1. Project Title: Integrative studies of vector-related field epidemiology for grapevine red blotch-associated virus and grapevine leafroll-associated virus in Oregon.

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Introduction:

Grapevine virus diseases are of serious concern for vineyard managers and winemakers in Oregon. The effects of diseases such as grapevine leafroll-associated virus (GLRaV) and grapevine red blotch-associated virus (GRBaV) impact grape berry qualities. Growers and scientists alike have noticed a consistently lower °Brix at harvest in symptomatic vines (Al Rwahnih et al. 2013, 2015). These observations have resulted in growers removing symptomatic

vines from vineyards in Oregon. Currently, studies of crop quality are being conducted in California and Washington State.

Our studies werecoordinated with other researchgroups dealing with vector transmission biology (Kent Daane), virology and non-crop hosts (Sudarshana Mysore, Frank Zalom), and viticulture (Rhonda Smith). Together with the study performed in Oregon, these studies aimto create a better understanding of the regional epidemiology and importance of GLRaV, GRBaV, and their respective potential insect vectors in Oregon. Our California collaborators establishedprotocols to identifythe most probable insect species that transmit the Geminiviridae (Ghanim et al. 2007, Chen and Gilbertson 2009, Cilia et al. 2012) including leafhoppers (Poojari et al. 2013) and treehoppers (Bahder et al. 2016) that may vector GRBaV. To help identify candidate vector species, we supplied our California collaborators with preserved insects collected in Oregon during 2015, and additional collections are planned for 2016. These samples will be tested for presence of GRBaV and temporally analyzed to determine likely vector species.

The epidemiology of GRBaV remains unresolved for Oregon, but new data from this study and also others nationwide have shed some light on this aspect. Here we summarize the current state of knowledge of the epidemiology of GLRaV and GRBaV through an Oregon perspective.
The goal of the work detailed in this report is to build on the current information available on the potential insect vectors, with focus on their seasonal presence and spatial distribution in Oregon vineyards.Ultimately, information from this projectwill direct future control programs for both GLRaV and GRBaV.

Further to the above, we specifically aimed to provide timely updates and management tools for wine grape growers regarding management of these two viruses and insect vectors. At this time, published information on the epidemiology of GRBaV in vineyards is lacking. It is not clear which insects may spread this virus in Oregon. Up to this reporting cycle, we did not know whether vector insects and virus infectionoriginated from infectedplant materials or from non-

crop host plants. The below data, however, indicate that insect vectors do play a role in spread of this virus.

The objectives of the 2014 and 2015 crop season specifically included:

1. Follow insect vector distribution and disease progression in established vineyard plots for grapevine red blotch-associated virus (GRBaV) and grapevine leafroll-associated virus (GLRaV) in Oregon.
2. Extension of information on the importance of vectors, GLRaV and GRBaV in Oregon vineyards.

Materials and methods

Seven vineyard sites represented Southern Oregon, the Willamette Valley, and Eastern Oregon and were surveyed for insects during 2013-2015 using sticky card sampling and sweep netting.All sampled vineyards contained at least 32 rows and 22 pole-to-pole "bays" (Charles et al. 2009) and 3-6 vines per bay. The temporal and spatial distributions of insects that are vectors of GLRaV strains in Oregonwere recently determined (Walton et al. 2013, Dalton et al. 2014), but this information is in its beginning stages for GRBaV (Bahder et al. 2016).

Leaf samples were taken yearly from two study blocks in Southern Oregonand were analyzed for both GLRaV and GRBaV from 2013-2015. Due to high incidence of both viruses, one study block was taken out of production following the 2013 season and was replaced with a separate vineyardsite in this region. In the Willamette Valley,oneblock wasanalyzed from 2013-2015 and an additional block was included for analysis during 2014. Two sites in the Columbia Basin were included in this study.

i. Insect vector distribution and species composition

Insects that may vector viruses were surveyed using sticky traps placed along the border (4 border traps/vineyard) and center (2 center traps/vineyard) of selected blocks. Trap collections were conducted once per month starting in May and continuing until September (5 sampling

dates), typically the period when most of the candidate insect vector species are present at higher population levels. Additional insect vector sampling was conducted using sweep-netting techniques during July-Septemberof 2015. Here we used a systematic sampling pattern in a minimum of 40 locations of each study vineyard. Sweep net-collected insects for each sampling location and date were directly placed into separate vials containing 95% ethyl alcohol. These samples were investigated under a stereomicroscope to determine species composition using morphological characters as highlighted in Saguez et al. (2014) and Beirne (1956). Collected insects are currently being identified in preparation for statistical analysis using standard ANOVA, similar to the methodology described in Walton et al. (2013).A subsample of vector insects was shipped to California entomologists for viral detection.

ii. Disease progression in established vineyard plots for grapevine red blotch-associated virus (GRBaV) and grapevine leafroll-associated virus (GLRaV) in Oregon.

