Comprehensive Real-Time RT-PCR Assays for the Detection of Fifteen Viruses Infecting Prunus spp.

Alfredo Diaz-Lara 1,†, Kristian Stevens 2,†, Vicki Klaassen 3, Deborah Golino 1 and Maher Al Rwahnih 1,*

1 Department of Plant Pathology, University of California-Davis, Davis, CA 95616, USA; adiazlara@ucdavis.edu (A.D.-L.); dagolino@ucdavis.edu (D.G.)
2 Department of Evolution and Ecology, University of California-Davis, Davis, CA 95616, USA; kastevans@ucdavis.edu
3 Foundation Plant Services, University of California-Davis, Davis, CA 95616, USA; vaklaassen@ucdavis.edu
* Correspondence: malrwahnih@ucdavis.edu
† These authors equally contributed to this work.

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Abstract: Viruses can cause economic losses in fruit trees, including Prunus spp., by reducing yield and marketable fruit. Given the genetic diversity of viruses, reliable diagnostic methods relying on PCR are critical in determining viral infection in fruit trees. This study evaluated the broad-range detection capacity of currently available real-time RT-PCR assays for Prunus-infecting viruses and developed new assays when current tests were inadequate or absent. Available assays for 15 different viruses were exhaustively evaluated in silico to determine their capacity to detect virus isolates deposited in GenBank. During this evaluation, several isolates deposited since the assay was designed exhibited nucleotide mismatches in relation to the existing assay’s primer sequences. In cases where updating an existing assay was impractical, we performed a redesign with the dual goals of assay compactness and comprehensive inclusion of genetic diversity. The efficiency of each developed assay was determined by a standard curve. To validate the assay designs, we tested them against a comprehensive set of 87 positive and negative Prunus samples independently analyzed by high throughput sequencing. As a result, all the real-time RT-PCR assays described herein successfully detected the different viruses and their corresponding isolates. To further validate the new and updated assays a Prunus germplasm collection was surveyed. The sensitive and reliable detection methods described here will be used for the large-scale pathogen testing required to maintain the highest quality nursery stock.

Keywords: Prunus spp.; viruses; genetic diversity; detection; real-time RT-PCR; high throughput sequencing

1. Introduction

Fruit trees are grown worldwide, mainly as a food source, and Prunus spp. are one of the most popular cultivated trees. The genus Prunus includes almonds, apricots, cherries, peaches, plums, and nectarines. In the United States, the main producers of Prunus spp. are the states of California, Washington, Oregon, South Carolina, Georgia, Michigan, and New Jersey (https://www.nass.usda.gov/index.php).

Although economic losses due to viral infection in Prunus spp. are difficult to quantify, viruses can cause losses by reducing plant vigor and growth, delaying fruit ripening, and causing graft and compatibility issues. Viruses can also remain latent, later, causing plants to grow slowly, produce smaller fruit, and have a reduced lifespan, but often these detrimental impacts may go unnoticed unless
crops are visibly damaged [1]. Reduction in yield and poor product quality from some viruses can be severe and lead to tree removal. Some major viruses of Prunus spp. include apple chlorotic leaf spot virus (ACLSV), cherry green ring mottle virus (CGRMV), cherry leaf roll virus (CLRV), little cherry virus-1 and -2 (LChV-1 and -2), prune dwarf virus (PDV), and Prunus necrotic ringspot virus (PNRSV). Cembali et al. [2] estimated that the United States sweet cherry and clingstone peach industries could save $11,191,460 and $5,580,877, respectively, adopting a virus protection program.

The genetic diversity of plant viruses is well known (reviewed in [3,4]). For example, divergent variants of LChV-1 and LChV-2 have been characterized via high throughput sequencing (HTS), which affects the epidemiology and symptomatology associated with these viruses [5,6]. PNRSV and PDV isolates can be classified in several phylogroups based on their coat protein (CP) or RNA-dependent RNA polymerase (RdRp) genes [7,8]. This genetic diversity makes it difficult to design and maintain sensitive assays that will reliably detect different virus isolates. Nucleic acid specific detection of viruses is a very useful technique to determine if a particular virus is present in a plant. Methods relying on PCR require sequence specific primers which may not amplify a virus sequence if the virus has nucleotide differences at the primer binding site. Given the genetic diversity of viruses, it is possible to miss detection of virus strains if the PCR assay is not specific for a variant present in nature.

Efficient and reliable laboratory diagnostic tests are critical in determining viral infection in Prunus spp. While several diagnostic methods are available for viral detection (e.g., biological indexing and ELISA), the advantages of using real-time reverse transcription PCR (RT-PCR) to detect viruses have been documented. The development of real-time RT-PCR assays led to superior sensitivity, speed, reproducibility, and limited risk of contamination compared to end-point RT-PCR [9,10]. The main feature of real-time RT-PCR is that DNA amplification is detected in real time as RT-PCR is in progress by the use of a fluorescent reporter, thus, the reporter signal strength is directly proportional to the number of amplified copies [11]. These characteristics often make it the method of choice in routine diagnostics. Virus testing of imported propagation materials into the United States has been the most important measure used to prevent the introduction and spread of viruses [2], and real-time RT-PCR is one of the diagnostic tools employed by inspection agencies. There are two types of real-time RT-PCR systems. The first is based on a generic non-sequence-specific double-stranded DNA-binding dye such as SYBR Green, and the second is based on sequence-specific DNA hydrolysis probes [11]. In this study, we used TaqMan hydrolysis probes with a FAM dye label on the 5′ end and a minor groove binder (MGB) and nonfluorescent quencher on the 3′ end.

