Prunus geminivirus A: A Novel *Grablovirus* Infecting *Prunus* spp.

Maher Al Rwahnih,[†] Department of Plant Pathology, University of California, Davis, 95616; **Olufemi J. Alabi**, Department of Plant Pathology & Microbiology, Texas A&M AgriLife Research and Extension Center, Weslaco 78596; and **Nathaniel M. Westrick** and **Deborah Golino**, Department of Plant Pathology, University of California, Davis

Abstract

Increased use of metagenomics for routine virus diagnosis has led to the characterization of several genus level geminiviruses from tree fruit long thought to exclusively host RNA viruses. In this study, the identification and molecular characterization of a novel geminivirus is reported for the first time in *Prunus* spp. The virus, provisionally named *Prunus geminivirus A* (PrGVA), was identified by Illumina sequencing from an asymptomatic plum tree. PrGVA was subsequently confirmed by rolling cycle amplification, cloning, and Sanger sequencing of its complete genome (3,174 to 3,176 nucleotides) from an additional 18 (9 apricot and 9 plum) field isolates. Apart from the nonanucleotide motif TAATATT↓AC present in its

virion strand origin of replication, other conserved motifs of PrGVA support its geminiviral origin. PrGVA shared highest complete genome (73 to 74%), coat protein amino acid (83 to 85%) and rep-associated amino acid (74%) identities with *Grapevine red blotch virus* (GRBV). PrGVA was graft but not mechanically transmissible. Quantitative polymerase chain reaction screening of *Prunus* spp. in the National Clonal Germplasm Repository collection using newly designed primers and probes revealed 69.4% (apricot), 55.8% (plum), and 8.3% (cherry) incidences of PrGVA. PrGVA is proposed as a novel member of the genus *Grablovirus* based on its close genome and phylogenetic relationship with GRBV.

The *Geminiviridae* family is characterized by viruses with small, circular, single-stranded, nonenveloped, mono- or bipartite DNA genomes (Brown et al. 2015). Until the last decade, the family consisted of members belonging to four genera that are largely typified by their insect vector. These are the leafhopper-transmitted members of genera *Mastrevirus* and *Curtovirus*, whitefly-transmitted members of genus *Begomovirus*, and the treehopper-transmitted members of genus *Topocuvirus* (Brown et al. 2015). Subsequently, diverse geminiviruses belonging to three other genera (*Becurtovirus*, *Turncurtovirus*, and *Eragrovirus*) were discovered, bringing the number of genera in the *Geminiviridae* family to seven. A common feature of viruses belonging to these seven genera is that they are primarily pathogens of economically important herbaceous hosts and weed species occurring mainly in the tropical and subtropical regions of the world. The situation changed over the last decade when several new geminiviruses were characterized from temperate and tropical woody plant species and additional herbaceous plants. These include *Citrus chlorotic dwarf-associated virus* (CCDaV) (Loconsole et al. 2012), *French bean severe leaf curl virus* (FbSLCV) (JX094280), *Grapevine red blotch virus* (GRBV) (Al Rwahnih et al. 2013; Krenz et al. 2012; Poojari et al. 2013), *Euphorbia caput-medusae latent virus* (EcmLV) (Bernardo et al. 2013), *Jatropha mosaic virus* (JaMV) (Polston et al. 2014), *Alfalfa leaf curl virus* (ALCV) (Bernardo et al. 2016; Roumagnac et al. 2015), *Mulberry mosaic dwarf-associated virus* (MMDaV) (Ma et al. 2015), *Apple geminivirus* (AGmV) (Liang et al. 2015), *Plantago lanceolata latent virus* (PILV) (Susi et al. 2017), and *Grapevine geminivirus A* (GGVA) (Al Rwahnih et al. 2017). These recent discoveries have led to a revisit of the *Geminiviridae* taxonomy, resulting in the establishment of two additional genera, *Capulavirus* and *Grablovirus* (Adams et al. 2017). The genus *Capulavirus* consist of ALCV, EcmLV, FbSLCV, and PILV while GRBV is the only member of the genus *Grablovirus*. Two other unclassified genera, consisting of CCDaV and MMDaV on the one hand and AGmV and GGVA on the other, await

recognition by the International Committee on Taxonomy of Viruses (ICTV) due to lack of information on their vectors or ability to form geminate particles characteristic of the family (Varsani et al. 2017).

