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Application of Next Generation Sequencing for Diagnostic Testing of Tree Fruit Viruses and Viroids

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Abstract

Conventional detection of viruses and virus-like diseases of plants is accomplished using a combination of molecular, serological, and biological indexing. These are the primary tools used by plant virologists to monitor and ensure trees are free of known viral pathogens. The biological indexing assay, or bioassay, is considered to be the "gold standard" as it is the only method of the three that can detect new, uncharacterized, or poorly characterized viral disease agents. Unfortunately, this method is also the most labor intensive and can take up to three years to complete. Next generation sequencing (NGS) is a technology with rapidly expanding possibilities including potential applications for the detection of plant viruses. In this study, comparisons are made between tree fruit testing by conventional and NGS methods, to demonstrate the efficacy of NGS. A comparison of 178 infected trees, many infected with several viral pathogens, demonstrated that conventional and NGS were equally capable of detecting known viruses and viroids. Comparable results were obtained for 170 of 178 of the specimens. Of the remaining eight specimens, some discrepancies were observed between viruses detected by the two methods, representing less than 5% of the specimens. NGS was further demonstrated to be equal or superior for the detection of new or poorly characterized viruses when compared with a conventional bioassay. These results validated both the effectiveness of conventional virus testing methods and the use of NGS as an additional or alternative method for plant virus detection.

Compared with most cellular pathogens, viruses can be difficult to detect and identify because they are obligate parasites unable to propagate outside of a host cell and are polyphyletic with no single gene shared by all viruses that could be used as a general virus test. Phytosanitary requirements for the importation of fruit trees are designed to ensure that materials entering the country are free of pathogenic viruses of concern. The list of quarantine viruses can vary depending on the status of each virus and the host crops that are grown in each individual country. In addition to known pathogenic viruses, new, potentially harmful viruses or virus strains are continuously being identified. It is important that these viruses be detected and prevented from entry before becoming established and causing potential harm. Over the years, regulatory measures have been developed and put in place to address these issues. One of the first techniques developed was biological indexing, or bioassay (for review, see Thompson et al. 2011). Material suspected of being infected with a virus is inoculated onto a sensitive indicator plant, and the indicator is observed for symptom development. Symptomology is characteristic for virus species/indicator species combinations. The process, while effective, is time consuming and labor intensive. Multiple indicator cultivars need to be inoculated and indicator plants need to be monitored at regular intervals during the growing season for up to three years. Test sites need to be isolated from commercial growing area and require restricted access. Regular mitigation procedures are required to minimize the potential movement or spread of viruses from the test site, along with ongoing care and maintenance of the plants. Highly trained personnel with specialized knowledge are needed to recognize often subtle symptoms and distinguish them from symptoms caused by other pests or environmental effects.

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To improve detection, additional methods have been employed, including electron microscopy to visualize virus particles (Hari and Das 1998). It wasn't until the development of polyclonal, and later monoclonal antibodies and their application in enzyme linked immunosorbant assays (ELISA) that rapid, accurate testing for known viruses became routine (López et al. 2006). Nucleic acid based test methods, most importantly polymerase chain reaction (PCR), evolved later, improving sensitivity and specificity (Hadidi et al. 1995). In today's post entry quarantine diagnostic laboratory, all of these methods are in regular use, each having unique advantages. Antibody and nucleic acid based methods are rapid and relatively inexpensive but require a priori knowledge of the virus in question. Biological indexing has the advantage of potentially detecting uncharacterized viruses for which specific molecular tests have not yet been developed, but is slow and labor intensive. Electron microscopy can also detect new viruses but is limited to characterizing gross particle morphology, is an expensive instrument to purchase and maintain, and is not effective at detecting low titer viruses or virionless viral infections.

Advances in next generation sequencing (NGS) technologies have allowed for a new metagenomic approach for the detection of viruses. Total nucleic acid extract from an infected sample corresponding to both host and pathogen(s) is sequenced and the viral sequences identified using bioinformatic tools. The rapid development of this approach has led to studies on the metagenomics analysis of virus infected plants (Coetzee et al. 2010; Jones 2014; Roossinck 2012; Roossinck et al. 2010). It is only natural that following the successful demonstration of NGS for new virus discovery, this method would eventually be applied to diagnostic testing of plant viruses. A rapid and accurate method to fully index viruses in a sample by inspection and quarantine services has been a long sought, but unattainable goal. With producer demand for access to the latest plant cultivars, there is a worldwide need for faster, more efficient methods to facilitate the movement of plants across borders while maintaining phytosanitary standards. NGS could replace many of the existing tools currently used by quarantine facilities (Martin et al. 2016). Several groups have been exploring the use of NGS for plant virus diagnostics. VirFind (Ho and Tzanetakis 2014) and VirusDetect (Zheng et al. 2017) are

specific workflows for plant virus detection and discovery from NGS data. The Food and Environmental Research Agency in the United Kingdom are also pursuing NGS workflows for plant virus detection (Adams et al. 2009). Recently, Al Rwahnih et al. (2015) published a manuscript on "Comparison of next generation sequencing vs. biological indexing for the optimal detection of viral pathogens in grapevine" that demonstrated equal or improved detection for NGS.

NGS is potentially an ideal, universal screening method for plant virus diagnostics (Martin et al. 2016). Multiple PCR, ELISA, and bioassays could be eliminated in favor of a single NGS assay, followed up with individual confirmatory testing for a positive identification. This would greatly reduce the time, effort, and cost associated with importing foreign planting material, enabling industry to more rapidly respond to market demands for new plant cultivars developed around the world.

Materials and Methods

Specimens. Plants utilized in this study are listed in Table 1. These plants are maintained in 2 to 7 gallon pots in screen houses as controls that are used for diagnostic testing and test development at the Canadian Food Inspection Agency (CFIA), Centre for Plant Health (CPH), Sidney Laboratory. This collection of plant specimens has been maintained and expanded since the mid-1960s (Table 1). Newly received specimens are graft inoculated onto several healthy young plants. Further propagations are made as required to replace plants that have overgrown their container or have died. Several plants corresponding to each specimen are maintained at CPH.