Here, currently available real-time RT-PCR assays for different Prunus-infecting viruses (Table 1) were evaluated, and in many cases updated or redesigned to accommodate additional sequence diversity that was not available at the time the assay was originally designed. In the case of viruses with no published real-time RT-PCR assay, a new assay was designed. Thus, 15 new or updated real-time RT-PCR assays were developed during this study. In most cases, these assays utilized multiple primers and probes for detecting all known virus variants. Comprehensive evaluation and compact design (i.e., use the minimum number of primers to cover the genetic diversity) of so many assays were made possible because of purpose built Python scripts. Subsequently, all assays were empirically validated using previously known infected plant material. Lastly, for additional validation, a Prunus germplasm collection, representing different accessions originating from 53 countries, was screened with the real-time RT-PCR assays.
Table 1. Prunus-infecting viruses included in this study and currently available real-time RT-PCR assays.

| Virus                                         | Acronym    | Assay Citation        |
|-----------------------------------------------|------------|-----------------------|
| Apple chlorotic leafspot virus                | ACLSV      | Osman et al. 2016 [10]|
| Cherry green ring mottle virus                | CGRMV      | Osman et al. 2016 [10]|
| Cherry leaf roll virus                        | CLRV       | Osman et al. 2014 [9] |
| Cherry necrotic rusty mottle virus            | CNRMV      | Osman et al. 2016 [10]|
| Cherry rasp leaf virus                        | CRLV       | Osman et al. 2016 [10]|
| Cherry rusty mottle-associated virus          | CRMaV      | NA                    |
| Cherry virus A                                | CVA        | Osman et al. 2016 [10]|
| Little cherry virus 1                         | LChV-1     | Katsiani et al. 2017 [12]|
| Little cherry virus 2                         | LChV-2     | Jelkmann et al. 2006 [13]|
| Nectarine stem pitting-associated virus       | NSPaV      | NA                    |
| Nectarine virus M                             | NVM        | NA                    |
| Peach mosaic virus                            | PcMV       | NA                    |
| Plum bark necrosis stem pitting-associated    | PBNSPaV    | Lin et al. 2013 [14]  |
| virus                                         |            |                       |
| Prune dwarf virus                             | PDV        | Osman et al. 2014 [9] |
| Prunus necrotic ringspot virus                 | PNRSV      | Osman et al. 2014 [9] |

Not available assay (NA).

2. Results

2.1. New or Updated Real-Time RT-PCR Assays That Accommodate Virus Genetic Diversity

Previously published real-time RT-PCR assays for targeted viruses (Table 1) were evaluated in silico to determine their capacity to detect the current virus isolates deposited in GenBank. In the case of ACLSV, CGRMV, cherry necrotic rusty mottle virus (CNRMV), cherry rasp leaf virus (CRLV), cherry virus A (CVA), LChV-1, LChV-2, plum bark necrosis stem pitting-associated virus (PBNSPaV), PDV, and PNRSV, our sequence analysis showed nucleotide mismatches between primers/probe sequences of corresponding assays and the alignment generated for each virus sequence. Mismatches observed during this analysis ranged from 1 to 10 nucleotides, highlighting the need to keep assays current with respect to known genetic diversity. In contrast, the CLRV assay did not display nucleotide mismatches, indicating that no modification was needed.

Additional primers or probes were added to the current ACLSV, CRLV, LChV-1, and PDV assays (Table 2; Figure S1) in order to cover all the known genetic diversity of the virus variants. Adjustments to these assays primarily involved one extra probe or up to two extra primers. Additionally, in the case of LChV-1, the degenerate oligonucleotide probe included in the original assay was replaced by two probes placed in a nearby conserved region.

Given the new sequence data available in GenBank, the in silico analysis revealed that the genomic regions targeted by the published CGRMV, CNRMV, CVA, LChV-2, PBNSPaV, and PNRSV assays were not as conserved as previously thought. As a consequence, new compact assays that amplified an alternative target were designed (Table 2; Figure S1).

