Phylogeny of Geminivirus Coat Protein Sequences and Digital PCR Aid in Identifying *Spissistilus festinus* as a Vector of Grapevine red blotch-associated virus

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ABSTRACT

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Grapevine red blotch-associated virus (GRBaV) is a single-stranded DNA virus, proposed to be a member of the family *Geminiviridae*, and is associated with grapevines showing red blotch symptoms in North America. The existence of the virus was reported in 2012, and subsequently detected in grapevines in major grape production regions. We investigated if a vector exists that can transmit GRBaV in vineyards. Phylogenetic analysis of the predicted amino acid sequence of coat protein (CP) of GRBaV with the CP of 23 geminiviruses representing all seven genera of the family *Geminiviridae* revealed that GRBaV-CP was most similar to that of *Tomato pseudo-curly top virus*, a geminivirus known to be transmitted by a treehopper (Membracidae), a family that is closely related to leafhoppers (Cicadellidae). To identify vectors of GRBaV, hemipteran species within and nearby wine grape vineyards where virus spread was suspected were collected and transmission assays were conducted. Among the species tested, the three-cornered alfalfa hopper *Spissistilus festinus* (Hemiptera: Membracidae) was able to both acquire the virus from a grapevine infected with GRBaV and transmit the virus to healthy grapevines in the laboratory. In commercial vineyards, lateral shoots of grapevines girdled due to feeding injury by the adult three-cornered alfalfa hopper also tested positive for the virus using digital PCR. These findings represent an important step in understanding the biology of GRBaV and develop management guidelines.

Additional keywords: *Erythroneura elegantula*, *E. variabilis*, *E. ziczac*.

Grapevine red blotch disease (GRBD) is a recently recognized disease of grapevines (Calvi 2011; Sudarshana and Fuchs 2015; Sudarshana et al. 2015). Symptoms of GRBD include reddening of primary, secondary, and tertiary veins as well as a red blotch pattern on the leaf surface in red-fruited varieties. In white-fruited varieties, symptoms include irregular chlorosis on leaf blades that may become necrotic in the autumn months (Sudarshana et al. 2015). Disease symptoms may resemble those of grapevine leafroll disease (GLD) and GRBD has also been referred to in the literature as simply a red leaf disease (Calvi 2011; Poojari et al. 2013).

Grapevine red blotch-associated virus (GRBaV) is a DNA virus that was discovered in symptomatic grapevines in California using a metagenomic approach (Al Rwahnih et al. 2012; Al Rwahnih et al. 2013). This virus has also been referred to as Grapevine Cabernet franc-associated virus (Krenz et al. 2012); Grapevine red leaf-associated virus (Poojari et al. 2013), and Grapevine geminivirus (Seguin et al. 2014). Despite its slightly larger genome size (~3,206 nucleotides), GRBaV shares several features of monopartite geminiviruses and has been proposed to be a member of the family *Geminiviridae*. Even though the virus was first reported from field samples in 2012, it was detected in herbarium specimens archived as early as 1940 (Al Rwahnih et al. 2015a).

Negative impacts of GRBD on wine grape quality have been reported (Calvi 2011; Poojari et al. 2013). Because of the potential economic impact of the disease and the presence of GRBaV in grapevines previously thought to be affected by leafroll disease but tested negative for all known leafroll-associated viruses (Sudarshana et al. 2015), GRBaV has become a major concern for wine grape producers in the United States. This virus also has been found in table grape accessions at the National Clonal Germplasm Repository in Winters, CA (Al Rwahnih et al. 2015b), but its significance to table grape producers is not known. Recently, GRBaV was detected in wild grapes relatively close to vineyards affected by GRBD in California, indicating the likelihood of plant-to-plant spread in nature (Bahder et al. 2016; Perry et al. 2016).

Members of the family *Geminiviridae* are transmitted by hemipteran insects. While members of the largest genus *Begomovirus* are transmitted by whiteflies (*Bemisia tabaci*, family Aleyrodidae), members of the other genera are transmitted by leafhoppers (Cicadellidae) and in one case, a treehopper (Membracidae) (Briddon et al. 1996). The only other geminivirus-like DNA virus that infects a woody plant and is known to exhibit horizontal transmission is *Citrus chlorotic dwarf-associated virus*, transmitted by the whitefly *Parabemisia myricae* (Loconsole et al. 2012). For GRBaV, Poojari et al. (2013) implicated the Virginia creeper leafhopper, *Erythroneura ziczac* Walsh, as a vector. However, the lack of reports of GRBaV spread in other regions of North America besides California, where *E. ziczac* is well-established together with the propensity of *E. ziczac* to feed on mesophyll, and the phloem limited nature of monopartite geminiviruses, suggests the more likely possibility for other insect species to transmit GRBaV under field conditions. *E. elegantula* Osborn and

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*The *e-X*tra logo stands for "electronic extra" and indicates that one supplementary figure and one supplementary table are published online.

