Insights Into the Ecology of *Grapevine red blotch virus* in a Diseased Vineyard

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ABSTRACT

Limited information is available on the spread of *Grapevine red blotch virus* (GRBV, genus *Grabovirus*, family *Geminiviridae*) in vineyards. To investigate ecological aspects of red blotch disease spread, sticky cards to catch flying insects were placed in 2015 (April to November) and 2016 (March to November) in a vineyard study site in California where disease incidence increased by nearly 20% between 2014 and 2016. Subsets of insect species or taxa were removed from sticky card traps and individual specimens were tested for the presence of GRBV by multiplex polymerase chain reaction. GRBV was consistently detected in *Spissistilus festinus* (Membracidae), *Colladonus reductus* (Cicadellidae), *Osbormellus borealis* (Cicadellidae), and a *Melanoliarus* sp. (Cixiidae). Populations of these four candidate vectors

peaked from June to September, with viruliferous *S. festinus* peaking from late June to early July in both years. An assessment of co-occurrence and covariation between the spatial distribution of GRBV-infected vines and viruliferous insects identified a significant association only with viruliferous *S. festinus*. These findings revealed the epidemiological relevance of *S. festinus* as a vector of GRBV in a vineyard ecosystem. Sequencing coat protein and replicase-associated protein gene fragments of GRBV isolates from newly infected vines and viruliferous vector candidates further suggested secondary spread primarily from local sources and occasionally from background sources.

Additional keywords: spatial analysis.

Red blotch is an emerging viral disease of grapevine (*Vitis* spp.) that is threatening vineyard profitability (Cieniewicz et al. 2017a; Sudarshana et al. 2015). In red-berried cultivars of *Vitis vinifera*, red blotch symptoms are similar to those elicited by leafroll disease, damage due to mite feeding, and nutritional disorders such as potassium or phosphorous deficiency. In white-berried *V. vinifera* cultivars, red blotch disease symptoms are similar to those of leafroll or magnesium deficiency. Delayed fruit ripening and reduced fruit qualities are also characteristic of red blotch disease (Cieniewicz et al. 2017a; Sudarshana et al. 2015). The estimated economic impact of the disease ranges from \$2,213 to \$68,548 per hectare over the 25-year lifespan of Cabernet Sauvignon and Merlot vineyards (Ricketts et al. 2017).

Grapevine red blotch virus (GRBV) was identified in diseased vines in 2011 (Al Rwahnih et al. 2013; Krenz et al. 2012) and later demonstrated as the causative agent of red blotch disease (Fuchs et al. 2015). GRBV is the type member of the genus *Grabovirus* in the family *Geminiviridae* (Varsani et al. 2017). Its genome consists of a single molecule of circular, single-stranded DNA (Cieniewicz et al. 2017a; Krenz et al. 2012; Sudarshana et al. 2015). Phylogenetic studies of GRBV isolates revealed the existence of two distinct clades, named 1 and 2, with nucleotide sequence differences of up to 8.5% (Krenz et al. 2014). GRBV is transmissible by grafting (Al Rwahnih et al. 2013; Poojari et al. 2013) and vegetative propagation, which may explain its distribution in all major viticulture regions of the United States (Krenz et al. 2014). GRBV has also been reported in Canada (Poojari et al. 2017), Switzerland (Reynard 2015), South Korea (Lim et al. 2016), and India (GenBank accession number KU522121).

The primary inoculum responsible for GRBV epidemics in most vineyards is likely attributed to dissemination of infected planting material (Krenz et al. 2014; Sudarshana et al. 2015). However, the mechanisms responsible for the secondary spread of GRBV within and between vineyards are unknown. A comprehensive understanding of the epidemiology of red blotch is critical for effective disease management. Short-distance spread has been observed in some vineyards in the western United States, and attributes of the spatiotemporal distribution of diseased vines suggest a new type of grapevine virus vector (Cieniewicz et al. 2017b). GRBV was also detected in free-living vines proximal to commercial vineyards in California (Perry et al. 2016). These results were subsequently confirmed in an independent study (Bahder et al. 2016b), providing additional evidence of spread by an insect vector. This hypothesis is strengthened by the very high nucleotide sequence identities of GRBV isolates recovered from free-living and proximal *V. vinifera* ‘Merlot’ and, to a lesser extent, *V. vinifera* ‘Cabernet franc’ vines (Perry et al. 2016).

