Discovery of Viruses and Virus-Like Pathogens in Pistachio using High-Throughput Sequencing

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Abstract

Pistachio (*Pistacia vera* L.) trees from the National Clonal Germplasm Repository (NCGR) and orchards in California were surveyed for viruses and virus-like agents by high-throughput sequencing (HTS). Analyses of sequence information from 60 trees identified a novel virus, provisionally named “Pistachio ampelovirus A” (PAVA), in the NCGR that showed low amino acid sequence identity (approximately 42%) compared with members of the genus *Ampelovirus* (family *Closteroviridae*). A putative viroid, provisionally named “Citrus bark cracking viroid-pistachio” (CBCVd-pis), was also found in the NCGR and showed approximately 87% similarity to *Citrus bark cracking viroid* (CBCVd, genus *Cocadviroid*,

family *Pospiviroidae*). Both PAVA and CBCVd-pis were graft transmissible to healthy UCB-1 hybrid rootstock seedlings (*P. atlantica* × *P. integerrima*). A field survey of 123 trees from commercial orchards found no incidence of PAVA but five (4%) samples were infected with CBCVd-pis. Of 675 NCGR trees, 16 (2.3%) were positive for PAVA and 172 (25.4%) were positive for CBCVd-pis by reverse-transcription polymerase chain reaction. Additionally, several contigs across multiple samples exhibited significant sequence similarity to a number of other plant virus species in different families. These findings require further study and confirmation. This study establishes the occurrence of viral and viroid populations infecting pistachio trees.

California is the leading pistachio (*Pistacia vera* L.) exporter worldwide, having surpassed Iran in recent years. California pistachio acreage continues to increase, with approximately 117,900 bearing hectares in 2015, located primarily in the San Joaquin Valley (NASS, USDA 2016). Limited acreage occurs in other hot, dry western states such as Arizona, Nevada, New Mexico, Texas, and Utah. In 2016, California produced a record 451,500 tons of pistachios (Administrative Committee for Pistachios 2017) relative to 146,174 tons produced the previous year (NASS, USDA 2016).

The pistachio tree is dioecious, with female trees requiring a male pollinizer. California’s pistachio industry relies almost entirely on the female cultivar Kerman, whose fruit matures from early September to mid-October (Kallsen et al. 2009). The UCB-1 hybrid rootstock (*P. atlantica* × *P. integerrima*), developed by the University of California, Berkeley, is one of the most widely used rootstocks due to its tolerance to salinity, frost, and Verticillium wilt and high yield scions grafted onto this rootstock (Ferguson et al. 2002).

An important resource of genetic material from *Pistacia* spp. is the National Clonal Germplasm Repository (NCGR). Located near Winters, CA, the NCGR contains approximately 700 pistachio trees consisting of 334 pistachio accessions, representing 14 taxa. Trees in the collection originate globally, including Afghanistan, China, France, India, Iran, Israel, Kazakhstan, Pakistan, Syria, Turkey, Turkmenistan, the United States, the former USSR, and Uzbekistan (<https://npgsweb.ars-grin.gov>). The overall phytosanitary status of the NCGR pistachio collection had never been evaluated due to the scarcity of information on infectious agents.

The only reported viral disease of pistachio is pistachio rosette virus, described on wild pistachio (*P. vera*) in Turkmenia, former

USSR, currently Turkmenistan. This virus was later eventually found in Uzbekistan, Tadjikistan, Afghanistan, and Iran (Kreutzberg 1940). Symptoms due to pistachio rosette virus included stunting of the tree, along with the formation of rosettes derived from modified twigs with swollen nodes and thin internodes. In addition, the occurrence of *Hop stunt viroid* (HSVd) was recently reported in multiple cultivars of pistachio (Elleuch et al. 2013). However, association of disease with HSVd infection has not been shown.

Recently, pistachio nurseries and growers have observed that both ungrafted clonally propagated UCB-1 trees and pistachio scion cultivars grafted on UCB-1 rootstock have occasionally become stunted. Stunted UCB-1 trees also have smaller trunk diameters, short internode lengths, and a more gray-green canopy color in comparison with asymptomatic, healthy-looking trees and the term “pistachio bushy top syndrome” has been used to describe the affected trees (Randall et al. 2014). The gram-positive plant-pathogenic bacterium *Rhodococcus fascians* has been isolated from several symptomatic plants sampled in affected orchards (Stamler et al. 2015). However, a recent study by Savory et al. (2017) showed that these virulence-gene-lacking *Rhodococcus* pistachio isolates failed to cause pistachio bushy top syndrome.

