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A Simulation of the Use of High Throughput Sequencing as Pre-Screening Assay to Enhance the Surveillance of Citrus Viruses and Viroids in the EPPO Region

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Abstract: Citrus are affected by many viruses and viroids, some globally widespread and some restricted to particular countries or areas. In this study, we simulated the use of high throughput sequencing (HTS) and the bioinformatic analysis of small interfering RNAs (siRNA) as a pre-screening method to guide bioindexing and molecular detection to enhance the surveillance survey of some key or emerging citrus viruses, such as non-European citrus tristeza virus isolates (non-EU CTV), citrus tatter leaf virus, citrus leprosis virus, citrus yellow mosaic virus, and citrus bark cracking viroid, present in the EPPO lists, and the citrus yellow vein clearing virus. The HTS's ability to detect other citrus viroids was also evaluated. The results demonstrate that HTS provides a comprehensive phytosanitary status of citrus samples either in single and multiple infections of viruses and viroids. It also provides effective information on citrus tristeza virus mixed infections despite not being able to identify the non-EU variants of the virus. Bioindexing checks each single virus infection but does not differentiate viroids on the Etrog citron indicator and is time-consuming. Molecular assays are valuable as confirmation tests of viruses and viroids but many pairs of primers are needed for a full screening and new or non-target pathogens remain undetected. In addition, the genomes of two isolates of the citrus yellow vein clearing virus and the citrus tatter leaf virus, detected in a sample from China, are described.

Keywords: citrus tristeza virus; citrus yellow vein clearing virus; citrus tatter leaf virus; non-EU viruses; genotype; genome coverage

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1. Introduction

The number of viruses and viroids affecting citrus has grown considerably in the last few decades [1]. The different global distribution and the presence of different strains and variants mean that their transborder movement among countries needs to be prevented. In line with the International Plant Protection Convention guidelines for surveillance [2], the main objectives are to monitor pests for quarantine purposes, alien invasive species, pests with a negative economic and/or farming impact, and the natural enemies of pests. In addition, biosecurity reasons may create phytosanitary targets that are not categorized as quarantine pests and which must be included in regular surveillance programs, the aim being the early detection of detrimental viruses [3]. This requires detection and identification methods that are in line with current international standards together with a statistically sound and risk-based pest survey approach [4].

According to the categorization of citrus virus and viroid diseases in the Euro-Mediterranean region, the European and Mediterranean Plant Protection Organization

(EPPO) has drawn up two lists of pests whose regulation is relevant for the entire EPPO region or large parts of it [5]. The citrus blight disease, citrus leprosis virus (CiLV), citrus tatter leaf virus (CTLV) and citrus yellow mosaic virus (CYMV), which are not present in the EPPO region, are included in the EPPO A1 List, whereas the citrus bark cracking viroid (CBCVd) and citrus tristeza virus (CTV) non-European isolates (non-EU CTV), and satsuma dwarf virus, are included in the EPPO A2 List. Each of the above viruses and viroids requires different tools and levels of detectability, and complex procedures are needed for reliable results. However, the satsuma dwarf virus affects mainly citrus varieties less relevant in the EPPO region and the causal agent of the citrus blight remains unknown, and no direct test is available [6].

The citrus leprosis syndrome is a disease complex severely affecting leaves, fruits and twigs associated with several known viruses [1]. CTLV (also known as apple stem grooving virus, ASGV) is the causal agent of tatter leaf, a graft incompatibility disease affecting plants grafted on trifoliate orange (*Poncirus trifoliata*) and its hybrids, now used in the EPPO region to prevent the citrus tristeza decline [7]. CYMV, closely related to the cacao swollen shoot virus [8], causes serious losses in pummelo and sweet orange [9].

For non-EU CTV isolates, in the EPPO A2 List, which are responsible for field stem pitting (SP) on sweet orange regardless of rootstock, and resistance-breaking (RB) of trifoliate orange and its hybrids [10], EU member states are required to plan annual surveys using a statistically sound and risk-based approach, in line with current international standards [4]. CBCVd, the smallest of the citrus viroids, a chimeric recombinant viroid between citrus exocortis viroid (CEVd) and hop stunt viroid (HSVd) [11], induces severe symptoms on trifoliate orange and Carrizo citrange [12,13].

Despite not being reported by the EPPO lists, an additional virus, namely the citrus yellow vein clearing virus (CYVCV) is of current concern worldwide. It is a member of the genus *Mandarivirus*, which affects lemon production in Turkey and China, as it is easily transmitted by aphids and mechanically [14].

All these viruses and viroids have been fully sequenced by high throughput sequencing (HTS) (Table 1) [15–28] and the potential use of HTS as a routine diagnostic tool for the phytosanitary control of citrus varieties is under evaluation in California [29]. Endemic viruses and viroids of citrus, and new species were confirmed in an Australian living pathogen collection using HTS methods [30]. National-based surveys using HTS have also been launched in Belgium [31]. A recent study has shown that it is possible to detect known and unknown CTV genotypes in mixed infections using an HTS assay [32]. In Italy, HTS has been used to evaluate the genetic structure of the CTV population leading to the discovery of a few infections of T36 strain [33] and the study of variants responsible for seedling yellow (SY) recovery [34]. Moreover, HTS has been used to investigate on a stem pitting isolate from China [35].

HTS has also been used to clarify the genetic differences of old and new citrus viroids [36,37]. All induce leaf bending and curling symptoms on the Etrog citron indicator. However, they are not sufficiently distinctive to be able to be differentiated. Despite the availability of many primers for molecular detection, accurate results are possible only after a bio-amplification on Etrog citron or by collecting specific bands from PolyAcrylamide Gel Electrophoresis (PAGE).

Thanks to the extremely high-throughput data generation, HTS is widely used in many projects dealing with different crops, supported by new bioinformatic instruments for handling the data produced [38]. Exciting results have been achieved in the discovery and characterization of new viruses, the detection of unexpected viral pathogens in plants, the investigation of viral diversity, evolution and spread, and the evaluation of the plant virome [39–41].

Table 1. Complete genome of citrus viruses sequenced by high throughput technologies.

