

ECOLOGY OF GRAPEVINE RED BLOTCH DISEASE

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Elizabeth Jeannette Cieniewicz

May 2019

© 2019 Elizabeth Jeannette Cieniewicz

ECOLOGY OF GRAPEVINE RED BLOTCH DISEASE

Elizabeth Jeannette Cieniewicz, Ph. D.

Cornell University 2019

Grapevine red blotch virus (GRBV) is the type member of a new genus- *Grablovirus* in the family *Geminiviridae*. GRBV causes grapevine red blotch disease, which was identified within the last decade as a threat to grape production in North America. GRBV is primarily spread via grafting and dissemination of infected planting material, but it can also be vectored under greenhouse conditions by the three-cornered alfalfa hopper (*Spissistilus festinus*). The epidemiology and ecology of this disease is not well understood. In order to gain a comprehensive understanding of GRBV spread in vineyards and the factors mediating secondary spread, we conducted several multi-year studies in vineyards with varying levels of GRBV incidence. We mapped red blotch symptoms for up to five years, and conducted insect surveys for two years in two vineyards in California and one vineyard in New York. These studies collectively demonstrated that GRBV is spreading in California vineyards, but not in New York. They also demonstrated that spread of GRBV is associated with the spatial distribution of *S. festinus*. Based on the frequent detection of GRBV in specimens from these surveys, three additional insect vector candidates were identified- *Colladonus reductus*, *Osbornellus borealis* (Cicadellidae) and a *Melanoliarius* spp. (Cixiidae), though these and *S. festinus* were not observed in the New York vineyard. GRBV and a related grablovirus, wild Vitis virus 1 (WVV1), were detected in wild grapevines throughout northern California, in higher density closer to grape production areas. The diversity of GRBV in wild grapevines and grape cultivars is similar but the

distribution and diversity patterns of GRBV in wild grapevines suggest that the direction of GRBV spread is predominantly from vineyards to wild grapevines. Studies on diversity of *S. festinus* throughout California and the southeastern U.S. revealed two distinct genotypes, in which the southeastern populations are distinct from the California populations, regardless of the host plant or time of collection. These studies on the epidemiology and ecology of red blotch disease have informed disease management recommendations with efforts that should be focused on removal of inoculum sources (i.e. infected vines and wild grapevines) rather than controlling *S. festinus* populations.

BIOGRAPHICAL SKETCH

Elizabeth (Libby) Cieniewicz was born and raised in Berks County, Pennsylvania and attended Lebanon Valley College in Annville, PA for her B.S. in Biology. Libby worked with several research mentors at Lebanon Valley in independent research projects. She participated in summer research experiences in plant pathology at Cornell University in Geneva, NY and in microbial marine ecology at the University of Delaware in Lewes, DE. Her professional interests are in plant virus ecology and virus-arthropod vector interactions, extension, and teaching. She is actively involved in the American Phytopathological Society. While at Cornell she has served as President and Vice President of the Student Association of the Geneva Experiment Station (SAGES), co-founded the first annual NYSAES Student Symposium in 2016, and participated in plant science outreach with elementary schools in Ithaca and Geneva, NY. She also mentored high school seniors in research rotations from the New Visions Medical Program in Geneva, NY and undergraduates in the Cornell Summer Scholars Program.

Dedicated to

my mother and father; to whom I owe everything
and Toby, the best boy ever

ACKNOWLEDGEMENTS

First and foremost, I am grateful to my mentor, Dr. Marc Fuchs for his unwavering support and guidance, for braving California traffic to collect wild grapevines, for inspiring my love of plant virus research and extension, and opening up endless opportunities to me. I thank my committee member Dr. Chris Smart for her perspectives and advice in my research, for supporting my interests in plant science outreach in schools, and for being the non-virologist voice of reason on my committee. Thank you also to my committee member Dr. Michelle Heck for her innovative ideas, and for treating me as one of her own.

I want to extend my appreciation to members of the Fuchs lab past and present who have supported me and this research, especially Yen Mei Cheung, Rosemary Cox, Fu-Wah Choi, Pat Marsella-Herrick, David Macumber, Madison Flasco, Alex Clarke, and Jess Choi. Special thank you to my friend Larissa Osterbaan, a patient and kind person to experience graduate school with. Thank you to the undergraduates who assisted with this research and taught me how to be a better mentor- Hannah Sweet, Anuli Onwumelu, Alex Clarke, Victoria Poplaski, Quinlan Corbett, and Melina Brunelli.

Thank you to those who inspired my research interests including Dr. Rebecca Urban, Dr. Allan Wolfe, Dr. John Gottula, Dr. Stewart Gray, Dr. Keith Perry, Dr. Greg Loeb, Dr. Brian Nault, Dr. Sarah Pethybridge, and Dr. Tim Martinson. Thank you to those who supported our research in vineyards, especially Morgan Brett, Kelsey Leslie, and all those at St. Supéry Estate Vineyards and Winery. Thank you to Alice Wise, Amanda Gardner, and Claudia Purita at One Woman Wines and Vineyards.

On a personal note, I extend my utmost appreciation to my friends at Cornell,

in particular Elizabeth Maloney, Katrin Ayer, Angela Kruse, Adrienne Gorny, Chase Crowell, Bill Weldon, and Chris Peritore. Thank you to my perfect parents, grandparents, and my constantly challenging and wonderful siblings (and in-laws) Annie, Romeo, Mary-Kate, Kyle, Danny, Carly, Patrick, and Matthew. Finally, thank you to Jessie Kriner, for keeping my spirits high, always.

This research was financially supported by USDA-NIFA Predoctoral Fellowship funds, Cornell CALS Extension/Outreach Assistantships, the California Grape Rootstock Research Foundation, the California Grape Rootstock Improvement Commission, the American Vineyard Foundation, the California Department of Food and Agriculture, the New York Grape and Wine Foundation, and USDA-National Institute of Food and Agriculture-Critical Agricultural Research and Extension.

TABLE OF CONTENTS

CHAPTER 1: GRAPEVINE RED BLOTCH- MOLECULAR BIOLOGY OF THE VIRUS AND MANAGEMENT OF THE DISEASE	1
ABSTRACT	1
INTRODUCTION	3
DISEASE SYMPTOMS AND ECONOMIC IMPACT.....	4
THE VIRUS GENOME STRUCTURE AND GENETIC VARIABILITY	7
FULFILLING KOCH’S POSTULATES	9
DETECTION AND DIAGNOSTICS	11
HOST RANGE AND GEOGRAPHIC DISTRIBUTION	12
EPIDEMIOLOGY AND TRANSMISSION.....	14
MANAGEMENT	19
ORIGIN OF THE VIRUS AND FUTURE PROSPECTS	19
REFERENCES	21
CHAPTER 2: SPATIOTEMPORAL SPREAD OF GRAPEVINE RED BLOTCH- ASSOCIATED VIRUS IN A CALIFORNIA VINEYARD	27
ABSTRACT	27
INTRODUCTION	29
MATERIALS AND METHODS	30
RESULTS	35
DISCUSSION.....	46
REFERENCES	49

CHAPTER 3: INSIGHTS INTO THE ECOLOGY OF GRAPEVINE RED BLOTCH VIRUS IN A DISEASED VINEYARD.....	56
ABSTRACT	56
INTRODUCTION	58
MATERIALS AND METHODS	61
RESULTS	68
DISCUSSION.....	85
REFERENCES	90
CHAPTER 4: PREVALENCE AND GENETIC DIVERSITY OF GRABLOVIRUSES IN FREE-LIVING <i>VITIS</i> SPP.....	97
ABSTRACT	97
INTRODUCTION	99
MATERIALS AND METHODS	101
RESULTS	106
DISCUSSION.....	126
REFERENCES	131
CHAPTER 5: SPREAD DYNAMICS OF GRAPEVINE RED BLOTCH VIRUS IN RELATION TO <i>SPISSISTILUS FESTINUS</i> ABUNDANCE IN VINEYARDS.....	138
ABSTRACT	138
INTRODUCTION	140
MATERIALS AND METHODS	142
RESULTS	147
DISCUSSION.....	162

REFERENCES	168
CHAPTER 6: TWO DISTINCT GENOTYPES OF <i>SPISSISTILUS FESTINUS</i> IN THE UNITED STATES	175
ABSTRACT	175
INTRODUCTION	177
MATERIALS AND METHODS	178
RESULTS	184
DISCUSSION	189
REFERENCES	191
CHAPTER 7: RED BLOTCH ECOLOGY: CONCLUSIONS, MANAGEMENT IMPLICATIONS, AND FUTURE DIRECTIONS	195
SPREAD OF GRBV AND PHENOLOGY OF <i>SPISSISTILUS FESTINUS</i> IN VINEYARDS	197
GRABLOVIRUSES: A RED BLOTCH DISEASE COMPLEX?	199
TRANSMISSION BIOLOGY	201
GRAPEVINE RED BLOTCH VIRUS HOST RANGE	204
REFERENCES	204
APPENDIX 1: GUIDELINES FOR REARING <i>SPISSISTILUS FESTINUS</i> IN A CONTROLLED ENVIRONMENT	209
JUSTIFICATION	209
FIELD COLLECTION OF <i>SPISSISTILUS FESTINUS</i>	210
REARING PROCEDURES IN A CONTROLLED ENVIRONMENT	210
PEST MANAGEMENT	213

<i>SPISSISTILUS FESTINUS</i> REARING: NOTABLE OBSERVATIONS	217
REFERENCES	220
APPENDIX 2: PROGRESS IN <i>SPISSISTILUS FESTINUS</i> TRANSMISSION	
BIOLOGY	221
JUSTIFICATION	221
PART 1: <i>SPISSISTILUS FESTINUS</i> GUT AND SALIVARY GLAND	
ANATOMY	223
PART 2: <i>SPISSISTILUS FESTINUS</i> BEHAVIOR ON GRAPEVINE	229
PART 3: ACQUISITION OF GRAPEVINE RED BLOTCH VIRUS BY	
<i>SPISSISTILUS FESTINUS</i>	234
PART 4: TRANSMISSION ASSAYS, TRIAL AND ERROR	239
PART 5: CONCLUDING REMARKS	246
REFERENCES	246
APPENDIX 3: EXPERIMENTAL HOST RANGE OF GRAPEVINE RED BLOTCH	
VIRUS	252
JUSTIFICATION	252
AGROINOCULATION METHOD	252
DETECTION OF SPLICED GRBV TRANSCRIPTS.....	253
AGROINOCULATION EXPERIMENTS: RESULTS	254
CONCLUDING REMARKS AND FUTURE DIRECTIONS	258
REFERENCES	259
APPENDIX 4: EXTENSION ARTICLES- STUDIES ON RED BLOTCH ECOLOGY	
INFORM DISEASE MANAGEMENT RECOMMENDATIONS.....	263

RED BLOTCH DISEASE: A NEW THREAT TO GRAPE	
PRODUCTION	264
EFFECTS OF GRBV ON VINE GROWTH AND FRUIT JUICE	
CHEMISTRY	267
SPREAD OF RED BLOTCH IN A NAPA VALLEY VINEYARD.....	267
SURVEY OF POTENTIAL INSECT VECTORS IN A CALIFORNIA	
VINEYARD	271
LIMITED SPREAD OF GRBV IN AN ADJACENT CALIFORNIA	
VINEYARD	272
SURVEY OF RED BLOTCH IN A NEW YORK VINEYARD.....	274
GRBV IS WIDESPREAD IN WILD GRAPEVINES IN CALIFORNIA.....	274
A NEWLY RECOGNIZED VIRUS RELATED TO GRBV IS PRESENT IN	
WILD VINES IN CALIFORNIA	275
ROLE OF COVER CROPS IN RED BLOTCH ECOLOGY	277
RED BLOTCH DISEASE MANAGEMENT RECOMMENDATIONS	279
REFERENCES	282

APPENDIX 5: GRAPEVINE LEAFROLL DISEASE: INTEGRATED PEST	
MANAGEMENT DISEASE FACT SHEET	285
INTRODUCTION	286
SYMPTOMS AND IMPACT	286
CAUSAL AGENTS	289
CONDITIONS FOR INFECTION.....	291
MANAGEMENT	291

APPENDIX 6: APPLE CHLOROTIC LEAF SPOT VIRUS: INTEGRATED PEST	
--------------------------------------------------------------	--

MANAGEMENT DISEASE FACT SHEET	293
INTRODUCTION	294
DISEASE AND CAUSAL AGENT	294
SYMPTOMS AND IMPACT	296
SPREAD	297
MANAGEMENT	297

APPENDIX 7: APPLE STEM PITTING VIRUS: INTEGRATED PEST

MANAGEMENT DISEASE FACT SHEET	299
INTRODUCTION	300
DISEASE AND CAUSAL AGENT	300
SYMPTOMS AND IMPACT	302
SPREAD	302
MANAGEMENT	304

LIST OF FIGURES

Figure 1-1	Foliar symptoms of red blotch disease on various grape cultivars.....	5
Figure 1-2	Genome organization of grapevine red blotch-associated virus.....	8
Figure 1-3	Aggregation of approximately 20 red blotch diseased vines followed by healthy vines in two adjacent rows of a Cabernet franc vineyard in California.....	15
Figure 1-4	Adult <i>Spissistilus festinus</i> , a vector of GRBaV, resting on a shoot of a <i>Vitis vinifera</i> cv. Cabernet Sauvignon vine in California.....	17
Figure 1-5	Girdle damage to a single leaf of a Cabernet Sauvignon shoot, which is suggestive of <i>Spissistilus festinus</i> feeding.....	18
Figure 2-1	Spatial pattern in 2014-2016 of symptomatic vines and PCR detection of grapevine red blotch-associated virus in a section of a ‘Cabernet franc’ vineyard near a riparian habitat.....	37
Figure 2-2	Spatial pattern of diseased vines in 2014-2016 in a ‘Cabernet franc’ vineyard affected by red blotch and the annual disease incidence plotted over the long axis in 5-vine increments.....	40
Figure 2-3	Z-statistics derived from testing for spatial aggregation of diseased vines within rows using ordinary runs analysis of a ‘Cabernet franc’ vineyard	41
Figure 2-4	Contour plots of the posterior parameter density of a spatiotemporal stochastic model fitted to the spread of grapevine red blotch-associated virus in a ‘Cabernet franc’ vineyard.....	45
Figure 3-1	Map of the ‘Cabernet franc’ vineyard infected with grapevine red blotch virus clade 2 isolates that was selected for this study, indicating	

	its relative location close to a riparian area, and a close-up of the vineyard area selected for this study with the layout of the insect survey overlaid with GRBV incidence	63
Figure 3-2	Diversity and abundance of insect taxa in a ‘Cabernet franc’ vineyard infected by grapevine red blotch virus in Rutherford, California in 2015 and 2016	72
Figure 3-3	Seasonal population dynamics of candidate insect vectors of grapevine red blotch virus in 2015 and 2016	75
Figure 3-4	Seasonal dynamics of ingestion of grapevine red blotch virus by candidate insect vectors in 2015 and 2016	76
Figure 3-5	Neighbor-joining phylogenetic tree indicating relationships among grapevine red blotch virus isolates from infected vines, candidate insect vectors, and nearby GRBV-infected vines	78
Figure 3-6	Spatial distribution of candidate insect vectors at the edge of a ‘Cabernet franc’ vineyard.....	84
Figure 4-1	Map of northern California counties showing the density of acreage planted to grapevine, along with the locations and grablovirus infection status of free-living vines	111
Figure 4-2	Map of New York counties showing the density of acreage planted to grapevine, along with the locations and grablovirus infection status of free-living vines.....	113
Figure 4-3	Maximum likelihood phylogeny of wild <i>Vitis</i> virus 1 full genome sequences of isolates from free-living <i>Vitis</i> spp.	117
Figure 4-4	Recombination events in wild <i>Vitis</i> virus 1 isolate NY1424	118
Figure 4-5	Maximum likelihood phylogeny of wild <i>Vitis</i> virus 1 diversity	

	fragment nucleotide sequences of isolates from free-living <i>Vitis</i> spp. in California.....	120
Figure 4-6	Maximum likelihood phylogeny of grapevine red blotch virus diversity fragment nucleotide sequences of isolates from free-living <i>Vitis</i> spp. in California.....	122
Figure 4-7	Pairwise identity matrix of grapevine red blotch virus and wild <i>Vitis</i> virus 1 diversity fragment sequences	124
Figure 4-8	Circular alignment of nucleotide sequences of representative grapevine red blotch virus and wild <i>Vitis</i> virus 1 isolates	125
Figure 5-1	Map of grapevine red blotch virus incidence in a 1.5-hectare Cabernet Sauvignon and a 2.0 hectare Cabernet franc vineyard within ten years-post planting	150
Figure 5-2	Graphical and pictorial depiction of four grapevine red blotch disease epidemics in a Merlot vineyard in New York and California	152
Figure 5-3	Neighbor-joining phylogeny of the genomic diversity fragment of grapevine red blotch virus isolates from vineyards in California and New York	154
Figure 5-4	Location of <i>Spissistilus festinus</i> caught on sticky cards in a Cabernet Sauvignon vineyard in California in 2017 and 2018.....	157
Figure 5-5	Seasonal abundance of <i>Spissistilus festinus</i> and other candidate insect vectors in a Cabernet Sauvignon vineyard in California in 2017 and 2018	159
Figure 6-1	Neighbor-joining phylogeny and pairwise identity matrix of mitochondrial cytochrome C oxidase I partial gene sequences from <i>Spissistilus festinus</i> populations from various locations, years, and crops throughout the U.S.....	185

Figure 6-2	Internal transcribed spacer 2 sequence analysis from several populations of <i>Spissistilus festinus</i>	186
Figure 6-3	Diagnostic assay for identification of specimens of the two <i>Spissistilus festinus</i> genotypes.....	188
Figure 7-1	Neighbor-joining phylogeny of 114 grapevine red blotch virus and 13 wild Vitis virus 1 full genome accessions	202
Figure 7-2	Maps of the United States and the world in which grapevine red blotch virus has been detected in vineyards	203
Figure A1-1	Egg, first instar, second instar, third instar, fourth instar, fifth instar, and adult <i>Spissistilus festinus</i>	212
Figure A1-2	Sign of two-spotted spider mite on alfalfa plants.....	215
Figure A1-3	<i>Spissistilus festinus</i> blue color morphs observed in the laboratory colony	219
Figure A2-1	Locations of major incisions for dissecting salivary glands and the gut from <i>Spissistilus festinus</i> adults.....	225
Figure A2-2	Proposed anatomical description of salivary glands of <i>Spissistilus festinus</i> adults	226
Figure A2-3	Proposed anatomical description of the guts of <i>Spissistilus festinus</i> adults.....	228
Figure A2-4	<i>Spissistilus festinus</i> eggs inserted into grapevine petiole, and first instar nymph hatched on grape leaf.....	230
Figure A2-5	<i>Spissistilus festinus</i> on potted grapevines in the greenhouse.....	231
Figure A2-6	Mortality curves and gut images of field-collected <i>Spissistilus festinus</i>	

	on potted alfalfa, and grapevine red blotch virus-positive and -negative potted grapevines in the greenhouse.....	233
Figure A2-7	Reduction in grapevine red blotch virus DNA in <i>Spissistilus festinus</i> over time after removal of exposure of the virus source, and higher overall copy number in nymphs compared to adults.....	238
Figure A3-1	Reverse transcription-PCR detection of grapevine red blotch virus transcripts following agro-inoculation	257
Figure A4-1	Reduction in fruit quality and ripening on a red blotch diseased compared to a healthy vine.....	265
Figure A4-2	Foliar symptoms of red blotch disease on several cultivars	266
Figure A4-3	Red blotch secondary spread in a gradient down the row of a Cabernet franc vineyard in Rutherford, California	269
Figure A4-4	Grapevine red blotch disease spread in a Cabernet franc vineyard in California over five years	270
Figure A4-5	Landscape view of red blotch spread in Cabernet franc and Cabernet Sauvignon vineyards in Rutherford, California.....	273
Figure A4-6	Map of wild vines in northern California surveyed for the presence of grapevine red blotch virus and wild Vitis virus 1.....	276
Figure A4-7	Three-cornered alfalfa hopper egg and first instar on a petiole of a <i>Vitis vinifera</i> cv. Syrah vine.....	278
Figure A5-1	Symptoms of grapevine leafroll disease in vineyards	288
Figure A5-2	Electron micrograph of a grapevine leafroll-associated virus particle isolated from a diseased vine.....	290

Figure A6-1	Declining Red Delicious/G.935 trees infected with apple chlorotic leaf spot virus and apple stem pitting virus in a nursery	295
Figure A7-1	Depressions on the woody cylinder of the trunk of a declining apple tree infected with apple stem pitting virus and apple chlorotic leaf spot virus	301
Figure A7-2	A declining apple tree infected by apple stem pitting virus and apple chlorotic leaf spot virus compared to healthy trees in a nursery	303

LIST OF TABLES

Table 2-1	Spatial aggregation of grapevines affected by red blotch disease in a vineyard in California.....	42
Table 2-2	Spatiotemporal analysis of a red blotch epidemic in a vineyard in California	43
Table 3-1	Grapevine red blotch virus detection in insects trapped in a ‘Cabernet franc’ vineyard.....	70
Table 3-2	Grapevine red blotch virus isolate sequence information	79
Table 3-3	Analysis of the spatial pattern of grapevine red blotch infected grapevines and candidate insect vector populations.....	82
Table 3-4	Association between spatial patterns of virus incidence and candidate insect vector populations	83
Table 4-1	California grapevine red blotch virus and wild <i>Vitis</i> virus 1 isolates collected in a survey of wild grapevines	108
Table 4-2	Grablovirus detection in free-living <i>Vitis</i> spp. in California by county	110
Table 4-3	Grablovirus detection in free-living <i>Vitis</i> spp. in New York by county	112
Table 5-1	Comparative red blotch disease progress in relation to <i>Spissistilus festinus</i> populations in three vineyards planted in 2008.....	151
Table 5-2	Grapevine red blotch virus detection in insects trapped in a diseased ‘Cabernet Sauvignon’ vineyard in California in 2017 and 2018.....	158

Table 5-3	Grapevine red blotch virus detection in insects trapped in a diseased ‘Merlot’ vineyard in New York in 2017 and 2018.....	161
Table 6-1	Sites of <i>Spissistilus festinus</i> collection, date of collection, and primers used for genomic characterization.....	180
Table A2-1	Summary of four transmission experiments using potted vines and individual <i>Spissistilus festinus</i>	242
Table A3-1	Results of agroinoculation experiments on potential alternative hosts of grapevine red blotch virus	256

LIST OF ABBREVIATIONS

- GRBaV Grapevine red blotch-associated virus: The virus name included “associated” before the virus was demonstrated as the causal agent of red blotch disease.
- GRBV Grapevine red blotch virus: The causative agent of red blotch disease.
- TCAH Three-cornered alfalfa hopper: Common name of *Spissistilus festinus*, the vector of GRBV.
- SADIE Spatial Analysis by Distance IndicEs: A statistical model used to test the spatial aggregation and associations between sets of spatial data.
- PCR Polymerase chain reaction: Molecular biology technique used to make many copies of specific DNA fragments

PREFACE

Grapevine red blotch virus (GRBV) is the first geminivirus discovered in grapevine. An understanding of the epidemiology and ecology of red blotch disease is needed in order to devise optimal management strategies in vineyards. In this dissertation the reader will gain an understanding of the ecology of red blotch disease and future directions of this research. Chapter 1 introduces the reader to the pathosystem and management of red blotch disease. Chapter 2 describes the spatiotemporal dynamics of a red blotch epidemic in a Cabernet franc vineyard in California between 2014 and 2016. Chapter 3 describes the insect vector phenology, GRBV ingestion, and spatial association with infected vines in the aforementioned Cabernet franc vineyard in 2015 and 2016. Chapter 4 is focused on the widespread distribution of GRBV and a related virus, wild Vitis virus 1, in wild grapevines in northern California, and the absence of these viruses from wild vines in New York. Chapter 5 follows up on chapters 2 and 3 and describes red blotch spread attributes in vineyards in California and New York. In Chapter 6 I describe the identification of two distinct genotypes of *Spissistilus festinus*, the vector of GRBV, one from the southeastern U.S. and one from California. In Chapter 7 I discuss perspectives of this research and future directions for research on red blotch epidemiology and GRBV biology. Appendix 1 describes best practices for rearing *S. festinus*. Appendix 2 outlines progress in understanding the transmission biology of GRBV by the vector *S. festinus*. Appendix 3 describes the progress in understanding the experimental host range of GRBV based on agro-inoculation experiments. Appendix 4 is an extension publication on management of red blotch disease in vineyards. Appendices 5 through 7 are disease factsheets developed for extension on grapevine leafroll disease, apple chlorotic leaf spot virus, and apple stem pitting virus, respectively.

CHAPTER 1

GRAPEVINE RED BLOTCH: MOLECULAR BIOLOGY OF THE VIRUS AND MANAGEMENT OF THE DISEASE

ABSTRACT

Red blotch is a recently recognized disease of grapevine for which the graft-transmissible grapevine red blotch-associated virus (GRBaV), a proposed member of a new genus within the family *Geminiviridae*, is the causal agent. The virus affects fruit quality, delays ripening and probably reduces yield and vigor. Estimated economic losses range from \$2,213 to \$68,548 per hectare over a 25-year productive lifespan of a vineyard. The genome of GRBaV is circular and consists of a single molecule of single-stranded DNA with seven predicted open reading frames. Foliar symptoms consist of red blotches that expand and coalesce late summer and fall, and irregular chlorotic areas that become necrotic later in the season in red-berried and white-berried cultivars, respectively. Visual diagnosis is often unreliable due to several confounding abiotic and biotic factors, including similarities with leafroll disease symptoms; therefore, PCR-based assays are recommended for an accurate diagnosis. Although red blotch disease was only recognized in 2008, GRBaV was detected in archival grapevine leaves sampled in 1940 in California and kept in a herbarium collection, suggesting the virus was present in vineyards more than 70 years prior to its identification. Surveys of vineyards revealed the occurrence of GRBaV in some of the major grape-growing regions in the USA and Canada. Outside of North America, the virus was found so far in Switzerland in material introduced from the USA and in

South Korea. An early account of the Virginia creeper leafhopper as a vector of GRBaV was not confirmed; instead, the three-cornered alfalfa treehopper was shown to be a likely vector of epidemiological significance. Disease management strategies almost exclusively rely on roguing and replacing vineyards using planting material derived from clean, virus-tested stocks. Advancing our understanding of disease epidemiology and viral gene expression are important future research topics for red blotch disease and GRBaV.

*This chapter was published in: Cieniewicz E.J., Perry K.L. & Fuchs M. (2017) Grapevine red blotch: molecular biology of the virus and management of the disease. Pages 303-314 in: "Grapevine viruses: molecular biology, diagnostics and management", B. Meng, G.P. Martelli, D. Golino and M. Fuchs (eds), Springer Verlag, Berlin, Germany.

INTRODUCTION

Red blotch is a relatively recently recognized viral disease of grapevine. It was first described in a Cabernet Sauvignon vineyard at a Research Station of the University of California-Davis in Oakville, California (Calvi, 2011). Symptoms of diseased vines were similar to leafroll disease symptoms but none of the diagnostic tools available for leafroll viruses would detect a virus. Concomitantly, several laboratories were handling material exhibiting leafroll-like symptoms for which the etiology was elusive, as all of them would test negative for the five known leafroll-associated viruses. Most of these vines were found later to be infected by the causal agent of red blotch disease after the virus was discovered and specific diagnostic tools were developed.

Research efforts at Cornell University (Krenz *et al.* 2012) and UC-Davis (Al Rwahnih *et al.* 2013) led to the identification and characterization of Grapevine red-blotch associated virus (GRBaV) in vines affected by red blotch disease. Subsequently, other groups described the same virus although it was initially named Cabernet franc-associated virus (Krenz *et al.* 2012), Grapevine redleaf-associated virus (Poojari *et al.* 2013) and Grapevine geminivirus (Seguin *et al.* 2014). Based on the characteristic symptoms initially observed on diseased vines, the name Grapevine red-blotch associated virus was retained and adopted by the grape virology community (Sudarshana *et al.* 2015).

The objectives of this chapter are to provide an overview of our current knowledge of the biology and ecology of red blotch disease, and to offer perspectives for future research.

DISEASE SYMPTOMS AND ECONOMIC IMPACT

Red blotch disease symptoms consist of foliar and fruit symptoms. Foliar symptoms first appear on older leaves at the base of the canopy in late spring to early summer. Symptoms are initially seen on older leaves (**Figure 1-1A**) and progressively observed toward the top of the canopy in late summer and fall. In red-berried cultivars, red blotches are observed early in the growing season; these typically coalesce with most of the leaf blade becoming red later in the season (**Figure 1-1B**). Shades of red vary from crimson to purple. Heavily symptomatic leaves often drop off prematurely late in the season. In white-berried cultivars, foliar symptoms consist of chlorotic areas that can become necrotic later in the season (**Figure 1-1C**). On fruits, delays in ripening, altered fruit juice chemistry indices, particularly of total soluble solids (-1° to -4° Brix) and lower anthocyanin contents in berry skin (**Figure 1-1D**), are characteristic of red blotch disease.

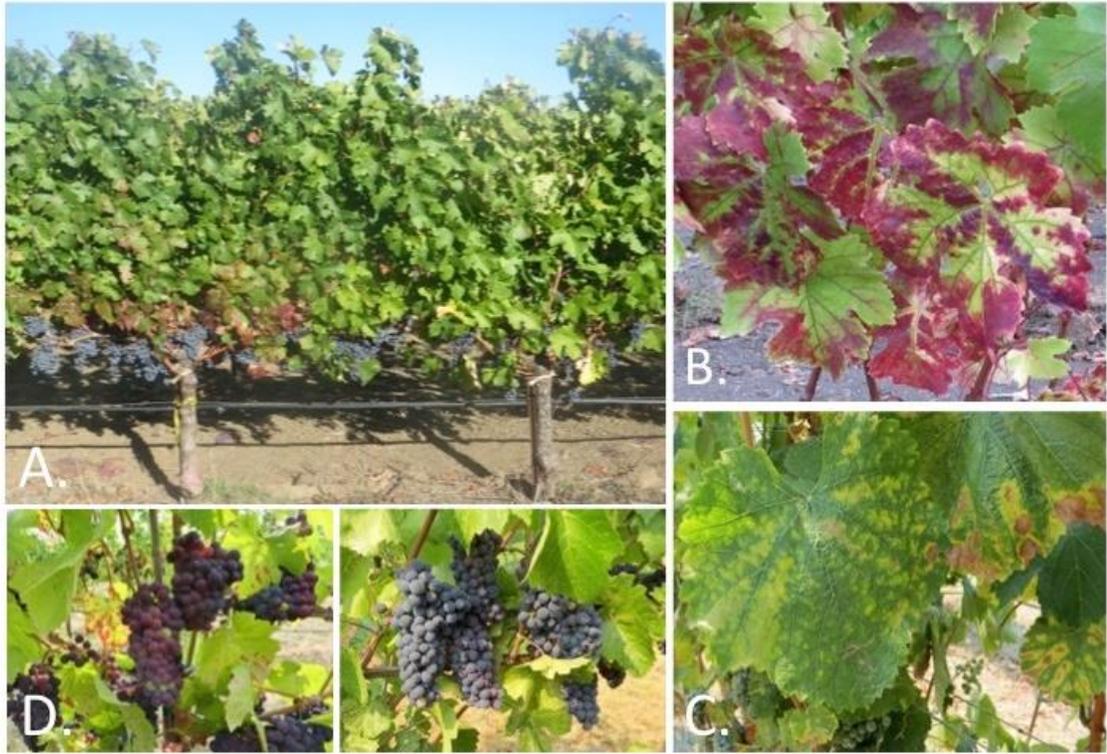


Figure 1-1 Foliar symptoms of red blotch (A) at the bottom of the canopy of a diseased (left) compared to a healthy (right) Cabernet franc; close-up of foliar symptoms on (B) Cabernet franc and (C) Chardonnay; and (D) fruit symptoms on a diseased (left) compared to a healthy (right) Pinot noir.

The severity of symptoms and their onset vary with cultivar, vineyard location and growing season. In addition, a visual diagnosis can be challenging due to similarities of foliar and fruit symptoms between red blotch and leafroll diseases. Similarities exist also between red blotch symptoms in red-berried cultivars and those caused by other biotic factors such as Pierce's disease, crown gall, and mite damage, and abiotic factors such as poor root health, shoot girdling due to insect damage, and trunk injury. Red blotch symptoms are also similar to symptoms elicited by nutrient deficiencies such as magnesium or potassium deficiency. These numerous confounding factors and the variation in symptom expression make a visual diagnosis of red blotch disease difficult; only PCR-based assays are reliable for an accurate diagnosis.

The economic cost of red blotch is estimated to range from \$2,213 to \$68,548 per hectare over a 25-year productive lifespan of Cabernet Sauvignon and Merlot vineyards, depending on the level of initial infection and price penalty for sub-optimal fruit quality (Ricketts *et al.* 2016). These estimates are more or less within the range previously determined for leafroll disease in Cabernet Sauvignon in California and New York (\$25,000 to \$226,405) (Atallah *et al.* 2012; Ricketts *et al.* 2015). The lower cost estimates for red blotch compared to leafroll are likely due to the limited information currently available on the effect of red blotch on fruit yield and on the rate of spread in vineyards. Therefore, an intrinsic lower impact of red blotch compared to leafroll should not be assumed. Studies on the impact of GRBaV on vigor and yield are underway, as are epidemiological studies. This research will be important to improve estimates of loss due to red blotch.

THE VIRUS GENOME STRUCTURE AND GENETIC VARIABILITY

GRBaV is a monopartite gemini-like virus with a genome of one single-stranded circular DNA element (**Figure 1-2**). The sequence of the genome has been used to predict encoded proteins and the overall organization of the genome (Al Rwahnih *et al.* 2013; Krenz *et al.* 2012, 2014; Poojari *et al.* 2013). There are six previously reported open reading frames (ORFs), all of which are, in part, overlapping. The circular genome is depicted with a conserved origin of replication at the top, with genome-sense ORFs clockwise to the right (V2, V1 and V3), and the complementary sense ORFs counterclockwise to the left (C1, C2 and C3). A recent analysis of transcription is consistent with the expression of a seventh ORF designated V0 (Perry *et al.* unpublished). The protein function is only clear for two of the viral products, as deduced from sequence conservation within the family *Geminiviridae*; these are the coat protein encoded by V1 and a replicase expressed from a spliced transcript spanning the C1 (RepA) and C2 ORFs. This gene expression strategy is seen in other members of the family *Geminiviridae*, with the splicing site confirmed in mapped GRBaV transcripts (Krenz *et al.* 2014).

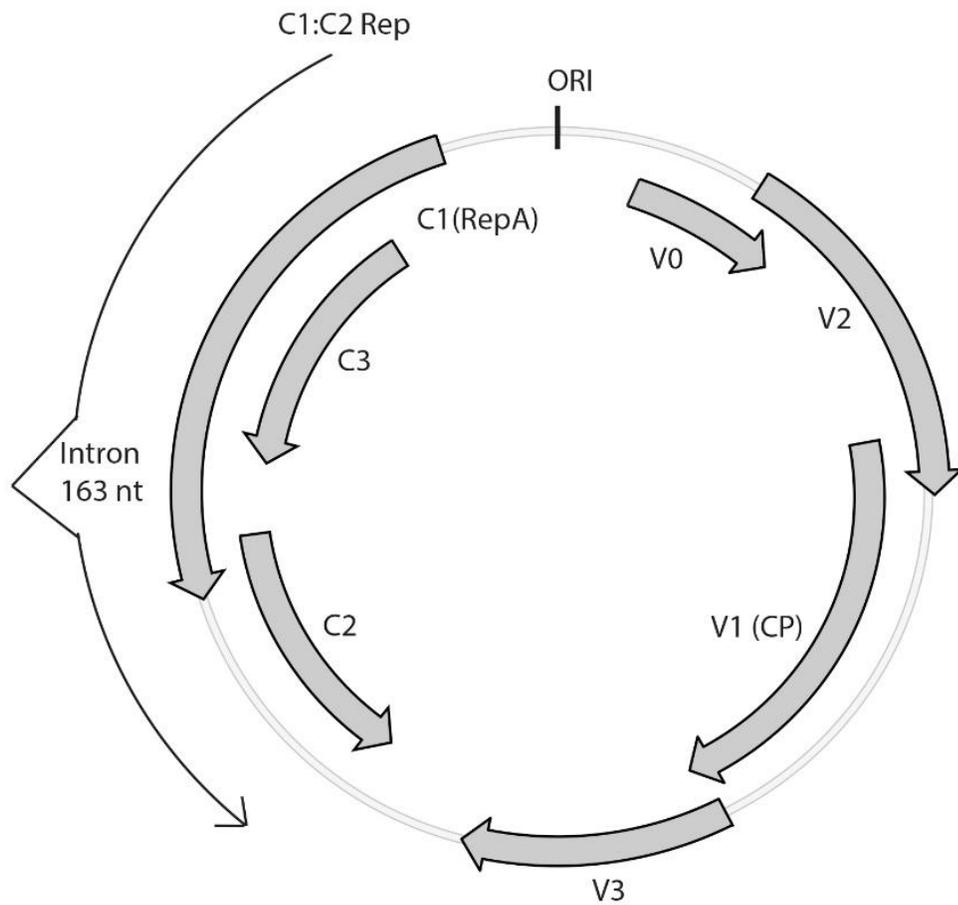


Figure 1-2 Genome organization of grapevine red blotch-associated virus (GRBaV). The circular single-stranded genome of 3,206 nucleotides is diagrammed with thick arrows indicating the open reading frames. Additional features are as described in the text.

The ORFs V0, V2, and V3 show no sequence similarity to other viral genes, but have been hypothesized to play a role in movement. Transient expression studies showed that protein V2 localizes in the nucleoplasm, Cajal bodies, and cytoplasm when fused to GFP; and protein V3 localizes in various unidentified subnuclear bodies when similarly fused to GFP (Guo *et al.* 2015). It is important to note that outside of the C1:C2 splice site, all other details of gene expression are entirely deduced from sequence analyses, and no encoded proteins have been detected. That said, analysis of sequence and codons in the 23 available GRBaV genomes clearly show that five of the six ORFs were under strong purifying selection (Perry *et al.* 2016), from which it can be inferred these proteins are functional and expressed. When first published, the identification and functionality of ORF C3 was uncertain, but surprisingly, this gene is under strong positive selection and is now assumed to play an essential role.

Genetic variability among isolates of GRBaV is sufficient to recognize two distinct clades with nucleotide variation of up to 9% (Krenz *et al.* 2014). Isolates within clade I show a maximum of 5% sequence heterogeneity, while those within clade II are relatively homogeneous with a 2% or less in nucleotide variation. These levels of variation are consistent with all isolates forming a single species. Phylogenetic analyses further reveal that GRBaV is the type member of a new genus within the family *Geminiviridae* (Varsani *et al.* 2014), tentatively named *Grablovirus*.

FULFILLING KOCH'S POSTULATES

To satisfy Koch's postulates, infectious GRBaV clones were engineered from partial dimer constructs of the genome of isolates NY175 and NY358 that belong to

phylogenetic clades I and II, respectively (Krenz *et al.* 2014). These clones were used in agroinoculation experiments using healthy, tissue culture-grown vines of *V. vinifera* cultivars and rootstock genotypes (Fuchs *et al.* 2015). Constructs of green fluorescent protein (GFP) and the two genomic RNAs of grapevine fanleaf virus (GFLV) were used as negative controls in agroinfiltration experiments.

A number of agroinfiltrated vines of Cabernet Sauvignon, Cabernet franc, Syrah, Pinot noir, Pinot gris and Chardonnay showed red blotch-like symptoms at 1-3 months post-treatment. Foliar symptoms consisted of interveinal reddening in red-berried cultivars and chlorotic spots in the white-berried cultivar Chardonnay. Unlike wine grape cultivars, agroinoculated rootstock SO4 (*V. berlandieri* x *V. riparia*) became symptomatic (chlorosis and cupping) only after one dormancy period, whereas agroinoculated rootstock 3309C (*V. riparia* x *V. rupestris*) remained asymptomatic (Fuchs *et al.* 2015). Some of the agroinfiltrated grapevines tested positive for GRBaV by multiplex PCR (Krenz *et al.* 2014) and all the PCR-positive plants were symptomatic, while the negative plants were asymptomatic. None of the plants treated with GFP and GFLV constructs, or untreated plants exhibited red blotch-like symptoms, nor did they test positive for GRBaV in PCR (Fuchs *et al.* 2015).

After one or two dormancy periods, the full-length genomic sequence of some of the GRBaV progeny was determined in a few selected agroinfected plants by rolling circle amplification, cloning and sequencing. The nucleotide sequence of the virus progeny was 99.6-99.9% identical to that of the partial dimer constructs of GRBaV isolates NY175 and NY358 used as inoculum in agroinfection assays, indicating that the recovered GRBaV variants are nearly-identical to the engineered

inoculum (Fuchs *et al.* 2015). These findings were consistent with our hypotheses that GRBaV is the causal agent of red blotch disease, satisfying Koch's postulates and demonstrating that GRBaV is the causative agent of red blotch disease (Fuchs *et al.* 2015).

Recently the three-cornered alfalfa treehopper, *Spissistilus festinus* (Say), was described as a vector of GRBaV (Bahder *et al.* 2016b). Some healthy Cabernet Sauvignon vines exposed to viruliferous treehoppers became infected and exhibited typical red blotch symptoms. This provided additional evidence that GRBaV is the causative agent of red blotch disease.

DETECTION AND DIAGNOSTICS

The diagnostic resources for the detection of GRBaV all rely on amplification of viral DNA sequences by polymerase chain reaction (PCR). Assays were designed as a simplex reaction (Al Rwahnih *et al.* 2013) or in a multiplex format with two primer pairs and an internal control (Krenz *et al.* 2014). Quantitative PCR (qPCR) assays are employed in foundation plant programs and by commercial testing services, but thus far there is only one literature report for a qPCR assay (Bahder *et al.* 2016b). An isothermal amplification technology (recombinase polymerase amplification; Piepenburg *et al.* 2006) is under commercial development for single use test kits and has the advantage of detecting virus in crude plant homogenates. In general, false negative results can be problematic and have been observed for field and greenhouse vines previously shown as infected. Sampling of older symptomatic leaves appears to be more reliable for virus detection, although the viral DNA can be recovered from

new growth at the tips of canes.

The visualization of virions within, or purified from, infected plants has remained elusive and no electron microscopic images have been obtained. Antibodies were produced against synthetic peptides and bacterially expressed coat protein. These antibodies recognize their cognate antigens, but show no differential reaction when tested against infected and uninfected plant extracts in both western blot assays and in an enzyme-linked immunosorbent assay (K.L. Perry, unpublished). The virus is assumed to be phloem limited and difficult to consistently detect, and it is not clear if antibody-based detection methods will ever be applicable.

HOST RANGE AND GEOGRAPHIC DISTRIBUTION

The cultivated grape *Vitis vinifera* and other *Vitis* sp. are the only reported hosts of GRBaV. Free-living vines were shown to harbor GRBaV (Bahder *et al.* 2016a; Perry *et al.* 2016) and some of them were determined to be *V. californica* × *V. vinifera* hybrids (Perry *et al.* 2016). The virus is widespread throughout some of the major grape growing regions of the USA (Krenz *et al.* 2014), British Columbia and Ontario in Canada (Poojari *et al.* 2016). By contrast, there are very few reports of the virus outside of this region. Five grapevine accessions in a Swiss experimental vineyard harbored GRBaV, and all of these were imported from California (Reynard, 2015; 2016). A recent report indicated GRBaV was present in cultivated vines in Korea, but the origin of the vines is not known (Lim *et al.* 2016). GRBaV was also detected in several table grape accessions established at the USDA-ARS clonal germplasm repository in Winters, California (Al Rwahnih *et al.* 2015a) and in a few

interspecific hybrids at the USDA-ARS cold hardy germplasm repository in Geneva, New York (K.L. Perry, unpublished). Interestingly, GRBaV was also detected in archival leaf samples of *V. vinifera* cv. Abouriou that were collected in Sonoma County of California in 1940 and kept in a herbarium at UC-Davis (Al Rwahnih *et al.* 2015b). This finding suggested that the virus was present in vineyards prior to the recognition of the disease in 2008 and prior to the characterization of the virus genome in 2011.

EPIDEMIOLOGY AND TRANSMISSION

GRBaV is graft-transmissible (Al Rwahnih *et al.* 2013; Poojari *et al.* 2013) and was detected in most of the grape-growing regions of the United States, which indicates a high likelihood of dissemination via infected propagation material. On a local scale, clustering of GRBaV-infected vines within healthy vineyards proximal to infected vineyards (**Figure 1-3**), and the spatiotemporal increase of infected, symptomatic vines in some vineyards located on the west coast of the USA (Bahder *et al.* 2016b) implicate an insect vector in the spread of GRBaV. In the northeastern United States, there is no evidence that GRBaV is spreading to or within vineyards. Similarly, there is no indication of GRBaV spread in Switzerland (Reynard, 2016). Interestingly, GRBaV was detected in some free-living vines in the vicinity of diseased commercial vineyards in California (Bahder *et al.* 2016a; Perry *et al.* 2016), further supporting the implication of an insect vector in the spread of GRBaV from cultivated to free-living grapes or vice-versa. The role of infected free-living vines as alternate host in disease epidemiology requires further investigation.



Figure 1-3 Aggregation of approximately 20 red blotch diseased vines followed by healthy vines in two adjacent rows of a Cabernet franc vineyard. Please note the changing color of the canopy between diseased and healthy vines.

Early on, the Virginia creeper leafhopper was reported as a vector of GRBaV in the greenhouse (Poojari *et al.* 2013) but this result was not confirmed (Bahder *et al.* 2016b). Instead, the three cornered alfalfa treehopper, *Spissistilus festinus* (Say) (**Figure 1-4**), was shown to transmit GRBaV under greenhouse conditions. Vines of *Vitis vinifera* cv. Cabernet Sauvignon exposed to *S. festinus* that fed on GRBaV-infected source material tested positive by digital PCR at five months post inoculation. Additionally, some of the exposed vines that tested GRBaV-positive developed foliar symptoms of red blotch disease around five months post inoculation (Bahder *et al.* 2016b).

While *S. festinus* is documented as a vector in the greenhouse, the extent to which it transmits GRBaV in the vineyard is to be determined. This treehopper can cause problems in soybean, peanut, alfalfa and other legumes in the southern United States, where it undergoes several generations per year and causes girdling damage to its hosts (Mitchell and Newsom, 1984). Although a generalist feeder, *S. festinus* is not known to reproduce on grape and is rarely considered a pest of grape. Notwithstanding, in red-berried grape cultivars, girdle damage to shoots or petioles is suggestive of *S. festinus* feeding (**Figure 1-5**), although other insects such as leafhoppers can cause similar damage.



Figure 1-4 Adult *Spissistilus festinus*, a confirmed vector of GRBaV, resting on a shoot of a *V. vinifera* cv. Cabernet Sauvignon in California.



Figure 1-5 Girdle damage to a single leaf of a Cabernet Sauvignon shoot, suggestive of *S. festinus* feeding.

MANAGEMENT

There are currently no methods for curing a vine of GRBaV in diseased vineyards, highlighting the importance of preventive measures to manage red blotch. Since GRBaV can be introduced to vineyards via infected propagation material, planting certified vines derived from virus-tested, clean stocks is critical in establishing healthy vineyards and preventing the introduction of the disease. Frequent scouting is also important for evaluating the presence and spread of GRBaV. Since symptoms can easily be confused with leafroll disease, mite damage, nutrient deficiency, and even mechanical damage, it is essential that symptom evaluation be confirmed with DNA-based detection assays.

Economic analyses suggest that roguing symptomatic vines and replanting with clean vines derived from virus-tested stocks minimize losses if red blotch incidence is low to moderate (below 30%), while a full vineyard replacement should be pursued if disease incidence is higher, generally above 30% (Ricketts *et al.* 2016). These findings should help vineyard managers in adopting appropriate management strategies. Control of the insect vector, *Spissistilus festinus*, using insecticides is currently not recommended. This approach may complement cultural management strategies once the phenology of this insect and its efficiency as a vector in vineyards are better understood.

IS RED BLOTCH AN EMERGING VIRUS? ORIGIN OF THE VIRUS AND FUTURE PROSPECTS

Grapevine red blotch is an emerging disease, having only been recognized as a

distinct malady since 2008. While the virus is known to have been present in California grapevines for over 70 years, it presumably was not present at sufficient levels to gain notice as a disease-causing agent distinct from leafroll-associated viruses. The most likely explanation is that GRBaV spread within nursery stocks over the past few decades. By the time the virus was discovered in 2011 and its association with disease recognized, it had already been effectively spread throughout grape growing regions of North America. The insect vector may play a role in the persistence of GRBaV in a viticultural setting by moving the virus among cultivated vines and to or from wild vines; thus far, the role of the vector in disease epidemiology is likely dwarfed by the movement of the virus in nursery stocks.

The geographical origin of GRBaV remains a mystery. One hypothesis is that GRBaV is globally distributed in most grape growing areas of the world, but is not associated with a sufficient level of disease to be recognized and reported. Due to the potential negative impact of a finding on a country's economy, there is a disincentive to look for and report the virus. It may be that the virus is normally very rare, but that in North America the presence of a vector in combination with nursery operations was responsible for its spread to the point of recognition and detection. An alternative hypothesis is that the virus emerged in North America and has not yet spread to other grape production areas. Consistent with this notion is the fact that it has largely only been described from North American vineyards. There are no reports of the virus from commercial grape production areas of Europe. A survey of 2700 vines in Switzerland did not reveal the presence of GRBaV (Reynard, 2016). GRBaV sequences were also found only to be present in high-throughput sequence datasets originating from North

America. The global Sequence Read Archive of the National Center for Biotechnology Information contains 2105 *Vitis* sequence databases submitted from around the world, with the majority being from Europe. GRBaV was found in 31 of these 2105 databases and all were from North America (Vargas and Perry, unpublished). The latter ‘survey’ is biased and has limitations, but the correlation is intriguing. A third hypothesis would be that GRBaV is primarily a virus of some yet to be discovered host and only relatively recently moved into *Vitis* sp. This might have been facilitated by adaptation to a new insect vector such as the three cornered alfalfa treehopper.

Grower awareness of red blotch disease and their desire to source GRBaV-free vines resulted recently in more widespread testing of nursery planting stocks and the establishment of new increase vineyard blocks. It appears this will be a disease that can be managed effectively through clean plant programs. As new reservoirs for the virus are identified, a better understanding of the epidemiology of red blotch will be essential to avoid further spread of virus and disease.

REFERENCES

Al Rwahnih, M., Ashita, D., Anderson, M., Rowhani, A., Uyemoto, J.K., Sudarshana, M.R. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103:1069-1076.

Al Rwahnih, M., Rowhani, A., Golino, D., Islas, C., Preece, J., Sudarshana, M.R. 2015a. Detection and genetic diversity of Grapevine red blotch-associated virus isolates in table grape accessions in the National Clonal Germplasm Repository in California. *Canadian Journal of Plant Pathology* 37:130-135.

Al Rwahnih, M., Rowhani, A., Golino, D. 2015b. First report of grapevine red blotch-associated virus in archival grapevine material from Sonoma County, California. *Plant Disease* 99:895.

Atallah, S., Gomez, M. Fuchs, M., Martinson, T. 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet franc in Finger Lakes vineyards of New York. *American Journal of Enology and Viticulture* 63:73-79.

Bahder, B.W., Zalom, F.G., Sudarshana, M.R. 2016a. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of Grapevine red blotch-associated virus. *Plant Disease* doi/pdf/10.1094/PDIS-02-16-0153-RE.

Bahder B, Zalom F, Jayanth M, Sudarshana M. 2016b. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* (Say) as a vector of Grapevine red blotch-associated virus. *Phytopathology* <http://dx.doi.org/10.1094>.

Calvi, B.L. 2011. Effects of red-leaf disease on Cabernet Sauvignon at the Oakville experimental vineyard and mitigation by harvest delay and crop adjustment. M.S. thesis, University of California, Davis, CA.

Fuchs, M., Krenz, B., Yepes, L.M., Thompson, J., McLane, H., Perry, K.L. 2015. Is Grapevine red blotch-associated virus the causal agent of red blotch disease? In: Proceedings of the 18th Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine. pp. 72-73.

Guo, T.W., Vimalasvaran, D., Thompson, J.R., Perry, K.L., Krenz, B. 2015. Subcellular localization of grapevine red blotch-associated virus ORFs V2 and V3. *Virus Genes* 51:156-158.

Krenz, B., Thompson, J., Fuchs, M., Perry, K.L. 2012. Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology* 86:7715.

Krenz, B., Thompson, J.R., McLane, H., Fuchs, M., Perry, K.L. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 102:1232-1240.

Lim, S. Igori, D., Zhao, F., Moon, J.S. 2016. First report of Grapevine red blotch-associated virus on grapevine in Korea. *Plant Disease* doi/pdf/10.1094/PDIS-03-16-0283-PDN.

Mitchell P., Newsom L. 1984. Seasonal history of the three cornered alfalfa hopper (Homoptera: Membracidae) in Louisiana. *Journal of Economic Entomology* 77:906-914.

Perry, K.L., McLane, H., Hyder, M.Z., Dangl, G.S., Thompson, J.R., Fuchs, M.F. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* sp. proximal to cultivated grapevines. *Phytopathology* 106:663-670.

Piepenburg, O., Williams, C.H., Stemple D.L., Armes, N.A. 2006. DNA detection using recombination proteins. *PLoS Biology* 4:1115–1121.

Poojari, S., Lowery, T., Schmidt, A-M., Rott, M., Mcfadden-Smith, W., Stobbs, L., Urbez-Torres, J.R. 2016. Red blotch and the virus in Canada. In: Webinar on red blotch disease, February 26, <http://www.ipmcenters.org/index.cfm/center-products/ipm-eacademy/upcoming-events/red-blotch-speakers/>

Poojari, S., Alabi, O.J., Fofanov, V.Y., Naidu, R.A. 2013. A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family geminiviridae implicated in grapevine redleaf disease by next-generation sequencing. *PLoS One* 8:e64194.

Reynard, J.S. 2015. Survey of emerging viruses in Switzerland. In: Proceedings of the 18th Congress of the International Council for the Study of Virus and Virus-like

Diseases of the Grapevine. pp. 223-224.

Reynard, J.S. 2016. Red blotch and the virus in Europe. IPM eAcademy webinar.

February 26, 2016.

<https://www.youtube.com/watch?v=LEGXd3iLBx4&list=PLK93eCG1nCqKFZWFWuMqKPyAvU-Qk--B3&index=7>

Ricketts, K.D., Gomez, M.I., Fuchs, M.F., Martinson, T.E., R.J. Smith, Cooper, M.L., Moyer, M., Wise A. 2016. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. Vineyards. *American Journal of Enology and Viticulture*, in revision.

Ricketts, K.D., Gomez, M.I., Atallah, S.S., Fuchs, M.F., Martinson, T., Smith, R.J., Verdegaal, P.S., Cooper, M.L., Bettiga, L.J., Battany, M.C. 2015. Reducing the economic impact of grapevine leafroll disease in California: Identifying optimal management practices. *American Journal of Enology and Viticulture* 66:138-147.

Seguin, J., Rajeswaran, R., Malpica-Lopez, N., Martin, R.R., Kasschau, K., Dolja, V.V., Otten, P., Farinelli, L., Poogin M.M. 2014. *De novo* reconstruction of consensus master genomes of plant RNA and DNA viruses from siRNAs. *PLoS One* 9:e88513.

Sudarshana, M.R., Perry, K.L., Fuchs, M.F. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105:1026-1032.

Varsani, A., Navas-Castillo, J., Moriones, E., Hernández-Zepeda, C., Idris, A., Brown, J.K., Murilo Zerbini, F., Martin, D.P. 2014. Establishment of three new genera in the family *Geminiviridae*: *Becurtovirus*, *Eragrovirus* and *Turncurtovirus*. Archives of Virology 159:2193-2203.

CHAPTER 2

SPATIOTEMPORAL SPREAD OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS IN A CALIFORNIA VINEYARD

ABSTRACT

Grapevine red blotch-associated virus (GRBaV), the causative agent of red blotch disease, is a member of the family *Geminiviridae* and the first known geminivirus of *Vitis* spp. Limited information is available on the epidemiology of red blotch disease. A 2-hectare *Vitis vinifera* cv. ‘Cabernet franc’ vineyard in Napa County, California, USA was selected for monitoring GRBaV spread over a three-year period (2014-2016) based on an initially low disease incidence and an aggregation of symptomatic vines at the edge of the vineyard proximal to a wooded riparian area. The incidence of diseased plants increased by 1-2% annually. Spatial analysis of diseased plants in each year using ordinary runs analysis within rows and Spatial Analysis by Distance IndicEs (SADIE) demonstrated aggregation. Spatiotemporal analysis between consecutive years within the association function of SADIE revealed a strong overall association among all three years ($X = 0.874-0.945$). Analysis of epidemic spread fitting a stochastic spatiotemporal model using the Monte Carlo Markov Chain method identified strong evidence for localized (within vineyard) spread. A spatial pattern consisting of a combination of strongly aggregated and randomly isolated symptomatic vines within 8-years post-planting suggested unique epidemic attributes compared to those of other grapevine viruses vectored by mealybugs and soft scales or by dagger nematodes for which typical within-row spread and small-scale

autocorrelation are well documented. These findings are consistent with the existence of a new type of vector for a grapevine virus.

*This chapter was published in: Cieniewicz E.J., Pethybridge S.J., Gorny A., Madden L.V., McLane H., Perry K.L. & Fuchs M. (2017) Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Research*, 241: 156-162.

INTRODUCTION

Red blotch is a relatively recently recognized viral disease of grapevine. It was first described in a ‘Cabernet Sauvignon’ vineyard at the University of California-Davis Oakville Research Field Station in 2008 (Calvi, 2011). Grapevine red blotch-associated virus (GRBaV) was later identified in symptomatic vines (Krenz *et al.* 2012; Al Rwahnih *et al.* 2013) and demonstrated as the causative agent of red blotch disease (Fuchs *et al.* 2015). GRBaV is the type member of the genus *Grablovirus* in the family *Geminiviridae* (Varsani *et al.* 2017). Its genome is a single component, consisting of a single-stranded circular DNA (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. Similarly, in white-berried cultivars of *V. vinifera*, red blotch disease symptoms are similar to those of leafroll or magnesium deficiency. Symptoms are therefore not a reliable indicator of GRBaV infection, which must be confirmed by sequence-based detection techniques such as polymerase chain reaction (PCR) (Sudarshana *et al.* 2015).