PCR primers were used to determine the genomic status and spatial distribution of GRBaV together with that of GLRaV in Oregon vineyards (Krentz et al. 2012, Rwahnih et al. 2013). In fall of 2013-15, two hundred vines pervineyardweresystematically sampled and plant materials placed in an -80°C freezer within 24 hours of collection. Nucleic acid extracts frompetioles of each of the sampled vines were annotated and shipped to California virologists for genomic verification of virus infection status. The spatial distribution from analyzed plant materials wasassessed using SADIE (Spatial Analysis by Distance Indices, Perry 1995, Perry 1996, Perry & Dixon 2002) in order to document any changes and rate of virus spreadover time. Non-parametric spatial analysis was conducted in order to describe general spatial trends of virus infection levels as indicated by the collected plant materials. The SADIE analytical procedure (Perry 1995) was used to determine the overall index of aggregation, I_a, of each type of virus. By this metric, it can be determined whether distribution of virus within a vineyard block is uniform or aggregated. The dimensionless index of clustering, v_{ij}, measured the degree of clustering in areas with above-average density of virus, i.e., patches, or areas with below-average density, i.e., gaps (Winder et al. 2001, 2012; Maestre and Cortina 2002; De Villiers 2006). Values of v_{ij} between -1.5 and 1.5 indicate randomness (Winder et al. 2001; Perry and Dixon

2002; De Villiers 2006). To test for non-randomness, the mean values of the clustering indices, $\bar{v}_i$ and $\bar{v}_j$ were used. Patches of relatively high virus density are indicated by SADIE coordinate output values larger than 1.5, and gaps are indicated by coordinate values less than -1.5. Significant clustering, random association, and gaps were visually illustrated by inputting SADIE cluster analysis into Surfer® v12 (Golden Software, Inc., Golden, CO) and using the inverse distance weighted method.

Results

i. Insect vector distribution and species composition

Current data illustrate that 76-98% of all insects collected on sticky cards in Oregon from 2010-2015 were *Erythroneura elegantula* (Western Grape Leafhopper, Table 1), one ofseveral candidate vector species of GRBaV (Table 2). In both 2014 and 2015, over 1,200 Western Grape Leafhoppers were trapped on sticky cards during the season (Figure 1, Dalton et al. in prep.). In Vineyard 2, this leafhopper made up 92% and 98% of all insects collected using sticky cards in 2014 and 2015, respectively. By contrast, sweep net sampling in Vineyard 2 and Vineyard 3 showed relatively higher proportions of *Deltocephalus grex*(Table 3, Figure 2)and*Exitianus exitiosus*(Table 3), other candidate vector species.Both species are known virus vectors inother crops. The first is only foundin Western USA regions and a vector of Chickpea Chlorotic Dwarf Virus, which is a Geminivirus (Nault and Madden1988, Horn et al. 1993, Manzoor et al. 2014). In Vineyard 2, pheromone-baited grape mealybug monitoring showed little evidence of grape mealybug presence, but did show populations of Gill's mealybug.*Ceresapacifica* (Figure 2), which is related to recently identified GRBaV vector*Spissistilus festinus*(Bahder et al. 2016), was additionally found in at least two locations unrelated to vineyards in Oregon.

Table 1. Historical dominance of *E. elegantula* in Oregon viticultural areas during 2010-2015, as indicated through sticky card collections.