Virus	Genus	Family	Host	Country	Reference
Citrus tristeza virus (CTV)	*Closterovirus*	*Closteroviridae*	Sweet orange, Mexican lime, sour orange	Spain	[15]
			C. sinensis, *C. macrophylla*	Florida	[16]
			C. sinensis, *C. reticulata*	California	[17]
			Grapefruit	South Africa	[18]
Citrus sudden death-associated virus (CSDaV)	*Marafivirus*	*Tymoviridae*	Sweet orange	Brazil	[19]
Citrus leaf blotch virus (CLBV)	*Citrivirus*	*Betaflexiviridae*	Sweet Cherry	China	[20]
Citrus psorosis ophiovirus (CPV)	*Ophiovirus*	*Aspiviridae*	Sweet orange	China	[unpublished]
Citrus tatter leaf virus (CTLV)	*Capillovirus*	*Betaflexiviridae*	Apple	China	[21]
Citrus yellow vein clearing virus (CYVCV)	*Mandarivirus*	*Alphaflexiviridae*	Lemon	Turkey	[22]
Citrus chlorotic dwarf-associated virus (CCDaV)	*Geminivirus*	*Geminiviridae*	Lemon	Turkey	[23]
Citrus vein enation virus (CVEV)	*Enamovirus*	*Luteoviridae*	Etrog citron	Spain	[24]
Citrus leprosis virus-N (CiLV-N)	*Dichoravirus*	*Rhabdoviridae*		Mexico	[25]
Citrus leprosis virus-C2 (CiLV-2)	*Cilevirus*	Unassigned		Colombia	[26]
Citrus concave gum-associated virus (CCGaV)	*Phlebovirus*	*Phenuiviridae*	Tarocco sweet orange	Italy	[27]
Citrus jingmen-like virus (CJLV)	*Flavivirus*	*Flaviviridae*	Valencia sweet orange	Brazil	[19]
Citrus virga-like virus (CVLV)	*Virgavirus*	*Virgaviridae*	Valencia sweet orange	Brazil	[19]
Satsuma dwarf virus (SDV)	*Sadwavirus*	*Secoviridae*	Sour orange and tangor	China	[28]

In this study, we evaluated the use of HTS as a pre-screening method combined with bioindexing and molecular detection in a simulated surveillance survey of citrus virus and viroids regulated by EU phytosanitary measures (Figure 1). The investigation was related to the presence of non-EU CTV isolates, sensu EFSA [4] and to the detection of some virus and viroids in the EPPO A1 and A2 Lists (CTLV, CiLV, CYMV, CBCVd) and to CYVCV. Other citrus viroids and the genetic structure of a local CTV population were also investigated. This involved the sequencing of small interfering RNAs (siRNA), produced in a high quantity by RNA silencing antiviral mechanisms in the plants [40]. The analysis was carried out using siRNA libraries of six field samples of citrus, three of them already analyzed only for the presence of CTV and here re-analyzed for all the viruses and viroids listed above, using a bioinformatic pipeline able to detect mixed virus and viroid infections.

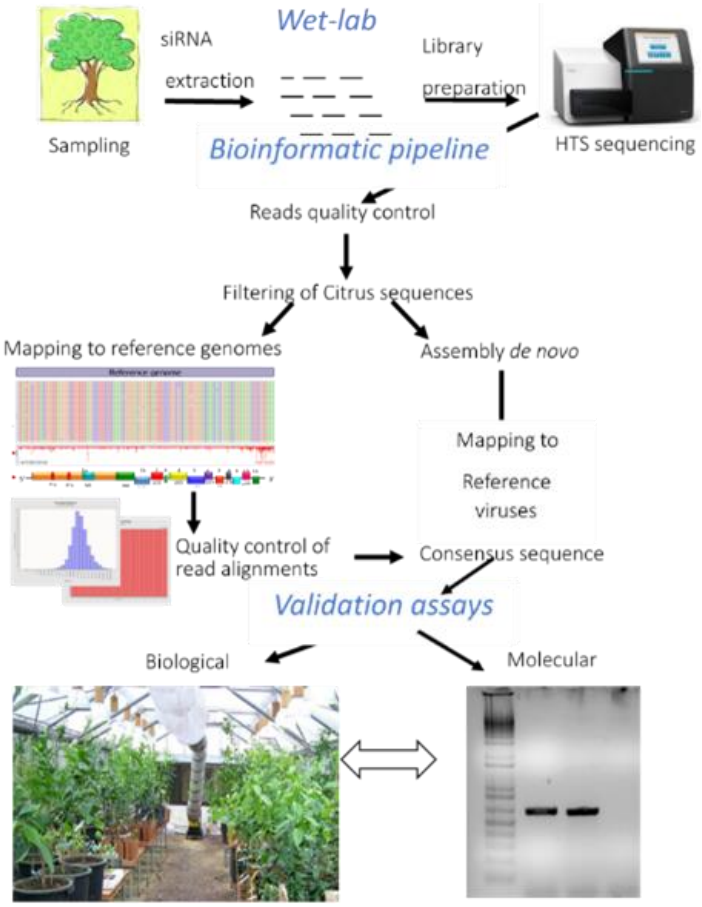

Figure 1. Schematic workflow of the procedure adopted for the detection of citrus viruses and viroids based on a multi-disciplinary approach in which HTS (including a wet-lab and a bioinformatic phase) is used as pre-screening analysis, whereas biological tests and molecular detection are used as validating assays.

Our results showed that the adoption of HTS for pre-screening can be effectively used for the simultaneous identification of virus and viroids and to detect the prevalent CTV genotypes. However, bioindexing is still necessary to characterize the phenotypic profiles of CTV isolates inducing stem pitting or resistance breaking as well as to confirm the presence of other viruses (i.e., CYVCV and CTLV), supported also by molecular detection. Preliminary results of this multi-disciplinary approach have been presented at the 2019 Joint IOCV XXI and IRCHLB VI [42].

2. Materials and Methods

2.1. Source Plants and Small-RNA Libraries

Six siRNA libraries generated from field sources, tested CTV-positive by enzyme-linked immunosorbent assay (ELISA), were selected for this study. The library of HU-PSTS isolates was generated from a field sweet orange tree in the Hunan province (Chenzou county, China) [35,43] (Table 2). The other libraries were generated from samples collected in a heavily infected CTV focus area in Sicily: Mac25, Mac39, and Mac101 were generated from three ungrafted seedlings of alemow [33,34], whereas the P3R1 and P3R3 libraries were obtained from two different branches of the same tree with different CTV titers (one CTV-ELISA positive and one negative), from a declining Tarocco Sant'Alfio sweet orange grafted on sour orange (Table 2). The Mac25, Mac39, Mac101, and HU-PSTS libraries were previously obtained and partially analyzed only for CTV [33,34,42] and are reanalyzed here with an additional upstream plant host filtering step. P3R1 and P3R3 were fully analyzed for the first time in this study.