While our understanding of red blotch epidemiology is still in its infancy, strides have been made in recent years toward an improved understanding of virus spread. GRBaV is transmissible by grafting (Al Rwahnih *et al.* 2013), which is likely the most important mode of dispersal (Sudarshana *et al.* 2015). Consequently, since its discovery in 2011, GRBaV has been detected in all major viticulture regions of the United States (Krenz *et al.* 2014), as well as in Canada (Poojari *et al.* 2016), Switzerland (Reynard 2015, 2016) and South Korea (Lim *et al.* 2016). While long

distance dispersal is most likely attributed to dissemination of infected propagation material, short distance dispersal within vineyards suggestive of spread by an insect vector has been observed in some areas, mainly on the west coast in the United States (M. Fuchs, personal observations). In addition, GRBaV has been detected in free-living vines close to diseased commercial vineyards in California (Perry *et al.* 2016). These findings were subsequently confirmed (Bahder *et al.* 2016a), consistent with short-distance spread of GRBaV to and from established vineyards, likely by an insect vector. The three cornered alfalfa treehopper, *Spissistilus festinus* (Say) transmits GRBaV under greenhouse conditions (Bahder *et al.* 2016b) and is currently the only known insect vector. More work is needed to determine if *S. festinus* is a vector of epidemiological significance in vineyards and whether other insects play a role in GRBaV transmission.

Monitoring the spatial distribution of diseased vines over time is important for a comprehensive understanding of the factors governing the rate of GRBaV spread and subsequently devising appropriate disease management strategies. The objective of this study was to document spread of GRBaV in a California vineyard and characterize the spatiotemporal attributes of a red blotch disease epidemic.

MATERIALS AND METHODS

Field site and data collection

A 2-hectare *Vitis vinifera* ‘Cabernet franc’ vineyard established in 2008 in Napa County, California, USA was selected for this study based on an initial aggregated pattern of diseased vines in a section adjacent to a wooded riparian area

although the overall disease incidence throughout the vineyard was low. The ‘Cabernet franc’ vineyard consisted of 44 rows and 114-195 vines per row, spaced 1.2 and 2.1 m within and between rows, respectively. The incidence of red blotch was assessed visually by inspection for the presence of red blotch symptoms on leaves in the lower part of the canopy (0 = asymptomatic vine; and 1 = symptomatic vine) in each October from 2014 to 2016 throughout the entire vineyard.

Association between symptoms and virus presence

To verify that foliar red blotch symptoms were associated with GRBaV infection in the ‘Cabernet franc’ vineyard, a subset of the vineyard nearest the riparian area spanning 10 rows, 25 vines per row was selected for PCR testing and visual inspection for symptoms annually. Six leaf samples were collected from the older portion of the canopy of each vine in October of 2014-2016. Dormant cane material was collected between December and February in 2013-2015. Total DNA was isolated from a 50 mg subsample of petioles and/or dormant cane scrapings using the H.P. Plant DNA Mini kit (OMEGA Biotek). Diagnostic multiplex PCR was performed as described by Krenz *et al.* (2014) using the HotStar HiFidelity Polymerase kit (Qiagen). To test for an association between PCR results and the presence of symptoms, the Kendall rank correlation coefficient τ (Kendall, 1938) was calculated in RStudio (version 3.1.1).

In addition to GRBaV testing, leaf samples from all 250 vines were also tested in 2016 for grapevine leafroll-associated virus 1 (GLRaV-1), grapevine leafroll-associated virus 2 (GLRaV-2), grapevine leafroll-associated virus 3 (GLRaV-3) and grapevine leafroll-associated virus 4 (GLRaV-4) by ELISA using commercial

antibodies (Bioreba AG, Reinach, Switzerland).

Spatial analyses

Ordinary runs. The spatial pattern of symptomatic vines in one dimension was determined using ordinary runs analysis (Gibbons, 1976; Madden *et al.* 1982).

Ordinary runs determines whether the pattern of diseased vines is aggregated or random. A run was defined as one or more vines of identical status (diseased or healthy). Individual rows of the vineyard in which disease incidence exceeded 5% were considered for ordinary runs analysis. A nonrandom sequence of diseased vines was concluded on a row by row basis if the Z -statistic, $Z_U \leq -1.64$ ($P \leq 0.05$); this occurred if the observed number of runs was significantly different from the expected number of runs under a null hypothesis of randomness (Madden *et al.* 1982).

SADIE. The spatial pattern of diseased vines was analyzed using the Spatial Analysis by Distance IndicEs (SADIE) algorithm (Li *et al.* 2012; Perry, 1995; 1998; Perry *et al.* 1999; Xu and Madden, 2005). The SADIE approach to spatial analysis uses calculation of the distance to regularity which is defined as the number of ‘moves’ that sampling units (i.e. vines) designated as diseased are required to produce a completely regular distribution across the area of interest. Significance of the distance to regularity is determined by randomizations using the same number of diseased sampling units in the observed dataset. Through the randomizations, the distribution and mean (expected value) distance to regularity for a random spatial process is determined. The index of aggregation (I_a) is equal to the ratio of the observed distance to regularity to the expected value for a random pattern. An index of aggregation equal

to one suggests a random spatial arrangement whereas an index of aggregation greater than one reflects an aggregated pattern. To quantify significance of the magnitude of the index of aggregation, the maximum number of randomizations within SADIE ($n = 5,967$) was used for each analysis to assess deviation from the null hypothesis of no spatial dependence (Perry, 1995; 1998). Due to computing constraints of SADIE, vine doublets were considered as single sampling points and the vineyard sampling area was truncated for this analysis and spanned 13 rows, i.e. rows 21-33, 134 vines in each row (67 doublets).

Spatiotemporal analyses

Temporal associations in spatial patterns of diseased vines between years were determined using the association function of SADIE (Version 1.22). The null hypothesis was a lack of association between spatial patterns of diseased vines over time. Local association (χ_k) was first quantified by performing comparisons between clustering indices for each assessment time using SADIE. The local clustering index for each location is a dimensionless metric of the magnitude of the spatial clump or gap at that location based on the raw data for that location and the distance to regularity determined from the randomizations. Overall association (X) was then calculated based on the correlation coefficient of the local clustering indices between pairs of years. Significance of X was tested by randomizations with values of local association at the scale of each sampling unit, with adjustment for small-scale spatial autocorrelation in the population at both time periods using the Dutilleul adjustment (Dutilleul *et al.* 2008).

The maximum number of 9,999 randomizations was used to assess significance within a two-tailed test (Winder *et al.* 2001).

Spatiotemporal patterns of infected grapevines between 2014 and 2015, and 2015 and 2016, were also analyzed using the spatiotemporal stochastic model described by Gibson (1997a, 1997b) and fit to the data using a Monte Carlo Markov Chain (MCMC) algorithm (Gibson 1997a, 1997b, Gibson and Austin 1986; Pethybridge and Madden 2003). In brief, this modeling approach assumes that a grapevine that is susceptible at a specific point in time may acquire GRBaV probabilistically at a rate dictated by a parameter vector, \vec{a} (a_1, a_2) (Gibson and Austin 1986). The parameter a_1 quantifies the rate at which a susceptible grapevine becomes infected from sources unrelated to the location of other infected plants in the vineyard, also referred to as “background” infection (Gibson 1997a; 1997b). Here the background infection rate is re-parameterized and presented as $b = [\log(1 + a_1)]^{1/2}$. The lower bound for b was 0, reflecting no background infection; and although there was no upper bound, a value of 3 is considered high. As b increases, the contribution of inoculum from “background” infection dominates disease increase over time (Gibson 1997a; 1997b). In contrast, the parameter a_2 quantifies the “local” infection rate at which a susceptible grapevine may become infected from GRBaV-infected plants within the vineyard. As described by Gibson (1997a), the probability of a grapevine becoming infected from other plants within the same defined local area (i.e. vineyard) decreases as a power function of the distance (r) between them, r^{a_2} . For example, a small a_2 is indicative of a shallow dispersal gradient and inferring disease spread over long distances. Alternatively, as a_2 increases, the dispersal gradient becomes steeper, indicating that disease spread is

highly localized. The procedures used to provide point estimates of the parameters were previously described (Gibson, 1997a). The upper and lower bounds for the “local” parameter interval used were 3.5 and 0, respectively. Similarly, the upper and lower bounds for the “background” parameter was 3.0 and 0, respectively. Posterior parameter densities were also determined to identify the most probable values of the point estimates. The same area of the vineyard selected for SADIE spanning 13 rows was used for the MCMC analysis.

RESULTS

Association between disease symptoms and presence of GRBaV

Suspected foliar red blotch symptoms were initially identified in the ‘Cabernet franc’ vineyard in fall of 2012, particularly at the edge proximal to the riparian area. The presence of GRBaV was confirmed in fall of 2013 in some of these vines (Perry *et al.* 2016). This result inspired a more extensive characterization of the association between symptoms and the occurrence of GRBaV. In 2014, disease incidence in the section of the vineyard proximal to the riparian area was 50.4% (126/250), as determined by symptom monitoring and testing for GRBaV by PCR in some vines (Perry *et al.* 2016). Testing each of the 250 vines in the same section of the vineyard showed an increase in disease incidence to 66.4% (166/250) in 2015 and 71.2% (178/250) in 2016 (**Figure 2-1**). In 2014, 60 of the 65 (92%) vines analyzed were symptomatic and PCR positive for GRBaV; in 2015 (166/166, 100%) and in 2016 (178/178, 100%), all the symptomatic vines tested positive for GRBaV by multiplex PCR. The presence of symptoms and PCR results were strongly correlated ($\tau = 0.894$,

$P < 0.0001$; $\tau = 0.957$, $P < 0.0001$; and $\tau = 0.963$, $P < 0.0001$ in 2014, 2015, and 2016, respectively).

These results provided a high level of confidence in the accuracy of visual disease assessment. Only a few vines (five each in 2014 and 2015; and three in 2016) showed a discrepancy between the PCR result for GRBaV and the presence of red blotch symptoms. These vines were asymptomatic but GRBaV was detected by PCR. The majority of vines in this category became symptomatic in the following season (**Figure 2-1**). Also, GLRaV-1 was detected in five vines, GLRaV-2 in two vines, GLRaV-3 in nine vines and GLRaV-4 in four vines. Some of the vines infected with GLRaVs were also infected by GRBaV but did not exhibit typical foliar leafroll symptoms, including leaf cupping and green veins (Naidu *et al.* 2014). Based on these findings, disease incidence was confidently evaluated throughout the whole vineyard by visual symptom monitoring.

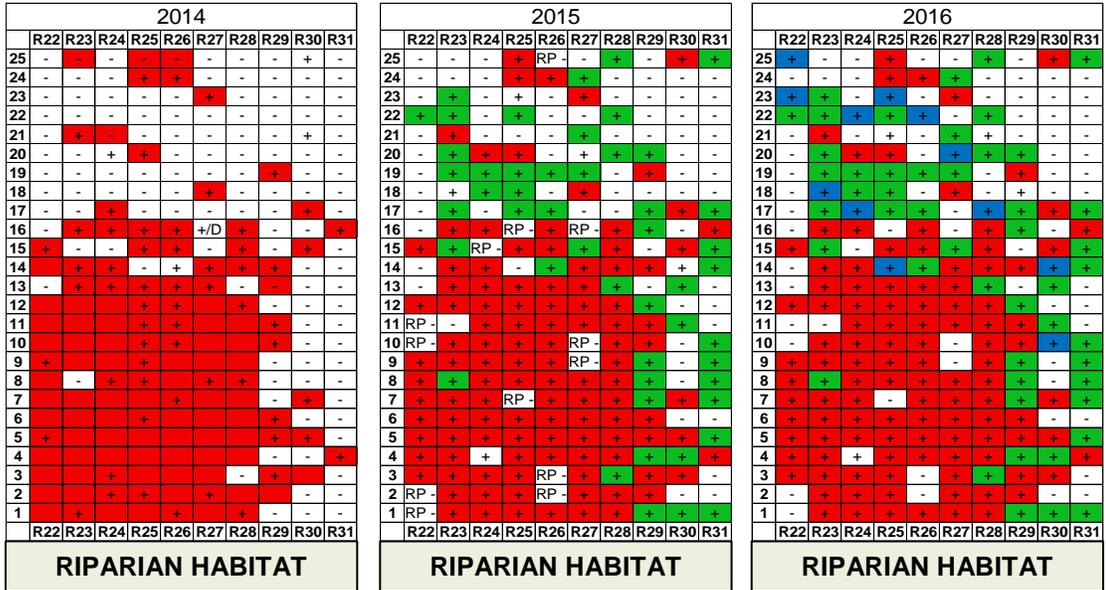


Figure 2-1 Annual spatial pattern of symptomatic vines and those in which grapevine red blotch-associated virus (GRBaV) was detected by PCR in a section of the vineyard, spanning 10 rows and 25 vines per row, adjacent to a riparian habitat. (+) indicates GRBaV detection by PCR, (-) indicates no detection of GRBaV by PCR and a blank cell (in 2014) indicates a vine that was not tested by PCR. Red, green, and blue shaded cells indicate newly symptomatic vines in 2014, 2015, and 2016 respectively, and white cells indicate symptomless vines. ‘RP’ indicates a vine that was re-planted in 2015.

Annual increase in red blotch disease incidence

Disease incidence was 3.9% (305/7,691 vines) in 2014, 6.0% (461/7,686 vines) in 2015 and 7.1% (547/7,679 vines) in 2016 throughout the entire 2-hectare vineyard. This result revealed an overall 2.1% and 1.1% increase in disease incidence in 2014-2015 and 2015-2016, respectively. The presence of symptoms was consistent over three years, i.e. the same vines showed symptoms each year, with new symptomatic vines observed every year.

The magnitude of the annual increase in number of symptomatic vines was most substantial in the section of the vineyard adjacent to the riparian habitat (**Figure 2-2a**). Plotting disease incidence along the long axis of the vineyard for each year highlighted two major points: i) the absolute magnitude of increase in red blotch incidence was greater between 2014 and 2015 (2.1%) than between 2015 and 2016 (1.1%), and ii) the annual increase in incidence was primarily localized to the section of the vineyard nearest the riparian area (**Figure 2-2b**). In this section, the disease incidence increased by 16% from 2014 to 2015, and 4.8% from 2015 to 2016.

Spatial analyses

Ordinary runs analysis indicated a significant aggregation ($Z_u \leq -1.64$) of diseased vines in at least two of the three years in rows 16, 21-33, 35, and 38, as well as randomly distributed diseased vines in the remaining rows of the 'Cabernet franc' vineyard. Aggregation was observed in 23%, 36%, and 39% of the rows analyzed in 2014, 2015, and 2016, respectively. The level of aggregation of diseased vines, as indicated by the magnitude of the Z-statistic, was higher in rows 21 through 33 than in

the other rows analyzed (**Figure 2-3**).

Based on the results of ordinary runs analysis, the spatial pattern of diseased vines in rows 21 through 33 was selected for analysis using SADIE (**Figure 2-2a**, gray shaded area). Aggregation of diseased vines was confirmed by SADIE ($P < 0.002$; **Table 2-1**). Moreover, the magnitude of the index of aggregation (I_a) increased in consecutive years (**Table 2-1**). These results revealed a combination of strongly aggregated and randomly isolated symptomatic vines within 8-years post-planting.

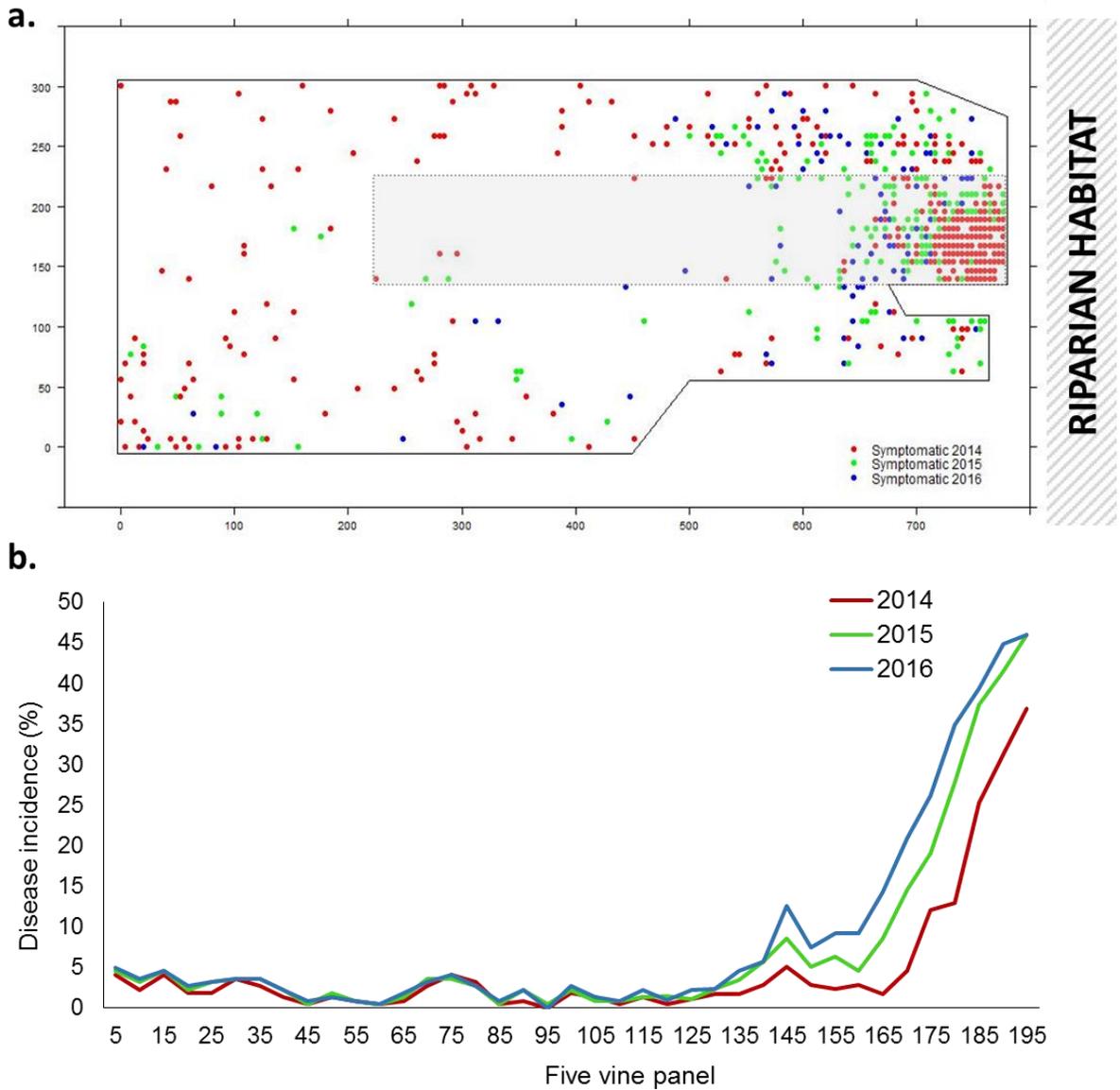


Figure 2-2 Spatial pattern of diseased vines over a three-year period (2014-2016) in a 2-hectare ‘Cabernet franc’ vineyard affected by red blotch. **(a)** Gray shaded area delineating the area included in Spatial Analysis by Distance IndicEs (SADIE) and MCMC spatiotemporal analysis. **(b)** Annual disease incidence plotted over the long axis in 5-vine panel increments.

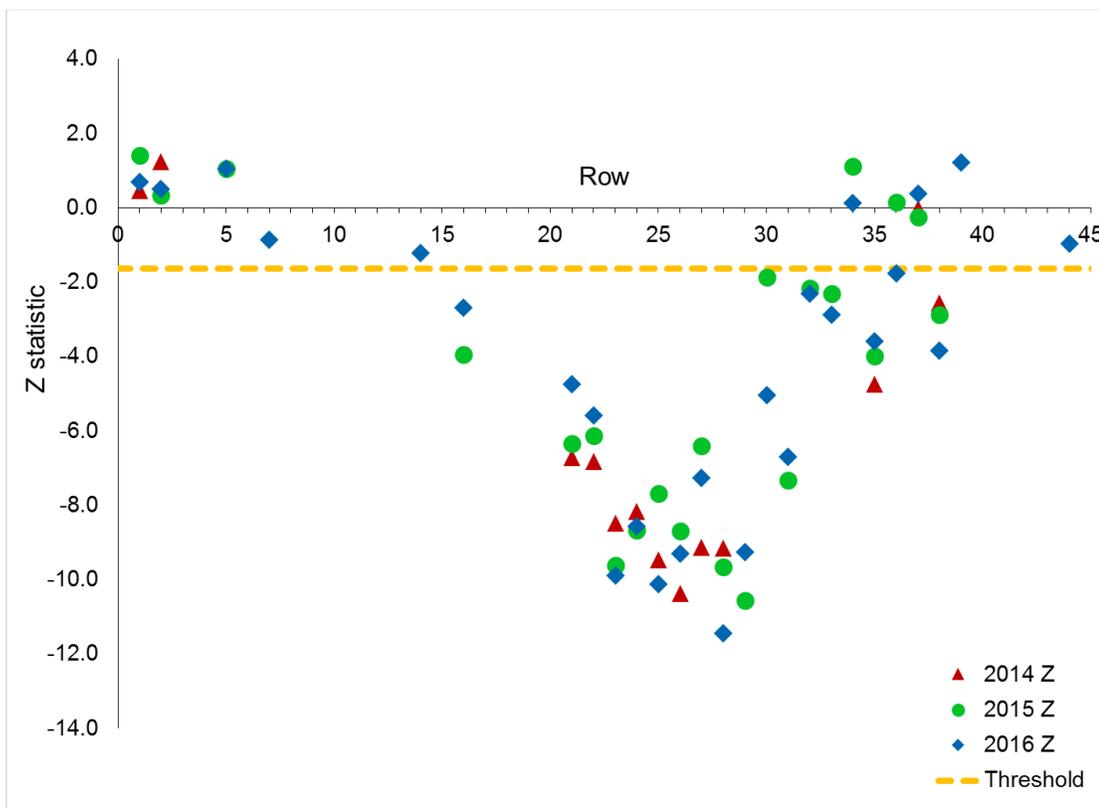


Figure 2-3 Z-statistics derived from testing for spatial aggregation of grapevine red blotch-associated virus (GRBaV) diseased vines within rows using ordinary runs analysis of a 2-hectare ‘Cabernet franc’ vineyard in California. Ordinary runs analysis was only implemented if disease incidence within an individual row was greater than 5%. Spatial aggregation of diseased vines was concluded if the Z_u was less than or equal to -1.64 (yellow horizontal dotted line).

Table 2-1 Spatial analysis of grapevines affected by red blotch disease in a Californian vineyard from 2014 to 2016 according to SADIE.

Year	<i>D</i>^a	<i>I_a</i>^b	<i>P</i>
2014	6560.77	10.43	<0.002
2015	9090.73	12.01	<0.002
2016	9994.97	12.52	<0.002

^a*D*: Distance to regularity.

^b*I_a*: Index of aggregation.

Table 2-2 Spatiotemporal analysis of a red blotch epidemic in a vineyard in California using the association function of the Spatial Analysis by Distance Indices (Winder *et al.* 2001).

Year 1	Year 2	Effective sample size	Overall association (X) ^a
2014	2015	438.7	0.894***
2015	2016	458.2	0.945***
2014	2016	509.7	0.874***

^aAsterisks indicate significance according to a two-tail test at $P < 0.0001$.

Spatiotemporal analyses

Significant spatial associations ($P < 0.001$) were detected between the local clustering indices between successive seasons (**Table 2-2**), suggesting the degree of spatial aggregation of diseased vines was associated with the spatial position of diseased vines in the previous year. This result indicated that GRBaV can potentially spread over time within and between rows in a vineyard area where diseased vines are aggregated.

Point estimates for a_2 were 1.225 and 1.05 in 2014-2015 and 2015-2016, respectively. Moreover, in both years, the point estimate for b was 0. Contours of the posterior density of the parameter vector, \bar{a} demonstrated that values of b were relatively low in both years (**Figure 2-4**). These results indicated that spread of disease predominantly originated from local sources, consistent with short-range movement of vectors within the vineyard.

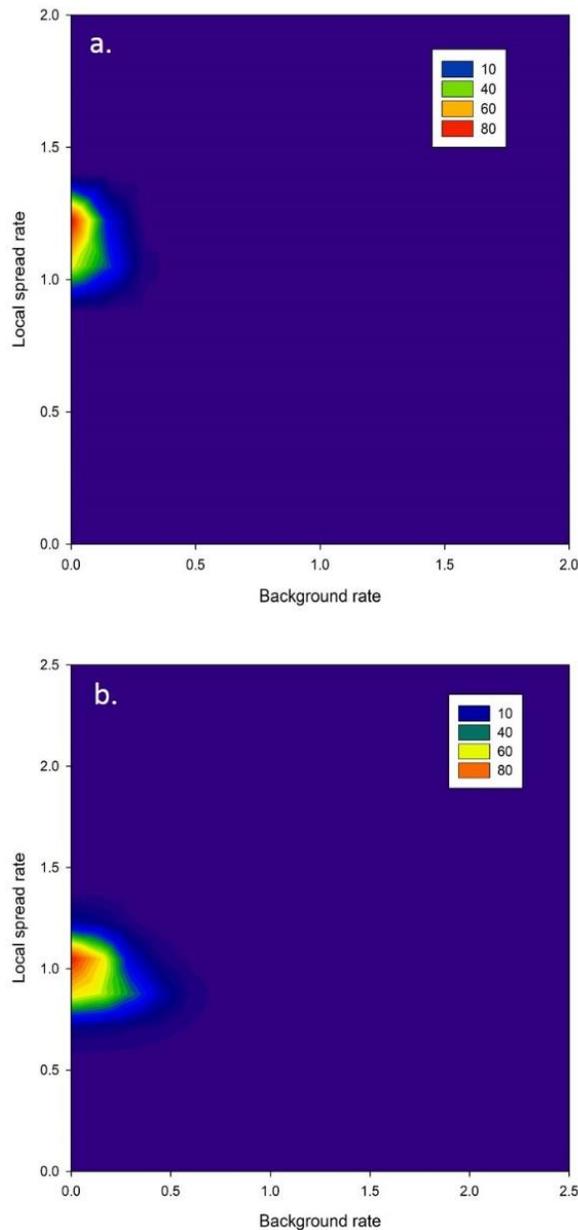


Figure 2-4 Contour plots of the posterior parameter density of a spatiotemporal stochastic model (Gibson, 1997a; Madden *et al.* 2007) fitted to the spread of grapevine red blotch-associated virus (GRBaV) in a ‘Cabernet franc’ vineyard in California between **(a)** 2014 and 2015, and **(b)** 2015 and 2016 using a Monte Carlo Markov Chain algorithm. Percentile ranges of the posterior density of the local and background infection parameters are indicated by colors. The dark blue represents the 10th and lower percentile, which is the parameter region without support in this analysis.

DISCUSSION

The ‘Cabernet franc’ vineyard described here was planted in 2008. Symptoms similar to red blotch disease were first noticed in 2012 at the edge proximal to the riparian area. This timing coincided with the discovery of GRBaV and the development of a diagnostic PCR assay for the virus (Krenz *et al.* 2012; Al Rwahnih *et al.* 2013). In 2013, the presence of GRBaV in symptomatic vines adjacent to the riparian area was confirmed by multiplex PCR (Krenz *et al.* 2014), and the site was selected for monitoring spread in 2014.

The presence of asymptomatic, GRBaV-infected vines that developed symptoms the following season in the area of the vineyard proximal to the riparian area suggested that the overall disease incidence estimated in the entire vineyard slightly under-represented the extent of GRBaV infection. Nonetheless, significant correlation between PCR detection of GRBaV and presence of symptoms ($\tau = 0.894$ - 0.963 , $P < 0.0001$) suggested that the foliar red blotch symptoms were strongly associated with GRBaV infection. Some of the GRBaV-infected vines also showed symptoms of Pierce’s disease with leaf scorching, immature shoots or vine death in this section of the vineyard (data not shown). A few vines were also infected with leafroll viruses. Although discriminating red blotch, leafroll and Pierce’s disease symptoms was sometimes challenging, the number of vines affected by leafroll and Pierce’s diseases was limited. Therefore, our assessment of red blotch disease incidence was not affected by the presence of other grapevine pathogens in the ‘Cabernet franc’ vineyard selected for this study.

The spatiotemporal pattern of GRBaV spread in this vineyard did not match

the pattern described for other grapevine viruses. For example, leafroll viruses are typically spread along vineyard rows (Habibi and Nutter, 1997; Pietersen, 2006), as a result of mealybug- and soft scale insect-mediated transmission (Naidu *et al.* 2014). A similar pattern has been described for vitiviruses that are transmitted by mealybugs (Rosciglione *et al.* 1983). In contrast, grapevine fanleaf virus and other nepoviruses are generally distributed in small foci in diseased vineyards, owing to their transmission by dagger nematodes (Andret-Link *et al.* 2004; Brown *et al.* 1993; Hewitt *et al.* 1958). The pattern of GRBaV spread illustrated in this study showed a combination of isolated and aggregated diseased vines eight years post-planting. Remarkably, the aggregation of diseased vines was more pronounced near the riparian habitat, with a gradient of disease that seemed to emanate from the riparian habitat. These spread attributes are unique when compared to those of other grapevine viruses. Therefore, a new type of grapevine virus vector is likely transmitting GRBaV. Recently, Bahder *et al.* (2016b) documented the transmission of GRBaV by *S. festinus* in the greenhouse. Populations of *S. festinus* were observed in the selected ‘Cabernet franc’ vineyard concurrently with our survey of GRBaV spread (Cieniewicz *et al.* unpublished). Follow-up investigations are needed to verify the epidemiological significance of *S. festinus* and determine if other insects are capable of transmitting GRBaV in vineyards.

The monocyclic portion of this epidemic could be attributed to an initial introduction of GRBaV via the planting material and subsequent local spread of GRBaV. However, this hypothesis is purely speculative because our surveys were only initiated six years after the ‘Cabernet franc’ vineyard was established, and

therefore the original source of inoculum is unknown. The introduction of inoculum to the area of the 'Cabernet franc' vineyard from outside of this area of interest was determined to be very limited by fitting a spatiotemporal model using a MCMC algorithm in 2014-2016. A 40 year-old *V. vinifera* cv. 'Merlot' vineyard directly southeast of the 'Cabernet franc' and free-living grapes in the riparian area proximal to the highly aggregated section of the vineyard could be potential external sources (Perry *et al.* 2016). Nonetheless, nearly identical sequence identity among 44 GRBaV isolates collected from the 'Cabernet franc' vineyard in 2014, and high, but not identical, sequence identity compared to GRBaV isolates recovered from the nearby free-living and 'Merlot' vines (Perry *et al.* 2016) support the results of the MCMC analysis, which suggest that spread of GRBaV in the 'Cabernet franc' vineyard originated from inoculum sources within the vineyard.

Effective management of red blotch relies on a comprehensive understanding of disease epidemiology and factors mediating the spread of GRBaV. An economic analysis of the effect of red blotch on vineyard profitability estimated the impact to range from \$2,213 per hectare in eastern Washington State under initial low disease incidence to \$68,548 per hectare in Napa County under high initial disease incidence and high quality penalty over a 25-year lifespan of a vineyard (Ricketts *et al.* 2017). This study further suggested that, under low to moderate disease incidence (<30%), roguing and replacing diseased vines with vines derived from certified, virus-tested stocks minimized the economic losses from the disease. In contrast, removing the entire vineyard and establishing vines derived from certified, virus-tested stocks is recommended under high disease incidence (>30%) (Ricketts *et al.* 2017). Our results

from the ‘Cabernet franc’ vineyard support the recommendation to (i) rogue and replant under low disease incidence (4.3 -7.1%) because spread was limited (1-2% per year), and (ii) eliminate the entire vineyard and replant under high disease incidence (50.4-71.2%) because spread was high (4.8 to 16% per year) in the section with diseased vines in small foci. These findings therefore strengthened the rationale underpinning current recommendations for management of red blotch disease that were developed at a time when no information was yet available on spread of GRBaV (Ricketts *et al.* 2017). Our findings stress the need to refine the economic models by taking into account the spread patterns described herein. Such efforts will likely result in optimized management options for red blotch based on a lower action threshold for the selection of infected vines for roguing versus removing and replanting the entire vineyard.

It is important to note that the spatiotemporal attributes of spread observed in the ‘Cabernet franc’ vineyard selected for this study may not be representative of the red blotch epidemics in other vineyards or in other viticulture regions. Similar epidemiology studies in other types of vineyards and other regions are therefore needed to further advance our understanding of the dynamics of red blotch disease epidemics and optimize economic models. Nonetheless, this study provided an important and sentinel perspective on the pattern of GRBaV spread within an otherwise healthy vineyard.

REFERENCES

Al Rwahnih, M., Ashita, D., Anderson, M., Rowhani, A., Uyemoto, J.K., Sudarshana,

M.R., 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103 (10): 1069-1076.

Andret-Link, P., Laporte, C., Valat, L., Laval, V., Ritzenthaler, C., Demangeat, G., Vigne, E., Pfeiffer, P., Stussi-Garaud, C., Fuchs, M., 2004. *Grapevine fanleaf virus*: still a major threat to the grapevine industry. *Journal of Plant Pathology* 86 (3): 183-195.

Bahder, B.W., Zalom, F.G., Sudarshana, M.R., 2016a. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of Grapevine red blotch-associated virus. *Plant Disease* 100 (8): 1571-1574.

Bahder B, Zalom F, Jayanth M, Sudarshana M., 2016b. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* (Say) as a vector of Grapevine red blotch-associated virus. *Phytopathology* 106 (10): 1223-1230.

Brown, D. J., Halbrendt, J. M., Robbins, R. T., Vrain, T. C., 1993. Transmission of nepoviruses by *Xiphinema americanum*-group nematodes. *Journal of Nematology* 25 (3): 349-354.

Calvi, B.L., 2011. Effects of red-leaf disease on Cabernet Sauvignon at the Oakville experimental vineyard and mitigation by harvest delay and crop adjustment. M.S. thesis, University of California, Davis, CA.

Dutilleul, P., Clifford, P., Richardson, S., Hemon, D., 2008. Modifying the t-test for assessing the correlation between two spatial processes. *Biometrics* 49 (1): 305-314.

Fuchs, M., Krenz, B., Yepes, L.M., Thompson, J., McLane, H., Perry, K.L., 2015. Is Grapevine red blotch-associated virus the causal agent of red blotch disease? In: *Proceedings of the 18th Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Ankara, Turkey*, pp.72-73.

Gibbons, J.D., 1976. *Nonparametric methods for quantitative analysis*. Holt, Rinehart and Winston. New York, 463 pp.

Gibson, G.J., 1997a. Investigating mechanisms of spatiotemporal epidemic spread using stochastic models. *Phytopathology* 87 (2): 139-146.

Gibson, G.J., 1997b. Markov chain monte carlo methods for fitting spatiotemporal epidemic stochastic models in plant pathology. *Applied Statistics* 46 (2): 215-233.

Gibson, G.J., Austin, E.J., 1986. Fitting and testing spatio-temporal models with applications in plant epidemiology. *Plant Pathology* 45 (2): 172-184.

Habili, N., Nutter, F.W. Jr., 1997. Temporal and spatial analysis of Grapevine leafroll-associated virus 3 in pinot noir grapevines in Australia. *Plant Disease* 81 (6): 625-628.

Hewitt, W. B., Raski, D.J., Goheen, A.C., 1958. Nematode vector of soil-borne fanleaf virus of grapevines. *Phytopathology* 48 (11): 586-595.

Kendall, M. G., 1938. A new measure of rank correlation. *Biometrika* 30 (1-2), 81-93.

Krenz, B., Thompson, J., Fuchs, M., Perry, K.L., 2012. Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology* 86 (14): 7715.

Krenz, B., Thompson, J., McLane, H., Fuchs, M., Perry, K.L., 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 102 (11): 1232-1240.

Li, B., Madden, L. V., Xu, X., 2012. Spatial analysis by distance indices: An alternative local clustering index for studying spatial patterns. *Methods Ecol. Evol.* 3: 368–377. doi:10.1111/j.2041-210X.2011.00165.x

Lim, S. I., Iori, D., Zhao, F., Moon, J.S., 2016. First report of Grapevine red blotch-associated virus on grapevine in Korea. *Plant Disease* 100 (9): 1957.

Madden, L. V., Hughes, G., van den Bosch, F., 2007. *The Study of Plant Disease Epidemics*, American Phytopathological Society, APS Press, St. Paul, MN.

- Madden, L.V., Louie, R., Abt, J.J., Knoke, J.K., 1982. Evaluation of tests for randomness of infected plants. *Phytopathology* 72 (2): 195-198.
- Naidu, R.A., Rowhani, A., Fuchs, M., Golino, D.A., Martelli, G.P., 2014. Grapevine leafroll: a complex viral disease affecting a high-value fruit crop. *Plant Disease* 98 (9): 1172–1185.
- Perry, J.N., 1995. Spatial Analysis by Distance Indices. *Journal of Animal Ecology* 64 (3): 303-314.
- Perry, J.N., 1998. Measures of spatial pattern for counts. *Ecology* 79 (3): 1008-1017.
- Perry, J.N., Winder, L., Holland, J.M., Alston, R.D., 1999. Red-blue plots for detecting clusters in count data. *Ecology Letters* 2 (2): 106-113.
- Perry, K.L., McLane, H., Hyder, M.Z., Dangl, G.S., Thompson, J.R., Fuchs, M.F., 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* sp. proximal to cultivated grapevines. *Phytopathology* 106 (6): 663-670.
- Pethybridge, S.J., Madden, L.V., 2003. Analysis of spatiotemporal dynamics of virus spread in an Australian hop garden by stochastic modeling. *Plant Disease* 87 (1): 56-62.

Pietersen, G., 2006. Spatio-temporal distribution dynamics of grapevine leafroll disease in Western Cape vineyards,” in Extended abstracts of the 15th Meeting of the International Council for the Study of Virus and Virus-like diseases of the Grapevine (ICVG), April 3–7, Stellenbosch, South Africa, pp.126-127.

Poojari, S., Lowery, T., Schmidt, A-M., Rott, M., McFadden-Smith, W., Stobbs, L., Urbez-Torres, J.R., 2016. Red blotch and the virus in Canada. In: Webinar on red blotch disease, February 26, <http://www.ipmcenters.org/index.cfm/center-products/ipm-eacademy/upcoming-events/red-blotch-speakers/>

Reynard, J.S., 2015. Survey of emerging viruses in Switzerland. In: Proceedings of the 18th Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Ankara, Turkey, pp. 223-224.

Reynard, J.S., 2016. Red blotch and the virus in Europe. IPM eAcademy webinar. February 26, 2016, <https://www.youtube.com/watch?v=LEGXd3iLBx4&index=7&list=PLK93eCG1nCcKFZWFwuMqKPyAvU-Qk--B3>

Ricketts, K.D., Gomez, M.I., Fuchs, M.F., Martinson, T.E., Smith, R.J., Cooper, M.L., Wise, A., 2017. Mitigating the economic impact of Grapevine Red Blotch: Optimizing disease management strategies in U.S. vineyards. *American Journal of Enology and Viticulture* 68 (1): 127-135.

Rosciglione, B., Castellano, M.A., Martelli, G. P., Savino, V., Canizzaro, G., 1983.

Mealybug transmission of grapevine virus A. *Vitis* 22 (4): 331-347.

Sudarshana, M.R., Perry, K.L., Fuchs, M.F., 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105 (7): 1026-1032.

Varsani, A., Roumagnac, P., Fuchs, M., Navas-Castillo, J., Moriones, E., Idris, I.,

Bridson, R.W. Rivera-Bustamante, R., Murilo Zerbini, F., Martin, D.P., 2017.

Capulavirus and *Grablovirus*: Two new genera in the family *Geminiviridae*. *Archives of Virology*, in press.

Winder, L., Alexander, C.J., Holland, J.M., Woolley, C., Perry, J.N., 2001. Modelling the dynamic spatio-temporal response of predators to transient prey patches in the field. *Ecology Letters* 4 (6): 568-576.

Xu, X., Madden, L. V, 2005. Interrelationships Among SADIE Indices for

Characterizing Spatial Patterns of Organisms. *Phytopathology* 95 (16): 874–883.

CHAPTER 3

INSIGHTS INTO THE ECOLOGY OF GRAPEVINE RED BLOTCH VIRUS IN A DISEASED VINEYARD

ABSTRACT

Limited information is available on the spread of grapevine red blotch virus (GRBV, genus *Grablovirus*, 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/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), *Osbornellus borealis* (Cicadellidae) and a *Melanoliarius* species (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 co-variation 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.

*This chapter was published in: Cieniewicz E.J., Loeb G., Pethybridge S., Perry K.L. and Fuchs M. (2018) Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology*, 108: 94-102.

INTRODUCTION

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 (Krenz *et al.* 2012; Al Rwahnih *et al.* 2013) and later demonstrated as the causative agent of red blotch disease (Fuchs *et al.* 2015). GRBV is the type member of the genus *Grablovirus* 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.* 2016a), 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 ‘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.* 2016b) 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.* 2016b). 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

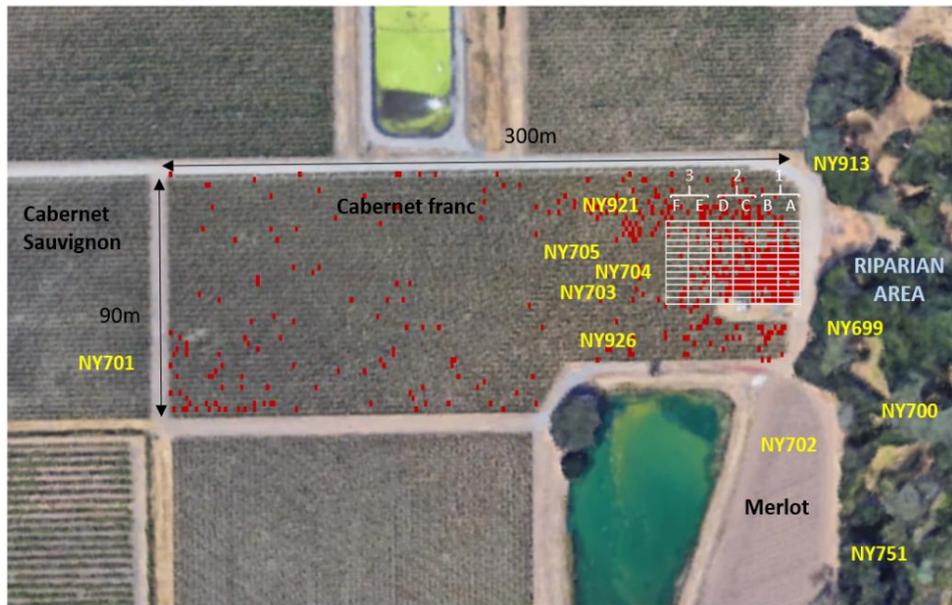
A 2-hectare *Vitis vinifera* ‘Cabernet franc’ vineyard established in 2008 in Napa County, CA was selected for this study. Selection of this vineyard was based on temporal changes in GRBV incidence with spatiotemporal analysis of epidemics suggesting the presence of secondary spread between 2014 and 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 cm by 12.7 cm) were placed on the middle trellis wire (1.2 m above the ground) throughout a sampling area that spanned twelve rows, and six 4-vine panels per row. The 4-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 (**Figure 3-1A**). 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 twelve rows contained three sticky cards in either A-C-E or B-D-F orientation (**Figure 3-1B**). The spacing of two sticky cards between two 4-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

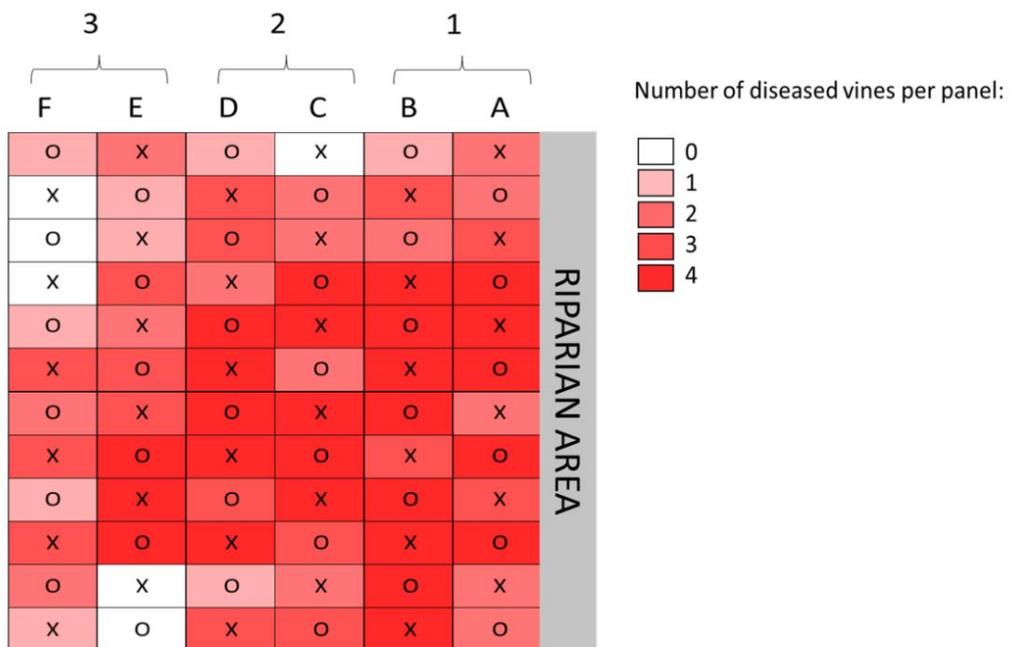
evaluation, and replaced with new sticky cards. The survey was conducted from April to November 2015, and March to November 2016 to span the entire growing season.

Figure 3-1 Map of the ‘Cabernet franc’ vineyard infected with grapevine red blotch virus (GRBV) clade 2 isolates that was selected for this study, indicating (A) its relative location close to a riparian area and a ‘Cabernet Sauvignon’ vineyard infected with GRBV clade 1 isolates (Perry *et al.* 2016). The layout of the sticky card insect survey is shown with a white grid at the edge of the vineyard proximal to the riparian area. Yellow NY letters followed by numbers represent the approximate location of randomly selected vines from which GRBV sequence information was previously obtained (Perry *et al.* 2016). (B) Close-up of the vineyard area selected for this study with the layout of the sticky cards that is overlaid with GRBV incidence (Cieniewicz *et al.* 2017b) illustrated with a heat map. Sticky card orientation during even and odd weeks is shown by (x) and (o), respectively. In A to F, each box represents a 4 vine-panel, and sections 1 to 3 represent two groups of adjacent rows.

A.



B.



Insect identification and specimen handling

Insects caught on sticky card traps were identified to genus/species when possible based on morphological characteristics. Specimens were identified and counted while still impacted on sticky cards. The number and identity of specimens was 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., 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) and/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). PCRs 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 1X 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/ LepR1 (Footit *et al.* 2014; Hebert *et al.* 2004) were used to amplify the barcode region of the later identified *Melanoliarus* sp. Primers LCO1490/ HCO2198 (Folmer *et al.* 1994) were used to amplify the barcode region of the later identified *Osbornellus borealis* and *Spissistilus festinus*. PCRs 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, and CPfor and CPrev were used to amplify GRBV *Rep* and *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 grapes (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 (Li *et al.* 2012; Perry 1995, 1998; Perry *et al.* 1999; Xu and Madden 2005; Cieniewicz *et al.* 2017b). 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 in scores of 0 to 4 based on the number of diseased vines in each 4-vine panel with 0 indicating no diseased vine in the panel and 4 indicating four diseased vines in the 4-vine panel (**Figure 3-1B**).

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 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* (Membracidae), *C. reductus* (Cicadellidae), *O. borealis* (Cicadellidae) and a *Melanoliarius* species (Cixiidae) by multiplex PCR (**Table 3-1**). 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 3-1**). GRBV was not detected in any of the *E. ziczac* specimens tested in 2016 (**Table 3-1**).

Table 3-1 Grapevine red blotch virus 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).

			GRBV detection in 2015		GRBV detection in 2016		Cumulative GRBV detection	
Hemiptera	Membracidae	<i>Spissistilus festinus</i>	12/25 ^a	48%	13/25	52%	25/50	50%
	Cixiidae	<i>Melanoliarus</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>Erythroneura ziczac</i>	- ^b	-	0/10	0%	0/10	0%
		<i>Erythroneura tricincta</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%

^aNumber of individual specimens in which GRBV was detected by multiplex PCR over the total number of specimens tested

^b(-) No specimen tested.

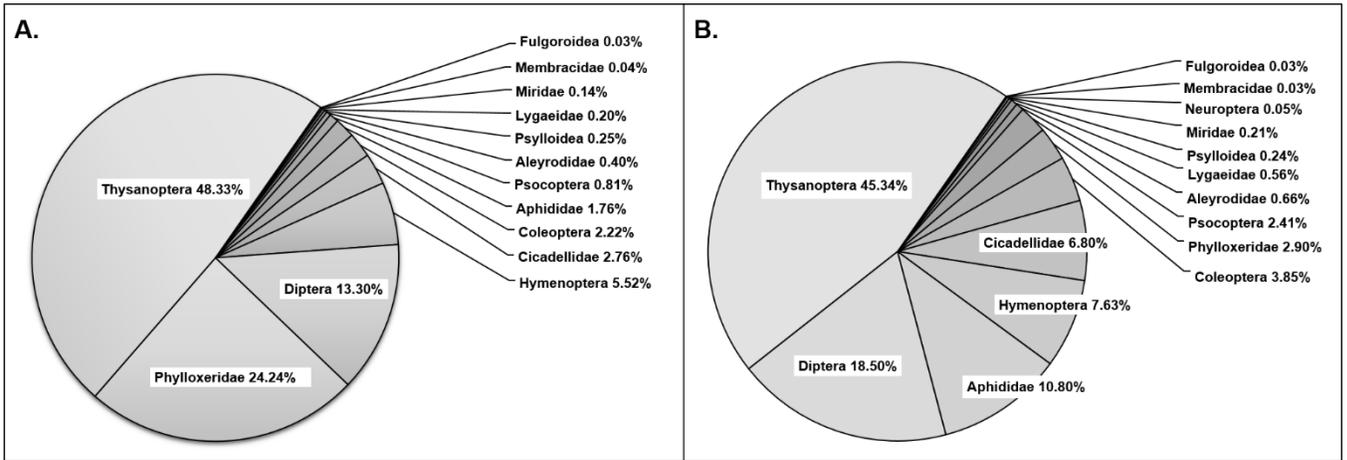


Figure 3-2 Diversity and abundance of insect taxa identified from sticky cards placed in a *Vitis vinifera* ‘Cabernet franc’ vineyard infected by grapevine red blotch virus in Napa County, California in (A) 2015 and (B) 2016.

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 (**Figure 3-2**). Thysanoptera were most abundant in both years and the abundance of Diptera was consistent across years (**Figure 3-2**). More Phylloxeridae were caught in 2015 than in 2016 while 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 (**Figure 3-2**).

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* species 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 (**Figure 3-3A**) and late June 2016 (**Figure 3-3B**), with populations quickly tapering after July. Populations of *C. reductus* peaked in August 2015 (**Figure 3-3A**) and in April and September 2016 (**Figure 3-3B**). Populations of *Melanoliarus* sp. peaked in July (**Figure 3-3**). *O. borealis* was captured infrequently in June and July, and increasingly in August and September of both years (**Figure 3-3**).

GRBV was not detected in vector candidates until June (**Figure 3-4**), with the exception of one *C. reductus* specimen that tested positive for GRBV in early May 2016 (**Figure 3-4F**). The incidence of viruliferous *S. festinus* was highest in July in 2015 (**Figure 3-4A**) and June in 2016 (**Figure 3-4B**). Viruliferous *O. borealis* were detected from July to November in 2015 (**Figure 3-4C**) and 2016 (**Figure 3-4D**), while viruliferous *Melanoliarus* sp. were captured on sticky cards only from July to September (**Figure 3-4G & 3-4H**).

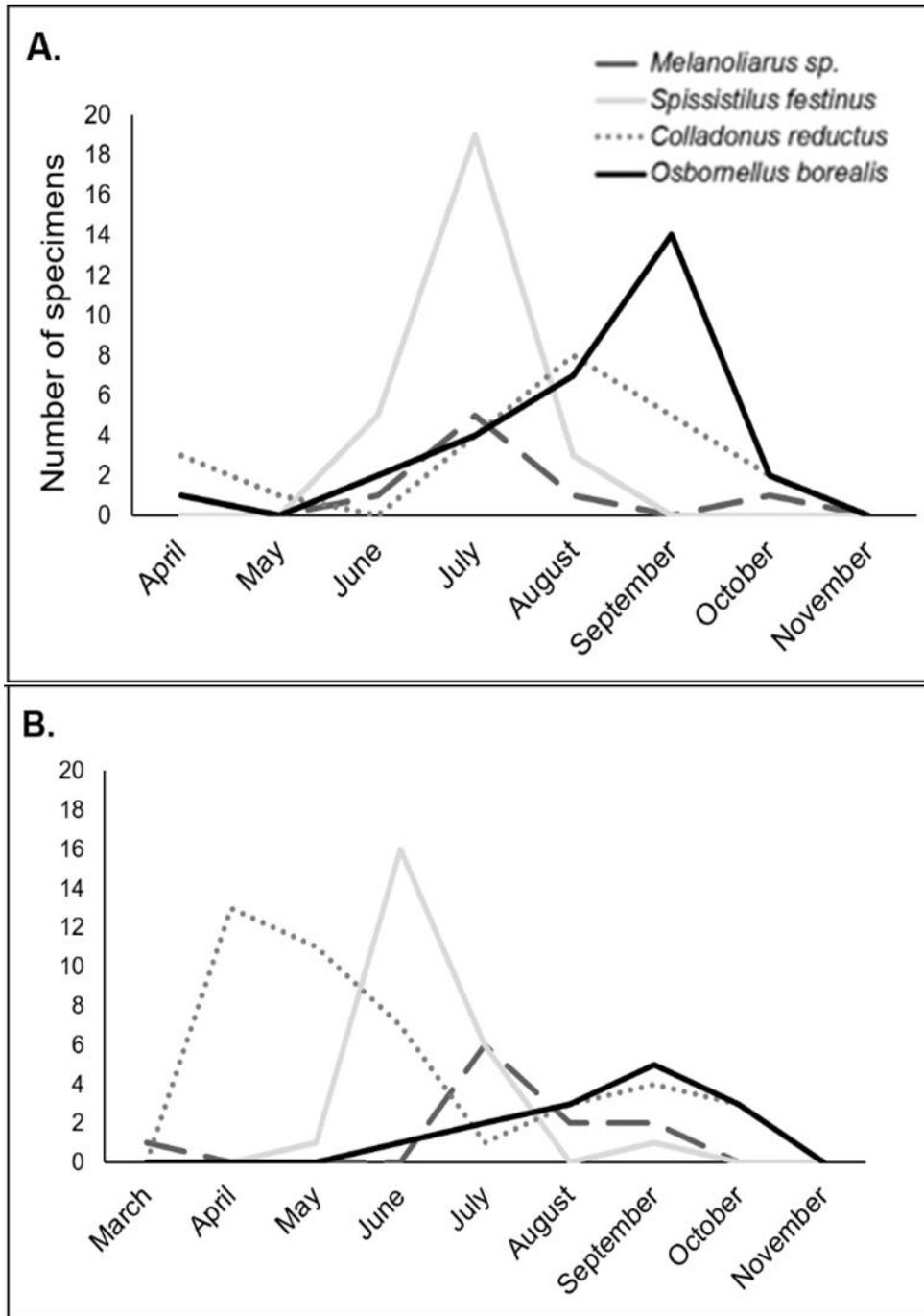


Figure 3-3 Seasonal population dynamics of candidate insect vectors of grapevine red blotch virus based on specimens captured on sticky cards in a diseased *Vitis vinifera* ‘Cabernet franc’ vineyard in Napa County, California in (A) 2015 and (B) 2016.

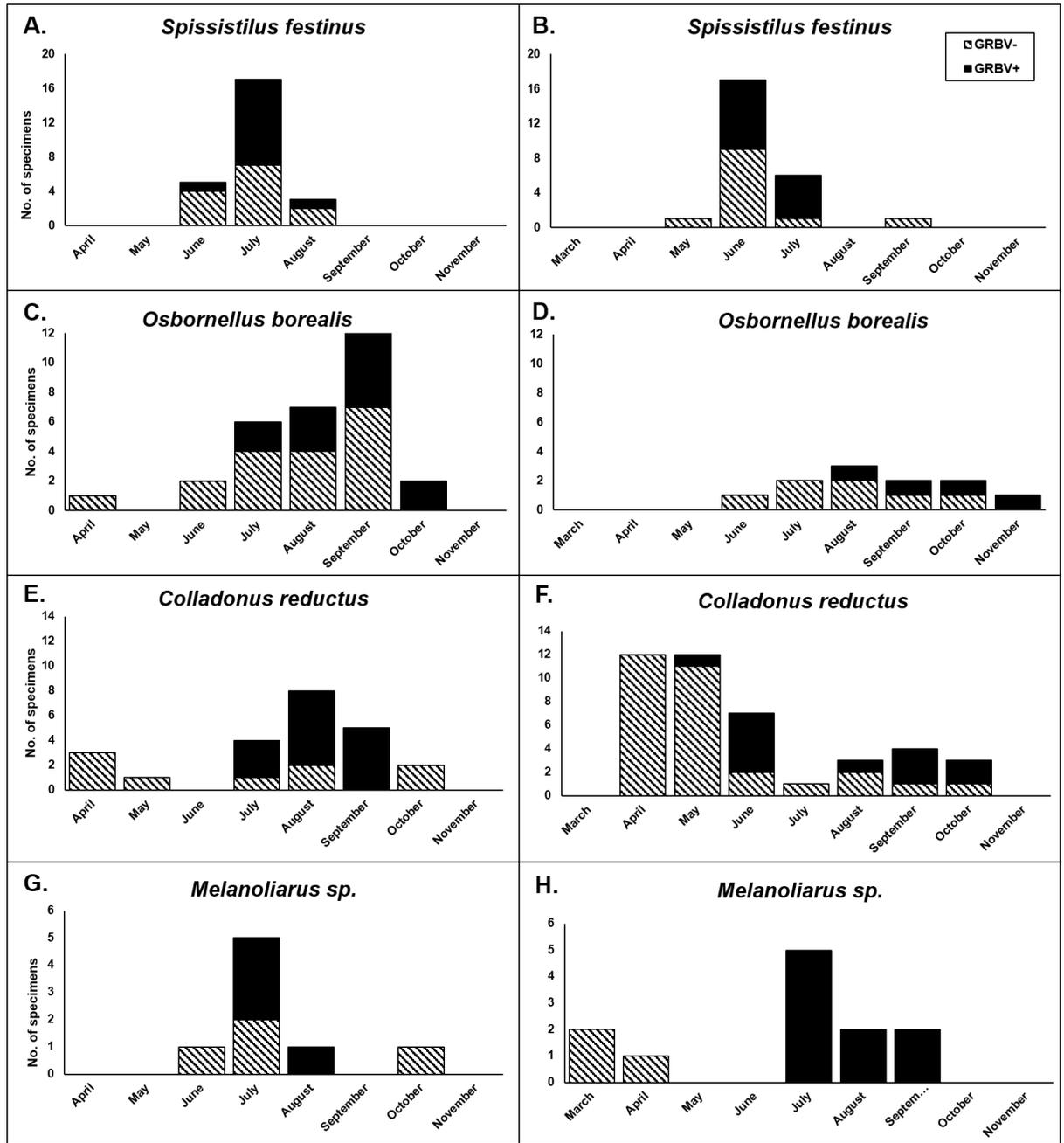


Figure 3-4 Seasonal dynamics of ingestion of grapevine red blotch virus (GRBV) by candidate insect vectors in a diseased *Vitis vinifera* 'Cabernet franc' vineyard in Napa County, California in (A, C, E, and G) 2015 and (B, D, F, and H) 2016. The presence of GRBV in individual candidate insect vector specimens was assessed by multiplex PCR.