To date, the genus *Grablovirus* consists of GRBV isolates from Canada, South Korea, and the United States. Because the *Grablovirus* genus has no additional related viruses, a genome-wide species demarcation threshold of <80% was proposed for members of the genus pending reports of new grabloviruses (Varsani et al. 2017). The discovery of a novel putative *Grablovirus*, provisionally named *Prunus geminivirus A* (PrGVA), is reported in this study. PrGVA has genome organization similar to that of GRBV and also shares very close phylogenetic relationship with GRBV based on analysis of complete genome, coat protein (CP) amino acid, and replicase (Rep) amino acid sequences. However, at 73.3 and 74.4% identities with GRBV isolates 3138-03 and NY137, respectively, its complete genome fell short of the current *Grablovirus* species demarcation threshold proposed by Varsani et al. (2017). These results, together with the discovery of yet another distantly related putative *Grablovirus*, Wild vitis virus 1 (WVV1), from a wild grapevine species in California (Perry et al. 2018), opens avenues for a revisit of the species demarcation criteria of the new genus.

Materials and Methods

Plant material. In 2013, a domestic plum variety, named FT7, grafted onto Marianna 2624 rootstock was received for inclusion in the Foundation Plant Services (FPS, University of California Davis) collection. The tree was grown in a screenhouse and assayed for known fruit tree pathogens, as described below.

Assays for known fruit tree pathogens. Total nucleic acid (TNA) was isolated from FT7 as described by Al Rwahnih et al. (2017). The TNA sample was analyzed by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) for a panel of 15 *Prunus*-infecting viruses belonging to six genera and two viroid species. The RT-qPCR assays were performed using TaqMan probes on the QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific, Carlsbad, CA) as described previously (Osman et al. 2012) (V. Klaassen and M. Al Rwahnih, unpublished data). The targeted pathogens included *Little cherry virus 1* (genus *Velarivirus*); *Little cherry virus 2* and *Plum bark necrosis stem pitting-associated virus* (genus *Ampelovirus*); *Apple chlorotic leaf spot virus* (genus *Trichovirus*); *Apple mosaic virus*, *American plum line pattern virus*, *Prune dwarf virus*, and *Prunus necrotic ringspot virus* in the genus *Ilarvirus*; *Cherry green ring mottle virus* (genus *Foveavirus*); *Cherry leaf roll virus*, *Peach rosette mosaic virus*, and *Tomato ringspot virus* in the genus *Nepovirus*;

[†]Corresponding author: M. Al Rwahnih; E-mail: malrwahnih@ucdavis.edu

*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary figures are published online.

Cherry necrotic rusty mottle virus (genus *Robigovirus*); *Cherry rasp leaf virus* (genus *Cheravirus*); *Cherry virus A* (genus *Capillovirus*); *Hop stunt viroid* (genus *Hostuviroid*); and *Peach latent mosaic viroid* (genus *Pelamoviroid*). FT7 was also screened using a universal phytoplasma assay (Hodgetts et al. 2009). Positive, negative, and no-template control samples were included for each pathogen assay.

Biological assays. As part of the FPS pipeline for routine testing of new germplasm introductions, several bioassays were performed on FT7. Bud chips from FT7 were grafted onto stone fruit indicator cultivars, including Elberta (peach), Tilton (apricot), Shiro (plum), Bing (cherry), Kwanzan (cherry), and Tomentosa (cherry), as previously described (Jelkmann 2004). Graft inoculation assays were performed during fall 2013 and the experimental trees were observed for presence of virus-like symptoms during fall and spring of two consecutive years. In addition, leaf petiole tissue from the FT7 tree was mechanically inoculated onto a panel of herbaceous indicator plants, including *Chenopodium amaranticolor*, *C. quinoa*, *Cucumis sativus*, and *Nicotiana glauca*, as previously described (Rowhani et al. 2005).

In 2017, bud chips from FT7 were grafted onto 12 GF305 peach seedlings, 4 Myrobalan 29C, and 4 Citation plum rootstocks. The grafted plants were maintained in the greenhouse. Mechanical inoculation of material from FT7 onto a panel of herbaceous was also repeated as described above.

High-throughput sequencing and sequence analysis. A TNA aliquot from FT7 was subjected to ribosomal RNA depletion and a cDNA library was constructed from the RNA template using a TruSeq Stranded Total RNA with Ribo-Zero Plant kit (Illumina, San Diego, CA). Sequencing was performed in-house on the Illumina NextSeq 500 platform and the raw high-throughput sequencing (HTS) reads were trimmed using the CLC Bio Genomic Workstation (v8.5.1; Qiagen, Hilden, Germany) trimming tool. Library construction, sequencing, sequence assembly, and analyses were performed using tools, programs, and conditions described by Al Rwahnih et al. (2017).