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E. variabilis Beamer, two other leafhopper species in the same genus as *E. ziczac*, are widespread in California vineyards, but their ability to transmit GRBaV has not been evaluated.

The primary objective of this study was to evaluate the ability of the three leafhopper species, *E. elegantula*, *E. variabilis*, *E. ziczac*, and other hemipteran insects that are commonly found in California vineyards to transmit GRBaV under greenhouse conditions.

MATERIALS AND METHODS

Evaluation of potential hemipteran vectors. To evaluate potential vectors of GRBaV, a phylogenetic analysis was conducted on the amino acid sequence of the coat protein (CP) of 23 geminiviruses from the seven genera of the family *Geminiviridae* (Supplementary Table S1) to determine the relationship of GRBaV to other geminiviruses. Sequence data were aligned using MEGA6 (Tamura et al. 2013) and the phylogenetic tree was constructed using the neighbor-joining method.

Plant material and virus isolates. Potted plants of cultivar Cabernet Sauvignon (CS) grafted on rootstock Freedom (courtesy Casa Cristal Nursery, Inc., Delano, CA) were used for insect colony establishment and as recipient plants in transmission assays. These plants were maintained in a greenhouse under a 16 h photoperiod at 26°C. Virus-infected source material used for virus acquisition were propagated from grapevines infected with the isolate CF214-1 (NC022002.1) (Al Rwahnih et al. 2013). This isolate represents clade II, one of two common GRBaV variants (Al Rwahnih et al. 2015b; Krenz et al. 2014). All GRBaV-free plants and insect colonies designated for transmission assays were maintained in insect-proof cages (MegaView Science, Taipei, Taiwan) in a greenhouse separate from that used to maintain virus-infected source plants.

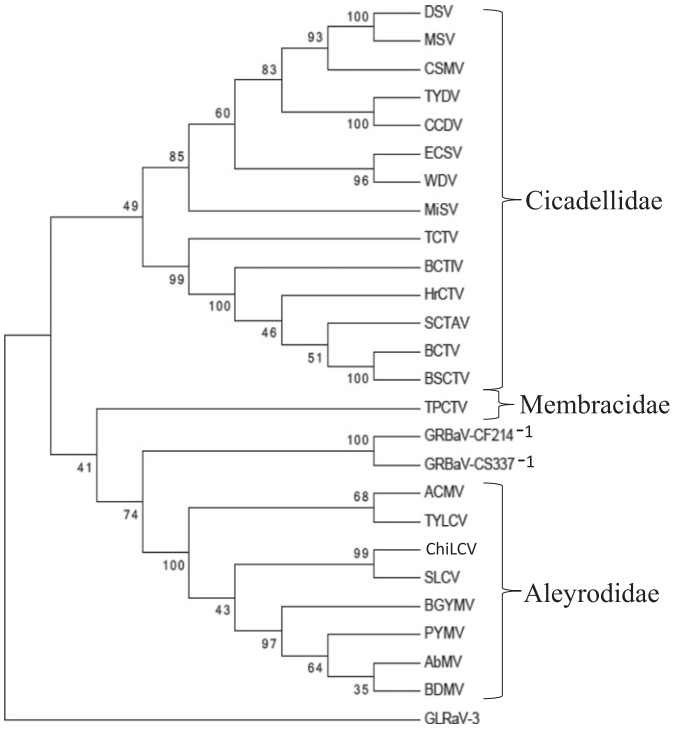

Fig. 1. Neighbor-joining tree indicating the relationship of amino acid sequence of coat protein (CP) of geminiviruses with Grapevine red blotch-associated virus (GRBaV). Isolates GRBaV-CF214-1 and GRBaV-CS337-1 represent two clades of GRBaV. CP of *Grapevine leafroll-associated virus 3* (GLRaV-3) was used as an outlier. Other viruses in the tree are as follows: *Abutilon mosaic virus* (AbMV), *African cassava mosaic virus* (ACMV), *Bean dwarf mosaic virus* (BDMV), *Bean golden mosaic virus* (BGYMV), *Beet curly top Iran virus* (BCTIV), *Beet curly top virus* (BCTV), *Beet severe curly top virus* (BSCTV), *Chickpea chlorotic dwarf virus* (CpCDV), *Chili leaf curl virus* (ChiLCuV), *Chloris striate mosaic virus* (CSMV), *Digitaria streak virus* (DSV), *Eragrostris curvula streak virus* (ECSV), *Horseradish curly top virus* (HrCTV), *Maize streak virus* (MSV), *Micanthus streak virus* (MiSV), *Potato yellow mosaic virus* (PYMV), *Spinach curly top Arizona virus* (SCTAV), *Squash leaf curl virus* (SLCV), *Tobacco yellow dwarf virus* (TYDV), *Tomato pseudo-curly top virus* (TPCTV), *Tomato yellow leaf curl virus* (TYLCV), *Turnip curly top virus* (TCTV), and *Wheat dwarf virus* (WDV).