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 (**Figure 3-5**). 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 out 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 (**Figure 3-5**). 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 (**Figure 3-5**). The phylogenetic lineages assembled from GRBV *CP* sequences was consistent with that obtained from GRBV *Rep* sequences (data not shown).

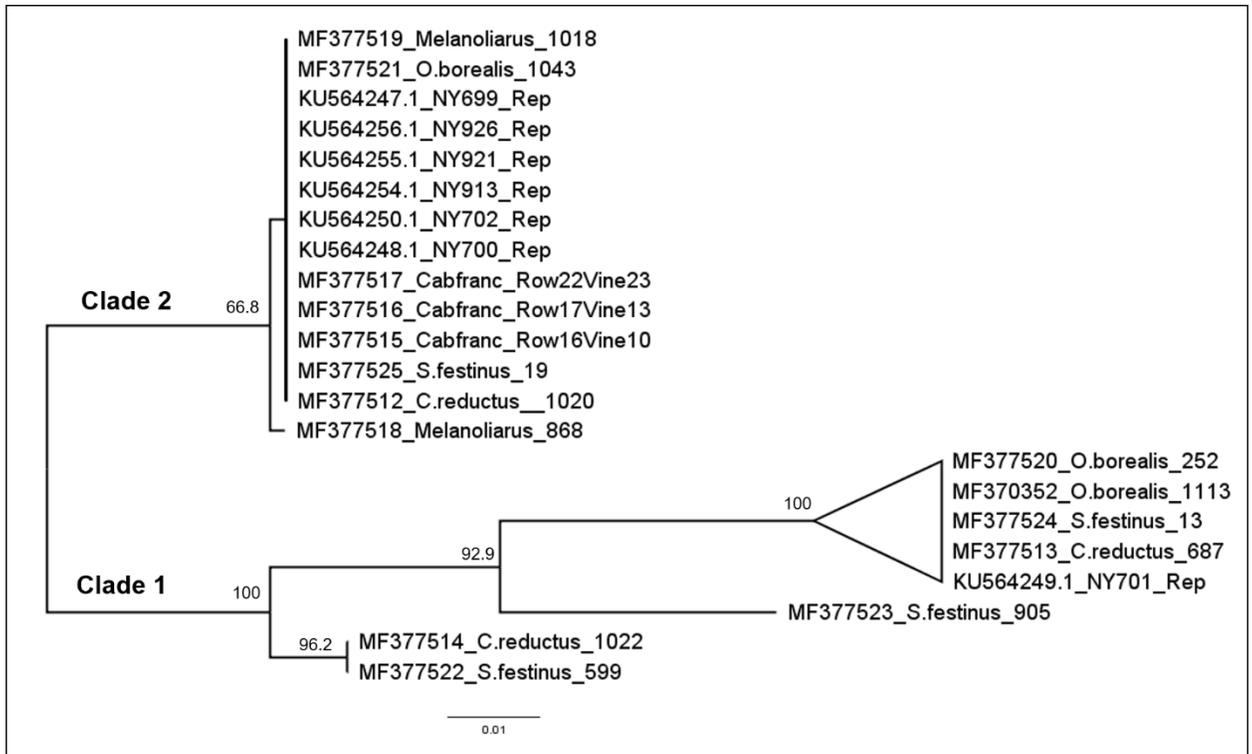


Figure 3-5 Neighbor-joining phylogenetic tree indicating relationships among grapevine red blotch virus (GRBV) Rep nucleic acid sequences recovered from infected ‘Cabernet franc’ vines, candidate insect vectors (*Melanoliarus*, *Spissistilus festinus*, *Colladonus reductus*, and *Osbornellus borealis*) 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 **Table 3-2**.

Table 3-2 Grapevine red blotch virus sequence information used in this study.

Sequence information	Isolate source	Collection Date	GenBank Accession Number	Reference
GRBV Rep ^a	<i>Vitis vinifera</i> ‘Cabernet franc’ (row 16, vine 10) ^c	11 Oct. 2016	MF377515	This study
GRBV Rep	<i>V. vinifera</i> ‘Cabernet franc’ (row 17, vine 13) ^c	11 Oct. 2016	MF377516	This study
GRBV Rep	<i>V. vinifera</i> ‘Cabernet franc’ (row 22, vine 23) ^c	11 Oct. 2016	MF377517	This study
GRBV Rep	<i>Colladonus reductus</i> #1020 ^d	26 Aug. 2015	MF377512	This study
GRBV Rep	<i>Colladonus reductus</i> #687	1 July 2015	MF377513	This study
GRBV Rep	<i>Colladonus reductus</i> #1022	16 Sept. 2015	MF377514	This study
GRBV Rep	<i>Melanoliarus</i> #868	16 July 2015	MF377518	This study
GRBV Rep	<i>Melanoliarus</i> #1018 ^e	12 Aug. 2015	MF377519	This study
GRBV Rep	<i>Osbornellus borealis</i> #252	10 Oct. 2016	MF377520	This study
GRBV Rep	<i>Osbornellus borealis</i> #1043 ^f	2 Sept. 2015	MF377521	This study
GRBV Rep	<i>Osbornellus borealis</i> #1113	23 Sept. 2015	MF370352	This study
GRBV Rep	<i>Spissistilus festinus</i> #13	29 June 2016	MF377524	This study
GRBV Rep	<i>Spissistilus festinus</i> #19 ^g	7 July 2016	MF377525	This study
GRBV Rep	<i>Spissistilus festinus</i> #905	29 July 2015	MF377523	This study
GRBV Rep	<i>Spissistilus festinus</i> #599	25 June 2015	MF377522	This study
GRBV NY699 Rep	Free living <i>Vitis</i> species	Oct. 2014	KU564247.1	Perry <i>et al.</i> (2016)
GRBV NY700 Rep	Free living <i>Vitis</i> species	Oct. 2014	KU564248.1	Perry <i>et al.</i> (2016)
GRBV NY913 Rep	Free living <i>Vitis</i> species	Oct. 2014	KU564254.1	Perry <i>et al.</i> (2016)
GRBV NY701 Rep	<i>V. vinifera</i> ‘Cabernet sauvignon’	Oct. 2014	KU564249.1	Perry <i>et al.</i> (2016)
GRBV NY702 Rep	<i>Vitis vinifera</i> ‘Merlot’	Oct. 2014	KU564250.1	Perry <i>et al.</i> (2016)
GRBV NY926 Rep	<i>Vitis vinifera</i> ‘Cabernet franc’	Oct. 2014	KU564256.1	Perry <i>et al.</i> (2016)
GRBV NY921 Rep	<i>Vitis vinifera</i> ‘Cabernet franc’	Oct. 2014	KU564255.1	Perry <i>et al.</i> (2016)
mt-coi barcode ^b	<i>Osbornellus borealis</i> #1043	2 Sept. 2015	MF414209	This study
mt-coi barcode	<i>Melanoliarus species</i> #1018	12 Aug. 2015	MF414210	This study
mt-coi barcode	<i>Spissistilus festinus</i> #599	25 June 2015	MF414211	This study

^a Sequence derived from the grapevine red blotch virus (GRBV) gene encoding a putative replicase-associated protein.

^b Sequence derived from the 3’ end of the gene encoding the mitochondrial cytochrome C oxidase subunit I (mt-coi).

^c 171 additional GRBV Rep sequences from *V. vinifera* ‘Cabernet franc’ vines showed greater than 99% identity, and are not shown.

^d 14 additional GRBV Rep sequences from *Colladonus reductus* specimens showed greater than 99% identity, and are not shown.

^e 11 additional GRBV Rep sequences from *Melanoliarus sp.* specimens showed greater than 99% identity, and are not shown.

^f 16 additional GRBV Rep sequences from *Osbornellus borealis* specimens showed greater

than 99% identity, and are not shown.

§18 additional GRBV Rep sequences from *Spissistilus festinus* specimens showed greater than 99% identity, and are not shown.

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 3-3**). 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 3-4**).

The spatial distribution of vector candidates on sticky cards also indicated a gradient of higher *S. festinus* (N= 50) and *O. borealis* (N=42) at the edge of the vineyard next to a riparian area and decreasing *S. festinus* and *O. borealis* populations distant from the edge (**Figure 3-6**). 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 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 (**Figure 3-6**).

Table 3-3 Spatial Analysis by Distance Indices (SADIE) 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> =
GRBV incidence	418.7	2.088	0.002**
<i>S. festinus</i> (total counts)	156.8	1.171	0.137 (ns)
<i>S. festinus</i> (viruliferous counts)	141.2	1.486	0.0099**
<i>O. borealis</i> (total counts)	218.9	1.549	0.007**
<i>O. borealis</i> (viruliferous counts)	136.3	1.702	0.0012**
<i>C. 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> (total counts)	64.4	0.853	0.833 (ns)
<i>Melanoliarius</i> (viruliferous counts)	58.5	0.7962	0.956 (ns)

^aTotal 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.

** Significance according to a two-tail test at *P* < 0.01.

Table 3-4 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 (SADIE) with an actual sample size of 72.

Variable ^a	Effective sample size	Overall association (X) ^b
<i>S. festinus</i> (total counts)	76.1	-0.0028 (0.5094; ns)
<i>S. festinus</i> (viruliferous counts)	72.0	0.1989 (0.0479*)
<i>O. 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>C. 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>Melanoliarius</i> (total counts)	71.4	0.075 (0.529; ns)
<i>Melanoliarius</i> (viruliferous counts)	72.2	0.083 (0.237; ns)

^aTotal counts and total viruliferous specimen counts are provided for each candidate insect vector species.

^bOverall association (X). Two-tail probability value presented parenthetically.

*Significance according to a two-tail test at $P > 0.05$.

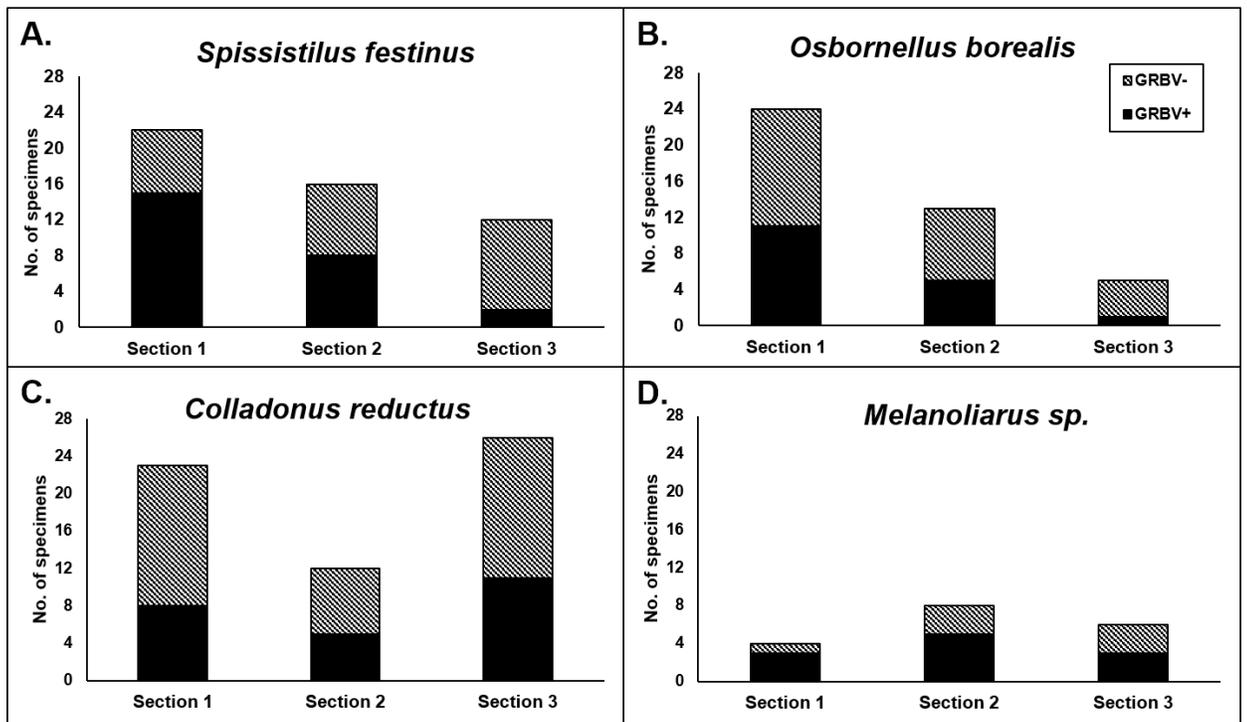


Figure 3-6 Spatial distribution of candidate insect vectors at the edge of a ‘Cabernet franc’ vineyard where grapevine red blotch virus incidence is aggregated proximal to a riparian habitat. The number of specimens in 2015 and 2016 are summed for each species. Sections 1 (0 to 10 m from the edge of the vineyard), 2 (10 to 20 m within the vineyard) and 3 (20 to 30 m within the vineyard) indicate partitions of two 4-vine panels, where section 1 is positioned closest to the edge and section 3 is furthest into the vineyard.

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.* 2016), 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 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 (intra-vineyard) and background (extra-vineyard) sources. It will be important to determine if 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.* 2016b). The population dynamics of *S. festinus* 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 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 (**Figure 3-6**). These results suggested a directional influx of aviruliferous *S. festinus* and *O. borealis* 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 *Melanoliarius* 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 *Melanoliarius* 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.* 2016b). 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 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 fruits 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 like *Melanoliarius* sp. also feed on phloem, and some can transmit phytoplasmas, such as the lethal yellows phytoplasma in palms (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.

While 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 non-exhaustive list of examples of phloem-feeders (Douglas 2005) 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 (*Scaphytopius magdalensis*, *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 (**Figure 3-1**), and potentially feeding and ingesting GRBV. The sticky card sampling method likely resulted in under-sampling populations of immature insects, as shown for other systems (Musser *et al.* 2004). Consequently, we cannot discount the possibility that some feeding insects were recalcitrant to capture on sticky cards, and hence potentially under-represented 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 co-variation 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.

REFERENCES

Al Rwahnih, M., Ashita, D., Anderson, M., Rowhani, A., Uyemoto, J. K., and Sudarshana, M. R. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103:1069-1076.

Bahder, B. W., Zalom, F. G., and Sudarshana, M. R. 2016a. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of Grapevine red blotch-associated virus. *Plant Dis.* 100:1571-1574.

Bahder, B. W., Zalom, F. G., Jayanth, M., and Sudarshana, M. R. 2016b. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* (Say) as a vector of Grapevine red blotch-associated virus. *Phytopathology* 106:1223-1230.

Cieniewicz, E. J., Perry, K. L., and Fuchs, M. F. 2017a. Grapevine red blotch: molecular biology of the virus and management of the disease. Pages 303-314 in: *Grapevine viruses: molecular biology, diagnostics and management*. B. Meng, G.P.

Martelli, D. Golino and M. Fuchs, eds. Springer Verlag, Berlin, Germany.

Cieniewicz, E. J., Pethybridge, S. J., Gorny, A., Madden, L. V., McLane, H., Perry, K. L., and Fuchs, M. F. 2017b. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Res.*

<http://dx.doi.org/10.1016/j.virusres.2017.03.020>.

Douglas, A E. 2005. Phloem-sap feeding by animals: Problems and solutions. *J. Exp. Bot.* 57(4): 747–54.

Dutilleul, P., Clifford, P., Richardson, S., and Hemon, D. 2008. Modifying the t-test for assessing the correlation between two spatial processes. *Biometrics* 49: 305-314.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *J. Exp. Mar. Bio. Ecol.* 3:294-299.

Footit, R. G., Maw, E., and Hebert, P. D. N. 2014. DNA barcodes for nearctic Auchenorrhyncha (Insecta: Hemiptera). *PLoS ONE* 9: e101385.

Fuchs, M. F., Krenz, B., Yepes, L. M., Thompson, J., McLane, H., and Perry, K. L. 2015. Is Grapevine red blotch-associated virus the causal agent of red blotch disease? In: *Proceedings of the 18th Congress of the International Council for the Study of*

Virus and Virus-like Diseases of the Grapevine, Ankara, Turkey, pp.72-73.

Hebert, P. D. N., Cywinska, A., Ball, S. L., and Dewaard, J. R. 2003. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond.* 270: 313–21.

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H., and Hallwachs, W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc. Natl. Acad. Sci. USA* 101:14812-14817.

Holzinger, W. E., Emeljanov, A.F., and Kammerlander, I. 2002. The family Cixiidae (Spinola) 1839 (Hemiptera: Fulgoromorpha) – a review. *Denisia* 4:113-138.

Jensen, D. D. 1957. Transmission of peach yellow leaf roll virus by *Fiebertiella florii* (Stal) and a new vector *Osbornellus borealis* (DeL. & M.) *J. Econ. Entomol.* 50:668-672.

Krenz, B., Thompson, J., Fuchs, M. F, and Perry, K. L. 2012. Complete genome sequence of a new circular DNA virus from grapevine. *J. Virol.* 86:7715.

Krenz, B., Thompson, J., McLane, H., Fuchs, M. F, and Perry, K. L. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 102:1232-1240.

Li, B., Madden, L. V., and Xu, X. 2012. Spatial analysis by distance indices: An alternative local clustering index for studying spatial patterns. *Methods Ecol. Evol.* 3:368-377.

Lim, S., Igori, D., Zhao, F., and Moon, J.S. 2016. First report of Grapevine red blotch-associated virus on grapevine in Korea. *Plant Dis.* 100:1957.

Mueller, A. J., and Dumas, B. A. 1975. Effects of stem girdling by the three-cornered alfalfa hopper on soybean yields. *J. Econ. Entomol.* 68:511-512.

Musser, F. R., Nyrop, J. P., and Shelton, A. M. 2004. Survey of predators and sampling method comparison in sweet corn. *J. Econ. Entomol.* 97:136-144.

Perry, J. N. 1995. Spatial Analysis by Distance Indices. *J. Anim. Ecol.* 64:303-314.

Perry, J. N. 1998. Measures of spatial pattern for counts. *Ecology* 79:1008-1017.

Perry, J. N., Winder, L., Holland, J. M., and Alston, R. D. 1999. Red-blue plots for detecting clusters in count data. *Ecol. Lett.* 2:106-113.

Perry, K. L., McLane, H., Hyder, M. Z., Dangl, G. S., Thompson, J. R., and Fuchs, M. F. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* sp. proximal to cultivated grapevines. *Phytopathology* 106:663-670.

Poojari, S., Lowery, D. T., Rott, M., Schmidt A. M. and Úrbez-Torres, J. R. 2017. Incidence, distribution and genetic diversity of Grapevine red blotch virus in British Columbia. *Can. J. of Plant Path.*, DOI:10.1080/07060661.2017.1312532

Poojari, S., Alabi, O. J., Fofanov, V. Y., and Naidu, R. A. 2013. A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family *Geminiviridae* implicated in grapevine redleaf disease by next-generation sequencing. *PLoS ONE* 8:1-17. 10.1371/journal.pone.0064194.

Reynard, J. S. 2015. Survey of emerging viruses in Switzerland. In: Proceedings of the 18th Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Ankara, Turkey, pp. 223-224.

Ricketts, K. D., Gomez, M. I., Fuchs, M. F., Martinson, T. E., Smith, R. J., Cooper, M. L., and Wise, A. 2017. Mitigating the economic impact of Grapevine Red Blotch: Optimizing disease management strategies in U.S. vineyards. *Am. J. Enol. Vitic.* 68:127-135.

Saguez, J., Lemoyne, P., Giordanengo, P., Olivier, C., Lasnier, J., Mauffette, Y., and Vincent, C. 2015. Characterization of the feeding behavior of three *Erythroneura* species on grapevine by histological and DC-electrical penetration graph techniques. *Entomol. Exp. Appl.* 157:227-240.

Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.

Sudarshana, M. R., Perry, K. L., and Fuchs, M. F. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105:1026-1032.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882.

Varsani, A., Roumagnac, P., Fuchs, M. F., Navas-Castillo, J., Moriones, E., Idris, I., Briddon, R. W., Rivera-Bustamante, R., Murilo Zerbini, F., and Martin, D. P. 2017. *Capulavirus* and *Grablovirus*: Two new genera in the family *Geminiviridae*. *Arch. Virol.* doi: 10.1007/s00705-017-3268-6

Winder, L., Alexander, C. J., Holland, J. M., Woolley, C., and Perry, J. N. 2001. Modelling the dynamic spatio-temporal response of predators to transient prey patches in the field. *Ecology Lett.* 4: 568-576.

Wolfe, H. R. 1955. Transmission of the Western X disease virus by the leafhopper *Colladonus montanus* (Van D.). *Plant Dis. Rep.* 39:298-299.

Wolfe, H. R., Anthon, E. W., and Jones, S. L. 1950. Transmission of Western X-disease of peaches by the leafhopper *Colladonus geminatus* (Van D.). *Phytopathology* 40:971.

Xu, X., and Madden, L. V 2005. Interrelationships among SADIE indices for characterizing spatial patterns of organisms. *Phytopathology* 95:874-883.

CHAPTER 4

PREVALENCE AND GENETIC DIVERSITY OF GRABLOVIRUSES IN FREE-LIVING VITIS SPP.

ABSTRACT

The distribution and diversity of grapevine red blotch virus (GRBV) and wild Vitis virus 1 (WVV1) (genus *Grablovirus*; family *Geminiviridae*) were determined in free-living *Vitis* species in northern California and New York from 2013 to 2017. Grabloviruses were detected by polymerase chain reaction in 28% (57 of 203) of samples from California but in none of the 163 samples from New York. The incidence of GRBV in free-living vines was significantly higher in samples from California counties with high compared to low grape production ($\chi^2 = 83.09$; $P < 0.001$), and in samples near (< 5km) to compared to far (> 5km) from vineyards ($\chi^2 = 57.58$; $P < 0.001$). These results suggested a directional spread of GRBV inoculum predominantly from vineyards to free-living *Vitis* species. WVV1 incidence was also significantly higher in areas with higher grape production acreage ($\chi^2 = 16.02$; $P < 0.001$). However in contrast to GRBV, no differential distribution of WVV1 incidence was observed with regard to distance from vineyards ($\chi^2 = 0.88$; $P < 0.3513$). Two distinct phylogenetic clades were identified for both GRBV and WVV1 isolates from free-living *Vitis* species, although the nucleotide sequence variability of the genomic diversity fragment was higher for WVV1 (94.3-99.8% sequence identity within clade 1 isolates; 90.1-100% within clade 2 isolates) than GRBV (98.3% between clade 1 isolates; 96.9 to 100% within clade 2 isolates). Additionally, evidence for intra-

specific recombination events was found in WVV1 isolates and confirmed in GRBV isolates. The prevalence of grabloviruses in California free-living vines highlights the need for vigilance regarding potential grablovirus inoculum sources in order to protect new vineyard plantings and foundation stock vineyards in California.

*This chapter was published in: Cieniewicz E.J., Thompson J., McLane H., Perry K.L., Dangi G.S., Corbett Q., Martinson T., Wise A., Wallis A., O'Connell J., Dunst R., Cox K., and Fuchs M. (2018) Prevalence and genetic diversity of grabloviruses in free-living *Vitis* spp. *Plant Disease*, 102: 2308-2316.

INTRODUCTION

Grapevine red blotch disease is emerging as one of the most important viral diseases of *Vitis* spp. in North America (Cieniewicz *et al.* 2017a). Red blotch disease was described for the first time in 2008 in California as reddening of leaves with reports of reduced fruit qualities, similar to both the symptoms and ripening effects of leafroll disease. However, symptomatic vines tested negative for all known leafroll-associated viruses. Grapevine red blotch virus (GRBV) was found associated with disease symptoms (Al Rwahnih *et al.* 2013) and later demonstrated as the causative agent of red blotch disease in *Vitis* spp. (Yepes *et al.* 2018). GRBV infection disrupts berry development, inhibits ripening pathways (Blanco-Ulate *et al.* 2017), and threatens the productivity and profitability of vineyards (Cieniewicz *et al.* 2017a, Ricketts *et al.* 2017, Sudarshana *et al.* 2015).

GRBV is the type member of the genus *Grabovirus* in the family *Geminiviridae* (Varsani *et al.* 2017). This virus is widespread in vineyards throughout the United States, likely due to its graft-transmissibility and dissemination via infected propagation material (Krenz *et al.* 2014). Additionally, GRBV is vectored by the three cornered alfalfa treehopper, *Spissistilus festinus*, as demonstrated in a greenhouse study (Bahder *et al.* 2016a). Spatiotemporal spread of red blotch disease was recently documented in a *V. vinifera* ‘Cabernet franc’ vineyard in California (Cieniewicz *et al.* 2017b) and the epidemiological role of *S. festinus* was illustrated (Cieniewicz *et al.* 2018).

GRBV was detected in free-living (feral) *Vitis* spp. nearby commercial vineyards in Napa County in California (Perry *et al.* 2016). Some of the infected free-

living vines were hybrids of *Vitis californica* and *V. vinifera* (Perry *et al.* 2016). The presence of GRBV in free-living *Vitis* spp. in Napa County was later corroborated in an independent study (Bahder *et al.* 2016b). The notion of free-living *Vitis* spp. serving as inoculum reservoirs has been demonstrated in other grapevine pathosystems (Beach *et al.* 2017, Canik Orel *et al.* 2017, Klaassen *et al.* 2011, Pacifico *et al.* 2016, Sabanadzovic *et al.* 2009). Although vector-mediated transmission of GRBV between free-living grapevines and commercial vineyards has not been confirmed, nor the direction of inoculum movement elucidated, the presence of GRBV in free-living *Vitis* spp. suggests that uncultivated grapevines may be sources of virus inoculum.

Since the identification of GRBV, several gemini-like viruses have been discovered in *Vitis* species, including wild Vitis virus 1 (WVV1) (Perry *et al.* 2018), grapevine geminivirus A (GGVA) (Al Rwahnih *et al.* 2016) and temperate fruit decay-associated virus (Basso *et al.* 2015). WVV1 is a putative member of the genus *Grablovirus* (Perry *et al.* 2018) along with GGVA and the type species, GRBV (Varsani *et al.* 2017). Unlike GRBV, WVV1 has only been detected in free-living *Vitis* spp.; it has not yet been detected in any *Vitis* cultivar or rootstock genotype. In addition, like GRBV, WVV1 is not associated with any disease symptoms in free-living *Vitis* spp. (Perry *et al.* 2016, 2018). To date, GRBV and WVV1 have only been described in free-living *Vitis* spp. in Napa County in California (Bahder *et al.* 2016b, Perry *et al.* 2016, 2018). Limited information is available on the distribution of both GRBV and WVV1 in free-living *Vitis* spp. in varied ecosystems. We investigated the prevalence and distribution of GRBV and WVV1 in free-living grape populations and hypothesized a directional movement of GRBV between cultivated and non-cultivated

plants. A related objective was to determine the genetic diversity of GRBV and WVV1 isolates in free-living *Vitis* spp. To address these objectives, we surveyed free-living grapevines in close proximity to vineyards and also far from vineyards in both California and New York.

MATERIALS AND METHODS

Collection of free-living *Vitis* spp. and genetic identification of California samples

Hardwood cuttings and leaf samples were collected from free-living *Vitis* spp. from 2013 to 2017 in the following counties in northern California: Butte, Glenn, Napa, Sacramento, Solano, Sonoma, and Sutter. These counties range from having high grape production (9.5 and 5.9% acreage planted to grapes in Napa and Sonoma counties) to very low grape production (0.006, 0.0999 and 0.017% acreage planted to grapes in Sutter, Glenn and Butte counties respectively). Samples were collected from free-living vines in or nearby riparian areas, primarily along the banks of the Sacramento River and Napa River, as well as along the banks of Maxwell Creek. A subset of California free-living *Vitis* spp. samples was genetically identified at Foundation Plant Services, University of California in Davis, California by using eight single-sequence repeat markers, as previously described (Dangl *et al.* 2015, Perry *et al.* 2016).

In New York, samples were collected from free-living *Vitis* spp. from March to August 2017 from the following counties: Chautauqua, Clinton, Ontario, Seneca, Steuben, Suffolk, Tompkins, Ulster, and Yates. These counties represent the major grape growing regions of New York, including the Finger Lakes Region, Hudson

Valley, Long Island, Champlain Valley, and Western New York. Sample collection in these counties was not restricted to riversides because free-living grapevines are more widely dispersed away from New York riparian areas compared to California.

An average of 20 free-living *Vitis* spp. samples were collected per county both in California and New York, except for Napa, Sacramento and Ulster counties for which more samples were collected. Both in California and New York, samples were selected either proximal to vineyards (less than 5 km) or away from vineyards (more than 5 km). No information was available on the health status of proximal vineyards when samples of free-living *Vitis* spp. were collected with a few exceptions in Napa County for which the presence of GRBV had been previously documented (Cieniewicz *et al.* unpublished). Lignified wood with at least 15 buds was collected from individual free-living grapevines and, if leaf tissue was available at the time of sampling, at least 10 leaves were collected in addition to lignified wood. Samples were collected from individual free-living grapevines at least 30 meters from other free-living grapevines to avoid redundancy in sampling. Samples were stored at 4°C until virus testing.

Virus detection by PCR

Cambium scrapings and, if available, petiole tissue were removed from free-living grapevine samples using a razor blade and total plant DNA was extracted using the H.P. Plant DNA Kit (OMEGA Biotek, Norcross, GA) and stored at -20°C. Samples were tested for GRBV using diagnostic triplex polymerase chain reaction (PCR) with primers targeting a coat protein (CP) gene fragment, a replicase-associated

protein (Rep) gene fragment, and a *Vitis* 16S rDNA internal control (Krenz *et al.* 2014). The Repfor and Reprev primers initially designed for the amplification for the *Rep* fragment from GRBV also amplify the *Rep* fragment from WVV1 (Perry *et al.* 2018). However, the CPfor and CPrev primers initially designed for the amplification of a *CP* fragment from GRBV do not produce any fragment from WVV1 in PCR (Perry *et al.* 2018). Therefore, samples that yielded only the *Rep* fragment in the GRBV multiplex PCR were then assayed specifically for WVV1 using primers WVV1_CPfor (5'-GATGAATCGAATTCTGAAAC-3') and WVV1_CPrev (5'-ATACATACTACTCACAGTCAATAC-3'). PCRs were carried out with HotStar Plus 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 1X Tris-acetate-EDTA buffer and stained with GelRED (Biotium, Fremont, CA).

Using PCR data, incidence of GRBV and WVV1 infection was determined for free-living grapevine populations in each county in California. The respective incidences of GRBV and WVV1 in free-living vines in each county were analyzed with regard to county grapevine production acreage and proximity to commercial grape production in an exploratory manner using Chi-Square tests in SAS (version 9.4; SAS Institute).

QGIS map construction

California and New York county maps with GPS locations of free-living grapevines collected for this study were constructed using QGIS desktop version

2.18.11. New York State civil boundary shapefiles were downloaded from New York GIS services website (New York State GIS, 2017). Percentage of acreage planted to grapes was calculated for each county in New York using data from the United States Department of Agriculture- National Agriculture Statistics Service/ New York Department of Agriculture and Markets 2012 census for acreage of grapes. For the California map, county shapefiles were downloaded from the US Census Bureau's 2016 Master Address File/Topologically Integrated Geographic Encoding and Referencing database (California Open Data Portal, 2017). Percentage of acreage planted to grapes was calculated for each county in California using data from the USDA-NASS/C DFA 2016 grape acreage report (USDA-NASS and C DFA, 2016).

For both California and New York, county shapefiles were styled with a graduated shading system using the percentage of acreage planted to grapes in each county. GPS locations of free-living vines were included as a delimited text layer using coordinate reference system EPSG:4326 WGS 84 and styled by color according to infection status. To avoid overlap, point displacement was used with the following parameters: Concentric ring placement method, point distance tolerance of 0.0009 (map units), no displacement ring outline and ring size adjustment of 0.05 mm.

Characterization of the full-length genome of WVV1 isolates

Total nucleic acids were extracted from cambium scrapings or leaf petioles of samples that tested positive for WVV1 in PCR with a cetyltrimethylammonium bromide buffer and used in rolling circle amplification (RCA) to amplify circular DNA, as previously described (Krenz *et al.* 2014). RCA products were digested by

restriction enzyme *KpnI* to produce a full-length 3.2 kb fragment that was cloned into plasmid pUC19 (New England Biolabs, Ipswich, MA). For a single co-infected vine, restriction enzymes *KpnI* and *AvaI* were used for the digestion of GRBV and WVV1 RCA products, respectively. The resulting clones were transformed into *Escherichia coli* and sequenced at the Genomics Facility of the Cornell University Biotechnology Resource Center.

Genetic variability among GRBV and WVV1 isolates

The genetic variability of a subset of GRBV isolates from infected free-living *Vitis* spp. was determined by sequencing PCR amplicons (744 bp) of the diversity fragment, i.e. a region spanning the origin of replication, with primers p1282-F and p1283-R (Perry *et al.* 2016). Primers WVV1divF (5'-GAGGGTATGTTAGGAAAA-3') and WVV1divR (5'-GCAGCAGGCAAAGATAAATCC-3') were designed to PCR-amplify a 739 bp diversity fragment from WVV1 isolates. GRBV and WVV1 DNA amplicons were resolved by electrophoresis, purified using ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems, Foster City, CA) and sequenced at the Genomics Facility of the Cornell University Biotechnology Resource Center. Sequences were assembled using the DNASTAR Lasergene software suite version 14.1.

GRBV and WVV1 sequences from this study or previously determined (Perry *et al.* 2016, Perry *et al.* 2018) are available in GenBank and listed in **Table 4-1**. Sequences were aligned using ClustalW (Larkin *et al.* 2007). Recombination detection analyses were performed on the WVV1 and GRBV diversity fragment alignments, as

well as the WVV1 full-length genome alignment, using the RDP4 software (Martin *et al.* 2015). Recombination events were considered to be significant if at least three of seven recombination detection methods found evidence for recombination with *P* values of <0.05 (Krenz *et al.* 2014). The reticulate nature of the phylogenies resulting from recombination was confirmed by computing delta scores as well as applying the Phi test for overall recombination detection in SplitsTree4 (Huson and Bryant, 2006). Delta score and Q-residual value equal 0 if distances between taxa fit a tree exactly, inferring no recombination; whereas delta score and Q-residual values equal 1 if relationships are absolutely reticulate in nature, inferring a high degree of recombination. Scores range from 0 (tree-like) to 1 (non-treelike) (Huson and Bryant, 2006).

Maximum likelihood phylogenies (RaxML) with recombinant regions removed were constructed in RDP4. To provide further indication of genetic variability among GRBV and WVV1, a pairwise identity matrix of WVV1 and GRBV diversity fragment sequences was constructed using the Sequence Demarcation Tool (SDT) version 1.2 (Muhire *et al.* 2014). Sequences were aligned using MUSCLE (Edgar 2004).

RESULTS

Grablovirus detection in free-living grapevines

In California, GRBV was detected in 21.2% (43/203) of the free-living *Vitis* spp. collected between 2014 and 2017 (**Table 4-2, Figure 4-1**). None of the infected samples exhibited disease symptoms. Genetic fingerprinting of a subset of GRBV-

infected samples from California identified them as hybrids of *V. californica* x *V. vinifera* or hybrids of *V. californica* x rootstock genotypes (**Table 4-1**). In New York, no grabloviruses were detected in any of the 163 free-living grapevine samples tested, regardless of their proximity to production vineyards (**Table 4-3, Figure 4-2**). Based on morphological characteristics, New York samples were *V. riparia*, *V. aestivalis*, *V. labrusca* or hybrids derived thereof.

Table 4-1 California GRBV and WVV1 isolates used in this study.

Virus	Isolate	County	Sequence type	GenBank accession number	Citation
GRBV	NY1290 ^a	Napa	Diversity fragment	MG976051	This study
GRBV	NY1292	Napa	Diversity fragment	MG976052	This study
GRBV	NY1314	Napa	Diversity fragment	MG976053	This study
GRBV	NY1319 ^b	Napa	Diversity fragment	MG976054	This study
GRBV	NY1361 ^c	Napa	Diversity fragment	MG976055	This study
GRBV	NY1425	Napa	Diversity fragment	MG976056	This study
GRBV	NY1465	Napa	Diversity fragment	MG976057	This study
GRBV	NY1467	Napa	Diversity fragment	MG976058	This study
GRBV	NY1614	Napa	Diversity fragment	MG976059	This study
GRBV	NY1615	Napa	Diversity fragment	MG976060	This study
GRBV	EFD1	Sacramento	Diversity fragment	MG976061	This study
GRBV	EFD2	Sacramento	Diversity fragment	MG976062	This study
GRBV	EFD5	Sacramento	Diversity fragment	MG976063	This study
GRBV	EFD6	Sacramento	Diversity fragment	MG976064	This study
GRBV	Elkhorn5	Sacramento	Diversity fragment	MG976065	This study
GRBV	ERP1	Sacramento	Diversity fragment	MG976066	This study
GRBV	F2	Sacramento	Diversity fragment	MG976067	This study
GRBV	IKEA2	Sacramento	Diversity fragment	MG976068	This study
GRBV	NY1606	Sacramento	Diversity fragment	MG976069	This study
GRBV	NY1607	Sacramento	Diversity fragment	MG976070	This study
GRBV	Solano_16	Solano	Diversity fragment	MG976071	This study
GRBV	Solano_17	Solano	Diversity fragment	MG976072	This study
GRBV	Solano_20	Solano	Diversity fragment	MG976073	This study
GRBV	Sonoma_25	Sonoma	Diversity fragment	MG976074	This study
GRBV	Sonoma_29	Sonoma	Diversity fragment	MG976075	This study
GRBV	Sonoma_34	Sonoma	Diversity fragment	MG976076	This study
GRBV	Sonoma_37	Sonoma	Diversity fragment	MG976077	This study
GRBV	Canal2	Sutter	Diversity fragment	MG976078	This study
GRBV	NY699	Napa	Full length genome	KU564247	Perry <i>et al.</i> 2016
GRBV	NY700	Napa	Full length genome	KU564248	Perry <i>et al.</i> 2016
GRBV	NY913	Napa	Full length genome	KU564254	Perry <i>et al.</i> 2016
GRBV	NY175	Napa	Full length genome	KF147916	Krenz <i>et al.</i> 2014
GRBV	NY358	Napa	Full length genome	JQ901105	Krenz <i>et al.</i> 2014
WVV1	Glenn_5	Glenn	Diversity fragment	MG976079	This study
WVV1	Sonoma_27	Sonoma	Diversity fragment	MG976080	This study
WVV1	Sonoma_33	Sonoma	Diversity fragment	MG976081	This study
WVV1	Solano_7	Solano	Full length genome	MG976082	This study
WVV1	Solano_8	Solano	Full length genome	MG976083	This study
WVV1	Solano_19	Solano	Full length genome	MG976084	This study
WVV1	NY1325 ^d	Napa	Full length genome	MF185004	Perry <i>et al.</i> 2018
WVV1	NY1358	Napa	Full length genome	MF185005	Perry <i>et al.</i> 2018
WVV1	NY1424 ^e	Napa	Full length genome	MF185006	Perry <i>et al.</i> 2018
WVV1	NY1466	Napa	Full length genome	MF185007	Perry <i>et al.</i> 2018
WVV1	NY1467	Napa	Full length genome	MF185008	Perry <i>et al.</i> 2018
WVV1	NY1468	Napa	Full length genome	NC035480	Perry <i>et al.</i> 2018

WVV1	NY1468	Napa	Full length genome	MF185009	Perry <i>et al.</i> 2018
WVV1	NY1616	Napa	Full length genome	MF185010	Perry <i>et al.</i> 2018

^a The plant host is an F1 hybrid of *Vitis californica* x rootstock Ruggeri 140 (*V. berlandieri* x *V. rupestris*)

^b The plant host is an F1 hybrid of *V. californica* x *V. vinifera* cv. Syrah or Durif

^c The plant host is an F1 hybrid of *V. californica* x rootstock *V. rupestris* St. George

^d The plant host is a *V. californica*

^e The plant host is an F1 hybrid of *V. californica* x *V. vinifera* cv. Cabernet Sauvignon

Table 4-2 Grablovirus detection in free-living *Vitis* spp. in California.

County	Distance ^a	GRBV ^b	WVV1 ^c	Co-infection ^d
Napa	<5km	24/83	9/83	1/83
	>5km	na	na	na
Sonoma	<5km	5/23	2/23	0/23
	>5km	na	na	na
Solano	<5km	2/8	1/8	0/8
	>5km	1/12	2/12	0/12
Sutter	<5km	1/5	0/5	0/5
	>5km	0/14	0/14	0/14
Sacramento	<5km	8/23	0/23	0/23
	>5km	2/8	0/8	0/8
Butte	<5km	na	na	na
	>5km	0/15	0/15	0/15
Glenn	<5km	na	na	na
	>5km	0/12	1/12	0/12

^a Relative distance of free-living *Vitis* spp. samples from vineyards: 0-5km (<5km) and more than 5km (>5km)

^b Number of free-living vines testing positive for grapevine red blotch virus (GRBV) over the total number of samples tested.

^c Number of free-living vines testing positive for wild Vitis virus 1 (WVV1) over the total number of samples tested.

^d Co-infection by GRBV and WVV1

na: not applicable

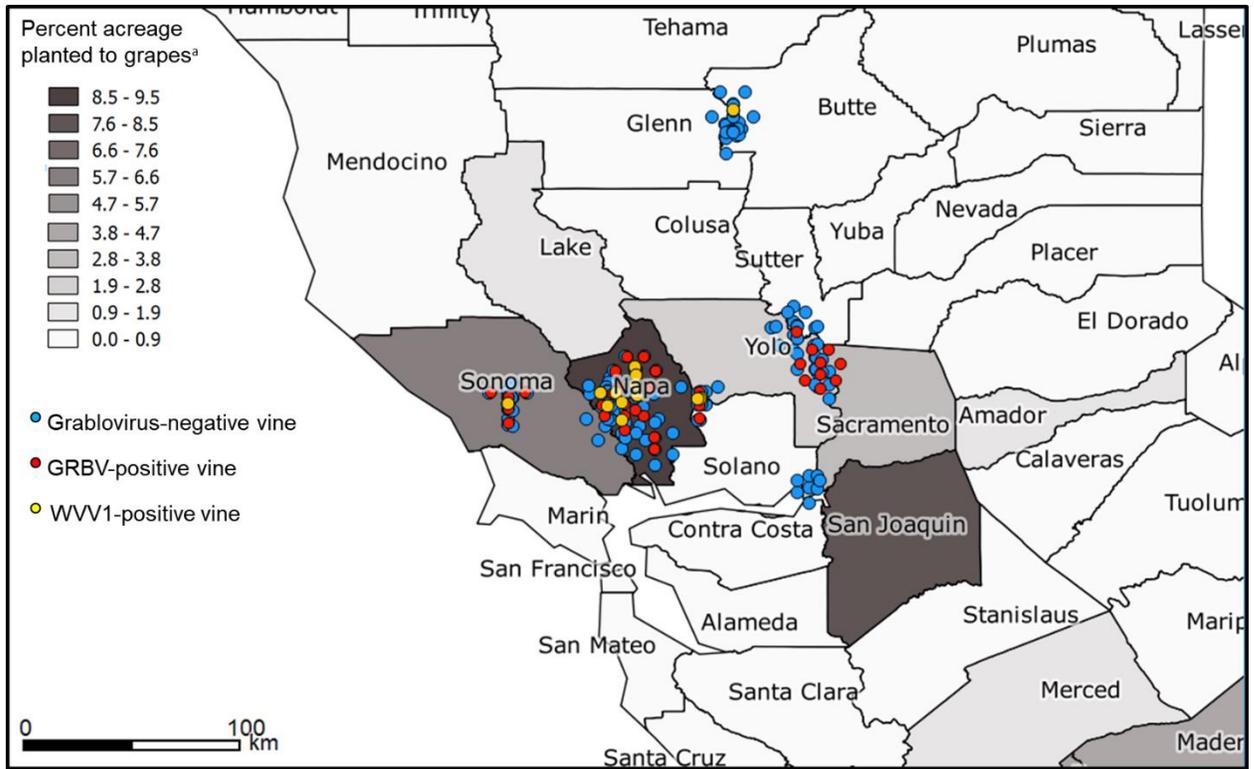


Figure 4-1 Map of northern California counties showing the density of acreage planted to grapevine, along with the locations and infection status of free-living vines collected for this study. Map was constructed in QGIS desktop version 2.18.11. Point displacement was used to avoid overlapping points for the locations of free-living vines.

Table 4-3 Grablovirus detection in free-living *Vitis* spp. in New York

<i>County</i>	<i>Distance^a</i>	<i>GRBV^b</i>	<i>WVVI^c</i>	<i>Co-infection^d</i>
<i>Clinton</i>	<5km	0/20	0/20	0/20
	>5km	na	na	na
<i>Ontario</i>	<5km	na	na	na
	>5km	0/20	0/20	0/20
<i>Chautauqua</i>	<5km	0/20	0/20	0/20
	>5km	na	na	na
<i>Yates</i>	<5km	0/18	0/18	0/18
	>5km	na	na	na
<i>Seneca</i>	<5km	0/13	0/13	0/13
	>5km	na	na	na
<i>Steuben</i>	<5km	0/14	0/14	0/14
	>5km	na	na	na
<i>Tompkins</i>	<5km	0/6	0/6	0/6
	>5km	na	na	na
<i>Ulster</i>	<5km	na	na	na
	>5km	0/31	0/31	0/31
<i>Suffolk</i>	<5km	0/21	0/21	0/21
	>5km	na	na	na

^a Distance of free-living *Vitis* spp. samples from vineyards: 0-5km (<5km) and more than 5km (>5km)

^b Number of free-living vines testing positive for grapevine red blotch virus (GRBV) over the total number of samples tested.

^c Number of free-living vines testing positive for wild *Vitis* virus 1 (WVVI) over the total number of samples tested.

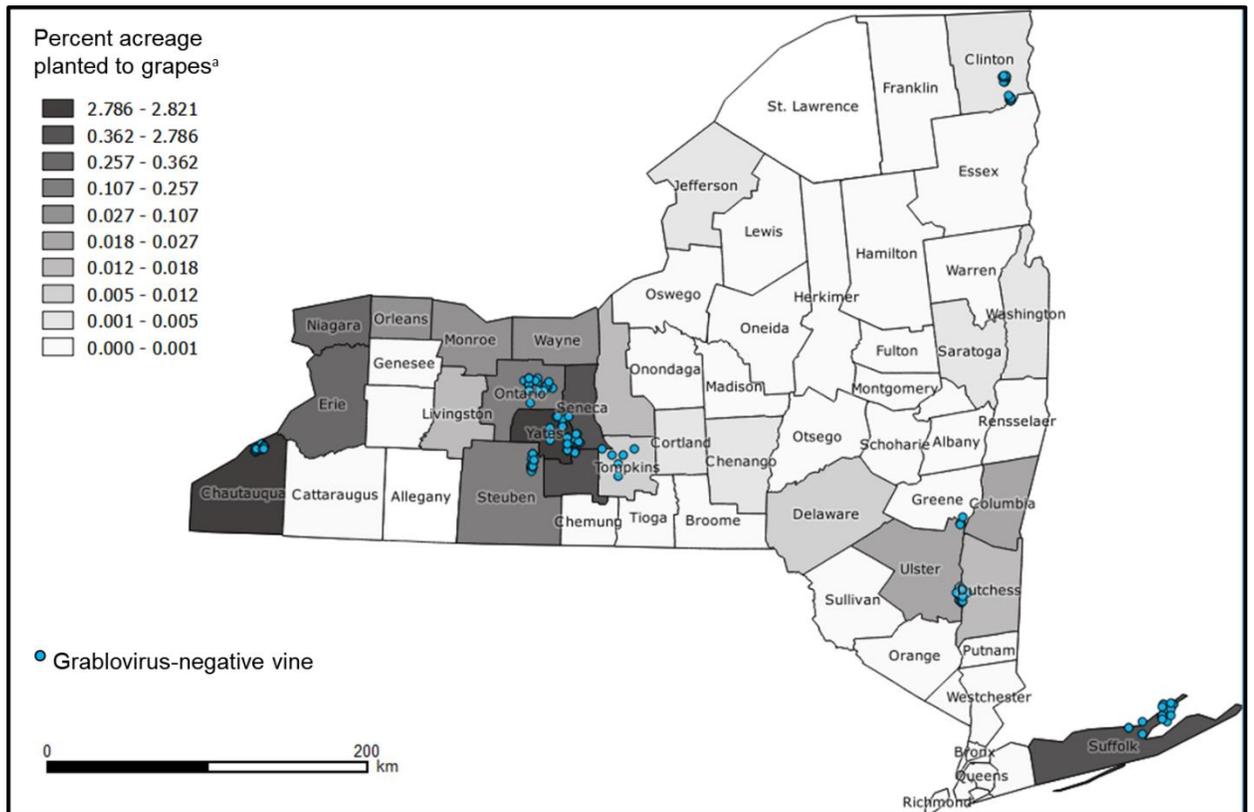


Figure 4-2 Map of New York counties showing the density of acreage planted to grapevine, along with the locations and infection status of free-living vines collected for this study. Map was constructed in QGIS desktop version 2.18.11. Point displacement was used to avoid overlapping points for the locations of free-living vines.

GRBV prevalence was higher in free-living grapevines in the California counties rated as having high commercial grapevine production (3.8-9.5% of total county acreage planted to grapes) than in counties rated as having low grapevine production (0.006-0.73% of total acreage planted to grapes) ($\chi^2 = 83.09$; $P < 0.001$) (**Figure 4-1**). When considering individual counties, GRBV incidence was significantly higher in Napa (29%, 24/83), Sonoma (22%, 5/23) and Sacramento (32%, 10/31) compared to Solano (15%, 3/20) and Sutter (5%, 1/19) counties ($\chi^2 = 22.79$; $P < 0.001$). Infected free-living *Vitis* spp. samples in Napa, Sonoma and Sacramento counties were identified both close to (within 30 m to 5 km) or far from (more than 5 km) vineyards, although the GRBV incidence was higher in samples near (28%, 40 of 142) compared to far from vineyards (5%, 3 of 61) ($\chi^2 = 57.58$; $P < 0.001$) (**Table 4-2**). GRBV was not detected in any of the free-living vines collected in Glenn and Butte counties, two counties with extremely low commercial grape production (less than 0.1% of the total acreage) and not bordering counties with major viticulture acreage (**Figure 4-1**). GRBV-infected samples in Solano County were less than 10 km away from vineyards in Napa County, a neighboring county with the highest acreage planted to grapes (**Figure 4-1**). The single GRBV-infected free-living *Vitis* spp. sample from Sutter County is an exception because it was far away from any production vineyard but close to nursery vineyards.

WVV1 was detected in 7% (15/203) of the California free-living grapevines collected between 2014 and 2017. Similar to GRBV incidence, WVV1 incidence was higher in counties with the highest commercial grapevine production ($\chi^2 = 16.02$; $P < 0.001$). None of the infected samples exhibited virus-like symptoms. The virus was

found in Napa (11%, 9/83), Sonoma (9%, 2/23) and Solano (15%, 3/20) but not in Sacramento, Sutter and Butte counties, and only one sample from Glenn County tested positive for WVV1 (**Table 4-2**). There were no differences in WVV1 incidence based on proximity to commercial vineyards ($\chi^2 = 0.88$; $P = 0.3513$). A single free-living *Vitis* spp. sample from Napa County was infected with both WVV1 and GRBV (**Table 4-2**). Based on morphological characteristics, the plant hosts were either *V. californica* or hybrids of *V. californica*. Subsequently, genetic fingerprinting of two WVV1-infected samples revealed that one sample was pure *V. californica* and the other was a hybrid of *V. californica* and *V. vinifera* cv. Cabernet Sauvignon (**Table 4-1**).

Two distinct lineages and evidence for recombination for WVV1 isolates

Phylogenetic analyses of the full-length genome sequence of 13 WVV1 isolates from California, including three new complete sequences determined in this study (**Table 4-1**), revealed two distinct lineages (**Figure 4-3**). The between-clade nucleotide sequence identity varies from 93.3 to 94.7%. The within-clade 1 nucleotide sequence identity ranges from 96.7 to 99.8%; and the within-clade 2 nucleotide sequence identity ranges from 94.7 to 99.6%. WVV1 isolates from clade 2 were from free-living grapevines sampled in Napa County, while clade 1 isolates were all derived from free-living grapevines in Solano County.

RDP4 analyses applied to the alignment of full-length WVV1 genome sequences revealed isolate Napa_NY1424 as a probable recombinant. Three of seven methods (MaxChi, CHIMAERA, and 3SEQ) found evidence of recombination event 1, and five (RDP, BOOTSCAN, MaxCHI, CHIMAERA, and 3SEQ) of seven methods

found evidence of recombination event 2 for isolate Napa_NY1424. The average P values for each recombination event were less than 0.05 (**Figure 4-4**). Recombination event 1 has a possible major parent in isolate Napa_NY1325 (96.1% similarity) and a minor parent isolate in Solano_7 (92.2% similarity) with probable breakpoints at nucleotide positions 74 and 474. Recombination event 2 has a possible major parent in isolate Napa_NY1466 (96.7% similarity) and a minor parent in isolate Solano_7 (95.4% similarity) with probable breakpoints at nucleotide positions 1,475 and 2,306 with Napa_NY1424 as the reference isolate (**Figure 4-4**). The Phi test in SplitsTree4 confirmed a high likelihood of recombination among WVV1 full-length genome sequences with a ($P < 0.01$). The delta score was 0.1881 and the Q-residual score was 0.002715, indicating reticulation among the sequences and supporting the presence of recombination.

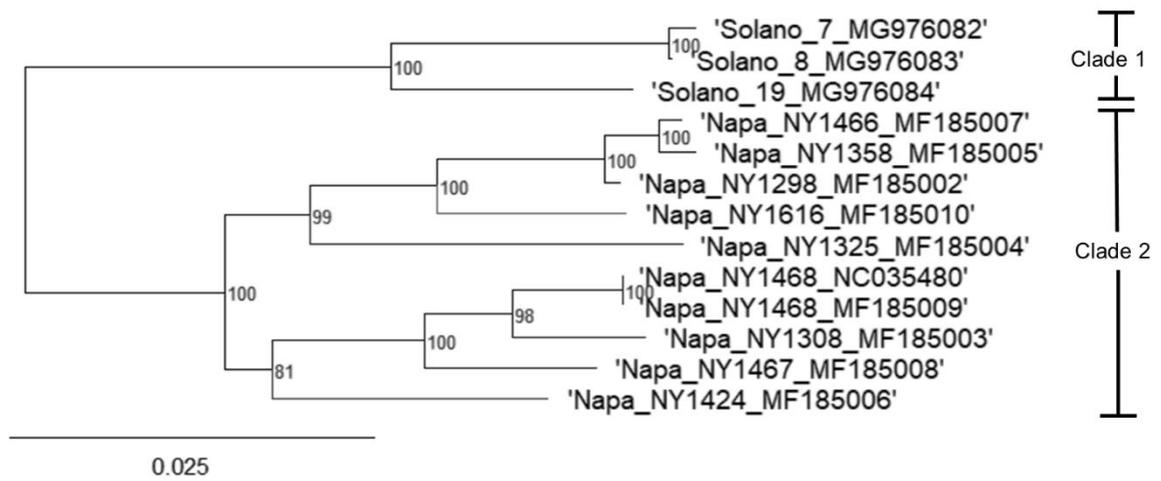


Figure 4-3 Maximum likelihood (RaxML) phylogeny of wild *Vitis* virus 1 (WVV1) full genome sequences of isolates from free-living *Vitis* spp. in California. Numbers at branches indicate bootstrap support (1000 bootstrap replicates). Detailed information on WVV1 isolates is provided in Table 1.