The Virginia creeper leafhopper (*Erythroneura ziczac* (Walsh)) (Poojari et al. 2013) and the three-cornered alfalfa treehopper (*Spissistilus festinus* (Say)) (Bahder et al. 2016a) have been shown to transmit GRBV from infected to healthy vines under greenhouse conditions. Neither of these findings has yet been confirmed in a vineyard ecosystem. Moreover, the transmission ability of *E. ziczac* was recently refuted (Bahder et al. 2016a). Therefore, the epidemiological significance of *S. festinus* and *E. ziczac* is unknown, stressing the need for vector ecology studies in diseased vineyards. The primary objectives of this study were to investigate ecological aspects of GRBV in a commercial vineyard in which spread was documented (Cieniewicz et al. 2017b) and to characterize the diversity and seasonal distribution of potential hemipteran insect vectors as well as their spatial association with GRBV-infected vines.

MATERIALS AND METHODS

Vineyard study site and experimental design. A 2-ha *V. vinifera* ‘Cabernet franc’ vineyard established in 2008 in Napa

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

County, CA was selected for this study. Selection of this vineyard was based on temporal changes in GRBV incidence, with spatio-temporal analysis of epidemics suggesting the presence of secondary spread between 2014 and 2016 by an unknown vector (Cieniewicz et al. 2017b). The spatial pattern of diseased vines was characterized by a significant edge effect adjacent to a wooded riparian area and randomly distributed, diseased vines throughout the remainder of the vineyard (Cieniewicz et al. 2017b).

In this Cabernet franc vineyard, vines are spaced 1.2 and 2.1 m within and between rows, respectively. The study area was positioned at the edge of the Cabernet franc vineyard adjacent to a riparian area. Yellow sticky cards (7.6 by 12.7 cm) were placed on the middle trellis wire (1.2 m above the ground) throughout a sampling area that spanned 12 rows and six four-vine panels per row. The four-vine panels in the sampling area were designated A to F, with panel A positioned at the edge of the vineyard and panel F furthest into the vineyard (Supplementary Fig. S1). In each row, a sticky card was placed in every other panel (A-C-E and B-D-F) in alternating rows, such that each of the 12 rows contained three sticky cards in either A-C-E or B-D-F orientation. The spacing of two sticky cards between two four-vine panels was approximately 10 m, and the spacing between the first and last sticky card in any given row was approximately 20 m. Sticky cards were removed weekly, placed in plastic bags, shipped overnight from the vineyard to the laboratory in Geneva, NY for evaluation, and replaced with new sticky cards. The survey was conducted from April to November 2015 and March to November 2016 to span the entire growing season.

Insect identification and specimen handling. Insects caught on sticky card traps were identified to genus and species when possible, based on morphological characteristics. Specimens were identified and counted while still attached to sticky cards. The number and identity of specimens were recorded for each sticky card to evaluate the abundance and diversity of flying insects. Of approximately 134,000 insects caught on sticky card traps in both years, 960 (700 and 260 in 2015 and 2016, respectively) were tested for GRBV by multiplex polymerase chain reaction (PCR) (Krenz et al. 2014). Insects in high abundance (*Erythroneura* spp. and members of families Phylloxeridae, Aleyrodidae, Aphididae, and Thysanoptera) were randomly sampled from sticky cards spanning the entire growing season for testing in both years. For hemipteran insects of low abundance (<0.25% of total specimens), every specimen was removed from sticky cards in 2015 and 2016 for PCR testing (Krenz et al. 2014). Specimens were individually removed from sticky cards using Goo Gone liquid degreaser (Weiman Products, Gurnee, IL) to dissolve the adhesive and loosen the specimens. Individual specimens were stored at -20°C until testing by multiplex PCR for GRBV detection (Krenz et al. 2014) or species identification by sequencing of the mitochondrial DNA barcode region (Hebert et al. 2003).

GRBV detection in insects. Total DNA was isolated from individual specimens removed from the sticky cards using the E.Z.N.A. Insect DNA kit (OMEGA Biotek, Norcross, GA) and stored at -20°C. Diagnostic multiplex PCR was performed using primers targeting a coat protein (*CP*) gene fragment and a replicase-associated protein (*Rep*) gene fragment (Krenz et al. 2014). PCR were carried out with HotStar HiFidelity polymerase (Qiagen, Carlsbad, CA) and manufacturer-suggested conditions in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). DNA amplification products were resolved by electrophoresis on 2% agarose gels in 1× Tris-acetate-EDTA buffer and stained with GelRED (Biotium, Fremont, CA). The detection of GRBV amplicons in species or taxa was assumed to result from virus ingestion. Species or taxa for which more than 40% of the specimens tested positive for GRBV in PCR were considered vector candidates.