Table 2. Virus isolate and source plants used to assess the virome by using high-throughput sequencing.

Virus Isolate	Field Plant	Origin	Age	Tissue Source
HU-PSTS	Sweet orange/*P. trifoliata*	Hunan, China	≈30 years	Bark, sour orange
P3R1	Sweet orange/sour orange	Sicily, Italy	≈22 years	Bark, sweet orange
P3R3	Sweet orange/sour orange	Sicily, Italy	≈22 years	Bark, sweet orange
Mac39	Alemow seedling	Sicily, Italy	≈2 years	Bark, sour orange
Mac101	Alemow seedling	Sicily, Italy	≈2 years	Bark, sour orange
Mac25	Alemow seedling	Sicily, Italy	≈2 years	Bark, sour orange

The small RNA libraries have been generated according to Licciardello et al., [43] from 200 mg of young bark tissues. The small RNA fraction was extracted using the MirPremier MicroRNA isolation kit (Sigma Aldrich, St. Louis, MO, USA) as instructed by the manufacturer and used as input for library preparation by the NEXT flex Small RNA Sequencing kit (Bioo Scientific, Austin, TX, USA). The resulting libraries were multiplexed, clustered, and sequenced on an Illumina HiSeq 2000 (San Diego, CA, USA) with a single-read 50 cycles sequencing protocol plus indexing. The sequencing runs were analyzed with the Illumina CASAVA pipeline (v1.8.2 San Diego, CA, USA) and small RNA adapters removed using the "Trim sequences" option of the CLC Genomics Workbench (v 6.0.4 CLC Bio, Qiagen, Aarhus, Denmark).

2.2. Data Evaluation by Bioinformatics

For the detection of viral genomes, a "map reads to reference" bioinformatic approach was used to assemble the read sequences to a selected group of known virus and viroid genomic sequences (Table 3). Quality trimmed data were imported into Bowtie2 v. 2.1.0 and a preliminary plant host filtering step was performed after alignment with the chromosomes of *Citrus sinensis* (NC_023046 to NC_023054), chloroplast (NC_008334) and miRNAs (retrieved from MiRBase database) using the default parameters. For the CTV genotype detection, the unmapped reads were aligned to six reference genomes representative of the six main CTV genotypes [16]. The mapping alignments were further measured by three metrics generated by Qualimap [44] analysis: read counts, percentage of reads count and percentage of genome fraction coverage (GFC) at 50X. Graphical representations showing the quality of the reads aligned against each reference genome, generated by Qualimap, were assessed for a fast evaluation of the unequivocal presence of the virus or viroid in a library.

The consensus sequence was generated by SAM tools 0.1.19 (BSD License, MIT License) and analyzed for similarity against other sequences in the GenBank by BLASTn (NCBI, Bethesda, MD, USA). In addition, a de novo assembly bioinformatic approach has been used for the Mac25 sample and for the CYVCV genome reconstruction in the HU-PSTS using a Velvet (1.2.10, Illumina, San Diego, CA, USA) assembler [45] at different k-mer ranges. Alignment files (.bam) were visualized by Tablet [46] and Integrative Genomics Viewer (IGV) [47].

2.3. Biological Indexing

All the trees were indexed by bark inoculation of Mexican lime (*Citrus aurantifolia*), sour orange (*C. aurantium*), Duncan and/or Oroblanco grapefruit (*C. paradisi*), Hamlin sweet orange (*C. sinensis*) grafted on sour orange, and Etrog citron (*C. medica*) grafted on *C. volkameriana*. Supplementary tests were carried out on Carrizo citrange (*P. trifoliata* x *C. sinensis*) seedlings for the detection of RB CTV [17] and CTLV searching and on lemon (*C. limon*) seedlings for detection of CYVCV. At least three plants were inoculated for each indicator and one of each was used as non-inoculated control. The indexing was carried out in a safe greenhouse, with heating and cooling systems, located near Catania (Sicily, Italy, 37°24′ N, 15°03′ E). Symptoms were visually assessed periodically over a 2-year period, according to Garnsey et al. [48].

Table 3. List of reference sequences of citrus tristeza virus (one representative genome of each genotype), non-EU viruses and viroids used for genome reconstruction by mapping reads of siRNA libraries.

Virus	Genotype	Accession	Isolate	Origin
Citrus tristeza virus	VT	KC748392	SG29	Italy
	T3	EU857538	NZ-M16	New Zealand
	T30	KC748391	Bau282	Italy
	T68	EU076703	B165	India
	RB	FJ525435	NZRB-M17	New Zealand
	T36	EU937521	T36 FS2-2	Florida
Citrus yellow vein clearing		JX040635	Y1	Turkey
		NC_026592	CQ	China
		KP313241	PK	Pakistan
Citrus tatter leaf virus		KC588948	MTH	China
		JX416228	PK	Taiwan
Citrus sudden death-associated virus		AY884005		Brazil
Citrus leprosis virus C	RNA1	NC_008169		Brazil
	RNA2	NC_008170		Brazil
Citrus yellow mosaic virus		DQ875213		India
Viroids				
Citrus exocortis viroid		AB054599	MA	Japan
Hop stunt viroid		AB211242	EX1	Japan
Citrus bent leaf viroid		NC001651	CBLVd-225	Israel
Citrus dwarfing viroid isolate		EU934031	E822	California
Citrus viroid V		NC010165		Spain
Citrus bark cracking viroid		NC003539		Israel

2.4. RT-PCR and Real-Time RT-PCR

Reverse transcription-polymerase chain reaction(RT-PCR) and real-time RT-PCR were used to validate the bioindexing or HTS results. The total RNA was extracted using Trizol reagent (Invitrogen S.r.l.; Milan, Italy), from 100 mg of bark tissues, previously pulverized with liquid nitrogen, according to the manufacturer's instructions. For CTV genotype characterization, RT-PCR (for T68 and T3 genotypes, according to Roy et al. [49]) and real-time RT-PCR using specific dual labeled-probes (for VT, T3, T36, T30) were evaluated [50,51]. CYVCV was detected using the primers 1fw and 921rev targeting 5′ region following the PCR conditions previously reported [22]. CTLV was detected using the TL3 and TL4 primer pair and PCR conditions described by Tatineni et al. [52]. For CEVd, HSVd, and citrus dwarfing viroid (CDVd) detection, real-time RT-PCR protocols were used [53,54]. For CBCVd (CVd-IV), the primers and PCR conditions described by Bernard and Duran-Villa [55] were used, whereas CVd-V-R and CVd-V- F primers were used for citrus viroid V (CVd-V) [56].