In this study, high-throughput sequencing (HTS) was used to conduct a survey of viral agents infecting pistachio, including trees exhibiting bushy top syndrome.

Materials and Methods

Plant material for HTS analysis. From 2014 to 2016, 35 samples (17 scion, 16 rootstock [12 clonally propagated], and 2 UCB-1 seedlings) were collected from 21 asymptomatic and 10 symptomatic pistachios (including two Pioneer Gold 1 seedlings of *P. integerrima*) from 18 orchards, primarily located in Kern County, CA (Table 1). Symptoms and abnormalities included stunting as well as galls and cracking at the graft union in clonal UCB-1 trees affected with the pistachio bushy top syndrome. One tree (samples F1 to F3) displayed symptoms of stem pitting. Twenty-five samples from asymptomatic pistachio trees were also collected from the NCGR collection (Table 2). Branches were collected, placed in individual labeled bags, and stored in a cooler with ice until placed in cold storage at 4°C. Two UCB-1 seedlings sourced from Foundation Plant Services (FPS) and tested negative for viruses and virus-like pathogens by HTS were used as healthy controls.

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Total nucleic acid extraction and HTS. Total nucleic acid (TNA) extracts were prepared from leaf petioles or bark tissue, as described by Al Rwahnih et al. (2012). Briefly, approximately 0.2 g of tissue was homogenized using a Homex grinder (Bioreba, South Bend, IN) and TNA extracts were prepared using a MagMAX-96 viral RNA isolation kit (Ambion, Austin, TX) as per the manufacturer's protocol. Aliquots of TNA samples from source trees were subjected to ribosomal RNA depletion and complementary DNA (cDNA) library construction using a TruSeq Stranded Total RNA with Ribozero Plant kit (Illumina, San Diego, CA). Sequencing was performed on the Illumina NextSeq 500 platform.

Assembly and sequence analysis. Illumina reads were adapter trimmed and, subsequently, de novo assembled into contiguous consensus sequences (contigs) of at least 200 bp in length using the CLC Bio Genomic Workstation (v8.5.1; Qiagen, Hilden, Germany). Contigs were annotated using a multistep process to generate candidate viral sequences. First, the initial list of contigs was generated using the tBLASTx program (v. 2.4.0) (Tatusova and Madden 1999) to compare contig sequences against the viral genome division of the National Center for Biotechnology Information RefSeq (<https://www.ncbi.nlm.nih.gov/genome/viruses/>). Contigs matching viral genomes with a combined E-value equal to or less than 10^{-4} were considered candidates.

Second, only viruses for which a known land plant host (Streptophyta), as determined by the Viral Host Database (Mihara et al. 2016), were considered further. Next, the reduced list of viral hits was compared against the complete nonredundant GenBank databases using BLASTx to compare sequences against protein (nr) and BLASTn to compare sequences against nucleotide (nt), providing the annotation ultimately used for viral agent identification. Specificity was further enhanced by considering the newly assembled genome sequence of pistachio (Palmer et al. 2017). Assembled transcripts were mapped to the genomes of *P. vera*, *P. atlantica*, and *P. integerrima* using the program GMAP (v. 6.2015) (Wu and Watanabe 2005). If the coverage at the top-ranked locus exceeded 70%, the candidate was not considered further. The annotation for each contig sequence was checked to ensure that either the representative nr and nt hits supported the initial plant virus homology, or a different plant virus homology was suggested. Although the screen was targeted at plant viruses and viroids, potential mycoviruses (as determined by the more exhaustive GenBank BLAST screens) were observed and noted.