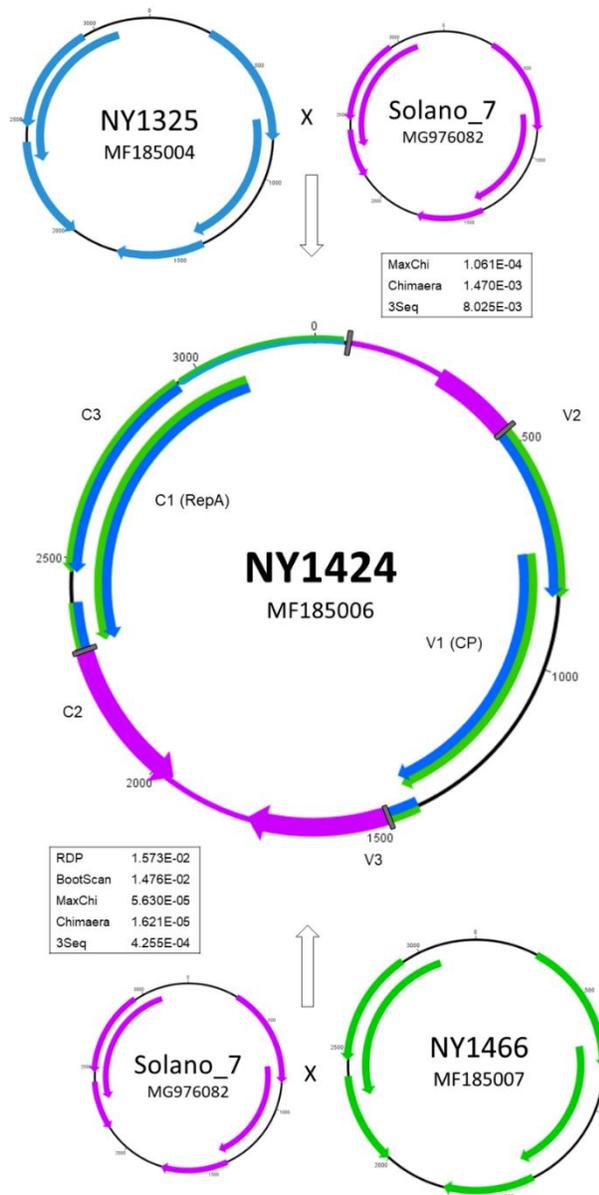


Figure 4-4 Recombination events in wild Vitis virus 1 (WVV1) isolate NY1424 (GenBank accession number MF185006). Recombination event number 1 is between major parental isolate NY1325 (larger, blue, MF185004) and minor parental isolate Solano_7 (smaller, purple, MG976082). Recombination event number 2 is between major parental isolate NY1466 (larger, green, MF185007) and minor parental isolate Solano_7 (smaller, purple, MG976082). The region flanking the origin of replication of WVV1 isolate NY1424 belongs to the major parent for each of the two recombination events. Recombination detection methods and associated *P* values are indicated in boxes next to the respective recombination events.

Phylogenetic analyses using the diversity fragment alignment containing three additional WVV1 isolates from Glenn and Sonoma counties resulted in a tree topology that reflected two distinct lineages of WVV1 isolates (**Figure 4-5**). This result was consistent with the phylogenetic tree obtained with full genome sequences. Two isolates from Sonoma County (Sonoma_25 and Sonoma_33) are in clade 2 with isolates from Napa County. The single isolate from Glenn County (Glenn_5) is in clade 1 with isolates from Solano County (**Figure 4-5**). The WVV1 genomic diversity fragments have 94.3 to 99.8% sequence identity among isolates within clade 1 and 90.1 to 100% sequence identity among isolates within clade 2.

RDP4 analyses support the chimeric nature of isolate Napa_NY1424 with isolate Napa_NY1467 (95.7% similarity) as a potential major parent and an unknown minor parent detected in three (MaxCHI, $P = 2.042E-04$; CHIMAERA, $P = 2.033E-03$; and 3SEQ, $P = 2.558E-03$) of seven recombination detection methods with breakpoints at nucleotide positions 194 and 542 in the alignment. RDP4 analyses further provided evidence for recombination in the diversity fragment sequence of isolate Napa_NY1325 with isolate Napa_NY1468 (94.5% similarity) as a potential major parent and isolate Sonoma_33 (96.5% similarity) as a potential minor parent in three of seven analyses (MaxCHI, $P = 1.008E-02$; CHIMAERA, $P = 1.077E-02$; and 3SEQ, $P = 3.442E-02$) with breakpoints at nucleotide positions 594 and 112 in the alignment. The Phi test in SplitsTree4 confirmed a high likelihood of recombination among WVV1 genomic diversity fragment sequences ($P < 0.01$). The delta score was 0.1426 and the Q-residual score was 0.02136, indicating reticulation among the sequences and supporting the presence of recombination.

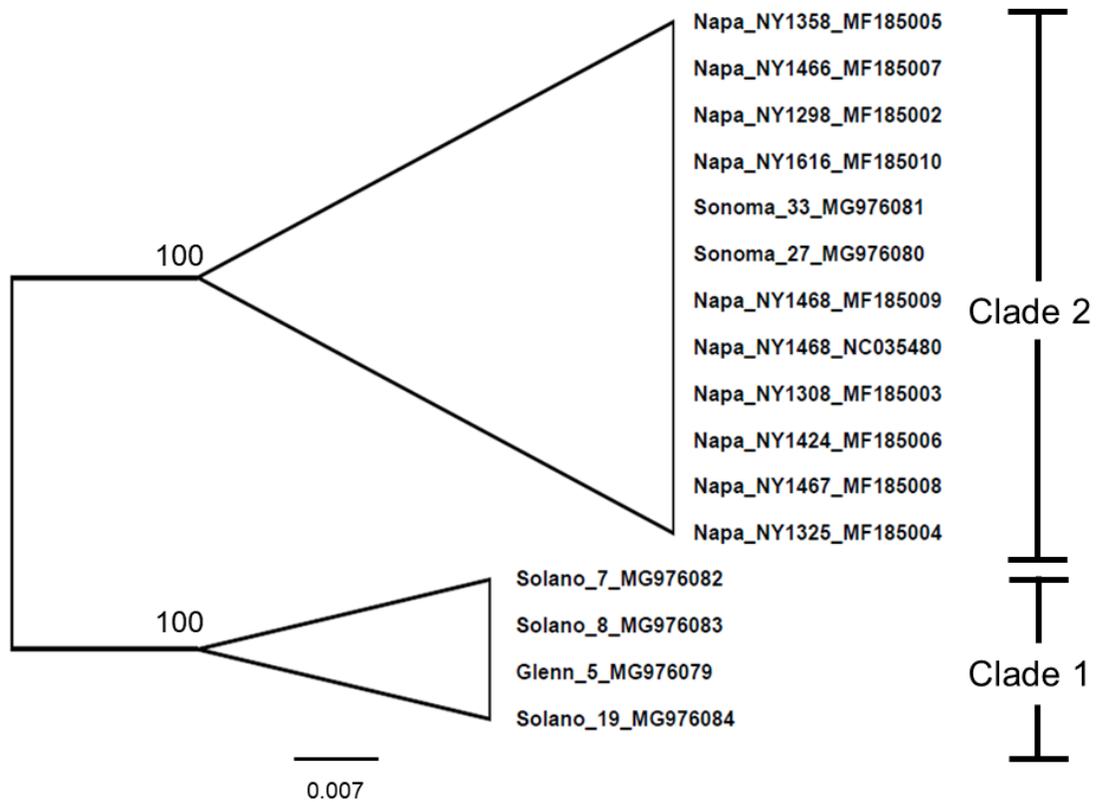


Figure 4-5 Maximum likelihood phylogeny of wild *Vitis* virus 1 (WVV1) diversity fragment nucleotide sequences of isolates from free-living *Vitis* spp. in California. Numbers at branches indicate bootstrap support (1000 bootstrap replicates, random seed=3). Branches with less than 70% bootstrap support were collapsed. Detailed information on WVV1 isolates is provided in **Table 4-1**.

Two distinct phylogenetic lineages of GRBV isolates, evidence for recombination

Phylogenetic analyses of the genomic diversity fragment nucleotide sequence of GRBV isolates from free-living *Vitis* spp. revealed two distinct clades (**Figure 4-6**). The majority of GRBV isolates, including all those from Napa, Sonoma and Solano counties and most from Sacramento County, belong to clade 2 in which the nucleotide sequence identity ranges from 96.9 to 100%. One isolate from Sacramento County and the only isolate from Sutter County grouped in clade 1, with 98.3% nucleotide sequence identity. The nucleotide sequence identity between clades 1 and 2 ranges from 88.3 to 90.9%. Evidence for recombination was detected in isolates Sonoma_34 and Sacramento_F2, both in clade 2. A single recombination event was detected in three (MaxCHI, $P = 1.645E-02$; SiScan, $P = 3.665E-02$; and 3SEQ, $P = 2.081E-02$) of seven RDP4 methods for the diversity fragment sequence of isolate Sonoma_34. The potential major parent is unknown (Napa_NY1290 was used for analyses) and the minor parent is isolate Napa_NY1361 (98.8% similarity) with breakpoints at nucleotide positions 210 and 472. Similarly, isolate Sacramento_F2 is a recombinant identified by four (MaxCHI, $P = 1.409E-04$; Chimaera, $P = 1.693E-03$; SiScan, $P = 5.530E-03$; and 3Seq $P = 1.835E-04$) of the seven RDP4 methods with isolate Napa_NY1361 (99.7% similarity) as a potential major parent and an unknown minor parent isolate (Sonoma_34 used for analyses), and breakpoints at nucleotide positions 216 and 449 in the alignment. The Phi test did find evidence of recombination among the GRBV genomic diversity fragment sequences ($P = 5.122E-9$). The delta score was 0.1664 and the Q-residual score was $4.359E-4$, indicating reticulation among the sequences and supporting the presence of recombination.

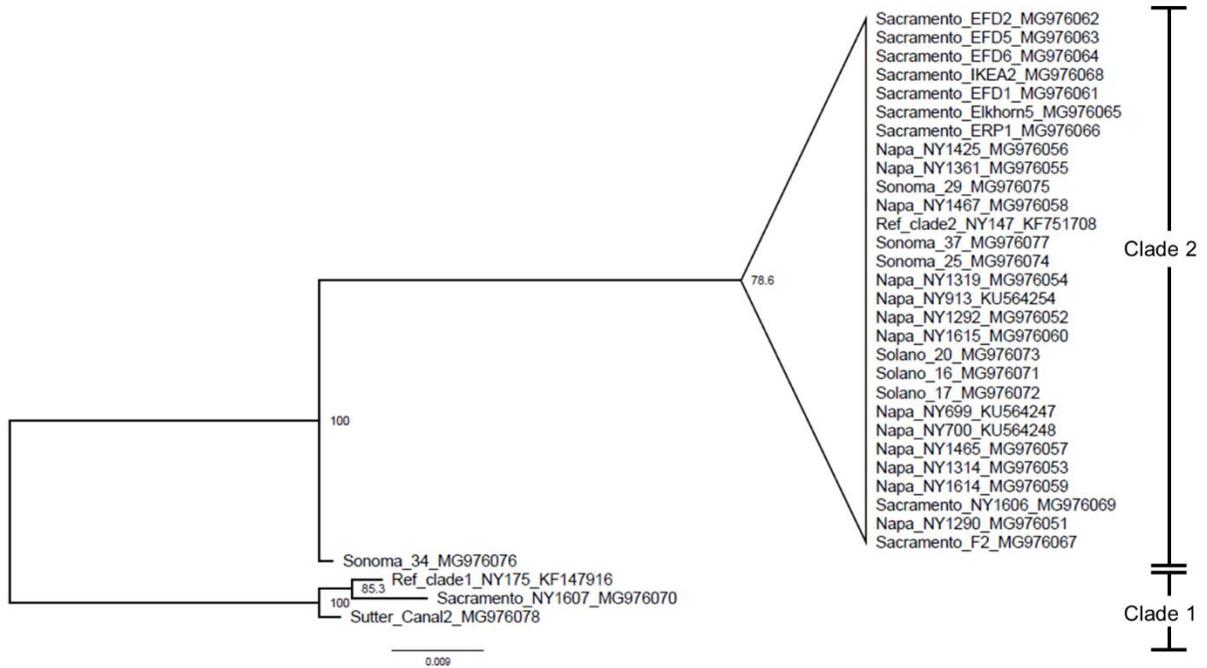


Figure 4-6 Maximum likelihood phylogeny of grapevine red blotch virus (GRBV) diversity fragment nucleotide sequences of isolates from free-living *Vitis* spp. in California. Recombinant regions corresponding to the contribution of the minor parent were removed from isolates Sonoma_34 and Sacramento_F2 for phylogenetic analyses. Numbers at branches indicate bootstrap support (1000 bootstrap replicates). Branches with less than 70% bootstrap support were collapsed. Detailed information on GRBV isolates is provided in **Table 4-1**.

Grablovirus diversity in free-living vines in California

A pairwise identity matrix including the genomic diversity fragments of WVV1 and GRBV together provided additional evidence for the presence of two groups of isolates for both WVV1 and GRBV. The pairwise identity matrix confirmed a higher nucleotide sequence variability for WVV1 compared to GRBV isolates (**Figure 4-7**).

When considering a representative isolate from each clade of WVV1 and GRBV, the V1 open reading frame (ORF), putatively encoding the coat protein, is the most highly conserved ORF of grabloviruses at the amino acid level (78.6-79%), as previously reported (Perry *et al.* 2018), and the second most highly conserved ORF at the nucleotide level (70.5-70.8%), with C3 being the most conserved ORF at the nucleotide level (**Figure 4-8**).

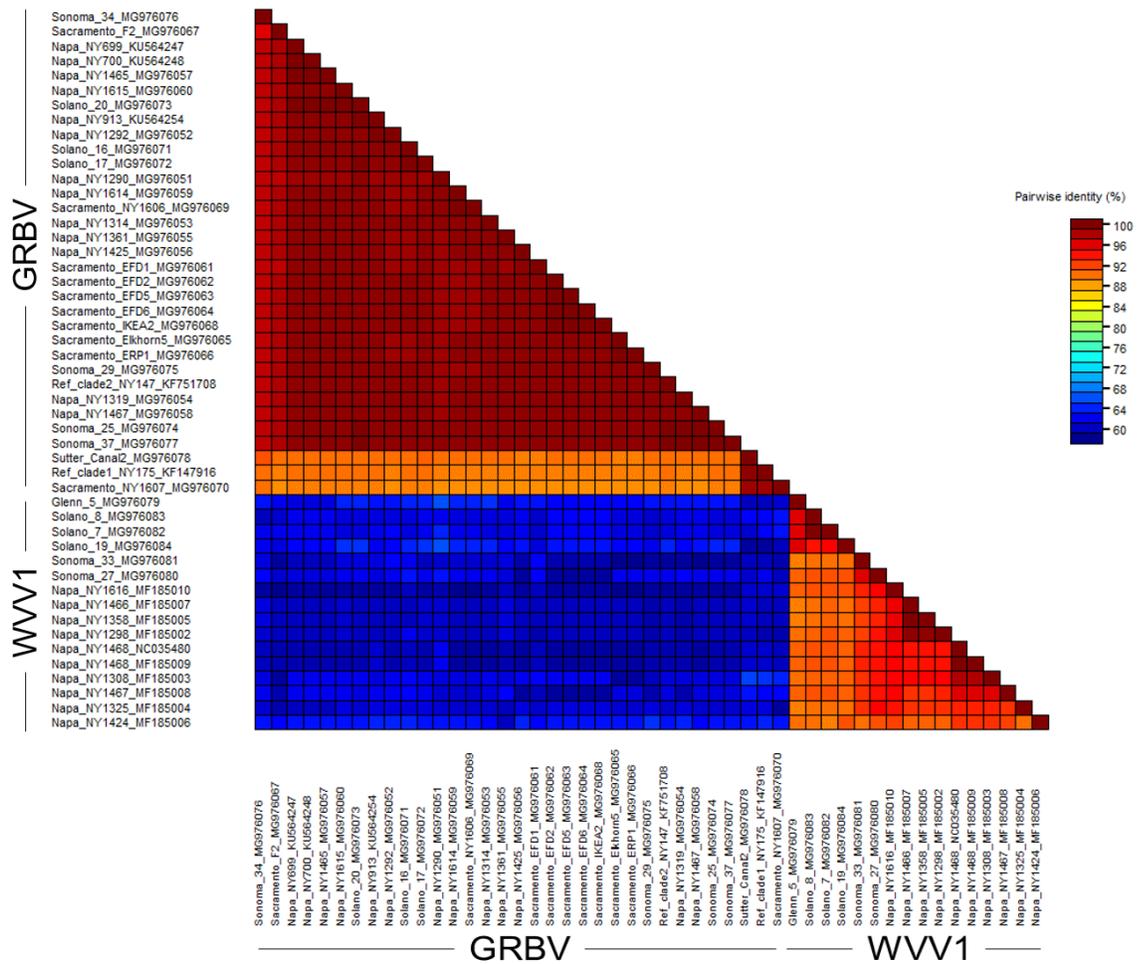


Figure 4-7 Pairwise identity matrix of grapevine red blotch virus (GRBV) and wild *Vitis* virus 1 (WVV1) diversity fragment sequences of isolates from free-living *Vitis* spp. in California. Sequences were aligned using MUSCLE.

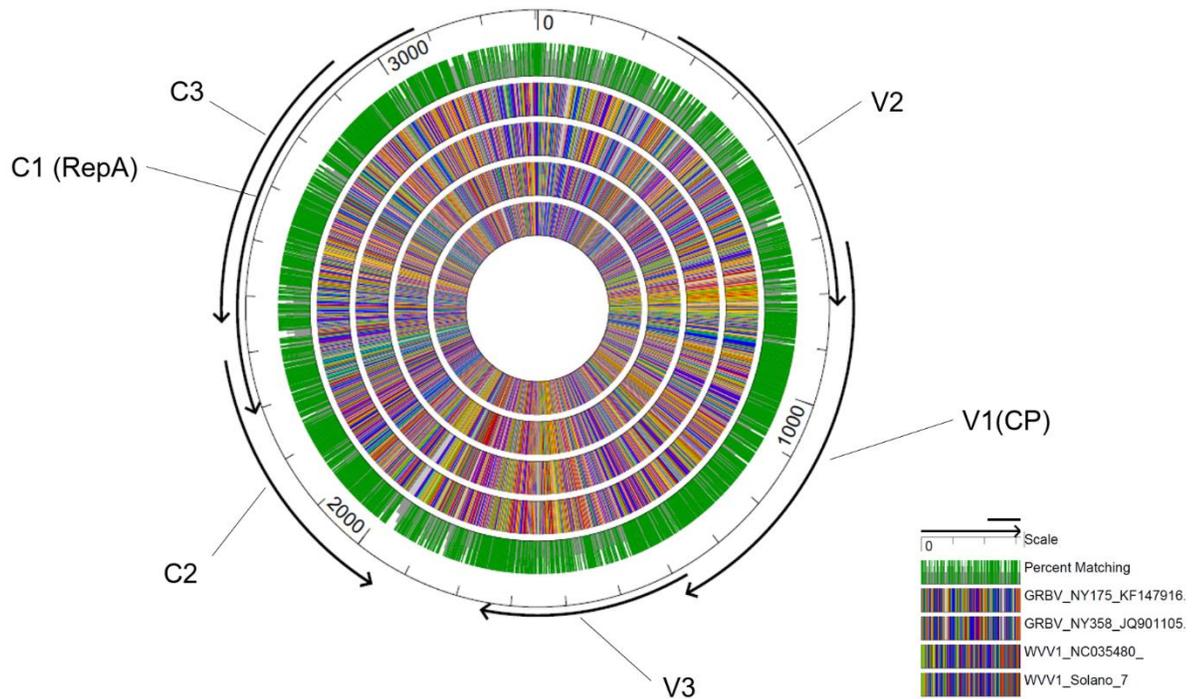


Figure 4-8 Circular alignment (ClustalW) of GRBV and WVV1 nucleotide sequences of isolates representative of each of the two clades for each virus. Percent identity is depicted on the outermost layer of the alignment, illustrating nucleotide sequence conservation (70.5-70.8%) within the V1 open reading frame (ORF). Numbers correspond to the position in the alignment and minor tick marks demarcate 100 bases. Colors in the inner four rings correspond to nucleotides, where adenine is red, cytosine is green, thymine is blue, guanine is yellow, and gray indicates a gap. ORF guidelines are representative of GRBV isolate NY358 (Krenz *et al.* 2014) to illustrate approximate ORF locations for grabloviruses.

DISCUSSION

This study expands on previous research reporting the detection of GRBV in free-living grapevines near a ‘Cabernet franc’ vineyard in Napa County, California (Perry *et al.* 2016) and in other areas of Napa County (Bahder *et al.* 2016b). Here, GRBV was detected in free-living *Vitis* spp. in most California counties surveyed, primarily in those where grape production is substantial (3.8-9.5% acreage planted to grapevine). Since spread of GRBV has been documented in California (Cieniewicz *et al.* 2017b), the discovery of GRBV in free-living vines stimulates questions on the direction of spread of GRBV between free-living *Vitis* spp. and vineyards, whether from commercial vineyards to uncultivated grapevines or vice versa. To approach this question, we sampled free-living grapevines throughout northern California counties both within major viticulture areas and also far from grape production areas in Glenn and Butte counties, where walnut, almond, and rice production predominate (CDFA, 2016).

Our findings suggest a directional movement of GRBV predominantly from commercial vineyards to free-living vines. This hypothesis is supported by the higher incidence of GRBV in free-living vines in counties with more grapevine production, as well as the lack of GRBV in counties far from vineyard production in California. It is also supported by a significantly higher GRBV incidence in uncultivated vines near (< 5km) vineyards (28%, 40 of 142) compared to far (> 5km) from vineyards (5%, 3 of 61). Additionally, characterizing the genetic variability of the genomic diversity fragments demonstrated that the majority of GRBV isolates were in phylogenetic clade 2 and only 2 isolates were in clade 1. A similar pattern of a dominant clade 2 was

described for GRBV isolates both from commercial vineyards (Krenz *et al.* 2014) and insect vector candidates in an infected vineyard (Cieniewicz *et al.* 2018).

It is plausible that GRBV clade 2 predominates in vineyards due to widespread dissemination of infected propagation material, with less widespread dissemination of GRBV clade 1 genetic variants. Finding a similar pattern of genetic variant dispersal among isolates from free-living vines supports the hypothesis that GRBV inoculum is moving predominantly from vineyards to free-living vines. In contrast, WVV1 has only thus far been detected in free-living grapevines, where the distribution of isolates would not be directly influenced by human activity (i.e. dissemination of infected propagation material). This might explain the higher degree of genetic diversity among WVV1 isolates, in comparison to GRBV. The hypothesized directional movement of GRBV predominantly from vineyards to free-living grapevines is further strengthened by the perceived absence of GRBV in free-living vines in New York, where GRBV incidence in vineyards is low, and there is no evidence of secondary spread (Cieniewicz *et al.* unpublished).

While the findings of this study allow us to postulate on the predominant direction of GRBV inoculum movement to be from commercial vineyards to free-living vines in California, the potential of free-living vines to serve as an inoculum source should not be discounted. This is particularly important to consider if efforts are made to remove an infected vineyard in California and re-plant with vines derived from clean, virus-tested stocks, as is currently recommended (Cieniewicz *et al.* 2017a). Free-living vines infected with GRBV could also be a source of inoculum if grown proximal to nursery operations, where even infrequent introductions of the

virus could potentially go un-noticed and result in large-scale dissemination in propagated vines.

WVV1 has thus far only been detected in free-living grapevines in Napa, Sonoma, Solano and Glenn counties in California. While *S. festinus* has been identified as a GRBV vector (Bahder *et al.* 2016a) of epidemiological significance (Cieniewicz *et al.* 2018), no insect vector has yet been identified for WVV1. Therefore, the method by which free-living *Vitis* spp. become infected with WVV1 remains unknown. The importance of this research is highlighted by the amino acid sequence identity of 78.6-79% between the presumptive GRBV and WVV1 coat proteins (CP) encoded by the V1 ORF (Perry *et al.* 2018). Since vector specificity and competency is dependent on the sequence of the CP in geminiviruses (Briddon *et al.* 1990), it is possible that WVV1 and GRBV are both vectored by *S. festinus*, or alternatively WVV1 is vectored by a related membracid. Transmission assays are needed to determine if WVV1 can be transmitted by an insect vector. Additionally, the possibility that grabloviruses are transmitted by seed cannot be discounted, knowing that some begomoviruses (*Geminiviridae*) are seed transmitted (Anabestani *et al.* 2017, Kil *et al.* 2016, 2017, Kim *et al.* 2015, Kothandaraman *et al.* 2016). Thus, it is possible that some free-living vines became infected by GRBV and/or WVV1 via seed transmission rather than vector transmission. More work is needed to determine if GRBV and WVV1 are seed-transmissible. Interestingly, a single case of mixed WVV1 and GRBV infection was identified in Napa County. Based on our current knowledge of grablovirus transmission, it is uncertain how this free-living vine became infected by GRBV and WVV1, but presumably by vector or seed transmission.

The implications of GRBV-infected free-living *Vitis* spp. in the epidemiology of red blotch disease are not well understood. Similarly, limited information is available on the phenology of the known insect vector, *S. festinus*, in vineyard ecosystems. In at least one instance in California, spread of GRBV occurs most rapidly in vineyard areas where initial inoculum is aggregated (Cieniewicz *et al.* 2017b). Management of red blotch disease is limited to preventive measures and the use of planting material derived from clean, virus-tested (negative) stocks. Effective sanitation practices in vineyards are better informed by a greater awareness of inoculum sources in and around vineyards. As previously described, frequent scouting for red blotch disease symptoms and removal of infected vineyards is crucial for prevention of GRBV spread (Cieniewicz *et al.* 2017a,b, 2018, Sudarshana *et al.* 2015). Results of this study suggest that removal of any free-living *Vitis* spp. proximal to vineyard sites should be considered for replants in Napa, Sonoma, Sacramento, and Solano counties. Similar recommendations apply to foundation stock vineyards in these counties.

It is unknown whether WVV1 causes disease in cultivated grapevines such as *V. vinifera*. Nonetheless considering the genetic relatedness of WVV1 with GRBV (Perry *et al.* 2018) and the impact of GRBV on vineyard profitability (Cieniewicz *et al.* 2017a, Ricketts *et al.* 2017, Sudarshana *et al.* 2015), learning more about the distribution, biology, and ecology of WVV1 is of interest to fill a gap in knowledge about grabloviruses. For example, determining the host range and transmission mode of both GRBV and WVV1 is critical for a comprehensive understanding of grablovirus epidemiology. Assuming WVV1 spread to cultivated vines can occur and

the virus has similar detrimental effects on production and fruit quality as its very close relative, GRBV, we may be provided with the first example of a grapevine virus (WVV1) with the potential for a proactive management approach, rather than a reactive response to a newly discovered and potentially emerging virus. In any event, eliminating free-living *Vitis* spp. proximal to production and foundation stock vineyards in Napa, Sonoma, Sacramento and Solano counties should become part of a comprehensive portfolio of grapevirus management strategies.

REFERENCES

Al Rwahnih, M., Ashita, D., Anderson, M., Rowhani, A., Uyemoto, J. K., and Sudarshana, M. R. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103:1069-1076.

Al Rwahnih, M., Alabi, O., Westrick, N., Golino, D. 2018. *Prunus* geminivirus A: A novel Grablovirus infecting *Prunus* spp. *Plant Dis.* doi: 10.1094/PDIS-09-17-1486-RE

Anabestani, A., Behjatnia, S., Izadpanah, K., Tabein, S., Accotto, G. 2017. Seed transmission of beet curly top virus and beet curly top Iran virus in a local cultivar of petunia in Iran. *Viruses* 9:299

Bahder, B. W., Zalom, F. G., Jayanth, M., and Sudarshana, M. R. 2016a. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* (Say) as a vector of Grapevine red blotch-associated virus. *Phytopathology* 106:1223-1230.

Bahder, B. W., Zalom, F. G., and Sudarshana, M. R. 2016b. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of Grapevine red blotch-associated virus. *Plant Dis.* 100:1571-1574.

Basso, M., da Silva, J., Fajardo, T., Fontes, E., Zerbini, F. 2015. A novel, highly divergent ssDNA virus identified in Brazil infecting apple, pear and grapevine. *Virus*

Res. 210:27-33.

Beach S., Kovens M., Hubbert L., Honesty S., Guo Q., Pap D., Dai R., Kovacs L. & Qiu W. 2017. Genetic and phenotypic characterization of Grapevine vein clearing virus from wild *Vitis rupestris*. *Phytopathology* 107:138-144.

Blanco-Ulate, B., Hopfer, H., Figueroa-Balderas, R., Ye, Z., Rivero, R.M., Albacete, A., Pérez-Alfocea, F., Koyama, R., Anderson, M.M., Smith, R.J., Ebeler, S.E., Cantu, D., 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *J. Exp. Bot.* 68:1225-1238.

Briddon, R. W., Pinner, M. S., Stanley, J., & Markham, P. G. 1990. Geminivirus coat protein gene replacement alters insect specificity. *Virology* 177:85-94.

California Department of Food and Agriculture. 2016. California Agricultural Statistics Review, 2015-2016. <https://www.cdfa.ca.gov/statistics/PDFs/2016Report.pdf>.

California Open Data Portal. 2017. CA Geographic Boundaries. <https://data.ca.gov/dataset/ca-geographic-boundaries>

Canik Orel, D., Reid, C.L., Fuchs, M., Burr, T.J. 2017. Identifying environmental sources of *Agrobacterium vitis* in vineyards and wild grapevines. *Am J Enol Vitic.* 68: 213-217.

Cieniewicz E.J., Loeb G., Pethybridge S., Perry K.L. & Fuchs M.F. 2018. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108:94-102.

Cieniewicz E.J., Perry K.L. & Fuchs M. 2017a. Grapevine red blotch: molecular biology of the virus and management of the disease. Pages 303-314 in: "Grapevine viruses: molecular biology, diagnostics and management", B. Meng, G.P. Martelli, D. Golino and M. Fuchs (eds), Springer Verlag, Berlin, Germany.

Cieniewicz E.J., Pethybridge S.J., Gorny A., Madden L.V., McLane H., Perry K.L., Fuchs M.F. 2017b. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Res.* 241:156-162.

Dangl, G.S., Mendum, M.L., Yang, J., Walker, M.L., Preece, J.E. 2015. Hybridization of cultivated *Vitis vinifera* with wild *V. californica* and *V. girdiana* in California. *Ecol. Evol.* 5:5671-5684.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792-1797.

Huson, D.H. and Bryant, D. 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* 23:254-267.

Kil, E.-J., Kim, S., Lee, Y.-J., Byun, H.-S., Park, J., Seo, H., Kim, C.-S., Shim, J.-K., Lee, J.-H., Kim, J.-K., Lee, K.-Y., Choi, H.-S., Lee, S. 2016. Tomato yellow leaf curl virus (TYLCV-IL): a seed-transmissible geminivirus in tomatoes. *Sci. Rep.* 6:19013.

Kil, E.-J., Park, J., Choi, E.-Y., Byun, H.-S., Lee, K.-Y., An, C.G., Lee, J.-H., Lee, G.-S., Choi, H.-S., Kim, C.-S., Kim, J.-K., Lee, S. 2017. Seed transmission of Tomato yellow leaf curl virus in sweet pepper (*Capsicum annuum*). *Eur. J. Plant Pathol.* 150:759-764.

Kim, J., Kil, E.-J., Kim, S., Seo, H., Byun, H.-S., Park, J., Chung, M.-N., Kwak, H.-R., Kim, M.-K., Kim, C.-S., Yang, J.-W., Lee, K.-Y., Choi, H.-S., Lee, S. 2015. Seed transmission of Sweet potato leaf curl virus in sweet potato (*Ipomoea batatas*). *Plant Pathol.* 64:1284-1291.

Klaassen, V.A., Sim, S.T., Dangl, G.S., Osman, F., Al Rwahnih, M., Rowhani, A., Golino, D.A. 2011. *Vitis californica* and *Vitis californica* × *Vitis vinifera* hybrids are hosts for Grapevine leafroll-associated virus-2 and -3 and Grapevine virus A and B. *Plant Dis.* 95:657-665.

Kothandaraman, S.V., Devadason, A., Ganesan, M.V., 2016. Seed-borne nature of a begomovirus, Mung bean yellow mosaic virus in black gram. *Appl. Microbiol. Biotechnol.* 100:1925-1933.

Krenz, B., Thompson, J., McLane, H., Fuchs, M.F, Perry, K.L. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 102:1232-1240.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G. 2007. ClustalW and ClustalX version 2. *Bioinformatics* 23:2947-2948.

Martin, D.P., Murrell, B., Golden, M., Khoosal, A., Muhire, B. 2015. RDP4: Detection and analysis of recombination patterns in virus genomes. *Virus Evol.* 1:vev003.

Muhire, B.M., Varsani, A., Martin, D.P. 2014. SDT: A virus classification tool based on pairwise sequence alignment and identity calculation. *PLoS ONE* 9:e108277.

New York State GIS. 2017. NYS Civil Boundaries.

Pacifico, D., Stigliano, E., Sposito, L., Spinelli, P., Garfi, G., Gristina, A.S., Fontana, I., Carimi, F. 2016. Survey of viral infections in spontaneous grapevines from natural environments in Sicily. *Eur. J. Plant Pathol.* 145:189-197.

Perry, K.L., McLane, H., Hyder, M.Z., Dangl, G.S., Thompson, J.R., and Fuchs, M.F. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* sp. proximal to cultivated grapevines. *Phytopathology* 106:663-670.

Perry, K.L., Mclane, H., Thompson, J.R., Fuchs, M. 2018. A novel grablovirus from non-cultivated grapevine (*Vitis* sp.) in North America. *Arch. Virol.* 163:259-262.

Ricketts, K., Gómez, M., Fuchs, M., Martinson, T., Smith, R., Cooper, M., Moyer, M., Wise, A. 2017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. *Am. J. Enol. Vitic* 68:127-135.

Sabanadzovic, S. 2009. Viruses of native *Vitis* germplasm in the Southeastern United States. Extended Abstracts of the 16th Meeting of International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine, pp. 32-35, August 31-September 4, Dijon, France, <http://icvg.org/data/ICVG-2012-Proceedings.pdf>

Sudarshana, M.R., Perry, K.L., Fuchs, M.F., 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105:1026-1032.

USDA National Agricultural Statistics Service, California Department of Food and Agriculture. 2017. Grape Acreage Report 2016 Crop.
https://www.nass.usda.gov/Statistics_by_State/California/Publications/Grape_Acreage/2017/201704gabt00.pdf

Varsani, A., Roumagnac, P., Fuchs, M., Navas-Castillo, J., Moriones, E., Idris, A.,
Bridson, R.W., Rivera-Bustamante, R., Murilo Zerbini, F., Martin, D.P. 2017.
Capulavirus and *Grablovirus*: two new genera in the family *Geminiviridae*. Arch.
Virol. 162:1819-1831.

Yepes, L.M. Cieniewicz, E., Krenz, B. McLane, H., Thompson, J.R., Perry, K.L., Fuchs,
M. 2018. Causative role of grapevine red blotch virus in red blotch disease.
Phytopathology, DOI: 10.1094/PHYTO-12-17-0419-R.

CHAPTER 5

SPREAD DYNAMICS OF GRAPEVINE RED BLOTCH VIRUS IN RELATION TO SPISSISTILUS FESTINUS ABUNDANCE IN VINEYARDS

ABSTRACT

Grapevine red blotch virus (GRBV) has emerged in the last decade as a threat to wine grape production in North America. This virus is disseminated through infected planting material, and can also be vectored by *Spissistilus festinus*, the three-cornered alfalfa hopper. Limited information is available on the dynamics of GRBV spread in vineyards. In this study, we investigated spread of GRBV in three diseased vineyards all planted in 2008 and analyzed the community of insects visiting these vineyards using sticky cards, with a major focus on *S. festinus*. In a 1.5-hectare Cabernet Sauvignon vineyard in Napa County in California, limited spread of GRBV by ten years post-planting was observed (estimated 0.12% annual increase in disease incidence, 1% increase between 2014-2018), despite a large initial source of inoculum (estimated 48% at planting). In an adjacent 2-hectare Cabernet franc vineyard, red blotch incidence increased 2% annually by ten years post-planting (10% between 2014 and 2018), despite an initially lower source of GRBV inoculum (estimated 1% at planting). Populations of *S. festinus*, including viruliferous specimens, were low in the two California vineyards, however, a ten-fold lower abundance was observed in the Cabernet Sauvignon vineyard compared to the Cabernet franc vineyard. In a 1.2-hectare Merlot vineyard in Suffolk County in New York, there was no evidence of secondary spread of GRBV over a five year period (2014-2018), despite a large initial

source of inoculum (estimated 40% at planting), and no *S. festinus* was found. These results suggest an association between spread dynamics of GRBV and abundance of *S. festinus* populations in vineyards.

*This chapter was submitted for publication in a special issue of *Viruses* on “Plant Virus Epidemiology and Control”: Cieniewicz E.J., Flasco M., Brunelli M., Onwumelu A., Wise A., and Fuchs M. (2019) Spread dynamics of grapevine red blotch virus in relation to *Spissistilus festinus* abundance in vineyards. *Viruses*, in review.

INTRODUCTION

Grapevine red blotch disease was described for the first time in 2008 on *Vitis vinifera* ‘Cabernet Sauvignon’ in California (Calvi 2011). Grapevine red blotch virus (GRBV) was initially identified in association with diseased vines (Krenz *et al.* 2012; Al Rwahnih *et al.* 2013) and later found to be the causative agent of red blotch disease (Yepes *et al.* 2018). GRBV is the type species of the genus *Grablovirus* in the family *Geminiviridae* (Varsani *et al.* 2017). Its genome consists of single-stranded circular DNA with seven putative bi-directional, overlapping open reading frames (Cieniewicz *et al.* 2017a). GRBV isolates comprise two distinct phylogenetic clades (Krenz *et al.* 2014). The biological implications of these two groups of virus genetic variants are unknown.

Red-berried grape cultivars infected with GRBV show symptoms of foliar reddening, similar to those of leafroll disease, mite feeding damage, and nutrient deficiencies (Cieniewicz *et al.* 2017a). Infected white-berried *V. vinifera* cultivars may show chlorosis and cupping, similar to leafroll or magnesium deficiency. Red blotch disease results in delayed ripening and reduced fruit qualities (Blanco-Ulate *et al.* 2017, Calvi 2011, Kurtural *et al.* 2019). Economic losses due to red blotch disease are estimated to range from \$2,213 to \$68,548 per hectare over the 25-year lifespan of Cabernet Sauvignon and Merlot vineyards (Ricketts *et al.* 2017).

Long distance spread of GRBV is attributed to dissemination of infected planting material. This has resulted in GRBV presence in viticulture regions of the United States (Krenz *et al.* 2014) and Canada (Poojari *et al.* 2017, Xiao *et al.* 2015). The virus was also described in Switzerland (Reynard *et al.* 2018), South Korea (Lim

et al. 2016), Mexico (Gasperin-Bulbarela *et al.* 2018), and India (GenBank accession no. KU522121.1). A three-year study of a 2-hectare *V. vinifera* cv. Cabernet franc vineyard in Napa County, California, USA revealed a 1-2% annual increase in disease incidence from 2014 to 2016 (Cieniewicz, *et al.* 2017b). The vineyard was planted in 2008 and began showing red blotch symptoms in 2012 at the edge proximal to an unmanaged riparian area surrounding the Napa River. The aggregation of symptomatic vines combined with randomly isolated symptomatic vines was suggestive of virus spread via a flying insect vector (Cieniewicz, *et al.* 2017b). GRBV was also detected in free-living *Vitis* spp. in Napa County (Bahder *et al.* 2016b, Perry *et al.* 2016), and in high frequency in free-living grapevines close to commercial vineyards throughout northern California (Cieniewicz *et al.* 2018a).

An insect survey in the area of the Cabernet franc vineyard in Napa County, California, where secondary spread of GRBV was first documented (Cieniewicz *et al.* 2017) showed that only four of the 43 insect species tested for GRBV from sticky cards qualified as potential vector candidates based on at least 30% of their specimens testing positive for GRBV (Cieniewicz *et al.* 2018). Indeed, GRBV was consistently detected in the three-cornered alfalfa hopper (*Spissistilus festinus* [Say], Membracidae), *Colladonus reductus* (Cicadellidae), *Osbornellus borealis* (Cicadellidae) and a *Melanoliarus* species (Cixiidae) over two growing seasons (Cieniewicz *et al.* 2017, 2018b). These four hemipteran vector candidates are not typically considered pests of grapevine but are all phloem-feeders (Jensen 1957; Wolfe 1955; Wolfe *et al.* 1950; Mueller and Dumas 1975; Holzinger *et al.* 2002). GRBV is transmitted by *S. festinus* in a greenhouse setting (Bahder *et al.* 2016a),

however the capacity for the other three insect vector candidates to transmit GRBV is unknown.

Spread of GRBV has only been documented in a single epidemic in California (Cieniewicz *et al.* 2017), and suggested in vineyards in Oregon by an unknown vector (Dalton *et al.* 2019). Secondary spread patterns need to be investigated in additional vineyards and regions to better understand factors influencing the spread dynamics of GRBV. The main objective of this study was to characterize the spread of GRBV in New York and California vineyards in conjunction with the populations of potential insect vectors, including *S. festinus*, the only vector currently known. We hypothesized an association between GRBV spread dynamics and abundance of *S. festinus*.

MATERIALS AND METHODS

Study vineyard selection in California and New York

Three vineyards, two in California and one in New York, with vines showing red blotch disease symptoms were chosen for this study. The 1.5-hectare *V. vinifera* ‘Cabernet Sauvignon’ vineyard in Napa County, California was planted in 2008 with vines derived from two different clones- CS4 and CS169- both grafted onto rootstock 101-14 Mgt (*V. riparia* x *V. rupestris*). This vineyard was chosen for this study because of (1) its proximity to a 2-ha Cabernet franc vineyard in which secondary spread of GRBV was documented and *S. festinus* was identified as a vector of epidemiological relevance (Cieniewicz *et al.* 2017; 2018), and (2) red blotch symptoms observed in nearly all of the vines derived from clone CS4 in the first-year

post-planting. We also expanded on previous research carried out in the 2-ha Cabernet franc vineyard in California from 2014 to 2016 (Cieniewicz *et al.* 2017; 2018) by assessing disease progress in 2017 and 2018. The Cabernet franc vines were grafted onto rootstock 101-14 Mgt. A 1.2-hectare *V. vinifera* ‘Merlot’ clone 181 vineyard planted in 2008 in Suffolk County, New York was also chosen for this study, due to an initial incidence of 40% of the vines showing foliar reddening symptoms. Merlot vines were also grafted onto 101-14 Mgt.

Survey for GRBV incidence in California and New York vineyards

The two California vineyards were visually surveyed for disease symptoms in October of 2017 and 2018. In October of 2017, leaf and petiole samples (6-8 leaves per vine, 3-4 from each side of the trunk) of Cabernet Sauvignon were collected from 34 symptomatic vines for GRBV testing by multiplex polymerase chain reaction (PCR) with primers targeting a coat protein (CP) gene fragment and a replicase-associated protein (Rep) gene fragment (Krenz *et al.* 2014). In October of 2018, vines of clone 169 showing red blotch disease symptoms for the first time were sampled for GRBV detection by multiplex PCR (Krenz *et al.* 2014).

The New York vineyard was not surveyed for visual red blotch disease symptoms. This is because aggressive leaf removal in the fruiting zone post-véraison by the vineyard manager confounded symptomatology of infected vines. Instead, leaf and petiole samples (6-8 leaves per vine, 3-4 from each side of the trunk) were collected from a subset of vines in late August or September of each year from 2014 to 2016. In 2017 and 2018, samples were collected from only vines which tested negative

for GRBV in the previous years. All petiole samples were tested for GRBV using multiplex PCR (Krenz *et al.* 2014).

Disease progress was assessed for each study vineyard by plotting the percentage of diseased vines in each year. A subsection of the Cabernet franc vineyard spanning 13 rows and 42 vines was considered separately. The area under the disease progress stairs (AUDPS, Simko and Piepho 2012) was calculated for each vineyard and the Cabernet franc vineyard subsection, using years after planting (6 to 10) on the ‘x’ axis and percent disease on the ‘y’ axis in R Studio package ‘agricolae’ (version 3.5.1).

Insect survey, handling, and removal

An insect survey was conducted in the Cabernet Sauvignon vineyard in Napa County, California from March to November in 2017 and 2018. Sticky cards were placed in 12 rows, in alternating panels spanning six four-vine panels per row so that each row contained three sticky cards. Each sticky card was collected and replaced with a new sticky card in the adjacent panel each week, so that sticky cards were switched from A-C-E orientation to B-D-F orientation each week, alternating the panel in every other row so that a checkerboard-like pattern was achieved. The vineyard area surveyed included six rows of clone CS4 and six rows of clone CS169. Sticky cards were shipped weekly to the laboratory in Geneva, New York.

An insect survey was also conducted in the Merlot vineyard in Suffolk County, New York. A total of 52 yellow sticky cards was placed on the lower catch wire throughout the vineyard in five rows, with one trap in alternate panels of six vines,

from April through August in 2017 and 2018. Traps were collected every two weeks, placed in clear plastic bags and shipped to the laboratory in Geneva, NY.

Hemipteran insects caught on sticky cards were identified based on morphological characteristics to the genus and species level when possible. The number of each specimen was recorded prior to their removal from the sticky card to identify abundance and diversity. A subset of specimens of high abundance (>60 during a growing season) were chosen at random to be tested for GRBV by multiplex PCR (Krenz *et al.* 2014), and those of low abundance (<60) were all tested. Removal of individual specimens from the yellow sticky cards was facilitated using Goo Gone liquid degreaser (Weiman Products, Gurnee, IL) to dissolve adhesive (Cieniewicz *et al.* 2018). Individuals were stored in microcentrifuge tubes at -20°C until testing. Insect species for which more than 30% of the specimens consistently tested positive for GRBV by multiplex PCR were considered vector candidates, as previously described (Cieniewicz *et al.* 2018).

GRBV detection in insects by polymerase chain reaction

Total DNA was extracted from individual insects removed from the sticky cards using the E.Z.N.A Insect DNA Kit (OMEGA Biotek, Norcross GA) and stored at -20°C. Specimens were tested for GRBV using multiplex PCR (Krenz *et al.* 2014). PCRs were carried out using HotStar Taq polymerase at manufacturer-suggested conditions in a C1000 Touch Thermal Cycler (Bio Rad, Hercules CA). DNA amplicons were resolved by electrophoresis on 2% agarose gels in 1x Tris-acetate-EDTA buffer and then stained with GelRED (Biotium, Fremont CA). Detection of

GRBV in specimens was assumed to be a result of virus ingestion.

Genetic diversity of GRBV isolates from infected grapevines

Total DNA was isolated from symptomatic grapevines in study vineyards using the H.P. Plant DNA kit (OMEGA Biotek, Norcross GA) and tested for GRBV using multiplex PCR (Krenz *et al.* 2014). To determine genetic relatedness of GRBV isolates recovered from infected vines in the New York vineyard and the Cabernet Sauvignon vineyard in California, a 750 -bp fragment spanning the GRBV origin of replication was amplified by PCR using primers GRBVdivFOR (5' GAGGGTATGTGAGGAAGAAG 3') and GRBVdivREV (5' GCAGAAGGCAACGATATATCC 3') (Perry *et al.* 2016). PCR products were then purified using ExoSAP-IT (Applied Biosystems, Foster City CA) and Sanger-sequenced at the Cornell University Biotechnology Resource Center (Ithaca NY). Sequences were assembled using the Lasergene software suite (version 15.1.1), and aligned using ClustalW (Thompson *et al.* 1994). Phylogenies were constructed considering 575 residues of the isolates characterized in this study and others for which sequence information is available in GenBank using the neighbor-joining method (Saitou and Nei, 1987). Branching confidence was estimated using 1000 bootstrap replicates.

Survey for GRBV in vineyard cover crop species

In order to investigate whether cover crops in vineyards serve as alternative GRBV inoculum source, cover crop samples were surveyed in 13 vineyards of

cultivars Petit Verdot, Cabernet Sauvignon, Cabernet franc, and Sauvignon blanc in Napa County, California. These vineyards reflected a variety of cultivars, levels of red blotch disease pressure (high to low), and terrains.

Cover crops were collected for GRBV testing each April/May from 2016-2018 prior to tilling or mowing of the cover crops. Samples were collected from 2-4 locations within each vineyard and pooled together in 11x13" plastic bags for each collection location. Samples were shipped overnight to the laboratory in Geneva, New York for testing. Each bag was sub-sampled to separate cover crop species. In 2016 and 2017, DNA was extracted from cover crop samples using the H.P. Plant DNA kit (OMEGA Biotek, Norcross GA). In 2018, total nucleic acids were isolated using the Kingfisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham MA) and the MAGMAX 96 Viral RNA isolation kit (Life Technologies, Carlsbad CA). Samples were tested for GRBV using multiplex PCR (Krenz *et al.* 2014).

RESULTS

Secondary spread of GRBV in California and New York vineyards

Disease incidence in the 2-hectare Cabernet franc vineyard in California increased from 3.9% (305 diseased vines of 7,691 vines surveyed) in 2014 to 7.1% (547 of 7,691 vines diseased) in 2016 (Cieniewicz *et al.* 2017), and then from 9% (696 of 7,691 vines diseased) in 2017 to 13.8% (1,058 of 7,691 vines diseased) in 2018 (this study). The increase in disease incidence in 2017-2018 was most pronounced in the area where disease incidence was initially aggregated (**Figure 5-1 & Figure 5-2b,c**), similar to trends observed in previous years (Cieniewicz *et al.* 2017). The

aggregation of diseased vines at the edge of the vineyard close to the riparian area is likely explained by the initial presence of the virus in the planting material, likely in the rootstock because disease symptoms were only observed four year post-planting (Cieniewicz *et al.* 2017). Spread of GRBV in the Cabernet franc vineyard is illustrated by high AUDPS values, whether throughout the entire vineyard or in a subsection proximal to the riparian area (Table 1).

In the adjacent 1.5-hectare Cabernet Sauvignon vineyard in California, disease symptoms were widespread throughout vines of clone 4 by one to two years post-planting (**Figure 5-1**), suggesting introduction of GRBV via infected planting material, and likely the scion due to early symptomatology. Based on visual assessment, red blotch symptoms were apparent in very few (<15) vines throughout clone 169 in the years between planting in 2008 and our survey in 2017 (**Figure 5-2d**). In the vineyard section established with clone 169, disease incidence increased from 0.86% (24 of 2799 vines infected) in 2017 to 1.2% (33 of 2799 vines infected) in 2018. This result was consistent with limited spread of GRBV in vines of clone 169 despite the availability of a large initial source of inoculum (estimated 48% at planting), confined primarily to adjacent vines of clone 4 (calculated 81% in 2017) (**Figure 5-2a,d**). Spread of GRBV in the Cabernet Sauvignon vineyard is illustrated by a low AUDPS value (**Table 5-1**).

In the 1.2-hectare Merlot vineyard in New York, red blotch disease symptoms were observed in 2009, the year after planting. However, disease incidence could not be reliably assessed visually because foliar symptomatology was confounded by extensive leaf removal in the fruiting zone by the vineyard manager at a post-véraison

development stage, thereby removing the oldest leaves where foliar red blotch symptoms are pronounced. Nonetheless, a cursory visual assessment suggested an overall 40% disease incidence at planting. Annual collection of leaf samples from 51 infected and 14 GRBV-negative vines in the Merlot vineyard followed by PCR testing in 2014-2018 provided no indication of spread. All vines that tested negative in 2014 continued to test negative for GRBV through the 2018 season (**Figure 5-2a, Table 5-1**). An AUDPS value of 0 in the Merlot vineyard is consistent with a lack of spread (**Table 5-1**).

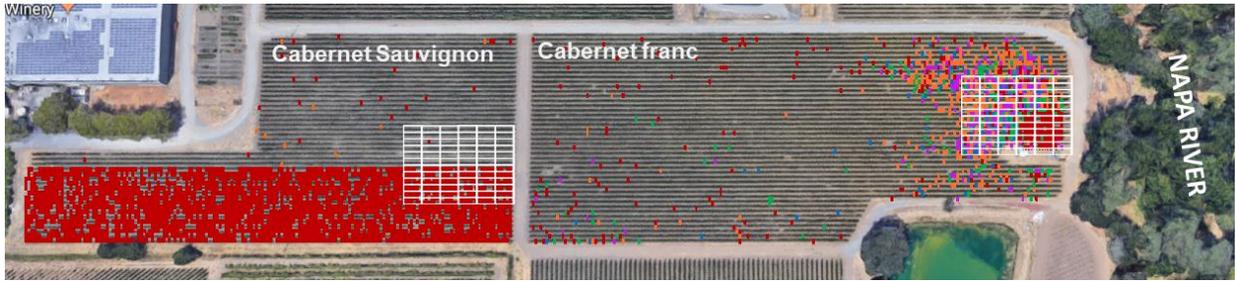


Figure 5-1 Map of grapevine red blotch virus (GRBV) incidence in a 1.5-hectare Cabernet Sauvignon and a 2.0 hectare Cabernet franc vineyard within ten years-post planting. In the Cabernet Sauvignon vineyard, red overlay indicates diseased vines in 2017 at the onset of the study and orange indicates new GRBV infections in 2018. In the Cabernet franc vineyard, red overlay indicates initial disease incidence at the onset of the study in 2014. Green, blue, purple, and orange indicate new infections in 2015, 2016, 2017, and 2018 respectively. Infections recorded in 2015 and 2016 were previously documented (Cieniewicz *et al.* 2017). The 2017 and 2018 infections are reported here for the first time. White grids indicate areas where insect survey was conducted in 2015 and 2016 in the Cabernet franc (Cieniewicz *et al.* 2018) and in 2017 and 2018 in the Cabernet Sauvignon (this study).

Table 5-1 Comparative red blotch disease progress in relation to *Spissistilus festinus* populations in three vineyards planted in 2008.

Vineyard	<i>Spissistilus festinus</i> abundance ^a	Disease incidence at planting (estimated)	Disease incidence 2014	Disease incidence 2018	AUDPS ^b
Cabernet franc, California (subsection)	25	10%	25.8%	78.4%	595.2
Cabernet franc, California (whole)	25	1%	3.9%	13.8%	126.2
Cabernet Sauvignon, California	2-3	48%	49.1%	50.1%	10.1
Merlot, New York	0	40%	40.0%	40.0%	0

^aAnnual count of *Spissistilus festinus* on sticky cards in a sub-portion of the vineyard (Cieniewicz *et al.* 2018; this study). *S. festinus* count may not be representative of the distribution of *S. festinus* in the entire vineyard.

^bAUDPS: Area under the disease progress stairs, calculated from 2014 to 2018

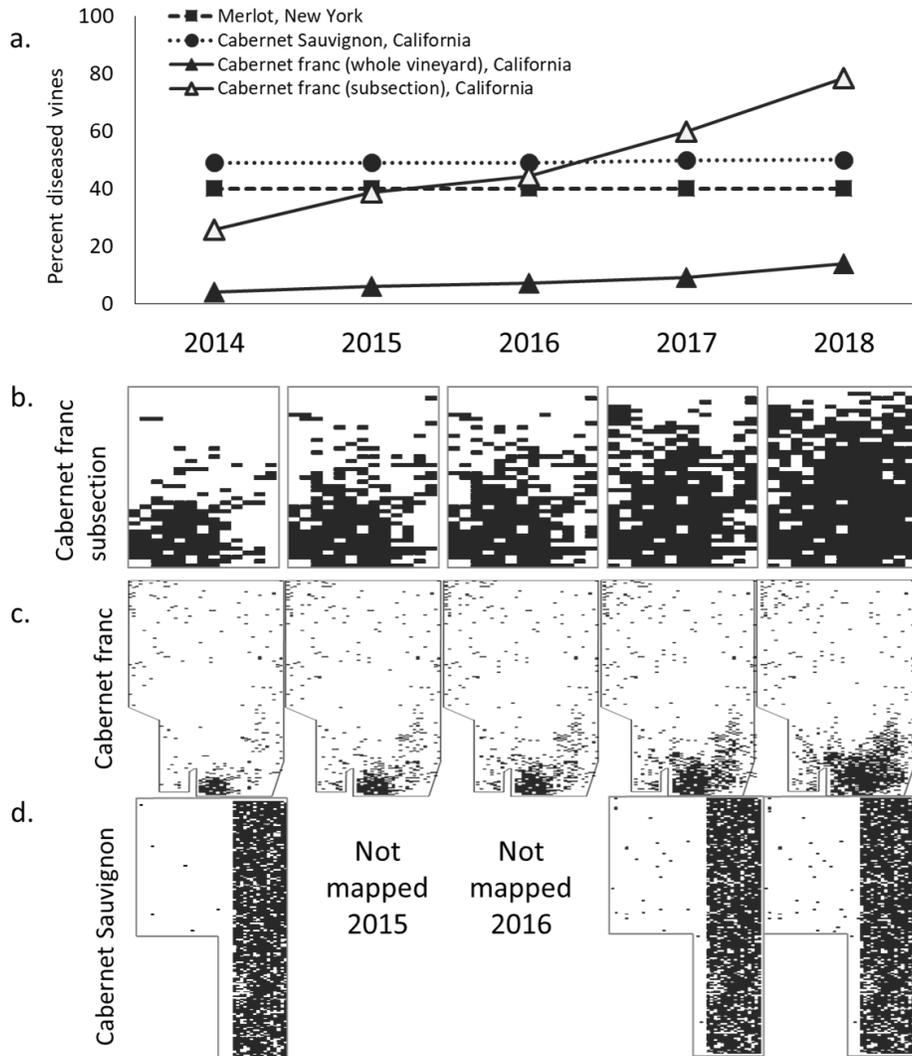


Figure 5-2 Depiction of (a) four grapevine red blotch disease epidemics in a Merlot vineyard in New York characterized by relatively high initial disease incidence (40%) but absence of *Spissistilus festinus*; a Cabernet Sauvignon vineyard with relatively high disease incidence (estimated 48% at planting) and very low *S. festinus* populations (N=5) over two years; a Cabernet franc vineyard with relatively low initial disease incidence (estimated 1% at planting) and relatively higher populations of *S. festinus* (N=50) over two years; and a subset of the Cabernet franc vineyard with initial relatively high disease incidence (25.8%) and relatively high *S. festinus* populations (N=50) (Cieniewicz *et al.* 2018). Annual distribution of GRBV incidence (diseased vines indicated in black, healthy vines indicated by white space) in (b) a subsection of the Cabernet franc vineyard (c) the whole Cabernet franc vineyard, and (d) the Cabernet Sauvignon vineyard. For the latter vineyard, the 2014 map is estimated based on cursory visual assessment of diseased vines, and the 2017 and 2018 maps are based on thorough mapping of disease symptoms.

Genetic diversity of GRBV in infected grapevines

In the Cabernet Sauvignon vineyard in California, GRBV isolates from vines of clone 4 represented phylogenetic clades 1 and 2 (**Figure 5-3**). This could mean that vines were likely infected with a mixture of both genetic variants of GRBV at the time of planting. In symptomatic vines of clone 169, GRBV isolates were similarly of both phylogenetic clades (**Figure 5-3**).

In the Merlot vineyard in New York, all GRBV isolates characterized from a subset of symptomatic vines were nearly identical to each other (>99% nucleotide sequence identity) and clustered in phylogenetic clade 2 (**Figure 5-3**). This result supported the hypothesis that the vines were infected at planting, likely because they were derived from Merlot vine stocks infected with the same isolate (**Figure 5-3**).