Sequence-based insect identification. To provide confirmatory evidence for the morphology-based identification of some insect species, mitochondrial cytochrome C oxidase gene I (COI) barcode-based identification was performed for identification of vector candidate species. A 650-bp region of the 3' end of the COI gene was

targeted for PCR amplification. Primers LepF2-t1 and LepR1 (Footitt et al. 2014; Hebert et al. 2004) were used to amplify the barcode region of the later identified *Melanoliarius* sp. Primers LCO1490 and HCO2198 (Folmer et al. 1994) were used to amplify the barcode region of the later identified *Osbornellus borealis* and *S. festinus*. PCR were performed and confirmed by gel electrophoresis, as described above. PCR products were sequenced at the Cornell Biotechnology Resource Center in Ithaca, NY and assembled using the DNASTAR Lasergene software suite, version 14.1.

GRBV sequence diversity from vector candidates and infected vines. In order to determine the genetic relatedness of GRBV isolates recovered from the vector candidates caught on sticky cards to the GRBV isolates recovered from newly infected vines in the Cabernet franc vineyard in 2016, the GRBV *Rep* and *CP* fragments were amplified from infected vines and insects carrying GRBV. Primers Repfor and Reprev or CPfor and CPrev were used to amplify GRBV *Rep* or *CP* fragments, respectively, in simplex PCR using conditions as described above. GRBV *Rep* and *CP* fragments were amplified from each vine in the Cabernet franc vineyard that tested GRBV positive in 2016 ($n = 174$), and each of the viruliferous vector candidate specimens captured in 2015 and 2016 ($n = 82$). Simplex PCR amplicons were sequenced as described above and aligned with previously sequenced *Rep* and *CP* fragments of GRBV isolates recovered from the same Cabernet franc vineyard and proximal free-living grape plants (Perry et al. 2016) using ClustalW (Thompson et al. 1997). Phylogenies were constructed using the neighbor-joining method (Saitou and Nei 1987) after bootstrapping (1,000 simulations) to estimate branching robustness in Megalign (DNASTAR Lasergene software suite, version 14.1).

Spatial distribution of GRBV-infected vines and insect vector candidates. The spatial pattern of diseased vines and GRBV-positive insect vector candidates was analyzed using the spatial analysis by distance indices (SADIE) algorithm (Cieniewicz et al. 2017b; Li et al. 2012; Perry 1995, 1998; Perry et al. 1999; Xu and Madden 2005). Spatial association between GRBV incidence and each of the individual candidate vectors was first evaluated with the local clustering index (χ_k) calculated through SADIE (Perry 1998). Insect count data were arranged by vine panel, corresponding to the location of the sticky card in each panel in the vineyard. Similarly, panels of vines were arranged by scores of 0 to 4 based on the number of diseased vines in each four-vine panel, with 0 indicating no diseased vine in the panel and 4 indicating four diseased vines in the four-vine panel. Clustering indices represent the net distance that individuals need to move at each sampling unit to achieve regularity. This index is a measure of the similarity between the clustering indices for the two variables measured “locally”, at the level of the sampling unit (Winder et al. 2001). Overall spatial association was calculated as the mean of the local clustering indices (X) between either of the two variables of interest. The significance of X was tested by 9,999 randomizations with values of the local clustering indices after allowing for small-scale spatial autocorrelation in the local clustering indices within either population (Dutilleul et al. 1993) and using a two-tailed test (Winder et al. 2001). The null hypothesis used for each test was a lack of association between spatial patterns of diseased vines and viruliferous insects.

RESULTS

GRBV detection in insects. GRBV was detected in at least 40% of *S. festinus* (family Membracidae), *Colladonus reductus* (family Cicadellidae), *O. borealis* (Cicadellidae), and a *Melanoliarius* sp. (family Cixiidae) by multiplex PCR (Table 1). This result revealed that specimens of these four hemipteran species visited the study vineyard and ingested GRBV over two consecutive years. The four insects are hereafter referred to as vector candidates. GRBV was not found by multiplex PCR in the majority of other insects tested over two consecutive years, or it was found in only a few specimens (3 to 8%) of a limited number of insects (Table 1). GRBV

was not detected in any of the *E. ziczac* specimens tested in 2016 (Table 1).

