Grapevine red blotch-associated virus is Present in Free-Living *Vitis* spp. Proximal to Cultivated Grapevines

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ABSTRACT

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Red blotch is an emerging disease of grapevine associated with grapevine red blotch-associated virus (GRBaV). The virus spreads with infected planting stocks but no vector of epidemiological significance has been conclusively identified. A vineyard block of red-blotch-affected *Vitis vinifera* ‘Cabernet franc’ clone 214 was observed in California, with a clustering of infected, symptomatic vines focused along one edge of the field proximal to a riparian habitat with free-living *Vitis* spp. No genetic heterogeneity was

observed in a 587-nucleotide region of the GRBaV genome in a population of 44 Cabernet franc clone 214 isolates. By contrast, genetic differences were observed in isolates from other cultivars and clones growing in adjacent blocks. GRBaV was confirmed infecting four free-living vines, two of which were shown to be *V. californica* × *V. vinifera* hybrids. The genomes of three free-living GRBaV vine isolates and seven from *V. vinifera* cultivars were compared; free-living vine isolates were shown to be more similar to each other and a ‘Merlot’ isolate than to the other cultivated vine isolates. The finding that GRBaV is present in free-living *Vitis* spp. indicates the virus can be spread by natural (nonhuman-mediated) means, and we hypothesize that in-field spread of GRBaV is occurring.

Grapevine red blotch is an emerging disease of grapevine in North America. The disease was first observed in 2008 in Napa Valley of California and described as a “red-leaf” disease, with symptoms resembling leafroll but testing negative for leafroll viruses (Calvi 2011).

Grapevine red blotch-associated virus (GRBaV), a gemini-like virus, was subsequently shown to be present in infected vines (Al Rwahnih et al. 2012; Krenz et al. 2012; Poojari et al. 2013), and inoculation experiments with GRBaV resulted in red blotch leaf symptoms, indicating that this is the causal agent of the disease (Fuchs et al. 2015). Many GRBaV-infected red cultivars of grapevine exhibit fairly distinctive leaf-reddening symptoms but, because these symptoms may also result from other disorders (e.g., nutrient deficiencies, infection by other viruses, or crown gall), the presence of GRBaV must be confirmed by nucleic acid assays (Sudarshana et al. 2015). GRBaV is widely distributed in grape-growing regions of the United States and has significantly compromised production (Krenz et al. 2014).

The single-stranded DNA genome of GRBaV resembles that of members of the family *Geminiviridae*. The single component genome of approximately 3,206 nucleotides (nt) encodes an estimated three proteins in the virion sense and three in the complementary sense (Krenz et al. 2012). A putative coat protein (CP)-encoding open reading frame (ORF) has been identified based on its position in the genome and a limited resemblance to other geminivirus CP genes but, thus far, neither the CP nor virions have been observed in infected plants. From the 16 sequenced genomes of GRBaV, only a limited amount of genetic variability has been observed (Al Rwahnih et al. 2015; Sudarshana et al. 2015). Phylogenetic analyses show isolates clustering into two clades. Members of clade 1 show

up to 5% nucleotide variation, and putative clade 1 recombinants have been described (Krenz et al. 2014). Members of clade 2 are more homogeneous, with a maximum of 2% nucleotide variation. The nucleotide variation between clades ranged from 7 to 9%, consistent with intraspecies variation within the family *Geminiviridae* (Varsani et al. 2014). An important consideration for the biology of GRBaV is that well-described members of the family *Geminiviridae* are transmitted by arthropod vectors, typically whiteflies or leafhoppers.

Two vexing questions surrounding this disease are: how does the virus spread and what is the origin of the virus? The widespread geographical detection of the virus in North America in a very short period (2012 to 2015) is most easily explained by its movement with propagation material (Sudarshana et al. 2015), and it has been found in certified planting stocks (Stamp and Wei 2014). The extent to which the virus is able to spread within and between vineyards remains unanswered, although observations of the spatial and temporal distribution of symptomatic vines in vineyards are consistent with vector-associated or other types of spread (Calvi 2011; Perry et al. 2015). Elucidating the origin of a virus such as GRBaV is problematic, because information on ancestral viruses and hosts is often lacking. *Vitis* spp. are the only known hosts for GRBaV, and there are no closely related viruses (Krenz et al. 2014; Sudarshana et al. 2015). Although recognition of red blotch as a disease is recent, it is likely that it has been present for some time but overlooked because the disease is confused with grapevine leafroll. The detection of GRBaV in an archival sample dating from 1940 is consistent with this notion (Al Rwahnih et al. 2015).