Conventional testing. Conventional test methods, defined here as one or a combination of biological indexing, ELISA, and/or PCR, have been performed over time on the collection of specimens to determine disease status. Table 2 is a summary of current methods being used to detect viruses/viroids or diseases thought to be caused by a virus/viroid at CPH. Some plants have undergone a "full range of testing" while others have been tested for only one or a few viruses/viroids. Table 2 represents a list of assays used in the past and present diagnostic program. Some of these are primary test assays; others are secondary assays used to confirm a primary test result. For example, nepoviruses are assayed initially by biological indexing and a positive result is confirmed using PCR or ELISA. While an original specimen may have been infected with multiple viruses/viroids, some of these may not be present in subsequent propagations. As a result, discrepancies can arise between the test results. A diagnostic summary of a specimen, based on conventional testing, is the sum of the individual test results performed on the original plant and all the plants propagated from it.

dsRNA extraction and Illumina sequencing. Double stranded RNA (dsRNA) was extracted from leaf material (Kesanakurti et al. 2016) at two seasonal time points and combined, the first when the plants had just broken dormancy, and the second in late summer. This was to increase the chances of detecting viruses that may vary in titer seasonally (Roossinck 2010). Ten to 15 μl of each dsRNA extract was sent to the National Research Council, Saskatoon Laboratory for sequencing on a HiSeq 2500 (Illumina). Libraries were constructed using the TruSeq Stranded mRNA Kit (Illumina). Since the kit was designed for the construction of libraries from mRNA, the initial steps of the protocol were modified and 5 μl of dsRNA was added to 14.5 μl of fragment, primer, and finish mix instead of 19.5 μl as described in the original protocol. Paired-end 100 base reads were generated with 24 samples per lane. Two to 20 million reads were obtained for each sample, with the majority of samples consisting of 6 to 10 million reads.

Virus identification. Each sample was screened for viruses using Virtool (www.virtool.ca), an in-house developed sample manager that can run multiple diagnostic analysis workflows. Samples were given a unique identification number and scored for virus presence/absence independent of the conventional test results. Paired FASTQ files imported into Virtool were trimmed using Skewer 0.2.2+ (Jiang et al. 2014) and quality assessed using FastQC 0.11.5+ (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). A workflow, based on PathoScope (Hong et al. 2014) using Bowtie2 2.2.3+ (Langmead and Salzberg 2012) to align NGS reads to a custom database of plant virus sequences derived from GenBank, was used to screen for known viruses. PathoScope provides a framework to rapidly and accurately quantify the proportion of reads from an individual virus present in the NGS data (Hong et al. 2014). A significant advantage of using a read mapping approach compared with contig assembly is that fewer reads are required, increasing the sensitivity of the assay and minimizing the costs of sequencing (Visser et al. 2016). A virus species was considered positive for a sample if the number of matching reads was greater than 1,000 and/or coverage was greater than 15%. Novel viruses were identified using the workflow NuVs in Virtool. NGS sample reads were first screened for known virus and host sequences using Bowtie2 and discarded. Remaining reads were assembled into contigs using SPAdes 3.8+ (Bankevich et al. 2012) and viral coding regions in the contigs predicted using the vFAM resource (Skewes-Cox et al. 2014) and HMMER 3.1b2+ (Eddy 2011). Novel viral sequences were assembled into full genomes using CLCbio Genomics Workbench (Qiagen). Further analysis is in progress to determine the significance of these sequences (data not shown).

Results

Leaf samples from 178 fruit tree specimens from the CPH, Sidney Laboratory, virus infected plant repository, were collected in May and again in September. dsRNA was extracted from each sample and the May and September extracts were combined and submitted for sequencing. NGS results for each specimen are summarized in Table 1. A variety of plant species were selected including *Malus domestica*, *Prunus avium*, *P. persica*, *P. domestica*, and *Pyrus communis*, with data based on conventional testing methods (ELISA, PCR, and bioassays) included for comparison. In many cases, the plants used for conventional testing were no longer available or the plant propagation available for NGS testing had been incompletely tested using conventional methods. This made a direct comparison difficult in situations where results differed between the two data sets. If a result differed, the plant used for NGS testing was retested by an independent NGS or PCR test for confirmation unless noted otherwise. Exceptions were made when the two methods differed with respect to closely related viruses, or for viruses that were not tested for by conventional methods.

Viruses and/or viroids were detected in most of the samples. In total, over 531 viral and 21 viroid sequences were identified. Both RNA and DNA viruses could be detected from the dsRNA extracts. Many of the specimens were infected with more than one virus or viroid. In at least 50 specimens, multiple genotypes were observed for one or more of the infecting viruses. For example, in sample 103, at least 12 distinct genotypes of *Apple stem pitting virus* (ASPV) were detected. Viruses and viroids were detected by mapping sample NGS reads to reference genomic sequences from GenBank. In most cases, thousands of reads were obtained covering 50 to 100% of the virus or viroid genome. If only a small portion of the genome was covered, for example 2%, with hundreds or even thousands of reads, this could be attributed to a region in the genome of low complexity (e.g., an internal poly A region) and was not considered a positive result. A very low level of sample cross contamination was observed in the NGS data; to filter out this low level of contamination, mappings with less than 100 reads and/or less than 15% genomic coverage were considered to be negative.

Comparison of viruses/viroids/symptomology detected by conventional and NGS testing. Conventional testing identified 341 viruses/viroid/diseases, while with NGS there were 342 viruses/viroid detections in the same 178 specimens. Of these, 313 (over 90%) were positively identified by both methods. Results from conventional and NGS testing can be broken down into several categories: equivalent (conventional and NGS gave the same results for the virus/viroid species detected), similar (one or more virus detected by both conventional and NGS, but NGS provided a more accurate identification of the virus species, or the conventional testing was incomplete), analogous (discrepancy between conventional and NGS testing likely due to testing of different plant propagations), and

different (one or more viruses detected by conventional or NGS not detected by the other method).

The majority of specimens, 139 or 76%, gave equivalent test results from conventional and NGS methods (specimens #1 to 139). These included *Malus*, *Pyrus*, and *Prunus* species infected with 19 different virus and four viroid species. No viruses or viroids were detected by either method for eight of these specimens (#132 to 139), which were obtained as known positive controls, indicating that they were not infected with the expected virus, or that the virus had been lost during propagation. Since the results agreed between the two methods, no follow up testing was performed for these specimens.

Similar results were obtained for 22 specimens (#140 to 161, Table 1). These could be subdivided into two groups. In the first group (#140 to 154), both methods detected similar or closely related virus species. The improved genomic sequence resolution provided by NGS enabled a more accurate identification of the infecting virus(es) in the specimen. For example, the degenerate PCR primers, TriFoCap (Foissac et al. 2005), designed to amplify virus species from the family *Betaflexiviridae*, genera *Trichovirus*, *Foveavirus*, and *Capillovirus*, gave a positive result for #140, #141, and #173. NGS detected *Cherry mottle leaf virus* (CMLV) in #140 and #141 and *Cherry virus A* in #173, all members of the *Betaflexiviridae*. Likewise, the prunus specimens (#142 to 154) were found to be infected with one or more unclassified, but closely related, viruses of the *Betaflexiviridae*: *Cherry green ring mottle virus* (CGRMV), *Cherry necrotic rusty mottle virus* (CNRMV), *Cherry rusty mottle virus* (CRMV), *Cherry twisted leaf virus* (ChTLV) (Villamor et al. 2015, 2016), or a new betaflexivirus species identified in this study (#152 and #153). In the second group (samples #155 to 161), a virus or viroid was detected by NGS and not by conventional testing. In most cases, conventional testing was incomplete on these specimens leading to the discrepancy. As mentioned previously, not all plants in the repository have undergone the full range of conventional testing. For most of these specimens, the virus or viroid identified by NGS was confirmed by a second NGS and/or PCR test. For #153 and #158, a recently identified *Prunus virus F* (PrVF) (Villamor et al. 2017) and geminivirus, respectively, were found by NGS. A positive bioassay result was obtained for the specimen infected with the geminivirus and a possible correlation between symptoms and the presence of this virus is ongoing. *Cherry leaf roll virus* (CLRV), a nepovirus, was identified in #153 by bioassay, and could not be detected by NGS, but PrVF was found associated with this specimen by NGS. Confirmatory tests for CLRV and pathogenicity studies need to be carried out to determine if PrVF or CLRV is the etiological agent in this case.