Genome and phylogenetic analysis. Identified genome sequences of two putative viral agents were analyzed using different bioinformatic tools. The search for potential protein encoding segments in the genome sequence of each agent was conducted using

Table 1. Field samples collected between 2014 and 2016

Field survey, sample ^a	Sample description	Cultivar or RS (scion/RS) ^b	Location	Year planted	Symptoms
2014					
F1	RS bark	RS (UCB-1 seedling)	Kern	2004	Scion stem pitting
F2	Roots	RS (UCB-1 seedling)	Kern	2004	Scion stem pitting
F3	Bark	Kerman/UCB-1 seedling	Kern	2004	Scion stem pitting
F4	Bark	Clonal UCB-1	Tulare	2012	Galls with multiple buds on stems; stem galls; cracking ^c
F5	Bark	Clonal UCB-1	Kern	2012	Stunting, stem-cracking ^c
F6	Bark	Clonal UCB-1	Kern	2012	Stunting, stem-cracking ^c
F7	Bark	Clonal UCB-1	Kern	2012	Stunting, stem-cracking ^c
F8	Bark	Clonal UCB-1	Kern	2012	Asymptomatic
F9	Bark	Clonal UCB-1	Kern	2012	Asymptomatic
F10	Bark	UCB-1 Seedling	FPS	N/A	Asymptomatic
2015					
F15-1	Scion bark	Kerman scion/PG1 RS	Kern	2003	Asymptomatic
F15-2	Scion bark	Kerman scion/PG1 RS	Kern	1992	Asymptomatic
F15-3	Scion bark	Kerman scion/PG1 RS	Kern	2010	Asymptomatic
F15-4	Scion bark	Kerman scion/Terebinthus RS	Kern	1970	Asymptomatic
F15-5	Scion bark	Kerman scion/Terebinthus RS	Kern	1970	Asymptomatic
F15-6	Scion bark	Kerman scion/Kresha RS	Kern	1995	Asymptomatic
F15-7	Scion bark	Kerman scion/PG1 RS	Kern	2002	Asymptomatic
F15-8	Scion bark	Experimental scion/UCB1	Kern	1997	Asymptomatic
F15-9	Scion bark	Atlantica Terebinthus RS	Kern	1970	Asymptomatic
F15-10	Scion bark	Kerman scion/PG1 RS	Kern	1997	Asymptomatic
F15-11	Scion bark	Kerman scion/PG1 RS	Kern	1997	Asymptomatic
F15-12	Bark	UCB-1 Seedling	FPS	N/A	Asymptomatic
2016					
FC-1	Bark	Clonal UCB-1	Kern	2015	Galls with multiple buds on stems; stem galls; cracking ^c
FC-2	Bark	Clonal UCB-1	Kern	2015	Galls with multiple buds on stems; stem galls; cracking ^c
FC-3	Bark	Clonal UCB-1	Kern	2015	Galls with multiple buds on stems; stem galls; cracking ^c
FC-4	Bark	Clonal UCB-1	Fresno	2016	Asymptomatic
FC-5	Bark	Kerman scion/Terebinthus RS	Kern	1970	Asymptomatic
FC-6	Bark	Kerman scion/Terebinthus RS	Kern	Unknown	Asymptomatic
FC-7	Bark	Kerman scion/Terebinthus RS	Kern	Unknown	Asymptomatic
FC-8	Bark	Experimental scion/UCB1 RS	Kern	Unknown	Asymptomatic
FC-9	Bark	Experimental scion/UCB1 RS	Kern	Unknown	Asymptomatic
FC-10	Bark	Atlantica Terebinthus RS	Kern	1970	Asymptomatic
FC-11	Bark	PG1 seedling	Kern	2015	Trunk die back; sucker proliferation
FC-12	Bark	PG1 seedling	Kern	2015	Trunk die back; bark cracking ^c
FC-13	Bark	Kerman scion/Terebinthus RS	Kern	1970	Asymptomatic

^a F1 to F3 are samples from the same tree.

^b RS = rootstock and PG1 = Pioneer Gold 1.

^c Symptoms associated with bushy top syndrome.

the program ORF Finder and translation products of each predicted gene were verified using BLASTp (v. 2.4.0) (Tatusova and Madden 1999). Sequences of representative members of the family to which each agent belong were used for phylogenetic analyses. Sequence alignments were done using MUSCLE (Edgar 2004). Later, maximum-likelihood phylogenetic trees were constructed using PhyML (Guindon and Gascuel 2003) with 500 bootstrap replicates; edges with less than 50% bootstrap support were suppressed. GenBank accessions used for phylogenetic analysis and pairwise sequence alignment are provided in Supplementary Table S1.