Given that GRBaV infects cultivated *Vitis* spp., it would seem likely that it would be able to infect free-living (noncultivated) grapevines, at least experimentally. From a disease-management perspective, free-living vines could serve as reservoirs for the pathogen. A well-recognized example of this is seen in the epidemiology of Pierce’s Disease, caused by the bacterial pathogen *Xylella fastidiosa*. This pathogen persists in California grapevine (*Vitis californica*) (and

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other hosts) bordering vineyards and is transmitted by arthropods (sharpshooters and spittlebug) to adjacent cultivated vines (Baumgartner and Warren 2005). A number of viruses have also been observed to be present in free-living vines adjoining commercial vineyards. *Grapevine leafroll-associated virus-2* (GLRaV-2), *Grapevine leafroll-associated virus-3* (GLRaV-3), *Grapevine virus A*, and *Grapevine virus B* have all been detected in *V. californica* and *V. californica* × *V. vinifera* hybrids in California, and the latter three viruses were thought to have been vector transmitted into nearby vineyards (Klaassen et al. 2011). By contrast, although GLRaV-2, GLRaV-3, and *Grapevine fleck virus* were shown to be present in cultivated vines and GLRaV-3 was seen in mealybug vectors in Virginia, the viruses were not detected in wild vines proximal to vineyards (Jones et al. 2015). In a survey of native *V. aestivalis* in the eastern United States, previously undescribed enamovirus-like and oryzavirus-like viruses were observed, although these viruses have not been reported from cultivated vines (Ghanem-Sabanadzovic and Sabanadzovic 2010). The epidemiology of grapevine viruses may differ in the eastern United States relative to western states.

In 2012, a vineyard in California showed an unusual apparent clustering of vines with severe red blotch. The number of symptomatic vines was greatest along one edge of the block, proximal to an uncultivated riparian habitat that included free-living vines (*Vitis* spp.). Focusing on a subsection of the vineyard, we mapped the distribution of GRBaV-infected vines and performed a genetic analysis of vines from within and proximal to this field. Our objectives in this study were threefold: to (i) assess the genetic diversity of GRBaV isolates from vines within a single block of the same cultivar and clonal line, (ii) determine whether GRBaV was present in free-living vines proximal to a vineyard with GRBaV-infected vines, and (iii) determine the relatedness of any GRBaV isolates from free-living vines and those present in an adjacent vineyard.

MATERIALS AND METHODS

Vineyard blocks, sampling, nucleic acid extraction, and identification of grapevines. Vines in adjacent blocks of ‘Cabernet franc’, ‘Cabernet Sauvignon’, and ‘Merlot’ were sampled at a single vineyard site in the Napa Valley of California. Three to five leaves, each collected from separate canes of a single vine, were pooled and processed for nucleic acid extraction (Krenz et al. 2014). Testing of vines for the presence of GRBaV followed the multiplex polymerase chain reaction (PCR)-based diagnostic methods described by Krenz et al. (2014). Vines scored as negative indicated that no virus-specific product was observed. Vines scored positive indicated that both the CP gene and replicase (Rep)-associated gene PCR products were obtained.

Free-living *Vitis* spp. vine identifications were determined at Foundation Plant Services, University of California, Davis (<http://fps.ucdavis.edu/dnain.cfm>). The genomes of vines were evaluated using eight simple-sequence repeat (SSR) markers using methods described previously (Dangl et al. 2015). The markers were VVMD 5 and VVMD 7 (Bowers et al. 1996); VVMD 27, VVMD 31, and VVMD 32 (Bowers et al. 1999); VrZAG 62 and VrZAG 79 (Sefc et al. 1999); and VVS2 (Thomas and Scott 1993). At these eight markers, some alleles are unique to or occur at a much higher frequency in *V. californica* than in other species. Considering the codominant nature of SSR markers, alleles were ascribed to *V. californica* or to another *Vitis* sp. (Dangl et al. 2015). Alleles not ascribed to *V. californica* were manually compared locus by locus to reference profiles to identify possible parents from among the relatively small number of *V. vinifera* and hybrid rootstock cultivars currently or historically grown in the Napa Valley.