Nucleic acid extraction and virus detection; standard PCR, qPCR, and digital PCR (dPCR) assays. Total DNA was extracted from insects and plants using the DNeasy Blood & Tissue Kit and DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA), respectively. For cohorts of recipient plants being extracted together, cross-sections of petioles were taken from each of the 15 leaves until approximately 100 mg of tissue was obtained.

Standard PCR assays to verify GRBaV in source plants and final tests on recipient plants were performed in 25-µl reactions using primers GVGF1 and GVGR1 as described previously (Al Rwahnih et al. 2013). The amplicons were visualized by running 2 µl of the PCR product on a 1% agarose gel. Amplicons of the expected size (556 bp) were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and sequenced using primers GVGF1 and GVGR1 at the DNA sequencing facility, University of California, Davis. The identity of the sequenced products was determined by BLAST searches against the GenBank database.

Detection of GRBaV by qPCR was performed in 20 µl reactions comprised of 1 µl of DNA extract, 0.15 µM of each of the primers GVGF1 and GVGR1, 2% polyvinyl pyrollidone-40, 0.3 µl of Stratagene Reference Dye (1:500), 10 µl of SoFast EvaGreen Supermix (Bio-Rad Inc., Hercules, CA) with the remaining volume made up with nuclease free water. Thermal cycling conditions were as follows: initial denaturation at 95°C for 2 min; 35 cycles of 95°C for 30 s, 62°C for 1 min, and 72°C for 1.5 min; on a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific Inc., Waltham, MA). Following amplification, high-resolution melting analysis was performed to confirm identity of the product amplified. Conditions for melting point analysis were 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s with continuous measurement of fluorescence. Samples were scored positive if a measureable cycle threshold (Ct) value was generated and the amplicon had a melting temperature comparable to that produced by a cloned GRBaV fragment (GVGF1/GVGR1 product) and a positive control source plant. The same procedure was used to detect GRBaV in insect specimens.

The dPCR assays were performed on plant cohorts at monthly intervals using TaqMan assays on a QuantStudio 3D Digital PCR System (www.thermofisher.com/us/en/home.html). The assays were performed using 6 µl of DNA template and 9.5 µl of reaction mixture (76.3% QuantStudio 3D Master Mix, 7.6% TaqMan Assay, and remaining volume made up with DEPC-treated water). The TaqMan probe used in the assay is 5′-FAM -AGA ACT GAA GTT GAA GAA TT-3′ and primers 5′-AAG AAT TGC ATT GAC TGA ACC TGA-3′ (forward) and 5′-CCT AGC TCC AGG TCC AGA CG-3′ (reverse). The reaction mix was loaded on a chip using QuantStudio 3D Digital PCR Chip Loader and run on a ProFlex Base PCR System. Thermal cycling conditions consisted of an initial denaturation at 95°C for 2 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. End point fluorescence data were collected and analyzed using QuantStudio 3D AnalysisSuite Cloud Software (https://www.thermofisher.com/us/en/home/life-science/pcr/digital-pcr/quantstudio-3d-digital-pcr-system/quantstudio-3d-software.html). The FAM calculated threshold for establishing positive fluorescence reactions was set at 3,000 relative fluorescence units based on a positive control, a cloned fragment of GRBaV genome, at a concentration of 10 copies/µl.

Insect collection, colony establishment, and test for virus in insects. Vineyard plots where GRBaV-infected grapevines were present and disease incidence was observed to increase over time, as well as vineyards where no noticeable increase in GRBD incidence was observed, were sampled over a 2-year period from March 2014 to November 2015. Yellow sticky traps were deployed and sampled weekly throughout the study period to determine what insect species

were present at each field site. Sweep-netting was also conducted biweekly to collect live individuals for colony establishment.

Live insect specimens collected from vineyards where GRBD was not observed were reared on grapevines, tested to be free from GRBaV in a greenhouse at 26°C under a 16 h photoperiod until used in transmission bioassays. Following failed attempts to rear *S. festinus* on Cabernet Sauvignon grapevines, *S. festinus* insects were collected from alfalfa fields on the University of California (UC)-Davis Agronomy Farm using sweep nets and maintained on alfalfa grown from seeds (AMERISTAND 901TS) in the greenhouse to establish a permanent colony of *S. festinus*. Subsets of 20 live individuals for each insect species were exposed to GRBaV-infected grapevines for 48 h and subsequently tested for the presence of the virus.