Finally, real-time RT-PCR assays for cherry rusty mottle-associated virus (CRMaV), nectarine stem pitting-associated virus (NSPaV), nectarine virus M (NVM), and peach mosaic virus (PcMV) were not available. Compact real-time RT-PCR assays were developed for these viruses as described below (Table 2; Figure S1).
| Virus  | Oligo Name  | Sequence (5′ to 3′) | 5′ Reporter | Probe Type | Target Region | Reference          |
|-------|-------------|---------------------|-------------|------------|--------------|-------------------|
| ACLSV | ACLSV-F1    | GCAGACCCCCCTCATAGGAAG |             |            |              | Osman et al., 2016 [10] |
|       | ACLSV-R1    | TCGGCTGCGAAGATGATGTC |             |            |              |                   |
|       | ACLSV-R2    | TTGCGGTCCGGAAGGAGGTAGTC |             |            |              |                   |
|       | ACLSV-R3    | TTGCGGTCCGGAAGGAGGTAGTC |             |            |              |                   |
|       | ACLSV-R4    | TTGCGGTCCGGAAGGAGGTAGTC |             |            |              |                   |
|       | ACLSV-R5    | GATGTTCAAATCAGGAGGAGGTAGTC |             |            |              |                   |
|       | ACLSV-P1    | CCATCTTCGCGGAAACAT | FAM         | MGB        |              |                   |
|       | ACLSV-P2    | CCATCTTCGCGGAAACAT | FAM         | MGB        |              |                   |
| CGRMV | CGRMV-F1    | GCTCTGGCTGCGGAAATAT |             | TGB1       |              | This study        |
|       | CGRMV-F2    | GCTCTGGCTGCGGAAATAT |             | TGB1       |              |                   |
|       | CGRMV-R1    | GGGGCTGAAAGTCTCTAAAG |             |            |              |                   |
|       | CGRMV-R2    | GGGGCTGAAAGTCTCTAAAG |             |            |              |                   |
|       | CGRMV-R3    | GGGGCTGAAAGTCTCTAAAG |             |            |              |                   |
|       | CGRMV-R4    | GGGGCTGAAAGTCTCTAAAG |             |            |              |                   |
|       | CGRMV-R5    | GGGGCTGAAAGTCTCTAAAG |             |            |              |                   |
|       | CGRMV-P1    | CTCTGGCTGCAAGAAGAAT | FAM         | MGB        |              |                   |
|       | CGRMV-P2    | CTCTGGCTGCAAGAAGAAT | FAM         | MGB        |              |                   |
| CLRV  | CLRV-F1     | TGGCGGACCGTTGAAACGG |             |            |              |                   |
|       | CLRV-R1     | TACCTACAAGACCCGCGC |             |            |              |                   |
|       | CLRV-R2     | TACCTACAAGACCCGCGC |             |            |              |                   |
|       | CLRV-P1     | GTTAAAGTCTGACTGTTG | FAM         | MGB        |              |                   |
|       | CLRV-P2     | TACCTGTAACGACTGTTG | FAM         | MGB        |              |                   |
| CNRMV | CNRMV-F1    | AATCCCACCTCAAGTCTTGAG |             |            |              | This study        |
|       | CNRMV-R1    | GTGTCGAAACCCGCAATGCA |             |            |              |                   |
|       | CNRMV-R2    | GTGTCGAAACCCGCAATGCA |             |            |              |                   |
| CRLV  | CRLV-F1     | TGCTTATGGAAGGACAAA |             | RdRp       |              | Osman et al., 2016 [10] |
|       | CRLV-R1     | TCCFGGGCGTAATCCTCCTC |             |            |              |                   |
|       | CRLV-R2     | AACATCTCCCGTATGCTCCTAC |             |            |              |                   |
|       | CRLV-P1     | TGCGTTATGGAAGGATATTTC |             |            |              |                   |
|       | CRLV-P2     | TGCTTATGGAAGGATATTTC |             |            |              |                   |
| CRMaV | CRMaV-F1    | TAATTTGCACTTTTGAGTCTG |             |            |              | This study        |
|       | CRMaV-F2    | TTATTTGCACTTTTGAGTCTG |             |            |              |                   |
|       | CRMaV-R1    | TCCGTAAGAGCACTGCTCTAAC |             |            |              |                   |
|       | CRMaV-R2    | TCCGTAAGAGCACTGCTCTAAC |             |            |              |                   |
|       | CRMaV-P1    | TGTTATCATAACAGCTCCCAG |             |            |              |                   |
|       | CRMaV-P2    | TGTTATCATAACAGCTCCCAG |             |            |              |                   |
| CVA   | CVA-F1      | CCGAGACCCTGAGAAGAATC |             |            |              | This study        |
|       | CVA-F2      | CCGAGACCCTGAGAAGAATC |             |            |              |                   |
|       | CVA-F3      | CCGAGACCCTGAGAAGAATC |             |            |              |                   |
|       | CVA-R1      | GCACACACTACACACACATGC |             |            |              |                   |
|       | CVA-R2      | GCACACACTACACACACACCA |             |            |              |                   |
|       | CVA-P1      | ACTGCAACATTCCTCAG | FAM         | MGB        |              |                   |
|       | CVA-P2      | ACTGCAACATTCCTCAG | FAM         | MGB        |              |                   |
| Virus   | Oligo Name | Sequence (5’ to 3’) | 5’ Reporter | Probe Type | Target Region | Reference               |
|---------|------------|---------------------|-------------|------------|--------------|-------------------------|
| LChV-1  | LChV1-F1   | CCAATGCACAAGCACATAATGA |             |            |              | Katsiani et al., 2017 [12] |
|         | LChV1-F2   | CCAATGCATAAAGCTCATAGCAT |             |            |              |                         |
|         | LChV1-F3   | CCGATGCAAAAGACATCAAT |             |            |              |                         |
|         | LChV1-F4   | CGATGCATAAGCTCATAGCTAGT |             |            |              |                         |
|         | LChV1-P1   | GATACTGATAGCTGCTGCTCG | FAM         | MGB        |              |                         |
|         | LChV1-P2   | GATACTGATAGCTGCTGCTCG | FAM         | MGB        |              |                         |
|         | LChV2-F1   | TTTGACCAGAATACCCTCCTCG |             |            | RdRp         | This study              |
|         | LChV2-F2   | AGTCAGCACCAGAAACTCTCTCTG |             |            |              |                         |
|         | LChV2-F3   | TACAAAAAGCTGTGCTGCTGACTACAGG |             |            |              |                         |
|         | LChV2-P1   | TCTGGAGATGATCTCATT | FAM         | MGB        |              |                         |
|         | LChV2-P2   | TCTAGAGAGCTGCCTCTT | FAM         | MGB        |              |                         |
| NSPaV   | NSPaV-F1   | AGCGAATGGAGCAAAATCTGGA |             |            |              |                         |
|         | NSPaV-F2   | AAAGCAAAATGGACGCAAAATCTGAT |             |            |              |                         |
|         | NSPaV-R1   | CAATGAGTGTGCAAGGTGATG |             |            | CP           | This study              |
|         | NSPaV-R2   | CAATGAGTGTGCAAGGTGATG |             |            |              |                         |
|         | NSPaV-P1   | TCGCTGGCGAATT | FAM         | MGB        |              |                         |
| NVM     | NVM-F1     | TGATTCCCTCCTCGACTACGGA |             |            | RdRp Polyprotein | This study              |
|         | NVM-R1     | AGGCCTTGATAGGCGTTCCA |             |            |              |                         |
|         | NVM-R2     | GAGGCTTAATGGCCTCCAC |             |            |              |                         |
|         | NVM-P1     | CCGGCAAGTCCGACCC |             |            |              |                         |
| PcMV    | PcMV-F1    | ACGAGGATGCTCCTGATGAGT |             |            | RdRp         | This study              |
|         | PcMV-R1    | ACAAACTCCTCCTCAAGCTCATC |             |            |              |                         |
|         | PcMV-R2    | GACCATCTCCTCAGCTCATC |             |            |              |                         |
|         | PcMV-P1    | TTCTGGAGATGAAACGC | FAM         | MGB        |              |                         |
|         | PBNSPaV-F1 | GGCTGTAAGTCTTGGCTTGGCTTCTG |             |            | 3’ UTR         | This study              |
|         | PBNSPaV-R1 | ACCACCGCGGACAGGTGATT |             |            |              |                         |
|         | PBNSPaV-P1 | CTTCTGAGACAGGATAA | FAM         | MGB        |              |                         |
| PDV     | PDV-F1     | TGATACCAAGGTTATACCGGAAATTG |             |            | CP           | Osman et al., 2014 [9] |
|         | PDV-F2     | TGATACCAAGGTTATACCGGAAATTG |             |            |              |                         |
|         | PDV-F3     | TGATACCAAGGTTATACCGGAAATTG |             |            |              |                         |
|         | PDV-F4     | TGATACCAAGGTTATACCGGAAATTG |             |            |              |                         |
|         | PDV-R1     | TGAACCTCTTACGGTTTGCAGGGGAT |             |            | CP           | Osman et al., 2014 [9] |
|         | PDV-R2     | AACAATTCTCCTCCTAGAGGGGATTT |             |            |              |                         |
|         | PDV-P1     | TCTACGGCACCTCTAAAGGTT | FAM         | MGB        |              |                         |
|         | PDV-P2     | TGTCTTACGGGACTCTAA | FAM         | MGB        |              |                         |
Table 2. Cont.