Nucleic acid sequence analysis. In experiments to assess the genetic variation of GRBaV isolates from within the vineyard, an 844-bp fragment was amplified using primers p1282-F (5′-TGTAACGACGCGCCAGTGAGGGTATGTGAGGAAGAAG-3′) specific to nucleotides 2,987 to 3,006 of reference isolate NY358

(GenBank accession JQ901105.2) and containing an M13 forward primer sequence (TGTAACGACGCGCCAGT) at its 5′ end, and p1283-R (5′-GCGGATAACAATTTACACAGGGCAGAAGGCAACGATATATCC-3′) specific to nucleotides 524 to 504 and containing M13 reverse primer sequence (GCGGATAACAATTTACACAGG) at its 5′ end. A typical 25-μl reaction contained 200 μM each dNTP, 20 pM each primer, 4% dimethyl sulfoxide, 100 to 150 ng of template DNA, and 1.0 μl of Herculanase II Fusion DNA polymerase (Stratagene, La Jolla, CA). Reactions were performed at a number of annealing temperatures; the most common thermocycling conditions were one cycle of 95°C for 3 min; 35 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s; and a final extension at 72°C for 20 min. The PCR products were purified using QiAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and commercially sequenced using M13 forward and reverse primers. The PCR product was sequenced in both directions to obtain data for both DNA strands. An internal 587-nt region of this PCR fragment was sequenced corresponding to nucleotides positions 2,991 through 1 to 371 of the circular genome of GenBank accession JQ901105.2. Sequences were assembled with the Vector NTI program (Life Technologies Inc., Grand Island, NY) and analyzed with BLAST (Altschul et al. 1997). If the sequence was identical to the reference genome of isolate NY703, the sequence was assumed to be representative of that isolate within the vine. If genetic variation was observed, a second, independent PCR product was generated to confirm that the variation was not an artifact of thermopolymerase error.

Phylogenetic and selection analysis. Sequences were aligned using T-Coffee (Di Tommaso et al. 2011). Substitution model selection and neighbor-joining, maximum-likelihood (PhyML) (Guindon et al. 2010), and Bayesian (MrBayes) (Ronquist and Huelsenbeck 2003) inferred phylogenetic trees were generated in TOPALi2 (Milne et al. 2009). Unrooted phylogenetic networks were generated in SplitsTree4 using the NeighborNet method (Huson and Bryant 2006). Prior to selection analyses, all alignments were screened for recombination using RDP4 (Martin et al. 2011) and GARD (Kosakovsky Pond et al. 2006) and partitioned when necessary. Negatively and positively selected codons were identified using the MEME (Murrell et al. 2013) and FUBAR (Murrell et al. 2013) algorithms. Global ratios of non-synonymous to synonymous nucleotide substitutions (d_N/d_S ratios) for each ORF were calculated using SLAC (Kosakovsky Pond et al. 2005).

RESULTS

A Cabernet franc block heavily infected with GRBaV. In 2012, a California vineyard of 6-year-old Cabernet franc clone 214 on rootstock 101-14 was observed showing symptoms of red blotch. A section of the vineyard was visually assessed in 2014 and approximately half of the vines (127 of 250; 51%) were symptomatic (Fig. 1). Of these vines, 187 (65 symptomatic and 122 asymptomatic) were tested for GRBaV by PCR. Of the 65 symptomatic vines tested, 60 (92%) were positive for both the CP and Rep gene fragments of GRBaV. Of the 122 asymptomatic vines tested, all but 5 were negative (117 of 122; 96%). Vines from the study site were also tested by enzyme-linked immunosorbent assay for GLRaV-1, -2, -3, -4, and related strains, and only 3 of 190 vines were infected with GLRaV-1 or GLRaV-4 (data not presented). Thus, there was a strong correlation between the presence of red blotch symptoms and the detection of GRBaV. A striking feature of this planting was that the symptomatic and infected vines of Cabernet franc clone 214 were clustered at one end of the block, proximal to a riparian habitat (Fig. 1B).

In adjoining plantings of other clones and cultivars, some vines showed symptoms of red blotch disease and were confirmed by PCR as being infected with GRBaV. One GRBaV-infected vine from each of these plantings was selected for further study; these are NY701 (Cabernet Sauvignon, clone 4), NY702 (Merlot, clone 30),

and NY921 and NY926 (Cabernet franc, clones 327 and 623, respectively) (Fig. 1A; Table 1).