Discrepancies were also observed for specimens #162 to 170. However, since different plants were used for the conventional and NGS methods, the differences could be due to uneven virus distribution during plant propagation, which resulted in the observed differences. This is not unexpected since during graft inoculation, not all viruses are transferred equally from the graft infected material to the new host plant. In most cases, the propagation was retested using NGS and/or a specific PCR to confirm the presence or absence of the virus or viroid in the NGS tested propagation.

Differing results were obtained for specific viruses in #171 to 178. For #171 and #172, a bioassay suggesting necrotic rusty mottle disease and prune dwarf disease symptoms, respectively, could not be confirmed in NGS and second NGS test; CVA and ChTLV were detected and confirmed by PCR. For #173, conventional and NGS detected *Asian prunus virus* (APV), *Plum bark necrosis stem pitting associated virus* (PBNSPaV), and NGS identified CVA was also detected using the TriFoCap PCR assay. NGS also detected *Tomato ringspot virus* (ToRSV), which was not detected by conventional methods, but was detected using a confirmatory PCR test for ToRSV following NGS. For #174, PBNSPaV was not detected by conventional testing, but was detected by both an initial and confirmatory NGS test. For #175, PDV was detected by conventional PCR but could not be detected with either an initial or confirmatory NGS test. For #176 and #177, NGS detected a novel tymo- and a novel leuteovirus, which were not detected by conventional testing. Finally, sample #178 was unique in that CVA was detected by NGS but not detected by conventional methods. A confirmatory PCR test to CVA was negative.

In total, 26 viruses and four viroid species were identified by NGS. Most of the viruses are single stranded RNA virus from the families *Bromoviridae*, *Betaflexiviridae*, *Closteroviridae*, *Leutoviridae*, *Potyviridae*, *Secoviridae*, and *Tymoviridae*, with several double stranded DNA viruses from the *Caulimoviridae* and *Totiviridae* families and a DNA virus of the *Geminiviridae*. Viroids from both the *Pospiviroidae* and *Avsunviroidae* families were detected.

Discussion

Conventional testing of fruits trees for virus and virus-like disease is an onerous proposition due to the various and lengthy testing procedures required. A rapid and cost effective method with equal or better sensitivity that could replace most or all conventional methods would be greatly beneficial. In this study, we were interested in comparing virus/viroid test results for infected plant specimens obtained by conventional testing to those obtained more recently by NGS. To do this, one plant per specimen was tested by NGS and the results compared with the range of conventional test procedures performed on multiple plants. It was expected that for some specimens, the results may not match for one of several possible reasons: 1) Incomplete transmission of viruses/viroids by grafting to new propagation, 2) incomplete or nonspecific virus/viroid testing by conventional methods, and 3) new virus discovery by NGS, not detectable by conventional methods. All three instances were observed, at times in combination for a particular specimen. Since it wasn't possible to validate a different result obtained from two different plants propagations if one of them was no longer available, any difference observed in this case was assumed to be due to first reason above. When results due to 1 and 2 above are eliminated from the comparison, very few differences were observed between the conventional and NGS testing. Comparable results were obtained from 170 of the 178 specimens or 96%. CNRMV and PDV suspected in #171 and #172 based on biological indexing, which had not been confirmed using another method, tested negative by NGS. More than likely, completion of all conventional testing would have concluded neither virus was present. ToRSV (#173) and PBNSPaV (#174) tested negative by conventional and positive by both NGS and a confirmatory PCR test, PDV (#175) tested positive by conventional and negative by both NGS and a confirmatory PCR test, and a novel tymovirus (#176) and leuteovirus (#177) positive by both NGS and confirmatory test was negative by conventional methods. These are the few instances where NGS provided a result that was distinctly different than obtained by conventional testing. The other major difference was the detection of CVA (#178) by NGS, which could not be confirmed by PCR. For NGS, one false positive and zero false negatives were confirmed and for conventional testing, one false positive and four false negatives out of over 340 viruses/viroids were confirmed.

Based on the results, NGS can be considered a direct equivalent to conventional testing with essentially 0% false negatives. A false positive, while less serious than a false negative, is still a concern and is an issue with NGS. We suggest that for NGS to be used as a diagnostic tool, the potential for false positives be recognized, and procedures implemented to confirm positive results by a secondary method using a fresh sample extract. This is already a common practice in diagnostic testing. While we used a cut off of greater than 100 reads and/or a coverage of greater than 15% to determine a positive result by NGS, these are somewhat arbitrary numbers designed to filter out low level sample cross contamination and could be reduced with better hygienic sample handling measures throughout the NGS procedure.

For a routine testing laboratory, NGS has some clear advantages. Compared with conventional testing, it is more rapid and more samples can be processed in the same amount of time. While some of the conventional testing used for the specimens in this study can take years to complete, NGS testing and analysis can be completed in weeks or months. A single NGS sample is required, compared with

Table 1. Conventional and NGS test results[a]