| Virus       | Oligo Name | Sequence (5’ to 3’) | 5’ Reporter | Probe Type | Target Region | Reference |
|-------------|------------|---------------------|-------------|------------|---------------|-----------|
| PNRSV       | PNRSV-F1   | ACCGAGAGGTGACAACGACAG |             |            |               |           |
| PNRSV       | PNRSV-F2   | ACACAGAGGTGACGACGA  |             |            |               |           |
| PNRSV       | PNRSV-F3   | ACCGAGAGGTGACGACGA  |             |            |               |           |
| PNRSV       | PNRSV-F4   | ACACGAGGTGACGACTCTG |             |            |               |           |
| PNRSV-R1    | CCTTAAGAACCCCTTCATAC |             |             |            |               |           |
| PNRSV-R2    | CCTTCAGAAAACCTCTCTAGACA |             |             |            |               |           |
| PNRSV       | PNRSV-P1   | CCGAATGAACTCTATGAGTT | FAM         | MGB        |               |           |
| PNRSV       | PNRSV-P2   | CCGAATGAACTCTCAAGAGG | FAM         | MGB        |               |           |

1 Virus name and corresponding acronym in Table 1. 2 Forward primer (F), reverse primer (R), and probe (P). 3 Sequences in bold represent the primers and probes included in previously published assays. 4 Coat protein (CP), RNA-dependent RNA polymerase (RdRp), triple gene block 1 (TGB1), untranslated region (UTR).
2.2. Detection of Targeted Viruses via High Throughput Sequencing

Select samples originating from the Foundation Plant Services (FPS) and the Clean Plant Center Northwest (CPCNW) collections of new Prunus introductions were analyzed for the viruses described in Table 1 using HTS. As a result of this inspection, multiple isolates were identified for all the viruses (Table S1), with the exception of NVM, PcMV, and CRMaV, which had only one isolate each. These Prunus samples were subsequently used to evaluate the updated/new assays (described below). If the HTS analysis determined that a sample was free of viruses or infected by not targeted viruses, the sample was used as a negative control.

2.3. Validation of Assay Design

Virus detection was validated by comparing real-time RT-PCR and HTS results for each virus-infected sample (Table S1). In all cases, real-time RT-PCR and HTS results agreed and Ct values were less than 28. No amplification was observed with healthy plant controls or plants infected by unrelated viruses, confirming the specificity of the assays. Since degenerate primers and probes with multiple sequence combinations (i.e., several possible bases in one or more positions) were not used to account for genetic diversity, the presence of all unique primers and TaqMan probes in the same reaction mixture was essential for the successful detection of all isolates in this study. The amplification efficiency varied among assays and ranged from 82% to 117% (Figure S2).