In the Cabernet franc vineyard, GRBV isolates from vines in the area of initial disease aggregation were previously characterized and shown to be nearly identical to each other (>99% nucleotide sequence identity) and clustered in phylogenetic clade 2 (Cieniewicz *et al.* 2017). This lack of genetic variability was consistent with the virus inoculum originating from the planting material and with the occurrence of spread from local sources (Cieniewicz *et al.* 2018).

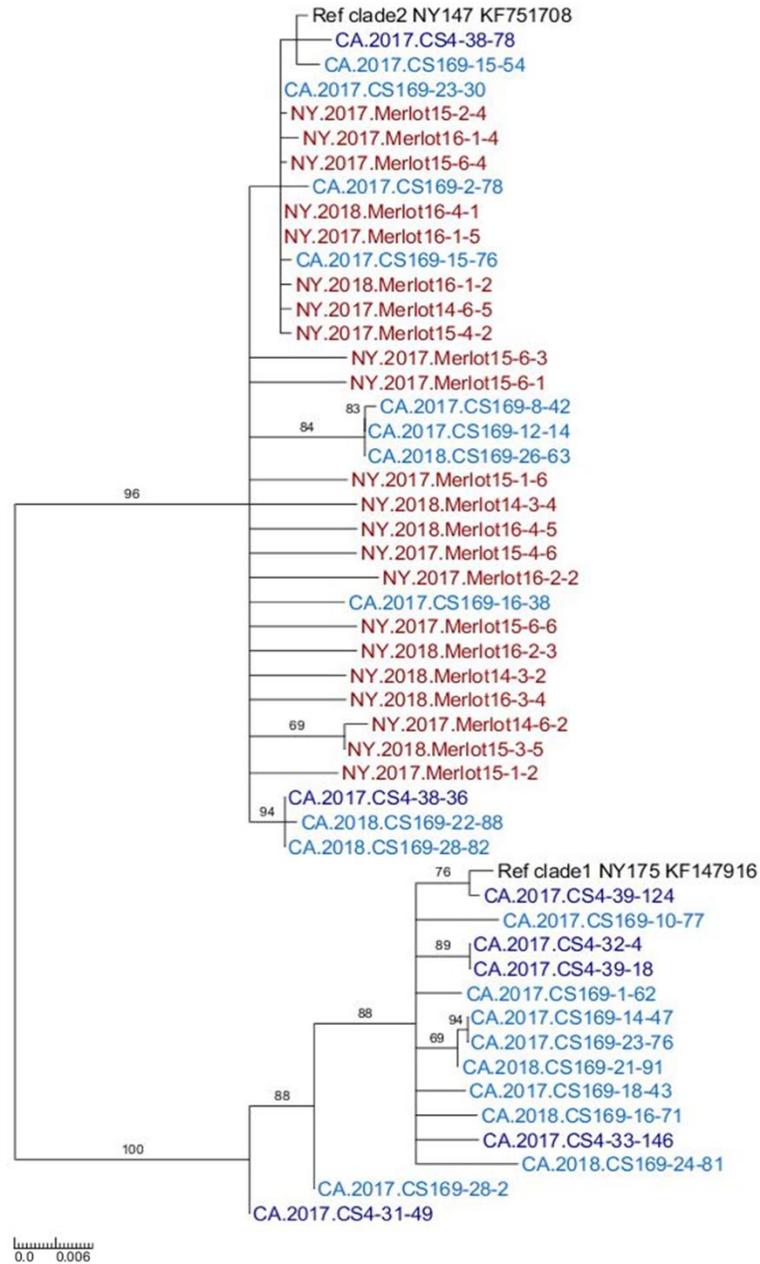


Figure 5-3 Neighbor-joining phylogenetic tree of a 575-nt genomic fragment spanning the origin of replication of grapevine red blotch virus (GRBV) isolates from the Cabernet Sauvignon vineyard in California and the Merlot vineyard in New York. Labels with red text indicate GRBV isolates from the New York vineyard; those with blue text indicate isolates from the California vineyard, where the darker blue specifies isolates from vines of clone 4 and lighter blue specifies isolates from vines of clone 169. Reference isolates for each phylogenetic clade are indicated in black. Numbers at nodes indicate bootstrap support (1000 replicates). Branches with less than 65% support have been collapsed.

Insect community and GRBV ingestion in the Cabernet Sauvignon vineyard in California

The four insect species identified as vector candidates in a previous study in the neighboring Cabernet franc vineyard in 2015 and 2016 (Cieniewicz *et al.* 2018) were present in the Cabernet Sauvignon vineyard in at least one year, but their abundance differed. *S. festinus* was rarely captured in the Cabernet Sauvignon vineyard. Additionally, only two of three specimens tested positive for GRBV in 2017, while the two specimens in 2018 tested negative for GRBV (**Table 5-2**). Of the five *S. festinus* found over two years in the Cabernet Sauvignon vineyard, one of the GRBV-positive specimens was found on the clone 4 side and one on the clone 169 side, whereas one of the GRBV-negative specimens was found on the clone 4 side and two of the GRBV-negative specimens was found on the clone 169 side (**Figure 5-4**). The low number of *S. festinus* represented a 10-fold lower population in the Cabernet Sauvignon vineyard (this study) compared to the Cabernet franc vineyard previously studied (Cieniewicz *et al.* 2018). Similarly, the abundance of *O. borealis* (minus 6-fold) and *Melanoliarus* sp. (minus 20-fold) was reduced in the Cabernet Sauvignon vineyard (this study) relative to the Cabernet franc vineyard (Cieniewicz *et al.* 2018) while it slightly increased for *C. reductus* (plus 1.3-fold). GRBV was also detected in previously identified vector candidates *O. borealis* and *C. reductus* but not in the single *Melanoliarus* sp. specimen (**Table 5-2**). A few specimens of other phloem-feeding insects, including *Scaphytopius magdalenis*, *Empoasca* spp., as well as some aphids and foliar phylloxera species, tested positive for GRBV (**Table 5-2**); however, none of these insects was considered a vector candidate due to the comparatively low

rate of GRBV ingestion.

Population dynamics of *S. festinus*, *O. borealis*, *C. reductus*, and *Melanoliarius* sp. were similar between 2017 and 2018, although the population peak was shifted earlier in 2018 compared to 2017 (**Figure 5-5**). *S. festinus* peaked in July in 2017 and in June of 2018; *O. borealis* was found in August of 2017 and July of 2018; *C. reductus* peaked in May and August of 2017, and in May and July in 2018; *Melanoliarius* sp. was not found in 2017 and only one specimen was found on sticky cards in June of 2018 (**Figure 5-5**). Total populations over the two years were low for *S. festinus*, *O. borealis*, and *Melanoliarius* sp. with five, eight, and one specimen respectively; in comparison *C. reductus* populations totaled 110 specimens over the two years (**Figure 5-5**).

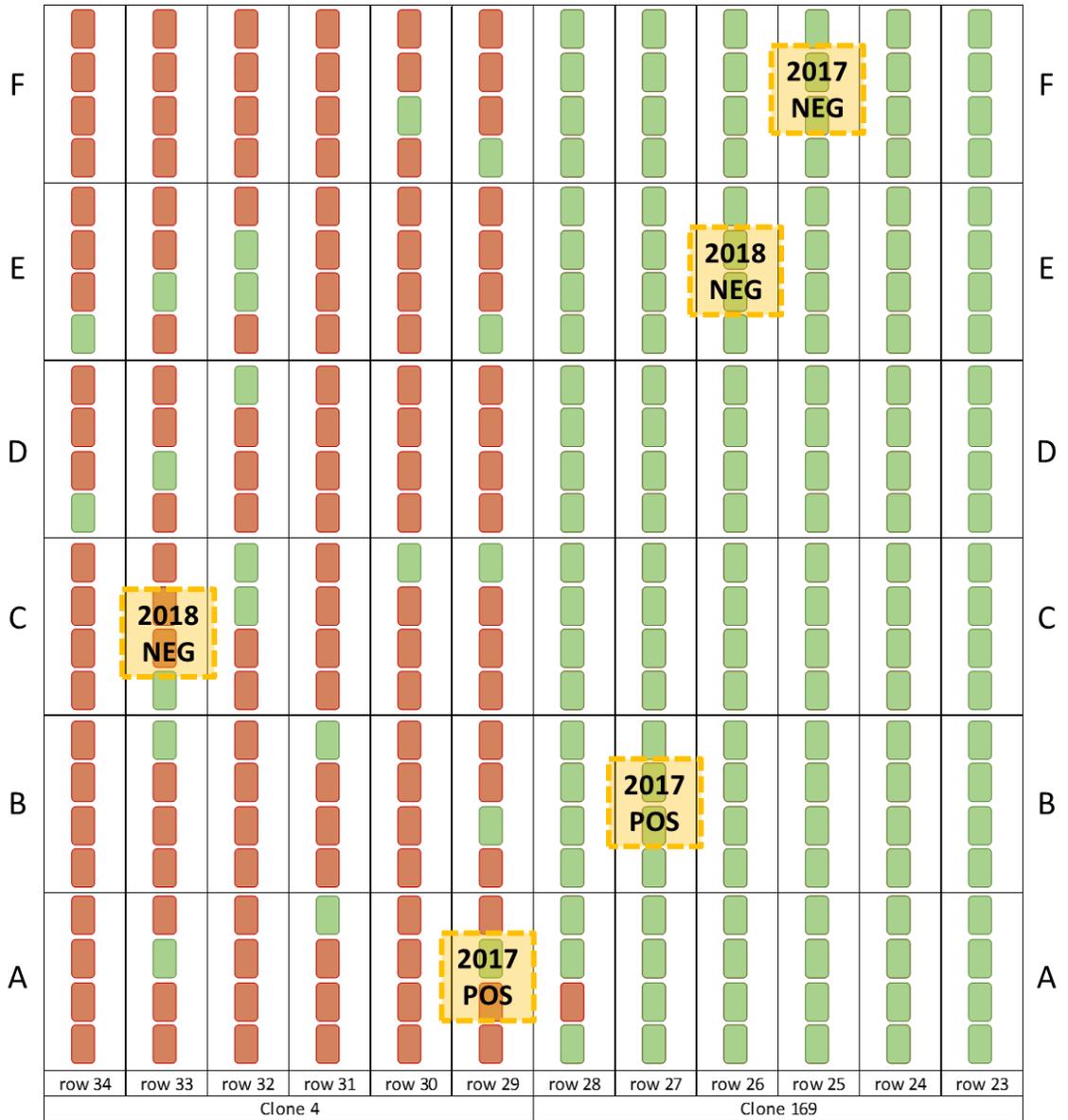


Figure 5-4 Location of *Spissistilus festinus* caught on sticky cards in a Cabernet Sauvignon vineyard in California in 2017 and 2018. Red rectangles indicate grapevine red blotch virus (GRBV)-infected vines; green rectangles indicate asymptomatic vines. Yellow boxes indicate locations, year, and GRBV-positive (POS) or -negative (NEG) *S. festinus* specimen. Letters A-F indicate the four-vine panel designation. In this survey, three yellow sticky cards were placed in each row, alternating from A-C-E orientation, and B-D-F orientation, and switched weekly from April through November in 2017 and 2018.

Table 5-2 Grapevine red blotch virus (GRBV) detection in insects trapped on yellow sticky cards in a diseased *Vitis vinifera* ‘Cabernet Sauvignon’ vineyard in California in 2017 and 2018 in which limited spread of GRBV has been observed.

Family, genus, species	GRBV detection ¹					
	2017		2018		Cumulative	
	N	%	n	%	n	%
Membracidae						
<i>Spissistilus festinus</i>	2/3	67	0/2	0	2/5	40
Cicadellidae						
<i>Colladonus reductus</i>	19/63	30	2/21	10	21/84	25
<i>Osbornellus borealis</i>	4/6	67	0/1	0	5/7	71
<i>Scaphytopius sp.</i>	10/50	20	8/44	18	18/94	19
<i>Euscelis sp.</i>	0/7	0	0/28	0	0/35	0
<i>Empoasca sp.</i>	0/18	0	3/66	5	3/84	4
<i>Erythroneura variabilis</i>	0/25	0	0/50	0	0/75	0
<i>Erythroneura elegantula</i>	0/15	0	0/49	0	0/64	0
<i>Erythroneura ziczac</i>	0/2	0	-	-	0/2	0
<i>Deltocephalus sp.</i>	0/8	0	0/3	0	0/11	0
<i>Japananus hyalinus</i>	0/1	0	0/8	0	0/9	0
<i>Xestocephalus sp.</i>	-	na	0/10	0	0/10	0
Cixiidae						
<i>Melanoliarus sp.</i>	-	na	0/1	0	0/1	0
Aphididae	1/28	4	2/67	3	3/95	3
Delphacidae	0/2	0	0/2	0	0/4	0
Phylloxeridae	0/8	0	3/31	10	3/39	8
Psylloidea	0/6	0	0/26	0	0/32	0
Miridae	0/12	0	-	na	0/12	0
Lygaeidae	0/2	0	-	na	0/2	0

¹ “n” = the proportion of individual specimens in which GRBV was detected by polymerase chain reaction; “-” indicates that no specimen was tested in that year; na: not applicable.

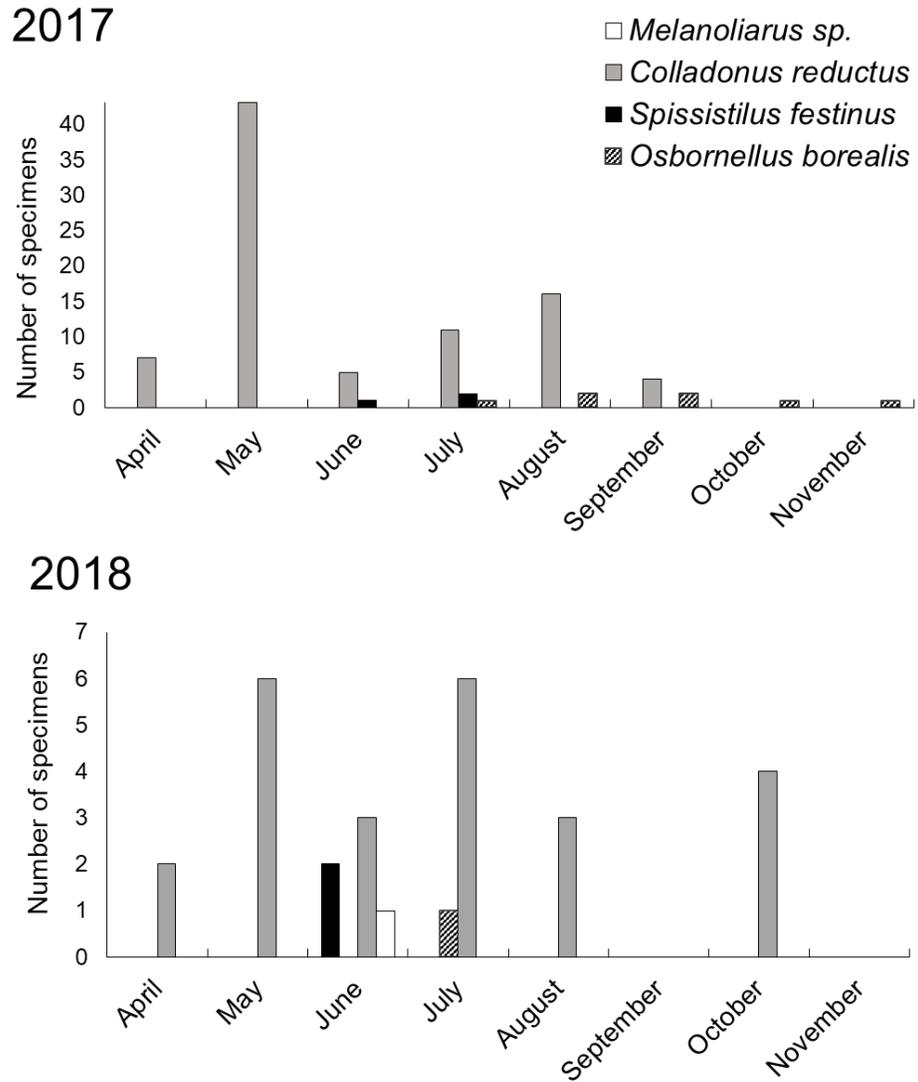


Figure 5-5 Monthly abundance of *Spissistilus festinus* and candidate insect vectors trapped on yellow sticky cards in a Cabernet Sauvignon vineyard in Napa County, California in 2017 (top) and 2018 (bottom).

Insect community and GRBV ingestion in the Merlot vineyard in New York

The insect community caught on sticky cards in the Merlot vineyard in New York differed from the community in the Cabernet Sauvignon vineyard in California. *Spissistilus festinus* was not detected on any of the sticky cards in 2017 or 2018 but other species of Membracidae (*Acutalis* sp., *Entylia* sp., *Campylenchia* sp. and *Stictocephala* sp.) were found in the Merlot vineyard (**Table 5-3**). None of the three vector candidates identified in the Cabernet Sauvignon vineyard (this study) and the Cabernet franc vineyard previously surveyed, i.e. *S. festinus*, *O. borealis*, *C. reductus*, and *Melanoliarius* sp. (Cieniewicz *et al.* 2018), was found in the Merlot vineyard in New York. Of the treehoppers, only *Entylia* sp. tested positive for GRBV: one of seven specimens in 2017; and one of 30 specimens in 2018 (**Table 5-3**). These rates of ingestion are extremely low; therefore, *Entylia* sp. was not considered as a vector candidate. Similarly to the Cabernet Sauvignon vineyard in California (this study) and the previously studied Cabernet franc vineyard in California (Cieniewicz *et al.* 2018), some phloem-feeding insects caught on sticky card traps also occasionally tested positive for GRBV, indicating ingestion of the virus while feeding on the vines (**Table 5-3**). However, none of them was considered a potential vector candidate due to the low rate of GRBV ingestion.

Table 5-3 Grapevine red blotch virus detection in insects trapped on yellow sticky cards in a diseased *Vitis vinifera* ‘Merlot’ vineyard in Suffolk County, New York in 2017 and 2018 in which no spread of GRBV has been observed.

Family, genus, species	GRBV detection ¹					
	2017		2018		Cumulative	
	n	%	n	%	n	%
Membracidae						
<i>Acutalis</i> sp.	0/11	0	0/4	0	0/15	0
<i>Entylia</i> sp.	1/7	14	1/30	3	2/37	5
<i>Campylenchia</i> sp.	0/1	0	-	na	0/1	0
<i>Stictocephala</i> sp.	0/3	0	-	na	0/3	0
Cercopidae						
<i>Philaenus</i> sp.	0/13	0	0/4	0	0/17	0
<i>Clastoptera</i> sp.	0/2	0	0/3	0	0/5	0
Cicadellidae						
<i>Cicadula</i> sp.	1/19	5	3/16	19	4/35	11
<i>Colladonus</i> sp.	0/2	0	0/1	0	0/3	0
<i>Deltocephalinae</i> sp.	0/15	0	0/14	0	0/29	0
<i>Draeculacephala minerva</i>	0/1	0	-	na	0/1	0
<i>Graphocephala coccinea</i>	0/37	0	1/24	4	1/61	2
<i>Empoasca</i> sp.	2/28	7	5/30	17	7/58	12
<i>Erythroneura</i> sp.	0/1	0	0/11	0	0/12	0
<i>Paraphlepsius</i> sp.	1/38	3	1/26	4	2/64	3
<i>Scaphoideus</i> sp.	1/6	17	4/20	20	5/26	19
<i>Scaphytopius</i> sp.	0/1	0	0/8	0	0/9	0
<i>Xestocephalus</i> sp.	-	na	0/10	0	0/10	0
Aleyrodidae	0/1	0	-	na	0/1	0
Aphididae	0/12	0	0/22	0	0/34	0
Fulgoroidea	-	na	0/2	0	0/2	0
Psylloidea	-	na	0/3	0	0/3	0

¹ “n” = the proportion of individual specimens in which GRBV was detected by polymerase chain reaction; “-” indicates that no specimen was tested in that year; na: not applicable.

GRBV absent from surveyed vineyard cover crop species in Napa County, California

Cover crop species in vineyard middle rows were surveyed for GRBV in spring in 13 vineyards in Napa County, California. Based on visual assessment of red blotch disease, four of the surveyed vineyards were heavily (>80%) symptomatic, three were moderately (10-50%) symptomatic, one was mostly asymptomatic (<5%), one was a recently planted vineyard, and four were of unknown disease incidence. Two of the vineyards were near water sources (<10 m away from a river or pond), and five of them were adjacent to forested habitat. In 2016 and 2017 cover crop species included only legumes such as fava beans (*Vicia faba*), purple vetch (*Vicia americana*), red and white clover (*Trifolium spp.*), and field peas (*Pisum sativum* subsp. *arvense*). In 2018 cover crops included the aforementioned species as well as mixed grasses (*Poaceae*) from nine of the vineyard sites. GRBV was not detected in any of the 476 total cover crop samples from Napa County in 2016, 2017 and 2018.

DISCUSSION

In this study we characterized spread of GRBV in three red blotch diseased vineyards, two in California and one in New York, all planted in 2008. Distinct spread dynamics were documented in the three vineyards with an annual disease increase of 0%, 0.12% and 2% in a 1.2-hectare Merlot vineyard in New York, a 1.5-hectare Cabernet Sauvignon vineyard in California and a 2-hectare Cabernet franc vineyard in California, respectively. The differential rate of annual disease increase was unrelated to the initial level of GRBV inoculum available (**Table 5-1**). Indeed, a 40% (Merlot

vineyard in New York), 48% (Cabernet Sauvignon vineyard in California) and 1% (Cabernet franc vineyard in California) initial infection rate was estimated at the time the vineyards were established. The highest increase of infected vines (2% annually) was observed in the Cabernet franc vineyard with the lowest virus inoculum at planting (1%), and the lowest increase of infected vines (0-0.12% annually) in the Merlot and Cabernet Sauvignon vineyards, respectively, with the highest virus inoculum at planting (40-48%).

We also described the abundance and GRBV ingestion rates of hemipteran insects in two of the three study vineyards. Our findings revealed distinct insect communities in a Cabernet Sauvignon vineyard in California (**Table 5-2**) and in a Merlot vineyard in New York (**Table 5-3**). Extremely low populations of *S. festinus*, currently the only confirmed insect vector of GRBV (Bahder *et al.* 2016), were found on sticky cards in the Cabernet Sauvignon vineyard in California, and no *S. festinus* was found in the Merlot vineyard in New York. The other three vector candidates previously identified, i.e. *O. borealis*, *C. reductus*, and *Melanoliarus sp.* (Cieniewicz *et al.* 2018), were found on sticky cards in the Cabernet Sauvignon vineyard in California but not in the Merlot vineyard in New York. With the exception of *S. festinus* (Bahder *et al.* 2016a) the ability of these insects to transmit GRBV is unknown, and should be tested.

The passive sampling method of yellow sticky cards may not be the ideal method of determining populations of insects in the vineyard (Cieniewicz *et al.* 2018; Preto *et al.* 2019). This is because immature life stages of hemipterans and/or crawling insects are likely under-sampled. Nonetheless, yellow sticky cards are a consistent

form of sampling, and allow for comparison of populations of flying insects between vineyards (Cieniewicz *et al.* 2018, this study).

Secondary spread of GRBV has not been reported in New York, thus far, although the virus is present in some vineyards in the Finger Lakes and Long Island regions. The lack of spread of GRBV in a five-year period (2014-2018) in the Merlot vineyard in the North Fork of Long Island, New York is consistent with the absence of *S. festinus*. The geographic range of *S. festinus* is throughout the southern United States and as far south as Costa Rica (Beyer *et al.* 2017, Caldwell 1949). To our knowledge, recent information on the geographic distribution of *S. festinus* in the U.S. is not available and this issue should be revisited, particularly with regard to changes in agricultural practices and climate change that occurred over the past 70 years. Nonetheless, despite the detection of four species of Membracidae on the sticky cards in the Merlot vineyard, only a single specimen each year of the *Entylia* species tested positive for GRBV. Using the criteria of at least 30% of insects testing positive in a vineyard survey to be considered vector candidates, established in a previous study (Cieniewicz *et al.* 2018a), we did not identify any insect vector candidates in the New York Merlot vineyard. The lack of observed secondary spread of GRBV in New York vineyards is also consistent with the absence of GRBV in free-living *Vitis* spp. throughout the state (Cieniewicz *et al.* 2018b).

Comparing the secondary spread dynamics of GRBV in the Cabernet franc (Cieniewicz *et al.* 2017, this study) and Cabernet Sauvignon (this study) vineyards in California demonstrated a marked difference, despite both vineyards being planted in 2008, and managed identically in terms of chemical management practices against

fungal diseases and cover crop rotations. Overall, secondary spread is occurring faster in the Cabernet franc vineyard (2% increase annually) compared to the Cabernet Sauvignon vineyard (0.12% increase annually) despite the small initial source of inoculum in the former (estimated 1% at planting) and large source of initial inoculum in the latter (estimated 48% at planting) vineyard. We attribute this inverse spread dynamic to the ten-fold higher population of *S. festinus* observed in the canopy of the Cabernet franc vineyard (N=25 in each year) compared to the Cabernet Sauvignon vineyard (N= 2-3 in each year). If few *S. festinus* visit GRBV-infected vines, it is unlikely that the rate of secondary spread will be high. Experiments in controlled conditions should be carried out to validate the observations made in diseased vineyards. Nonetheless, the vineyard observation validated our hypothesis on the association between GRBV spread dynamics and abundance of *S. festinus* populations.

Spread dynamics of GRBV in the two study vineyards in California are similar to accounts of Pierce's Disease (PD) outbreaks in California (reviewed in Redak *et al.* 2004). For example, in northern and coastal California, the blue-green sharpshooter (*Graphocephala atropunctata*) is associated with PD outbreaks near riparian habitats. Similarly, in vineyards in the Central Valley of California, PD outbreaks commonly occur near irrigated fields and water sources, even if the sharpshooter vectors are not colonizers of grape (Redak *et al.* 2004). Whereas *Xylella fastidiosa*, the causal agent of PD, can be transmitted from several plant hosts in these habitats, GRBV has thus far only been found in *Vitis* spp., and no other environmental reservoirs are known. Himalayan blackberry (*Rubus armeniacus*) was reported as an alternative host of GRBV in winter and spring in northern California (Bahder *et al.* 2016); however, this

finding has yet to be confirmed in independent studies. Free-living *Vitis* spp. near vineyards may serve as a source of GRBV inoculum for vector-mediated transmission to cultivated vines (Cieniewicz *et al.* 2018b; Perry *et al.* 2016, Bahder *et al.* 2016b). However, free-living vines are likely a minor source of inoculum considering GRBV is widespread in vineyards in California (Krenz *et al.* 2014).

None of the vineyard middle-row cover crop species, including legumes, tested positive for GRBV over three consecutive years (2016-2018). This suggested that cover crops do not likely serve as alternative GRBV inoculum source in a vineyard ecosystem. Although *S. festinus* can reproduce on *Vitis* spp., it does not complete its life cycle on this host (Preto *et al.* 2018a). Instead, *S. festinus* prefers to reproduce on hosts in the *Fabaceae* family (Preto *et al.* 2018b). This has profound implications for disease management. The relatively higher population of *S. festinus* caught on sticky cards in the Cabernet franc vineyard (Cieniewicz *et al.* 2018) compared to the Cabernet Sauvignon vineyard (this study) could be explained by its proximity to a riparian area. Preto *et al.* (2019) posit that *S. festinus* overwinter outside of vineyards and then reproduce in vineyard ground cover (cover crops or leguminous weeds), and feed on grapevine when the herbaceous groundcover dries up or is tilled. Additionally, higher levels of *S. festinus* aggregation were observed at the vineyard edge adjacent to an irrigation ditch (Preto *et al.* 2019). In our study, *S. festinus* populations in the vineyard canopy were reduced by ten-fold at 250 m from the edge of the Napa River riparian habitat into the Cabernet Sauvignon vineyard (**Figure 5-1**). This may be attributed to preferred hosts of *S. festinus* or a more suitable environment within the forested/riparian habitat. The significance of water sources to *S. festinus* phenology is

unknown and should be further investigated.

Management practices to minimize spread of viruses by controlling insect populations are typically only effective when the insect vector is also a colonizer of the crop (Halbert 2008), which is not the case for *S. festinus* and *Vitis* spp. (Preto *et al.* 2018a). Moreover, depending on chemical control of vectors to control spread of viruses in perennial crops like grape is usually not warranted, as a single missed or sub-optimal application can result in new virus infections (Halbert 2008). Therefore, although our study demonstrates that relatively higher *S. festinus* populations are associated with faster rates of GRBV spread, our results taken together with behavioral studies of *S. festinus* (Preto *et al.* 2018a,b, 2019) reinforce recommendations that red blotch disease management efforts should focus on removing sources of virus inoculum rather than reducing populations of *S. festinus*. However, cultural controls to reduce *S. festinus* habitat may help in slowing spread if replanting is not desired.

More research is needed to improve our understanding of red blotch disease epidemiology. First, more secondary spread studies are needed in vineyards in California and in other regions to assess whether our findings translate into similar spread patterns and how local viticulture practices and environment might affect secondary spread of GRBV. Second, the factors mediating the efficiency of GRBV transmission by *S. festinus* need to be investigated. For example, determining the required feeding times for virus acquisition and inoculation, cultivar effects on transmission efficiency, and virus factors involved in transmission will all help to advance our understanding of spread. Third, the capacity of *O. borealis*, *C. reductus*,

and *Melanoliarus sp.* at transmitting GRBV should be investigated. Additionally, the role of free-living *Vitis sp.* as GRBV reservoir for *S. festinus*-mediated transmission to cultivated grapevines should be examined. Finally, further investigating the phenology of *S. festinus* in vineyard ecosystems is important to refine disease management recommendations.

REFERENCES

Al Rwahnih, M., Dave, A., Anderson, M.M., Rowhani, A., Uyemoto, J.K., Sudarshana, M.R., Rwahnih, A. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103:1069–1076.

Bahder, B.W., Zalom, F.G., Jayanth, M., Sudarshana, M.R. 2016a. Phylogeny of Geminivirus Coat Protein Sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology* 106:1223–1230.

Bahder, B.W., Zalom, F.G., Sudarshana, M.R. 2016b. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Dis.* 100:1571–1574.

Beyer, B.A., Srinivasan, R., Roberts, P.M., Abney, M.R. 2017. Biology and management of the three-cornered alfalfa hopper (Hemiptera: Membracidae) in alfalfa, soybean, and peanut. *J. Integr. Pest Manag.* 8:1-10.

Blanco-Ulate, B., Hopfer, H., Figueroa-Balderas, R., Ye, Z., Rivero, R.M., Albacete, A., Pérez-Alfocea, F., Koyama, R., Anderson, M.M., Smith, R.J. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *J. Exp. Bot.* 68:1225–1238.

Caldwell, J.S. 1949. A generic revision of the treehoppers of the tribe Ceresini in America north of Mexico based on a study of the male genitalia. *Proc. United States Natl. Museum* 98:491–521.

Calvi, B. L. 2011. Effects of red-leaf disease on Cabernet Sauvignon at the Oakville experimental vineyard and mitigation by harvest delay and crop adjustment. M.S. thesis, University of California, Davis.

Cieniewicz, E., Perry, K., Fuchs, M. 2017. Grapevine Viruses: Molecular Biology, Diagnostics and Management. In *Grapevine Viruses: Molecular Biology, Diagnostics and Management*, Meng, Baozhong, Martelli, Giovanni, Golino, Deborah, Fuchs, Marc, Ed., Springer Verlag: Berlin, Germany, pp. 303–314 ISBN 978-3-319-57704-3.

Cieniewicz, E., Thompson, J.R., McLane, H., Perry, K.L., Dangl, G.S., Corbett, Q., Martinson, T., Wise, A., Wallis, A., O’Connell, J., Cox, K., Fuchs, M. 2018. Prevalence and genetic Diversity of grabloviruses in free-living *Vitis* spp. *Plant Dis.* 102:2308–2316.

Cieniewicz, E.J., Pethybridge, S.J., Gorny, A., Madden, L. V., McLane, H., Perry, K.L., Fuchs, M. 2017. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Res.* 241:156–162.

Cieniewicz, E.J., Pethybridge, S.J., Loeb, G., Perry, K., Fuchs, M. 2018. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108:94–102.

Dalton, D.T., Hilton, R.J., Kaiser, C., Daane, K.M., Sudarshana, M.R., Vo, J., Zalom, F.G., Buser, J.Z., Walton, V.M. 2019. Spatial associations of vines infected with grapevine red blotch virus in Oregon vineyards. *Plant Dis.* PDIS-08-18-1306-RE.

Gasperin-Bulbarela, J., Licea-Navarro, A.F., Pino-Villar, C., Hernández-Martínez, R., Carrillo-Tripp, J. 2019. First report of grapevine red blotch virus in Mexico. *Plant Dis.* PDIS-07-18-1227.

Halbert, S. 2008. Management of insect-vectored pathogens of plants. In *Encyclopedia of Entomology*, 2nd ed., Capinera, J. L. Ed., Springer: Netherlands, pp. 1336–1337.

Holzinger, W. E., Emeljanov, A. F., Kammerlander, I. 2002. The family Cixiidae (Spinola) 1839 (Hemiptera: Fulgoromorpha)—A review. *Denisia* 4:113-138.

Jensen, D.D. 1957. Transmission of peach yellow leaf roll virus by *Fiebertiella florii* and a new vector, *Osbornellus borealis*. J. Econ. Entomol. 50:668–672.

Krenz, B., Thompson, J.R., Fuchs, M., Perry, K.L. 2012. Complete genome sequence of a new circular DNA virus from grapevine. J. Virol. 86:7715.

Krenz, B., Thompson, J.R., McLane, H., Fuchs, M., Perry, K.L. 2014. Grapevine red blotch-associated virus is widespread in the United States. Phytopathology 104:1232–1240.

Kurtural, K., Martínez-Lüscher, J., Brillante, L., Yu, R., Plank, C., Smith R.J., Cooper, M., Oberholster, A. 2019. Grapevine red blotch virus may reduce carbon translocation leading to impaired grape berry ripening. J. Agric. Food Chem. in press.

Lim, S., Igori, D., Zhao, F., Moon, J.S., Cho, I.-S., Choi, G.-S. 2016. First report of grapevine red blotch-associated virus on grapevine in Korea. Plant Dis. 100: 1957.

Mueller, A. J., Dumas, B. A. 1975. Effects of stem girdling by the three- cornered alfalfa hopper on soybean yields. J. Econ. Entomol. 68:511-512.

Perry, K.L., McLane, H., Hyder, M.Z., Dangl, G.S., Thompson, J.R., Fuchs, M.F. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* sp. proximal to cultivated grapevines. Phytopathology 106:663–670.

- Poojari, S., Lowery, D.T., Rott, M., Schmidt, A.M., Úrbez-Torres, J.R. 2017. Incidence, distribution and genetic diversity of grapevine red blotch virus in British Columbia. *Can. J. Plant Pathol.* 39:201–211.
- Preto, C.R., Bahder, B.W., Bick, E.N., Sudarshana, M.R., Zalom, F.G. 2019. Seasonal dynamics of *Spissistilus festinus* (Say) (Hemiptera: Membracidae) in a Californian vineyard. *J. Econ. Entomol.* In press.
- Preto, C.R., Sudarshana, M.R., Bollinger, M.L., Zalom, F.G. 2018. *Vitis vinifera* (Vitales: Vitaceae) as a reproductive host of *Spissistilus festinus* (Hemiptera: Membracidae). *J. Insect Sci.* 18:1-7.
- Preto, C.R., Sudarshana, M.R., Zalom, F.G. 2018. Feeding and reproductive hosts of *Spissistilus festinus* (Say) (Hemiptera: Membracidae) found in Californian vineyards. *J. Econ. Entomol.* 111:2531–2535.
- Redak, R.A., Purcell, A.H., Lopes, J.R.S., Blua, M.J., Mizell III, R.F., Andersen, P.C. 2004. The biology of xylem fluid- feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. *Annu. Rev. Entomol.* 49:243–270.

Reynard, J.S., Brodard, J, Dubuis, N., Zufferey, V., Schumpp, O., Schaerer, S., Gugerli, P. 2018. Grapevine red blotch virus: Absence in Swiss vineyards and analysis of potential detrimental effect on viticultural performance. *Plant Disease* 102:651-655.

Ricketts, K.D., Gómez, M.I., Fuchs, M.F., Martinson, T.E., Smith, R.J., Cooper, M.L., Moyer, M.M., Wise, A. 2017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. *Am. J. Enol. Vitic* 68:127-135.

Saitou, N., Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–25.

Simko, I., Piepho, H.P. 2012. The area under the disease progress stairs: Calculation, advantage, and application. *Phytopathology* 102:381-389.

Thompson, J.D., Higgins, D.G., Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.

Varsani, A., Roumagnac, P., Fuchs, M., Navas-Castillo, J., Moriones, E., Idris, A., Briddon, R.W., Rivera-Bustamante, R., Murilo Zerbini, F., Martin, D.P. 2017.

Capulavirus and *Grablovirus*: two new genera in the family *Geminiviridae*. Arch. Virol. 162:1819–1831.

Wolfe, H. R. 1955. Transmission of the Western X disease virus by the leaf-hopper *Colladonus montanus* (Van D.). Plant Dis. Rep. 39:298-299.

Wolfe, H. R., Anthon, E. W., Jones, S. L. 1950. Transmission of western X-disease of peaches by the leafhopper *Colladonus geminatus* (Van D.). Phytopathology 40:971.

Xiao, H., Kim, W.S., Meng, B. 2015. Comparison and improvement of methodologies for isolation of quality RNA from diverse woody plant species and utilization in detection of viral pathogens. Virology Journal 12: 171.

Yepes, L.M., Cieniewicz, E.J., Krenz, B., McLane, H., Thompson, J.R., Perry, K.L., Fuchs, M. 2018. Causative role of grapevine red blotch virus in red blotch disease. Phytopathology 108:902-909.

CHAPTER 6
**TWO DISTINCT GENOTYPES OF *SPISSISTILUS FESTINUS* IN THE
UNITED STATES**

ABSTRACT

Spissistilus festinus is a frequent pest of leguminous crops in the southern United States, and a vector of grapevine red blotch virus. There is currently no information on the genetic diversity of *S. festinus*. In this study, populations of *S. festinus* were collected from various crops and geographic locations in 2015-2017, and fragments of the mitochondrial cytochrome C oxidase 1 (mt-COI) gene and the nuclear internal transcribed spacer 2 (ITS2) region were characterized by PCR and sequencing. Sequence analyses of the mt-COI and ITS2 genomic regions revealed two distinct genetic *S. festinus* lineages with all of the specimens from California comprising one phylogenetic clade, and all specimens from the southeastern U.S. comprising another clade, regardless of host and year of collection. The nucleotide sequence variation of the mt-COI gene fragment revealed up to 12.1% sequence divergence between the two phylogenetic clades. A diagnostic polymerase chain reaction was developed to distinguish between specimens of the two genotypes of *S. festinus*. These results suggest the existence of two genotypes, and potentially cryptic species, within *S. festinus*.

*This chapter is in preparation for submission as a short communication to the journal *Insects*: Cieniewicz E.J., Poplaski, V., Brunelli M., Dombroskie, J., and Fuchs M.

(2019) Two distinct genotypes of *Spissistilus festinus* in the United States. *Insects*, in preparation.

INTRODUCTION

The three-cornered alfalfa hopper, *Spissistilus festinus* (Say) (Membracidae), is an agricultural pest of legume crops, in particular peanuts, soybeans, and alfalfa in the southern United States (Beyer *et al.* 2017). *Spissistilus festinus* transmits grapevine red blotch virus (GRBV) from infected to healthy grapevines in the greenhouse (Bahder *et al.* 2016) and is also associated with red blotch disease spread in vineyards in California (Cieniewicz *et al.* 2018). Although *S. festinus* oviposites in green grapevine tissue, it does not survive to adulthood on grapevines (Preto *et al.* 2018) and is not considered a direct pest of grapevine. An improved understanding of the biology of *S. festinus* is critical to progressing research on treehopper-virus-host interactions.

Molecular markers have been widely used to determine phylogenetic relationships among insects. For sub-species phylogenetic characterization, several markers have been applied but the mitochondrial cytochrome C oxidase (mtCOI) “barcode” region is the most widely used (Caterino *et al.* 2000). For example, the mtCOI is routinely used to resolve sub-species phylogenetic relationships and cryptic species identification within *Bemisia tabaci*, one of the most agriculturally important hemipteran pests globally (Frohlich *et al.* 1999; Dinsdale *et al.* 2010; Perring *et al.* 2018). Awareness of subspecies variation and cryptic species is important because *B. tabaci* biotypes may have different virus transmission efficiencies (Bedford *et al.* 1994; Chowda-Reddy *et al.* 2012; Shi *et al.* 2017) and susceptibility to insecticides (Costa *et al.* 2019; Luo *et al.* 2010; Horowitz *et al.* 2005).

As *S. festinus* is a long-known pest of leguminous crops in the southeastern U.S. and a recently identified problem for grape production as a vector of GRBV in

California, understanding population structure of this treehopper is important to better inform the management of this pest in agricultural production systems. In this study we investigated genetic diversity of *S. festinus* populations collected over multiple years, geographic locations, and from various crops. We characterized the genetic variation of a 710-nt ‘barcode’ region of the mitochondrial cytochrome C oxidase subunit 1 (mt-COI) as well as the 500-nt nuclear internal transcribed spacer 2 region (ITS2) of representative specimens of each *S. festinus* population.

MATERIALS AND METHODS

***Spissistilus festinus* specimen collection and DNA extraction**

S. festinus adults were collected from various crops (clover, alfalfa, soybean, peanut, grape, and weeds) in multiple states in 2015, 2016, and 2017 (**Table 6-1**). At each site, between 12 and 25 *S. festinus* were captured. *Spissistilus festinus* adults collected from *Vitis vinifera* ‘Cabernet franc’ in Napa County, California were collected on yellow sticky cards in June and July of 2015 and 2016 (Cieniewicz *et al.* 2018). All remaining specimens were collected by sweep netting. Specimens from Alabama, Mississippi, Georgia, North Carolina, and Virginia were preserved in 100% ethanol upon collection. Ethanol was drained for shipment to the laboratory in Geneva, New York and specimens were then stored at -20°C until nucleic acid extraction. Specimens from Parlier, Lodi, Knights Landing, and Davis in California were immediately shipped following collection and stored at -20°C until nucleic acid extraction. Total DNA was extracted from individual specimens using the E.Z.N.A Insect DNA kit (OMEGA Biotek) and DNA was stored at -20°C for polymerase chain

reaction (PCR) using specific primers.

Table 6-1 Sites of *Spissistilus festinus* collection, date of collection and mt-COI primers used for PCR characterization.

Location	Latitude	Longitude	Host	Date Collected	Primers
Davis, California	38.5381	-121.8813	Clover	October 2017	HCO1298/LCO1490 ^a
Knights Landing, California	38.7914	-121.7338	Alfalfa	October 2015	HCO1298/LCO1490
Knights Landing, California	38.7894	-121.7259	Alfalfa	October 2016	HCO1298/LCO1490
Knights Landing, California	38.7660	-121.7911	Alfalfa	October 2016	HCO1298/LCO1490
Lodi, California	38.1152	-121.4343	Alfalfa	October 2017	HCO1298/LCO1490
Rutherford, California	38.4572	-122.4103	Grape	June-July 2015	HCO1298/LCO1490
Rutherford, California	38.4572	-122.4103	Grape	June-July 2016	HCO1298/LCO1490
Parlier, California	36.5956	-119.5117	Alfalfa	November 2015	HCO1298/LCO1490
Auburn, Alabama	32.5927	-85.4858	Soybean	October 2016	SETCAHfor/LepR1 ^b
Painter, Virginia	37.5859	-75.7821	Weeds	July 2017	SETCAHfor/LepR1
Pantego, North Carolina	35.6172	-76.7568	Soybean	July-August 2017	SETCAHfor/LepR1
Starkville, Mississippi	33.4815	-88.7841	Peanut	October 2016	SETCAHfor/LepR1
Tifton, Georgia	31.4887	-83.5413	Alfalfa	August 2017	SETCAHfor/LepR1

^aPrimers HCO1298 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') were developed by Folmer *et al.* (1994).

^bPrimer SETCAHfor (5'-TTTCTACAAGCCACAGGGATATTGG-3') was developed in this study, and primer LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') was developed by Hebert *et al.* (2004).

PCR-amplification and sequencing of *S. festinus* genomic fragments

For the California samples, primers ‘LCO1490’ and ‘HCO1298’ were used to amplify a 710-bp fragment from the mitochondrial cytochrome C oxidase subunit 1 gene (mt-COI) (Folmer *et al.* 1994) by PCR. HotStar taq polymerase (Qiagen) was used for all PCRs at manufacturer-suggested conditions, with an annealing temperature of 54°C. Unexpectedly, no mt-COI barcode region was amplified with these primers for the *S. festinus* populations from Alabama, Mississippi, Georgia, North Carolina, and Virginia.

To recover the mt-COI barcode region from the southeastern U.S. *S. festinus* population, tRNA-based primers ‘tRWF2_t1’ and ‘tRWF2_t1’ (both containing the universal primer sequence of ‘M13F’ at the 5’ end) were used in a cocktail as forward primers in combination with the alternative universal reverse primer ‘LepR1’ to amplify a larger region and recover the region in which the HCO1298/LCO1490 primers failed (Park *et al.* 2010). A specimen from the Alabama collection was used for this assay. The resulting 900-bp amplicon was directly Sanger-sequenced at the Cornell University Biotechnology Resource Center (Ithaca, NY) using the primers ‘M13F’ and ‘LepR1’. Based on the 900-bp sequence, a forward primer was then designed to anneal at the same region as the ‘LCO1490’ primer to amplify a fragment of the mt-COI barcode region from *S. festinus* populations from the southeastern United States by PCR. For these *S. festinus* specimens, the primers ‘SETCAHfor’ (5’-TTTCTACAAGCCACAGGGATATTGG-3’) and ‘LepR1’ (Hebert *et al.* 2004) were used to amplify a 710 bp mt-COI barcode fragment.

All PCRs were resolved using electrophoresis on 2% agarose gels. PCR

products were then purified using ExoSAP-IT (Applied Biosystems, Foster City, CA) and directly sequenced in both directions using the same primers. Sequences were obtained from between 8 and 24 specimens per *S. festinus* population, depending on the number collected for each population. Sequences were assembled, manually edited, and aligned (ClustalW) using the Lasergene software suite (version 15.1.1). Sequences from each population were aligned and the resulting consensus sequence was then aligned with other consensus sequences. Based on quality of sequences, 547 nucleotides were considered in the mt-COI alignment. Phylogenies were constructed using the neighbor-joining method (Saitou and Nei 1987) and branching confidence was estimated using 1000 bootstrap replicates. A mt-COI sequence from *S. festinus* collected in Arizona was retrieved from Genbank (KF919668.1) to determine its relationship with mt-COI sequences determined in this study. Accession KF919668.1 was the only *S. festinus* mt-COI sequence in Genbank that was long enough to be included in the alignment.

The internal transcribed spacer 2 (ITS2), a non-coding region between the genes encoding the 5.8S and 28S ribosome subunit was also amplified by PCR and sequenced for at least two specimens from each *S. festinus* population. Primers ‘Cas5p8Fc’ and ‘Cas28b1d’ were used to amplify a 500 bp fragment containing the ITS2 region (Ji *et al.* 2003). Sequences were analyzed as described above. Primer sequences were removed from the resulting alignment and a phylogeny was constructed as described above, with 445 nucleotides considered.

Diagnostic PCR for distinction of *S. festinus* genotype

Based on regions of the mt-COI alignment that were highly variable between the two *S. festinus* genotypes, PCR primers were designed for specific detection of each genotype. The primers designed to amplify a 496 bp fragment of the mt-COI gene of the “West” genotype of *S. festinus* in PCR are ‘TCAHcoiWestF’ (5'-GAATTGGGACAACCAGGACC-3') and ‘TCAHcoiWestR’ (5'-AACTGGAAGAGACATGAGG-3'). Primers designed for the “East” genotype of *S. festinus* are ‘TCAHcoiEastF’ (5'-CCTCCGTCTATAATTCTACTCCTTA-3') and ‘TCAHcoiEastR’ (5'-CCTGCGTAAGTG TAGGGAGAAAATGGCG-3'), amplifying a 145 bp region in PCR. These primers were combined for 12.5 µL PCR reactions with the following reaction setup: 2.5 µL of 10X PCR Buffer (Qiagen), 1.0 µL of each primer at 10 µM, 0.25 µL of dNTP mix (10 mM each nucleotide) (Thermo Scientific), 0.125 µL of HotStar Taq polymerase (Qiagen), and 4.625 µL of nuclease-free water. PCRs were run at the following temperature cycling protocol: 95°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 59°C for 60 seconds, 72°C for 60 seconds; 72°C for 10 minutes. PCR products were resolved by gel electrophoresis on 2% agarose gels in 1X tris acetate EDTA buffer at 100 volts for 35 minutes, and then stained with GelRED (Biotium) and imaged under UV light.

The sensitivity and specificity of the diagnostic PCR was validated by testing for cross-amplification of *S. festinus* genotypes and for amplification of the mt-COI gene fragments from other hemipteran insects, respectively. The DNA extracts from various hemiptera were available in a laboratory collection from previous studies in California and New York vineyards (Cieniewicz *et al.* 2018, 2019).

RESULTS

Two distinct *S. festinus* genotypes based on geography

Populations of *S. festinus* had low within-population nucleotide sequence variation (between 0 and 1%) within the 547 bp mt-COI genomic region. Phylogenetic analyses of mt-COI sequences revealed two distinct clades (**Figure 6-1**). The populations from California and the specimen from Arizona formed one clade and were indistinguishable from each other, regardless of source crop, location within California, or time of collection. Similarly, the populations from the southeastern U.S. also formed one clade and were indistinguishable from each other. The nucleotide sequence variation within the “West” clade ranged from 0.0-0.9% and 0.0-0.4% in the “East” clade. Nucleotide sequence divergence between these two clades ranges from 11.4-12.1%, within the 547 nucleotides considered (**Figure 6-1**). The two distinct clades of mt-COI sequences suggested two genotypes of *S. festinus*.

Based on ITS2 sequences, populations of *S. festinus* also grouped into two distinct phylogenetic clades, one with all the specimens from California and the Arizona accession and one with specimens from the southeastern U.S. In comparison to the mt-COI sequences, there was higher nucleotide sequence conservation among the ITS2 sequences. Nucleotide differences were only observed at three positions in the 445 nt-long alignment considered, within the ITS2 region (**Figure 6-2**).

Additionally, the 5.8S and 28S motifs and the predicted ITS2 region were detected by the annotation function in the ITS2 database (**Figure 6-2**) (Ankenbrand *et al.* 2015). The two distinct clades of ITS2 sequences were consistent with two genotypes of *S. festinus*.

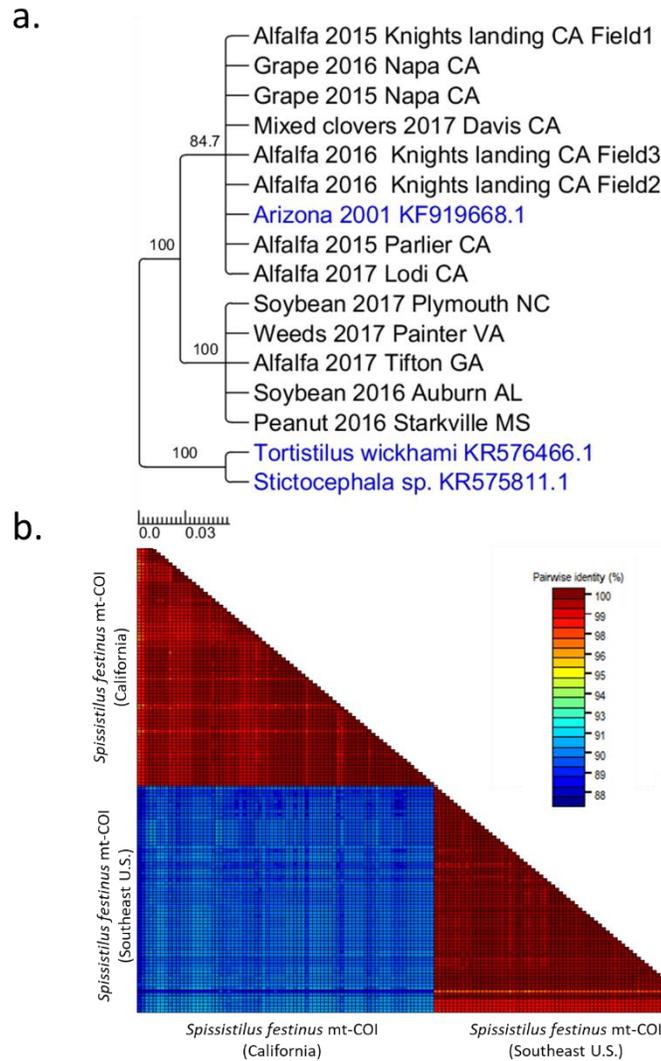


Figure 6-1 (a) Neighbor-joining phylogenetic tree of mitochondrial cytochrome C oxidase I (mt-COI) representative sequences from *Spissistilus festinus* populations collected from various locations, years, and crops in California and the southeastern U.S, indicating two distinct genotypes based on geographic location. *Stictocephala* sp. and *Tortistilus wickhami* mt-COI were used as outgroups. Sequences derived from Genbank are denoted in blue. (b) Pairwise identity matrix based on ClustalW alignment of mt-COI sequences from individual specimens from this study. Each square depicts a pairwise comparison, where red indicates high (>98% sequence identity) and blue indicates lower (88-90% sequence identity). This indicates high homogeneity among sequences within populations, and a distinction between mt-COI sequences from *S. festinus* specimens from the western and eastern U.S.

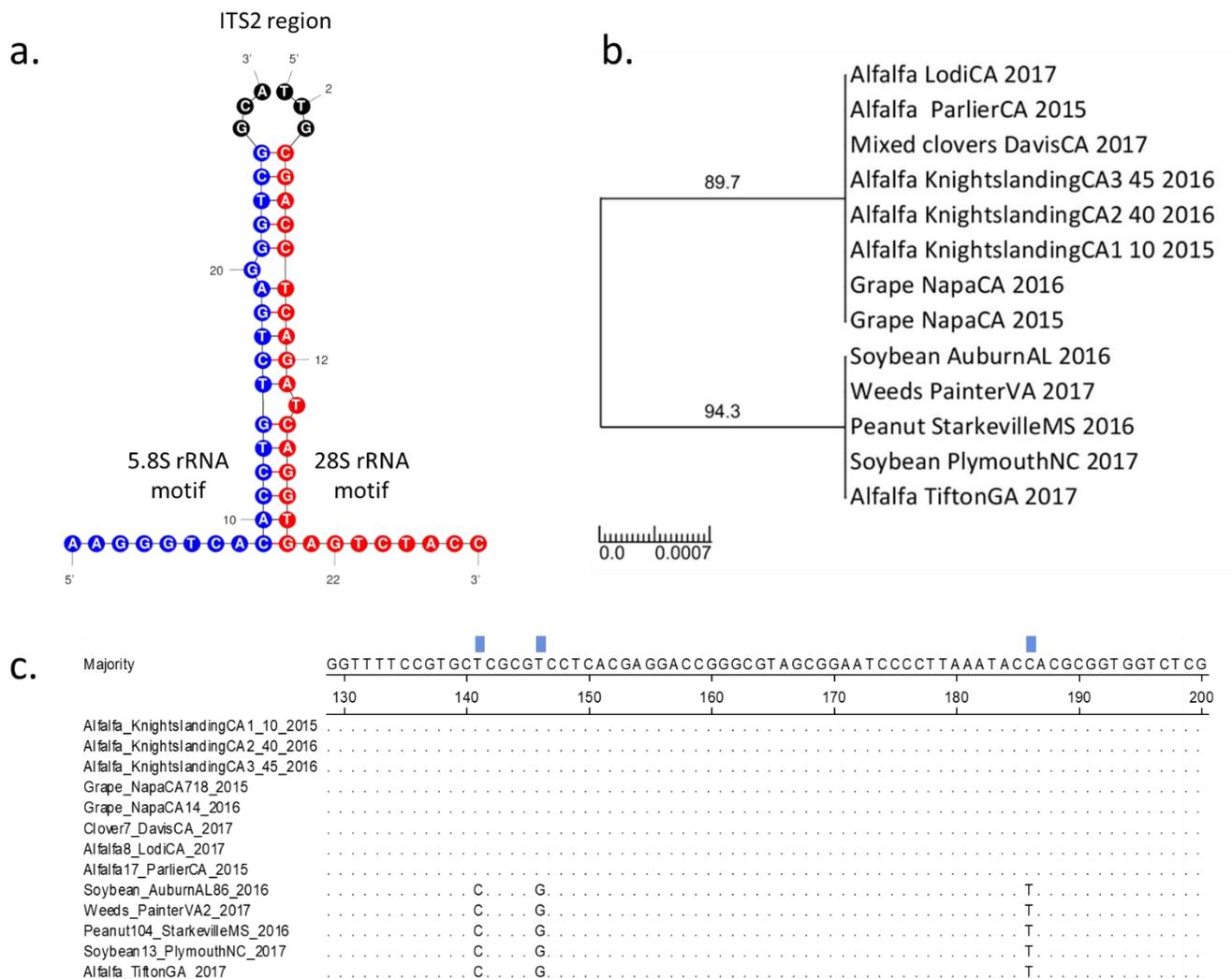


Figure 6-2 Internal transcribed spacer 2 (ITS2) (a) depiction of 5.8S (blue) and 28S (red) motifs denoting the boundaries of the ITS2 region (black) used as a nuclear marker for subspecies genetic variation among populations of *Spissistilus festinus*. (b) Neighbor-joining unrooted phylogenetic tree of ITS2 sequences of *S. festinus* specimens collected from various crops, years, and locations indicated in the node names. Numbers at branches indicate bootstrap support (1000 bootstrap replicates). (c) Segment of ClustalW alignment of ITS2 sequences (495 residues considered) in which the three nucleotide differences at positions 141, 146, and 186 were observed between *S. festinus* specimens from California and specimens from the southeastern U.S.

