggplant latent viroid, High Plains wheat mosaic virus, prunus necrotic ringspot virus, arabis mosaic virus small satellite, CLRV RNA1, and CLRV RNA2 were selected as references for sample B21+B5 to perform map-to-reference analysis. Rice stripe virus RNA 3 was selected as a reference for sample B23 to perform map-to-reference analysis. Only CLRV RNA1 and CLRV RNA2 had a high match according to the CLC Workbench. CLRV RNA 1 had 29,574 reads matched with the B21+B5 sample in BLASTn with CLC Genomic Workbench, and CLRV RNA 2 had 405 reads matched with the B21+B5 sample in BLASTn with CLC Genomic Workbench.

Based on the results from BLASTx, CLRV RNA 1 and CLRV RNA 2 were chosen as references for sample B21+B5 for map-to-reference analysis. Grapevine virus A was selected as a reference for sample B13, while CMV RNA 3 was chosen for sample B23. Among these, only CLRV RNA 1 and CLRV RNA 2 had a high match with the B21+B5 sample in the BLASTx analysis conducted with CLC Genomic Workbench. CLRV RNA 1 had 29.574 reads matched with the B21+B5 sample in BLASTx with CLC Genomic Workbench, and CLRV RNA 2 had 405 reads matched with the B21+B5 sample in BLASTx with CLC Genomic Workbench. Additionally, CMV RNA 3 showed a minor match with the B23 sample in the same analysis. CMV RNA 3 had 4 reads that matched with B23 sample. Notably, no viral hits were identified in the BLASTx analysis for sample B22.

Confirmatory Analysis Results

According to the PCR test results, the CLRV was detected in only one sample (B21), which had a size of 431 bp (Figure 3). This sample was collected from the Ulukışla-Porsuk area, known for its extensive cherry tree cultivation in the Ulukışla district of Niğde. While CLRV primarily affects cherry trees, it can also impact other hosts. Notably, studies conducted in the Van region of Türkiye have shown that CLRV was detected in walnuts (Ozturk et al., 2008).

CONCLUSION

The primary factor contributing to the rising prevalence of diseases affecting walnut (*Juglans regia*) orchards is the absence of comprehensive agricultural insurance coverage among the majority of the orchards analyzed in this study. This lack of financial protection limits farmers' ability to implement effective disease management strategies, such as timely fungicide applications, integrated pest management (IPM) practices, and orchard sanitation measures. Furthermore, insufficient knowledge regarding walnut cultivation, including optimal pruning techniques, soil health management, and disease prevention protocols, exacerbates the susceptibility of

orchards to pathogenic infections. This knowledge gap significantly hinders early diagnosis and intervention, facilitating the rapid spread of fungal, bacterial, and viral pathogens, thereby compromising overall orchard productivity and sustainability.