HTS validation and determination of geminiviral genome sequences. A TNA aliquot from FT7 was enriched for geminivirus genome targets via rolling cycle amplification (RCA) (Inoue-Nagata et al. 2004) using a TempliPhi Amplification Kit (GE Healthcare Life Sciences, Uppsala, Sweden), as described in the manufacturer's protocol. Two pairs of abutting primers (Table 1) were designed based on the HTS-derived geminiviral sequence obtained from FT7 and used in conventional PCR with RCA or TNA aliquots as template to drive the amplification of circular single-stranded (ss)DNA genomic DNA fragment present in the sample. The PCR were carried out in 25- μ l reaction volumes using the PrimeSTAR GXL DNA Polymerase (Clontech Laboratories, Inc., Palo Alto, CA), according to the manufacturer's rapid protocol recommendations. Obtained DNA amplicons (Fig. 1) were gel eluted with the Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA), A-tailed, cloned into pCR2.1 TOPO-TA vector (ThermoFischer Scientific), and Sanger sequenced as two overlapping DNA fragments in a commercial facility.

Genome and phylogenetic analysis. Blastn analysis was performed for the assembled complete viral ssDNA genome sequence to ascertain its viral origin and to identify sequences producing significant alignments to isolate FT7. The search for potential protein-encoding segments in the complete genome sequence was conducted using the program ORF Finder and translation products of each predicted open reading frame (ORF) were verified using the SMART BLAST algorithm (<https://www.ncbi.nlm.nih.gov/orffinder/>). The program FGENESH+ v.2.6 (Solovyev 2007) was used to predict the C1:C2 fusion protein based on similarity with its geminivirus homologs (http://www.softberry.com/berry.phtml?topic=fgenes_plus&group=programs&subgroup=gfs). For this purpose, the complete genome of the new virus was queried separately with the C1:C2 protein sequences of GRBV isolate NY137 and an isolate of *Chickpea chlorosis virus-B*. The predicted protein was considered acceptable if both independent queries yielded the same amino acid sequences. Multiple sequence alignments were performed for complete genome and gene-specific nucleotide and protein sequences of the virus and other geminiviruses using the MUSCLE alignment program (<https://www.ebi.ac.uk/Tools/msa/muscle/>). The alignment files were used to generate sequence identity matrices using the SDT v.1.2 program (Muhire et al. 2014) and for phylogenetic analysis using the MEGA v.7.0 program (Kumar et al. 2016). Sequences of representative members of all nine recognized and two unclassified genera in the family *Geminiviridae* were included in these analyses. The genome architecture of the new virus was drawn using the SnapGene Viewer 4.0.2 software (GSL Biotech; <http://www.snapgene.com/>) and its CP-encoded nuclear localization signal (NLS) motif was predicted using the program NucPred (Brameier et al. 2007). The Rep protein N-terminal double-stranded (ds)RNA binding motifs of the new virus were identified based on homology with corresponding amino acid sequences of other members of the family *Geminiviridae*.

Development of conventional PCR and qPCR diagnostic assays. An alignment file of all the newly derived sequences (plum = 9 and apricot = 9) was used to design primers for routine detection of the novel virus in epidemiological studies by conventional PCR and qPCR (Table 1). The Primer Express software (v3.0.1; ThermoFisher Scientific, Grand Island, NY) was used for the design of primers and probe and the minimal criteria for the design of qPCR assays (Bustin et al. 2009) was followed in developing the qPCR assay. All qPCR probes were MGB probes, labeled with 5' FAM reporters and a nonfluorescent quencher. The amplification conditions for the conventional PCR assay consisted of one cycle of 94°C for 2 min; 35 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 45 s; and one cycle of 72°C for 5 min. Cycling conditions for qPCR were 50°C for 2 min and 95°C for 20 s, then 40 cycles at 95°C for 1 s and 60°C for 20 s. Assay optimization and analysis of the qPCR data were conducted as previously described (Al Rwahnih et al. 2017). The virus specificities of DNA amplicons obtained in conventional PCR and qPCR assays were verified via direct sequencing of gel-eluted DNA fragments of

Table 1. Primers and probes designed in this study for the molecular characterization and routine diagnosis of *Prunus geminivirus A* (PrGVA)^a

Primer name ^b	Genomic region ^c	Sequence (5'-3') ^d	Temperature (°C)	Amplicon size (bp)
PrGVAv157	LIR	<u>CgATTggACAg</u> AggTACTTACAgT	56	3,175
PrGVAc165	LIR	<u>gTCCAATCgCAg</u> AACTCCATAC	...	...
PrGVAv1679	V3	<u>ggTCAACATAgACCAg</u> ACAgTCCgT	56	3,175
PrGVAc1682	V3	<u>gACCAgAACCAAgTTCTAAgTCC</u>	...	...
PrGVAv2527	C1	<u>TTggTTAgCAAAgTATTC</u> AAgTCg	56	815
PrGVAc165	LIR	<u>gTCCAATCgCAg</u> AACTCCATAC	...	...
PrGVAv1514	V3	<u>ACgTTgAAgAgACgTCg</u> TTCTg	60	83
PrGVAc1596	V3	<u>TCTTCATCAg</u> gATCAACCATCACT	...	...
Probev1552	V3	<u>TTCCAACCTgAAgCg</u> AAg	...	...