Diagnostic polymerase chain reaction for *S. festinus* genotypes

A diagnostic PCR assay was designed to distinguish between the “West” and “East” genotypes of *S. festinus* with the amplification of a 496 bp and 145 bp DNA amplicon, respectively (**Figure 6-3a**). The sensitivity of this assay was determined through the specific amplification of DNA targets only from *S. festinus* specimens (**Figure 6-3a**). The specificity of the assays was verified through a lack of amplification of DNA targets from other Membracidae (*Acutalis* sp., *Stictocephala* sp., *Campylenchia* sp., *Entylia carinata*), Cercopidae (*Philaenus* sp., *Clastoptera* sp.), Fulgoroidea (Delphacidae sp., Cixiidae sp.), Cicadellidae (*Colladonus reductus*, *Osbornellus borealis*, *Scaphytopius* sp., *Empoasca* sp., *Erythroneura elegantula*, *Euscelis* sp.), Aphididae, Aleyrodidae, and Psylloidea (**Figure 6-3b**).

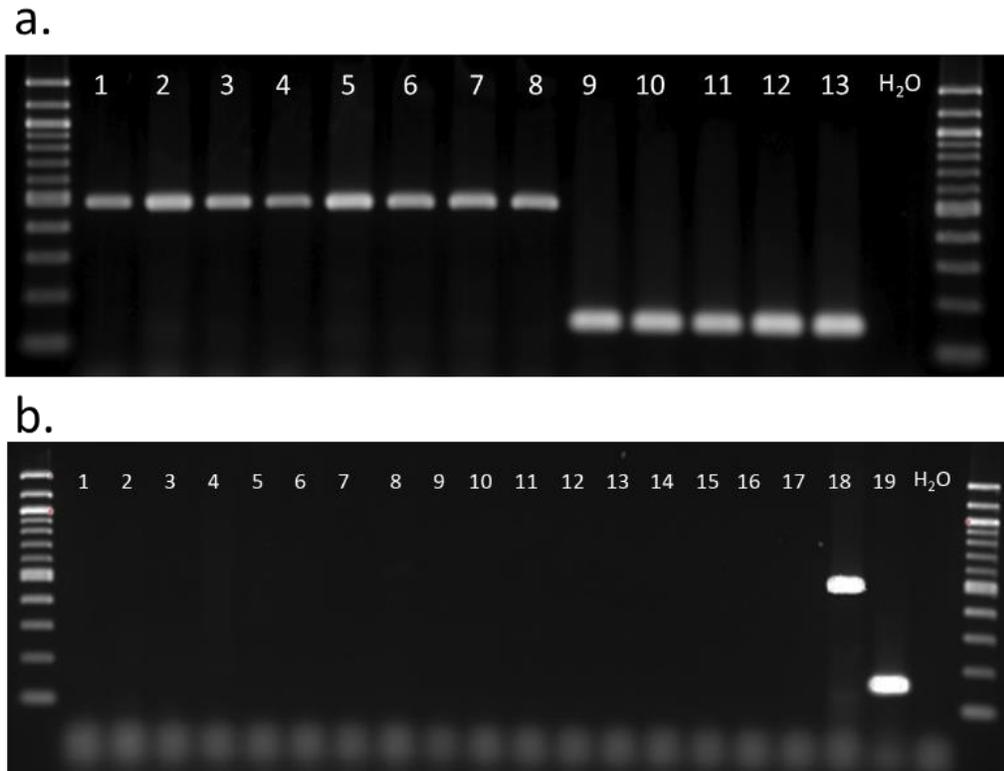


Figure 6-3 (a) Diagnostic polymerase chain reaction for DNA sequence-based identification of specimens of the *S. festinus* genotype from California (lanes 1-8) and of the *S. festinus* genotype from the southeastern U.S. (lanes 9-13). (b) Test of specificity of diagnostic PCR assay against other Membracidae (lanes 1-4), Cercopidae (lanes 5-6), Fulgoroidea (lanes 7-8), Cicadellidae (lanes 9-14), Aphididae (lane 15), Aleyrodidae (lane 16), Psylloidea (lane 17) compared to the “West” *S. festinus* genotype (lane 18) and the “East” *S. festinus* genotype (lane 19).

DISCUSSION

In this study we collected populations of *Spissistilus festinus* from various geographic locations, crops, and in different years. We analyzed genetic variation within and between populations by sequencing a PCR-amplified fragment of the mt-COI gene, and the nuclear noncoding ITS2 region, downstream of the 5.8S rRNA region and upstream of the 28S rRNA region. For both genetic markers there was no detectable variation within populations, defined as collections from the same location at the same time. Based on both genetic markers, two distinct genotypes of *S. festinus* were observed, in which the populations from the southeastern United States grouped in one clade, and all populations from California grouped into another clade.

The two genetic markers in this study were selected due to their widespread use in insect systematics and availability of degenerate PCR primers (Hwang and Kim 1999; Park *et al.* 2011; Ji, Zhang, and He 2003). We acknowledge that there may be more accurate genetic markers or methods, such as single nucleotide polymorphism (SNP) datasets for resolving population genetics and phylogeography (Kjer *et al.* 2016; Wosula *et al.* 2017). However, although the ITS2 region displayed very low variability between *S. festinus* genotypes (only three nucleotide substitutions in a 445-nt ITS2 region), the variability in the ITS2 region did support the mt-COI results and strengthened the identification of two *S. festinus* genotypes.

There is currently no known biological difference between the “West” and “East” *S. festinus* genotypes. It is also unknown if these two genotypes are reproductively compatible. This should be evaluated to further resolve the taxonomy of *S. festinus*. Moreover, determining the genotype dispersal in other areas, for

example in the central U.S. and South America would further inform the subspecies genetic variation for *S. festinus* and may shed light on dispersal patterns. The diagnostic PCR assay reported in this study could facilitate *S. festinus* genotype identification and help to refine the actual geographic range of *S. festinus*.

The geographic range of *S. festinus* is reportedly throughout the southern U.S. and in South America (Caldwell 1949; Beyer *et al.* 2017), however the northern limits of its range are not well described, and should be further investigated. Nonetheless, the Cornell University Insect Collection has a single *S. festinus* specimen from Long Island, New York collected in 1933 although we did not find it in a yellow sticky card survey in a vineyard on Long Island in 2017 or 2018 (Cieniewicz *et al.* 2019). Therefore, it is important to be vigilant for the potential for *S. festinus* to travel north or east, or to be carried in shipments of plant materials, even transiently. Additionally, the geographic range of *S. festinus* may shift in light of climate change and changes in agricultural practices.

Understanding the implications of different *S. festinus* genotypes, and potential cryptic species, on biological factors is of interest. For example, these two genotypes should be further evaluated for differences in GRBV transmission capability and efficiency, insecticide resistance, and host range. In vineyards in Napa County, California, *S. festinus* appear to be localized toward the edges of vineyards, primarily reproducing in cover crop species (Preto *et al.* 2019), and at higher populations at vineyard edges near water sources (Cieniewicz *et al.* 2018; Preto *et al.* 2019). To gain a comprehensive understanding of the impact of *S. festinus* in vineyards, information on the phenology of *S. festinus* and spread of GRBV in multiple vineyard ecosystems

is needed. The diagnostic assay reported herein could also assist in these studies.

REFERENCES

- Ankenbrand, M. J., Keller, A., Wolf, M., Schultz, J., and Forster, F. 2015. ITS2 Database V: Twice as much. *Mol. Biol. Evol.* 32:3030–3032.
- Bahder, B. W., Zalom, F. G., Jayanth, M., and Sudarshana, M. R. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology*. 106:1223–1230.
- Bedford, I. D., Briddon, R. W., Brown, J. K., Rosell, R. C., and Markham, P. G. 1994. Geminivirus transmission and biological characterisation of *Bemisia tabaci* (Gennadius) biotypes from different geographic regions. *Ann. Appl. Biol.* 125:311–325.
- Beyer, B. A., Srinivasan, R., Roberts, P. M., and Abney, M. R. 2017. Biology and management of the threecornered alfalfa hopper (Hemiptera: Membracidae) in alfalfa, soybean, and peanut. *J. Integr. Pest Manag.* 8:1–10.
- Caldwell, J. S. 1949. A generic revision of the treehoppers of the tribe Ceresini in America north of Mexico based on a study of the male genitalia. *Proc. United States Natl. Museum.* 98:491–521.
- Caterino, M. S., Cho, S., and Sperling, F. A. H. 2000. The current state of insect molecular systematics: A thriving Tower of Babel. *Annu. Rev. Entomol.* 45:1–54.

- Chowda-Reddy, R., Kirankumar, M., Seal, S. E., Muniyappa, V., Valand, G. B., Govindappa, M., and Colvin, J. 2012. *Bemisia tabaci* phylogenetic groups in India and the relative transmission efficacy of tomato leaf curl Bangalore virus by an Indigenous and an exotic population. *J. Integr. Agric.* 11:235–248.
- Cieniewicz, E. J., Pethybridge, S. J., Loeb, G., Perry, K., and Fuchs, M. 2018. Insights into the ecology of *grapevine red blotch virus* in a diseased vineyard. *Phytopathology.* 108:94–102.
- Costa, H. S., Brown, J. K., Sivasupramaniam, S., and Bird, J. 2019. Regional distribution, insecticide resistance, and reciprocal crosses between the A and B biotypes of *Bemisia tabaci*. *Insect Sci. Applic.* 14:255–266.
- Dinsdale, A., Cook, L., Riginos, C., Buckley, Y. M., and De Barro, A. P. 2010. Refined global analysis of *Bemisia tabaci* (Hemiptera: Sternorrhyncha: Aleyrodoidea: Aleyrodidae) mitochondrial cytochrome oxidase 1 to identify species level genetic boundaries. *Ann. Entomol. Soc. Am.* 103:196–208.
- Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 3:294–299.
- Frohlich, D. R., Torres-Jerez, I., Bedford, I. D., Markham, P. G., and Brown, J. K. 1999. A phylogeographical analysis of the *Bemisia tabaci* species complex based on mitochondrial DNA markers. *Mol. Ecol.* 8:1683–1691.

- Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H., and Hallwachs, W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astrartes fulgerator*. *Proc. Natl. Acad. Sci.* 101:14812–14817.
- Horowitz, A. R., Kontsedalov, S., Khasdan, V., and Ishaaya, I. 2005. Biotypes B and Q of *Bemisia tabaci* and their relevance to neonicotinoid and pyriproxyfen resistance. *Arch. Insect Biochem. Physiol.* 58:216–225.
- Hwang, U.-W., and Kim, W. 1999. General properties and phylogenetic utilities of nuclear ribosomal DNA and mitochondrial DAN commonly used in molecular systematics. *Korean J. Parasitol.* 37:215–228.
- Ji, Y. J., Zhang, D. X., and He, L. J. 2003. Evolutionary conservation and versatility of a new set of primers for amplifying the ribosomal internal transcribed spacer regions in insects and other invertebrates. *Mol. Ecol. Notes.* 3:581–585.
- Kjer, K., Borowiec, M. L., Frandsen, P. B., Ware, J., and Wiegmann, B. M. 2016. Advances using molecular data in insect systematics. *Curr. Opin. Insect Sci.* 18:40–47.
- Luo, C., Jones, C. M., Devine, G., Zhang, F., Denholm, I., and Gorman, K. 2010. Insecticide resistance in *Bemisia tabaci* biotype Q (Hemiptera: Aleyrodidae) from China. *Crop Prot.* 29:429–434.
- Park, D.-S., Footitt, R., Maw, E., and Hebert, P. D. N. 2011. Barcoding bugs: DNA-based identification of the true bugs (Insecta: Hemiptera: Heteroptera). *PLoS One.* 6:1–9.

- Park, D.-S., Suh, S.-J., Oh, H.-W., and Hebert, P. D. 2010. Recovery of the mitochondrial COI barcode region in diverse Hexapoda through tRNA-based primers. *BMC Genomics*. 11:1–7.
- Perring, T. M., Stansly, P. A., Liu, T. X., Smith, H. A., and Andreason, S. A. 2018. Sustainable management of arthropod pests of tomato whiteflies: Biology, ecology, and management. *Sustain. Manag. Arthropod Pests Tomato*. :73–110.
- Preto, C. R., Bahder, B. W., Bick, E. N., Sudarshana, M. R., and Zalom, F. G. 2019. Seasonal dynamics of *Spissistilus festinus* (Hemiptera: Membracidae) in a Californian vineyard. *J. Econ. Entomol.* doi:10.109.
- Preto, C. R., Sudarshana, M. R., Bollinger, M. L., and Zalom, F. G. 2018. *Vitis vinifera* (Vitales: Vitaceae) as a reproductive host of *Spissistilus festinus* (Hemiptera: Membracidae). *J. Insect Sci.* 18:1–7.
- Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–25.
- Shi, X., Tang, X., Zhang, X., Zhang, D., Li, F., Yan, F., Zhang, Y., Zhou, X., and Liu, Y. 2017. Transmission efficiency, preference and behavior of *Bemisia tabaci* MEAM1 and MED under the influence of tomato chlorosis virus. *Front. Plant Sci.* 8:2271.
- Wosula, E. N., Chen, W., Fei, Z., and Legg, J. P. 2017. Unravelling the genetic diversity among cassava *Bemisia tabaci* whiteflies using NextRAD sequencing. *Genome Biol. Evol.* 9:2958–2973.

CHAPTER 7

RED BLOTCH ECOLOGY: CONCLUSIONS, MANAGEMENT IMPLICATIONS, AND FUTURE DIRECTIONS

Since 2014 when these studies were initiated, we have amassed substantial information on the epidemiology and ecology of grapevine red blotch disease in California and New York. These studies have collectively contributed to data-driven management recommendations for grape growers. For example, we have demonstrated that GRBV is spreading in vineyards in California (Cieniewicz *et al.* 2017), and that spatiotemporal spread is associated with the spatial patterns of *Spissistilus festinus* (Cieniewicz *et al.* 2018a). These vineyard studies have provided biological context for the recommendation by Ricketts *et al.* (2017) to rogue and remove infected vines if disease incidence is less than 30%, but to remove the entire vineyard and re-plant with vines derived from virus-tested nursery stock if red blotch disease incidence is greater than 30%. Spread occurs at a faster rate (5-10% annually) when initial disease incidence is high (greater than 25%), but slower spread (1-2% annually) occurs if initial disease incidence is low (less than 10%) (Cieniewicz *et al.* 2017, 2019). We have also provided evidence that *S. festinus* is in low abundance in vineyards, and stays near the edge of a vineyard near a water source (Cieniewicz *et al.* 2018a, 2019), a trend which was corroborated in an independent study focused on under-canopy cover crops in California vineyards (Preto *et al.* 2019). The scarcity of *S. festinus* in vineyards, despite occurrence of spread, strengthens the rationale behind recommendations to focus on removal of GRBV inoculum sources, rather than

reducing *S. festinus* abundance.

Our study on the distribution and diversity of grapevine viruses in free-living grapevines in California suggests that the direction of spread of GRBV is predominantly from commercial vineyards to wild vines, rather than wild vines serving as a substantial inoculum source (Cieniewicz *et al.* 2018b). Though, wild grapevines should still be considered as potential sources of GRBV inoculum near newly planted vineyards and nursery operations in areas where GRBV is spreading, like Napa County, California.

In California, GRBV is spreading in vineyards, is spatially associated with *S. festinus* in vineyards, and is prevalent in wild grapevines near vineyards. In contrast, GRBV spread has not been observed in New York, *S. festinus* is extremely rare, no other vector candidates have been identified, and GRBV was not detected in wild grapevines in an expansive survey. These patterns may be different in time with climate change and changes in viticulture practices. Nonetheless, current management strategies in California should be focused on frequent scouting for GRBV symptoms and spread and removal of inoculum sources. In New York, it appears to be less critical to manage GRBV spread, but considering the detrimental effect of GRBV on fruit quality, planting vines derived from virus-tested nursery stocks should be prioritized in all grape growing regions.

To date, 114 accessions of full GRBV genome sequences have been deposited in GenBank. There are two major phylogenetic clades. The majority of sequences (74 of 114) are in phylogenetic clade 2, which has lower intra-clade variability (0-4.6% divergence). Forty of the sequences are in clade 1, in which the intra-clade variability

is 0-6.1%. Interclade variability ranges from 3.7-9.2% divergence (not accounting for recombination). There is no geographic or varietal specificity of these two genetic variants, and currently there is no known biological difference between the two variants. We have infectious bitmer clones representing both clades (Yepes *et al.* 2018), so experiments to test the differences between the two genetic variants are feasible. For example, we could investigate differences in symptom induction and severity, GRBV titer and rate of systemic movement, host range, transmission efficacy of the different GRBV genetic variants in replicated experiments.

Though we have learned a lot about GRBV epidemiology and ecology in recent years, more questions have arisen. Some important questions remain unanswered, in particular on the topics of transmission biology, host range, and red blotch ecology in other viticulture regions. Very little is known about genome expression and replication of grabloviruses, and information from other genera of geminiviruses may not be applicable to virus species of this divergent genus. It is also important to explore the potential for wild *Vitis* virus 1 (WVV1) to cause red blotch disease in grape cultivars. These potential research areas are discussed in this chapter and in more detail in Appendices II and III.

SPREAD OF GRBV AND PHENOLOGY OF SPISSISTILUS FESTINUS IN VINEYARDS

Studies on red blotch epidemiology have thus far been focused in Napa County, California and Suffolk County, New York (Cieniewicz *et al.* 2017, 2019). A recent study suggested that secondary spread of GRBV is occurring in at least two

locations in Oregon, though by an unknown vector (Dalton *et al.* 2019). Limited information on the ecology and phenology of *S. festinus* in vineyards in Napa County is available (Cieniewicz *et al.* 2018a; Preto *et al.* 2019). Regional environmental factors and differences in viticulture practices likely impact GRBV epidemiology. Therefore, studies on secondary spread and *S. festinus* ecology in vineyards are needed in other viticulture regions in order to devise optimal management strategies to mitigate GRBV spread. GRBV has been detected throughout North America in most areas where grapes are grown (Krenz *et al.* 2014, **Figure A7-2**). Other areas to consider for studies on secondary spread of GRBV would be more southern regions, where *S. festinus* is known to be abundant in legume crops (Beyer *et al.* 2017).

A major limiting factor in large-scale studies and diagnostics for commercial purposes is that detection assays for GRBV can be cost-prohibitive. Currently, only nucleic-acid based methods of virus testing, such as PCR (Krenz *et al.* 2014) or recombinase polymerase amplification technology (Li *et al.* 2017), are available to growers. Therefore, reliance on evaluation of symptoms and infrequent virus testing can contribute to the exacerbation of GRBV epidemics. A high throughput and less expensive method of indexing vines for GRBV would be useful for growers and for researchers. Thus far, attempts to develop a serological test for GRBV have not been successful. However, transcriptome (Vargas-Ascencio *et al.* 2019) and proteome studies (Buchs *et al.* 2018) may provide new opportunities for producing antibodies. Preliminary research suggests that loop-mediated isothermal amplification (LAMP) is a sensitive detection method for GRBV, and may have applicability for on-farm testing at a much lower cost than methods currently available (J. Thompson and K.

Perry, personal communication). Although extremely promising, this assay needs to be validated on-farm to assess its usefulness. Another potential avenue for large scale studies of GRBV spread is remote sensing. However, there is much research needed in order to make remote sensing feasible for red blotch surveillance. For example, identification of detectable and specific GRBV signatures, imaging mechanics in different environmental conditions, and differences in cultivar and training systems are obstacles that need to be addressed before remote sensing is applicable to GRBV surveillance.

GRABLOVIRUSES: A RED BLOTCH DISEASE COMPLEX?

Wild Vitis virus 1 (WVV1) is another member of the genus *Grablovirus*. It was discovered in free-living grapevines during a survey of wild grapevines for GRBV. The multiplex PCR used for GRBV detection uses primers flanking a 315-bp region within the C1 (RepA) open reading frame (ORF) and another set of primers flanking a 230-bp region within the V1 (coat protein) ORF. For several wild vines sampled from Napa County, California, the multiplex PCR was yielding only the RepA amplicon, but not the CP amplicon. These samples were then subjected to rolling circle amplification, and cloning and sequencing of the circular DNA products. This revealed a novel virus with 57-59% sequence identity to GRBV (Perry *et al.* 2018). WVV1 has thus far only been detected in wild grapevines, but is widespread throughout northern California in Napa, Sonoma, Solano, and Glenn counties (Cieniewicz *et al.* 2018b).

It is important to determine if WVV1 can cause red blotch disease. Many of

the major grapevine virus diseases are associated with multiple virus species. Leafroll disease is associated with five virus species, GLRVaV-1, 2, 3, 4, 7 (Martelli *et al.* 2012) and -13 (Ito and Nakaune, 2016). Fanleaf degeneration and decline is associated with grapevine fanleaf virus and other viruses of the family *Secoviridae* (Martelli 2014). The rugose wood complex is associated with multiple viruses of the genus *Vitivirus* (Martelli 2014). We should therefore consider the possibility that red blotch disease may be caused by more than one virus.

Experiments should be conducted to determine if WVV1 causes red blotch disease symptoms in commercial cultivars. In these experiments, infectious clones of WVV1 could be engineered as previously described (Yepes *et al.* 2018) and agro-inoculated into healthy grapevines, that then would be monitored for symptom development and virus replication. Another potential experiment would be to graft WVV1-infected material onto healthy scion and monitor for symptoms conferred to the scion. In collaboration with the Perry lab at Cornell, infectious clone engineering is in progress. We have also propagated cuttings from WVV1-infected wild grapevines in the greenhouse.

Another important question to address is whether WVV1 is transmitted by *S. festinus* or another membracid. The virus is distributed throughout wild grapevines in northern California, and therefore some distribution mechanism must be at play. If WVV1 can spread to grape cultivars, and especially if it has similar detrimental effects on production and fruit quality as GRBV, this may be the first example of a grapevine virus (WVV1) with the potential for a proactive management approach, rather than a reactive response to a new and emerging virus (Cieniewicz *et al.* 2018b).

TRANSMISSION BIOLOGY

Spissistilus festinus is currently the only known vector of GRBV (Bahder *et al.* 2016). It is important that this finding is confirmed in an independent study, as there has been confusion due to an early report of *Erythroneura ziczac* as a vector of GRBV (Poojari *et al.* 2013) though this result was not confirmed by others (Bahder *et al.* 2016; Daane *et al.* 2017). These studies are discussed in more detail in Appendix 2. Additionally, preliminary research to confirm *S. festinus* as a vector of GRBV and characterize the mode of transmission are reported in Appendix 2. Future directions are briefly discussed here.

Two distinct genotypes of *S. festinus* have been identified (Chapter 6). There is no known biological significance to the identification of the “East” and “West” genotypes. However, it will be important to test whether the two genotypes can transmit GRBV, and if there are any differences in transmission efficiency. We currently have a colony of *S. festinus* of the “West” genotype, so a colony of the “East” genotype would need to be established. Additionally, the discovery of a blue color morph in the colony is a potential factor to explore in the future (Appendix 1). It is unknown whether different color morphs exist in natural environments, but color morphs have shown differences in transmission in other pathosystems (Tahmasebi *et al.* 2012; Ammar *et al.* 2018). Also, the rearing host can influence transmission efficiency (Pinheiro *et al.* 2017). In order to test factors affecting transmission in the *S. festinus*-GRBV system, an optimized transmission assay also needs to be developed. Progress in development of a transmission assay is discussed in Appendix 2.

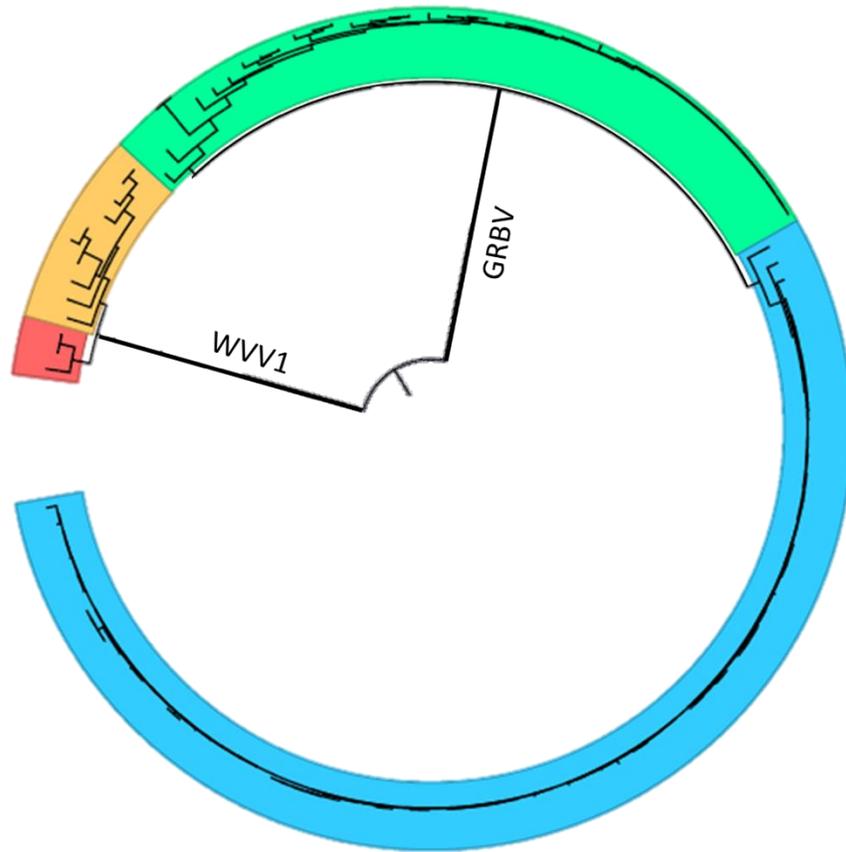


Figure 7-1 Neighbor-joining phylogeny of 114 grapevine red blotch virus (GRBV) and 13 wild *Vitis* virus 1 (WVV1) full genome accessions available in Genbank as of March 2019. WVV1 clade I is indicated in pink, clade II in orange. GRBV clade I is indicated in green, clade II in blue. Branches with less than 65% bootstrap support (1000 replicates) have been collapsed.

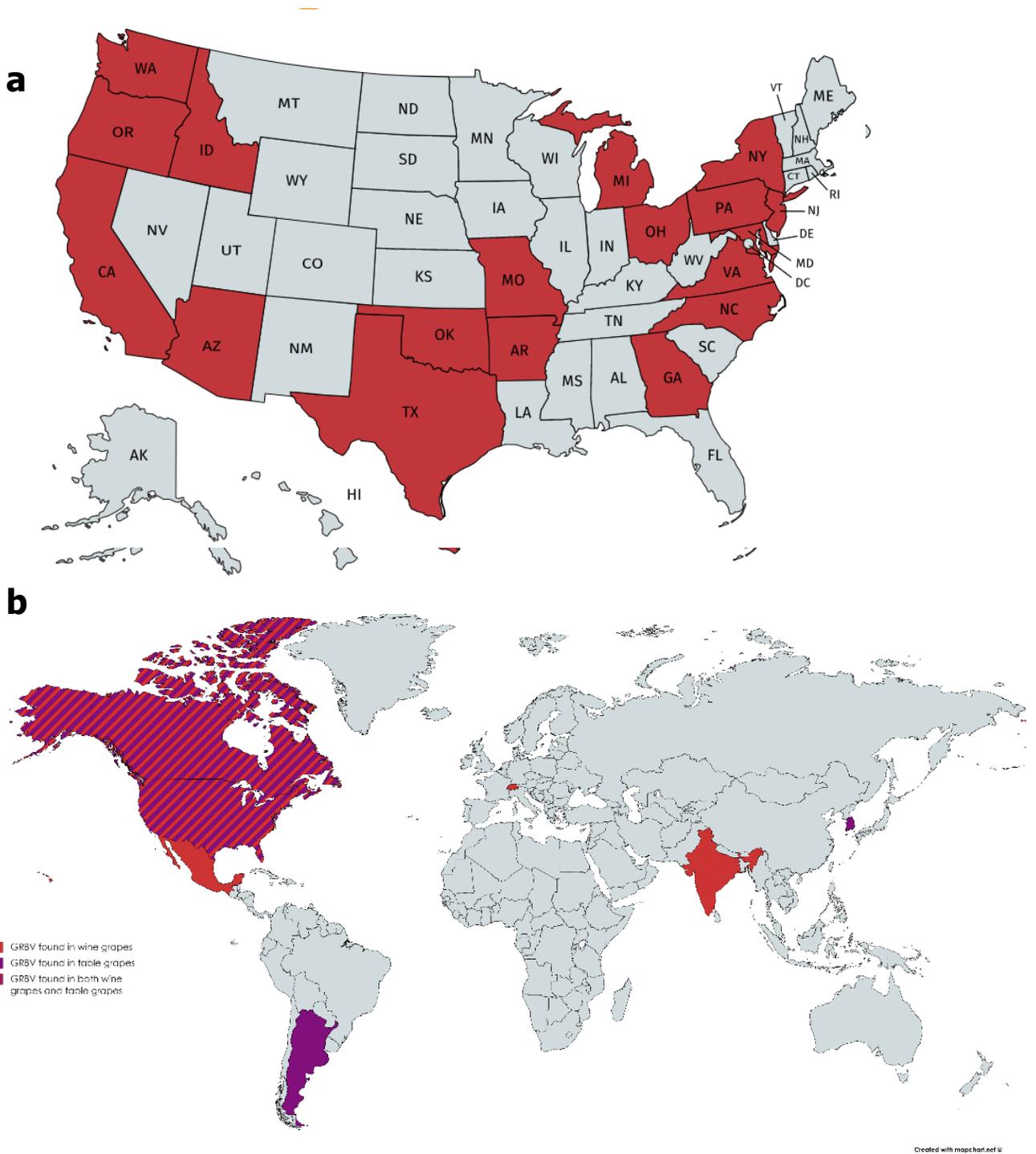


Figure 7-2 a) Map of the United States in which grapevine red blotch virus has been detected in vineyards. GRBV has been detected in grapevines in states in red. b) Global map indicating countries in which GRBV has been detected in wine grapes (red), table grapes (purple) or both (red/purple striped).

GRAPEVINE RED BLOTCH VIRUS HOST RANGE

Grapevine red blotch virus has thus far only been detected in *Vitis* spp. Though geminiviruses often have multiple hosts, sometimes with very wide host ranges (more than 300 host species) (Rojas *et al.* 2018). Though red blotch disease epidemiology may not be substantially affected by alternative annual hosts since grapevine is a perennial host, it is still important to determine the natural host range of GRBV to be vigilant for potential environmental reservoirs. If any alternative hosts are identified, recommendations for cover cropping strategies and weed management in vineyards may be adjusted.

Identifying the experimental host range of GRBV can also be useful in studies on understanding genome expression, virus movement, and transmission of GRBV by insect vectors. Progress in developing the tools for inoculation experiments and detection of replicating GRBV in plants are discussed in Appendix 3.

REFERENCES

Ammar, E.-D., Hall, D. G., Hosseinzadeh, S., and Heck, M. 2018. The quest for a non-vector psyllid: Natural variation in acquisition and transmission of the Huanglongbing pathogen ‘*Candidatus Liberibacter asiaticus*’ by Asian citrus psyllid isofemale lines. PLoS One. 13:e0195804.

Bahder, B. W., Zalom, F. G., Jayanth, M., and Sudarshana, M. R. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. Phytopathology 106:1223–1230.

Beyer, B. A., Srinivasan, R., Roberts, P. M., and Abney, M. R. 2017. Biology and management of the threecornered alfalfa hopper (Hemiptera: Membracidae) in alfalfa, soybean, and peanut. *J. Integr. Pest Manag.* 8:1–10.

Buchs, N., Braga-Lagache, S., Uldry, A.-C., Brodard, J., Debonneville, C., Reynard, J.-S., and Heller, M. 2018. Absolute quantification of grapevine red blotch virus in grapevine leaf and petiole tissues by proteomics. *Front. Plant Sci.* 9: doi: 10.3389.

Cieniewicz, E. J., Pethybridge, S. J., Gorny, A., Madden, L. V., McLane, H., Perry, K. L., and Fuchs, M. 2017. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Res.* 241:156–162.

Cieniewicz, E. J., Pethybridge, S. J., Loeb, G., Perry, K., and Fuchs, M. 2018a. Insights into the ecology of *grapevine red blotch virus* in a diseased vineyard. *Phytopathology.* 108:94–102.

Cieniewicz, E., Thompson, J. R., McLane, H., Perry, K. L., Dangel, G. S., Corbett, Q., Martinson, T., Wise, A., Wallis, A., O’Connell, J., Dunst, R., Cox, K., and Fuchs, M. 2018b. Prevalence and genetic diversity of grabloviruses in free-living *Vitis* spp. *Plant Dis.* 102:2308–2316.

Cieniewicz, E.J., Flasco, M., Brunelli, M., Wise, A., and Fuchs, M. 2019. Spread

dynamics of grapevine red blotch virus in relation to *Spissistilus festinus* abundance in vineyards. *Viruses* (In Review).

Daane, K. M., Almeida, R. P. P., Cooper, M. L., Golino, D. A., Wilson, H., and Anderson, J. 2017. Searching for potential vectors of grapevine red blotch-associated virus. In *CDFFA Pierce's Disease Research Symposium*, p. 1–12.

Dalton, D. T., Hilton, R. J., Kaiser, C., Daane, K. M., Sudarshana, M. R., Vo, J., Zalom, F., Buser, J., and Walton, V. 2019. Spatial associations of vines infected with grapevine red blotch virus in Oregon vineyards. *Plant Dis.* doi: PDIS-08-18-1306-RE.

Ito, T., and Nakaune, R. 2016. Molecular characterization of a novel putative ampelovirus tentatively named grapevine leafroll-associated virus 13. *Arch. Virol.* 161:2555–2559.

Krenz, B., Thompson, J. R., McLane, H., Fuchs, M., and Perry, K. L. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104:1232–1240.

Li, R., Fuchs, M. F., Perry, K. L., Mekuria, T., and Zhang, S. 2017. Development of a fast AmplifyRP Acceler8 diagnostic assay for grapevine red blotch virus. *J. Plant Pathol.* 99:657–662.

Martelli, G. P., Ghanem-Sabanadzovic, N.A., Agranovsky, A.A., Al Rwahnih, M., Dolja, V. V., Dovas, C. I., Fuchs, M.F., Gugerli, P., Hu, J. S., Jelkmann, W., Katis, N.I., Maliogka, V.I., Melzer, M.J., Menzel, W., Minafra, A., Rott, M.E., Rowhani, A., Sabanadzovic, S., Saldarelli, P. 2012. Taxonomic revision of the family *Closteroviridae* with special feference to the grapevine leafroll-associated members of the genus *Ampelovirus* and the putative species unassigned to the family. J. Plant Pathol. 94:7–19.

Martelli, G.P. 2014. Directory of virus and virus-like diseases of the grapevine and their agents. Journal of Plant Pathology 96 (1S): 1-136.

Perry, K. L., Mclane, H., Thompson, J. R., and Fuchs, M. 2018. A novel grablovirus from non-cultivated grapevine (*Vitis* sp.) in North America. Arch. Virol. 163:259–262.

Poojari, S., Alabi, O. J., Fofanov, V. Y., and Naidu, R. A. 2013. A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family *Geminiviridae* implicated in grapevine redleaf disease by next-generation sequencing. PLoS One. 8:e64194.

Preto, C. R., Bahder, B. W., Bick, E. N., Sudarshana, M. R., and Zalom, F. G. 2019. Seasonal dynamics of *Spissistilus festinus* (Hemiptera: Membracidae) in a Californian vineyard. J. Econ. Entomol. doi:10.109.

Ricketts, K. D., Gómez, M. I., Fuchs, M. F., Martinson, T. E., Smith, R. J., Cooper, M. L., Moyer, M., and Wise, A. 017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. *Am. J. Enol. Vitic.* 68:127–135.

Rojas, M. R., Macedo, M. A., Maliano, M. R., Soto-Aguilar, M., Souza, J.O., Briddon, R.W., Kenyon, L., Rivera Bustamante, R.F., Zerbini, F.M., Adkins, S., Legg, J.P., Kvarnheden, A., Wintermantel, W.M., Sudarshana, M.R., Peterschmitt, M., Lapidot, M., Martin, D.P., Moriones, E., Inoue-Nagata, A.K., and Gilbertson, R.L. 2018. World management of geminiviruses. *Annu. Rev. Phytopathol.* 56:637–677.

Tahmasebi, A., Dizadji, A., Farhoudi, F., Allahyari, H., and Koochi-Habibi, M. 2012. Comparative transmission of two cucumber mosaic virus isolates by two color morphs of *Acyrtosiphon pisum* (Harris). *Acta Virol.* 56:139–143.

Vargas-Asencio, J., Liou, H., Perry, K. L., and Thompson, J. R. 2019. Evidence for the splicing of grablovirus transcripts reveals a putative novel open reading frame. *J. Gen. Virol.* DOI 10.109.

Yepes, L. M., Cieniewicz, E. J., Krenz, B., McLane, H., Thompson, J. R., Perry, K. L., and Fuchs, M. 2018. Causative role of grapevine red blotch virus in red blotch disease. *Phytopathology.* 108:902–909.

APPENDIX 1

GUIDELINES FOR REARING SPISSISTILUS FESTINUS IN A CONTROLLED ENVIRONMENT

JUSTIFICATION

Spissistilus festinus (Hemiptera: Membracidae) is the only confirmed vector of grapevine red blotch virus (GRBV) (Bahder *et al.* 2016). In order to confirm this finding and to determine the mode of transmission, transmission efficiency, and other transmission biology factors, it is important to have a reproducing colony of *S. festinus*. While *S. festinus* can oviposit on grape, and first instars can hatch from grape tissues (Preto *et al.* 2018), they don't survive past the second instar on grape. Therefore, a colony of *S. festinus* on *Vitis* spp. does not seem feasible.

Caldwell (1949) reported that the geographic range of *S. festinus* is the southern United States and South America at least as far south as Costa Rica. Interestingly, the Cornell University Insect Collection contains one *S. festinus* specimen from Long Island, New York collected in 1933. Though this is the sole report of *S. festinus* to our knowledge in New York and *S. festinus* is likely not abundant in New York, this hemipteran species is abundant in alfalfa fields in California in the late summer/fall months. We consistently found *S. festinus* in high abundance (~15 adults per sweep) in alfalfa fields in Yolo and San Joaquin Counties, California. In October of 2015 *S. festinus* were collected from alfalfa in California, transferred to the laboratory in Geneva, New York, and reared on alfalfa in a controlled environment chamber. Rearing conditions were adapted from those

previously described (Meisch and Randolph 1965). The guidelines for rearing *S. festinus* and managing pests in the controlled environment are described hereafter.

FIELD COLLECTION OF SPISSISTILUS FESTINUS

Spissistilus festinus adults were collected in alfalfa fields in Knights Landing, California and Lodi, California. The population in our colony was first collected in October 2015, and then supplemented with field collected adults from alfalfa fields in October of 2016 and 2017. Aerial sweep nets were used to collect adults, and they were transferred to plastic pretzel containers with cheesecloth lids. Alfalfa stems were included for temporary feeding stores during overnight shipment to the laboratory in Geneva, New York. Containers were unpacked in secondary cages in the greenhouse, and *S. festinus* were sorted into clean cages using a battery-powered aspirator to avoid other insects.

REARING PROCEDURES IN A CONTROLLED ENVIRONMENT

Insects are reared on alfalfa plants growing in 6-inch pots. Alfalfa seeds inoculated with *Rhizobia* are planted weekly, with three plants per pot, and grown in the greenhouse for at least six weeks until flowering. Alfalfa plants are then transported to the rearing chamber. Rearing conditions were adapted from those outlined by Meisch and Randolph (1965). Conditions are intended to break/prevent reproductive diapause and promote nymph development. The conditions are: 85% relative humidity, 26.5°C, photoperiod of 14 light: 10 dark. Soft-sided cages are used for ease of cleaning and replacement.

Life stages are separated for rearing purposes. Adults are caged with potted alfalfa plants, which are refreshed weekly with plants from the greenhouse. At reproductive maturity (approximately seven weeks post-eclosion) gravid *S. festinus* adult females oviposit in stems. After one week, alfalfa plants with eggs are refreshed with fresh plants. Alfalfa plants with eggs are kept in insect-proof cages on shelves in the rearing chamber with access to light for at least three weeks, and sometimes four depending on the health of the plant. During this time, alfalfa plants with eggs are monitored every other day for eclosed first instar nymphs. Nymphs are transferred to new alfalfa plants using a fine paintbrush, and caged with other nymphs. The nymph cages are refreshed with new alfalfa as needed based on health of the plants. Nymphs molt through five instar stages (**Figure A1-1**). When alate adults emerge, they are transferred using a mechanical aspirator to the adult cage to mate and lay eggs. The approximate egg-to-egg generation time (time from oviposition to oviposition of next generation) is 55-60 days (Meisch and Randolph, 1965). Cages are replaced every other week. The used alfalfa plants and soil are autoclaved and composted.

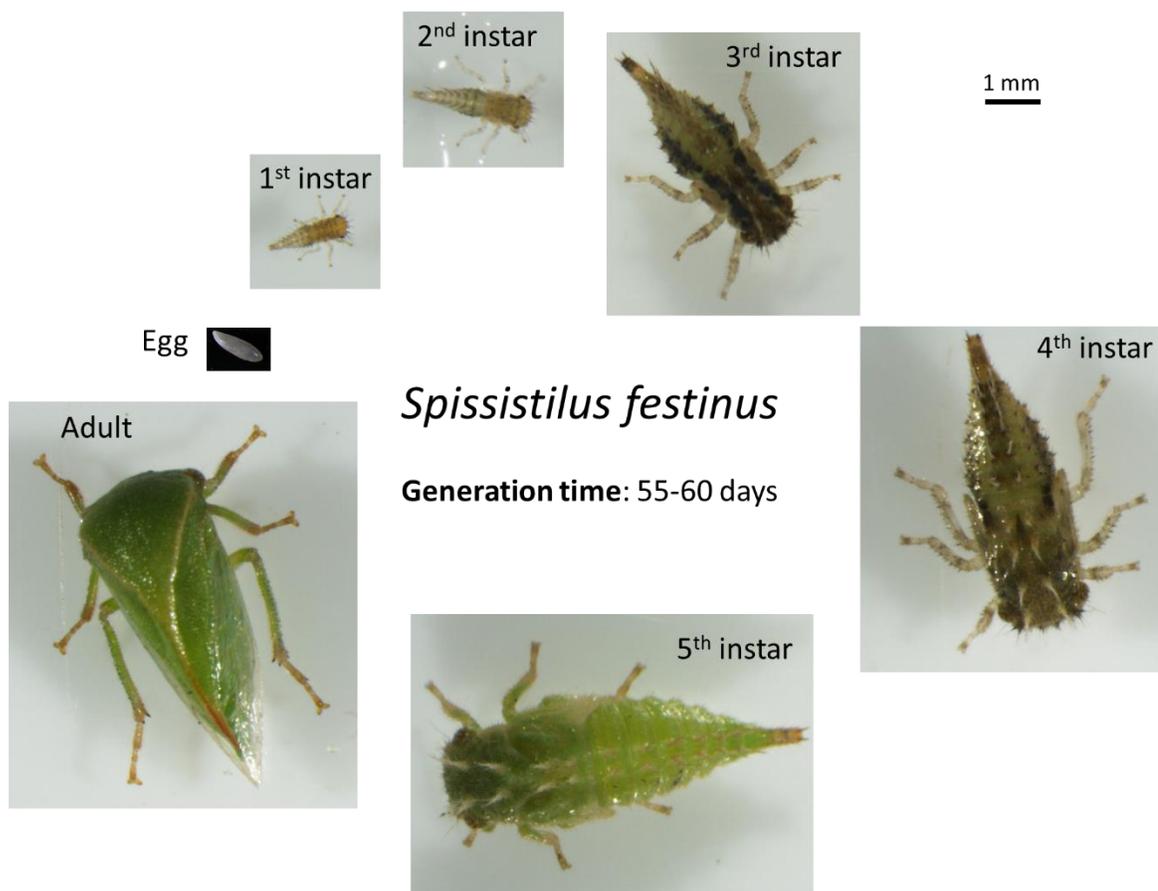


Figure A1-1 Egg, first instar, second instar, third instar, fourth instar, fifth instar, and adult *Spissistilus festinus*. Generation time (oviposition to oviposition) is 55-60 days in our colony.

PEST MANAGEMENT

Rearing of *S. festinus* on live plants grown in the greenhouse can introduce some pests into the *S. festinus* colony. These pests can have deleterious effects on the *S. festinus* colony. Unfortunately, pest management options in the greenhouse are limited. In addition, insecticide sprays and beneficial predators intended to manage greenhouse pests may impact *S. festinus* development, and therefore options are extremely limited. Typically alfalfa flowers are removed in order to minimize attraction of thrips (Thysanoptera spp.). Plants are also lightly shaken onto a tray with soap and water to dislodge and then kill thrips and the two-spotted spider mite (*Tetranychus urticae*). Finally, beneficial insects that are solely soil-dwellers and have no efficacy against *S. festinus* can be used to reduce greenhouse pests. Here I outline the major pest problems experienced while establishing and monitoring the *S. festinus* colony, and how we manage them.

Two-spotted spider mite (*Tetranychus urticae*)

This pest poses the largest problem to the *S. festinus* colony due to the damage it inflicts on the alfalfa plants. *T. urticae* feeds and reproduces directly on the leaves, reducing the alfalfa health too quickly before *S. festinus* nymphs eclose and can be transferred to new plants. Therefore, once early signs of *T. urticae* are observed, it is critical to go through the alfalfa plants and remove any leaves with *T. urticae* on them. The signs of *T. urticae* are small white spots on the upper side of the leaf, and often at least one adults with eggs are apparent on the underside of the leaf (**Figure A1-2**). If a plant is heavily damaged (signs of *T. urticae* on more than 5-10 leaves) then the entire

plant should be discarded. During *T. urticae* outbreaks, the cages should be replaced weekly and thoroughly cleaned, and hard surfaces should be cleaned with 95% ethanol as well.



Figure A1-2 Sign of two-spotted spider mite *Tetranychus urticae* on alfalfa plants. White spots on leaves are indicative of *T. urticae* feeding damage.

Thrips (Thysanoptera species)

Thrips are an important greenhouse pest, and can be extremely difficult to manage due to their tendency to burrow into flowers and other plant parts, rendering insecticide applications largely ineffective. In relation to the *S. festinus* colony, they inevitably get into the colony with the alfalfa plants. They damage the leaves by feeding on the mesophyll, reduce the photosynthetic capability, and eventually cause complete decline of the plant. However, in the rearing chamber the alfalfa plants need to be viable for a total of four weeks. Thrips rarely do lethal damage to the alfalfa in this time.

To reduce thrips populations, predatory mites like *Amblyseius degenerans* and *Amblyseius cucumeris* can be used in the greenhouse. These species do not attack *S. festinus* nymphs or adults. Removing flowers from the alfalfa plants and manually beating alfalfa plants over soapy water also aids in reducing populations of thrips. In contrast, although the minute pirate bug, *Orius laevigatus*, is effective as a biological control against thrips in greenhouses, it also feeds on *S. festinus* and reduces populations substantially. Occasionally, *Orius* adults or nymphs are found in the alfalfa, either flying in from outside or from adjacent greenhouses. When observed in the *S. festinus* colony, they must be immediately removed because of the severe damage they can do the *S. festinus* colony.

Fungus gnats (Diptera: Sciaridae)

Fungus gnat larvae thrive in wet soil. Therefore, over-watering the alfalfa plants can result in higher populations of fungus gnats. They very rarely damage the

alfalfa plants, unless populations are extremely dense and the larvae damage the alfalfa roots. They are effectively managed by taking care to not over-water the alfalfa. However, humid environments also help to reduce spider mite infestations, so an adequate balance of water is necessary for alfalfa health and pest management.

SPISSISTILUS FESTINUS REARING: NOTABLE OBSERVATIONS

Blue color morphs

Blue color morphs of *S. festinus* were occasionally observed in the colony (**Figure A1-3**). The blue morphs were always males, and when caged with females (initially reproductively immature) the progeny were all green morphs. The blue color morphs were rare (estimated 1 in every 500 adults) and did not occur in every generation. It is unknown if the blue color morphs exist in the natural environment, nor if there are any fitness differences between distinct *S. festinus* color morphs.

Sensitivity to light changes

The *S. festinus* colony nearly collapsed in spring of 2015. We temporarily used another rearing chamber for five weeks. The environmental conditions were identical for humidity, temperature, and photoperiod. However the lights were noticeably dimmer in the new rearing chamber. Eclosion stopped after two weeks in this chamber, and the adults and 1st-5th instars died off to within 10% of the original population within four weeks. The colony was moved to the original chamber, where

lights were slightly brighter. The surviving *S. festinus* reproduced again and the colony rebounded within one generation.

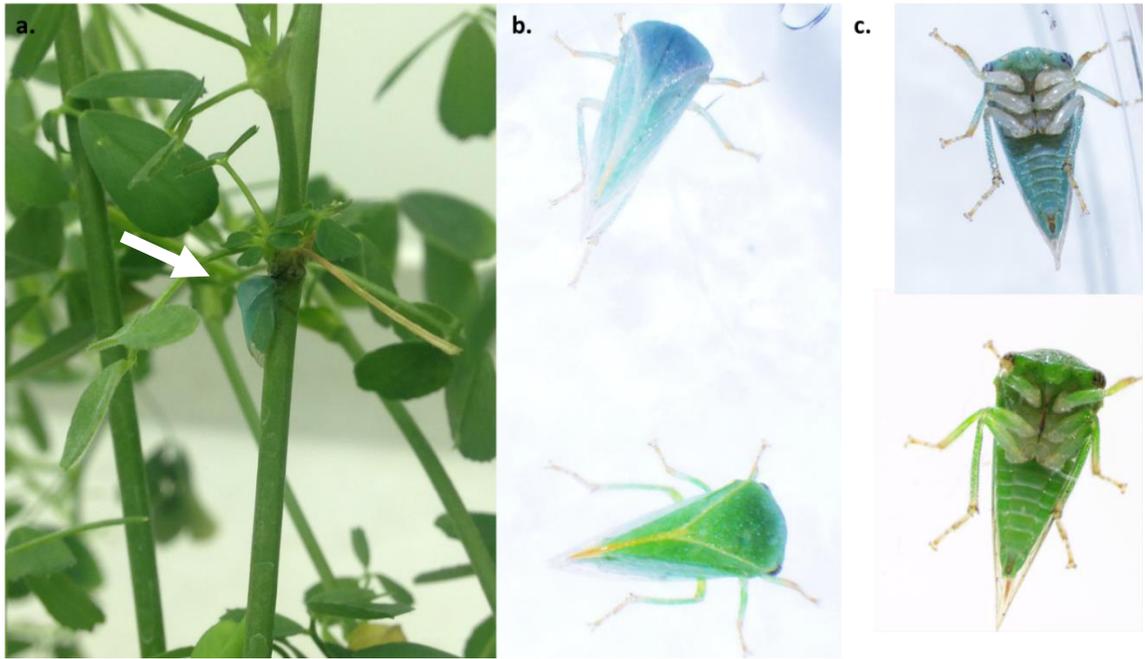


Figure A1-3 *Spissistilus festinus* blue color morph observed in the laboratory colony and indicated by a white arrow. (a) Male blue color morph on alfalfa, (b) Dorsal view of blue (top) and green (bottom) color morphs, and (c) Ventral view of the blue (top) and green (bottom) color morphs.

REFERENCES

Bahder, B. W., Zalom, F. G., Jayanth, M., and Sudarshana, M. R. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology*.

106:1223–1230

Caldwell, J. S. 1949. A generic revision of the treehoppers of the tribe Ceresini in America north of Mexico based on a study of the male genitalia. *Proc. United States Natl. Museum*. 98:491–521.

Meisch, M. V., and Randolph, N. M. 1965. Life-history studies and rearing techniques for the three-cornered alfalfa hopper. *J. Econ. Entomol.* 58:1057–1059.

Preto, C. R., Sudarshana, M. R., Bollinger, M. L., and Zalom, F. G. 2018. *Vitis vinifera* (Vitales: Vitaceae) as a reproductive host of *Spissistilus festinus* (Hemiptera: Membracidae). *J. Insect Sci.* 18:1–7

APPENDIX 2

PROGRESS IN SPISSISTILUS FESTINUS TRANSMISSION BIOLOGY

JUSTIFICATION

Spissistilus festinus is a vector of grapevine red blotch virus (GRBV) under greenhouse conditions (Bahder *et al.* 2016), and is associated with secondary spread of GRBV in California vineyards (Cieniewicz *et al.* 2018). GRBV is a recently ratified member of the family *Geminiviridae*, one of the largest and most important plant virus families globally, and the type member of the genus *Grablovirus* (Varsani *et al.* 2017). Members of the family *Geminiviridae* are transmitted in a circulative, non-propagative manner by insect vectors (Rojas *et al.* 2018).

Since the coat protein (CP) is involved in transmission, viruses within the family *Geminiviridae* are typically transmitted by hemipteran vectors in a genus-specific manner by *Bemisia tabaci* whiteflies (begomoviruses), leafhoppers (mastreviruses, curtoviruses, becurtoviruses, turncurtoviruses) and, until GRBV, a single treehopper-vectored geminivirus (tomato pseudo curly top virus vectored by *Micrutalis malleifera*) (reviewed by Varsani *et al.* 2017). Recently, the cowpea aphid (*Aphis craccivora*) was reported as a vector of alfalfa leaf curl virus (genus *Capulavirus*), though the transmission mode was not determined (Roumagnac *et al.* 2015), and the CP sequence did not provide clues as to what the vector may be (Varsani *et al.* 2017). The following year, *S. festinus* was shown to transmit GRBV (Bahder *et al.* 2016). These authors reported that phylogenetic analysis of the GRBV CP aided in identification of *S. festinus* as a vector because the GRBV CP was in the

same phylogenetic clade as the CP of TPCTV (vectored by *M. malleifera*). However, in a separate and more rigorous study, the CP of GRBV did not group with any other geminivirus CP (Varsani *et al.* 2017). These conflicting results suggested a need to confirm the capacity of *S. festinus* to transmit GRBV.

In recent years, more geminiviruses have been ratified as species by the International Committee on the Taxonomy of Viruses (ICTV) and several putative geminiviruses have been identified. Between the 9th and 10th ICTV reports on the family *Geminiviridae*, the number of geminivirus species more than doubled (218 virus species in 2011; 441 virus species in 2018) (Brown *et al.* 2009, Zerbini *et al.* 2017). Moreover, several virus genomes have been reported as putative geminiviruses, including two new putative grabloviruses that are closely related to GRBV such as wild Vitis virus 1 (Perry *et al.* 2018) and prunus geminivirus A (Al Rwahnih *et al.* 2018).

The rapid expansion in research on geminiviruses has raised questions about vector transmission. In particular, very little is known about treehopper transmission of geminiviruses, or treehopper transmission of plant pathogens in general. *Micrutalis malleifera*, vector of TPCTV, has not been extensively studied since more advanced genomics tools have been developed, though the transmission mode is characterized as persistent based on a latent period of more than 24 hours (Simons and Coe 1958). Although the virus movement through the insect vector has been extensively studied in begomoviruses and *B. tabaci* (reviewed by Rosen *et al.* 2015), the mode of transmission and the tissue tropism of the virus throughout the vector body have not been studied in treehopper-vectored pathosystems.

Understanding the mode of transmission will contribute to new insights into treehopper vector biology. *Spissistilus festinus* does not complete its life cycle on grapevine, nor does it seem to infest vineyards (Preto *et al.* 2018a). Rather, it reportedly prefers legumes as both a feeding and reproductive host (Preto *et al.* 2018b). The transmission mode of GRBV is unknown, and *S. festinus* has only been reported as a vector as a result of a single experiment. It is critical to confirm in an independent study that *S. festinus* can vector GRBV in replicated experiments. Moreover, establishing a reliable transmission assay will allow for further experiments to test differences between *S. festinus* genotypes and GRBV genotypes in transmission efficiency. It will also allow for investigating transmission to other potential GRBV hosts.

In this Appendix, I report on several experiments aimed at understanding *S. festinus*-GRBV interactions. The work reported herein warrants further replication and optimization, however, the insights gained from these experiments help to lay the groundwork for future research. This appendix is divided into the following five sections: 1) *S. festinus* gut and salivary gland anatomy, 2) *S. festinus* behavior on grapevine in the greenhouse, 3) Acquisition of GRBV by *S. festinus*, and 4) Transmission assays, trial and error, and 5) Concluding remarks.

PART 1: SPISSISTILUS FESTINUS GUT AND SALIVARY GLAND ANATOMY

The internal anatomy of Membracidae is not well described, and has never been described for *Spissistilus festinus*. Dissections were performed in order to locate the salivary glands and describe the gut anatomy of *S. festinus* using forceps, pins, and

a stereomicroscope. Insects can be anesthetized either by exposure to acetone, or by freezing at -80°C for 3 minutes. Specimens should be kept on ice until dissection. Fine forceps are helpful in separating the body segments (i.e. head, thorax, abdomen). Insect pins attached to plastic pipettors as handles (adapted from the Heck Lab, Boyce Thompson Institute) are useful for manipulating soft tissues (salivary glands, guts).

The dissection technique to remove the salivary glands is as follows: Insects are anesthetized, and placed on their side in a 20 µL droplet of 1X phosphate buffered saline (PBS). The head can be removed by loosening it from the thorax and pulling it off using fine forceps (**Figure A2-1**, incision 1). The salivary glands are typically attached to the head, and can be teased away using the insect pin tool (**Figure A2-2**). The salivary glands of another membracid *Tricentrus brunneus* contain four major lobes, with three in the anterior portion of the principal gland and one in the posterior portion of the principal gland, and also two elbow-shaped accessory glands (Zhong *et al.* 2015). In *S. festinus*, a tentative description of internal anatomy based on comparative morphology is suggested in Figure A2-2.

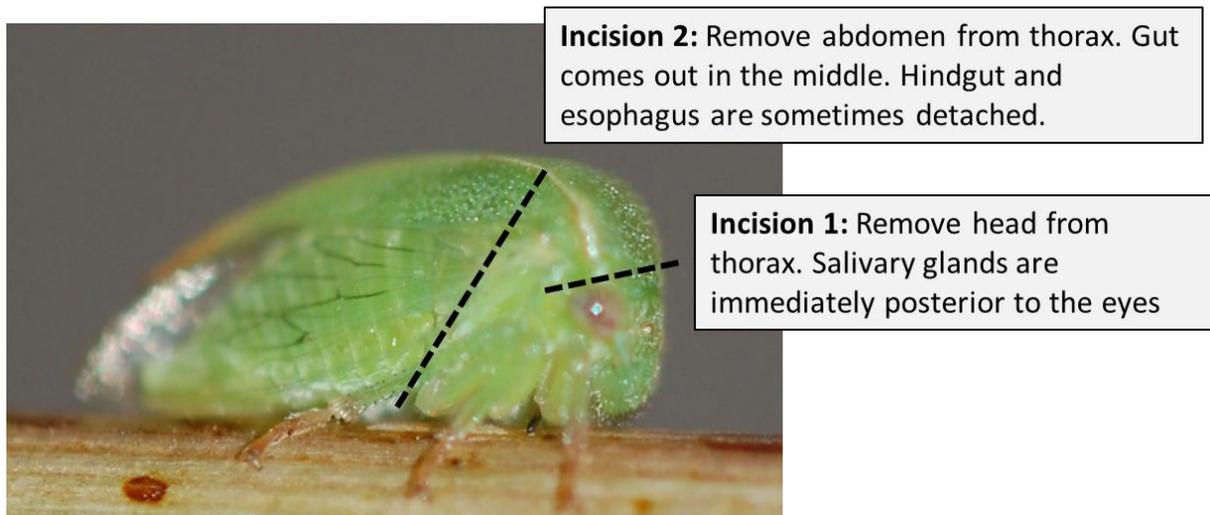


Figure A2-1 Locations of major incisions for dissecting salivary glands and the gut from *Spissistilus festinus* adults.