2.4. Screening of the Prunus Germplasm Collection

The real-time RT-PCR assays in Table 2 were used to evaluate the occurrence of viruses in a Prunus germplasm collection of diverse provenances at the National Clonal Germplasm Repository (NCCR). As a result of this survey, ACLSV, CGRMV, CNRMV, CVA, LChV-1, LChV-2, NSPaV, NVM, PcMV, PBNSPaV, PDV, or PNRSV were detected in 182 out of 333 trees or 54.6% of the tested accessions (Table 3; Table S2).

Table 3. Viruses identified during the survey in the National Clonal Germplasm Repository.

| Virus     | Number of Infected Trees |
|-----------|--------------------------|
| ACLSV     | 19 (5.7%)                |
| CGRMV     | 20 (6%)                  |
| CLRV      | 0 (0%)                   |
| CNRMV     | 4 (1.2%)                 |
| CRLV      | 0 (0%)                   |
| CRMaV     | 0 (0%)                   |
| CVA       | 39 (11.7%)               |
| LChV-1    | 10 (3%)                  |
| LChV-2    | 3 (0.9%)                 |
| NSPaV     | 4 (1.2%)                 |
| NVM       | 10 (3%)                  |
| PcMV      | 2 (0.6%)                 |
| PBNSPaV   | 33 (9.9%)                |
| PDV       | 29 (8.7%)                |
| PNRSV     | 127 (38.1%)              |

3. Discussion

This study exhaustively evaluated the genetic diversity represented by currently available Prunus fruit tree virus assays. We developed new and updated real-time RT-PCR assays to improve representation of current genetic diversity. These assays were designed with the dual goals of being compact, and at the same time, incorporating a complete picture of the known genetic diversity for high efficiency and sensitivity. New assays were designed to the most conserved region present in each virus species, which may involve the CP, RdRp, triple gene block 1, or the 3’ untranslated region.
Among virus isolates, pairwise sequence similarity within assay regions varied between 88.5% to 100% (Table 4). In order to generate all these assays, custom scripts were utilized to accelerate and simplify assay design (e.g., sequence alignment and primer/probe design were completed in a single process). This also allowed us to use multiple variant matched primers and probes instead of single degenerate pairs. The smaller and more uniform primers in these assays are an attempt to ameliorate the lower efficiency degeneracy can lead to [12]; additionally, the use of degenerate primers may result in a major number of primers per reaction in comparison with our new assays.

Table 4. Comparison between genome regions amplified by each real-time RT-PCR assay and GenBank accessions included in assay design.

| Assay  | Amplicon Size | Identity of Target Region | Number of Accessions Included in Design |
|--------|---------------|----------------------------|----------------------------------------|
| ACLSV  | 218 bp        | 88.5%                      | 247                                    |
| CGRMV  | 62 bp         | 96.5%                      | 35                                     |
| CLRV   | 83 bp         | 97.3%                      | 46                                     |
| CNRMV  | 120 bp        | 97.3%                      | 80                                     |
| CRLV   | 72 bp         | 93%                        | 6                                      |
| CRMaV  | 139 bp        | 97%                        | 23                                     |
| CVA    | 107 bp        | 96.6%                      | 67                                     |
| LChV-1 | 115 bp        | 90.3%                      | 13                                     |
| LChV-2 | 147 bp        | 94.1%                      | 6                                      |
| NSPaV  | 62 bp         | 98.9%                      | 6                                      |
| NVM    | 59 bp         | 98.9%                      | 4                                      |
| PcMV   | 152 bp        | 95.4%                      | 7                                      |
| PBNSPaV| 71 bp         | 100%                       | 10                                     |
| PDV    | 127 bp        | 95.6%                      | 122                                    |
| PNRSV  | 216 bp        | 95.6%                      | 230                                    |

Prunus samples from two collections, FPS and CPCNW, were used as virus sources to evaluate the updated or new assays. HTS analyses indicated that most of these samples (i.e., 69 out of 87) were infected with at least one of the 15 targeted viruses, revealing mixed infections in several samples (i.e., 20 samples). As a result of this evaluation, an agreement between real-time RT-PCR assay and HTS was obtained. To further validate the real-time RT-PCR assays, we collected and tested samples from the NCGR, which includes Prunus spp. accessions from a wide range of geographical regions. Although the actual virus diversity in the NCGR samples was not characterized by HTS, we detected 12 of the 15 viruses in 54.6% of the trees, suggesting that the PCR-based assays are robust. Thus, all the updated or new assays were tested against multiple isolates of each virus, except for CRMaV, which was identified in only one instance by both real-time RT-PCR and HTS.

During the initial validation of the real-time RT-PCR assays using samples previously analyzed by HTS, Ct values ranged from 12 to 28 and similar Ct values were obtained during the survey in the NCGR (Table S2). For CGRMV, CLRV, and PNRSV assays, there were a few cases where Ct values were >30. These samples were re-analyzed (i.e., extraction and testing were repeated) and confirmed to be negative. We hypothesize that these high Ct values were due to cross-contamination from strongly positive samples that were present in the initial processing. Consequently, any amplification after 30 cycles should be further investigated and verified.