^a Additional primer-walking primers (data not shown) were designed to complete the virus genome based on sequences fragments generated with the abutting primers.

^b Primer orientation (v = virion-sense and c = complementary-sense) and locations of their first nucleotides on virus genome are indicated.

^c LIR = large intergenic region, C1 = C1 protein, and V3 = V3 protein.

^d Underlined nucleotides indicate sequence overlaps between the pair of abutting primers used for amplification of complete genome of PrGVA.

representative samples at the UC Davis sequencing facility (<http://dna-seq.ucdavis.edu>).

Determination of virus incidence. A survey was conducted to determine the incidence of the newly characterized virus in the *Prunus* spp. collection of the National Clonal Germplasm Repository (NCGR) at Winters, CA. The survey was carried out during spring 2017 and leaf petiole samples were collected from 215 trees. Selected trees represented a diverse array of *Prunus* spp. (peach = 58, cherry = 36, apricot = 49, plum = 52, and almond = 20) sourced originally from worldwide geographical regions, several of which have been previously surveyed and screened by Osman et al. (2012). Based on the test results, the complete ssDNA genome of the new virus was determined from additional nine plum and nine apricot samples using abutting primers (Table 1) and assays described above.

Results

Biological assays. No visible virus-like symptom was observed on the original FT7 source tree or the graft-inoculated indicators cultivars Elberta (peach), Tilton (apricot), Shiro (plum), Bing (cherry), Kwanzan (cherry), and Tomentosa (cherry) during the spring and fall seasons of 2014 and 2015. Also, none of the four mechanically inoculated herbaceous plants produced virus-like symptoms. Sample FT7 was advanced for HTS diagnosis in order to exclude the presence of unknown virus or virus-like agents in the sample and to rule out the presence of known viruses at concentrations undetectable with traditional assays.

A novel geminivirus identified in FT7 via HTS. Approximately 28 million reads (each 76 nucleotides [nt] in length) were recovered from the Illumina sequencing of FT7 cDNA library. The assembly of these reads yielded 42,037 contigs. The annotation and tblastx analyses of the contigs against the reference virus genomes in the National Center for Biotechnology Information resulted in the identification of a single 3,175-nt contig (GenBank accession number MF579394) showing 76 to 78% homology with several GRBV isolates at 74 to 76% query coverage. A scan of this contig revealed the

presence of the nonanucleotide motif TAATATT↓AC and a blastx analysis produced significant alignments with several replication-associated putative conserved domains of known geminiviruses (data not shown). These results indicated the presence of a novel geminivirus in sample FT7, for which the tentative name *Prunus geminivirus A* (PrGVA) is assigned.

Complete genome of PrGVA determined via PCR, cloning, and Sanger sequencing. The two pairs of newly designed abutting primers pairs (Table 1) each amplified approximately 3-kb DNA bands from FT7 (Fig. 1). Cloning and Sanger sequencing of each DNA fragment resulted in the assembly of complete genome of PrGVA of 3,175 bp (GenBank accession number MF504029). Complete genome sequences of PrGVA were similarly obtained from additional isolates from plum ($n = 9$) and apricot ($n = 9$) trees (Table 2) with the primer pair PGVAv157 and PGVAc165 (Table 1). The length of these complete genome sequences ranged from 3,174 to 3,176 nt, with variations in genome length due to presence of indels in the large intergenic region (LIR) of some PrGVA isolates (GenBank accession numbers MF504030–40 and MF5104408–14). These results provide validation for the HTS data and revealed the occurrence of PrGVA in several apricot (*Prunus armeniaca*) and plum (*Prunus* spp.) trees in the NCGR collection.

PrGVA is graft but not mechanically transmissible. TNA prepared from petiole and bark scrapings of 20 graft-inoculated trees (GF305 peach seedlings = 12, Myrobalan 29C = 4, and Citation plum rootstocks = 4) were screened by qPCR 3 and 6 months postinoculation using primers and conditions described above (Table 1). Mechanically inoculated herbaceous host plants were similarly tested 3 weeks postinoculation. PrGVA was detected in petiole and bark scrapings of only one GF305 peach (*P. persica*) rootstock at 6 months post graft inoculation. The presence of PrGVA in this rootstock material was confirmed through multiple repeated tests. All nongrafted GF305 seedlings and healthy grafted controls tested negative for PrGVA. Furthermore, the complete genome of PrGVA was amplified from the infected GF305 material using the primers PGVAv157 and PGVAc165 (Table 1). Analysis of the Sanger-sequenced isolate of PrGVA from GF305 (GenBank accession number MF579393)

Fig. 1. Amplification of complete single-stranded DNA genome of *Prunus geminivirus A* (PrGVA) by conventional polymerase chain reaction from plum isolate FT7 using a pair of newly designed abutting primers, PrGVAv157 and PrGVAc165. Lane FT7 = originally infected plum tree, lane W = water, lane H = healthy control, and lane M = 1-kb-plus ladder.