Diversity of insects on sticky card traps. Insect vector candidates (*S. festinus*, *C. reductus*, *O. borealis*, and a *Melanoliarius* spp.) collectively comprised only 0.14% of specimens on sticky cards in both years (87 of 62,128 in 2015 and 99 of 72,242 in 2016), and 0.4% (87 of 18,525) and 0.6% (99 of 16,060) of Hemiptera on sticky

cards in 2015 and 2016, respectively (Fig. 1). Thysanoptera were most abundant in both years and the abundance of Diptera was consistent across years (Fig. 1). More Phylloxeridae were caught in 2015 than in 2016, whereas the abundance of Aphididae was higher in 2016 (11%) than in 2015 (<2%). Overall, Hemiptera comprised less than 23 and 30% of specimens on sticky cards in 2015 and in 2016, respectively (Fig. 1).

TABLE 1. Grapevine red blotch virus (GRBV) detection in insects trapped on sticky cards in 2015 and 2016 in a *Vitis vinifera* ‘Cabernet franc’ vineyard in which secondary disease spread was previously documented (Cieniewicz et al. 2017b)

Order, family, genus, species	GRBV detection ^a					
	2015		2016		Cumulative	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Hemiptera						
Membracidae						
<i>Spissistilus festinus</i>	12/25	48	13/25	52	25/50	50
Cixiidae						
<i>Melanoliarius</i> sp.	4/8	50	10/12	83	14/20	70
Cicadellidae						
<i>Osbornellus borealis</i>	13/31	42	4/11	36	17/42	40
<i>Colladonus reductus</i>	14/23	61	12/41	29	26/64	41
<i>Scaphytopius magdalensis</i>	3/45	7	2/17	12	5/62	8
<i>Empoasca</i> sp.	1/28	4	1/16	6	2/44	5
<i>Graphocephala atropunctata</i>	1/23	4	0/14	0	1/37	3
<i>Erythroneura variabilis</i>	0/22	0	0/22	0	0/44	0
<i>Euscelis</i> sp.	0/33	0	0/11	0	0/44	0
<i>Erythroneura elegantula</i>	0/41	0	0/24	0	0/65	0
<i>Japananus hyalinus</i>	0/4	0	–	–	0/4	0
<i>Deltocephalus</i> sp.	0/15	0	–	–	0/15	0
<i>Sophonia orientalis</i>	0/5	0	–	–	0/5	0
<i>Draeculacephala minerva</i>	0/4	0	–	–	0/4	0
<i>Xestocephalus</i> sp.	0/5	0	–	–	0/5	0
<i>E. ziczac</i>	–	–	0/10	0	0/10	0
<i>E. tricineta</i>	–	–	0/2	0	0/2	0
<i>Typhlocyba</i> sp.	0/5	0	0/6	0	0/11	0
Unidentified species 1	0/23	0	0/6	0	0/29	0
Unidentified species 2	0/16	0	–	–	0/16	0
Unidentified species 3	0/4	0	–	–	0/4	0
Unidentified species 4	0/1	0	0/1	0	0/1	0
Unidentified species 5	0/7	0	–	–	0/7	0
Unidentified species 6	0/4	0	–	–	0/4	0
Unidentified species 7	0/3	0	–	–	0/3	0
Unidentified species 8	0/2	0	–	–	0/2	0
Unidentified species 9	0/2	0	–	–	0/2	0
Unidentified species 10	0/12	0	–	–	0/12	0
Unidentified species 11	0/1	0	–	–	0/1	0
Delphacidae						
Unidentified species	0/10	0	0/9	0	0/19	0
Cercopidae						
Unidentified species	0/2	0	–	–	0/2	0
Aleyrodidae						
Unidentified species	0/52	0	0/17	0	0/69	0
Aphididae						
Unidentified species	1/46	2	1/12	8	2/58	3
Phylloxeridae						
Unidentified species	0/22	0	0/10	0	0/32	0
Psyllidae						
Unidentified species	0/25	0	0/5	0	0/30	0
Miridae						
<i>Lygus</i> sp.	1/16	6	–	–	1/16	6
Lygaeidae						
<i>Nysius raphanus</i>	0/8	0	–	–	0/8	0
Thysanoptera						
Unidentified species	0/36	0	–	–	0/36	0
Psocoptera						
Unidentified species	0/12	0	–	–	0/12	0
Coleoptera						
Unidentified species	0/24	0	–	–	0/24	0
Diptera						
Unidentified species	0/24	0	–	–	0/24	0
Hymenoptera						
Unidentified species	0/24	0	–	–	0/24	0

^a *n* = number of individual specimens in which GRBV was detected by multiplex polymerase chain reaction over the total number of specimens tested; – indicates no specimen tested.