In the United States, growers have adopted different methods for the control of viral diseases in fruit trees, including (i) the adoption of virus-tested propagation material and (ii) the eradication of infected trees [2]; all the viruses here investigated are part of the clean plant certification program. In that sense, new advances in real-time RT-PCR have significantly improved the detection of pathogens, allowing quick, sensitive, and precise identification compared to other historically used detection methods (e.g., end-point PCR, ELISA, and biological indexing). Moreover, real-time RT-PCR can be used to determine the number of virus copies present in a sample (i.e., virus quantification). In addition,
it has the potential to be multiplexed with other assays, increasing testing efficiencies by identifying different viruses during the same reaction or by including an internal control. Thus, the development of highly sensitive real-time RT-PCR assays with broad-range detection capacity is needed for large scale testing of Prunus species that may be infected by the genetically diverse viruses included in this study. The assays developed here can help the clean stock programs and the fruit tree industry by facilitating early detection of virus-infected material. Likewise, Fotiou et al. [15] just published a new real-time RT-PCR for plum pox virus, which is considered as one of the most important pathogens in fruit trees and currently quarantined in the United States.

4. Materials and Methods

4.1. In Silico Analysis and Update of Available Real-Time RT-PCR Assays

The exhaustive evaluation, update, and design of 15 assays against the current version of GenBank was facilitated by purpose-built scripts implementing some of the procedures described below. For each of the viruses listed in Table 1, the most recently published real-time RT-PCR assay was first evaluated against all virus sequences deposited in GenBank. First, we used a BLAST [16] database search to identify and obtain all GenBank sequences overlapping the current assay region. To maximize sensitivity, a tBLASTn translated alignment exploiting codon redundancy was used. Highly divergent variants were further individually confirmed by separate BLAST analysis against GenBank to eliminate the possibility of misidentification. Once target sequences were collected and their species identification confirmed, all existing primers and probes were aligned to all target sequences from GenBank covering the assay region. This alignment was accomplished using a Perl script that used an end-gap-free nucleotide alignment to identify the best matching probe, forward and reverse primer sequences to each GenBank variant. In each case, the variant sequences corresponding to the matching oligos were collected and analyzed for divergence. Thus, all unique candidate sequence variants were inspected for total or partial divergence to an existing primer/probe sequence. The location and quantity of nucleotide differences and the frequency of the sequence in GenBank were also determined and assays were updated with extra primers or probes. One probe or one primer was added when more than two nucleotide mismatches were detected during the sequence comparison.

4.2. Development of New Compact Real-Time RT-PCR Assays

In a handful of cases where a real-time RT-PCR assay did not exist (i.e., CRMaV, NSPaV, NVM, and PcMV) or, on inspection of the current genetic diversity, the previous assay was impractical to extend and update (i.e., CGRMV, CNRMV, CVA, LChV-2, PBNSPaV, and PNRSV), a new assay was designed using an exhaustive approach that proposed a compact assay covering existing genetic diversity. To accomplish this objective, a Python script was used to minimize the size of the assay, with respect to the probe, the forward and reverse primer(s) sequences. First, an input multiple alignment \( M \) was determined. Conservation and depth of public sequence information across the virus genome was evaluated using a MUSCLE [17] multiple alignment of all virus sequences deposited in GenBank.

Let \( M \) be the \( m \times n \) matrix containing the multiple sequence alignment considered for assay design. The matrix \( M \) contains \( n \) nucleotides or gap characters from each of \( m \) virus isolates. We define \( M(i,w) \) as the \( w \) adjacent columns of \( M \) starting at column \( i \), and \( S(M,i,w) \) as the number of unique rows, aka sequences, in \( M(i,w) \). We wish to minimize \( S \) given the constraints of the design. For a proposed real-time RT-PCR probe width \( w_p \), an optimal location for the probe was determined by exhaustive search:

\[
\min_{0 \leq i \leq n} S(M, i, w_p)
\]

Following optimal probe placement, we then considered the window of 100 bp to the left and right to obtain optimal forward and reverse primers. Let \( i_{\min} \) be the optimal probe location, and \( w \) be
the width of the proposed RT-PCR forward and reverse primers. Best candidate locations $j_{\text{min}}$ and $k_{\text{min}}$ for the forward and reverse primers were determined by sequential exhaustive searches:

$$
\min_{\text{imin} - 100 \leq j \leq \text{imin}} S(M, j, w) \quad \min_{\text{imin} + 100 \leq k \leq \text{imin} + 150} S(M, k, w)
$$

Final determination of primer and probe sequences for a region included an additional more precise primer length adjustment step to the correct melting temperature ($T_m$) according to the parameters for real-time RT-PCR with MGB probes employing the Primer Express software (ThermoFisher Scientific, Foster City, CA, USA). Primer–primer and primer–probe interactions were also evaluated using the same software. Finally, primers and probes were ranked by their frequency in the database. Considering the list of primers/probes in order of decreasing frequency, all primers and probes contributing two or more additional nucleotides, or one nucleotide within two bases of the 3’ end, were included in the final assay design. In general, the nucleotide sequence identity among virus isolates included in the assay regions varied between 88.5% to 100% (Table 4).

4.3. Virus Screening via High Throughput Sequencing

Plant material reported or suspected to be infected by the studied viruses was obtained from FPS (University of California-Davis) and the CPCNW (Washington State University); both Prunus collections include foreign and domestic introductions. In total, 87 Prunus samples (Table 5) were obtained and included in the virus screening via HTS. Briefly, total nucleic acid (TNA) extracts from Prunus samples were prepared following the methodology described by Al Rwahnih et al. [18]. Later, TNA aliquots were subjected to ribosomal RNA (rRNA) depletion and complementary DNA library construction using the TruSeq Stranded Total RNA with Ribo-Zero Plant kit (Illumina, San Diego, CA, USA). HTS analysis for known viruses was accomplished as described in [19], but the de novo assembly was completed by SPAdes v3.11 [20].