Table 2. GenBank accession numbers of complete genome sequences of *Prunus geminivirus A* (PrGVA) isolates derived in this study

Source, crop, isolate	Species	Variety	Country	Accession
USDA NCGR ^a				
Apricot				
AP4	<i>Prunus armeniaca</i>	No. 279/II-3	Poland	MF504030
AP6	<i>P. armeniaca</i>	Almon	Pakistan	MF504031
AP9	<i>P. armeniaca</i>	Palsteyn	South Africa	MF504032
AP10	<i>P. armeniaca</i>	Nonno	Italy	MF504033
AP11	<i>P. armeniaca</i>	Luizet	France	MF504034
AP12	<i>P. armeniaca</i>	KAZ 95 17-07	Kazakhstan	MF504035
AP13	<i>P. armeniaca</i>	Rakovsky BU 33	Hungary	MF504036
AP14	<i>P. armeniaca</i>	Santa Clara Sweet	United States	MF504037
AP16	<i>P. armeniaca</i>	DPRU 1787	Turkey	MF504038
Plum				
PL1	<i>Prunus</i> spp.	Botanky	India	MF504039
PL2	<i>P. simonii</i>	Simon	China	MF504040
PL4	<i>P. salicina</i>	Formosa	United States	MF510408
PL6	<i>P. domestica</i>	Reine Claude de Bavay	Unknown	MF510409
PL8	<i>P. domestica</i>	Pervenec 36-23	USSR	MF510410
PL9	<i>P. domestica</i>	Purple Gage	Unknown	MF510411
PL10	<i>P. salicina</i>	Wickson	Unknown	MF510412
PL11	<i>P. salicina</i>	QLG 087	China	MF510413
PL13	<i>P. salicina</i>	Abundance	United States	MF510414

^aUnited States Department of Agriculture National Clonal Germplasm Repository.

revealed 97 to 99% complete genome identities with virus isolates from plum and apricot. The results showed that PrGVA is graft transmissible, albeit at low frequency. All mechanically inoculated herbaceous plants tested negative for PrGVA.

Genome organization and molecular properties of PrGVA. Like most geminiviruses, PrGVA possesses the nonanucleotide motif TAATATT↓AC in its virion strand origin of replication (Fig. 2). Three virion-sense (V1, V2, and V3) and three complementary-sense (C1, C2, and C3) putative translation products were identified in the circular ssDNA genome of PrGVA (Fig. 2). In addition, a C1:C2 fusion protein that is potentially expressed from spliced C1 and C2 transcripts of PrGVA is predicted using the FGENESH+ 2.6 (http://www.softberry.com/berry.phtml?topic=fgenes_plus&group=programs&subgroup=gfs) program. This 38.3-kDa C1:C2 re-associated fusion protein spans nucleotide positions 1,877 to 2,264 and 2,428 to 3,017 of isolate FT7 (Fig. 2). The virion-sense and complementary-sense genes are separated by a 415-nt LIR encompassing the virion strand origin of replication and a short intergenic

region that is 159 nt in length. The CP-encoded NLS of PrGVA was predicted to be “KRRPSRRYRRATKKRSGRSSQKRRRARPR” (NucPred score = 0.68; Supplementary Fig. S1). The N-terminal re-associated protein sequences of PrGVA also contains the dsRNA-binding motifs FLTYP (motif I), HLH (motif II), and YVSKD (motif III) together with the so-called GRS domain (Supplementary Fig. S2) (Nash et al. 2011). In this respect, the dsRNA-binding motifs and GRS domain of PrGVA most resemble those of GRBV and WVV1 compared with other species in the family *Geminiviridae*.