Sequenced-based insect species identification. A representative specimen of each of *O. borealis* (GenBank accession number MF414209), *S. festinus* (MF414211), and *Melanoliarius* sp. (MF414210) produced PCR amplicons of 648 to 708 bp in the COI “barcode” region. A specimen of the *Melanoliarius* sp. was identified to the genus level, with 94% nucleotide sequence identity to sample ID BIOUG02202-A06 in the BOLD Systems database (www.boldsystems.org). The *O. borealis* specimen had 99.3% identity to sample ID BIOUG12705-A04 in the database, which meets the diversity cut-off for species identification. Similarly, the *S. festinus* specimen revealed 100% identity to sample ID BIOUG02202-G07 in the database, verifying species identification. Specimens of *C. reductus* repeatedly failed to produce PCR products with the primer sets selected for this study.

Population dynamics of the vector candidates. The abundance of the four vector candidates captured on sticky cards was low relative to total insect abundance, with only 87 and 99 specimens in 2015 and 2016, respectively. Populations of the four vector candidates peaked between June and September during both years. Populations of *S. festinus* peaked during early July 2015 (Fig. 2A) and late June 2016 (Fig. 2B), with populations quickly tapering after July. Populations of *C. reductus* peaked in August 2015 (Fig. 2A) and in April and September 2016 (Fig. 2B). Populations of the *Melanoliarius* sp. peaked in July (Fig. 2). *O. borealis* was captured infrequently in June and July and increasingly in August and September of both years (Fig. 2).

GRBV was not detected in vector candidates until June (Fig. 3), with the exception of one *C. reductus* specimen that tested positive

for GRBV in early May 2016 (Fig. 3F). The incidence of viruliferous *S. festinus* was highest in July 2015 (Fig. 3A) and June 2016 (Fig. 3B). Viruliferous *O. borealis* insects were detected from July to November 2015 (Fig. 3C) and 2016 (Fig. 3D), whereas the viruliferous *Melanoliarius* sp. was captured on sticky cards only from July to September (Fig. 3G and H).

GRBV sequence diversity from vector candidates and infected vines. The sequences of GRBV *Rep* fragments recovered from infected vines in the sampled area of the selected vineyard were nearly identical (>99% identity at the nucleotide level) to each other and belonged to phylogenetic clade 2 (Fig. 4). Similar results were obtained for the GRBV *CP* fragments recovered from infected Cabernet franc vines (data not shown). These data were consistent with previous findings from the same Cabernet franc vineyard (Perry et al. 2016). The majority of GRBV *Rep* fragments (89%, 56 of 63) recovered from the four vector candidates in this vineyard also showed high nucleotide sequence identity (>99%) to the corresponding fragments from infected vines (Fig. 4). This result revealed the genetic relatedness of GRBV isolates in infected vines and most of the viruliferous insect vector candidate specimens. However, a few GRBV *Rep* fragments (11%, 7 of 63) recovered from vector candidate specimens of *S. festinus*, *O. borealis*, and *C. reductus* differed from the corresponding GRBV nucleotide sequence from infected vines in the Cabernet franc vineyard and belonged to clade 1 (Fig. 4). The phylogenetic lineage assembled from GRBV *CP* sequences was consistent with that obtained from GRBV *Rep* sequences (data not shown).

Fig. 1. Diversity and abundance of insect taxa identified from sticky cards placed in a *Vitis vinifera* ‘Cabernet franc’ vineyard infected by *Grapevine red blotch virus* in Napa County, CA in **A**, 2015 and **B**, 2016.

Fig. 2. Seasonal population dynamics of candidate insect vectors of *Grapevine red blotch virus* based on specimens captured on sticky cards in a diseased *Vitis vinifera* ‘Cabernet franc’ vineyard in Napa County, CA in **A**, 2015 and **B**, 2016.

Spatial association of GRBV incidence in vines and vector candidates. Spatial pattern analyses indicated aggregated patterns of GRBV-infected vines and populations of *S. festinus* and *O. borealis*. No significant aggregation was found for *C. reductus* and the *Melanoliarius* sp. (Table 2). Moreover, there was a significant spatial association between the distribution of infected vines and viruliferous *S. festinus*. No significant spatial associations were identified between populations of alternative insect vector candidates and GRBV-infected vines (Table 3).