A lack of sequence diversity among GRBaV isolates from vines of a single *V. vinifera* clone. In order to assess the genetic variation of GRBaV isolates from within a single vineyard, a 587-nt genomic region was amplified and sequenced from multiple vines. This region includes noncoding sequences bounding the origin of replication and is typically the most variable region of geminivirus genomes (Brown et al. 2012). Nucleic acid extracts were prepared from 44 vines of the Cabernet franc clone 214 block, and the sequence from all isolates proved to be identical to that of the clade 2 reference isolate NY703 (GenBank accession KU564251; described below). For comparison, the same genomic region from four GRBaV isolates from cultivars and clones in adjoining plantings were also sequenced. The sequences of the clade 2 isolates NY702, NY921, and NY926 differed from those of NY703 (Cabernet franc clone 214) at between two and four nucleotide positions, as shown in the alignment in Figure 2. NY701 proved to be a clade 1 isolate and differed from the clade 2 isolates at a total of 60 sites when compared with NY703. Thus, the evaluated population of GRBaV within the single planting of Cabernet franc clone 214 is homogeneous in this region of the genome but differs in sequence from that observed in other clones and cultivars in adjoining plots.

Free-living *Vitis* spp. from a riparian habitat harbor GRBaV. Given the observed clustering of GRBaV-infected vines adjacent to the riparian habitat, it was of interest to sample free-living vines to determine whether they also harbored the virus. Woody canes from 10 free-living vines were sampled, and nucleic acid extracts were tested by PCR. Four of the vines (isolates NY699, NY700, NY751, and NY913) tested positive for both the CP and Rep gene fragments of GRBaV (Fig. 1A; Table 1). Sequencing of the Rep gene PCR products from these isolates revealed that they were all clade 2 isolates, similar to the majority of isolates from the cultivated vines (data not shown).

Free-living *Vitis* spp. are hybrids. The identity of two of the four free-living vines infected with GRBaV (NY699 and NY700) was evaluated using eight SSR markers for *Vitis* spp. The two vines shared an identical profile at these eight markers. The profile was clearly that of a *V. californica* × *V. vinifera* hybrid. At each marker, one allele could readily be ascribed to *V. californica* and the other to *V. vinifera*. At five markers, the alleles ascribed to *V. vinifera* have not been observed in *V. californica*. The second allele at two of these markers was exclusive to *V. californica*. The markers VVMD 7 and VVMD 31 appeared homozygous for a single high-frequency *V. vinifera* allele. Both loci have a high frequency of null alleles in *V. californica* (Dangl et al. 2015). Scoring these markers as heterozygous, with a high-frequency *V. vinifera* allele and a *V. californica*-derived null, there were *V. californica*-exclusive alleles at five of eight markers. When compared with reference SSR profiles for *V. vinifera* and hybrid rootstock cultivars currently or historically grown in the area, only ‘Sauvignon Blanc’ could account for the alleles ascribed to

V. vinifera at each of the eight markers. These eight markers are not sufficient to prove paternity. However, the results show that the vines are *V. californica* × *V. vinifera* hybrids, not pure native *V. californica*, and that they are, most likely, first-generation hybrids with Sauvignon Blanc as the *V. vinifera* parent.

Genomic sequence diversity. In order to better understand the relationship between the GRBaV isolates in the free-living vines and those in cultivated vines, we sequenced the genome of 10

Fig. 1. Layout of the vineyard planting blocks in relation to the adjacent riparian habitat. **A**, There are three planted blocks in the commercial vineyard in Napa Valley, CA: that of Merlot (clone 30), Cabernet Sauvignon (clone 4), and Cabernet franc (clones 327, 214, and 623). Vines from which Grapevine red blotch-associated virus (GRBaV) isolates were obtained are indicated by circles and the numbered designations correspond to the virus isolates. Sizes of the planting blocks are to scale, as indicated. The relative positions of the vines are diagrammatic (not to scale). **B**, Plot layout, vine symptoms, and virus status of vines of a section of the Cabernet franc clone 214 block close to the riparian area are depicted. Each cell corresponds to a planted vine and the shading of individual cells indicates that the vine exhibited symptoms of red blotch disease. A minus (–) or plus (+) symbol indicates that the vine tested negative or positive for GRBaV, respectively; the absence of a symbol indicates that the vine was not tested. The orientation is the same as in A, with the bordering riparian habitat area at the top of the figure facing east.