3. Results

After quality trimming, all the libraries analyzed in this study generated a high number of single reads, respectively, 38.7 M (HU-PSTS), 40.8 M (P3R1), and 29.3 M (P3R3). The total reads were retained for a preliminary host filtering step to remove the majority of the host reads (chromosomes, chloroplasts and miRNAs) which significantly reduced (at least 50%) the number of reads to be analyzed downstream as follows: 19.6 M (HU-PSTS),

8.5 M (P3R1), 4.1 M (P3R3). The unmapped reads were analyzed to search for the presence of non-EU CTV isolates, as well as CLVCV, CTLV, CiLV, CYMV and CBCVd, and other viruses and viroids. From the samples Mac25, Mac39, and Mac101, the total number of reads was 19.6 M, 17.8 M and 9.9 M, respectively. After the host filtering step, the total number of reads was 4.1 M (Mac25), 10.4 M (Mac39), 3.6 M (Mac101), reducing the reads by 20% to 58% of the original siRNA reads.

3.1. Searching for Non-EU CTV Isolates

The single reads retained after the host filtering were aligned with reference genomes of six CTV isolates representative of the main six genotypes [17] (Table 3). All the libraries showed a high number of read counts with SG29 (VT) corresponding to 71% of the entire library of P3R1, 58% of P3R3 and 84% in HU-PSTS, and 100% of genome coverage in all these libraries. Alignments with NZM16 (T3), B165 (T68), and Bau282 (T30) gave fewer reads ranging from 66% to 44%, with a coverage of about 95%, 85% and 90%, respectively (Figure 2), whereas alignments with the RB and T36 strains gave less than 35% read counts and a low percentage of coverage (50–45%) (Figure 2). The quality of alignments of the P3R1 library for each reference genome is shown as "genome histograms" and "genome fraction coverage" in Figure 3. Assuming that a 95% of genome coverage can be indicative of the presence of additional CTV strains in mixed infections and a maximum of 92% can be obtained for non-target genomes [32,57], the samples P3R1, P3R3 and HU-PSTS were further characterized by RT-PCR and real-time PCR [49–51] for CTV strain identification. P3R1 and P3R3 were positive only for VT, whereas HU-PSTS reacted positively to VT, T3 and T30 probes, and negatively to T36 and T36NS. On the other hand, although the RT-PCR detection for T3 and T68 strains were negative, the high homology of nucleotide sequences between VT and T3 cannot completely exclude the potential presence of T3 in the HU-PSTS.

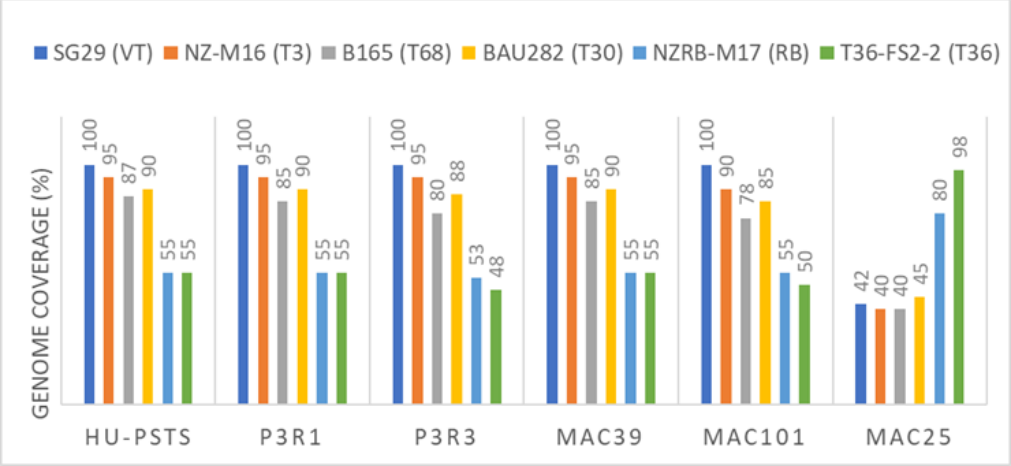

Figure 2. Graphical illustration of genome coverage per representative genome reference of six citrus tristeza virus genotypes after read mapping the siRNA library of each sample individually.

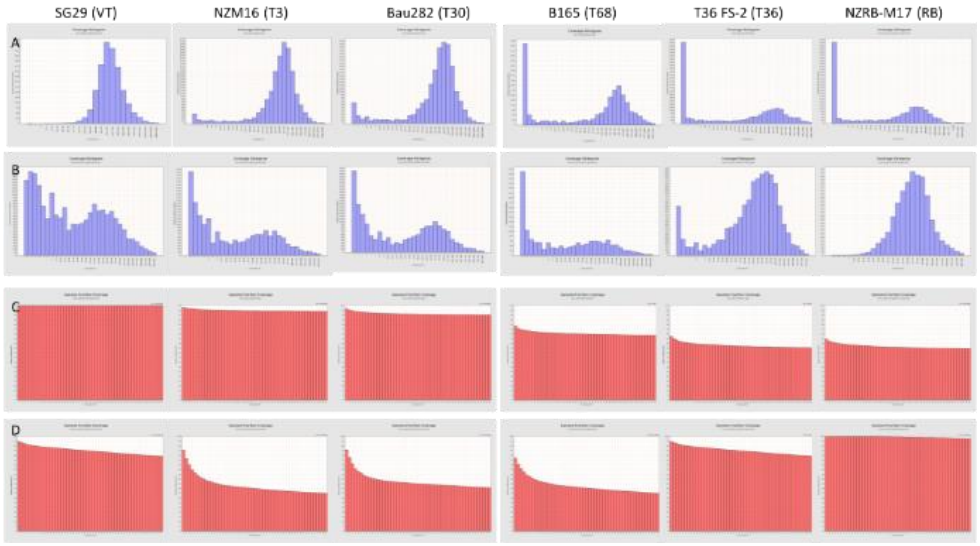

Figure 3. Quality alignments exemplified as genome histograms (blue) and genome fraction coverage (red) per reference genome representative of six citrus tristeza virus genotypes of P3R1 (VT) (**A**,**B**) and Mac25 (T36) (**C**,**D**) siRNA libraries, using Qualimap [44].