Specimin no.	Virus	Con	NGS	Confirm	R	Comments
1	PDV	√	√	N/A	E	
2	RRSV	√	√	N/A	E	
3	RRSV, ACLSV	√	√	N/A	E	
4	LChV-2, CVA	√	√	N/A	E	NGS: 2 LChV-2 and 2 CVA genotypes
5	CNRMV, CVA	√	√	N/A	E	
6	CVA, CGRMV	√	√	N/A	E	
7	PNRSV	√	√	N/A	E	
8	LChV-1	√	√	N/A	E	
9	CVA	√	√	N/A	E	NGS: 3+ CVA genotypes
10	PDV	√	√	N/A	E	
11	CMLV, PDV	√	√	N/A	E	
12	CRMV, CVA, PDV	√	√	N/A	E	NGS: 2 CVA genotypes
13	CRMV	√	√	N/A	E	
14	PLMVd	√	√	N/A	E	
15	PLMVd, ACLSV	√	√	N/A	E	
16	PLMVd	√	√	N/A	E	
17	PNRSV	√	√	N/A	E	
18	PNRSV	√	√	N/A	E	
19	PLMVd	√	√	N/A	E	
20	ACLSV	√	√	N/A	E	
21	PLMVd, CNRMV	√	√	N/A	E	
22	PLMVd	√	√	N/A	E	
23	PLMVd	√	√	N/A	E	
24	PLMVd, PcMV	√	√	N/A	E	
25	CVA	√	√	N/A	E	
26	PNRSV, GRMV, PDV, ACLSV, CVA	√	√	N/A	E	
27	PDV, PNRSV	√	√	N/A	E	
28	CGRMV, CVA, PNRSV, CMLV	√	√	N/A	E	NGS: 2 CVA genotypes
29	ACLSV	√	√	N/A	E	
30	PDV, PNRSV	√	√	N/A	E	
31	CVA, CNRMV, PDV	√	√	N/A	E	
32	PLMVd	√	√	N/A	E	
33	PLMVd	√	√	N/A	E	
34	PDV, PNRSV	√	√	N/A	E	
35	PDV, PNRSV	√	√	N/A	E	
36	APV	√	√	N/A	E	NGS: 2 APV genotypes
37	ToRSV	√	√	N/A	E	
38	PcMV	√	√	N/A	E	
39	PcMV	√	√	N/A	E	
40	PcMV	√	√	N/A	E	
41	PcMV	√	√	N/A	E	
42	PLMVd	√	√	N/A	E	
43	CRMV	√	√	N/A	E	
44	CVA, CGRMV, LCHV-2	√	√	N/A	E	NGS: 2 LCHV-2 genotypes
45	CVA, CGRMV	√	√	N/A	E	NGS: 3 CVA genotypes
46	CVA, LCHV-2	√	√	N/A	E	NGS: 2+ LCHV-2 and 3 CVA genotypes
47	ACLSV	√	√	N/A	E	
48	ACLSV	√	√	N/A	E	NGS: multiple ACLSV genotypes
49	CNRMV, CVA	√	√	N/A	E	
50	CNRMV, PNRSV, CVA	√	√	N/A	E	NGS: 2 CVA and 3 PNRSV genotypes
51	CVA	√	√	N/A	E	
52	LCHV-1, ACLSV	√	√	N/A	E	
53	CNRMV, CVA, PDV	√	√	N/A	E	NGS: 2 CNRMV and 3 PDV genotypes
54	PNRSV, CVA	√	√	N/A	E	
55	CRMV, LCHV-1	√	√	N/A	E	

(*continued on next page*)

[a] Con = conventional testing, NGS = next generation sequencing testing, Confirm = confirmatory testing of plant used for NGS testing, R = result, √ = positive test, X = negative test, Bio+= tested positive by bioassay, TriFoCap = positive by universal PCR assay for tricorna-, fovea-, and capilloviruses, sus = suspicious test from ELISA or PCR, NT = not tested, E = equivalent, S = similar, A = analogous, D = different. NGS(+/-) indicates that a follow up NGS test was either positive or negative, PCR(+/-) indicates that a follow up PCR test was either positive or negative, N/A = not analyzed further. If a comment refers to NGS or conventional testing, it is indicated as such. Number of identified species variants within a specimen are indicated in the Comments section. Under comparison, identical refers to specimens that tested positive by conventional and NGS methods to the same viruses. Abbreviations: American plum line pattern virus (APLPV), Apple chlorotic leaf spot virus (ACLSV), Apple dimple fruit viroid (ADFVd), Apple scar skin viroid (ASSVd), Apple stem grooving virus (ASGV), Apple stem pitting virus (ASPV), Apple mosaic virus (ApMV), Arabis mosaic virus (ArMV), Asian prunus virus 1 (APV1), Asian prunus virus 2 (APV-2), Cherry virus A (CVA), Cherry green ring mottle virus (CGRMV), Cherry mottle leaf virus (CMLV), Cherry rasp leaf virus (CRLV), Cherry necrotic rusty mottle virus (CNRMV), Cherry rusty mottle virus (CRMV), Cherry twisted leaf virus (ChTLV), Dapple apple viroid (DAVd), Hop stunt viroid (HSVd), Little cherry virus 1 (LChV-1), Little cherry virus 2 (LChV-2), Peach latent mosaic viroid (PLMVd), Peach mosaic virus (PcMV), Plum pox virus (PPV), Plum bark necrosis stem pitting associated virus (PBNSPaV), Prune dwarf virus (PDV), Prunus necrotic ringspot virus (PNRSV), Prunus virus F (PrVF), Quince yellow mosaic (QYM), Tomato blackring virus (TBRV), Tomato ringspot virus (ToRSV), Raspberry ringspot virus (RRSV).

Table 1. (*continued from preceding page*)