Year	% *E. elegantula*
2010	75.95
2011	83.02
2012	96.60
2014	94.78
2015	98.35

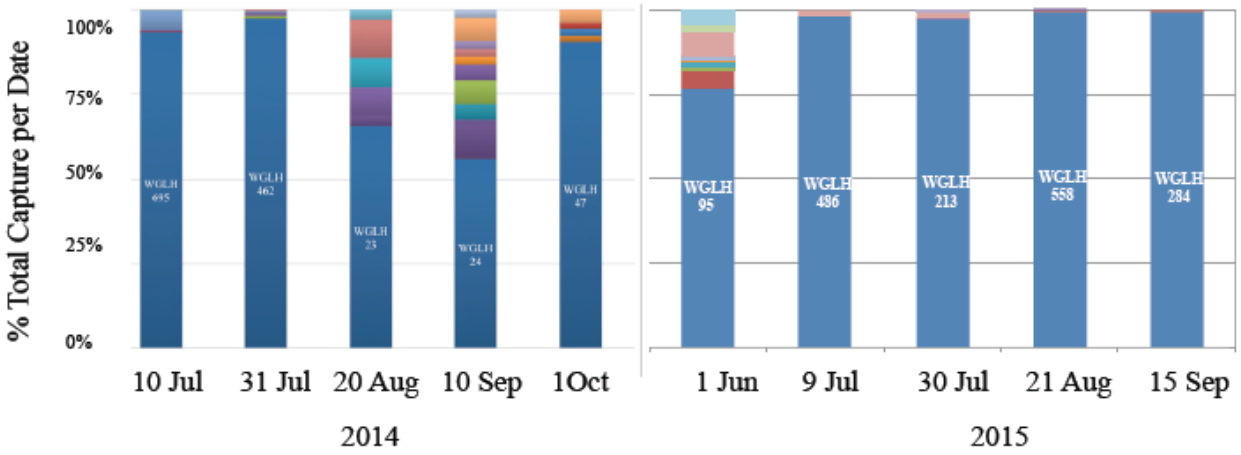

Figure 1. Prevalence of *Erythroneura elegantula* (western grape leafhopperlabeled bars) in Vineyard 2 in Southern Oregon in 2014 and 2015.

Figure 2. Two candidate vector insects, *Ceresa pacifica*(left) was not found in locations related to vineyards, but is related to *Spissistilus festinus*, a known vector of GRBaV (Bahder et al. 2016). *Deltocephalus grex*(right) was found in all vineyards in lower numbers in Oregon during 2013-2015.*D. grex* is a known vector of a related virus (Nault and Madden 1988, Horn et al. 1993).

Table 2. Potential GRBaV insect vector species found on sticky cards in Oregon vineyards during 2014-2015.

Species (common name)	**Percent**
Erythroneura elegantula (western grape leafhopper)	96.000
Deltocephalus grex	1.872
Empoasca spp.	0.730
Exitianus exitiosus	0.720
Cercopoidea (froghoppers/spittlebugs)	0.328
Aphids	0.112
Fieberiella florii	0.104
Helochara deltoides	0.084
Athysanus argentarius	0.028
Deltocephalus spp.	0.008
Empoasca fabae (potato leafhopper)	0.008
Gyponana praelonga	0.004

Table 3. Leafhopper species collected in 2015 in Southern Oregon vineyard blocks using sweep net sampling techniques.

Southern Oregon			Number of leafhoppers per sample				
Site/Date	**N samples**	**Mean N species**	***Deltocephalus grex***	***Erythroneura elegantula***	***Exitianus exitiosus***	***Sorhoanus* sp.**	**Total Leafhoppers**
Vineyard 2	66	3.4	32.1	26.0	2.1	0.2	64.6
9-Jul-15	39	4.5	54.2	10.9	3.4	0.3	76.1
15-Sep-15	27	1.9	0.1	48.0	0.3	0.0	48.0
Vineyard 3	79	2.7	6.4	0.4	0.6	0.4	12.3
10-Jul-15	39	3.4	12.6	0.6	1.0	0.2	19.6
14-Sep-15	40	2.0	0.5	0.2	0.3	0.6	5.1

ii. Disease progression in established vineyard plots for grapevine red blotch-associated virus (GRBaV) and grapevine leafroll-associated virus (GLRaV) in Oregon.