isolates from the study site. This included three genomes of isolates from Cabernet franc clone 214 (NY703, NY704, and NY705), three genomes from the free-living vines (NY699, NY700, and NY913; additional material of NY751 was not available for sequencing), and four additional genomes, one from each of the other cultivars or blocks (NY701, NY702, NY921, and NY926) (Table 1). Consistent

TABLE 1. Grapevine red blotch-associated virus isolates from this study

Host ^a	Isolate	GenBank sequence accession
Cabernet franc clone 214	NY703	KU564251
	NY704	KU564252
	NY705	KU564253
	NY921	KU564255
Cabernet franc clone 327	NY926	KU564256
Cabernet franc clone 623	NY701	KU564249
Cabernet Sauvignon clone 4	NY702	KU564250
Merlot clone 15	NY699	KU564247
Free-living <i>Vitis</i> spp.	NY700	KU564248
	NY913	KU564254
	NY751	Not determined

^a Cultivars of *Vitis vinifera*, except where noted. All isolates are clade 2 members, with the exception of the clade 1 isolate NY701.

with the 100% identity among the partial sequences for the 44 vines of the Cabernet franc clone 214, the genomes of the clone 214 isolates NY703 and NY705 were identical and only differ from NY704 at one nucleotide position (A2820G). These genomes were quite similar to those of the other clade 2 isolates NY702, NY921, and NY926 (>99.6% identity, differing at up to 13 nucleotide positions). When genomic nucleotide sequences of the above six cultivated vine isolates were aligned with those of the genomes of the three free-living vines, a comparable level of identity was observed; however, the free-living vine isolates were more similar to each other than to the cultivated vine isolates, with one exception. The free-living vine isolate NY913 most closely resembled the isolate from the Merlot clone 15 vine; the sequence of NY913 was identical to that of NY702, except at the noncoding nucleotide position (G116A). The genomes of free-living vine isolates NY699 and NY700 were identical but differed from the third free-living vine isolate NY913 at 4 of 3,206 nucleotide positions. Additional DNA from NY751 was not available to complete the whole-genome sequencing. When compared with the other cultivated vine genomes (NY703, NY704, NY705, NY921, and NY926), the genomes of free-living vine isolates differed at between 12 and 14 positions. NY701 proved to be a clade 1 isolate with <92% identity to the clade 2 isolates

Fig. 2. Nucleotide sequence alignment of a 587-nucleotide genomic region of Grapevine red blotch-associated virus bounding the origin of replication. The consensus sequence is shown at the top of the alignment; in the alignment below, dots indicate identity while the nucleotides shown are those that differ from the consensus. Of the five sequences being compared, the sequence of the clade 2 isolate NY703 at the top is identical to that of 44 sequenced isolates from Cabernet franc clone 214. NY702, NY921, and NY926 are clade 2 isolates from adjoining blocks planted with other cultivars. The more divergent clade 1 isolate NY701 is at the bottom.

described above. These relationships can be seen in the neighbor-joining-derived phylogenetic tree of the complete genome sequences of the 10 isolates of GRBaV in this study together with other GRBaV GenBank database sequences (Fig. 3A). Evidence for recombination in full-length alignments using RDP3 was identified for a number of isolates and was consistent with previous findings of genetic exchange in the viral sense genes both within clade 1 and between clades (Krenz et al. 2014). The ambiguity in the phylogenetic signal as a result of this exchange is depicted in the reticulate tree (Fig. 3B), where clade 2 contains a range of node widths.

Selection in GRBaV. Predicted coding regions across GRBaV genomes were analyzed at both a vineyard scale (within the genome of the nine clade 2 isolates from this study) and a global scale (13 previously reported genomes plus all genome sequences from this study: 9 from clade 2 plus the clade 1 isolate NY701.) (Fig. 4). For the global sample set, five of the six ORF analyzed were calculated

to be under strong purifying selection. The CP gene (V1) is under the most stringent purifying selection (d_N/d_S ratio = 0.14), followed by the V2 (0.23) and C2 (0.25). The ORF encoding the putative movement protein (V3) had the highest d_N/d_S ratio (0.38) among the partially overlapping ORF, while the internal C3 was the only example of a positively selected ORF (d_N/d_S ratio = 1.73). Specific examination of individual codons within the global samples shows that the complimentary-sense ORF C1 and C3 exhibit the largest number of sites under positive selection, with five in each ORF. Because of the low diversity across the vineyard study site (except for the single clade 1 isolate), comparisons with trends in selection observed in the global population were limited to the observed polymorphisms present in the clade 2 isolates only. Consistent with the high purifying selection in the V1 (CP) of global samples, of the seven polymorphisms present, all but one (amino acid 25) were synonymous mutations. In addition, synonymous sites of amino acids 61 and 73 were also identified as being under significant