Table 5. Prunus samples analyzed by high throughput sequencing during this study.

| Prunus Tree | Number of Samples |
|-------------|------------------|
| Almond      | 3                |
| Apricot     | 2                |
| Cherry      | 45               |
| Nectarine   | 10               |
| Peach       | 25               |
| Plum        | 2                |

4.4. Initial Validation of Assay Design and Efficiency

All the assays described in Table 2 were challenged against the set of 87 Prunus samples previously analyzed by HTS; such set included different viruses and multiple isolates of each virus, except for NVM, PcMV, and CRMaV with one isolate only. In addition, samples free of targeted viruses were considered in the analysis as negative controls.

Real-time RT-PCR reactions were completed in the QuantStudio 6 real-time PCR system using the TaqMan Fast Virus 1-Step Master Mix (ThermoFisher Scientific, Foster City, CA) and following the recommended protocol. Each reaction (10 µL final volume) included 2 µL of TNA and final primer and probe concentrations of 900 and 250 nM, respectively. The thermocycler conditions were as follows: 50 °C for 5 min, 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Additionally, the assays were multiplexed with a previously published 18S rRNA assay [21] to verify the presence of high-quality RNA during the reaction.

The efficiency of each real-time RT-PCR assay was determined using serial dilutions (1:1 to 1:1,000,000) of TNA extracts in water and run in triplicate. Standard curves were calculated using the QuantStudio 6 real-time PCR software.
4.5. Survey in the NCGR

The NCGR, a United States Department of Agriculture genetic resource, is located near Winters, California, and contains approximately 4000 Prunus trees representing different accessions (https://npgsweb.ars-grin.gov). Trees in this collection originate globally and contain almonds, apricots, cherries, peaches, plums, and nectarines. Using a random methodology but taking in consideration different types of Prunus material and countries of origin, 333 accessions (Table 6) were sampled and later tested via real-time RT-PCR.

Table 6. Prunus accessions included in the survey and corresponding country of origin.

| Country of Origin          | Number of Accessions |
|----------------------------|----------------------|
| Afghanistan                | 2                    |
| Albania                    | 3                    |
| Armenia                    | 4                    |
| Australia                  | 3                    |
| Azerbaijan                 | 3                    |
| Belgium                    | 1                    |
| Bosnia and Herzegovina     | 1                    |
| Brazil                     | 5                    |
| Bulgaria                   | 4                    |
| Canada                     | 7                    |
| China                      | 17                   |
| Czech Republic             | 2                    |
| Former Serbia and Montenegro| 7              |
| France                     | 7                    |
| Georgia                    | 5                    |
| Germany                    | 4                    |
| Greece                     | 2                    |
| Guatemala                  | 1                    |
| Hungary                    | 7                    |
| India                      | 8                    |
| Iran                       | 2                    |
| Israel                     | 1                    |
| Italy                      | 11                   |
| Japan                      | 10                   |
| Kazakhstan                 | 5                    |
| South Korea                | 3                    |
| Kyrgyzstan                 | 1                    |
| Latvia                     | 1                    |
| Lebanon                    | 1                    |
| Malta                      | 1                    |
| Mexico                     | 5                    |
| Morocco                    | 3                    |
| Nepal                      | 4                    |
| Netherlands                | 3                    |
| New Zealand                | 23                   |
| Pakistan                   | 23                   |
| Poland                     | 6                    |
| Romania                    | 10                   |
| Russian Federation         | 8                    |
| Serbia                     | 1                    |
| South Africa               | 7                    |
| Spain                      | 3                    |
| Sweden                     | 2                    |
| Switzerland                | 2                    |
| Syria                      | 1                    |
| Taiwan                     | 5                    |
| Thailand                   | 4                    |
| Turkey                     | 7                    |
| Turkmenistan               | 4                    |
| Ukraine                    | 5                    |
| United Kingdom             | 7                    |
| United States              | 75                   |
| Uzbekistan                 | 7                    |
| Unknown                    | 7                    |
Supplementary Materials: The following material are available online at http://www.mdpi.com/2223-7747/9/2/273/s1. Figure S1: Alignments of genomic regions present in different Prunus-infecting viruses and amplified by the real-time RT-PCR assays. Figure S2: Amplification plot and standard curve generated from new/updated real-time RT-PCR assays. Table S1: Prunus samples analyzed by HTS and included in the initial validation of assays. Table S2: Information of samples collected at the NCGR and tested positive for viruses during the survey.