PCR tools (Krentz et al. 2012;Al Rwahnih et al. 2013, 2015) allowed tissue analysis of the trials conducted during 2013-2015. Analysis of these tissues show that the two key viruses GLRaV and GRBaV do spread in Oregon vineyards. For GLRaV, the epidemiology is understood based on the phenology of its insect vector, the grape mealybug, *Pseudococcus maritimus* (Walton et al. 2013). Ecological mapping of GRBaV-positive vines, as verified by PCR during 2013-2015, also showed a significant trend of virus spread in two viticulturalareas studied in Oregon (Table 4; Figure 3). These are the first documented studies showing spread in this region. The distribution patterns and spread of the virus within vineyards suggests the presence of an insect vector (e.g. Figure 3; Perry & Dixon 2002; Dalton et al. in prep.). In Vineyard 1 in the Willamette Valley and in Vineyard 2 in Southern Oregon, vines infected with GRBaVnoticeably increased between years of leaf sampling (Table 4). In two sites (Vineyards 3 and 4), numbers of infected plants appeared to decrease. One possible explanation of this unexpected result is that incipient infection that occurred late in the season was pruned out of the vines before the virus could become systematic within host plants. This would further implicate responsibility of a vector insect species because non-systemic transmission of virus would have to occur during the

growing season.Similar mapping (Fig. 4, Dalton et al. in prep.) of GLRaVillustrated that the virus distribution is independent of GRBaV. Also, thespread of GRBaV is clearly not associated with the distribution patterns ofgrape mealybug, the vector of GLRaV (Dalton et al. 2014).

The detailed field distribution and temporal increase of GRBaV infection strongly suggests that an insect vector plays a role. Specific to this topic, our data indicate that GRBaV-infected plants radiate from vines identified with GRBaV in previous years of PCR testing (Figure 3a, b).The described pathogen-vector complex is of significant economic importance. During the past six years, eight vineyards wereinitially selected for virus-vector (GLRaV and GRBaV) studies. Since initiation of the viral studies in 2010, three blocks were either fully (2011, 2013) or partially (2015-2016) removed in Southern Oregon due to perceived losses of fruit quality. Vines in these blocks displayed heavy symptoms of virus prior to removal.

Table 4. Temporal GRBaV virus infectionin three Oregon grape-growing regions as determined by PCR from 2013 to 2015. Vines sampled in 2013 and 2014 were re-tested for GRBaV in 2015.

Location	Year	Positive vines	Assayed vines	% Infection
Willamette Valley	2013 & 2014	133	374	35.6%
(Vineyard 1)	2015	172	374	46.0%
S. Oregon	2014	11	194	5.7%
(Vineyard 2)	2015	58	194	29.9%
S. Oregon	2014	55	193	28.5%
(Vineyard 3)	2015	33	193	17.0%
E. Oregon	2013 & 2014	4	396	1.0%
(Vineyard 4)	2015	0	396	0.0%

Our collaborating growers in all regions have noticed that vines symptomatic of one virus or the other consistently had longer ripening times and lower °Brix as harvest approached. Currently,

studies of crop quality are being conducted in California and Washington state. In Oregon,preliminary evidence shows a negative impact of virus presence associated with vine photosynthesis (Pagay pers. comm.).