The spatial distribution of vector candidates on sticky cards also indicated a gradient of higher *S. festinus* ($n = 50$) and *O. borealis* ($n = 42$) populations at the edge of the vineyard next to a riparian area and decreasing *S. festinus* and *O. borealis* populations distant from the edge (Fig. 5). Additionally, both *S. festinus* and *O. borealis* populations of section 1 near the edge of the vineyard (0 to 10 m from the edge of the vineyard) had a higher proportion of viruliferous insects than the inner-vineyard section 2 (10 to 20 m within the vineyard) and section 3 (20 to 30 m within the vineyard), in which

Fig. 3. Seasonal dynamics of ingestion of Grapevine red blotch virus (GRBV) by candidate insect vectors in a diseased *Vitis vinifera* 'Cabernet franc' vineyard in Napa County, CA in **A, C, E, and G**, 2015 and **B, D, F, and H**, 2016. The presence of GRBV in individual candidate insect vector specimens was assessed by multiplex polymerase chain reaction.

the proportions of viruliferous specimens were lower. The spatial distribution of *C. reductus* ($n = 64$) and *Melanoliarius* ($n = 20$) was not dependent upon proximity to the edge of the vineyard (Fig. 5).

DISCUSSION

We investigated the ecological aspects of GRBV in a commercial Cabernet franc vineyard for which secondary spread was previously documented (Cieniewicz et al. 2017b). In addition to *S. festinus*, a recognized vector of GRBV (Bahder et al. 2016a), three additional potential GRBV insect vectors were identified and their diversity, relative abundance, and seasonal distribution was described. To our knowledge, this is the first research on the epidemiological relevance of *S. festinus* as a vector of GRBV. It is also the first report on the seasonal acquisition of GRBV by *S. festinus* in a vineyard ecosystem. This study was initiated in 2015, before there was any information on secondary spread of red blotch disease. Spatiotemporal analysis of spread in the same vineyard indicated a 1 to 2% increase in disease incidence annually, with the highest rate occurring near a riparian area (Cieniewicz et al. 2017b). In addition, the analysis of spread attributes of the epidemic indicated a limited influx of GRBV into the vineyard from background sources; rather, spread in this vineyard was due to local, within-vineyard sources of inoculum (Cieniewicz et al. 2017b). These conclusions were strengthened by the extremely high identity among GRBV sequences (99 to 100% nucleotide identity) recovered from newly infected vines in this vineyard in 2016 and the majority (89%) of the viruliferous vector candidates trapped on sticky cards in 2015 and 2016. These results suggested that most of the vector candidate specimens ingested GRBV from the Cabernet franc vineyard where they were captured. An alternative explanation is that candidate vectors carried the same GRBV genetic variant as was present in infected Cabernet franc vines but acquired it before entering the vineyard. However, a few (11%) specimens of *S. festinus*, *O. borealis*, and *C. reductus* carried GRBV *Rep* sequences that were distinct (88.7 to 94% nucleotide sequence identity) from GRBV *Rep* sequences in the infected vines and the majority of vector candidate specimens. This suggested that some vector candidates ingested GRBV from sources outside of the Cabernet franc vineyard and then visited the Cabernet franc vineyard to feed. A potential source of GRBV clade 1 isolates is a nearby Cabernet Sauvignon vineyard (Perry et al. 2016).

Altogether, our results indicated that GRBV can be ingested by vector candidates from both local (intravineyard) and background (extra-vineyard) sources. It will be important to determine whether comparable studies in other viticulture regions will lead to similar conclusions.

GRBV was only detected in the four vector candidates starting in June (with the exception of one *C. reductus* specimen in May 2016), suggesting that virus uptake and transmission is unlikely to occur prior to June in this vineyard. Additional studies are needed to verify whether findings from this Cabernet franc vineyard translate into similar trends in population dynamics in other vineyards. The disparity between 2015 and 2016 in the proportion of *C. reductus* that tested positive for GRBV is attributed to the higher number of specimens in April 2016, which all tested negative for GRBV, than the previous year. Additionally, the survey was initiated earlier in 2016 (March) than 2015 (April) and selection of specimens for GRBV testing by PCR preferentially targeted vector candidates in 2016.