HTS validation. Specific polymerase chain reaction (PCR) primers were designed from the viral contigs of interest (Supplementary Table S2). Because the viroid genome does not encode proteins, primers for the putative novel viroid were designed to amplify a 199-bp region of the viroid's 283-bp genome. Oligonucleotides for the putative novel virus were based on sequences of the coat protein (CP) and the heat shock protein 70 homolog (HSP70h) regions. Amplification of the target sequences via one-step reverse-transcription (RT)-PCR involved the use of SuperScript II reverse transcription (Life Technologies, Gaithersburg, MD) and GoTaq DNA Polymerase (Promega Corp., Madison, WI). Thermocycling conditions were 1 cycle of 52°C for 30 min; 35 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 1 min; and 1 cycle of 72°C for 7 min. The amplified products were analyzed by agarose gel electrophoresis in Tris-acetate-EDTA buffer. Bands with expected size were cut from the gel and sequenced for verification by Sanger sequencing in both directions.

Graft transmission. Twigs from six trees (W1, W2, W8, W9, W10, and W11; Table 2) in the NCGR collection were collected and chip bud grafted onto healthy UCB-1 seedling rootstocks sourced from FPS. In all, 10 to 12 graft replicates per source plant were used (at least two buds/plant). Healthy controls included nonchip-budded plants and grafted buds from plants known to be free of the pistachio viruses under study. The inoculated plants were kept in the greenhouse for 1 month to let the graft heal and then transferred to a shadehouse. At 6 to 10 months after grafting, TNA was extracted from the leaf petiole tissue of the inoculated UCB-1 seedling and

healthy controls. RT-PCR assays for the two putative novel agents were conducted as described above. Graft transmission was considered successful if the targeted virus was detected in the newly grafted UCB-1 seedling.

Surveys for new putative pathogens via RT-PCR. To evaluate the prevalence of the two new pathogens, samples from 34 trees were collected from selected orchards in Kern County, CA in 2016. In addition, 89 healthy looking samples were collected across 25 orchards in the San Joaquin Valley, CA and sent to FPS for analysis. Samples were also collected from the entire NCGR collection (675 trees in blocks A, B, C, and D containing 152, 143, 214, and 166 trees, respectively). In total, 798 trees were screened for the presence of the two putative novel agents via RT-PCR, as described above. All field-collected samples were also tested for the presence of HSVd using real-time quantitative RT-PCR (M. Al Rwahnih, unpublished data).

Results

Pistachio virome. HTS yielded between 18 and 75 million raw reads (Supplementary Tables S3 and S4) per cDNA library. Subsequent analysis identified sequences of several diverse viruses and viroids in the transcriptome of both asymptomatic or symptomatic sampled pistachio trees (Table 3). No viral agents were detected in the samples displaying stem-pitting symptoms.

Putative novel viroid of *Pospiviroidae*. A viroid not previously known in pistachio was discovered in sample W11 (cultivar Iran Large). The presence of this viroid was also verified in samples W8, W9, W10, W16, and W18. Bioinformatic analysis of a subset of infected trees yielded identical 283-bp contigs representing the entire circular RNA genome of the viroid. In-depth bioinformatic analysis demonstrated that the annotated contigs were not found in the *P. vera* host genome sequence. Expanded BLAST searches against the complete nonredundant set of GenBank nucleotide sequences confirmed the authenticity of the annotated contigs. Additionally, fresh RNA was extracted from three of the abovementioned trees (W9, W10, and W11) and RT-PCR was used with specific primers to confirm the viroid infection (Supplementary Fig. S1). Sanger sequencing

Table 2. Samples collected from the National Clonal Germplasm Repository (NCGR) pistachio collection between 2014 and 2015