From a biological point of view, the three sources (P3R1, P3R3, and HU-PSTS) showed a typical seedling yellow (SY) reaction in sour orange (Figure 4), associated with leaf blade malformation and more severe yellowing in HU-PSTS, due to the coinfection of CYVCV (Figure 5). As previously described [35–43], the Chinese source induced a severe stem pitting on Duncan grapefruit and a mild stem pitting on Hamlin sweet orange, which makes the isolate compatible with biotype 5 [48]. On the other hand, P3R1 and P3R3 induced stem pitting only on grapefruit compatible with biotype 4. Parallel inoculation of Carrizo citrange gave a negative detection of CTV by ELISA test, revealing an absence of CTV replication, thus excluding the presence of RB isolates in the three sources.

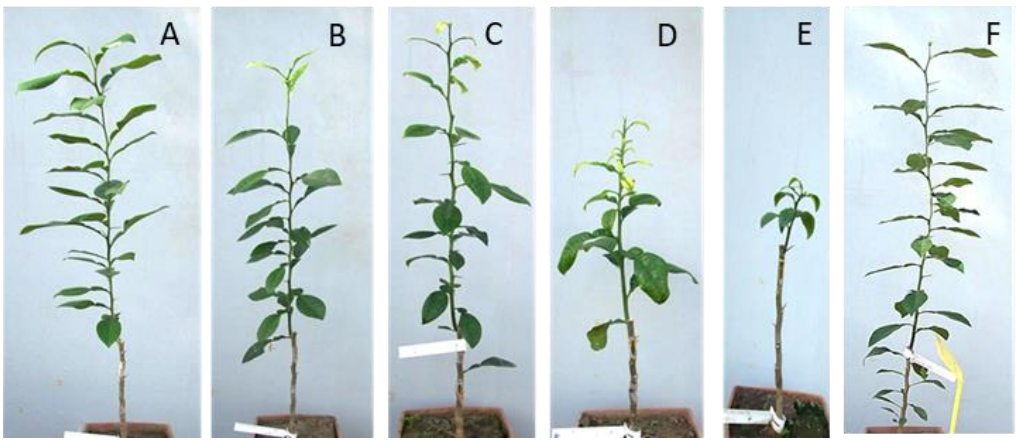

Figure 4. Symptomatic responses of sour orange seedlings inoculated with Mac39 (**A**), Mac101 (**B**), Mac25 (**C**), P3R1 (**D**), P3R3 (**E**), 6 months post-inoculation in comparison to non-inoculated control (**F**).

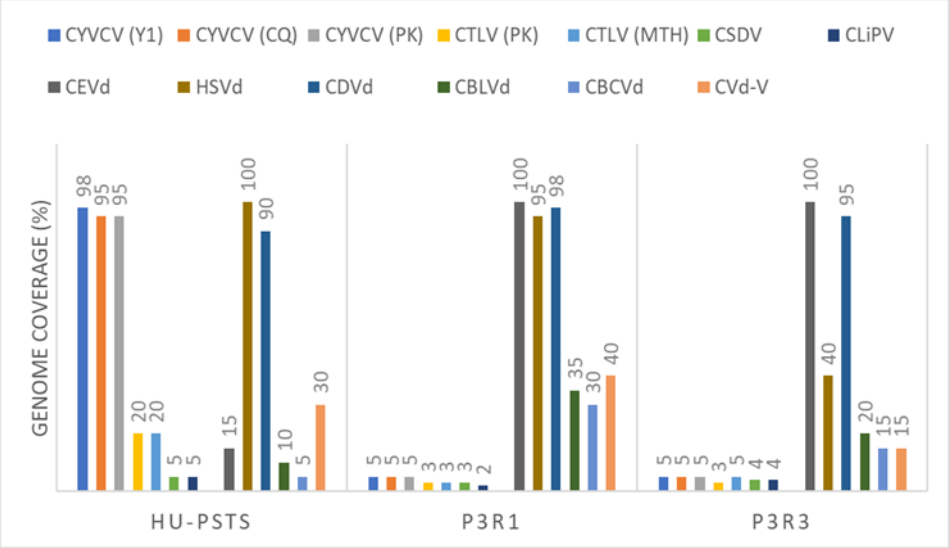

Figure 5. Graphical illustration of genome coverage per representative genome reference of non- EU viruses and viroids after read mapping the siRNA library of each sample individually.

Based on HTS and molecular and biological assays, the results show that P3R1 and P3R3 were infected only by one VT isolate, whereas HU-PSTS was a mixed isolate including at least two genotypes, VT and T30, although the presence of T3 cannot be excluded.

The highest number of reads was generated from the libraries of Mac39 and Mac101 seedlings mapped with SG29 (VT), the prevalent isolate in Sicily [43] after depletion of the host citrus genome. Approximately 9 M (Mac39) and 3.9 M (Mac101) reads were generated (corresponding to 86% and 91% of the entire libraries, respectively), which led to the full genome reconstruction with 100% coverage (Figure 2). The resulting consensus sequences of the assembled genomes were deposited under accession numbers KJ790175 (Mac39) and MW689620 (Mac101). Both genomes showed 99% similarity with SG29.

The Mac25 library, which was reported earlier to contain a T36 CTV isolate (KR263170) [33], was revisited in this study to check the potential presence of CTV isolates biologically non-EU sensu EFSA [4]. When the 4.1 M reads were aligned with CTV reference genomes, the highest number of read counts (18% of the entire library) was obtained after alignment with T36 FS-2 (T36) associated with 98% genome coverage (Figure 2). Alignment with the NZRB-M17 (RB) genome gave 80% coverage, which is not surprising since T36 and RB genotypes are genetically similar (90% nucleotide identity). The percentage coverage with the remaining reference genomes was under 40%. Graphs representing quality alignments for each reference genome are shown in Figure 3. Real-time RT-PCR by Ruiz-Ruiz et al. [50] confirmed a previous analysis by using the protocol of Yokomi et al. [33,51].