Collection of girdled grapevine shoots in vineyards. Grapevines in vineyards with apparent spread exhibited feeding damage in the form of girdled petioles and stems. A set of 10 girdled regions were sampled from one vineyard that had recent feeding damage and where the girdled region had not yet become necrotic, and were subsequently evaluated for the presence of GRBaV by dPCR.

Virus transmission assays. For all transmission assays, insects were exposed to GRBaV-infected source plants for an acquisition access period of 48 h and 15 individuals of each species were subsequently transferred to individual healthy recipient CS plants for an inoculation access period of 48 h. For each species, five individuals were moved directly from proven healthy plants to healthy recipient plants to serve as controls. After 48 h of feeding on recipient plants, insects were collected and preserved in 85% ethanol at room temperature to serve as vouchers.

Transmission efficiency of the insects was assessed by extracting DNA from the recipient plants and testing for GRBaV using dPCR at 2 weeks, and then at monthly intervals for up to 5 months postinoculation. To test for the presence of GRBaV in the recipient plants, single new-growth/terminal leaves of all plants were collected and pooled into a composite sample for analysis. DNA was extracted and tested for the presence of GRBaV using dPCR. When a composite sample tested positive for GRBaV, all the plants within that composite were tested individually at 5 months postinoculation using standard PCR to determine the number of plants infected by GRBaV. Amplicons from individual recipient plants testing positive for GRBaV were sequenced at the UC-Davis DNA sequencing facility to verify the nucleotide identity of the GRBaV isolates in the recipient plants.

RESULTS

Evaluation of potential vectors. Based on the neighbor-joining tree produced using CP amino acid sequences of geminiviruses representing all seven genera of the family *Geminiviridae*, GRBaV

Fig. 2. *Erythroneura* leafhoppers evaluated for their ability to transmit Grapevine red-blotch associated virus: *Erythroneura elegantula* (left), *E. variabilis* (middle), and *E. ziczac* (right).

TABLE 1. Status of grapevine red blotch-associated virus (GRBaV) spread in vineyards in California counties and occurrence of candidate insect vectors

Site	County	Spread of GRBaV in vineyard	*Spissistilus festinus*	*Erythroneura elegantula*	*E. variabilis*	*E. ziczac*
1	Amador	+	+	+	+	+
2	Mendocino	+	+	+	+	+
3	Napa	+	+	+	+	−
4	Napa	−	−	+	+	−
5	Napa	−	−	+	−	−
6	Napa	+	+	+	+	−
7	Sacramento	−	−	+	+	+

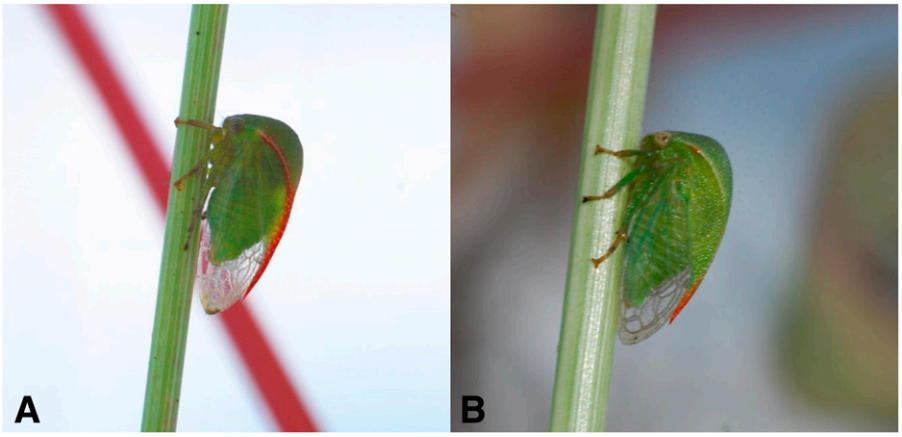

Fig. 3. *Spissistilus festinus* adult **A,** male and **B,** female feeding on alfalfa plants.

was most closely related to *Tomato pseudo-curly top virus* (TPCTV) (Fig. 1). The vector of TPCTV is the nightshade treehopper (*Micrutalis malleifera*, family Membracidae). The phylogenetic tree presented in Figure 1 shows four clades, with the top clade containing geminiviruses transmitted by leafhoppers (Cicadellidae), the two middle clades containing TPCTV, vectored by a membracid, and GRBaV. The last clade contains geminiviruses transmitted by whiteflies and have bipartite genomes with the exception of *Tomato yellow leaf curl virus*, which has a monopartite genome. These data show geminiviruses are transmitted by hemipteran insects that belong to either the Auchenorrhyncha (leafhoppers and treehoppers) or Sternorrhyncha (whiteflies) and GRBaV belongs to a clade of geminiviruses that is closest to a clade transmitted by Auchenorrhyncha.