PrGVA is a putative *Grabovirus* sp. In pairwise comparisons, PrGVA was most (73 to 74%) identical to GRBV isolates on a whole-genome basis. The virus also shared 69% complete genome identities with WVV1, another recently characterized putative grablovirus from noncultivated *Vitis* spp. sampled from California (Perry et al. 2018). Interestingly, WVV1 shared 68% complete genome identity with GRBV isolates, indicating that PrGVA and GRBV are more identical to each other than either is to WVV1 (Table 3). Analysis of gene-specific nucleotide and amino acid

Fig. 2. Genome map of the circular single-stranded DNA molecule of *Prunus geminivirus A* (PrGVA). Arrows denote the virion-sense (V) and complementary-sense (C) geminiviral genes encoded by the virus. LIR = long intergenic region sequences, SIR = short intergenic region sequences, V1 = coat protein, V2 = precoat protein, V3 = open reading frame V3, C1 = repA protein, C2 = transcriptional activator protein, C3 = replication enhancer protein, and C1:C2 = rep-associated protein. The stem-loop in the LIR of PrGVA contains the conserved geminiviral nonanucleotide sequence “TAATATT↓AC” present in the virion strand origin of replication. The map is based on PrGVA isolate FT7 (GenBank accession number MF504029).

sequences showed that PrGVA shared greater CP (76 to 77% nt and 83 to 85% amino acid [aa]) and Rep (75% nt and 74% aa) gene identities with GRBV compared with WVV1 (70 nt and 78% aa CP and 70 nt and 68% aa Rep identities) (Table 3). However, the predicted C2 and C3 cistrons of PrGVA were more identical to corresponding sequences of WVV1 than to GRBV (Table 3). As shown in the color-coded sequence identity matrix (Supplementary Fig. S3) and the derived phylogenetic trees (Fig. 3), PrGVA isolates clustered in the *Grablovirus* clade along with GRBV and WVV1 isolates based on complete genome (Fig. 3A) as well as CP (Fig. 3B) and Rep (Fig. 3C) amino acid sequences. In this clade, PrGVA isolates formed a distinct subclade closer to the GRBV subclade and distant from the subclade formed by WVV1 (Fig. 3). These results provide strong support for the assignment of PrGVA into the genus *Grablovirus* and justifies a revisit of the current <80% genome-wide pairwise identity species threshold (Varsani et al. 2017) in favor of a lower species demarcation cut-off value for the genus.

PrGVA is relatively widespread in the NCGR collection. A sensitive diagnostic qPCR assay was successfully developed for routine detection of PrGVA (Supplementary Fig. S4). The specificity of the assay was confirmed by Sanger sequencing of an amplified DNA fragment (data not shown). Using this assay, freshly collected samples from a total of 215 trees of diverse *Prunus* spp. (almond = 20, apricot = 49, cherry = 36, peach = 58, and plum = 52) were screened for the presence of PrGVA. In all, 66 (30.7%) of the tested trees (apricot = 34 of 49 or 69.4%, cherry = 3 of 36 or 8.3%, and plum = 29 of 52 or 55.8%) were positive for PrGVA (Table 4). All almond and peach trees tested negative for PrGVA (Table 4), indicating the absence of the virus in these plant species under natural field conditions. A breakdown of the 66 PrGVA-positive trees by origin indicated that they were originally introduced into the NCGR collection from 22 countries (Table 2), thus indicating a wide distribution of the novel *Grablovirus* sp. No visible virus symptoms were observed on the positive trees.

Discussion

The recovery of PrGVA in FT7 further underscores the benefits of HTS as a versatile tool for the culture-independent detection

of novel viruses that may have escaped traditional virus diagnostic techniques and may also be present as asymptomatic infections. As previously discussed (Al Rwahnih et al. 2017), this versatile tool enabled the discovery of the majority of geminiviruses characterized from woody perennials in recent years. Therefore, it seems inevitable that HTS will be adopted globally for certification purposes in plant certification programs in the coming years.