Specimin no.	Virus	Con	NGS	Confirm	R	Comments
56	CNRMV, CVA	√	√	N/A	E	NGS: 2 CVA genotypes
57	PNRSV, CVA, LCHV-1	√	√	N/A	E	
58	CMLV	√	√	N/A	E	
59	CMLV	√	√	N/A	E	
60	CGRMV, PDV, CVA	√	√	N/A	E	
61	CGRMV	√	√	N/A	E	
62	PNRSV, PDV	√	√	N/A	E	
63	CNRMV, CVA	√	√	N/A	E	NGS: 2+ CVA genotypes
64	LCHV-1, CVA	√	√	N/A	E	
65	CVA	√	√	N/A	E	
66	PPV	√	√	N/A	E	
67	CVA	√	√	N/A	E	
68	CVA	√	√	N/A	E	
69	LCHV-1, CVA	√	√	N/A	E	NGS: 3 CVA genotypes
70	PNRSV, PDV	√	√	N/A	E	
71	LChV-1, LChV-2, PNRSV, PDV, ACLSV, CVA, CGRMV	√√	√√	N/AN/A	E	NGS: 2 LChV1 3 PNRSV, 3 PDV and 4 CVA genotypes
72	ACLSV, ASPV	√	√	N/A	E	NGS: 2 ASPV and 2 ACLSV genotypes
73	ASGV, ASPV	√	√	N/A	E	
74	ASPV, ASGV, ACLSV	√	√	N/A	E	NGS: 2 ASGV and 2 ASPV genotypes
75	ASPV, ACLSV	√	√	N/A	E	
76	ASGV, ASPV, ACLSV	√	√	N/A	E	NGS: 9 ASPV and 2+ ACLSV genotypes
77	ASPV, ACLSV	√	√	N/A	E	NGS: 6+ ASPV genotypes
78	ACLSV	√	√	N/A	E	
79	ACLSV	√	√	N/A	E	
80	ASGV, ASPV, ACLSV	√	√	N/A	E	
81	ASPV, ACLSV	√	√	N/A	E	NGS: 6+ ASPV and 2+ ACLSV genotypes
82	ACLSV, ASPV	√	√	N/A	E	
83	ASPV, ACLSV	√	√	N/A	E	NGS: 4+ ASPV and 5+ ACLSV genotypes
84	ASPV, ACLSV	√	√	N/A	E	NGS: 2+ ASPV and 2+ ACLSV genotypes
85	ACLSV, ASGV, ASPV, ApMV	√	√	N/A	E	NGS: 2+ ASPV genotypes
86	ASPV, ACLSV	√	√	N/A	E	
87	ACLSV, ASPV	√	√	N/A	E	NGS: 3 ACLSV and 5 ASPV genotypes
88	ACLSV, ASPV	√	√	N/A	E	NGS: 3 ASPV genotypes
89	ASPV	√	√	N/A	E	
90	ASGV	√	√	N/A	E	
91	ACLSV, ApMV, ASGV, ASPV	√	√	N/A	E	NGS: 2 ASPV genotypes
92	ASGV	√	√	N/A	E	NGS: 2 ASGV genotypes
93	ASPV, ASGV	√	√	N/A	E	NGS: 2 ASPV and 3 ASGV genotypes
94	ASGV	√	√	N/A	E	NGS: 2 ASGV genotypes
95	ASPV	√	√	N/A	E	NGS: 6 ASPV genotypes
96	ACLSV	√	√	N/A	E	
97	PPV	√	√	N/A	E	
98	PPV	√	√	N/A	E	
99	PPV	√	√	N/A	E	
100	ACLSV, ASPV	√	√	N/A	E	NGS: 3 ACLSV and 9+ ASPV genotypes
101	ACLSV	√	√	N/A	E	
102	ASGV, ACLSV, ASPV	√	√	N/A	E	NGS: 2 ACLSV and 8+ ASPV genotypes
103	ASGV, ACLSV, ASPV	√	√	N/A	E	NGS: 12+ ASPV genotypes
104	ASGV, ACLSV, ASPV	√	√	N/A	E	NGS: 5+ ASPV genotypes
105	ACLSV, ASPV	√	√	N/A	E	NGS: 2 ACLSV and 8 ASPV genotypes
106	ASPV	√	√	N/A	E	
107	ASGV, ASPV	√	√	N/A	E	
108	ASGV, ACLSV	√	√	N/A	E	
109	ASPV	√	√	N/A	E	
110	ASGV	√	√	N/A	E	
111	ACLSV, ASPV	√	√	N/A	E	NGS: 3 ASPV genotypes
112	ACLSV, ASPV	√	√	N/A	E	NGS: 2 ACLSV and 9 ASPV genotypes
113	ACLSV	√	√	N/A	E	
114	ADFVd	√	√	N/A	E	
115	ADFVd	√	√	N/A	E	
116	ACLSV, ASPV	√	√	N/A	E	NGS: 7 ASPV genotypes
117	ALSV, ASPV, ACLSV, ASGV	√	√	N/A	E	
118	ACLSV, ASPV	√	√	N/A	E	NGS: 2 ACLSV and 5+ ASPV genotypes

(*continued on next page*)

Table 1. (*continued from preceding page*)

Specimin no.	Virus	Con	NGS	Confirm	R	Comments
119	ADFVd	√	√	N/A	E	
120	ACLSV, ASGV, ASPV	√	√	N/A	E	
121	ACLSV, ASPV	√	√	N/A	E	
122	AFCVd	√	√	N/A	E	
123	PBCVd	√	√	N/A	E	
124	CNRMV, PNRSV, CVA, LCHV-1,	√	√	N/A	E	NGS: 2 CVA genotypes
125	ACLSV, ASPV	√	√	N/A	E	NGS: 2 ACLSV and 7+ ASPV genotypes
126	ACLSV, ASPV, ASGV, ApMV	√	√	N/A	E	NGS: 4+ ACLSV and 6+ ASPV genotypes
127	ADFVd	√	√	N/A	E	
128	ASPV, ASGV, ACLSV, ASSVd	√	√	N/A	E	
129	CVA, ACLSV, CGRMV, PNRSV, PDV, CRLV	√√	√√	N/AN/A	E	
130	PBNSPaV, APV, PLMVd, ACLSV	√	√	N/A	E	NGS: detected both APV1 and APV2
131	LChV-1, CVA	√	√	N/A	E	NGS: 2 CVA genotypes
132	PLMVd	X	X	N/A	E	Obtained by CPH as pos control for PLMVd
133	PDV	X	X	N/A	E	Obtained by CPH as pos control for PDV
134	QYM	X	X	N/A	E	Obtained by CPH as pos control for QYM
135	QYM	X	X	N/A	E	Obtained by CPH as pos control for QYM
136	QYM	X	X	N/A	E	Obtained by CPH as pos control for QYM
137	QYM	X	X	N/A	E	Obtained by CPH as pos control for QYM
138	PDV, CLRV	X	X	N/A	E	Obtained by CPH as pos control for PDV and CLRV
139	ACLSV	X	X	N/A	E	Obtained by CPH as a positive control for ACLSV
140	CMLV	TriFoCap	√	N/A	S	Conventional: TriFoCap primers are positive for CMLV
141	CMLV	TriFoCap	√	N/A	S	Conventional: TriFoCap primers are positive for CMLV
142	CRMVCNRMV, ChTLV	Bio+Bio+	√	N/A	S	Conventional: ChTLV/CRMV/CNRMV can cross react
			X			NGS: more accurate species identification
143	CMLV, PDV, CGRMV	√	√	N/A	S	Conventional: Suspicious symptoms from bioassay, CRMV/CGRMV can cross react
	CRMV	Bio+	X			
144	CRMV	√	√	N/A	S	Conventional: CHTLV/CRMV/CNRMV can cross react
	CNRMV	√	X			NGS: more accurate species identification
	ChTLV	Bio+	X			
145	CVA, CGRMV	√	√	N/A	S	Conventional: CNRMV/CGRMV can cross react
	CNRMV	sus	X			NGS: more accurate species identification
146	CNRMV	√	X	N/A	S	Conventional: CNRMV/CRMV can cross react
	CRMV	X	√			NGS: more accurate identification of CRMV
147	CVA	√	√	N/A	S	Conventional: CGRMV/CNRMV/ CRMV can cross react
	CGRMV, CMLV, CNRMV	√	X			NGS: more accurate species identification
	CRMV	X	√			NGS: 5 CVA genotypes
148	PDV, CVA, CNRMV, ACLSV	√	√	N/A	S	NGS: 3 CVA genotypes
	CGRMV	√	X			Conventional: CNRMV/CGRMV can cross react
149	PNRSV, CGRMV, ACLSV, LChV-1	√	√	N/A	S	Conventional: CGRMV/CNRMV can cross react
	CNRMV	X	√			
150	CGRMV, PDV, PNRSV, CVA, CMLV	√	√	N/A	S	Conventional: CGRMV/CNRMV can cross react
	CNRMV	X	√			
151	CVA, ACLSV, LChV-1	√	√	N/A	S	NGS: 2 ACLSV genotypes
	CRMV	√	X			Conventional: CRMV related to new betaflexivirus and could cross react
	novel betaflexivirus	X	√			
152	CVA, ACLSV, LCHV-1	√	√	N/A	S	NGS: 2 ACLSV genotypes
	CRMV	√	X			Conventional: CRMV related to new betaflexivirus and could cross react
	novel betaflexivirus	X	√			
153	PDV, CVA	√	√	N/A	S	NGS: 2 CVA genotypes
	CLRV	Bio+	X			PrVF and CLRV members of Secoviridae