Insect collection, colony establishment, and virus detection after feeding. The three *Erythroneura* leafhoppers of interest here (Fig. 2) were collected from vineyards and reared on healthy Cabernet Sauvignon vines in the greenhouse. Both *E. elegantula* and *E. variabilis* were common in most of the vineyard sites that were sampled over a 2-year period, while *E. ziczac* was found in three of these vineyards (Table 1). However, a membracid, *Spissistilus festinus* (Say) (Fig. 3) was found in all vineyards where virus spread was suspected, and absent from vineyards where there was no evidence of spread. Live specimens of *S. festinus* were collected from vineyards with no incidence of GRBaV and were reared on healthy grapevines for colony establishment, but *S. festinus* was unable to reproduce on them.

When testing for the presence of GRBaV in insects after feeding on GRBaV-infected plants, the three leafhopper species and *S. festinus* all tested positive, with *S. festinus* providing the lowest Ct values (higher virus concentration) as well as highest percentage (75%) of positive individuals (Table 2). None of the species exposed to healthy grapevines tested positive for GRBaV. All three *Erythroneura* species that tested positive for GRBaV but had much higher Ct values (lower viral titer) and a lower percentage of positive individuals (Table 2).

Lateral shoots of grapevines that had girdling injury caused by *S. festinus* (Fig. 4) at vineyards with suspected spread of GRBaV also tested positive using dPCR. Out of the 10 shoot samples tested, two had weak, but detectable levels of GRBaV at the injured site (Fig. 5).

Transmission assays. All composite samples of recipient plants from transmission assays for the three *Erythroneura* species did not test positive for GRBaV throughout the study period using dPCR (Table 3). Also, all recipient plants exposed to *Erythroneura* leafhoppers tested

TABLE 2. Number of insects testing positive for the presence of Grapevine red blotch-associated virus (GRBaV) in qPCR tests after feeding on GRBaV-infected grapevine plants

Species	Number of positive/number tested	Mean cycle threshold (Ct) ± SE	Mean virus quantity/µl ± SE	Mean melting temperature (°C) ± SE
Erythroneura elegnatula	2/20	28.9 ± 0.81	1.79E + 01	83.3 ± 0.0
E. variabilis	1/20	32.1 ± 0.0	1.00E + 01	83.3 ± 0.0
E. ziczac	1/20	26.5 ± 0.0	1.55E + 01	83.3 ± 0.0
Spissistilus festinus	15/20	16.1 ± 0.34	1.86E + 05	83.3 ± 0.02
GRBaV-infected grapevine[a]	5/5	14.1 ± 0.63	1.34E + 06	83.3 ± 0.03
Healthy grapevine[a]	0/5	No Ct	N/A	52.4 ± 2.4
Buffer control[a]	0/5	No Ct	N/A	62.1 ± 5.4

[a] Plant and buffer controls used in qPCR tests.

Fig. 4. Grapevine **A,** leaf petiole and **B,** shoot girdled due to feeding injury by *Spissistilus festinus* in vineyards.

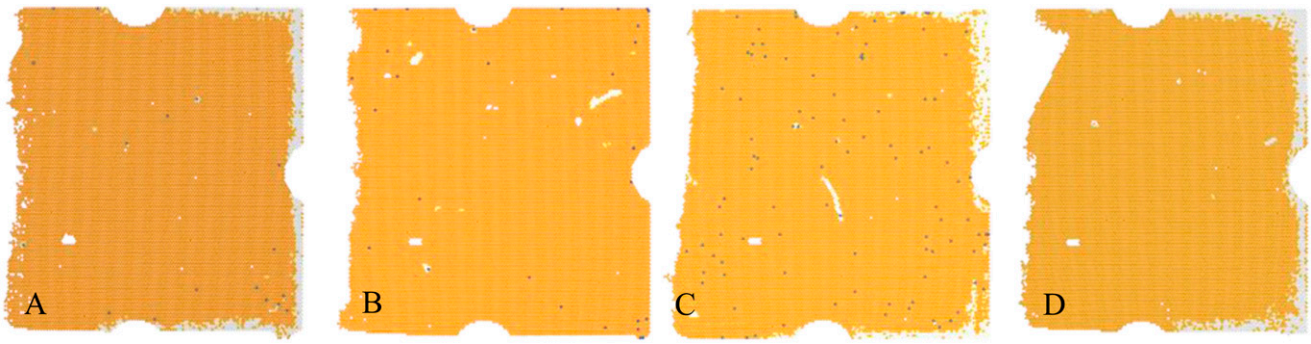

Fig. 5. Scans of chips used for digital PCR assay using QuantStudio 3D digital PCR system for the detection of Grapevine red blotch-associated virus (GRBaV) in the girdled regions of grapevines. **A and B,** Nucleic acid extracts from girdled shoot samples testing positive for GRBaV; **C,** nucleic acid extract from grapevines infected with GRBaV diluted to 0.001% of original extract; and **D,** nucleic acid extract from healthy grapevine control. Dots are reactions positive for GRBaV.