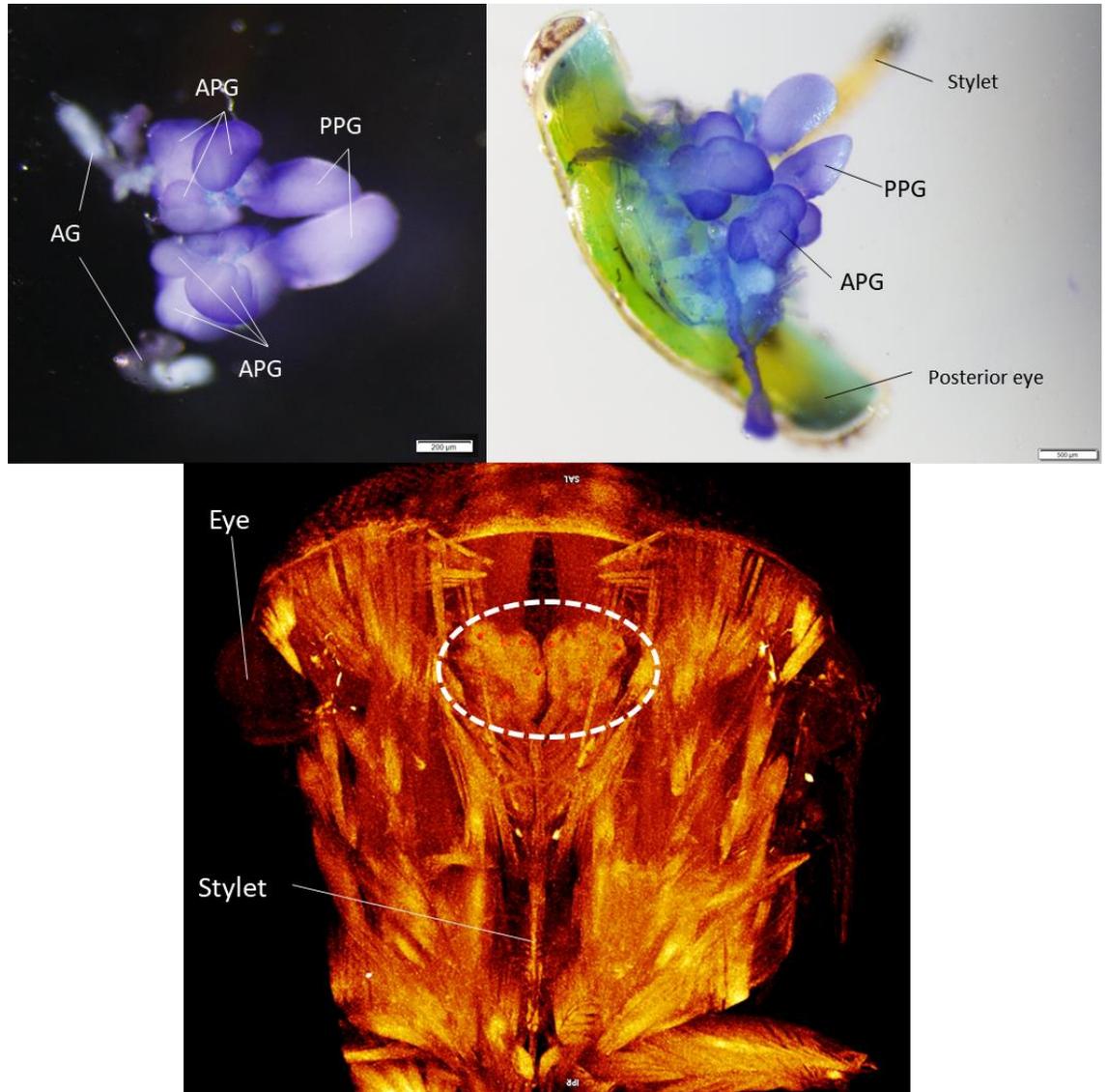


Figure A2-2 Proposed anatomical description of salivary glands of *Spissistilus festinus*. The top left figure shows the accessory glands (AG) in the background, out of focus, and the two major lobes of the principal salivary glands, the anterior principal gland (APG) and posterior principal gland (PPG), which are each paired and stained in toluidine blue. The top right figure shows the attachment of the salivary glands, just posterior and central to the eyes, also stained in toluidine blue. The bottom figure is a nano-computed tomography (nano-CT) rendering of the *S. festinus* head, from the viewpoint of directly in front of the head. The salivary glands are denoted within the white circle, dorsal to the stylet.

To dissect the gut, the wings can then be removed or bent to expose the juncture of the thorax and abdomen. The abdomen is then detached from the thorax by gently pulling the two segments apart (**Figure A2-1**). The gut comes out in between the thorax and abdomen. The hindgut easily comes detached, but can be gently coaxed out of the abdomen intact. Similarly, the esophagus may be detached in the thorax, but can be gently removed intact with the midgut (**Figure A2-3**). The proposed gut anatomy description is detailed (**Figure A2-3**), where the ingestion pathway begins with the esophagus, to the anterior midgut, middle midgut, posterior midgut, filter chamber, hindgut. The four Malpighian tubules appear to coil around the filter chamber, and then branch out into the hemocoel and re-attach at the hindgut (**Figure A2-3**).

This is the first attempt at a description of *S. festinus* internal anatomy. The anatomical description proposed here is based only on gross anatomy under a stereomicroscope. If GRBV is transmitted by *S. festinus* in a circulative mode like other virus species in the family *Geminiviridae*, then a comprehensive anatomical study is warranted for *S. festinus* to understand the pathway of the virus within the different insect organs. Histological examination of the salivary glands and guts would help to more accurately describe the cell types and infer anatomical functions.

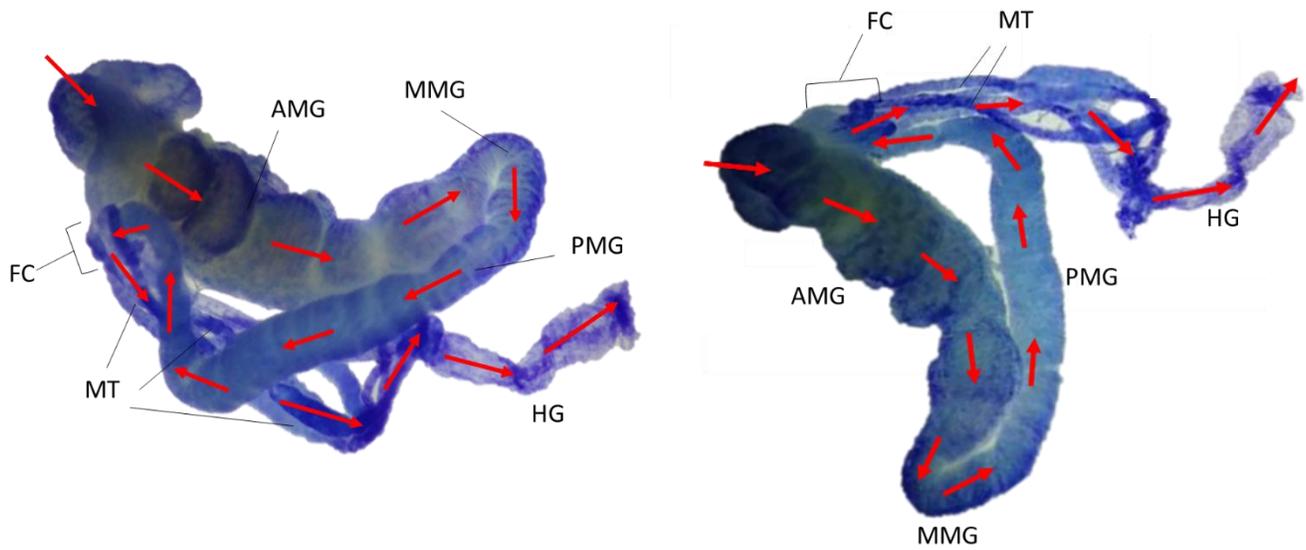


Figure A2-3 Proposed description of *Spissistilus festinus* gut anatomy. (Top) Toluidine blue-stained views of the gut in which the red arrows indicate the digestion pathway, originating from the esophagus (detached) through the anterior midgut (AMG), middle midgut (MMG), posterior midgut (PMG), filter chamber (FC), and hindgut (HG). (Bottom) Natural coloring of the guts after dissection, with no staining. The four Malpighian tubules (MT) are coiled around the filter chamber (left two images). These images are from independent dissections.

PART 2: SPISSISTILUS FESTINUS BEHAVIOR ON GRAPEVINE

Since *S. festinus* is not a pest of grapevine, its behavior on grapevine is largely unknown. Studies in vineyards have demonstrated an edge effect for the presence of *S. festinus* in vineyards (Cieniewicz *et al.* 2018, Preto *et al.* 2019). In addition, grapevine is not a preferred feeding host (Preto *et al.* 2018a), and though *S. festinus* will oviposit in green tissues in grapevines, it does not survive past the 2nd instar (Preto *et al.* 2018b, **Figure A2-4**). Understanding *S. festinus* behavior on grapevine will inform experimental design for replicated transmission experiments. In this section I report my observations of the behavior of *S. festinus* on potted grapevines in the greenhouse, and the results of an experiment to test the relative mortality rates of *S. festinus* on GRBV-positive grapevine, GRBV-negative grapevine, and alfalfa.

S. festinus adults feed on grapevine, where they feed on the phloem in a ring around petioles or green shoots (**Figure A2-5a and 5b**) both in the vineyard and in the greenhouse. In greenhouse conditions, they ingest GRBV (test PCR-positive) at a nearly 100% rate when caged with GRBV-infected, potted grapevine for at least two days. First and 2nd instar nymphs exhibit a high mortality rate (>90% mortality) on potted grapevines in greenhouse conditions. In contrast, 3rd through 5th instars and adults have a high survival rate (typically less than 10% mortality) on potted grapevines in the greenhouse. Fourth and 5th instar nymphs will often migrate toward the base of the trunk on potted grapevines for an unknown reason (**Figure A2-5c**).



Figure A2-4 (Top) *Spissistilus festinus* eggs (denoted by black arrows) inserted into grapevine petiole. (Bottom) First instar nymph hatched on grape leaf. *Spissistilus festinus* nymphs do not survive past the second instar on grape.

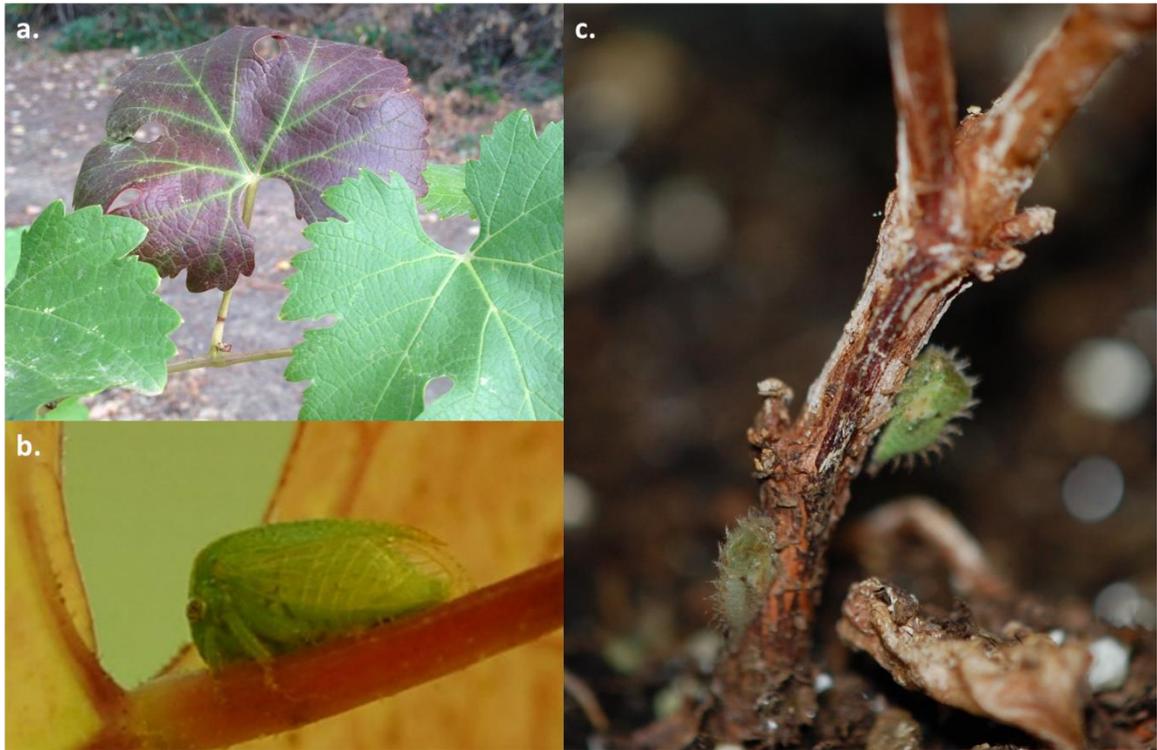
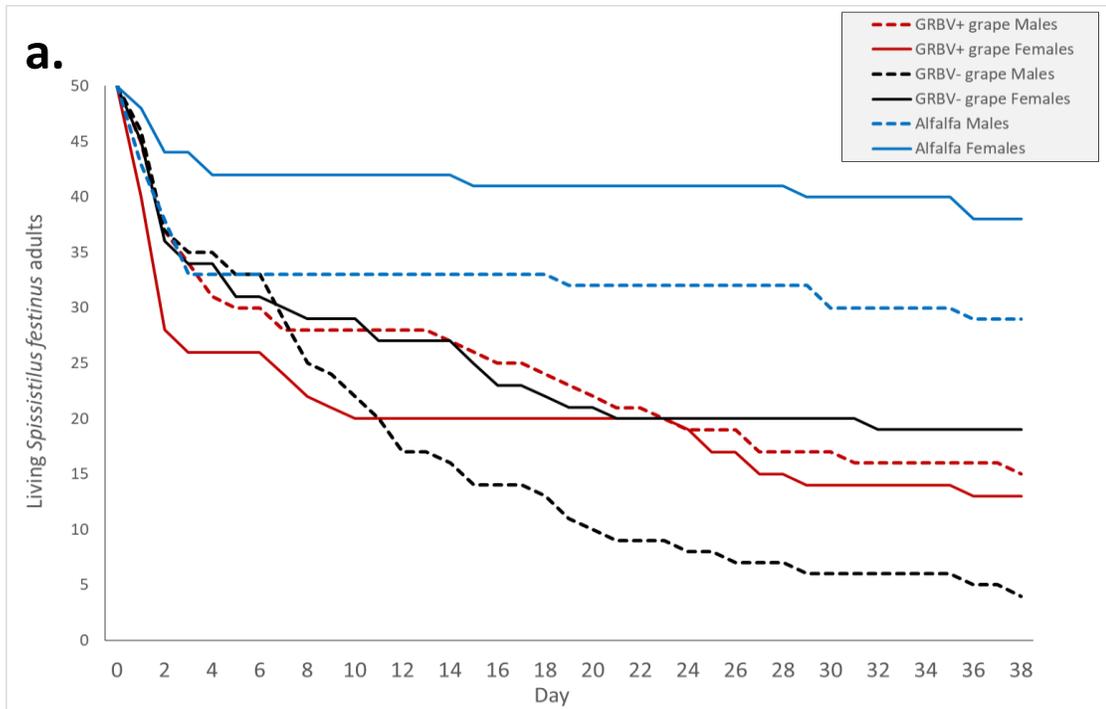


Figure A2-5 *Spissistilus festinus* (a) adults girdle grapevine petiole or small shoots by feeding in a ring, (b) adults feed preferentially on petiole or small shoots in the greenhouse, and (c) 4th and 5th instar nymphs often migrate to the base of the trunk in potted grapevines in the greenhouse.

To test the mortality rates of *S. festinus* on grapevine compared to alfalfa, we collected *S. festinus* adults from a roadside alfalfa patch surrounded by vineyards in Lodi, California. They were shipped overnight to the laboratory in Geneva, New York where they were sorted from other insects and plant material from the field collection. I caged 100 adults (50 females/50 males) in three groups with either (1) potted alfalfa plants, (2) GRBV-positive grapevine, or (3) GRBV-negative grapevines, and monitored the populations every other day for 38 days. Overall, *S. festinus* showed substantially higher survival on the alfalfa than both grapevine cages. In the GRBV-negative grapevine and alfalfa cages, the males showed higher mortality throughout most of the experiment, whereas in the GRBV-positive grapevine the females had slightly higher mortality than the males (**Figure A2-6a**).

The experiment was ended when the grapevines and alfalfa plants began to decline in health, at 38 days after introduction of the *S. festinus*. From a subset of the remaining *S. festinus* adults, the guts were dissected in order to determine if signs of digestive stress could be visually assessed. Orange pigmentation was observed in *S. festinus* anterior midguts in all three groups, though with slightly less pigmentation in the alfalfa group (**Figure A2-6b**). It is unknown if the orange pigmentation is indicative of digestive stress. The insects in this experiment were older adults (collected as adults from the field, shipped, and caged for 38 days) so the stress caused by shipment and age are likely factors contributing to digestive stress.

This experiment should be replicated using *S. festinus* from the colony. The digestive pigmentation as an indicator of digestive stress should also be further evaluated in future experiments.



b.

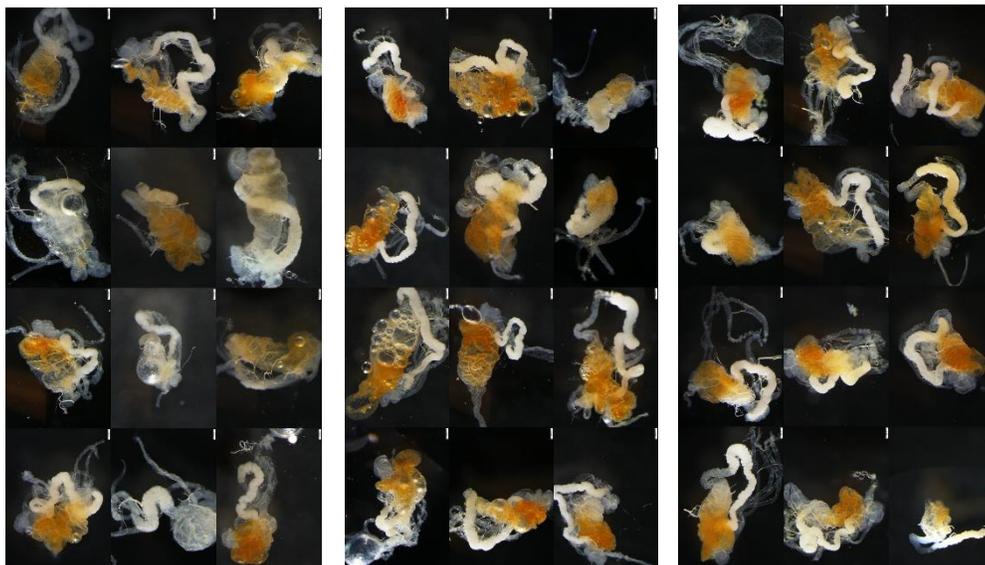


Figure A2-6 (a.) Mortality curves of field-collected *Spissistilus festinus* on potted alfalfa, and grapevine red blotch virus (GRBV)-positive and -negative potted grapevines in the greenhouse. (b.) Dissected *S. festinus* guts from alfalfa (left), GRBV-negative grapevine (middle), and GRBV-positive grapevine (right).

PART 3: ACQUISITION OF GRAPEVINE RED BLOTCH VIRUS BY

SPISSISTILUS FESTINUS

GRBV is ingested by almost 100% of *S. festinus* that feed on infected grapevine for at least two days. To provide preliminary insights into the transmission mode of GRBV by *S. festinus*, gut clearing experiments were carried out. In two experiments, *S. festinus* adults were caged on GRBV-positive grapevines for one week as an acquisition access period (AAP). Adults were then transferred to cages with alfalfa (assumed non-host of GRBV) for two weeks to clear their guts. Whole insects were then tested by multiplex PCR (Krenz *et al.* 2014). In the first experiment, 12 of 20 (60%) *S. festinus* tested GRBV-positive, and in the second experiment, 6 of 11 (55%) *S. festinus* tested GRBV-positive. Retaining the virus after a two-week gut clearing period suggests acquisition of GRBV into the hemolymph, through the gut, which is the first major barrier in circulative transmission. However, these experiments should be replicated using more insects. Additionally, although alfalfa is not suspected as a host of GRBV, it has not been shown to be a definitive non-host of GRBV, and therefore this should be confirmed in inoculation experiments.

Gut-clearing experiments to confirm acquisition of GRBV by *S. festinus* should be coupled with additional methods of testing acquisition. This could include PCR-testing of individual insect tissues following an acquisition access period of several days. Time course studies were performed in which the *S. festinus* adults and nymphs fed on infected grapevine for variable acquisition access periods (2, 3, 4, 5, 6, 7, 9, 12, and 23 days), and then transferred to alfalfa for 1 day to clear their esophagus. After esophagus clearing, the insects were decapitated to separate the salivary glands

from the gut, and the head and gut were tested separately by PCR for GRBV. Two negative control *S. festinus* from the alfalfa colony (not exposed to GRBV) were included at each time point. With a 2, 3, and 4 day AAP, 0/10 heads tested positive at each time, and 4/10, 8/10, and 7/10 guts tested positive, respectively. With a 5 day or greater AAP, 100% of the guts tested positive, suggesting that ingestion rates reach 100% with at least a 5 day AAP. Listed here are the results of testing of heads for a 5-day or greater AAP: (5-day, 0/5; 6-day, 1/8; 7-day, 1/7; 9-day, 1/15; 12-day, 1/8; 23-day, 2/13). In summary, at least a 6-day AAP resulted in 10-20% of *S. festinus* heads testing positive. These experiments should be repeated. This is more so since *S. festinus* tissues were dissected in a droplet of PBS, and it is possible that the GRBV-positive heads were a result of GRBV leaking from the gut into the head by dissection error or imprecision.

The results of these experiments highlight the importance of testing acquisition using multiple methods. Determining the tissue tropism of GRBV within *S. festinus* is important in testing the hypothesis that GRBV follows the circulative pathway of transmission. Another way of testing this hypothesis is fluorescence *in situ* hybridization (FISH). Procedures described by Kliot *et al.* (2014) could be adapted to the *S. festinus*-GRBV pathosystem to address the mode of transmission.

In brief, short (20-25 nt) probes conjugated to fluorophores such as Cy5 or Cy3 would be designed to hybridize to GRBV DNA fragments. *Spissistilus festinus* would be caged on GRBV-positive grapevine for an acquisition period of at least a week. Fixation and tissue preparation would need optimization. I envision two possibilities: 1) Specimens could be fixed, and then sectioned longitudinally, or 2) Specimens could

be dissected to isolate the salivary glands and gut. Following tissue preparation and fixation, the hybridization with GRBV probes would be performed as previously described (Kliot *et al.* 2014), and analyzed by confocal fluorescence microscopy. These FISH experiments should be performed when the optimal acquisition conditions are determined by time course experiments, as described above.

Experiments are underway to test the hypothesis that the GRBV transmission is circulative. By analogy with other members of the family *Geminiviridae*, we also hypothesize that GRBV does not replicate within *S. festinus*. In order to test this hypothesis, I conducted a time course experiment with both adults and 4th-5th instar nymphs. In this experiment, 32 nymphs and 32 adults were caged on GRBV-infected grapevines for an AAP of 2 weeks. They were then all transferred to alfalfa to remove access to GRBV. The day of transfer was time 0 (T₀). At 3-day time intervals (T₁=3 days, T₂=6 days, T₃=9 days, T₄=12 days), cohorts of six adults which acquired GRBV as nymphs and six which acquired GRBV as adults were removed and immediately frozen at -80°C. DNA was extracted from individual specimens, and diluted to 25 ng/μL.

Quantitative PCR (qPCR) using SYBR green was then performed with primers ‘qREP2s’ (5’-ACATCTCTGGGTTTGGTGATATT-3’) and ‘qREP2as’ (5’-CTACACGCCTTGCTCATCTT-3’) for detection of GRBV (Setiono *et al.* 2018), and ‘Sf18SFor’ (5’-GTGAGGTCTTCGGACTGGTG-3’) and ‘Sf18SRev’ (5’-GGTTCACCTACGGAAACCTTG-3’) for detection of *S. festinus* 18S rRNA. Fifty ng of DNA was added to each reaction, and qPCRs were run in triplicate. An external standard was included on each qPCR plate using a dilution series containing 1x10⁹ to

1x10² copies of the GRBV monomer, in order to obtain a standard curve of GRBV copy number. GRBV was quantified using the comparative Cq method and standard curve method described by Setiono *et al.* (2018). Variance among groups was estimated by ANOVA in R studio. Both quantification methods showed the following trends: 1) Higher GRBV titer in *S. festinus* which ingested GRBV as nymphs compared to adults, and 2) Decreasing GRBV titer over time in both *S. festinus* which ingested GRBV as nymphs and adults (**Figure A2-7**). These data suggest that nymphs may acquire more GRBV than adults. They also show that *S. festinus* retain GRBV after molting, as GRBV was detected in adults which fed on GRBV-infected vines as nymphs, but then molted into adults on alfalfa, after the GRBV source was removed. They also suggest that GRBV titer decreases over time after GRBV-exposure is removed, and therefore it is unlikely that GRBV replicates in *S. festinus*. Together, these preliminary results suggest a non-propagative transmission mode of GRBV by *S. festinus*.

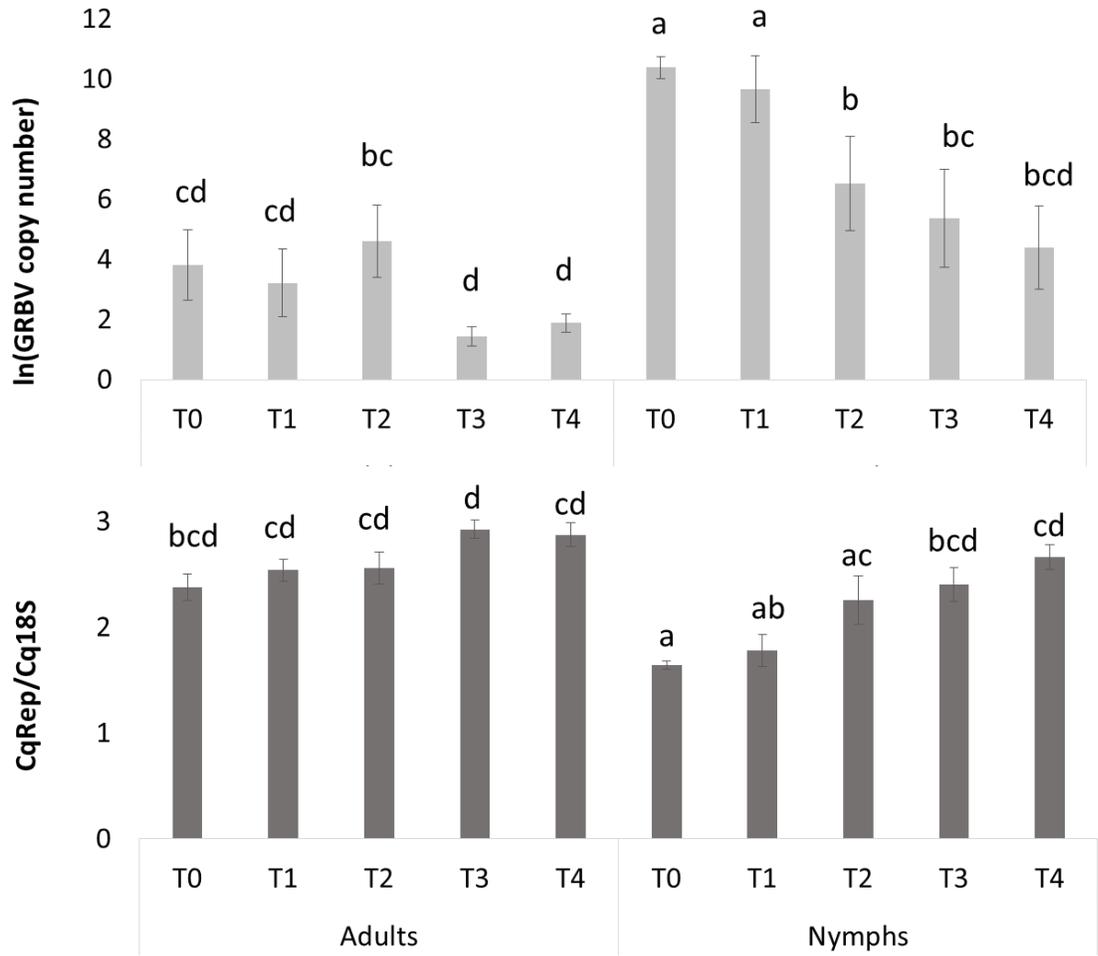


Figure A2-7 Reduction in grapevine red blotch virus (GRBV) DNA in *Spissistilus festinus* over time after exposure to GRBV source was removed. *S. festinus* were exposed to GRBV either as adults (left) or nymphs (right). Two methods of quantitative PCR analysis were used: (Top) GRBV copy number compared to external reference and (Bottom) Comparative Cq method of ‘Rep’ (GRBV target) and ‘18S’ (*S. festinus* target). Following feeding on GRBV-infected grapevine for two weeks, specimens were transferred to alfalfa and tested immediately (T0), and at three (T1), six (T2), nine (T3) and 12 (T4) days post-transfer. Letters above standard error bars indicate significance groups, as determined by ANOVA in R.

PART 4: TRANSMISSION ASSAYS, TRIAL AND ERROR

Spissistilus festinus collected from vineyards can reportedly transmit GRBV from infected *Vitis vinifera* ‘Cabernet Sauvignon’ (CS) vines to healthy CS vines under greenhouse conditions (Bahder *et al.* 2016). In this experiment, 15 *S. festinus* adults were collected from an alfalfa field near Davis, California and exposed to GRBV-infected CS vines for a 48-hour AAP, and then transferred to healthy CS vines for an IAP of 48 hours. The recipient plants were then tested for GRBV monthly for five months by dPCR, and started to test positive at the 3-month test, with increasingly more wells testing positive at months 4 and 5, and by month 5 GRBV was detectable by standard PCR. These authors also observed mild red blotch symptoms on two of the recipient vines by five months post-inoculation (Bahder *et al.* 2016).

The Bahder *et al.* (2016) study was paramount to advancing understanding of red blotch disease. However, it is important that these results be replicated in independent studies. Vineyard studies on the ecology of GRBV support *S. festinus* as the major vector of GRBV, at least in California (Cieniewicz *et al.* 2017, 2018). Nonetheless, controlled transmission assays should be carried out to confirm the capacity of *S. festinus* to transmit GRBV, especially since, to our knowledge, no other group has yet been able to replicate the transmission studies by Bahder *et al.* (2016).

There was initially confusion because the Virginia creeper leafhopper (*Erythroneura ziczac*) was reported as a vector of GRBV with 100% transmission efficiency (Poojari *et al.* 2013). Several concerns were immediately raised with this study. Importantly, *E. ziczac* is a mesophyll feeder, and GRBV is a phloem-limited geminivirus. Therefore, a transmission efficiency of 100% was subject to skepticism.

Additionally, there was no information at the time on GRBV spread in vineyards. However, there were anecdotal reports of GRBV spread occurring in the Napa Valley, where *E. ziczac* is rarely found. Nonetheless, these data could not be reproduced by groups at UC-Davis (Bahder *et al.* 2016) or UC-Berkeley (Daane *et al.* 2017).

In this section, I will summarize preliminary transmission experiments. In brief, I have tried a similar approach to the Bahder *et al.* (2016) study, using individual adults and potted vines. I have also attempted detached leaf inoculation assays using individual insects and detached grapevine leaves as recipients. Finally, I will describe the experiments in progress and future plans.

Experiments using potted grapevines

Transmission experiments using varying acquisition access periods (AAP) and inoculations access periods (IAP) and potted grapevines as both donor plants and recipient plants are summarized in **table A2-1**. In summary, all experiments using potted grapevines failed to repeat the results obtained by Bahder *et al.* (2016). Though, we learned several important experimental factors from these initial experiments. First, we learned that *S. festinus* will ingest GRBV and retain it after a feeding period of several days on clean plants (IAP). *S. festinus* does significant damage to plantlets from tissue culture by girdling the green shoots, resulting in high mortality of small (4-5 leaf) plantlets. Also, high *S. festinus* mortality (85%) was observed when 2nd and 3rd instars were caged on potted grapevine. It became apparent that using potted grapevines, although relevant for reproducing the UC-Davis study (Bahder *et al.* 2016), may not be ideal for future studies to determine the transmission efficiency and

the minimum requirements for successful transmission.

Table A2-1 Summary of four transmission experiments using potted vines and individual *S. festinus*

Exp.	AAP material ^a	IAP material	AAP time	IAP time	GRBV test results ^b	Notes
1	Agroinfected <i>V. vinifera</i> (Clade 2 clone)	Tissue culture plantlets	5 days	8 days	2/8 <i>S. festinus</i> + 0/4 plantlets + at 1 year post-inoculation	50% of the plantlets died due to <i>S. festinus</i> damage
2	Agroinfected <i>V. vinifera</i> (Clade 2 clone)	Tissue culture plants (one year in greenhouse)	6 days	5 days	5/7 <i>S. festinus</i> + 0/2 plants + at 1 year post-inoculation	Adults and 5 th instars used
3	Agroinfected <i>V. vinifera</i> (Clade 2 clone)	Tissue culture plantlets (12 weeks in greenhouse)	5 days	8 days	13/15 <i>S. festinus</i> + 0/4 plants + at 1 year post-inoculation	High mortality of 2 nd -3 rd instars (85% mortality)
4	Agroinfected <i>V. vinifera</i> (Clade 1 clone)	Tissue culture plantlets (>5 months in greenhouse)	4 days	2 days	7/8 <i>S. festinus</i> + 0/6 plants + at one year post-inoculation	4 th and 5 th instar nymphs used

^aGRBV-infected grapevines obtained by agroinoculation with clade 1 or clade 2 GRBV infectious clones (Yepes *et al.* 2018) were used as donor plants.

^bGRBV was tested in *S. festinus* and grapevines by multiplex PCR (Krenz *et al.* 2014).

Detached leaf inoculation assay

To address the limitations of potted grapevines for transmission assays, a detached leaf assay was attempted for *S. festinus* and GRBV. This assay was based on an excised leaf assay that is performed routinely in the Heck lab (Boyce Thompson Institute) for *Candidatus Liberibacter asiaticus* (CLAs) and its psyllid vector, *Diaphorina citri*, and was adapted from Raiol-Junior *et al.* (2017). In these assays, viruliferous psyllids are fed on excised citrus leaves in 50-mL conical tubes with the petiole submerged in sterile water, and the leaf blade exposed for psyllids to feed and inoculate CLAs. We adapted this assay for use in the *S. festinus*-GRBV-grape leaf system.

In our assay, two designs were attempted. In all experiments, *S. festinus* were caged on infected grapevines in the greenhouse for an AAP of at least one week. In the first experimental design, grape leaves were excised from GRBV-negative vines. The petiole was partially submerged in sterile water in 2-mL collection vials, with parafilm covering the water and a hole poked for the petiole. The leaf and 2-mL collection vial were placed in a magenta box, the viruliferous *S. festinus* were introduced to the magenta boxes, and covered with cheesecloth to prevent escape. IAPs lasted two days. *Spissistilus festinus* girdle the petioles, and therefore IAPs had to be relatively short to prevent the leaf from declining too quickly. Tissue could be harvested from the base of the petiole throughout the experiment at weekly intervals. In the first experiment, 1 of 12 detached leaves tested GRBV-positive by one week post inoculation, and continued to test positive at 2 and 3 weeks post inoculation. This experiment was replicated three more times, with 15, 22, and 20 recipient detached leaves respectively. In the fourth

experiment, two *S. festinus* were placed in each magenta box. Experiments 2-4 did not reproduce the results of the first experiment. Further, a control was missing from the first experimental design. Although the detached leaves were harvested from a plant that had previously tested negative, the individual leaves were not tested for GRBV prior to the IAP. Therefore, these experiments should be replicated.

Larger quart-sized containers with clip-lids were purchased to assist in the detached leaf assays. Therefore, a 5th experiment was attempted. In this experiment, the plant sources and tissue preparation were the same as described above, but the petiole was submerged in sterile water in 50-mL conical tubes, and there was more space for the wide grape leaves in the new containers. However, in this experiment, none of the 14 detached leaves tested positive for GRBV up to 4 weeks post-inoculation.

Experiments in progress, future plans

Based on transmission assays and detached leaf assays to date, we have not found convincing evidence of GRBV transmission by *S. festinus*. Several experiments are in progress or planned to test the hypothesis that GRBV is vectored by *S. festinus*. Based on excised leaf assays using CLAs and *D. citri*, at least 10 psyllids are needed for consistent inoculation of a single leaf (Michelle Heck, personal communication). The challenge with our experimental assay is that *S. festinus* damage the grape leaves by girdling the petiole, so having 10 *S. festinus* would likely result in a very short life of the leaf (<1 week). It is likely important to allow GRBV time to replicate in the inoculated leaf before it is detectable by PCR or qPCR. In an ongoing experiment, we

used eight *S. festinus* per leaf, and submerged the whole petiole, leaving the *S. festinus* to access only the veins on the leaf blade. It will be interesting to find out whether a higher number of *S. festinus* will increase the transmission rate and level of infection of excised grapevine leaves.

In some pathosystems undergoing the circulative transmission pathway, transmission is more efficient if the pathogen is acquired by the insect as nymphs (Ammar *et al.* 2016, Rotenberg *et al.* 2015). However this is dependent on the individual pathosystem. For example, in leafhopper transmission of maize fine streak virus, nymphs support higher viral titers than adults, but the developmental stage of the insect at the time of acquisition did not affect transmission rates (Todd *et al.* 2010). In the tomato-TPCTV-*M. malleifera* pathosystem, both adults and nymphs can efficiently vector the virus, and nymphs retain TPCTV after molting (Ammar and Nault 2002, Simons and Coe 1958). While comparison to other systems can be useful, the ability of *S. festinus* nymphs to transmit GRBV needs to be tested independently. Therefore, in the aforementioned ongoing detached leaf assay, the AAP spanned the developmental stages of *S. festinus* from 4th instar to adults (2 weeks in duration).

Another potential factor in our inability to reproduce the results demonstrating *S. festinus* as a vector in controlled conditions is rearing host effects on transmission. Pinheiro *et al.* (2017) elegantly demonstrated that transmission of potato leafroll virus (PLRV) by *Myzus persicae*, the green peach aphid, is significantly reduced dependent on the rearing host. PLRV titer was lower in turnip-reared aphids compared to physalis-reared aphids, owing to up-regulation of lysosomal enzymes in turnip-reared aphids. In viruses undergoing the circulative transmission pathway, understanding host

effects on gut physiological responses and virus transmission in the vector are critical. *Spissistilus festinus* are polyphagous, but the host impacts on gut physiology are unknown. A colony of *S. festinus* on snap bean (*Phaseolus vulgaris* var. ‘HiStyle’) has been established and has undergone 4-5 generations. Insects from this colony could be tested in future transmission assays and detached leaf inoculation assays.

PART V: CONCLUDING REMARKS

Altogether, the experiments reported in this appendix have increased our knowledge of *S. festinus* anatomy, behavior on grapevine, and acquisition of GRBV. This research has provided a foundation for future experiments. The development of a transmission assay is critical to be able to reproduce findings by Bahder *et al.* (2016), and also to further understand the biology of the *S. festinus*-GRBV system. Treehopper transmission of viruses is poorly understood, but is important for furthering the field of vector biology and developing novel methods of control of treehopper-vectored pathogens like GRBV.

REFERENCES

- Al Rwahnih, M., Alabi, O. J., Westrick, N. M., and Golino, D. 2018. Prunus geminivirus A: A novel *Grablovirus* infecting *Prunus* spp. *Plant Dis.* 102:1246–1253.
- Ammar, E.-D., and Nault, L. R. 2002. Virus transmission by leafhoppers, planthoppers and treehoppers (Auchenorrhyncha, Homoptera). *Adv. Bot. Res.* 36:141–167.

Bahder, B. W., Zalom, F. G., Jayanth, M., and Sudarshana, M. R. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology*. 106:1223–1230.

Brown, J.K., Fauquet, C.M., Briddon, R.W., Zerbini, M., Moriones, E. and Navas-Castillo, J. 2009. ICTV virus taxonomy profile: Geminiviridae. Ninth Report; 2009 Taxonomy Release.

Cieniewicz, E. J., Pethybridge, S. J., Gorny, A., Madden, L. V., McLane, H., Perry, K. L., and Fuchs, M. 2017. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Res.* 241:156–162.

Cieniewicz, E. J., Pethybridge, S. J., Loeb, G., Perry, K., and Fuchs, M. 2018. Insights into the ecology of *grapevine red blotch virus* in a diseased vineyard. *Phytopathology*. 108:94–102.

Kliot, A, Kontsedalov, S., Lebedev, G., Brumin, M., Cathrin, P. B., Marubayashi, J. M., Skaljac, M., Belausov, E., Czosnek, H., and Ghanim, M. 2014. Fluorescence in situ hybridizations (FISH) for the localization of viruses and endosymbiotic bacteria in plant and insect tissues. *J. Vis. Exp.* :e51030.

Krenz, B., Thompson, J. R., McLane, H., Fuchs, M., and Perry, K. L. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology*. 104:1232–1240.

Perry, K. L., Mclane, H., Thompson, J. R., and Fuchs, M. 2018. A novel grablovirus from non-cultivated grapevine (*Vitis* sp.) in North America. *Arch. Virol.* 163:259–262.

Poojari, S., Alabi, O. J., Fofanov, V. Y., and Naidu, R. A. 2013. A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family geminiviridae implicated in grapevine redleaf disease by next-generation sequencing. *PLoS One*. 8:e64194.

Preto, C. R., Bahder, B. W., Bick, E. N., Sudarshana, M. R., and Zalom, F. G. 2019. Seasonal dynamics of *Spissistilus festinus* (Hemiptera: Membracidae) in a Californian vineyard. *J. Econ. Entomol.* doi:10.109.

Preto, C. R., Sudarshana, M. R., Bollinger, M. L., and Zalom, F. G. 2018a. *Vitis vinifera* (Vitales: Vitaceae) as a reproductive host of *Spissistilus festinus* (Hemiptera: Membracidae). *J. Insect Sci.* 18:1–7.

Preto, C. R., Sudarshana, M. R., and Zalom, F. G. 2018b. Feeding and reproductive hosts of *Spissistilus festinus* (Say) (Hemiptera: Membracidae) found in Californian

Vineyards. *J. Econ. Entomol.* 111:2531–2535.

Raiol-Junior, L. L., Baia, A. D. B., Luiz, F. Q. B. F., Fassini, C. G., Marques, V. V., and Lopes, S. A. 2017. Research improvement in the excised citrus leaf assay to investigate inoculation of “*Candidatus Liberibacter asiaticus*” by the asian citrus psyllid *Diaphorina citri*. *Plant Dis.* 101:409–413.

Rojas, M. R., Macedo, M. A., Maliano, M. R., Soto-Aguilar, M., Souza, J. O., Briddon, R. W., Kenyon, L., Bustamante, R.F.R., Murilo Zerbini, F., Adkins, S., Legg, J.P., Kvarnheden, A., Wintermantel, W.M., Sudarshana, M.R., Peterschmitt, M., Lapidot, M., Martin, D.P., Moriones, E., Inoue-Nagata, A.K., Gilbertson, R.L. 2018. World management of geminiviruses. *Annu. Rev. Phytopathol.* 56:637–677.

Rosen, R., Kanakala, S., Kliot, A., Cathrin Pakkianathan, B., Farich, B. A., Santana-Magal, N., Elimelech, M., Kontsedalov, S., Lebedov, G., Cilia, M., and Ghanim, M. 2015. Persistent, circulative transmission of begomoviruses by whitefly vectors. *Curr. Opin. Virol.* 15:1–8.

Rotenberg, D., Jacobson, A. L., Schneweis, D. J., and Whitfield, A. E. 2015. Thrips transmission of tospoviruses. *Curr. Opin. Virol.* 15:80–89.

Roumagnac, P., Granier, M., Bernardo, P., Deshoux, M., Ferdinand, R., Galzi, S., Fernandez, E., Julian, C., Abt, I., Filloux, D., Mesleard, F., Varsani, A., Blanc, S.,

- Martin, D.P., and Peterschmitt, M. 2015. Alfalfa leaf curl virus: an aphid-transmitted geminivirus. *J. Virol.* 89:9683–9688
- Setiono, F. J., Chatterjee, D., Fuchs, M., Perry, K. L., and Thompson, J. R. 2018. The distribution and detection of grapevine red blotch virus in its host depend on time of sampling and tissue type. *Plant Dis.* 102:2187–2193.
- Simons, J. N. 1962. Life history and behavioral studies on *Micrutalis malleifera*, a vector of pseudo curly top virus. *J. Econ. Entomol.* 55:363–365.
- Simons, J. N., and Coe, D. M. 1958. Transmission of pseudo-curly top virus in Florida by a treehopper. *Virology.* 6:43–48.
- Todd, J. C., Ammar, E.-D., Redinbaugh, M. G., Hoy, C., and Hogenhout, S. A. 2010. Plant host range and leafhopper transmission of *Maize fine streak virus*. *Phytopathology.* 100:1138–1145.
- Varsani, A., Roumagnac, P., Fuchs, M. F, Navas-Castillo, J., Moriones, E., Idris, I., Briddon, R. W. Rivera-Bustamante, R., Murilo Zerbini, F., and Martin, D. P. 2017. *Capulavirus* and *Grablovirus*: Two new genera in the family *Geminiviridae*. *Arch. Virol.* 162:1819–1831.
- Yepes, L.M. Cieniewicz, E.J, Krenz, B. McLane, H., Thompson, J.,R. Perry, K.L. and

Fuchs, M. 2018. Causative role of grapevine red blotch virus in red blotch disease. *Phytopathology*, 108: 902-909.

Zerbini, F. M., Briddon, R. W., Idris, A., Martin, D. P., Moriones, E., Navas-Castillo, J., Rivera-Bustamante, R., Roumagnac, P., and Varsani, A. 2017. ICTV Virus Taxonomy Profile: Geminiviridae. *J. Gen. Virol.* 98:131–133.

Zhong, H., Zhang, Y., and Cong, W. 2015. Morphology and ultrastructure of salivary glands of male treehopper *Tricentrus brunneus* Funkhouser (Hemiptera: Membracoidea). *Entomol. Fenn.* 26:201–212.

APPENDIX 3

EXPERIMENTAL HOST RANGE OF GRAPEVINE RED BLOTCH VIRUS

JUSTIFICATION

Grapevine red blotch virus (GRBV) is widespread in *Vitis* spp. in commercial vineyards (Krenz *et al.* 2014), as well as in wild vines near infected vineyards in northern California (Bahder *et al.* 2016; Perry *et al.* 2016; Cieniewicz *et al.* 2018). Similarly to other geminiviruses, the host range of GRBV may not be limited to *Vitis* spp. Since *S. festinus* can be a damaging pest of legumes (Beyer *et al.* 2017) and legumes are often sown in vineyard row middles as a cover crop, it is important to evaluate potential alternative hosts of GRBV in the vineyard.

Determining the experimental host range of GRBV can help to target surveys for GRBV within and around vineyards. Identifying herbaceous alternative hosts of GRBV can also be useful for studying interactions among the virus, vector, and host. Herbaceous hosts generally grow faster than *Vitis* spp., and can generally be more easily managed in greenhouse and growth chamber settings. Therefore, studies aimed at better understanding genome expression, virus movement, and transmission of GRBV by insect vectors may be aided by alternative hosts.

AGROINOCULATION METHOD

Infectious bitmer clones, or partial tandem repeats of GRBV isolates (NY175 and NY358) representing both phylogenetic clades of GRBV were engineered in the Perry lab at Cornell University and cloned into *Agrobacterium tumefaciens* strain C58C1 (Yepes *et al.* 2018). For each experiment, seedlings of various herbaceous hosts were inoculated by the pin-prick method, in which sterile insect pins were used to transfer recombinant *A. tumefaciens* cultures on solid agar medium to the host, directly inoculated to the stem and petioles of at least two leaves. Agroinoculation

experiments were conducted in three hosts- *Nicotiana benthamiana*, *Solanum lycopersicum* ‘Florida Lanai,’ and *Phaseolus vulgaris* ‘HiStyle’. These three plant species were selected for these experiments because *N. benthamiana* is a common herbaceous host used in plant virology studies (Goodin *et al.* 2008), *Solanum lycopersicum* ‘Florida Lanai’ has been described as an optimal model host for studying geminiviruses of tomato (Rajabu *et al.* 2018) and preliminary work has shown that *Phaseolus vulgaris* ‘HiStyle’ may be a host of GRBV. A construct using the β -glucuronidase (GUS) reporter gene under control of the cauliflower mosaic virus 35S promoter was used as a positive control for T-DNA transfer by *A. tumefaciens* into test plants. These experiments were all conducted in walk-in growth chambers with a 14:10 light to dark photoperiod, 50% humidity, and 25°C.

DETECTION OF SPLICED GRBV TRANSCRIPTS

In order to definitely identify a plant as an alternative host of GRBV, we need a method of detecting virus replication in the plant following inoculation. There is not yet a serological test available for GRBV despite several attempts by both industry and academic labs to develop an antibody to recognize GRBV proteins in infected grapevines (Buchs *et al.* 2018; K.L. Perry, M. Sudarshana, R. Gilbertson, A. Wei, personal communication). Such a test would be helpful to detect the accumulation of GRBV proteins in infected plants. Conventional PCR cannot distinguish between the DNA of an infectious clone and the replicating virus genome in infected plants. Krenz *et al.* (2014) predicted a splicing event in which two open reading frames (ORFs) in the complementary sense orientation, C1 (RepA) and C2 (unknown function), fuse to form a replication-associated protein (Rep). Recently, RNAseq, RT-PCR, and transient expression in *N. benthamiana* (C1-C2 construct with read-through to 6x Histidine tract) confirmed the hypothesized splicing event in C1-C2 (Vargas-Asencio

et al. 2019). RT-PCR primers bridging the splice site in the C1-C2 ORF can be used to amplify both spliced and unspliced transcripts as a result of GRBV genome expression during replication (Yepes *et al.* 2018).

For RT-PCR, total nucleic acids are extracted from leaves/petioles using the Plant RNA kit (OMEGA Biotek) and used in the OneStep Ahead kit (Qiagen) with primers flanking the C1-C2 splice site. The forward primer ‘GRBVSplFor’ (5’-TTACAAGGCAAATATTGGAATG-3’) is used for both the NY175 (clade 1) and NY358 (clade 2) GRBV isolates. Reverse primers ‘GRBVClade1Rev’ (5’-CAAAATGAACTCTTCGTGGATC-3’) and ‘GRBVClade2Rev’ (5’-CAAAACGAACTCTACGTGGAAG-3’) are used for plants inoculated with the NY175 (clade 1) and NY358 (clade 2) GRBV isolates, respectively. The expected size for GRBV amplicons of spliced Rep transcripts and unspliced Rep transcripts (or viral genomic DNA) is 206 bp and 369 bp, respectively (**Figure A3-1**).

In agroinoculation experiments, seedlings (4-5 leaf stage) were inoculated with either the NY175 or NY358 construct. Negative controls included a mock inoculated (sterile needle) and non-inoculated plant. At seven days post-inoculation (dpi) the inoculated leaves were collected and tested by RT-PCR. At 14 and 21 dpi, leaves were collected from apical (non-inoculated) leaves to test for systemic movement of GRBV. RT-PCRs were run at the following thermocycling conditions: 50°C for 10 minutes, 95°C for 5 minutes, 40 cycles of 95°C for 15 seconds, 55°C for 25 seconds, 72°C for 25 seconds, and 72°C for 2 minutes. RT-PCR products were resolved by gel electrophoresis on 2% gels in 1X tris acetate EDTA buffer, run for 40 minutes at 100 V, and visualized under UV after staining with GelRed.

AGRO-INOCULATION EXPERIMENTS: RESULTS

The same general trends were observed in agroinoculation experiments with

three different hosts- *Nicotiana benthamiana*, *Phaseolus vulgaris* var. ‘HiStyle’, and *Solanum lycopersicum* var. ‘Florida Lanai.’ For each host, GRBV spliced transcripts (**Figure A3-1**) were observed in inoculated leaves by 7 dpi for both GRBV infectious clones (**Table A3-1**). However, throughout the duration of these experiments, GRBV was not detected in apical (non-inoculated) leaves (**Table A3-1**). This suggests that GRBV is replicating locally in inoculated leaves, but not moving systemically.

Table A3-1 Results of agroinoculation experiments on potential alternative hosts of grapevine red blotch virus.

Plant Species	Local infection ^a (at 7 dpi)		Systemic infection ^b (at 14 and 21 dpi)		GUS control
	NY175	NY358	NY175	NY358	
<i>Nicotiana benthamiana</i>	5/5	3/5	0/5	0/5	-
<i>Solanum lycopersicum</i>	5/5	4/5	0/5	0/5	-
<i>Phaseolus vulgaris</i>	6/8	5/8	0/6	0/6	+

^aLocal infection was detected in agroinoculated leaves of test plants

^bSystemic infection was detection in uninoculated, apical leaves of test plants

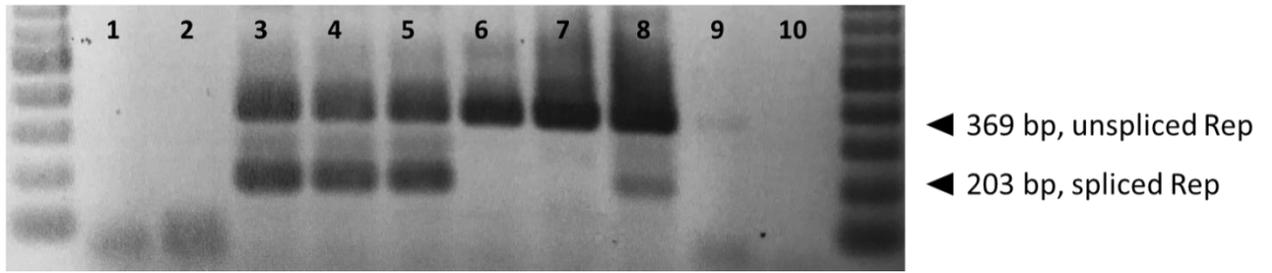


Figure A3-1 RT-PCR detection of grapevine red blotch virus spliced and unspliced Rep transcripts using NY175-specific primers. Lane 1- Mock inoculated control; Lane 2- Apical tissue of agroinoculated *N. benthamiana*, 14 dpi; Lanes 3-4, Agroinoculated *N. benthamiana* at 14 days post inoculation; Lane 5- Agroinoculated *N. benthamiana* at 7 days post inoculation. Lane 6- Agroinoculated *N. benthamiana* immediately following inoculation; Lane 7- Agro-GRBV construct (colony PCR); Lane 8- Agroinfected grapevine more than 2 years post-inoculation; Lane 9- NY358-agroinoculated *N. benthamiana* immediately following inoculation; Lane 10- No template (water) control.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

A tractable system to conduct experiments on GRBV would help us to better understand the biology of this divergent geminivirus. Additionally, an alternative host for GRBV could guide further study on the biology of other grabloviruses such as wild *Vitis virus 1* (Perry *et al.* 2018) and *prunus geminivirus A* (Al Rwahnih *et al.* 2018). For example, herbaceous hosts such as *N. benthamiana* would be a more tractable system than grapevine to study GRBV genome expression and carry out proteomics studies that could be useful in developing a serological test for GRBV. These experiments suggest that GRBV can replicate in locally inoculated leaves, but not move systemically in *N. benthamiana*, *S. lycopersicum*, and *P. vulgaris*.

In future research, several experimental variables could be tested to optimize systemic GRBV infection in herbaceous hosts. It is possible that the experimental conditions used thus far are not optimal for virus replication and/or systemic movement. For example, agroinoculation experiments could be done in growth chambers at various temperatures. Alternatively, it is possible that GRBV cannot move systemically in these hosts because it is lacking a host factor that is available in grapevine.

Geminivirus movement has mostly been studied in bipartite begomoviruses; there is little information available on movement of monopartite geminiviruses, like GRBV. In monopartite geminiviruses, the coat protein (CP) acts as a nuclear shuttle protein (NSP). Movement protein function in tomato yellow leaf curl virus, a monopartite begomovirus, is mediated by V2 (CP) alone or in complex with the protein encoded by the C4 ORF (Rojas *et al.* 2001). There are examples of begomoviruses that can move long distances as both virions and as ribonucleoprotein (RNP) complexes. For mastreviruses and curtoviruses (monopartite leafhopper-vectored geminiviruses), long distance movement is only known to occur as virions

(reviewed by Hipper *et al.* 2013). The mechanisms underlying cell-to-cell movement and systemic movement of GRBV are unknown.

To further test herbaceous alternative hosts, both agroinoculation and inoculation via the insect vector- *Spissistilus festinus*- could be used. Transmission experiment parameters optimized in grapevine, detailed in Appendix 2 of this dissertation, would be useful in testing alternative hosts of GRBV. Additionally, it would be interesting to conduct quantitative RT-PCR following agroinoculation of herbaceous hosts to determine if concentration of GRBV transcripts increases over time, even just locally. I would also test more herbaceous hosts, including model species such as *Medicago truncatula* and *Nicotiana tabacum*, and species commonly included in vineyard cover crops, such as *Medicago sativa*, *Pisum sativum*, *Trifolium* spp., *Vicia faba*, and *Lotus corniculatus*.