Fig. 3. A, Neighbor-joining (NJ)-derived phylogenetic tree of the complete genome sequences of Grapevine red blotch-associated virus. Sequences analyzed include those of the 10 isolates from Napa Valley, CA obtained in this study (in bold) and 13 previously reported isolates. All branches below 70% bootstrap support were collapsed. Additional support for the remaining branches was tested by both maximum likelihood (ML) and Bayesian inference (BI). Support at each node is depicted as NJ only = bootstrap percentages, NJ + BI = white circles, NJ + ML = white circles with bold border, and NJ + BI + ML = black circles. Asterisk = riparian habitat vines. **B,** Unrooted phylogenetic network generated in Splitstree4 using the NeighborNet method. Isolates from this study are depicted in bold. Parallel edges between branches show when there are conflicting phylogenetic signals indicative of recombination. Sequence accession numbers of previously reported isolates are KF147917 (NY135), KF147918 (NY137), KF751708 (NY147), KF147916 (NY175), KF47915 (NY271), JQ901105 (NY358), JX559642 (Canada), KC896623 (CF214), KC896624 (CS337), KC896625 (Z1A), KC427993 (GRLaV-WA-1), KC427995 (GRLaV-WA-MR), and KC427996 (GRLaV-WA-CF). Roman numerals indicate each defined clade. Bars show genetic distance.

purifying selection in the global analyses. Curiously, within C1, four of the five polymorphisms fall within the region predicted to include the intron (positions 2,450 to 2,288 of reference genome JQ901105.2) (Krenz et al. 2014). Of the polymorphisms seen within the vineyard population of GRBaV, three positions (296, 2,407, and 2,820 [in C3]) are also under positive selection in the global samples.

DISCUSSION

The original observation leading to this study was a clustered focus of vines with red blotch symptoms with a gradient of symptomatic vines outward from this area, a pattern consistent with the spread of a pathogen. Given the finding that GRBaV is present in free-living *Vitis* spp., one can infer that the virus can be spread by natural (nonhuman-mediated) means. Possible mechanisms by which a virus might spread to or among free-living *Vitis* spp. include pollen, seed, or vector transmission; in principle, local spread might

also occur via root grafting. At the vineyard study site described, we hypothesize that there has been spread of GRBaV but we are not able to ascertain the direction of movement, if any, between free-living and cultivated vines. Given the near identity of the genome of isolates from Merlot (NY702) and a free-living vine (NY913), and of the proximity of these vines, a single isolate may have moved from Merlot to the free-living vines (or vice-versa). Consistent with this notion, the Merlot vines were the oldest of the cultivated vines (approximately 35 years old), whereas the Cabernet franc blocks had been planted within the last decade. No further analysis of the Merlot vines could be undertaken, because this block was removed during the study period. The vineyard block studied most intensively was planted with Cabernet franc clone 214, and the sequence identity seen in isolates from 44 vines is consistent with infection from a single inoculum source. The most likely explanation is that the virus originated with the planting stock and has subsequently spread.

Fig. 4. Schematic showing genetic variation in open reading frames (ORF) across Grapevine red blotch-associated virus (GRBaV) genomes at both a vineyard scale (the 10 isolates from this study only) and a global scale (all 23 reported genomes). The inner circle shows the genome organization of GRBaV, with each ORF depicted as a gray arrow. Numbers within the name box for each ORF show the d_N/d_S ratio and the number of amino acids (in parentheses) per ORF. Numbers on the middle circle show the positions of positively selected sites and their associated amino acids for all known sequences, as determined by MEME (Murrell et al. 2012). Numbers with an asterisk highlight the amino acid position of negatively selected residues, as determined by FUBAR (Murrell et al. 2013). Numbers on the external circle show all polymorphisms detected in the vineyard population (this study) with amino acid and nucleotide (parentheses) positions. Synonymous polymorphisms are underlined. Blackened circles highlight codon positions in which both positive selection in the global samples and a nonsynonymous change in the vineyard samples were identified. In contrast, open circles highlight codon positions in which both negative selection in the global samples and a synonymous change in the vineyard samples were identified. Ori = origin of replication.