negative by standard PCR at 6 months (data not shown). Plant cohorts exposed to *S. festinus* that had been feeding on virus-infected source material began testing positive by dPCR for GRBaV at 3 months postinoculation (Table 3, Fig. 6). The percentage of positive reactions in this plant cohort increased at both 4 and 5 months postinoculation (Table 3, Fig. 6). The virus also was detectable using standard PCR at 5 months postinoculation (Fig. 7). Of the 15 receiving plants exposed to *S. festinus*, three plants were found infected with GRBaV at 5 months postinoculation, and two of these three recipient plants also exhibited mild red blotch leaf symptoms (Fig. 8). The negative control samples for *S. festinus* tested negative throughout the study period by dPCR, and all control plants tested negative using standard PCR after 5 months postinoculation (Fig. 6). The amplicons obtained from the three recipient plants matched the nucleotide sequence of the amplicon obtained from the corresponding source plant (Supplementary Fig. S1).

TABLE 3. Digital PCR results for monthly testing of recipient plant cohorts for the presence of Grapevine red blotch-associated virus

	Percentage of positive wells in dPCR assay		
Species	3 months	4 months	5 months
Erythroneura ziczac	0.0%	0.0%	0.0%
E. elegantula	0.0%	0.0%	0.0%
E. variabilis	0.0%	0.0%	0.0%
Spissistilus festinus	0.1%[a]	0.3%[b]	1.6%[c]

[a] 21/18,874 positive.
[b] 47/16,216 positive.
[c] 248/15,768 positive.

DISCUSSION

GRBaV is an emerging problem to grape production in North America (Sudarshana et al. 2015). While its exact taxonomic status has not been determined, it is most likely to be included in the family *Geminiviridae* based on the size and genome organization. Year-to-year increase in new GRBaV infections has been noticed in vineyards affected by GRBD in California (M. R. Sudarshana, *unpublished data*) indicating the prevalence of a vector that can transmit GRBaV in vineyards. The Virginia creeper leafhopper (*E. ziczac*) was reported to be a vector in greenhouse conditions (Poojari et al. 2013). However, *E. ziczac* is not widely distributed in California, and was not always found in vineyards where GRBD appeared to be spreading (Table 1). As a result, we revisited the possible transmission of GRBaV by this species and two more common *Erythroneura* leafhoppers frequently found in California vineyards, as well as by other hemipteran insects present in the vineyards where GRBaV spread was suspected.

Specificity between the CP of plant viruses and their insect vectors reflects an evolutionary history between the vector and the virus such as seen with the *Grapevine leafroll-associated virus* complex and the Coccoidea (Tsai et al. 2010). It seems likely that this would also be the case for geminiviruses, including GRBaV, and their vectors. We first examined the phylogenetic relationship of the putative CP of GRBaV with the CP of 23 geminiviruses, representing all seven genera of the family *Geminiviridae* (Fig. 1). The CP was selected because this is the protein encoded by the virus that determines vector specificity (Briddon et al. 1990). It is likely this protein interacts with a receptor protein(s) lining the insect gut