Winters Germplasm Survey, sample	Sample description	Cultivar or rootstock (scion/rootstock)	Location	Origin ^a
2014				
W1	Bark	Peters	NCGR	United States
W2	Bark	Damghan	NCGR	Iran
W3	Bark	Sfax	NCGR	Tunisia
W4	Bark	Trabonella	NCGR	Italy
W5	Bark	Red Aleppo	NCGR	Syria
W6	Bark	Kerman	NCGR	Iran
W7	Bark	Lassen	NCGR	Iran
W8	Bark	Aegina	NCGR	N/A
W9	Bark	Castel	NCGR	N/A
W10	Bark	Nazareth	NCGR	N/A
W11	Bark	Iran Large	NCGR	Iran?
W12	Bark	UCB-1 (healthy)	NCGR	United States
2015				
W13	Bark	Unknown	NCGR	Iran
W14	Bark	Pistaebadami/Op seedling	NCGR	Afghanistan
W15	Bark	Unknown	NCGR	India
W16	Bark	Unknown	NCGR	Turkey
W17	Bark	Pistaebadami Op seedling	NCGR	Iran
W18	Bark	Pistaebadami Op seedling	NCGR	Iran
W19	Bark	Bronte	NCGR	Italy
W20	Bark	Unknown	NCGR	Former USSR
W21	Bark	Unknown	NCGR	Former USSR
W22	Bark	Unknown	NCGR	Former USSR
W23	Bark	Unknown	NCGR	China
W24	Bark	Dangarinka × Op	NCGR	Turkmenistan
W25	Bark	UCB-1 (healthy)	NCGR	United States

^a N/A = not available.

of the amplicons yielded sequences 99% identical to the original contig obtained by HTS. The viroid displayed 87% identity (100% query coverage and 9e-55 E-value) to *Citrus bark cracking viroid* (CBCVd, genus *Cocadviroid*, family *Pospiviroidae*) in BLASTn analysis, and was provisionally identified as a strain of CBCVd, named “Citrus bark cracking viroid-pistachio” (CBCVd-pis; GenBank accession number MF198463). Phylogenetic analysis also confirmed the close relation of CBCVd-pis with CBCVd (Fig. 1). The four fully characterized pistachio isolates of CBCVd-pis from orchard and germplasm trees shared 100% identity with one another and 87% identity with the closest related isolate of CBCVd (GenBank: JN903762). Although the identity is lower than the 90% sequence identity threshold for new species demarcation (Owens et al. 2012), biological studies are required to confirm CBCVd-pis as a novel viroid species.

HTS analysis also revealed the presence of HSVd in multiple samples from the field and NCGR (data not shown).

Putative novel virus of *Closteroviridae*. The tBLASTx search revealed a new pistachio-infecting virus that was isolated from sample W10 (cultivar Nazareth) in the NCGR collection. The virus was represented in one large contig of 17,357 bp (GenBank accession number MF198462), which represents nearly the full genome of the virus. The predicted genomic organization was composed of 12 open reading frames (ORF) (Fig. 2), including major functional proteins (Table 4) typical of members of the genus *Ampelovirus* within the family *Closteroviridae* (Martelli et al. 2002). For example, a partial polyprotein sequence (1,447 amino acids [aa]) was identified at the 5' end of the virus genome, which resembled (38% amino acid identity) the polyprotein of *Little cherry virus 2* (LChV-2). The second predicted ORF (487 aa) encoded an RNA-dependent RNA polymerase with 46% amino acid identity with LChV-2. Putative HSP70h (535 aa) and HSP90h (496 aa) proteins identified in the central portion of the viral genome exhibited amino acid homology with corresponding sequences in *Grapevine leafroll-associated virus 3* (GLRaV-3) (45 and 24%, respectively). Finally, a CP (362 aa) and a minor CP (549 aa) were predicted with 44 and 32% amino acid identity, respectively, with the *Grapevine leafroll-associated virus 1* orthologs. Phylogenetic evidence was also obtained for the assignment of the new pistachio-infecting virus to the genus *Ampelovirus* (Fig. 3). The novel ampelovirus was provisionally named “Pistachio ampelovirus A” (PAVA) because no consistent symptoms are associated with its presence in infected trees at this time.

In order to confirm the existence of PAVA in the plant material (including sample W10) used for HTS, new TNA extracts were prepared, and RT-PCR was run with specific primers to amplify the

CP of PAVA. Additionally, trees adjacent to samples W8 and W10 were also included in the validation test. Thus, samples W8, W9, W10, and W11 and the tree adjacent to W8 (A8T8) tested positive for PAVA.