No apparent close sequence relationships were seen among the GRBaV clade 2 isolates from the free-living vines (three genomes) and the cultivated vines of Cabernet franc (clones 214, 327, and 624; five genomes total) and Cabernet Sauvignon (one genome). A limiting factor in this type of analysis is the possibility that sequence relationships may be obscured by selection pressures in different grapevine cultivars or species. That said, nonsynonymous changes were present for more than half of the polymorphisms (10 of 19, including those in overlapping ORF) observed among the 10 vineyard site genomes. Despite this limited variability, there is a consistency in the location of detected polymorphisms that extends out to the global GRBaV samples and other gemini-like viruses. The V1 (CP) ORF is under the most negative selection pressure, an observation made for both mastreviruses and begomoviruses (Duffy and Holmes 2009; Hadfield et al. 2012; Krabberger et al. 2012; Yang et al. 2014), followed by the replication-associated C1 and C2. More specifically, the CP ORF region close to the N terminus (amino acids 40 to 73) appears to be under the strongest negative selection, as demonstrated by the number of significantly negatively selected sites (six) in the global samples and synonymous substitutions (four) in the clade 2 vineyard site samples. The observation that C3 is under strong positive selection is remarkable, because the C3 ORF is in a different reading frame from and internal to C1 but C1 is under negative selection. Although the significance of this observation and the function of C3 are not known, it is very similar to what is seen in the begomovirus *Tomato yellow leaf curl virus*, wherein the C4 ORF is also internal to the Rep-encoding C1, and C4 is under strong positive selection while C1 is under negative selection (Yang et al. 2014). Additional evidence that C3 is a functional ORF is the context of the translational start site (ACAATGGC), consistent with the consensus identified for plant systems (Lütcke et al. 1987).

Uncertainty in the exact identity of the parents and recombinants and the position of breakpoints with the expanded and realigned dataset in this study made a reticulate phylogenetic tree a useful means for showing sequence affinities (Fig. 3B). In addition to the standard neighbor-joining tree (Fig. 3A), the reticulate tree further highlights the distinctive qualities of each clade. Clade 2 is compact, with low diversity, while clade 1 can be viewed as an expanding population. The reasons for this expansion are presently unclear. The ambiguity of phylogenetic signals as shown by the parallel branches is likely due, in part, to genetic exchange, evidence for which is present both within clade 1 and between clades. This suggests that GRBaV coinfections occur within the same cell (despite the assumed limited tissue tropism of this virus to the phloem), providing opportunities for novel variants to readily emerge.

In the genetic analysis of the free-living vines, the SSR profile shared by both tested vines was clearly that of a *V. californica* × *V. vinifera* hybrid. At these eight markers, it is unlikely that an identical profile would be shared by two different, seed-derived individuals. More likely, the shared profile results from sampling two vines of the same individual generated through natural clonal propagation (Dangl et al. 2015). Indeed, the two vines harboring GRBaV with identical viral genome sequences were growing within 20 m of each other. It remains to be determined whether pure *V. californica* and other free-living *Vitis* spp. in North America are susceptible to GRBaV.

The symptoms induced in red grapevine cultivars by GRBaV and leafroll viruses can be confused, depending on the specific cultivar–virus isolate–environment interactions and the timing of the observations. Nonetheless, in this study, we found a strong correspondence between our symptom ratings and detection of the virus. No obvious symptoms were observed in any of the free-living vines although, if symptoms were present, they might have gone undetected due to the vine growth habit and dense habitat.

As for all virus diseases of grapevine, the most effective strategy for management is to select and plant nursery stocks propagated from vines that test free of virus. GRBaV has presumably spread

with nursery stocks, because the red blotch disease was recognized within the western United States and across the country over a relatively short period of time, from 2008 to 2014 (Sudarshana et al. 2015). Nonetheless, the longer-term management of this virus will also require an understanding of any means by which natural spread can occur. Proving GRBaV spread in the field will require the deployment of rigorously tested sentinel vines (or new plantings) with the maintenance of control vines, presumably at an independent site. Alternatively, observations and circumstantial evidence for the role of a specific vector could lead to controlled vector transmission studies to demonstrate a mechanism of spread. A better understanding of the epidemiology of this newly emerging disease problem is needed to ensure the health of an industry that is continually replanting.

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