Parallel biological indexing showed that the three alemow seedlings had a different SY and SP reaction. Mac25 induced only slight chlorosis on sour orange, whereas Mac39 and Mac101 were asymptomatic (Figure 4). Mac39 and Mac101 caused stem pitting on grapefruit but not on sweet orange, whereas Mac25 did not cause any stem pitting.

HTS screening discriminated the CTV components of the six samples. The results were confirmed by RT-PCR and real-time PCR. Based on biological assays, only the source HU-PSTS met the requirements of a biologic non-EU CTV isolate because of the stem pitting induced on sweet orange [4]. On the other hand, the lack of replication on Carrizo citrange confirms that there was no RB present.

3.2. Searching for Non-EU Virus and Viroids

HTS sequencing of the HU-PSTS source highlighted that the siRNA library contains some viruses and viroids selected for this study. The read alignments with CYVCV isolates from Turkey (Y1) [22], China (CQ), and Pakistan (PK) revealed from 171,807 to 159,685 read counts with 98–95% genome coverage (Figure 5). Moreover, by de novo assembly, a total of

16 contigs, ranging from 821 nt to 1219 nt, were obtained using Kmer-13 and N50. The full genome was reconstructed after mapping the contigs to the CQ reference genome (Figure 6). The resulting consensus sequence of the assembled genome was deposited under accession number KT124646 (HU). BLASTn showed 99% identity with CYVCV CQ (KP313240) and CYVCV GX-GXP (KX156741) isolates from China, 98% with CYVCV RL (KP120977) and CYVCV PK (KP313241) from China and Pakistan, respectively, and 97% with CYVCV Y1 (JX040635). In addition, a 75% identity was found with the closely related Indian citrus ringspot virus (ICRSV) K1 (AF406744) with partial 66% query coverage. This result was also confirmed by read alignments with ICRSV K1 which revealed about 14,000 mapped reads, likely due to the common ancestor [22].

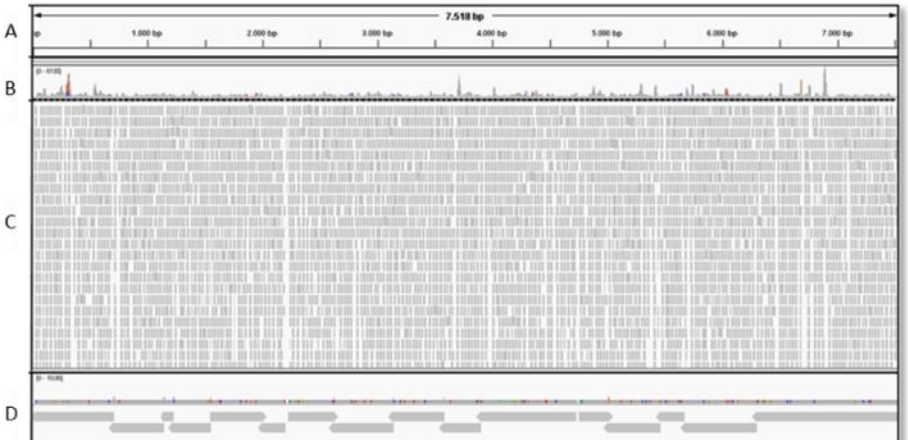

Figure 6. Visualization of the consensus alignments of citrus yellow vein clearing HU generated by both "map to reference" alignment of siRNA reads and de novo assembled contigs. (**A**) CYVCV CQ reference genome; (**B**) coverage obtained by siRNA reads alignment; (**C**) siRNA library (171,807 reads) which allowed the CYVCV-HU full genome reconstruction; (**D**) consensus obtained by alignments of 16 contigs. For the visualization the Integrative genome viewer (IGV) software has been used [47].

The presence of CYVCV was confirmed by the specific amplicons with the expected size of 921 bp of the 5′ region [22] (Figure S1A). Inoculation of the source HU-PSTS on sour orange and lemon revealed the typical clearing of lateral leaf veins, yellow flecks, and transient ring-spot-like symptoms on spring and autumn flushes, leaf crinkling and warping, and browning of the midribs described for the citrus yellow vein clearing disease [58] (Figure 7).

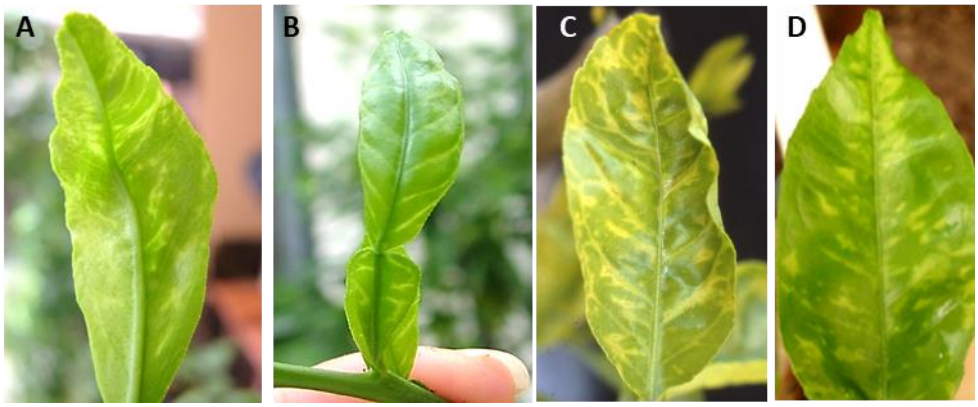

Figure 7. Leaf symptoms of citrus yellow vein clearing induced by inoculation of HU-PSTS on sour orange (**A**,**B**) and lemon (**C**,**D**) about 4–5 months post-inoculation.