The discovery of PrGVA (this study) and WVV1 (Perry et al. 2018), and the assignment of both viruses into the genus *Grablovirus*, opens avenues to address pertinent questions with respect to the species demarcation criteria in this new genus and the origin and evolutionary history of GRBV, its type species. Since its first identification and characterization (Al Rwahnih et al. 2013; Krenz et al. 2012; Poojari et al. 2013), GRBV has garnered intense interest among the global community of grapevine virologists and the wider viticultural community due to its documented economic impacts (Ricketts et al. 2015). As a consequence, tremendous efforts and resources have been devoted to gaining a better understanding of its biology, molecular biology, and epidemiology. Among other significant breakthroughs, Koch's postulates were successfully demonstrated for GRBV (Fuchs et al. 2015), the virus was detected in a *Vitis vinifera* herbarium sample archived since 1940 (Al Rwahnih et al. 2015) and in free-living vines proximal to commercial vineyards (Bahder et al. 2016b; Perry et al. 2016), and the three-cornered alfalfa hopper (*Spissistilus festinus* (Say)) was identified as its bona fide vector (Bahder et al. 2016a). A combination of these layers of evidence coupled with the unique genome organization and distinct phylogeny of GRBV isolates prompted the establishment of a new *Grablovirus* genus in the family *Geminiviridae* (Varsani et al. 2017) and its subsequent ratification by the ICTV (Adams et al. 2017). Because GRBV is the type and only member of this new genus, Varsani et al. (2017) proposed a <80% genome-wide pairwise identity as the species demarcation threshold for grabloviruses, pending when additional full genome sequences of related viruses are determined. The reliability of such a genome-wide pairwise-identity-based classification system is dependent upon the depth of available sequence data to rationally distinguish species and strains. Thus, the identification and characterization of WVV1 (Perry et al. 2018) and PrGVA (this study) provide opportunity for a better refinement of the *Grablovirus* taxonomy. Using the genome-wide pairwise-identity approach and recommended suite of programs (Muhire et al. 2013), a quick analysis of a total of 81 complete genome sequences of grabloviruses (GRBV = 53, WVV1 = 9, and PrGVA = 19) resulted in a pairwise identity scores distribution with notable "peaks" at approximately 68 to 69, 73 to 74, and 92 to 100% and clear "valleys" at approximately 70 to 72 and 75 to 91% (Fig. 4). Taking into consideration the assertion that placing the species demarcation threshold within one of the valleys would yield a classification scheme with minimal conflicts whereas placing it at one of the peaks would result in a scheme with maximal conflicts (Varsani et al. 2014), it seems rational to propose a species demarcation threshold of >72% genome-wide pairwise identity for grabloviruses. This threshold will need to be revisited as more *Grablovirus* sequences become available. In addition, this threshold should be considered along with one or more of other criteria such as (i) similarities in the genome organization of the putative member with existing grabloviruses, (ii) close phylogenetic relationship relative to members of other *Geminiviridae* genera (Fig. 3), (iii) similarity in number and arrangement of encoded genes (Fig. 2; Table 3), and (iv) high levels of CP- and Rep-associated protein nucleotide and amino acid identities (Table 3).

On the origin of grabloviruses, three hypotheses were proposed by Cieniewicz et al. (2017) for GRBV, its type species. The first postulated a global distribution for GRBV, with implications for its possible evolution from any of the viticultural regions of the world. The second proposed a North American origin for GRBV and its subsequent spread to other viticultural regions via movement of contaminated vegetative cuttings. In the third hypothesis, GRBV or its progenitor are thought to have originated from a yet-to-be-identified host from which it moved between hosts via possible arthropod

Table 3. Complete genome and gene-specific nucleotide (nt) and amino acid (aa) sequence comparisons among one definitive and two putative *Grablovirus* species

Region (nt/aa)	Grablovirus ^a	Length (nt/aa)	ID (nt/aa) (%)
Genome	PrGVA	3,175	
	GRBV	3,206	73–74
	WVV1	3,237	69
V2	PrGVA	531/176	
	GRBV	522/171	60–62/47
	WVV1	564/187	48/39
V1/CP	PrGVA	675/224	
	GRBV	678/225	76–77/83–85
	WVV1	672/223	70/78
V3	PrGVA	489/162	
	GRBV	375/124	58–59/49
	WVV1	372/123	44/34
C3	PrGVA	477/158	
	GRBV	480/159	36–38/9–12
	WVV1	465/154	73/59
C1	PrGVA	789/262	
	GRBV	786/261	72–74/63
	WVV1	795/264	68/58
C2	PrGVA	396/131	
	GRBV	450/149	33–35/8–9
	WVV1	483/160	60/64
C1:C2	PrGVA	978/325	
	GRBV	975/324	75/74
	WVV1	984/327	70/68

^a PrGVA = prunus geminivirus A, GRBV = grapevine red blotch virus, and WVV1 = wild vitis virus 1.

Fig. 3. Unrooted neighbor-joining phylogenetic trees reconstructed from the alignment of **A**, the complete genome nucleotide sequences as well as **B**, amino acid sequences of coat protein (CP) and **C**, rep-associated protein (Rep) of *Prunus geminivirus A* (PrGVA) and representative members of the family *Geminiviridae*. The trees were constructed using the MEGA7 program (Kumar et al. 2016) and percent bootstrap values (1,000 replicates) are indicated at the branch internodes. AGV = Apple geminivirus, BCTV = Beet curly top virus, HrCTV = Horseradish curly top virus, SCTAV = Spinach curly top Arizona virus, BCTIV = Beet curly top Iran virus, TCTV = Turnip curly top virus, ECSV = Eragrostis curvula streak virus, WDV = Wheat dwarf virus, MSV = Maize streak virus, PanSV = Panicum streak virus, TPCTV = Tomato pseudo-curly top virus, GRBV = Grapevine red blotch virus, FbSLSV, French bean severe leaf curl virus, EcmLV = Euphorbia caput-medusae latent virus, GGVA = grapevine geminivirus A, MMDaV = Mulberry mosaic dwarf-associated virus, CCDaV = Citrus chlorotic dwarf-associated virus, TLCCNV = Tomato leaf curl China virus, ELCV = Euphorbia leaf curl virus, ToLCMGV = Tomato leaf curl Madagascar virus, EACMV = East African cassava mosaic virus, TYLCCNV = Tomato yellow leaf curl China virus, AYV = Ageratum yellow vein virus, and ToLCHaV = Tomato leaf curl Hainan virus.