(*continued on next page*)

Table 1. (*continued from preceding page*)

Specimin no.	Virus	Con	NGS	Confirm	R	Comments
	PrVF	NT	√			
154	CGRMV	√	√		S	NGS: 3+ CNRMV genotypes
	CMLV	√	√			Conventional: CGRMV and CNRMV can cross react
	CNRMV	NT	√			Propagation tested by NGS was not initially tested for CNRMV by conventional methods.
	CVA	NT	√			
155	CMLV	√	√	N/A	S	Conventional: not initially tested for PLMVd
	PLMVd	NT	√			
156	PcMV	√	√		S	Conventional: not initially tested for ACLSV
	ACLSV	NT	√	PCR(+)		
157	LChV-2	√	√	N/A	S	Conventional: not initially tested for PLMVd
	PLMVd	NT	√			
158	CVA	NT	√	PCR, NGS(+)	S	Conventional: not initially tested for CVA
	novel geminivirus	Bio+	√	NGS(+)		
159	PPV, CVA	√	√	NGS (+)	S	Conventional: not initially tested for LChV-1
	LCHV-1	NT	√	NGS(+)		
160	CGRMV, CVA, PNRSV, CNRMV	√	√		S	Conventional: not initially tested for PDV
	PDV	NT	√	PCR(+PDV)		
161	LChV-1	NT	√	NGS(+)	S	Conventional: not initially tested for LChV-1
162	CVA, LChV-2, APLPV	√	√	NGS(+)	A	Conventional: not initially tested for LChV-1
	LChV-1	NT	√	NGS(+)		Different propagations tested by conventional versus NGS
	CNRMV	Bio+	X	NGS, PCR (-)		
163	TBRV	sus	X	NGS (-)	A	Propagation tested by NGS was not initially tested for PLMVd.
	PLMVd	NT	√	NGS(+)		Different propagations tested by conventional versus NGS
164	PPV	√	√	N/A	A	Different propagations tested by conventional versus NGS
	PLMVd	√	X	N/A		
165	CRMV	Bio+	X	NGS(-)	A	Different propagations tested by conventional versus NGS
	CVA	√	X	NGS(-)		
166	PLMVd	√	X	N/A	A	Different propagations tested by conventional versus NGS
167	CVA, CGRMV	√	X	PCR(-CVA) NGS(-all)	A	Different propagations tested by conventional versus NGS
168	PNRSV, PLMVd	√	X	NGS(-)	A	Different propagations tested by conventional versus NGS
169	ACLSV	√	√		A	Different propagations tested by conventional versus NGS
	ASPV	√	X	PCR(-)		
170	ASGV	√	√	NGS(+)	A	Different propagations tested by conventional versus d NGS
	ACLSV	√	X	NGS(-)		
171	CVA	√	√	NGS(+)	D	NGS: multiple CVA genotypes
	CNRMV	Bio +	X	NGS(-)		Conventional: Suspicious symptoms from bioassay
172	ChTLV	√	√	NGS(+)	D	Conventional: CNRMV/CGRMV can cross react
	PDV, CNRMV	Bio +	X	NGS(-)		Conventional: Suspicious symptoms from bioassay
173	APV	√	√		D	Propagation tested by NGS was not initially tested for CVA
	PBNSPaV	sus	√			
	CVA	TriFoCap	√			
	ToRSV	X	√			
174	ACLSV, CGRMV, CNRMV	√	√	NGS(+)	D	NGS: 2 PBNSPaV genotypes
	PBNSPaV	X	√	NGS(+)		
175	LCHV-2, CVA, ACLSV	√	√	NGS(+)	D	NGS: 3 CVA genotypes
	PDV	√	X	NGS(-)		
176	CNRMV, PPV	√	√	NGS(+)	D	
	novel tymovirus	X	√	NGS(+)		
177	PLMVd	√	√	NGS(+)	D	Conventional: not initially tested for TBRV
	TBRV	NT	√	NGS(+)		
	novel leuteovirus	X	√	NGS(+)		
178	PPV	√	√		D	Conventional: not initially tested for PNRSV, CVA, LChV-1 or ACLSV
	PNRSV, LChV-1, ACLSV	NT	√	PCR(+)		
	CVA	NT	√	PCR(-)		

Table 2. Test methods used for viruses, viroids, and suspected virus diseases of *Malus*, *Prunus*, and *Pyrus*

Virus species/disease	Host	Test method			
		ELISA	PCR	Woody bioassay[a]	Herbaceous bioassay[a]
American plum line pattern virus	*Prunus*			Shiro plum	*C. sativus*
Apple chat fruit	*Malus*			Lord Lambourne	
Apple chlorotic leafspot virus	*Malus*	X[b]	X[c]	R 12740 7A	*C. amaranticolor*
	Prunus	X[b]	X[c]	Spy 227	*N. occidentalis 37B*
	Pyrus	X[b]	X[c]	GF 305	*N. occidentalis obliqua*
				P. tomentosa	*C. quinoa*
				P. veitchii	
Apple dimple fruit viroid	*Malus*		X[d]		
Apple fruit crinkle viroid	*Malus*		X[e]		
Apple green crinkle viroid	*Malus*		X[f]	Cox's Orange Pippin	
				Golden Delicious	
				Granny Smith	
Apple leaf pucker	*Malus*			Golden Delicious	
				Spartan	
Apple latent spherical virus	*Malus*		X[g]		*C. quinoa*
Apple mosaic virus	*Malus*	X[h]	X[c]	Golden Delicious	
	Prunus	X[h]	X[c]	Lord Lambourne	
				GF 305	
Apple rasp leaf	*Malus*			Golden Delicious	
Apple ringspot	*Malus*			Cox's Orange Pippin	
				Golden Delicious	
				Granny Smith	
Apple rough skin	*Malus*			Golden Delicious	
Apple rubbery wood	*Malus*			Lord Lambourne	
	Pyrus			Lord Lambourne	
Apple scar skin viroid	*Malus*		X[d]	Lord Lambourne	
	Pyrus		X[d]	Spartan	
				Lord Lambourne	
Apple stem grooving virus	*Malus*		X[c,i]	Virginia Crab	*C. amaranticolor*
	Pyrus		X[c,i]	*P. veitchii*	*N. occidentalis 37B*
				Virginia Crab	*N. occidentalis obliqua*
					C. quinoa
Apple stem pitting virus	*Malus*		X[c]	Spy 227	*N. occidentalis 37B*
	Pyrus			Virginia Crab	*N. occidentalis obliqua*
				Jules D'Airolles	
				P. veitchii	
				Virginia Crab	
Apricot latent virus	*Prunus*		X[i,j]		
Apricot leaf pucker	*Prunus*			Tilton	
Arabis mosaic virus	*Malus*	X[b]	X		*C. amaranticolor*
	Pyrus	X	X	GF 305	*C. quinoa*
	Prunus	X[b]		Bing	*N. benthamiana*
Asian prunus virus	*Prunus*		X[i,k]		
Bark split	*Pyrus*			Bartlett	
Cherry albino	*Prunus*			Lambert	