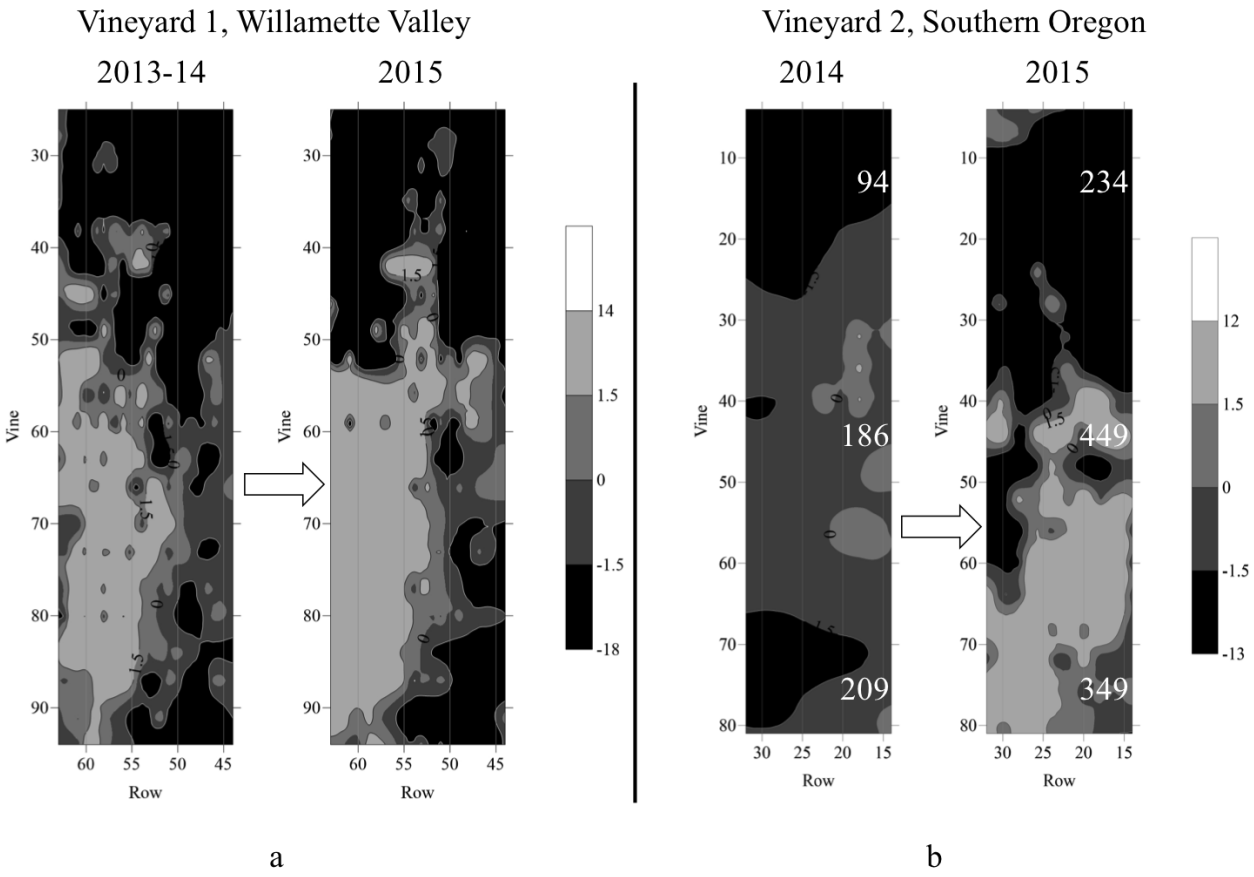

Figure 3 (a). Distribution and spread of Red Blotch in the Willamette Valley. **(b)**.Distribution and spread of Red Blotch in Southern Oregon. Data were generated using PCR analysis and ecologically-significant distribution. Light-colored areas indicate virus and dark-colored no virus distribution. White numbers indicate the mean number of western grape leafhopper in sticky card samples in each location of the vineyard (From Dalton et al. in prep).