Among the four vector candidates identified in this study, only *S. festinus* is known to transmit GRBV in the greenhouse (Bahder et al. 2016a). The population dynamics of *S. festinus* insects and their seasonal ingestion of GRBV were consistent between 2015 and 2016, with peaks in late June and early July. In addition, only viruliferous *S. festinus* populations were significantly associated

TABLE 2. Spatial analysis by distance indices of the spatial pattern of Grapevine red blotch virus (GRBV)-infected grapevines and candidate insect vector populations in 2015 and 2016

Variable ^a	<i>D</i> ^b	<i>I_a</i> ^c	<i>P</i> ^d
GRBV incidence	418.7	2.088	0.002**
<i>Spissistilus festinus</i> (total counts)	156.8	1.171	0.137 (ns)
<i>S. festinus</i> (viruliferous counts)	141.2	1.486	0.0099**
<i>Osbornellus borealis</i> (total counts)	218.9	1.549	0.007**
<i>O. borealis</i> (viruliferous counts)	136.3	1.702	0.0012**
<i>Colladonus reductus</i> (total counts)	157.3	0.931	0.613 (ns)
<i>C. reductus</i> (viruliferous counts)	127.1	1.305	0.052 (ns)
<i>Melanoliarius</i> sp. (total counts)	64.4	0.853	0.833 (ns)
<i>Melanoliarius</i> sp. (viruliferous counts)	58.5	0.7962	0.956 (ns)

^a Total counts and total viruliferous specimen counts are provided for each candidate insect vector species.
^b *D* = distance to regularity.
^c *I_a* = index of aggregation.
^d Asterisks (**) indicate significance according to a two-tail test at $P < 0.01$; ns = not significant.

Fig. 4. Neighbor-joining phylogenetic tree indicating relationships among Grapevine red blotch virus (GRBV) replicase-associated protein (Rep) nucleic acid sequences recovered from infected Cabernet franc vines, candidate insect vectors (*Melanoliarius* sp., *Spissistilus festinus*, *Colladonus reductus*, and *Osbornellus borealis*) isolated from sticky cards, and nearby GRBV-infected free-living and commercial vines from a previous study (Perry et al. 2016). Branches with less than 65% bootstrap support were collapsed. Information on the source of GRBV Rep sequence information is provided in Supplementary Table S1.

with the spatial pattern of infected vines, although the spatial pattern of viruliferous *O. borealis* showed a nearly significant ($P = 0.089$) association with the spatial pattern of infected vines. Also, a higher proportion of viruliferous *S. festinus* and *O. borealis* specimens were caught toward the edge of the vineyard and decreased further into the vineyard (Fig. 5). These results suggested a directional influx of aviruliferous *S. festinus* and *O. borealis* insects and a subsequent ingestion of GRBV primarily from infected vines located at the edge, proximal to a riparian area. No significant association was found for any of the other vector candidate populations. Although the lack of spatial association between infected vines and *C. reductus*, *O. borealis*, and the *Melanoliarus*

TABLE 3. Association analysis between spatial patterns of virus incidence and total candidate insect vector populations or viruliferous candidate insect vector populations using spatial analysis by distance indices with an actual sample size of 72

Variable ^a	Effective sample size	Overall association (X) ^b
<i>Spissistilus festinus</i> (total counts)	76.1	-0.0028 (0.5094; ns)
<i>S. festinus</i> (viruliferous counts)	72.0	0.1989 (0.0479*)
<i>Osbornellus borealis</i> (total counts)	73.0	0.1600 (0.089; ns)
<i>O. borealis</i> (viruliferous counts)	67.8	0.1643 (0.091; ns)
<i>Colladonus reductus</i> (total counts)	65.8	0.0810 (0.263; ns)
<i>C. reductus</i> (viruliferous counts)	68.8	-0.0895 (0.769; ns)
<i>Melanoliarus</i> sp. (total counts)	71.4	0.075 (0.529; ns)
<i>Melanoliarus</i> sp. (viruliferous counts)	72.2	0.083 (0.237; ns)

^a Total counts and total viruliferous specimen counts are provided for each candidate insect vector species.
^b Overall association (X). Two-tail probability value presented parenthetically. An asterisk (*) indicates significance according to a two-tail test at $P < 0.05$; ns = not significant.

sp. does not exclude these insects as vectors, this result supports the hypothesis that *S. festinus* is the predominant vector responsible for secondary spread of GRBV within this Cabernet franc vineyard. No information is available on the vector potential of *O. borealis*, *C. reductus*, and the *Melanoliarus* sp., providing an impetus for controlled GRBV transmission assays. However, this field study advances our understanding of the vector biology of this pathosystem, for which limited information is available. A previous report suggested *E. ziczac* as a vector of GRBV (Poojari et al. 2013) but this finding was later refuted (Bahder et al. 2016a). In our study, this leafhopper was not captured on sticky card traps in 2015 and none of the 10 specimens caught in 2016 tested positive for GRBV. Additionally, *E. ziczac* is a mesophyll feeder (Saguez et al. 2015), which differs from the phloem-feeder vector candidates identified empirically in this study. Collectively, our observations provide vineyard-based evidence that does not support the supposition that *E. ziczac* is a vector of GRBV.