(*continued on next page*)

[a] Thompson et al. 2011. Pages 299-302 in Virus and Virus-Like Diseases of Pome and Stone Fruits, APS Press, St. Paul, MN.
[b] Bioreba.
[c] Menzel et al. 2002. J. Virol. Meth. 99:81-92.
[d] Di Serio et al. 2002. J. Plant Pathol. 84:27-34.
[e] In house developed assay.
[f] James et al. 2013. Arch. Virol. 158:1877-1887.
[g] Yoshikawa et al. 2006. Arch Virol. 151:837-848.
[h] Agdia.
[i] Foissac et al. 2005. Phytopathology 95:617-625.
[j] Nemchinov et al. 2000. Arch. Virol. 145:1801-1813.
[k] Marais et al. 2004. Acta Hortic. 657:87-92.
[l] Bertolini et al. 2001. J. Virol. Meth. 96:33-41.
[m] James et al. 2000. Arch. Virol. 145:995-1007.
[n] James et al. 2001. Plant Dis. 85:47-85.
[o] Astruc et al. 1996. Eur. J. Plant Pathol. 102:837-846.
[p] Vitushkina et al. 1997. Eur. J. Plant Pathol. 103:803-808.
[q] Rott and Jelkmann 2001. Phytopathology 91:261-297.
[r] James et al. 2006. Phytopathology 96:137-143.
[s] Hernandez et al. 1992. J. Gen. Virol. 73:2503-2507.
[t] Rwahnih et al. 2007. Arch. Virol. 152:2197-2206.
[u] Plant Print Diagnostics.
[v] Agritest.
[w] Durviz.
[x] Candresse et al. 1995. Acta Hortic. 386:357-369.
[y] Ochoa-Corona et al. 2005. Page 259 in: 15th Biennial Australasian Plant Pathology Society Conference Handbook.
[z] Phyto Diagnostics.
[aa] Griesbach 1995. Plant Dis. 79:1054-1056.

Table 2. (*continued from preceding page*)

Virus species/disease	Host	Test method			
		ELISA	PCR	Woody bioassay[a]	Herbaceous bioassay[a]
Cherry green ring mottle virus	*Prunus*		X[e,i]	Kwanzan	
				P. tomentosa	
Cherry leafroll virus	*Prunus*		X[l]	GF 305	*C. quinoa*
				Bing	*C. amaranticolor*
					N. occidentalis 37B
					N. benthamiana
Cherry mottle leaf virus	*Prunus*	X[e]	X[i,m]	GF 305	*C. quinoa*
				Bing	
Cherry necrotic rusty mottle virus	*Prunus*		X[e,i]	Bing	
				Lambert	
				Sam	
Cherry rasp leaf virus	*Prunus*		X[n]	Bing	*C. quinoa*
					C. amaranticolor
					N. occidentalis 37B
					N. benthamiana
Cherry rusty mottle virus	Prunus		X[e,i]	Bing	
				Lambert	
				Sam	
Cherry short stem	*Prunus*			Bing	
				Lambert	
Cherry twisted leaf virus	*Prunus*		X[e,i]	Bing	
				Tilton	
Cherry virus A	*Prunus*		X[e]		
Flat Limb	*Malus*			Gravenstein	
Freckle pit	*Pyrus*			Bosc	
Hop stunt viroid	*Prunus*		X[o]		
Little cherry virus-1	*Prunus*		X[p]	Sam	
Little cherry virus-2	*Prunus*	X[e]	X[q]	Sam	
				Tilton	
Myrobalan latent virus	*Prunus*			GF 305	*C. quinoa*
					C. amaranticolor
					N. occidentalis 37B
					N. benthamiana
Peach asteroid spot	*Prunus*			GF 305	
Peach latent mosaic viroid	*Prunus*		X[e]	GF 305	
Peach mosaic virus	*Prunus*		X[r]	GF 305	
Pear blister canker viroid	*Pyrus*		X[s]	Bartlett	
Plum bark necrosis stem pitting virus	*Prunus*		X[t]		
Plum pox virus	*Prunus*	X[u,v,w]	X[x]	GF 305	*N. benthamiana*
				P. tomentosa	
Prune dwarf virus	*Prunus*	X[b,h]		GF 305	*C. sativus*
				P. tomentosa	
Prunus necrotic ringspot virus	*Prunus*	X[b]		GF 305	*C. quinoa*
				P. tomentosa	*C. sativus*
Raspberry ringspot virus	*Malus*	X[b]	X[y]		*C. amaranticolor*
				Bing	*C. quinoa*
	Prunus	X[b]	X[y]	GF 305	*N. benthamiana*
Rough bark	*Pyrus*			Bartlett	
Russet ring	*Malus*			Spartan	
				Golden Delicious	
Russet wart	*Malus*			Cox's Orange Pippin	
				Golden Delicious	
Star crack	*Malus*			Cox's Orange Pippin	
				Golden Delicious	
Strawberry latent ringspot virus	*Malus*	X[b]		Bing	*C. amaranticolor*
	Prunus	X[b]		GF 305	*C. quinoa*
Stock prune	*Prunus*			GF 305	
Stony pit	*Pyrus*			Bosc	
Tobacco ringspot virus	*Prunus*		X[e]	GF 305	*C. quinoa*
					C. amaranticolor
					N. occidentalis 37B
					N. benthamiana
Tomato blackring virus	*Malus*	X[b]	X[e]		*C. amaranticolor*
				GF 305	*C. quinoa*
	Prunus	X[b]	X[e]	Bing	*N. benthamiana*
Tomato bushy stunt virus	*Prunus*			Bing	*C. quinoa*
					C. sativus
Tomato ringspot virus	*Malus*	X[h,z]	X[aa]		*C. amaranticolor*
				GF 305	*C. quinoa*
	Prunus		X[aa]	*P. tomentosa*	*N. benthamiana*

multiple samples required to perform the many comparable conventional test methods, reducing material and personnel costs significantly per specimen tested. For practical diagnostic laboratory testing, this can be very significant. Extrapolating the results from the positive control specimens used in this study to samples, discrepancies can be avoided between conventional testing of multiple plant propagations, if NGS were to be used directly on the sample submitted for testing without the need for multiple propagations to be generated. Maintaining infected control material through the process of repropagation would also be greatly simplified using NGS to monitor complete virus/viroid transmission. It remains to be determined if NGS can be used to test the original source plant specimen directly as opposed to testing of plant propagations from a specimen, or if one sample point is sufficient for the detection of all viruses/viroids. NGS does require new levels of training for diagnostic staff. NGS library construction and sequencing requires additional equipment and training, or it can be outsourced to one of many laboratories providing this service. Data interpretation is critical and workflows such as Virtool and others help to simplify this process and make it more readily available.