Fig. 1. Maximum-likelihood phylogenetic tree of genus *Cocadviroid* includes all *Citrus bark cracking viroid* (CBCVd) isolates from GenBank and the three most closely related viroids to CBCVd. *Citrus bark cracking viroid-pistachio* (CBCVd-pis) shares a most recent common ancestor with CBCVd.

Table 3. Viral families for which tBLASTx annotation of detected contigs showed homology

Family	Number of samples detected	Sample ID	Host
<i>Alfalexiviridae</i>	2	W(18, 21)	Plants
<i>Betaflexiviridae</i>	4	F15-(1) W(7, 18, 21)	Plants
<i>Bromoviridae</i>	7	F15-(1, 4, 5, 7, 8, 9) W(6)	Plants
<i>Caulimoviridae</i>	16	F15-(3, 9) FC-(2, 4, 5, 6, 8, 9, 10, 12) W(14, 15, 18, 21, 22, 24)	Plants
<i>Closteroviridae</i>	8	F15-(1, 4, 5) W(6, 9, 10, 11, 21)	Plants
<i>Genomoviridae</i>	1	W(6)	Plants, fungi, and vertebrates
<i>Ophioviridae</i>	1	W(6)	Plants
<i>Ourmiavirus</i> (unclassified)	29	F15-(1, 4, 5, 6, 7, 8, 9, 10, 11) FC-(10) W(1-5, 7, 9, 10, 11, 14-22, 24)	Plants and fungi
<i>Partitiviridae</i>	11	F15-(1, 3, 5, 7, 10, 11) FC-(7, 13) W(6, 21, 24)	Plants and fungi
<i>Phenuiviridae</i>	2	W(4, 7)	Plants and insects
<i>Pospiviroidae</i>	29	F15-(2, 4, 5, 6, 8, 9, 10, 11) FC-(3, 5-10, 13) W(1-5, 7, 8, 9, 10, 11, 15, 16, 18, 19)	Plants
<i>Potyviridae</i>	2	F15-(4, 9)	Plants
<i>Sobemovirus</i> (unclassified)	5	F15-(3, 4, 8) FC-(1, 9)	Plants
<i>Tombusviridae</i>	17	F15-(2, 3, 8, 10) FC-(1, 3, 8, 9) W(1, 4, 7, 9, 10, 18, 21, 22, 24)	Plants and fungi
Unclassified	7	F15-(7, 9, 10) FC-(7, 13) W(7, 24)	Plants
<i>Virgaviridae</i>	6	F15-(1, 4, 7, 8, 9) W(6)	Plants

Detection of agents belonging to other taxonomic groups. Sequence analysis led to the identification of viral contigs belonging to several other plant-pathogenic families (Table 3). The final species identification and prevalence of these agents is still in progress. Notably, the number of mycovirus-like contigs identified in the HTS analysis was greater than the corresponding numbers for plant viruses. Their BLAST identifications were guided by GenBank reference sequences of homologous mycoviruses from nonpistachio sources. Consequently, the alignments through which these viruses were identified often contained multiple mismatches. No viral contigs were detected by HTS in the UCB-1 seedlings used as healthy controls.

PAVA and CBCVd-pis are graft transmissible. The virus was detected in petioles of four of five UCB-1 rootstocks grafted with PAVA-infected material (Fig. 4A) and the viroid was detected in petioles of two of five UCB-1 rootstocks grafted with CBCVd-pis-infected material (Fig. 4B). Both assays were run with healthy UCB-1 seedling and *P. vera* controls, as well as a water control, all of which showed no reaction in RT-PCR to either set of primers (Fig. 4).

Survey of PAVA and CBCVd-pis via RT-PCR. Of the 675 NCGR trees sampled and tested using RT-PCR, 16 trees tested positive for PAVA, 172 trees tested positive for CBCVd-pis, and 10 trees tested positive for both. For CBCVd-pis, these data reflects a 67% infection rate in block A, a 48% infection rate in block B, no infection in block C, and a 0.6% infection rate in block D. For PAVA, trees in blocks B, C, and D were free of the virus but block A had an infection rate of approximately 10%. PAVA was primarily detected in *P. vera*, with a few exceptions from *P. terebinthus* and *P. mexicana* (Table 5). The distribution of infected species for CBCVd-pis varied across the species *P. vera* (33.5%), *P. chinensis* (15.8%), *P. atlantica* (10.0%), *P. mexicana* (1.2%), *P. terebinthus* (5.8%), and other hybrids (33.5%).