The HU-PSTS library shared a total of 12,772 read counts with two citrus tatter leaf virus genome references and generated a consensus sequence of almost 6495 nt in length,

although with some gaps. However, the detection of CTLV was confirmed by RT-PCR (Figure S1B) and by the inoculation of Carrizo citrange seedlings, which showed typical CTLV symptoms, such as strong yellow mottle or blotch leaf spotting, followed by curling and twisting (i.e., tatter leaf) and a reduction in leaf blade size (Figure 8).

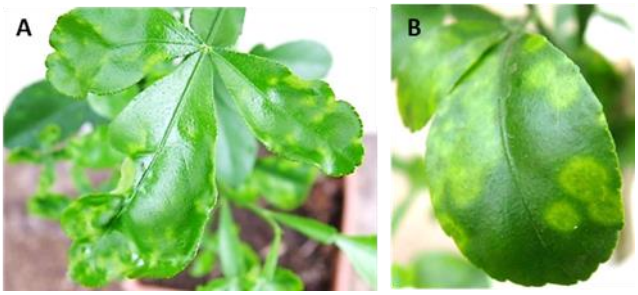

Figure 8. Symptoms of citrus tatter leaf on young (**A**) and mature (**B**) leaves of Carrizo citrange induced by bark inoculation of sour orange HU-PSTS infected, about 4–5 months post-inoculation.

The alignments with the other two citrus viruses listed by EPPO (CiLV and CYMV), investigated in this study, revealed 5900 read counts to CiLV1, 10,500 to CiLV2, but only 4500 to CYMV. The corresponding genome fraction coverage was also low. Other viruses were not explored as they were not of surveillance interest. The libraries of P3R1, P3R3, Mac25, Mac101, and Mac39, showed a low number of read counts and non-significant genome coverage with all the non-EU virus genome references.

The screening for citrus viroids was performed by aligning the siRNA libraries of the six samples against six citrus viroid reference genomes (Table 4). The HU-PSTS source provided a high number of mapped reads with CDVd (32,392) and HSVd (21,939) allowing the full genome reconstruction of these viroids (Table 4, Figure 9). On the other hand, CEVd, CBLVd and CVd-V, had fewer than 5000 reads, suggesting incomplete coverage or the absence of viroids. Real-time RT-PCR analysis confirmed the presence of CDVd and HSVd, and the absence of CEVd, CBLVd and CVdV. Indexing carried out on Etrog citron confirmed the presence of viroids. However, the overlapping symptoms observed, such as leaf epinasty and rugosity, petiole wrinkle and necrosis, midvein necrosis, and browning of the tip of the leaf blade [58,59], mean that it was not possible to differentiate between present and absent viroids.

A considerable number of reads was obtained after alignment of the P3R1 library with CEVd (57,780), CDVd (32,317) and HSVd (15,430), whereas the P3R3 library generated 23,522 reads with CEVd and 17,250 reads with CDVd. An even lower number of reads in the Mac101, Mac39, and Mac25 libraries mapped with viroid genomes references (Table 4). These data, along with those of the P3R1 and P3R3 libraries, were confirmed by real-time PCR and bioindexing in Etrog citron.

Table 4. Number of read counts obtained after alignments of siRNA libraries with viroids reference genomes. Positive identifications are in bold, considering a threshold limit of 15,000 reads.

	Read Count (RC)					
Viroid	**Mac39**	**Mac101**	**Mac25**	**P3R1**	**P3R3**	**HU-PSTS**
Citrus exocortis viroid	29	208	459	**57,780**	**23,522**	602
Hop stunt viroid	42	498	738	**15,400**	5146	**21,939**
Citrus bent leaf viroid	74	237	472	6859	3600	829
Citrus dwarfing viroid	178	118	228	**32,317**	**17,250**	**32,393**
Citrus viroid V	191	309	132	7497	5058	5374
Citrus bark cracking viroid	16	115	227	11,036	4932	199

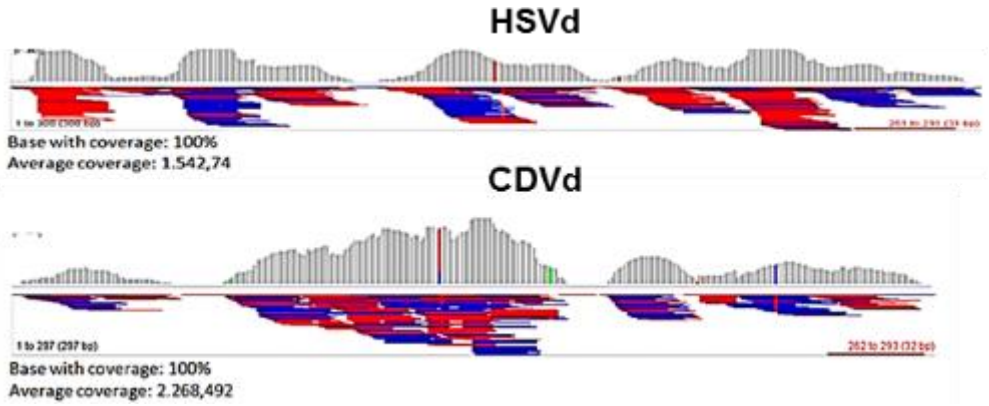

Figure 9. Genome reconstructions of the hop stunt viroid (HSVd) and citrus dwarfing viroid (CDVd) by alignment of the siRNA reads of HU-PSTS library with reference sequences.

4. Discussion

The surveillance of citrus pests is essential to preserve the citriculture worldwide, in accordance with the recommendations of regional phytosanitary services. Conventional diagnostic technologies used to detect all the listed pests include bioindexing and molecular tests. Bioindexing is the most reliable, but it is costly and time-consuming [60]. Although molecular diagnosis is fast and highly specific, many pairs of primers and PCR runs are needed for a full screening. Moreover, new or non-target pathogens remain undetected [40]. Multiplex PCR designed for many viruses and viroids speeds up the lab work, but in some cases may cause problematic interactions.

HTS is the most broad-spectrum diagnostic tool in the field of virus discovery [39,60–62]. However, as far as we know, HTS has not been explored for surveillance applications likely due to the costs involved [40].

Given its advantages, we explored sequential HTS pre-screening of samples, complemented by conventional bioindexing and molecular diagnostic technologies, to enhance the surveillance survey of citrus viruses and viroids. A bioinformatic pipeline for the identification of CTV single or mixed genotype infections was designed and used to determine the parameters to discriminate between false positive read mappings and true genotype-specific genome coverage.