NGS was able to provide a more accurate species identification for 19 specimens (#140 to #154, #172, #173, #177, and #178). Noteworthy among these was the identification of three new virus species, a betaflexivirus related to CGRMV, CNRMV, CRMV, and ChTLV that cross reacts with these viruses by biological indexing (#151 and #152), a geminivirus that produced novel symptoms on an herbaceous indicator (#158), and specimen #153, where a novel virus was detected that has recently been characterized as *Prunus virus F* (PrVF). In these situations, while conventional testing was unable to identify the correct virus species, a disease was detected. In a diagnostic setting, this specimen would be noted as infected and treated accordingly. In two specimens, NGS was also able to detect a novel tymovirus (#177) and leuteovirus (#178). These were obtained as positive controls for PPV and were not submitted for full range conventional testing. These results highlight the ability of NGS to more easily detect novel viruses. While it is possible that NGS is a better tool for the discovery of novel plant viruses, the initial detection of a novel virus-like sequence should be considered the start of further characterization studies, including transmission, host range, and pathology.

The novel viruses mentioned above can be graft transmitted to a healthy host, indicating that they are infectious agents of plants (data not shown). This is in contrast to several other viruses also detected by NGS, including toti-, birna-, and reoviruses. Totiviruses are dsRNA viruses belonging to the *Totiviridae* with fungi serving as natural hosts. Almost half of the 178 samples that were tested by NGS were also positive for a reovirus and or birnavirus. BLAST analysis indicates they are most closely related to insect virus species and were associated with both *Malus* and *Prunus* species. None of these viruses could be graft transmitted to a new host and are likely not plant infecting viruses (data not shown). It is likely that these viruses were detected from fungal and insect contamination of the collected leaf material. These results demonstrate not only the very high sensitivity of NGS for virus detection but also the potential pitfalls for using NGS as a diagnostic method. In particular, when a novel viral genomic sequence is detected, it is important to determine whether the virus is an infectious plant virus, a contaminant, or associated with another organism in association with the plant. While the toti-, reo-, and birnaviruses are obvious examples, an overanalysis of an NGS data set from any plant sample in this study will generate small fragments of virus-like sequences (data not shown). Most of these are presumably host plant genome contaminants. Plant RNA viruses contain conserved genes for proteins, e.g., RNA-dependent RNA polymerase, putative RNA helicase, chymotrypsin-like and/or papain-like proteases, and methyltransferases (Koonin and Dolja 1993), which are also homologous to genes of the host plant genome. It is important not to confuse these for a plant virus by using stringent bioinformatic analysis and experimental confirmation. As mentioned, some of these sequences may be from viruses associated with the plant but are not infectious of the plant itself. This brings up a further interesting point. Infectivity of a virus is defined by its ability to move between different cells of the host. It has been demonstrated that some insect viruses can replicate within a plant cell, but are noninfectious (Selling et al. 1990). Therefore, it is possible that some of these low frequency, virus-like fragments are insect viruses that have been transferred to isolated plant cells during the course of feeding, and may be replicating in individual plant cells but are not able to move from cell to cell.

Fifty samples were infected with multiple virus species variants indicated in the comments section of Table 1. For example, in #78, nine distinct sequence variants of ASPV could be identified and at least two ACLSV sequences. An advantage to dsRNA as a starting material for NGS is the high number of viral specific reads that can be obtained. Combined with a large number of paired-ended reads, it is easier to assemble and identify multiple sequence variants. However, in many cases it wasn't possible to identify the true number of variants, which is indicated with a "+" after the number of confirmed variants in Table 1. Several viruses, such as ASPV and ACLSV, were particularly prone to the presence of multiple variants. ASPV is a foveavirus and ACLSV is a trichovirus, both members of the *Betaflexiviridae*, infecting *Malus*. Whether these viruses are more prone to generating variants or this is an artifact of normal apple propagation practices is unclear.

The majority of plant viruses have RNA genomes (Murphy et al. 1995). Recently Basso et al. (2015) identified a group of highly divergent circular single stranded (ssDNA) viruses infecting apple, pear, and grapevine in Brazil, the first DNA viruses known to infect tree fruits. Extraction of dsRNA was chosen in this study because all known tree fruit viruses, at the start of the project, were RNA viruses, either ssRNA or dsRNA genomes. Extracts of high molecular weight dsRNA enriches for RNA viral sequences (Ralph 1969). What was unexpected was the discovery of a novel circular DNA virus in #158 from dsRNA extracts. In studies with grapevine viruses, we have been able to detect Grapevine red blotch virus, also a circular DNA virus, from dsRNA extracts (publication submitted). It is possible that circular DNA viruses with ambisense genomes that express RNA transcripts from both strands are unique and produce genomic length dsRNA by-products that are detectable using the methods described here. However, other studies have also found DNA viruses associated with dsRNA (Son et al. 2015). More work is required to determine whether dsRNA extractions can be universally applied for RNA and DNA viruses infecting plants.

In summary, NGS is a viable method for the detection and identification of virus and virus-like agents infecting tree fruits with advantages over conventional testing and some limitations. As a method, NGS cannot compare with PCR or ELISA where the goal is the detection of one or a few specific viruses, such as surveying for *Plum pox virus* (Thompson et al. 2001). NGS is most useful as a screening method for a large number of viruses and/or where there is a need to identify a novel causal agent of a disease with virus-like symptoms, or simply a need to identify all viruses that may be present as is the case for national and international certified import/export programs and breeding programs. The potential reduction in cost and time for these activities is considerable and it is only a matter of time before NGS becomes an integral method with the potential of replacing the bioassay. For research, the ability for new virus discovery and to better characterize the variability of viral sequences in an infected plant are unprecedented (Adams et al. 2009; Al Rwahnih et al. 2015; Barba et al. 2014; Barzon et al. 2011; Candresse et al. 2014; Coetzee et al. 2010; Kreuze et al. 2009; Martin et al. 2016; Massart et al. 2014; Mokili et al. 2012; Roossinck et al. 2010). Caution, however, needs to be exercised and NGS should be used in conjunction with a confirmatory method. The high sensitivity of NGS to detect very low virus concentrations also makes it highly sensitive to cross contamination of samples, or samples contaminated with mycoviruses, insect viruses, or others. It is important to not overanalyze the NGS data but to develop effective workflows to handle the large data sets to efficiently detect viruses of interest and eliminate spurious results.

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