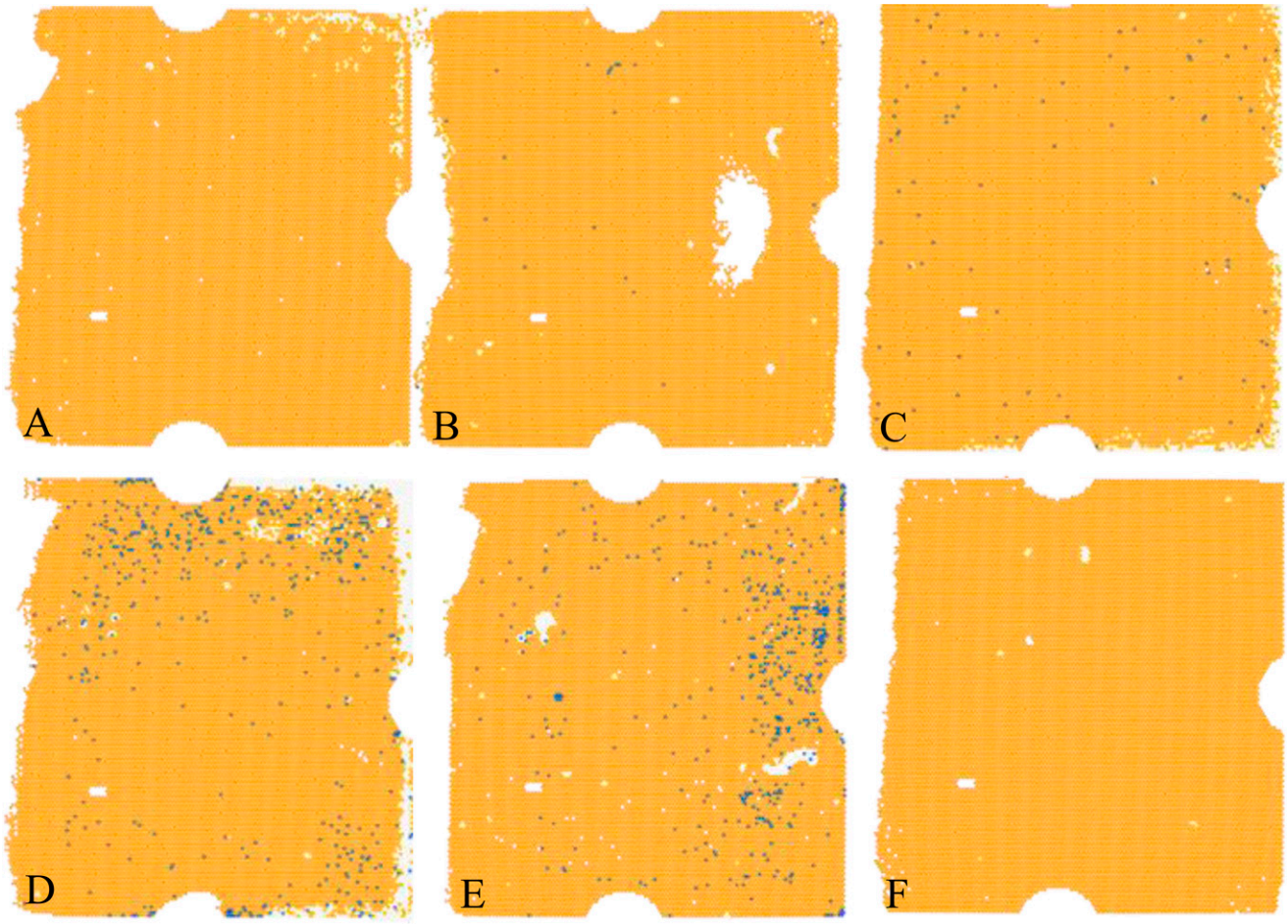

Fig. 6. Digital PCR results for cohorts of recipient plants exposed to *Spissistilus festinus*. **A,** Baseline test pre-inoculation; **B,** 3 month test; **C,** 4 month test; **D,** 5 month test; **E,** positive control diluted to 0.001% original concentration; and **F,** water control.

and salivary gland, and thus determines the specificity of insect vector–viral interactions. The close relationship of GRBaV to TPCTV based on CP amino acid sequence is consistent with findings by Varsani et al. (2014), which illustrated that GRBaV is closely related to TPCTV and belonged to the clade of geminiviruses that are vectored by auchenorrhychan insects. Based on this, it may be expected that GRBaV would be transmitted by an insect species within this suborder.

For the detection of GRBaV in receiving plants, new growth was selected because (i) the exact feeding sites could not be observed, (ii) removal of new growth relative to inoculation date would ensure inoculum was not removed before GRBaV became systemic, and (iii) the potential for contamination due to residual virions in fecal matter on the plant surface could be avoided. Adults of *S. festinus* were allowed to feed freely on recipient plants rather than being restricted to individual leaves. This is because the feeding behavior that causes girdling is a mechanism to block photosynthates and create a nutrient sink (Andersen et al. 2002) that could cause the virus to be isolated on the inoculated leaf, thus preventing it from infecting the plant systemically.

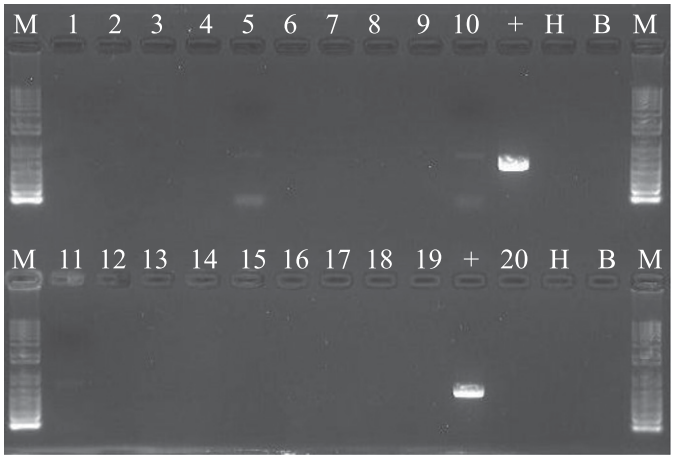

Fig. 7. Agarose gel image for recipient plants exposed to *Spissistilus festinus* at 5 months postinoculation; Lane M, 1-kb+ ladder; lanes 1 to 15, transmission assays from virus-infected plants; lanes 16 to 20, negative control transmission assays from healthy plants; lane +, positive control from virus-infected source plant; lane H, healthy control; and lane B, buffer control.