There were no positives in the 34 samples collected in the 2016 commercial field survey for either PAVA or CBCVd-pis. Among the 89 commercial field samples received from growers, none was positive for PAVA but 5 trees tested positive for CBCVd-pis. The presence of CBCVd-pis was confirmed via Sanger sequencing (data not shown). HSVd was more prevalent in commercial fields, with incidences of 91.1% (31 of 34) and 48.3% (43 of 89) in samples from the 2016 field survey and samples sent by growers, respectively.

Discussion

HTS analysis is faster and more comprehensive than the other classical diagnostic techniques for plant pathogen identification (Al Rwahnih et al. 2015; Rott et al. 2017). In this study, HTS was used to detect and characterize viruses and virus-like agents in pistachio. Our work showed the existence of many virus sequences related to several known plant-pathogenic virus families in pistachio but additional studies are needed for confirmatory purpose. This is the first documented report of the virome of a pistachio crop. HTS analysis also revealed a cohort of mycoviruses in pistachio, which appeared to be benign microorganisms.

In this work, specific contigs from the families *Closteroviridae* and *Pospiviroidae* were more extensively investigated. HTS was

employed to obtain the genomes of PAVA (a novel ampelovirus) and an unknown variant of CBCVd. Previously, Elleuch et al. (2013) reported the occurrence of HSVd in multiple cultivars of pistachio in Tunisia. Therefore, the detection of PAVA, CBCVd, and HSVd in pistachio in this study represents the first report of viral infections of this crop in North America.

CBCVd (genus *Cocadviroid*, family *Pospiviroidae*) is known to be associated with bark-cracking symptoms in citrus and was first discovered in citrus trees in California and subsequently in the southern United States, Europe, Africa, and Asia (EPPO 2015). CBCVd was recently reported for the first time on a crop outside of the *Citrus* genus as an aggressive pathogen on hop (Jakse et al. 2015). Phylogenetic analysis placed CBCVd-pis within the genus *Cocadviroid* in close relationship with other CBCVd isolates. The presence of a strain of CBCVd in pistachio is important because previous studies (Osman et al. 2017; Semancik and Vidalakis 2005) showed that CBCVd can be transmitted via artificial inoculation to a number of hosts and cause a range of symptoms. The identification of new natural hosts of CBCVd may help clarify the epidemiology of this emergent pathogen of hops (Jakse et al. 2015). From a taxonomic point of view, this viroid will be considered a strain of CBCVd in the absence of biological data to clarify its status as a novel viroid species. It should be noted that the classification of CBCVd in the genus *Cocadviroid* has been questioned, based on a suggestion for its reclassification into the genus *Pospiviroid* (Semancik and Vidalakis 2005).

Based on its genome organization, sequence homology, and phylogeny, PAVA is a tentative member in the genus *Ampelovirus*, whose type member (GLRaV-3) is an economically important pathogen to grapevine (Martelli et al. 2002). Other members of the genus such as LChV-2, *Pineapple mealybug wilt-associated virus 2*, and *Plum bark necrosis stem pitting-associated virus* are known to cause severe symptoms in other crops (Martelli et al. 2012).

Further studies will be needed to determine the prevalence of PAVA and CBCVd-pis in California nurseries and orchards, evaluate their potential natural spread, and assess their effects on tree performance and crop quality. Because these agents are graft transmissible, there is an increased risk of spread via infected propagation material.