According to Bester et al. [32], using simulating data, a genome coverage above 90% was assumed indicative of the presence of a specific genotype, and a genome coverage between 50–90% indicative of the presence of genotype variants not represented in the read mapping reference list. In compliance, 95% genome coverage is indicative of the presence of a genotype when 10,000 single and simulated reads mapped to a specific genotype [57], showing that the genome coverage threshold and depth of the libraries impacted the level of non-target genomes. We used the HTS libraries of six citrus sources to investigate the presence of some virus and viroids of interest for the surveillance according to EPPO A1 List (CiLV and CTLV) and A2 List (non-EU CTV isolates and CBCVd), as well CYVCV, a virus that is currently spreading rapidly in China and Turkey [14]. One source was from China (HU-PSTS), which was assumed to be infected by a non-EU virus, and five were from Sicily, two branches of the same sweet oranges grafted on sour orange (P3R1 and P3R3) and three as ungrafted seedlings (Mac25, Mac39, and Mac101), respectively.

Assuming a genome coverage threshold up to 90%, the read mapping on HU-PSTS, P3R1, and P3R3 sources were indicative of the presence of VT, T3 and T30 CTV genomes. However, only in HU-PSTS the presence of VT and T30 strains was confirmed also by molecular assays, whereas the presence of T3 remained doubtful due to the high homology with VT strains in several parts of the genome [16] and the high non-target genome coverage which can be obtained for the genotypes VT and T3 [32]. Previous results demonstrated that HU-PSTS contained mixed CTV strains [63] and further preliminary HTS analysis led

to the conclusion that T3, T68 and T30 could be potentially present in association to the VT strain [35].

On the other hand, in the samples P3R1,P3R3, Mac39, and Mac101, only the VT CTV genotype was confirmed, with a genome coverage of above 99%. The Mac25 source showed the presence of the T36 (98% genome coverage).

HTS was able to identify the genetic differences of the CTV components in each sample but did not explain whether the investigated CTV isolates induce stem-pitting sensu EFSA [4]. However, the subsequent testing with molecular and biological methods helped obtain a more conclusive diagnosis. Real-time RT-PCR and RT-PCR were necessary to support the genetic differences, whereas biological indexing led to a detailed phenotyping of the CTV isolates infecting the six samples. Regarding HU-PSTS, the stem pitting induced in sweet orange and grapefruit enabled us to identify it as a non-EU CTV isolate, biotype 5, whereas the lack of replication in Carrizo citrange meant that RB genotypes were excluded from the components of the isolate. Unfortunately, both biological tests need at least 6–10 months and are costly.

The presence of CYVCV and CTLV in the same source was easily detectable with all three technologies, biological, molecular, and genomic. The data provided by HTS were prompt and detailed, revealing genetic similarity with more than one reference genome, whereas qPCR was faster. The symptomatic detection on sour orange and Carrizo citrange took four and six months after inoculation, respectively.

Mixed viroid infections associated with CTV were detected by HTS in the three field sources (HU-PSTS, P3R1, and P3R3), individually confirmed by molecular testing. A non-conclusive result was obtained for citrus bark cracking viroid, which was highlighted with more than 11,000 reads in the source HU-PSTS. Unfortunately, although the number of reads was much higher than for the other viroids, our attempts to reconstruct the full genome of the viroid were unsuccessful and molecular analysis did not detect it. Moreover, bioindexing on Etrog citron generated undistinguishable and overlapping symptomatic reactions, thus showing that this test was inadequate to identify a specific viroid in mixed infections. Regarding the local field sources (P3R1 and P3R3) and alemow seedlings (Mac25, Mac39, and Mac101), the results of HTS screening were confirmed by biological and molecular tests.

Overall, each of the three detection methods—bioindexing, PCR, and HTS—was helpful in excluding and/or confirming the presence/absence of specific viruses and contributed to the final diagnosis. In most cases they helped to produce additional information. Molecular assays were more suitable than biological indexing regarding the viroid detection, while HTS would have been more adequate if there had been a higher number of reads. The results therefore indicate that the testing strategy needs to be selected based on the pathogen being searched for. The integration of the methods produces faster and more conclusive results.

Our results show that the sequencing depth, host filtering and the selection of reference genomes are crucial for the accurate detection of low titer viruses and viroids. RT-qPCR and HTS and mapping reads had a comparable level of sensitivity for the targeted detection of CTV, CTLV, CYVCV, CDVd, CEVd, and HSVd. They also provided information on the genetic similarity with single strains in GenBank. Similar results obtained for potato viruses demonstrated that a bioinformatics approach improved the sensitivity 10-fold [64].

The HTS analysis was equivalent or superior to the standard bioassay in detecting the viruses and viroids evaluated in the present study, including virus infections at low titers [40,60]. Moreover, our study highlights that it allows to review previous results when a new virus occurs or a different purpose is involved, as in this study. However, despite the stringent conditions used to differentiate genetic variants [57], HTS failed to detect non-EU CTV stem pitting isolates, identified only by bioindexing tests.

Our study shows that in HTS prescreening the number of mapped reads is strictly linked to the titer of the virus or viroid and is related to the total number of reads. For virus and viroid detection in CTV-infected plants, a threshold limit of at least 15,000 reads

mapped against the reference with a genomic coverage of 95% is recommended. Variable mapping depths may be acceptable if the presence of the virus or viroid is confirmed by other methods.

5. Conclusions

In conclusion, despite the cost, the use of HTS could provide a comprehensive phytosanitary status of citrus samples, thus reducing the greenhouse footprint, labor, time, and costs. It may enhance the surveillance of citrus viruses and viroids, identify CTV genotypes and enhance phylogenetic studies in mixed infections. Further comparisons, including those aimed at determining limits of detection [65], are evidently needed to clarify the analytical sensitivity required in HTS focused on surveillance surveys and to define specific details of the protocol. This will further contribute to improving the procedure, and the use will support its validation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11050400/s1, Figure S1. PCR detection of citrus yellow vein clearing virus (A) and citrus tatter leaf virus (B) RNAs isolated from sour orange (lanes 1, 2) and *P. trifoliata* (lane 3). Positive (+) and negative (−) controls have been added. M1: 100-bp molecular ladder, M2: 1Kb plus molecular ladder (Invitrogen).

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