In 2013, it was reported that GRBaV was detectable in all recipient plants at 4 months postinoculation as well as in individuals of *E. ziczac* tested for the presence of GRBaV by standard PCR (Poojari et al. 2013). Five percent of *E. ziczac* in our study tested positive for GRBaV as well; however, our results did not confirm the successful transmission of GRBaV by *E. ziczac*. Regardless of percentage, detection of GRBaV in *E. ziczac* does not provide any evidence that it is a vector, merely that it was feeding on an infected plant and residual virions passing through the gut are likely what is being detected (Lett et al. 2002). Poojari et al. (2013) stated that leaves where *E. ziczac* had fed, as well as newly emerging leaves tested positive for GRBaV. Presence of GRBaV at the feeding site as reported by Poojari et al. (2013) could be explained by residual viral particles being excreted by *E. ziczac* onto the leaf surface, a phenomenon documented in the whitefly *Bemisia tabaci* and another geminivirus, *Squash leaf curl virus* by Rosell et al. (1999). We also found no evidence of GRBaV transmission by *E. elegantula* and *E. variabilis* which are more widespread in California vineyards, although very low numbers of both species tested positive for the virus.

While transmission of GRBaV under greenhouse conditions with the *Erythroneura* leafhoppers failed, we confirmed successful transmission of GRBaV by the membracid *S. festinus*. It is interesting to

Fig. 8. A, Symptomatic leaves from recipient plant testing positive for Grapevine red blotch-associated virus after exposure to viruliferous *Spissistilus festinus* and **B,** healthy leaf from negative control transmission assay.

note the close phylogenetic relationship of GRBaV with TPCTV, and the transmission of both geminiviruses by membracids. *S. festinus* is native to the southeastern United States, and a common though usually minor pest of alfalfa, soybean, peanut, and various vegetable crops (Andersen et al. 2002). Its range extends throughout the southern United States to California, but can also be found in Northeastern United States extending as far north as Canada. The greater incidence of *S. festinus* in California (Wistrom et al. 2010), relative to other major grape growing regions in North America, is consistent with the lack of GRBD spread in regions such as New York (M. Fuchs, Cornell University, NY, *personal communication*) and Washington (D. Walsh, Washington State University, WA, *personal communication*) states. The fact that *S. festinus* is native to North America and not reported from other countries where wine grapes are produced and GRBD incidence is extremely low, also supports that it is the most likely vector of GRBaV in California. Despite successful transmission of GRBaV under greenhouse conditions by *S. festinus* and its absence from regions where no spread is documented, it is not yet known if *S. festinus* is the only species capable of transmitting GRBaV, whether in the greenhouse or in a natural setting. Other species within the genus *Spissistilus* as well as other closely related taxa of treehoppers that feed on grapes may still be competent vectors as well and should be the focus of future research on the epidemiology of GRBaV. While not considered a serious pest of wine grapes, *S. festinus* is known to cause feeding damage on leaf petioles and lateral shoots that results in a characteristic girdle (Fig. 4), blocking photosynthates, and ultimately turning the affected leaf red (Smith 2013). The detection of low levels of GRBaV by dPCR in girdled regions from vineyards exhibiting GRBD supports the hypothesis that *S. festinus* may be responsible for spread of the virus under field conditions. That only low levels of virus was detected in girdled areas of the petiole is not surprising because once virus is released into the phloem, virus particles that reach the sieve tubes would likely be quickly translocated through the phloem network, and the damaged tissue associated with the girdling might fail to support virus replication.

The ability of dPCR technology to detect extremely low amounts of virus greatly assisted in demonstrating GRBaV transmission by *S. festinus*. While this technology is just beginning to gain entry in plant virus diagnostics, it is becoming increasingly popular in the detection of human viral pathogens (Sedlak et al. 2014; Strain et al. 2013). dPCR has also been found to be more sensitive than qPCR in the detection of *Human immunodeficiency virus* and it can provide absolute quantitation without a need for standards (Strain et al. 2013).

Based on our results, *S. festinus* is a vector of GRBaV under strict laboratory conditions with some evidence to support its transmission in the field. Conversely, under the same conditions, *E. elegantula*, *E. variabilis*, and *E. ziczac* failed to successfully transmit GRBaV and could not be confirmed as vectors of GRBaV. These findings represent an important step in the understanding of the epidemiology of red blotch disease, and serve as a baseline for approaching management of the pathogen in the environment. Future studies to provide direct evidence for transmission of GRBaV by *S. festinus* under field conditions and understand the biology of *S. festinus* behavior in vineyards are needed to develop successful GRBD management programs.

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