None of the four candidate vector species identified in our study is recognized as a pest of grapevine. However, their traits are consistent with predictions of a new type of insect vector for a grapevine virus based on the spatial attributes of a GRBV epidemic (Cieniewicz et al. 2017b). The four candidate insect vectors identified in this study feed preferentially on phloem or belong to genera of known phloem feeders. *O. borealis* has been implicated as a vector of the X-disease phytoplasma (ribosomal group 16sRIII-I), affecting stone fruit and some reservoir hosts (Jensen 1957). Other species within the genus *Colladonus* (*C. geminatus* and *C. montanus*) also transmit the X-disease phytoplasma (Wolfe 1955; Wolfe et al. 1950). *S. festinus* is a phloem feeder that girdles stems of plants on which it feeds (Mueller and Dumas 1975). Cixiid planthoppers such as the *Melanoliarus* sp. also feed on phloem, and some can transmit phytoplasmas, such as the lethal yellows phytoplasma in palm (Holzinger et al. 2002). By analogy with other geminivirids, GRBV is anticipated to be phloem restricted, adding significance to the identification of the four phloem-feeding vector candidates.

Fig. 5. Spatial distribution of candidate insect vectors at the edge of a Cabernet franc vineyard where Grapevine red blotch virus incidence is aggregated proximal to a riparian habitat. Numbers of specimens in 2015 and 2016 are summed for each species. Sections 1 (0 to 10 m from the edge of the vineyard), 2 (10 to 20 m within the vineyard), and 3 (20 to 30 m within the vineyard) indicate partitions of two four-vine panels, where section 1 is positioned closest to the edge and section 3 is furthest into the vineyard.

Although the vector candidates are all phloem feeders, GRBV was not detected or not consistently detected in each of the phloem-feeding insects that were present on sticky card traps. A nonexhaustive list of examples of phloem feeders (Douglas 2006), in which GRBV was rarely or never detected by PCR, includes *Scaphytopius magdalensis* and *Empoasca* sp. leafhoppers, delphacid planthoppers, whiteflies, aphids, psyllids, and lygaeids. This could be attributed to a lack of or a lower rate of feeding on the infected Cabernet franc vines or an inability to ingest GRBV. Although GRBV was detected in a small percentage (3 to 8%) of some of these phloem-feeders (*S. magdalensis*, the *Empoasca* sp., and aphids), the four vector candidates were highlighted as potential vectors, with the proportion of viruliferous specimens exceeding 40%.

Yellow sticky cards were chosen as the sampling method to accomplish the goal of catching flying insects visiting the vines in the selected area of the study vineyard, including healthy and infected vines, and potentially feeding and ingesting GRBV. The sticky card sampling method likely resulted in undersampling populations of immature insects, as shown for other systems (Musser et al. 2004). Consequently, we cannot discount the possibility that some feeding insects were resistant to capture on sticky cards and, hence, potentially underrepresented in these findings. Therefore, the list of candidate vectors obtained here may not be exhaustive. Similarly, sticky cards may not be ideal for an accurate estimate of insect population densities. Other approaches such as sweep netting, vacuum traps, or UV light trapping may have added further information on vector candidate abundance. Nonetheless, our study suggested low population densities of vector candidates, with consistency over two consecutive years.

This study integrated unbiased survey outputs of GRBV ingestion by insects in a disease vineyard over two growing seasons and covariation analysis to reveal *S. festinus* as a vector of epidemiological significance. This research also revealed three additional candidate vectors of GRBV, which should be further evaluated for vectoring capabilities in controlled transmission assays to fundamentally advance vector biology. To our knowledge, the host range of the four vector candidates is poorly characterized. It will be critical to identify reproductive hosts in a vineyard ecosystem and determine whether some of them overlap with hosts of GRBV. This research is essential to advance our understanding of GRBV epidemiology and design optimal disease management strategies.

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