Table 4. Percentage (%) of amino acid identities between Pistachio ampelovirus A (PAVA) and some members of the genus *Ampelovirus*, family *Closteroviridae*^a

Protein	GLRaV-1	GLRaV-3	PMWaV-2	BVBaV	LChV-2
Polyprotein*	33	34	28	36	38
RdRp	43	43	44	44	46
HSP70h	41	45	38	43	35
HSP90h	23	24	23	20	20
CP	44	41	34	38	27
CPm	32	30	28	32	26

^a GLRaV-1 = Grapevine leafroll-associated virus 1, GLRaV-3 = Grapevine leafroll-associated virus 3, PMWaV-2 = Pineapple mealybug wilt-associated virus 2, BVBaV = Blackberry vein-banding associated virus, LChV-2 = Little cherry virus 2, RdRp = RNA-dependent RNA polymerase, HSP70h = heat shock protein 70 homolog, HSP90 = heat shock protein 90 homolog, CP = coat protein, and CPm = minor coat protein.

Fig. 2. Near complete genome organization of Pistachio ampelovirus A (PAVA) in reference to *Grapevine leafroll-associated virus 3* (GLRaV-3). Identified open reading frames and their products are described. MET = methyltransferase, HEL = helicase, RdRp = RNA-dependent RNA polymerase, HSP = heat shock protein, CP = coat protein, and CPm = minor coat protein.

Fig. 3. Maximum-likelihood phylogenetic tree of family *Closteroviridae* reconstructed from coat protein sequences places Pistachio ampelovirus A (PAVA) in a subclade within the genus *Ampelovirus*.

Fig. 4. A, Agarose gel showing the reverse-transcription polymerase chain reaction (RT-PCR) assay using Pistachio ampelovirus A (PAVA) coat protein primers, expected amplicon size 474 bp. **B,** Agarose gel showing the RT-PCR assay using Citrus bark cracking viroid-pistachio (CBCVd-pis) detection primers, expected amplicon size 199 bp. Lanes 1 to 5 = UCB-1 rootstocks grafted with PAVA- or CBCVd-pis-infected buds, VC = *P. vera* healthy control, RC = UCB-1 rootstock healthy control, W = water control, Po = positive control, and L = 1-kb Plus DNA ladder.

Table 5. Information describing the species, cultivar, and country of origin of all plants which tested positive for Pistachio ampelovirus A (PAVA) at the National Clonal Germplasm Repository^a

Location (block, row, tree)	Species	Cultivar	Country of origin
A 2 2	<i>Pistacia mexicana</i>	N/A	N/A
A 8 3	<i>P. terebinthus</i>	N/A	Iran
A 8 4	<i>P. terebinthus</i>	N/A	Iran
A 8 8	<i>P. vera</i>	N/A	USSR
A 8 9	<i>P. vera</i>	Pi 12815 × Pollen 3	United States
A 8 10	<i>P. vera</i>	Pi 12815 × Pollen 3	United States
A 9 4	<i>P. vera</i>	Iran Large	N/A
A 9 5	<i>P. vera</i>	Nazareth 1975	N/A
A 9 6	<i>P. vera</i>	Nazareth 1975	N/A
A 9 7	<i>P. vera</i>	Castel	N/A
A 9 8	<i>P. vera</i>	Castel	N/A
A 9 9	<i>P. vera</i>	Aegina	N/A
A 9 11	<i>P. vera</i>	N/A	Iran
A 9 12	<i>P. vera</i>	N/A	Iran
A 9 13	<i>P. vera</i>	Rashti	N/A
A 10 5	<i>P. vera</i>	N/A	Iran

^a N/A = not available.

Although a few overt disease symptoms such as galls and bark cracking were observed in productive pistachio orchards, no symptoms were apparent in the NCGR. However, due to the vast collection of genetically diverse germplasm present in the NCGR, there are admittedly increased chances of finding a greater variety of viruses in this collection. Further studies will be needed to correlate the presence of micro-organisms with tree fitness or orchard yields. The results presented here suggest that bushy top syndrome is unlikely of viral origin.

Knowledge of the nature of the virome or viral agent load carried by pistachio will move management options from a reactive to a proactive stance. PCR-based analysis of cellular and subcellular micro-organisms are essential components of best-management practices for routine testing and field screening for the presence of pistachio viruses in the orchard. The RT-PCR diagnostic tools designed in this study will also be useful in the screening of clean propagation material. Using certified clean stock, which has made a significant impact on the health status of other clonally propagated crops by providing the highest quality virus-tested material for propagation, will be an option for the pistachio industry to prevent the spread of these newly discovered viral agents and provide the means for the long-term elimination of disease from orchards.

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