vectors capable of feeding on these hosts. These hypotheses could readily be extended to the genus given the considerable relationship between GRBV, PrGVA, and WVV1. On the one hand, the combination of recent detection of the GRBV in free-living vines

Table 4. Prevalence and species distribution of *Prunus geminivirus A* (PrGVA) in the National Clonal Germplasm Repository collection, Winters, CA^a

Crop, species	Number tested	Number infected	Positive (%)
Plum			
<i>Prunus americana</i>	3	0	0.0
<i>P. bokhariensis</i>	3	2	66.7
<i>P. cerasifera</i>	2	1	50.0
<i>P. domestica</i>	20	11	55.0
<i>Prunus</i> hybrid	4	3	75.0
<i>P. maritima</i>	1	1	100.0
<i>P. munsoniana</i>	1	0	0.0
<i>P. salicina</i>	10	7	70.0
<i>P. simonii</i>	1	1	100.0
<i>Prunus</i> spp.	6	2	33.3
<i>P. spinosa</i>	1	1	100.0
Apricot			
<i>P. armeniaca</i>	42	32	76.2
<i>Prunus</i> hybrid	1	0	0.0
<i>P. mandshurica</i>	1	1	100.0
<i>P. mume</i>	5	1	20.0
Cherry			
<i>P. avium</i>	20	0	0.0
<i>P. cerasus</i>	10	2	20.0
<i>P. mahaleb</i>	1	0	0.0
<i>P. serotina</i>	1	0	0.0
<i>Prunus</i> spp.	1	0	0.0
<i>P. tomentosa</i>	1	1	100.0
<i>P. virginiana</i>	1	0	0.0
<i>Prunus</i> × <i>yedoensis</i>	1	0	0.0

^a In all, 20 almond and 58 peach trees were also screened for PrGVA but all tested negative for the virus.

proximal to commercial grapevine red blotch disease-affected vineyards in California (Perry et al. 2016), the North American origin of all characterized GRBV isolates to date, and the characterization of WVV1 from noncultivated grapevine (Perry et al. 2018) would favor the second hypothesis. Moreover, the discovery of PrGVA and its identification in several exotic *Prunus* accessions sourced from countries outside North America would favor the first hypothesis. Regardless of the geographical origin of these viruses, the discovery of WVV1 (Perry et al. 2018) and PrGVA (this study) favors the argument of a plausible evolution of grabloviruses from non-*V. vinifera* hosts and subsequent host jump of these viruses into cultivated hosts enabled by polyphagous arthropod vectors. The future identification of insect vectors for WVV1 and PrGVA and further studies on the ecology of all three grabloviruses will shed more light on the evolutionary pathway of members of this genus.

Graft transmissibility is a key feature of plant viruses in establishing disease etiology (Rowhani et al. 2005). Transmission of PrGVA was verified by grafting chip buds of three diseased source vines on test plants of GF 305 maintained under greenhouse conditions.

Systemic movement of the virus into the leaves distal to the grafted sites was detected at 6 months after inoculation by PCR assays. However, the grafted plants did not show any visible virus symptoms, similar to the source FT7 plant. Given the relationship of PrGVA to GRBV, it will be interesting to investigate the response of grapevine to this novel *Grablovirus* sp. enabled by the development of infectious clones for PrGVA. Furthermore, the investigation of a potential vector for PrGVA will shed further light on salient aspects of its biology and epidemiology.

In conclusion, this study reports the detection and characterization of PrGVA, a graft-transmissible putative *Grablovirus* spp. infecting *Prunus* spp. This discovery also represents the first identification of a geminivirus from *Prunus* spp. From a practical standpoint, the qPCR assay developed in this study will be useful for investigating the incidence, spread, and epidemiology of PrGVA across *Prunus* orchards worldwide.

Fig. 4. Distribution of pairwise identities of comparisons among complete genome sequences of 81 isolates of three grabloviruses (*Grapevine red blotch virus* = 53, Wild vitis virus 1 = 9, and *Prunus geminivirus A* = 19). Pairwise identities were calculated using SDT v1.2 (Muhire et al. 2014) with the MUSCLE-alignment option. Percent pairwise identities are indicated on the horizontal axis while the proportions of pairwise identities are shown on the vertical axis. According to Muhire et al. (2013), the valleys between peaks in the plot indicate percent pairwise identities that would make relatively conflict-free pairwise-identity-score-based taxonomic demarcation thresholds.

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