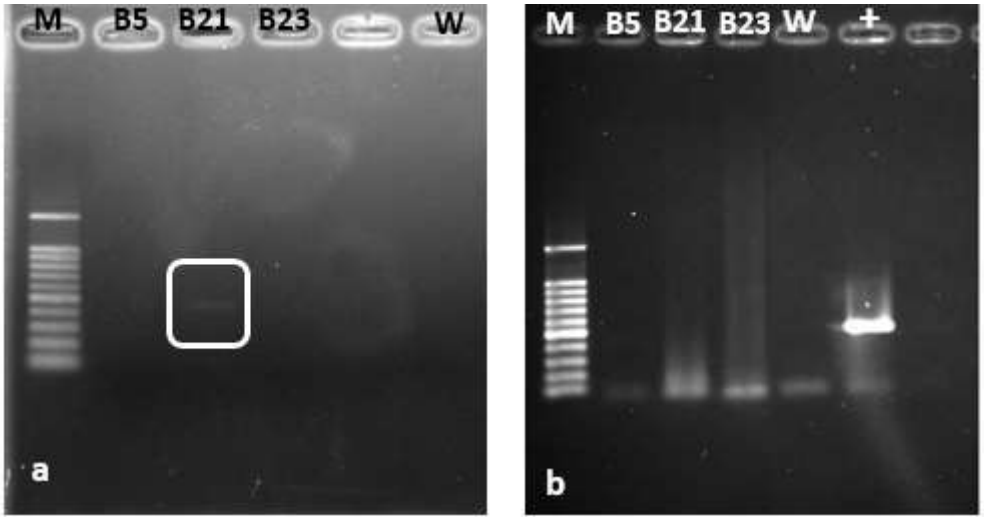

Figure 3. RT-PCR analysis of total RNA from walnut samples for CLRV (a) and CMV (b). M: 100 bp DNA Ladder (Thermo Scientific), +: CMV positive control, W non-template control.

Şekil 3. Ceviz örnekleri toplam RNA'dan CLRV (a) ve CMV (b) RT-PCR analiz sonucu. M: 100 bp DNA Ladder (Thermo Scientific), +: CMV pozitif control, W templete içermeyen kontrol.

In this study, walnut (*Juglans regia*) samples collected from the Ulukışla region in Niğde, an area predominantly known for its intensive cherry (*Prunus avium*) cultivation, were examined for the presence of viral disease symptoms. Previous studies have reported the occurrence of cherry leaf roll virus (CLRV) in walnuts from Van province (Öztürk et al., 2008; Sipahioğlu et al., 2011; Yegül and Baloğlu, 2019), highlighting its potential threat to walnut production. Given that CLRV is a polyphagous virus capable of infecting both walnut and cherry trees, the coexistence of these host species in various locations provided a suitable environment for investigating its spread and impact. Symptomatological analysis of walnut samples revealed characteristic viral disease manifestations, including irregular leaf development, chlorotic vein discoloration, and generalized foliar yellowing, all of which suggest possible CLRV infection or coinfection with other viral pathogens. These findings underscore the necessity for further molecular diagnostics to confirm viral identity and assess its epidemiological significance in walnut-growing regions.

Comparative analyses were conducted using Geneious Prime and CLC Genomics Workbench to evaluate their performance in bioinformatics workflows. CLC Genomics Workbench demonstrated superior efficacy, generating a higher number of contigs, which facilitated more accurate sequence assembly and improved reference matching. Furthermore, a comparative assessment of BLASTn and BLASTx revealed that BLASTx served as an effective validation tool for BLASTn, leading to enhanced accuracy in sequence identification. BLASTx, which focuses on protein-level homology, proved particularly advantageous for detecting novel viruses, as it enables the identification of conserved protein domains even when nucleotide-level similarity is low. The bioinformatics analysis identified a significant sequence match for cherry leaf roll virus (CLRV) in the B21+B5 sample, further substantiating the presence of viral infection.

Cherry leaf roll virus (CLRV) is primarily transmitted through vegetative propagation methods such as grafting, leading to the development of a characteristic symptom known as 'blackline' at the graft union. This necrotic reaction disrupts vascular connectivity, ultimately affecting tree vigor and productivity. Given that CLRV infection can cause substantial reductions in walnut (*Juglans regia*) yields, it is crucial for growers to implement effective disease management strategies. Since no chemical treatments are available for viral infections due to the high mutation rates and ability of plant viruses to evade host defense mechanisms, integrated management approaches are recommended. These include the use of virus-free, certified planting materials, rigorous sanitation of grafting tools, control of insect and weed vectors that may facilitate viral transmission, and systematic removal of infected trees to prevent further spread within orchards. Implementing these preventive measures is essential for maintaining the long-term sustainability of walnut production.

Author's Contribution

The authors declare that BMT: collected the samples, performed the extractions, PCR, and bioinformatics analyses, and writing the manuscript; SÖ: performed the bioinformatics analyses and editing the manuscripts; CUS: collecting the samples, preparing the samples for HTS and writing-editing the manuscripts.

Conflict of interests/Competing interests

The authors declare that there is no conflict of interest.

Ethics approval

The authors declare that there is no ethical issue.

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