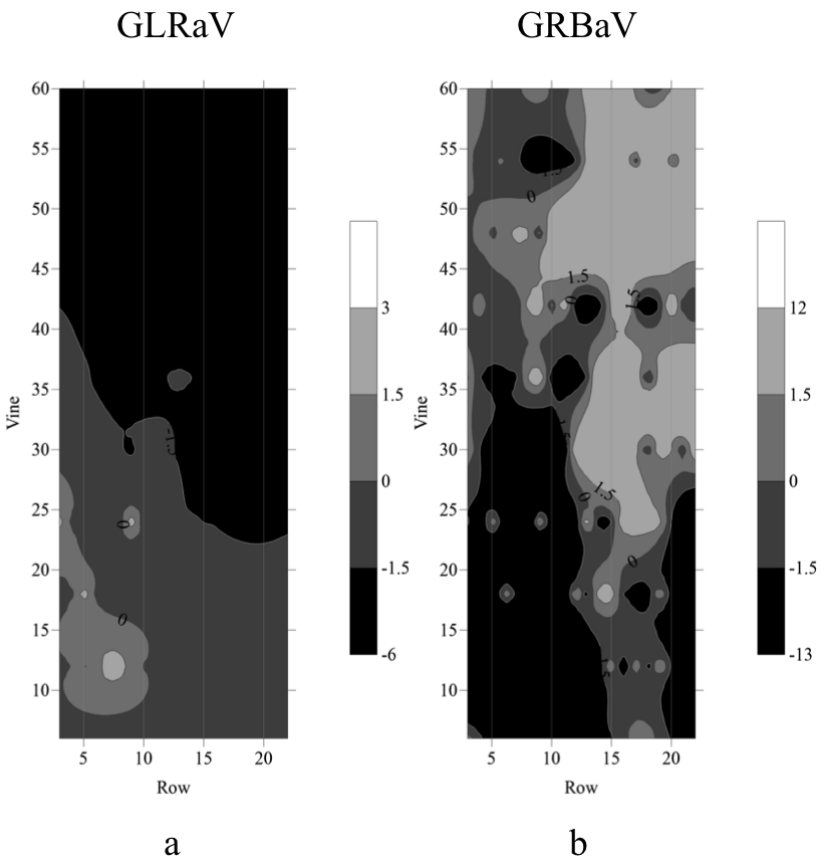

Figure 4 (a). Distribution of grapevine leafroll-associated virus (GLRaV) in 2013 as indicated by Spatial Analysis by Distance Indices (SADIE) from a vineyard in Southern Oregon. **(b).** Distribution of grapevine red blotch-associated virus (GRBaV) in 2013 as indicated by SADIE from the same vineyard in Southern Oregon.Light-colored areas indicate virus and dark-colored no virus distribution. White numbers indicate the mean number of western grape leafhopper in sticky card samples in each location of the vineyard (From Dalton et al. in prep).

Objective 2. Extension of information on the importance of vectors, GLRaV and GRBaV in Oregon vineyards.

Results were provided to growers, grape industry representatives and OSU Extension personnel through grower reports and seminars. In addition, information from this work was held at a regional vineyard workshop in the Willamette Valley during 2015. Additional winter meetings were held in Medford (organized by Rick Hilton) and Milton-Freewater (organized by Clive

Kaiser) as well as in Roseburg (organized by Steve Renquist). We presented a Spanish session on the epidemiology of Red Blotch at the annual Oregon Wine Industry Conference in Portland during February 2016. We also held a national webinar (ca. 350 attendees) to detail work conducted in Oregon during 2013-2015 to growers (see Grapevine Red Blotch YouTube video linked here). An annual Scouting workshop was held in Medford during July 2015 (60 growers) and in Milton-Freewater during May 2016. Monthly conference calls organized by Drs. Patricia Skinkis and Bob Martin (April 4, 2016 meeting with industry and other stakeholders)shared new information on virus epidemiology and vector status with extension agents in Oregon. Posters displaying research findings have been displayed annually since 2013 (2013 - 2016)at the OWRI Grape Day held in March in Corvallis.

Discussion

Vector insect collections compared to those in California show differences in Oregon vineyards. Information coming from insect virus transmission is encouraging with new revelations that *S.festinus*is a confirmed vector insect (Bahder et al. 2016). No clarity currently exists for potential vectors of GRBaV in Oregon. Our sampling in Oregon vineyards however shows a clear distinction from those in California. We have to date not found the confirmed vector of GRBaV (Bahder et al 2016), *S. festinus*. In Oregon we did however, collect specimens of the closely related treehopper, *C. pacifica*,near Medford and also in the Willamette Valley during 2015, but these findings were not made in locations that were in close proximity to vineyards.Two possible additional vector species include *E. exitiosus* and *D. grex*. Samples from our collections are currently being analyzed for virus in the salivary glands. The first of these two insects is only found in Western USA regions and both species are phylogenetically related to the vector of Chickpea Chlorotic Dwarf Virus (Knight 1976). It is important to note that GRBaV is only known to spread in Western USA vineyards, with no recordings of spread in vineyards in the eastern USA production regions. For this reason, we believe that we should focus our efforts on insect species that occur predominantly in the Western USA. One such species is *S.festinus*,but possible other species may include *E. exitiosus, D. grex* and *C. pacifica*.

As of yet, knowledge of field epidemiology of this disease is emerging for Oregon vineyards. Thespatial analysis of vine-virus status shows that distribution patterns of virus are not regularly distributed within vineyard blocks and that clusters of virus-infected plants can be found in nearly all vineyards that were sampled in Oregon. For these reasons we believe that the distribution of both virusessuggests vector dispersal, while the primary vector species of GLRaV is the grape mealybug (Walton et al. 2013).The differing distribution patterns of the two viruses found within vineyards suggest different vector insects are associated with each of the viruses. This spatial distribution describes statistically significant patching and gaps of both virus types in the affected Oregon vineyards, and that spread can be found in the Willamette Valley and Southern Oregon. Our data indicate that GRBaV-infected plants are concentrated toward the edge of the sampled vineyard blocks. In some cases it is clear that infected plants may have served as the source of initial infection. In one case, we found a one-year-old vine seedling in surrounding vegetation with GRBaV, suggesting a highly mobile insect vector. Radiation of virus-infected plants may have occurred over time. Genomic analysis of four of the most likely vector insectswill hopefully provide more clarity on this aspect of virus epidemiology. The possible impactsof alternative host plants and their role on spread of GRBaV need to be further investigated.

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