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References
1. Hadidi, A.; Barba, M.; Candresse, T.; Jelkmann, W. Virus and Virus-Like Diseases of Pome and Stone Fruits; American Phytopathological Society: St. Paul, MN, USA, 2011; ISBN 0-89054-396-8.
2. Cembali, T.; Folwell, R.J.; Wandschneider, P.; Eastwell, K.C.; Howell, W.E. Economic implications of a virus prevention program in deciduous tree fruits in the US. Crop Prot. 2003, 22, 1149–1156. [CrossRef]
3. Schneider, W.L.; Roossinck, M.J. Genetic Diversity in RNA Virus Quasispecies Is Controlled by Host-Virus Interactions. J. Virol. 2001, 75, 6566–6571. [CrossRef] [PubMed]
4. Moury, B.; Desbiez, C.; Jacquemond, M.; Lecoq, H. Genetic Diversity of Plant Virus Populations: Towards Hypothesis Testing in Molecular Epidemiology. In Advances in Virus Research; Plant Virus Epidemiology; Academic Press: Cambridge, MA, USA, 2006; Volume 67, pp. 49–87.
5. Katsiani, A.; Maliogka, V.I.; Katis, N.; Svanella-Dumas, L.; Olmos, A.; Ruiz-García, A.B.; Marais, A.; Faure, C.; Theil, S.; Lotos, L.; et al. High-Throughput Sequencing Reveals Further Diversity of Little Cherry Virus 1 with Implications for Diagnostics. Viruses 2018, 10, 385. [CrossRef] [PubMed]
6. Tahzima, R.; Foucart, Y.; Peusens, G.; Beliën, T.; Massart, S.; De Jonghe, K. High-Throughput Sequencing Assists Studies in Genomic Variability and Epidemiology of Little Cherry Virus 1 and 2 infecting Prunus spp. in Belgium. Viruses 2019, 11, 92. [CrossRef] [PubMed]
7. Cui, H.G.; Liu, H.Z.; Chen, J.; Zhou, J.F.; Qu, L.N.; Su, J.M.; Wang, G.P.; Hong, N. Genetic diversity of Prunus necrotic ringspot virus infecting stone fruit trees grown at seven regions in China and differentiation of three phylogroups by multiplex RT-PCR. Crop Prot. 2015, 74, 30–36. [CrossRef]
8. Kinoti, W.M.; Constable, F.E.; Nancarrow, N.; Plummer, K.M.; Rodoni, B. The Incidence and Genetic Diversity of Apple Mosaic Virus (ApMV) and Prune Dwarf Virus (PDV) in Prunus Species in Australia. Viruses 2018, 10, 136. [CrossRef] [PubMed]
9. Osman, F.; Al Rwahnih, M.; Rowhani, A. Improved detection of ilaviruses and nepoviruses affecting fruit trees using quantitative RT-qPCR. J. Plant Pathol. 2014, 96, 577–583.
10. Osman, F.; Al Rwahnih, M.; Rowhani, A. Real-time RT-qPCR detection of cherry rasp leaf virus, cherry green ring mottle virus, cherry necrotic rusty mottle virus, cherry virus a and apple chlorotic leaf spot virus in stone fruits. J. Plant Pathol. 2016, 99, 279–285.
11. Jia, Y. Chapter 3—Real-Time PCR. In Methods in Cell Biology; Laboratory Methods in Cell Biology; Conn, P.M., Ed.; Academic Press: Cambridge, MA, USA, 2012; Volume 112, pp. 55–68.
12. Katsiani, A.T.; Pappi, P.; Olmos, A.; Efthimiou, K.E.; Maliogka, V.I.; Katis, N.I. Development of a Real-Time RT-PCR for the Universal Detection of LChV1 and Study of the Seasonal Fluctuation of the Viral Titer in Sweet Cherry Cultivars. Plant Dis. 2017, 102, 899–904. [CrossRef] [PubMed]
13. Jelkmann, W.; Leible, S.; Rott, M. Little cherry closteroviruses-1 and-2, their genetic variability and detection by Real-Time-PCR. In Proceedings of the XX International Symposium on Virus and Virus-Like Diseases of Temperate Fruit Crops-Fruit Tree Diseases 781, Antalya, Turkey, 22 May 2006; pp. 321–330.
14. Lin, L.; Li, R.; Bateman, M.; Mock, R.; Kinard, G. Development of a multiplex TaqMan real-time RT-PCR assay for simultaneous detection of Asian prunus viruses, plum bark necrosis stem pitting associated virus, and peach latent mosaic viroid. *Eur. J. Plant Pathol.* **2013**, **137**, 797–804. [CrossRef]

15. Fotiou, I.S.; Pappi, P.G.; Efthimiou, K.E.; Katis, N.I.; Maliogka, V.I. Development of one-tube real-time RT-qPCR for the universal detection and quantification of Plum pox virus (PPV). *J. Virol. Methods* **2019**, **263**, 10–13. [CrossRef] [PubMed]

16. Tatusova, T.A.; Madden, T.L. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol. Lett.* **1999**, **174**, 247–250. [CrossRef] [PubMed]

17. Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, **32**, 1792–1797. [CrossRef] [PubMed]

18. Al Rwahnih, M.; Alabi, O.J.; Westrick, N.M.; Golino, D. Prunus geminivirus A: A Novel Grablovirus Infecting *Prunus* spp. *Plant Dis.* **2017**, **102**, 1246–1253. [CrossRef] [PubMed]

19. Al Rwahnih, M.; Rowhani, A.; Westrick, N.; Stevens, K.; Diaz-Lara, A.; Trouillas, F.P.; Preece, J.; Kallsen, C.; Farrar, K.; Golino, D. Discovery of Viruses and Virus-Like Pathogens in Pistachio using High-Throughput Sequencing. *Plant Dis.* **2018**, **102**, 1419–1425. [CrossRef]

20. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Pyshentski, A.D.; et al. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *J. Comput. Biol.* **2012**, **19**, 455–477. [CrossRef] [PubMed]

21. Osman, F.; Leutenegger, C.; Golino, D.; Rowhani, A. Real-time RT-PCR (TaqMan®) assays for the detection of Grapevine Leafroll associated viruses 1–5 and 9. *J. Virol. Methods* **2007**, **141**, 22–29. [CrossRef] [PubMed]