REFERENCES

- Al Rwahnih, M., Alabi, O. J., Westrick, N. M., and Golino, D. 2018. Prunus geminivirus A: A novel *Grablovirus* infecting *Prunus* spp. *Plant Dis.* 102:1246–1253.
- Bahder, B. W., Zalom, F. G., and Sudarshana, M. R. 2016. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Dis.* 100:1571–1574.
- Beyer, B. A., Srinivasan, R., Roberts, P. M., and Abney, M. R. 2017. Biology and

management of the threecornered alfalfa hopper (Hemiptera: Membracidae) in alfalfa, soybean, and peanut. *J. Integr. Pest Manag.* 8:1–10.

Buchs, N., Braga-Lagache, S., Uldry, A.-C., Brodard, J., Debonneville, C., Reynard, J.-S., and Heller, M. 2018. Absolute quantification of grapevine red blotch virus in grapevine leaf and petiole tissues by proteomics. *Front. Plant Sci.* 9: doi: 10.3389.

Cieniewicz, E., Thompson, J. R., McLane, H., Perry, K. L., Dangl, G. S., Corbett, Q., Martinson, T., Wise, A., Wallis, A., O'Connell, J., Dunst, R., Cox, K., and Fuchs, M. 2018b. Prevalence and genetic diversity of grapevine red blotch viruses in free-living *Vitis* spp. *Plant Dis.* 102:2308–2316.

Goodin, M. M., Zaitlin, D., Naidu, R. A., and Lommel, S. A. 2008. *Nicotiana benthamiana* : Its history and future as a model for plant-pathogen interactions. *Mol. Plant-Microbe Interact.* 21:1015–1026.

Hipper, C., Brault, V., Ziegler-Graff, V., and Revers, F. 2013. Viral and cellular factors involved in phloem transport of plant viruses. *Front. Plant Sci.* 4: doi: 10.3389

Krenz, B., Thompson, J. R., McLane, H., Fuchs, M., and Perry, K. L. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology.* 104:1232–1240.

Perry, K. L., McLane, H., Hyder, M. Z., Dangl, G. S., Thompson, J. R., and Fuchs, M. F. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* sp. proximal to cultivated grapevines. *Phytopathology*. 106:663–670.

Perry, K. L., Mclane, H., Thompson, J. R., and Fuchs, M. 2018. A novel grablovirus from non-cultivated grapevine (*Vitis* sp.) in North America. *Arch. Virol*. 163:259–262.

Rajabu, C. A., Kennedy, G. G., Ndunguru, J., Ateka, E. M., Tairo, F., Hanley-Bowdoin, L., Ascencio-Ibáñez, J.T. 2018. Lanai: A small, fast growing tomato variety is an excellent model system for studying geminiviruses. *J. Virol. Methods*. 256:89–99.

Rojas, M. R., Macedo, M. A., Maliano, M. R., Soto-Aguilar, M., Souza, J.O., Briddon, R.W., Kenyon, L., Rivera Bustamante, R.F., Zerbini, F.M., Adkins, S., Legg, J.P., Kvarnheden, A., Wintermantel, W.M., Sudarshana, M.R., Peterschmitt, M., Lapidot, M., Martin, D.P., Moriones, E., Inoue-Nagata, A.K., and Gilbertson, R.L. 2018. World management of geminiviruses. *Annu. Rev. Phytopathol*. 56:637–677.

Vargas-Asencio, J., Liou, H., Perry, K. L., and Thompson, J. R. 2019. Evidence for the splicing of grablovirus transcripts reveals a putative novel open reading frame. *J. Gen. Virol*. DOI 10.109.

Yepes, L. M., Cieniewicz, E. J., Krenz, B., McLane, H., Thompson, J. R., Perry, K. L., and Fuchs, M. 2018. Causative role of grapevine red blotch virus in red blotch disease. *Phytopathology*. 108:902–909.

APPENDIX 4

EXTENSION ARTICLES- STUDIES ON RED BLOTCH ECOLOGY INFORM DISEASE MANAGEMENT RECOMMENDATIONS

*This article was published in March of 2019 in Wine Business Monthly as:
Cieniewicz E.J., Wise A, Cooper M, Smith R, Martinson T, & Fuchs M. (2019)
Studies on red blotch ecology inform disease management recommendations. *Wine
Business Monthly*, March Issue, pp. 92-102.

An article of similar content was published in Appellation Cornell in November 2018
as: Cieniewicz E.J. & Fuchs M. (2018) Red blotch disease ecology and management.
Appellation Cornell Research Focus 2018-4. <https://grapesandwine.cals.cornell.edu>

Both articles were intended to translate findings of our research on red blotch disease
ecology into disease management recommendations for grape growers throughout the
United States.

RED BLOTCH DISEASE: A NEW THREAT TO GRAPE PRODUCTION

Red blotch disease has emerged in the last decade as one of the major virus diseases of grapevine in North America. Grapevine red blotch virus (GRBV), the causal agent of red blotch disease (Yepes *et al.* 2018), is widespread in vineyards throughout the United States (Cieniewicz *et al.* 2017a, Krenz *et al.* 2014, Sudarshana *et al.* 2015). GRBV affects the profitability of vineyards by reducing fruit quality and ripening (Blanco-Ulate *et al.* 2017) (**Figure A4-1**), resulting in losses up to \$170,000 per acre over the lifespan of a vineyard, depending on the initial disease incidence, cultivar, region, and price penalty for low quality fruit (Ricketts *et al.* 2017). Optimal management of red blotch requires a comprehensive understanding of disease ecology in vineyard ecosystems.

Red blotch disease is difficult to visually identify in vineyards because (i) symptoms are similar to those of leafroll disease and some nutrient disorders, and (ii) the timing of symptom onset and severity can vary greatly between growing seasons. In red cultivars, red blotches forming on the leaves are early indicators of the disease, and these may coalesce across the leaf blade (**Figure A4-2**). In white cultivars, irregular chlorotic areas of the leaves may form and become necrotic (**Figure A4-2**). Foliar symptoms typically appear first on older leaves at the base of the canopy, progress up the shoot toward younger leaves, and peak near harvest. Poor fruit coloration in red cultivars (**Figure A4-1**), and delayed fruit ripening in red and white cultivars can also be indicative of red blotch. GRBV often causes asymptomatic infections in rootstocks.



Figure A4-1 Reduction in fruit quality and ripening on (top, right) a red blotch diseased compared to (top, left) an asymptomatic Pinot noir vine in Oregon, and (bottom, left) red blotch diseased next to (bottom, right) an asymptomatic Cabernet franc vine on Long Island, New York. (Photos courtesy of M. Fuchs and A. Wise).

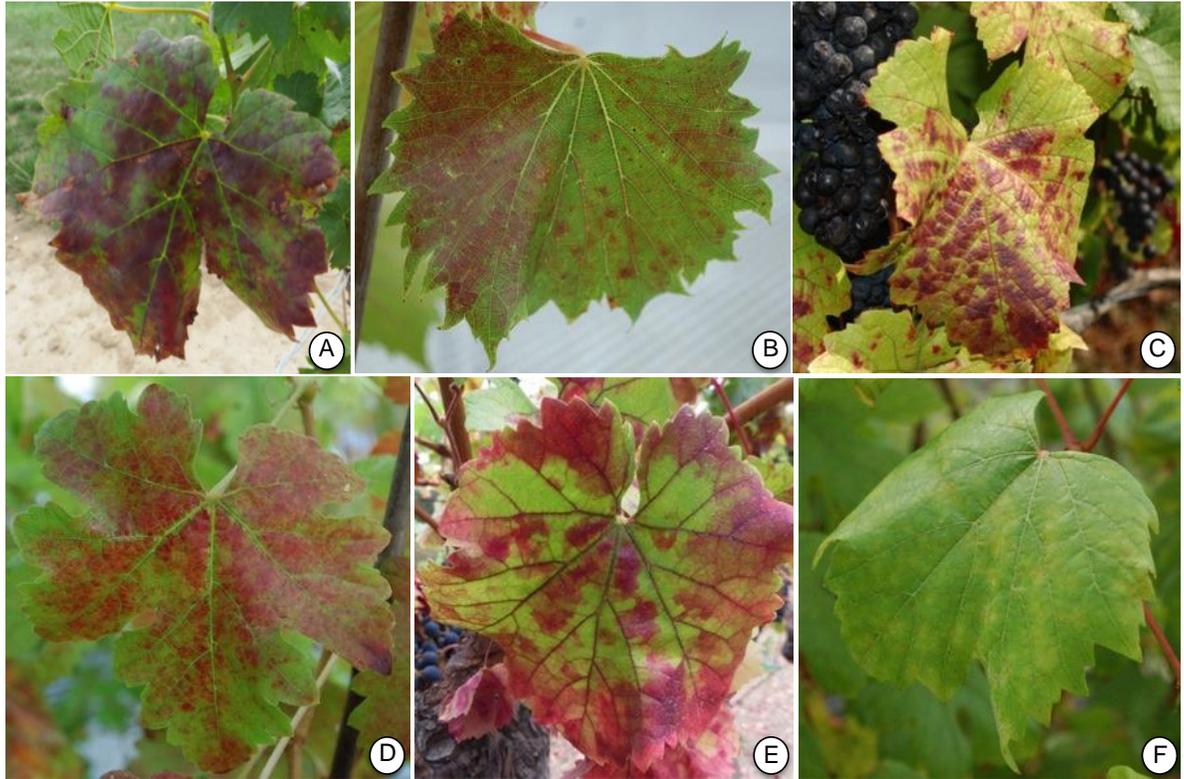


Figure A4-2 Close up of foliar symptoms of red blotch on Cabernet franc (A), Chambourcin (B), Pinot noir (C), Syrah (D), Cabernet Sauvignon (E) and Chardonnay (F) (Photos courtesy of M. Fuchs).

Although GRBV has likely been present for decades, red blotch disease remained elusive for a long time (Al Rwahnih *et al.* 2015). Currently, the only detection techniques available for GRBV are DNA-based tests such as the polymerase chain reaction (PCR) and its variations (Cieniewicz *et al.* 2017a, Sudarshana *et al.* 2015). To accurately assay for GRBV, older, basal leaf blades with petioles attached should be sampled late summer-early fall (Setiono *et al.* 2018). Alternatively, the basal region of canes should be collected in the dormant season, preferably from shoots originating in the head area of the vine.

EFFECTS OF GRBV ON VINE GROWTH AND FRUIT JUICE CHEMISTRY

Red blotch disease has a detrimental effect on vegetative growth and fruit juice chemistry (Calvi 2011). Specifically, growth of infected Merlot vines on Long Island in New York as measured by pruning weight was reduced by 4-5%, fruit yield by 11% and sugar content by up to 2.2 Brix. GRBV also increased titratable acidity by up to 1.2 g/L. Similar trends were observed for Chardonnay. A California study, conducted in 2013 and 2014 in Chardonnay and Cabernet Sauvignon highlighted the variable effects of GRBV. Total soluble solids were consistently reduced and titratable acidity tended to be elevated in infected vines in both cultivars; however, yield and vegetative growth were reduced in Chardonnay but not in Cabernet Sauvignon.

SPREAD OF RED BLOTCH IN A NAPA VALLEY VINEYARD

Every October from 2014-2018, red blotch incidence and spread were recorded in a 5-acre Cabernet franc vineyard that was planted in 2008, prior to the discovery of GRBV. By 2012 the vineyard manager noticed a gradient of vines exhibiting foliar reddening symptoms at the edge of the vineyard next to a riparian area (**Figure A4-3**). In 2013, we confirmed the presence of GRBV by PCR in a subset of the symptomatic

vines in this area.

The disease incidence (percentage of diseased vines) over the whole vineyard was 4.0% (305/7,691 vines diseased) in 2014, 6% in 2015, 7.1% in 2016, 9.1% in 2017 and 13.8% in 2018 (Figure 4). While disease incidence in the whole vineyard increased by only 10% over five years, the spread was much more rapid (40% over five years) in the area where infected vines were initially aggregated (**Figure A4-4**) (Cieniewicz *et al.* 2017b).

We applied spatiotemporal models to show that a vine was more likely to become infected if it was close to an infected vine, and that disease spread mostly from localized, within-vineyard sources rather than inoculum sources outside of the vineyard. The predictions from these models were confirmed by determining the genotypes of GRBV populations. Together, this work revealed that the virus inoculum likely originated from the planting material, probably the rootstock, and secondary spread occurred via an insect vector (Cieniewicz *et al.* 2017b, 2018a).



Figure A4-3 Red blotch secondary spread in a gradient down the row of a Cabernet franc vineyard in Rutherford, California (background). GRBV is transmitted in vineyards by the three-cornered alfalfa hopper (foreground) (Photo courtesy of E. Cieniewicz).

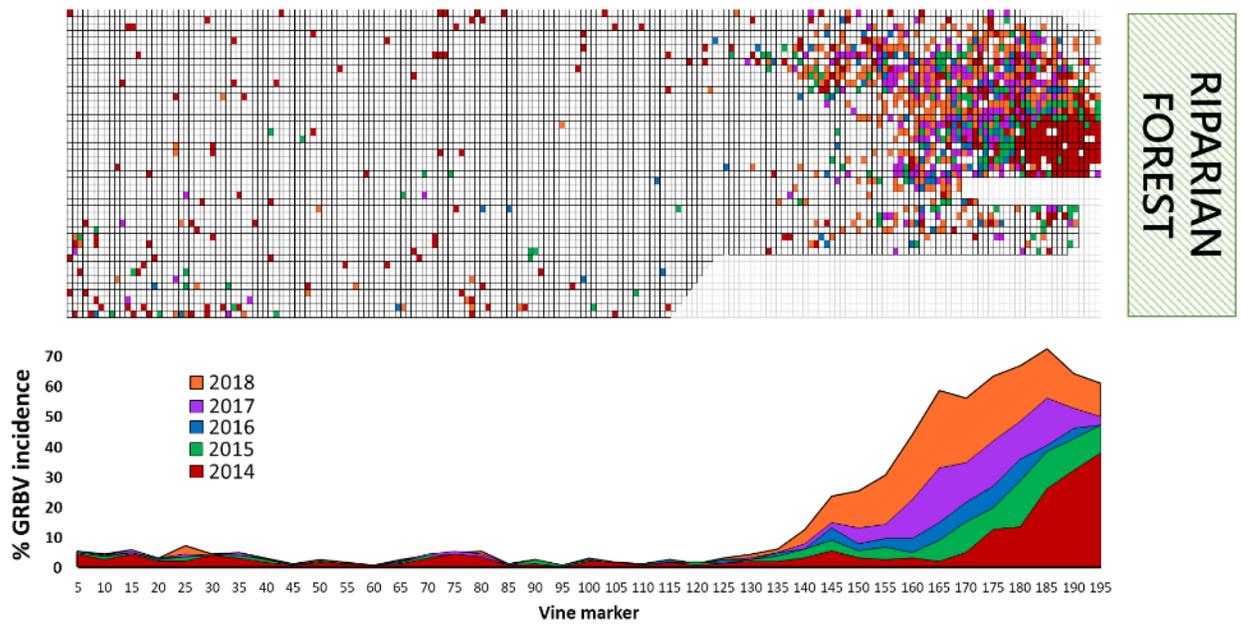


Figure A4-4 GRBV spread in a 5-acre Cabernet franc vineyard in California over 5 years. The top graph shows the entire study vineyard with each cell representing a single vine that is asymptomatic (blank) or symptomatic (colored). The bottom graph shows the distribution of diseased vines in 5-vine panels across rows.

SURVEY OF POTENTIAL INSECT VECTORS IN A CALIFORNIA VINEYARD

We conducted insect surveys in 2015 and 2016 in the area of the Cabernet franc vineyard with extensive spread. We hung 36 yellow sticky traps (3x5 inches) in a grid pattern spanning twelve rows, and six 4-vine panels per row from April through November (**Figure A4-5**). We refreshed traps weekly, and tested the insects for GRBV to determine which species are capable of ingesting the virus by feeding on the vines. Although this does not prove the insects are vectors, it does narrow down the pool of potential insect vector candidates and provides insights into timing of feeding and potential transmission.

In 2015, we tested over 700 insect specimens from 40 different species/taxa. Of the 40 taxa evaluated, only four species consistently tested positive for GRBV. In 2016, we tested fewer specimens (n=271) but results were consistent with the 2015 survey. The four insects identified as vector candidates are *Spissistilus festinus* (three-cornered alfalfa hopper -TCAH), currently the only confirmed vector of GRBV (Bahder *et al.* 2016a), two leafhoppers (*Colladonus reductus* and *Osbornellus borealis*) and a planthopper (*Melanoliarius* spp.) (Cieniewicz *et al.* 2018a).

These insects are all phloem-feeders, and were all found in low relative abundance. Collectively they comprised less than 0.14% of the total number of insects trapped over the two-year survey period. The populations of the four insect vector candidates peaked at different times in the season, but the population dynamics were consistent between 2015 and 2016. Populations of the TCAH in this vineyard peaked in late June- early July, and were higher in the vineyard edge in proximity to a riparian area (Cieniewicz *et al.* 2018a). Even during the weeks with highest density of TCAH, specimens were sparse, i.e. only 2-20 TCAH were found.

LIMITED SPREAD OF GRBV IN AN ADJACENT CALIFORNIA VINEYARD

The pattern of spread observed in the Cabernet franc vineyard may not be typical of other vineyards with GRBV-infected vines. For instance, immediately to the west of the Cabernet franc vineyard is a 5-acre Cabernet Sauvignon vineyard, also planted in 2008 but with two clones that were sourced from distinct nurseries (**Figure A4-5**). Spread of GRBV in the northern portion of this vineyard established with one clone has been very limited over time (less than 1% over 10 years) despite being adjacent to a large source of virus inoculum (40%) in the southern portion established with the other clone (**Figure A4-5**). Why is the rate of spread lower in the Cabernet Sauvignon vineyard than in the Cabernet franc vineyard in spite of the availability of a very high inoculum source (40%) in the former and a very low inoculum source (1%) in the latter at planting? Could a difference in vector population or behavior account for a distinct spread pattern between the two vineyards?

Insect surveys conducted in 2017 and 2018 showed that, although many of the same insects were present in both vineyards, the relative abundance of many of the insects differed. For example, we found fewer TCAH in the Cabernet Sauvignon vineyard (N=5) compared to the Cabernet franc vineyard (N=50) over the two-year survey periods. Similarly, there were fewer *Osbornellus borealis* and *Melanoliarus* spp. but the abundance of *Colladonus reductus* was higher in the Cabernet Sauvignon vineyard. The difference in insect abundance, particularly of the TCAH, could explain the differential spread of GRBV in the two study vineyards. Additionally, the difference in insect populations, specially of the TCAH, could be due to the proximity of the Cabernet franc vineyard to the wooded natural area, compared to the Cabernet Sauvignon vineyard, which is about 800 feet from the riparian habitat.

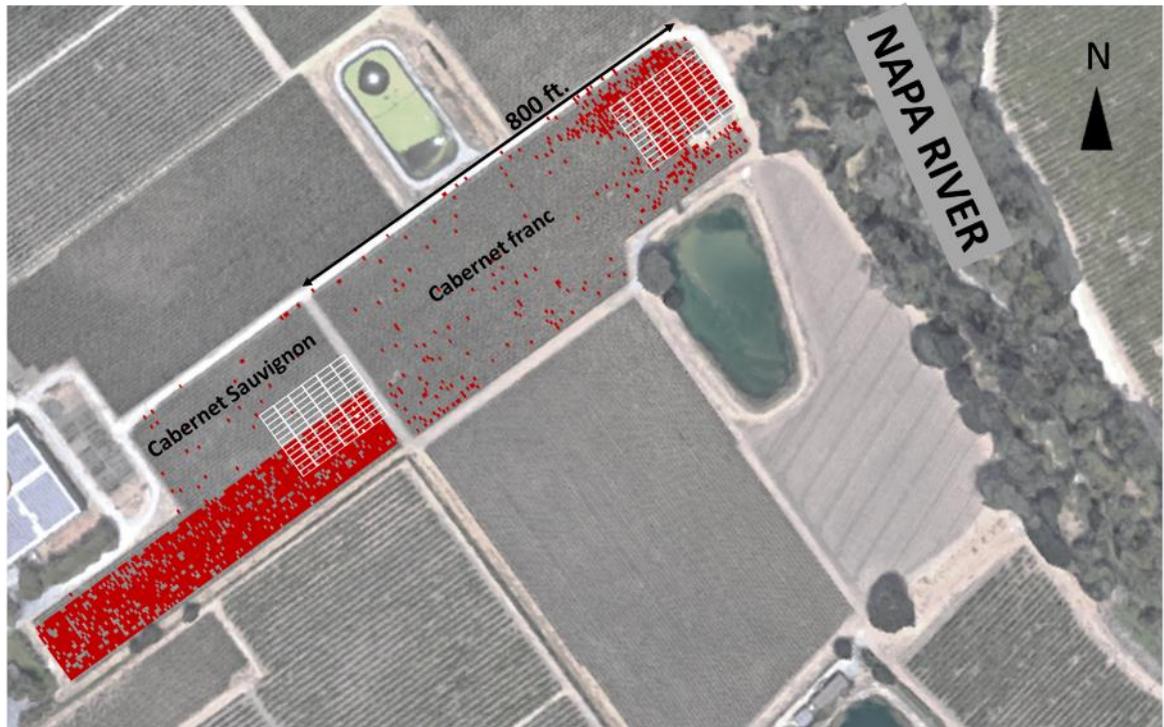


Figure A4-5 Landscape view of GRBV spread in Cabernet franc and Cabernet Sauvignon vineyards in Rutherford, California. Both vineyards were planted in 2008. Red overlay indicates GRBV-infected vines in 2018. White grids indicate area of surveys for insects in 2015-16 in the Cabernet franc vineyard and 2017-18 in Cabernet Sauvignon vineyard (Photo credit: Google earth satellite imagery and E. Cieniewicz).

SURVEY OF RED BLOTCH IN A NEW YORK VINEYARD

Concurrent with the California study, we surveyed a 3-acre Merlot vineyard on Long Island in New York during the 2014 to 2018 growing seasons. This vineyard was planted in 2008 and had a high GRBV incidence (60%), suggesting that the virus most likely resulted from infected planting material. Over the five years of sampling, negative vines consistently tested negative, indicating that no apparent secondary spread occurred in the Merlot vineyard.

This conclusion is strengthened by the results of an insect survey conducted in 2017 and 2018 parallel to the California studies. Although we found several phloem-feeding treehoppers and leafhoppers, none of them consistently tested positive for GRBV. The TCAH was not found in the Merlot vineyard, nor has it yet been reported in any vineyards in New York. To our knowledge, there is no evidence that GRBV is spreading by insect vectors on the east coast.

GRBV IS WIDESPREAD IN WILD GRAPEVINES IN CALIFORNIA

GRBV was detected in wild *Vitis* spp. near commercial vineyards in Napa County, including in the riparian area next to the Cabernet franc vineyard (Perry *et al.* 2016) and other locations in Napa Valley (Bahder *et al.* 2016b). To determine whether GRBV is widespread in wild vines, we collected samples in Northern California and New York, assayed them for GRBV and analyzed the genetic diversity of the virus populations.

In California, we found GRBV in 21% (43 of 203) of the wild *V. californica* and *V. californica* hybrid vines sampled. The virus was more prevalent in counties with more grape production (Napa, Sonoma, Sacramento) than in counties with less or no grape production (Solano, Sutter, Butte, Glenn) (**Figure A4-6**). The virus populations in wild vines genetically matched those in adjacent commercial vineyards.

This suggested that the predominant direction of spread of the virus is from commercial vineyards to adjacent wild vines, rather than the opposite (Cieniewicz *et al.* 2018b). Nonetheless, our surveys covered only four recent years (2014-2017), so we cannot rule out that GRBV may have originated from wild vines in California in the more distant past.

In New York, 163 wild *V. riparia*, *V. aestivalis* and *V. labrusca* vines from western NY, the Finger Lakes region, Champlain Valley, Hudson Valley, and Long Island that were assayed tested negative for GRBV.

A NEWLY RECOGNIZED VIRUS RELATED TO GRBV IS PRESENT IN WILD VINES IN CALIFORNIA

During the initial surveys of wild vines in California for GRBV, another virus closely related to GRBV was identified (Perry *et al.* 2018). This virus is named wild Vitis virus 1 (WVV1). We do not know whether WVV1 can infect *Vitis vinifera* cultivars and rootstocks, nor whether it is involved in red blotch disease. Nonetheless, extensive surveys showed WVV1 in 7% (15 of 203) of the wild vines surveyed in California. Similar to GRBV, the incidence of WVV1 was higher in California counties with high grape production but the virus was also found in Glenn county (far from grape production) (**Figure A4-6**). Like GRBV, WVV1 was not found in wild vines in New York (Cieniewicz *et al.* 2018b). WVV1 populations from wild vines show higher genetic diversity than GRBV populations from wild vines, likely because WVV1 has been spread by natural means through a yet to be identified insect vector rather than through vegetative propagation like GRBV (Cieniewicz *et al.* 2018b).

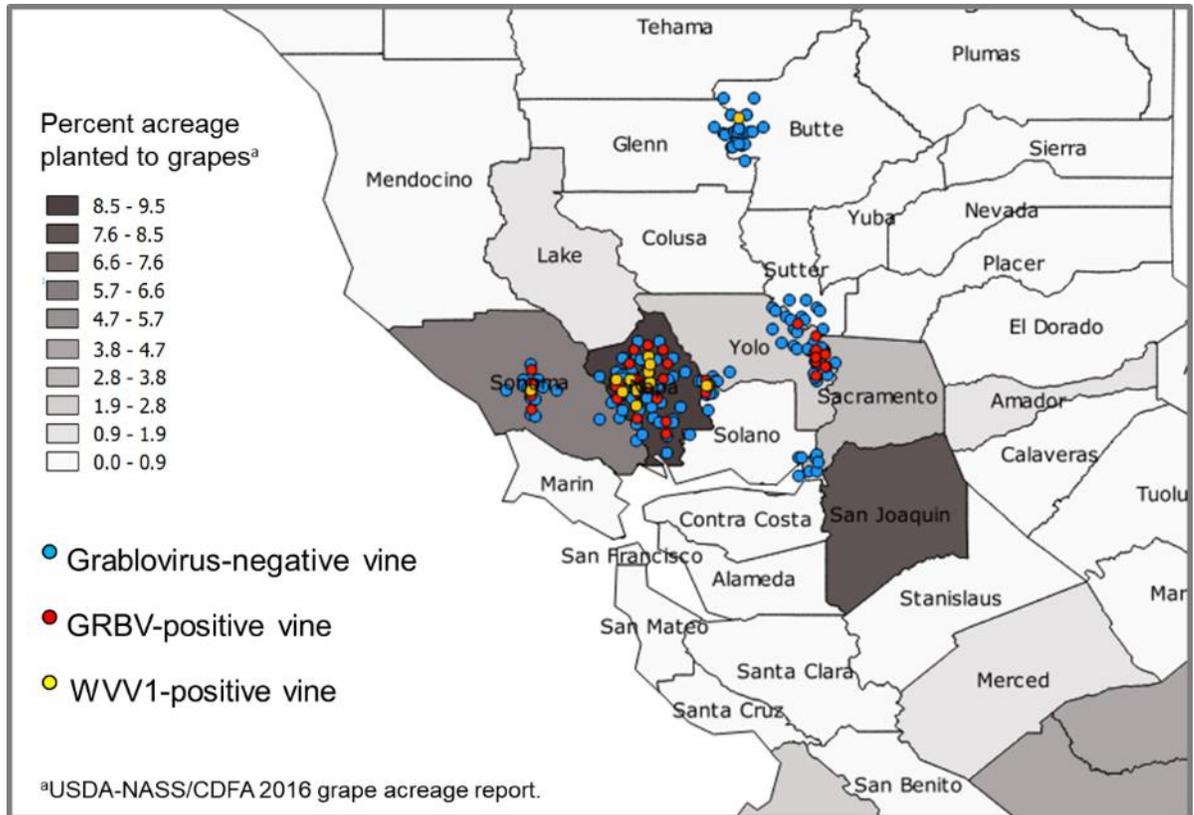


Figure A4-6 Map of wild vines in northern California surveyed for the presence of grapevine red blotch virus (GRBV) and wild Vitis virus 1 (WVV1). Counties are colored according to the percentage of acreage planted to grapevines.

ROLE OF COVER CROPS IN RED BLOTCH ECOLOGY

TCAH is not considered a pest of grapes. Although it can lay eggs in grape if caged only with grape as a host (**Figure A4-7**), it does not appear to complete its reproductive cycle on grapevines. Rather it prefers legumes as reproductive hosts (Preto *et al.* 2018).

To determine whether leguminous cover crops sown in vineyards serve as habitat for TCAH or a reservoir for GRBV, we sampled cover crops from nine vineyards in Napa Valley in the spring (2016-2018) before they were tilled. All cover crop samples (clover, medics, field peas, fava beans, poppy, grasses) tested negative for GRBV. Although TCAH are observed in these vineyards in summer months, no TCAH was found in springtime surveys of these cover crops. Therefore, legumes in row-middles are likely not contributing to GRBV inoculum or within-vineyard spread of the virus in the study vineyards in Napa Valley, where the ground is typically left bare in March-November. However, this may not hold true for other vineyards in Napa Valley or other regions where vegetation management practices differ, offering season-long refuges for TCAH.

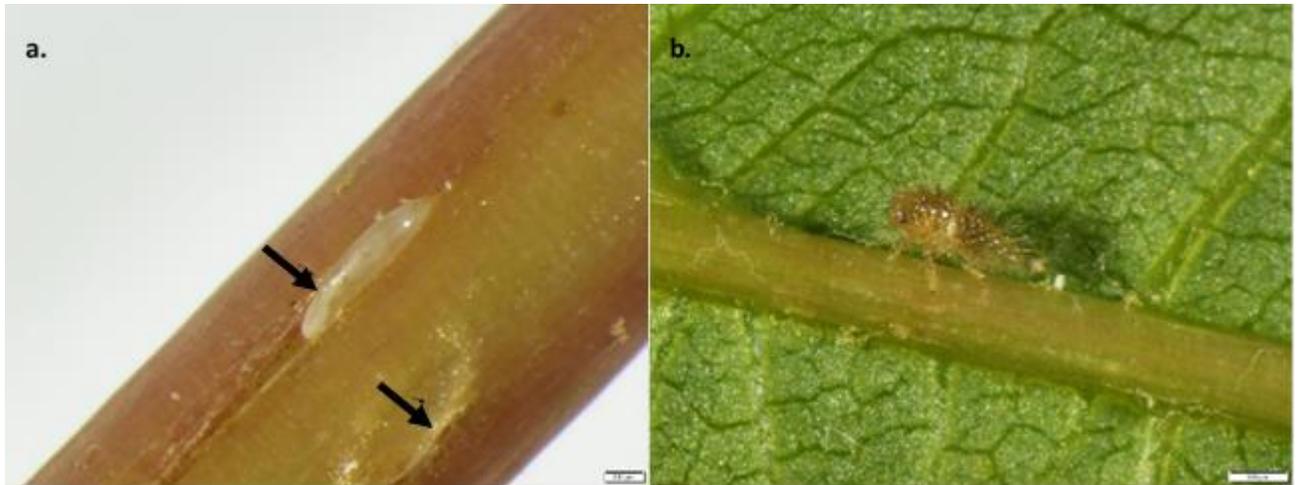


Figure A4-7 (a) Three-cornered alfalfa hopper (TCAH) eggs in a petiole of a *Vitis vinifera* cv. Syrah vine and b) First instar TCAH nymph hatched on a Syrah leaf. We have not observed TCAH survival past the first instar on grape in greenhouse conditions (Photos courtesy of E. Cieniewicz).

RED BLOTCH DISEASE MANAGEMENT RECOMMENDATIONS

Disease ecology studies are foundational for the development of disease management strategies. Patterns of GRBV spread, distribution and abundance of insect vectors, effects of GRBV on vine growth and fruit development, preferred hosts of insect vectors, and alternate hosts of GRBV, as well as lessons from economic analyses, are vital to inform science-based disease management programs.

In California, current evidence suggests that the TCAH is relatively infrequent in vineyards and does not reproduce on or to prefer grapevine as a host. Therefore, insecticide applications in vineyards targeting TCAH are not likely to produce significant reductions in insect populations and could produce detrimental secondary effects. Vegetation management practices that reduce the season-long incidence of TCAH's preferred breeding hosts could reduce insect populations, but this has not been demonstrated in replicated studies, nor is it clear what effect this would have on disease spread. Efforts to promptly identify and remove virus inoculum sources, i.e., infected production vines and wild vines adjacent to vineyard and nursery plantings, is critical since secondary spread of GRBV is occurring. Roguing is recommended if disease incidence is less than 30%, and entire vineyard removal is advised if disease incidence is generally higher than 30% (Ricketts *et al.* 2017). However, regional differences should be considered for the adoption of this cost-minimizing threshold as not all vineyards with a red blotch disease incidence higher than 30% sell grapes at reduced prices (Ricketts *et al.* 2017).

In New York and on the east coast, all evidence to date points toward the introduction of GRBV in vineyards solely through infected planting stocks with no vine to vine spread of GRBV via insect vectors. Nonetheless, growers should remain vigilant and frequently scout for disease symptoms. GRBV-infected vines will likely not attain optimum fruit maturity, so it makes sense to rogue and replace them as soon

as it is feasible if disease incidence is less than 30% (Ricketts *et al.* 2017).

For roguing of red cultivars, diseased vines should be marked immediately after harvest for removal at the earliest convenience, following confirmation of GRBV presence by PCR. Roguing efforts of white cultivars are more challenging because symptoms are subtle and a reliable identification of infected vines requires a PCR diagnosis. White cultivars are often favored in areas where spread of GRBV is occurring because expectations for the maturity of white and red cultivars differ; nonetheless, it is important to note that white cultivars can serve as sources of inoculum for GRBV spread. This is important to consider when planning re-plant strategies. As is the case for other virus diseases, prevention is the key to managing red blotch disease and planting vines derived from virus-tested nursery stock is critical.

Management recommendations of red blotch disease to reduce sources of GRBV inoculum:

- Frequently scout for red blotch disease symptoms beginning late in the season. Note that red blotch symptoms are easily confused with other diseases and nutrition disorders, and PCR tests are necessary for an absolute diagnosis.
- Determine red blotch disease incidence by counting the number of infected vines in a given area of a vineyard and dividing this number by the total number of vines inspected. An increase in infected vines over time (2-3 years) is indicative of spread. However, in young vines (<5th leaf) planted with infected material, symptoms may develop asynchronously over time as the vine matures, particularly if the rootstock is the source of the virus inoculum. In that case, an increase of diseased vines over time may not result from secondary spread.

- Rogue GRBV-infected vines if red blotch disease incidence is less than 30%
- Consider removing entire vineyards or vineyard areas if red blotch disease incidence is more than 30%
- Consider removing inoculum sources near new plantings, such as symptomatic and asymptomatic infected vines, following confirmation of the presence of GRBV by PCR (Test, don't guess!), as well as wild vines in forested areas near vineyards in Northern California, pending an environmental permit is secured from the California Department of Fish and Wildlife for vegetation management in riparian corridors.
- Plant vines derived from virus-tested scion and rootstock mother vines
- If spread of GRBV is suspected, it may be useful to monitor for the TCAH in vineyards to be aware of the presence of the vector of GRBV. Place yellow sticky insect traps on the middle trellis wire in the area of suspected spread. In a vineyard in Napa, TCAH populations peaked in late June and early July, however the TCAH behavior in vineyards likely varies depending on the site. Rotate traps weekly and identify TCAH using specific morphological traits.

We acknowledge singularities among estates and grape-growing regions in terms of vineyard management practices and tolerance to red blotch disease. Therefore, our disease management recommendations should be considered as guidelines to strategically devise a customized actionable list of corrective measures. We also acknowledge that our recommendations will need to be refined overtime according to our collective understanding of GRBV spread and behavior of the TCAH in vineyard ecosystems.

REFERENCES

- Al Rwahnih, M., Rowhani, A., and Golino, D. 2015. First report of grapevine red blotch-associated virus in archival grapevine material from Sonoma County, California. *Plant Disease* 99:895.
- Bahder, B.W., Zalom, F.G., Jayanth, M., and Sudarshana, M.R. 2016a. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* (Say) as a vector of Grapevine red blotch-associated virus. *Phytopathology* 106:1223-1230.
- Bahder, B.W., Zalom, F.G., and Sudarshana, M.R., 2016b. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Disease* 100:1571–1574.
- Blanco-Ulate, B., Hopfer, H., Figueroa-Balderas, R., Ye, Z., Rivero, R.M., Albacete, A., Pérez-Alfocea, F., Koyama, R., Anderson, M.M., Smith, R.J., Ebeler, S.E., and Cantu, D., 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *Journal of Experimental Botany* 68:1225-1238.
- Calvi, B. 2011. Effects of red-leaf disease on Cabernet sauvignon at the Oakville Experimental Vineyard and mitigation by harvest delay and crop adjustment. MS thesis. UC-Davis, CA.
- Cieniewicz, E.J., Perry, K.L., and Fuchs, M.F. 2017a. Grapevine red blotch: molecular biology of the virus and management of the disease. Pages 303-314 in: *Grapevine*

viruses: molecular biology, diagnostics and management. B. Meng, G.P. Martelli, D. Golino and M. Fuchs, eds. Springer Verlag, Berlin, Germany.

Cieniewicz E.J., Pethybridge S.J., Gorny A., Madden L.V., McLane H., Perry K.L., and Fuchs M.F. 2017b. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Research* 241:156-162.

Cieniewicz E.J., Loeb G., Pethybridge S., Perry K.L., and Fuchs M.F. 2018a. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108:94-102.

Cieniewicz E.J., Thompson J., McLane H., Perry K.L., Dangl G.S., Corbett Q., Martinson T., Wise A., Wallis A., O'Connell J., Dunst R., Cox K., and Fuchs M. 2018b. Prevalence and genetic diversity of grabloviruses in free-living *Vitis* spp. *Plant Disease* 102:2308-2316.

Krenz, B., Thompson, J., McLane, H., Fuchs, M., and Perry, K.L., 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 102:232-1240.

Perry, K.L., McLane, H., Hyder, M.Z., Dangl, G.S., Thompson, J.R., and Fuchs, M.F. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* sp. proximal to cultivated grapevines. *Phytopathology* 106:663-670.

Perry, K.L., McLane, H., Thompson, J.R., and Fuchs, M. 2018. A novel grablovirus from non-cultivated grapevine (*Vitis* sp.) in North America. *Archives of Virology*

163:259–262.

Preto, C.R., Sudarshana, M.R., and Zalom, F.G. 2018. Feeding and reproductive hosts of *Spissistilus festinus* (Say) (Hemiptera: Membracidae) found in Californian vineyards. doi:10.1093/jee/toy236/5069541

Ricketts, K., Gómez, M., Fuchs, M., Martinson, T., Smith, R., Cooper, M., Moyer, M., and Wise, A. 2017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. *American Journal of Enology and Viticulture* 68:127-135.

Setiono, F.J., Chatterjee, D., Fuchs, M., Perry, K.L., and Thompson, J.R. 2018. The distribution and ability to detect grapevine red blotch virus in its host depends on time of sampling and tissue type. *Plant Disease* 102:2187-2193.

Sudarshana, M.R., Perry, K.L., and Fuchs, M.F. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105:1026-1032.

APPENDIX 5

***GRAPEVINE LEAFROLL DISEASE: INTEGRATED PEST MANAGEMENT
DISEASE FACT SHEET***

*This article was published in 2014 on the Cornell University Integrated Pest Management website in response to a substantial taxonomic revision of the grapevine leafroll-associated viruses as: Cieniewicz E.J. & Fuchs M. (2014) Grapevine leafroll disease. NYS IPM Disease fact sheet,

<https://ecommons.cornell.edu/handle/1813/43103>

INTRODUCTION

Leafroll is one of the most important virus diseases of grapevine. It occurs in every major grape-growing region of the world. Grapevine leafroll disease can affect all native and *Vitis vinifera* cultivars, hybrids, and rootstocks, although symptoms are not always expressed on infected vines. The disease was described as early as the 19th century in Europe, but its graft-transmissibility was not demonstrated until 1937. In 1979 a specific type of flexuous and filamentous virus was reported in a leafroll-affected vine. Shortly thereafter, in 1983, the capacity of mealybugs to transmit one of the viruses associated with this disease was shown.

SYMPTOMS AND IMPACT

Leafroll-affected vines are less vigorous than healthy vines. Older leaves are cupped with the principal veins remaining green in late summer and fall. Symptoms are usually most conspicuous in red-fruited cultivars of *Vitis vinifera*, with reddening of leaves in addition to cupping (**Figure A5-1A, B**), although the major veins of leaves remain green (**Figure A5-1C**). In white-fruited *V. vinifera* cultivars, symptoms are less pronounced, consisting of slight chlorosis and cupping (**Figure A5-1D**). Infected native cultivars, hybrids, and rootstocks usually remain symptomless.

Leafroll disease causes significant yield losses and delays fruit ripening (**Figures A5-1E, F**). Reduced soluble solids and increased titratable acidity are also often reported. Without any control measures, the estimated economic impact of leafroll disease can range from approximately \$25,000 per hectare (for a 30% yield reduction and no grape quality penalty) to \$40,000 per hectare (for a 50% yield reduction and a 10% penalty for poor fruit quality).

Lower vigor associated with virus infection increases vine susceptibility to adverse environmental factors, such as cold winter temperatures, resulting in a higher level of mortality in virus-infected vines. Consequently, the cost of vineyard maintenance increases due to more frequent vine replacements.

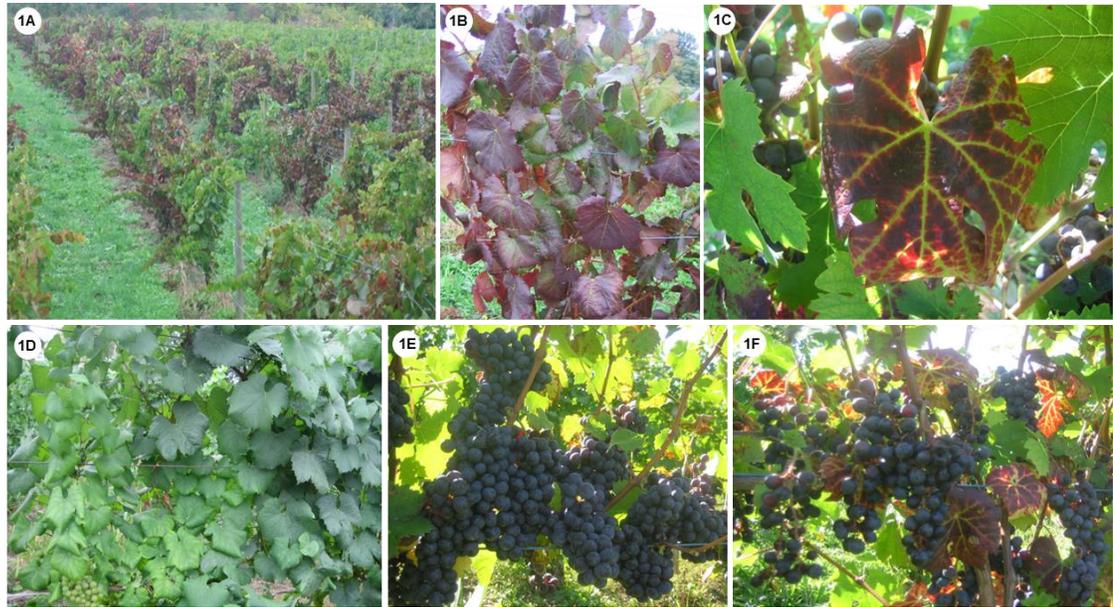


Figure A5-1 (A) High incidence of leafroll disease in a *Vitis vinifera* cv. Lemberger vineyard with conspicuous leaf reddening; (B) Close-up views of Lemberger and (C) Cabernet franc leaves with cupping and reddening while main veins remain green; (D) Leafroll symptoms (small, cupped, chlorotic leaves) on a Chardonnay vine (left) compared to a healthy vine (right); (E) Fruit yield and quality of a healthy and (F) leafroll-affected *Vitis vinifera* cv. Cabernet franc. Photos courtesy of Marc Fuchs.

CAUSAL AGENTS

To date, five distinct distinct filamentous viruses (**Figure A5-2**) identified as Grapevine leafroll-associated viruses (GLRaVs) have been isolated and characterized from leafroll-infected grapevines. They include GLRaV-1, -2, -3, -4, and -7. These viruses are serologically unrelated and their particle length ranges from 1,400 to 2,200 nanometers. Other than *Vitis* species, no wild or cultivated plant species are known to serve as alternate hosts for leafroll-associated viruses.

Most of these viruses can be detected by wood or green grafting onto indicator vines of *V. vinifera* cv. Pinot noir, Cabernet franc or Gamay. Lab tests including serological assays, such as double antibody sandwich enzyme-linked immunosorbent assay (ELISA) and molecular assays based on polymerase chain reaction (PCR) can also be used to detect GLRaVs in grapevine tissue. These tests are more reliable than indexing to diagnose leafroll viruses.

Among the five viruses associated with leafroll disease, GLRaV-1, GLRaV-2, and GLRaV-3 usually prevail in leafroll-affected grapevines. Additionally, GLRaV-2 also incites severe graft-incompatibility syndrome and decline of scions grafted on certain rootstocks, including Kober 5BB, 3309C, 5C, 1103P, Harmony and Freedom.

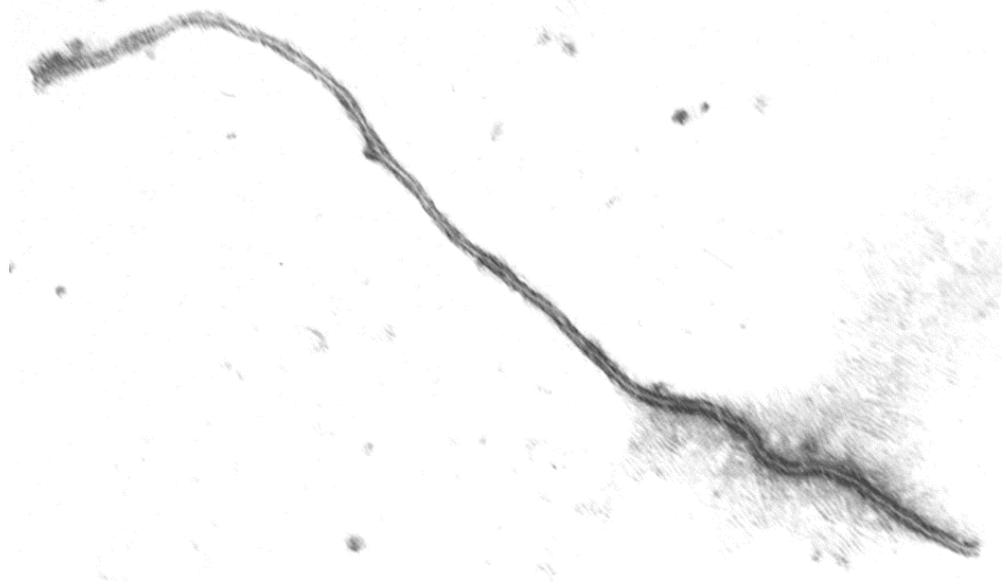


Figure A5-2 Electron micrograph of a GLRaV particle isolated from a leafroll-diseased vine. Photo by Marc Fuchs.

CONDITIONS FOR INFECTION

The most efficient means of transmitting leafroll-associated viruses is through vegetative propagation and grafting. GLRaVs can be moved across long distances in planting and propagation materials. In addition, insect vectors in two homopteran insect families (mealybugs – *Pseudococcidae* – and soft scales – *Coccidae*) can also transmit three (GLRaV -1, -3, -4) of the five GLRaVs. Mealybugs and soft scales feed on a wide range of host plants and can be serious pests of woody plants and shrubs, including *Vitis*. Most species overwinter as eggs and young instar nymphs beneath the bark of the trunk or underground on the roots. Females are wingless but can exhibit limited mobility, while adult males, which lack mouthparts, and hence, cannot feed and transmit the viruses, are winged. Because of their small size, crawlers and adults can be readily wind-blown.

Several such vectors are known from New York and other states in the eastern US: *Parthenolecanium corni* or brown apricot scale, *P. persicae* or grapevine scale, *Pulvinaria vitis* or woolly vine scale, *Neopulvinaria innumerabilis* or cottony maple scale, and *Pseudococcus maritimus* or grape mealybug. Another important pest of grapes and a known vector of GLRaV, *Planococcus ficus* or vine mealybug, has not been reported from the eastern US but is a potential invader. A census of mealybugs and soft scale insects in New York vineyards suggests that populations tend to be low, except in a few isolated cases where outbreaks have occurred.

MANAGEMENT

To date, the only way to manage grapevine leafroll disease and secure a

healthy and high quality crop is to ensure that the planting material originates from virus-tested, clean vine stocks and that factors contributing to infestation via insect vectors are well controlled. The importance of establishing new blocks with clean planting material cannot be over-emphasized because, once a vine is infected, there is no cure in the vineyard. No sources of resistance against any of the GLRaVs have been identified in wild or cultivated grapes. Therefore, conventional breeding is not a viable option to develop GLRaV-resistant material. Research is ongoing to develop resistant material through genetic engineering.

Roguing- the elimination of infected vines – can reduce the economic impact of leafroll disease to \$3,000–\$23,000 per hectare when disease prevalence is moderate (1–25%). When disease prevalence levels are greater than 25%, replacing the entire vineyard is economically preferable.

In nurseries and foundation blocks, the use of virus-tested material followed by regular and routine monitoring the disease, its causal agents, and insect vectors is paramount for providing planting material of high phytosanitary standards. Stocks in nurseries and mother blocks should be tested regularly for GLRaVs and such blocks should be isolated from commercial vineyards to avoid further infection through vector transmission. Also, mealybugs and soft scales should be regularly surveyed and managed.

APPENDIX 6

***APPLE CHLOROTIC LEAF SPOT VIRUS: INTEGRATED PEST
MANAGEMENT DISEASE FACT SHEET***

*This article was published in 2016 on the Cornell University Integrated Pest Management website in response to problems in New York with latent apple viruses as: Cieniewicz E.J. & Fuchs M. (2016) Apple chlorotic leaf spot virus. NYS IPM Disease fact sheet, <https://ecommons.cornell.edu/handle/1813/43945>

INTRODUCTION

Apple chlorotic leaf spot virus (ACLSV) infects pome and stone fruits. It can elicit diverse symptoms although, in most cultivars the virus is latent, which means that infected trees do not manifest observable symptoms. *Apple chlorotic leaf spot virus* is often detected in co-infection with other latent viruses such as *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus*. *Apple chlorotic leaf spot virus* can have a devastating effect on apple growth and productivity.

DISEASE AND CAUSAL AGENT

Implicated in several diseases of pome and stone fruits such as apple top working disease, apple russet ring disease, and pear ring pattern mosaic, ACLSV is one of the most widely distributed viruses of fruit trees. It is present in cultivated, ornamental and wild species of the Rosaceae. In addition to apple, ACLSV affects pear and quince, and stone fruits like peach, plum, apricot, and cherry. Although ACLSV exhibits latent infection in most trees, it causes symptoms of variable severity in susceptible cultivars, certain scion/rootstock combinations, or in co-infection with other latent viruses. This virus has a single-strand positive-sense RNA genome that is encapsulated in flexuous filamentous-shaped particles. A high degree of genetic variability exists among isolates of ACLSV.



Figure A6-1 Declining Red Delicious/G.935 trees infected with *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV) in a nursery in fall 2015. The budwood used for grafting was the source of the two viruses. Note the stunted growth, browning of leaves, reduced terminal growth or terminal dieback of six infected trees (left of the wooden post) compared to seven healthy trees (right of the wooden post). Photo credit: D. I. Breth and E. M. Tee.

SYMPTOMS AND IMPACT

Pome fruits

One of the first described viral diseases of apple, top working disease, is associated with ACLSV infection of the scion. Following budding, ACLSV translocates from a latently infected scion to a susceptible rootstock, resulting in a decline of grafted trees, generally 1-2 years after trees are top grafted. Tree decline can result from ACLSV infection alone or in association with another latent virus such as ASPV (**Figure A6-1**). Apple russet ring disease, in which yellow rings appear on foliage and russet rings appear on fruits, may result from ACLSV infection. Symptomatic fruits are unmarketable. In susceptible apple cultivars, foliar symptoms can include chlorotic leaf spots and line patterns, premature leaf drop, stunting, terminal dieback, blackening (necrosis) of inner bark and local bark necrosis around grafted, diseased buds. Up to 30% reduction in yield has been reported for ACLSV in combination with other latent viruses. Additionally, pear ring pattern mosaic symptoms may also be attributed to ACLSV.

Stone fruits

In stone fruits, some isolates of ACLSV can elicit foliar and fruit symptoms. Trunk symptoms are rare, but certain apricot cultivars can exhibit graft incompatibility and bud necrosis in association with ACLSV infection. Some ACLSV isolates can induce bark splitting in plum, leaf deformation and chlorosis in peach, and fruit necrosis, decline, and bark splitting in cherry.

SPREAD

Apple chlorotic leaf spot virus is disseminated through vegetative propagation, grafting and top working. There is no evidence of vector-borne, seed-borne, or pollen-borne transmission, although the virus can be mechanically transmitted, with some difficulty, from infected pome and stone fruit hosts to experimental herbaceous hosts. Therefore, the widespread distribution of ACLSV in pome and stone fruit trees worldwide has resulted from unintentional and careless use of scion budwood collected from infected trees for propagation or top working, and from infected rootstock liners. The absence of obvious symptoms on most infected trees increases the risk of unintentional propagation and distribution of ACLSV-infected stock.

MANAGEMENT

Like other viruses of woody crops, there is no cure for ACLSV in an established orchard and there is no direct measure to combat the virus besides removing infected trees. The best way to control the virus is to prevent its introduction in new orchards. Since ACLSV is latent in many cultivars and can be transmitted via grafting, the use of infected propagation material is culpable for most infections. A careful selection of clean, virus-tested (negative) planting material and budwood used for top working is the best method of preventing the introduction of ACLSV into new orchards. To ensure a healthy, long-lived orchard producing good yields and high quality fruit, only trees derived from clean, virus-tested buds and rootstock liners should be planted.

Propagation material should be sourced only from clean, virus-tested trees to prevent the dissemination of ACLSV. Laboratory tests such as serological assays and nucleic acid-based assays, including reverse transcription polymerase chain reaction (RT PCR), can reliably identify ACLSV in apple and facilitate the identification of clean propagation material. Therapeutic methodologies such as heat- and chemotherapy can be used in the laboratory to regenerate clean propagation material.

APPENDIX 7

APPLE STEM PITTING VIRUS: INTEGRATED PEST MANAGEMENT DISEASE FACT SHEET

*This article was published in 2016 on the Cornell University Integrated Pest Management website in response to problems in New York with latent apple viruses as: Cieniewicz E.J. & Fuchs M. (2016) Apple stem pitting virus. NYS IPM Disease fact sheet, <https://ecommons.cornell.edu/handle/1813/43945>.

INTRODUCTION

Apple stem pitting virus (ASPV) is a latent virus of pome fruits with worldwide distribution. In addition to apple, ASPV can affect pear and quince. Similarly to other latent viruses of pome fruits, infections with ASPV are often symptomless and therefore go mostly undetected.

DISEASE AND CAUSAL AGENT

Although ASPV is frequently found in apple, no symptom is associated with infection on most scion/rootstock combinations. However, in susceptible cultivars, extensive pitting of the woody cylinder can impair functions of the vascular system. Fruit yield can be reduced, particularly when ASPV is co-infecting with other latent viruses such as *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus*. *Apple stem pitting virus* is the type species of the genus *Foveavirus* and has a positive-sense single strand RNA genome encapsulated in flexuous, filamentous particles.



Figure A7-1 Depressions on the woody cylinder of the trunk of a declining Red Delicious/G.935 tree infected with *Apple stem pitting virus* and *Apple chlorotic leaf spot virus* observed after bark removal. Photo credit: D. I. Breth and E. M. Tee.

SYMPTOMS AND IMPACT

Apple stem pitting virus does not cause observable symptoms on most cultivars although pits can develop on the woody cylinder (**Figure A7-1**). When elicited, symptoms vary depending on viral isolate and cultivar. In susceptible apple cultivars ‘Charden’ and ‘Spy 227’, symptoms may include xylem pitting, leaf epinasty or downward growth and tree decline. In symptomatic trees, ASPV is often detected in mixed infection with other latent viruses of apple, particularly with ACLSV in ‘Red Delicious’ (**Figure A7-2**). In susceptible pear cultivars ‘Beurre Hardy’, ‘Nouveau Poiteau’, and ‘Jules d’Airroles’, ASPV infection is often accompanied by vein yellowing, red mottling, black or necrotic spotting, and pitted and deformed fruit or “stony fruit”. Symptomatic fruits are unmarketable.

SPREAD

Apple stem pitting virus occurs worldwide, wherever apple, pear and quince are cultivated. There is no reported insect vector and the virus is not seed transmitted. The major means of dispersal is via vegetative propagation, grafting and top working. Therefore, the presence of ASPV in infected trees results from the unintentional, careless use of scion budwood collected from infected trees for propagation or top working, and from infected rootstock liners. The absence of obvious symptoms of ASPV on most trees increases the risk of its unintentional distribution. Transmission, to some extent, of ASPV by root contact has been reported, highlighting the need to distance nursery material from infected trees.



Figure A7-2 A declining Red Delicious/G.935 tree infected by *Apple stem pitting virus* and *Apple chlorotic leaf spot virus* (foreground) compared to healthy trees (background) in a nursery in fall 2015. Photo credit: D. I. Breth and E. M. Tee.

MANAGEMENT

Like other viruses of woody crops, there is no cure for ASPV in an established orchard and there is no direct measure to combat the virus besides removing infected trees. The best way to control the virus is to prevent its introduction in new orchards. Since there is no vector that will introduce the virus to new plantings and ASPV is transmitted to new trees via grafting, selecting clean trees to source budwood from is paramount to mitigate its impact on tree performance. To ensure a healthy, long-lived orchard producing good yields and high quality fruit, only trees derived from clean, virus-tested buds and rootstock liners should be planted.

Propagation material should be selected and collected only from clean, virus-tested trees to prevent the dissemination of ASPV. To eliminate ASPV from elite scion cultivars and rootstock genotypes, therapeutics based on heat therapy at 37°C for 60-80 days can be used in the laboratory to regenerate clean trees.