Genetic Diversity and Distribution of *Grapevine leafroll-associated virus* in California

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ABSTRACT

Grapevine leafroll disease, caused by different species of *Grapevine leafroll-associated viruses* (*GLRaV,* Family *Closteroviridae*), has detrimental economic impacts on vineyards by decreasing the quality and quantity of the crop. Studies of infected vineyards continue to find new species and strains of *GLRaV*, but the genetic diversity of each these species is not well understood. This study identifies the species and strains which are present in Amador, El Dorado, San Luis Obispo and Lodi, California. I used a total of 548 samples collected from 24 vineyards and extracted high quality RNA from each isolate followed by RT-PCR for virus detection. The isolates identified as a single infection of *GLRaV-3* were sequenced and compared with sequences in GenBank and, along with control isolates, aligned to create a phylogenetic tree. Six species of *GLRaV* and five clades of *GLRaV-3* were present within the 24 vineyards. *GLRaV-2* and *GLRaV-3* were the most common species of virus infecting vineyards in these regions of California. The genetic distance between clades and individual samples in the phylogenetic tree support a large divergence between strains of *GLRaV-3*. The genetic information I identified will help researchers find the conserved viral sequences for *GLRaV*, which will improve future studies of the virus, and will help vineyard managers understand the spread of the virus.

KEYWORDS

Closteroviridae, mealybug, grapevine viruses, divergence, certification.

INTRODUCTION

Viral diseases in agricultural crops have detrimental economic impacts. Of the many viruses that infect grapevines, the most widespread and economically destructive is *Grapevine leafroll-associated virus* (*GLRaV*) (Rayapati, O'Neal, Walsh, 2008). These distinct virus species in the family *Closteroviridae* all cause similar disease symptoms known as grapevine leafroll disease (GLD) (Martelli et. al., 2002). Symptoms of GLD were observed over one hundred years ago and include: leaves rolling down and turning red, smaller and fewer clusters of fruit, and decreased sugar content (Namba, 1979). *GLRaV* is one of the most widespread species of viral diseases affecting grapevines in all regions of the world. It causes severe damage to wine industry production (Walker, Charles, Froud & Connolly, 2004) and has been associated with grapevine yield losses of 20-40% (Osman, Leutenegger, Golino & Rowhani, 2007). RNA viruses, such as *GLRaV*, cause many emerging and re-emerging diseases; therefore, the evolutionary processes that allow them to move through species boundaries with new vectors for transportation need to be understood (Holmes, 2009a).

All species of *GLRaV* can be spread by contaminated plant material and some species can be spread by insect vectors (Golino, 2008). The recent discovery of the presence of insect vectors has changed management strategies to control the virus. Vineyards that use contaminated plant material when grafting rootstock and scion can spread the virus, particularly if they then propagate the new plant (Cabaleiro, Segura & Garcia-Berrios, 1999). Research on *GLRaV* is necessary to ensure the best quality of certified grape nursery stock in California to prevent such spread (Rowhani & Golino, 1995). Studying the genome of the species and strains of the virus is important to understand how it diversifies and to improve certification testing to prevent its spread throughout vineyards.

There are currently over ten identified species of *Grapevine leafroll-associated virus;* however, the number of species of *GLRaV* is still uncertain and the genetic diversity of each species is not well understood (Golino & Almeida, 2008). Each species is identified numerically such as, *GLRaV-1, GLRaV-2* and so on. Nine of the species are ampeloviruses, one is a closterovirus (*GLRaV-2*) and one remains unassigned (*GLRaV-7*) (Martelli et. al. 2002). Multiple species or strains can be present in one vine; different symptoms can manifest depending on what combination of species are present in a vine and the variety of grape, and require different

management strategies. Although the symptoms caused by each virus may vary they are still grouped as GLD. However, how *GLRaV* causes the disease and manifests different symptoms is not well understood (Maree, Freeborough & Burger, 2008). Mealybugs spread *GLRaV-3* while *GLRaV-2* currently has no known evidence of vector spread. This information is critical for virus management. Only four species of one strain have been sequenced (Wang, Sharma, Duff & Almeida, 2010) and other isolates have limited sequence information (Turturo et. al, 2005). One way to learn more about the emergence and evolution of RNA viruses is to use sequence data (Holmes, 2009b). Genomic sequencing provides a way of determining different strains of the virus that have unique characteristics (Holmes, 2009b; Wang, 2010). GLD has been on the rise in California. Understanding the distribution and divergence between species and strains is critical to control of the virus and to create and implement management strategies.

In this study, I determined which species and strains of *GLRaV* cause grapevine leafroll disease in California's Amador County, El Dorado County, San Luis Obispo County and Lodi City. I hypothesized that multiple species of the virus are present in these regions. Although I expected there would be variation between each region in terms of which species are present, I expected *GLRaV-3* would be the most prevalent. With *GLRaV-3* as the dominant species, I hypothesized that there are many strains of *GLRaV-3* in each vineyard. The presence of multiple strains in a vineyard indicates that there were multiple virus introduction events by mealybugs and contaminated plant material.

METHODS

Sample collection

To determine the genetic diversity of the *GLRaVs* in vineyards in California I used a total of 548 samples collected from grapevines in Amador, El Dorado, San Luis Obispo and Lodi. Two hundred and thirty two samples were taken from 11 vineyards in Amador and El Dorado, 149 samples were taken from 5 vineyards in San Luis Obispo, and 167 samples were taken from 8 vineyards in Lodi. The samples were collected on two dates in October and November 2010 (Appendix A). Sites were selected based on the visible presence of foliar symptoms of GLD and

samples were taken from visibly infected plants, which showed curling, red leaves, and dying stalks. Fifteen to thirty samples were tested in each vineyard.

To examine vector spread based on the spatial distribution of diseased plants, multiple samples were taken from each row. The samples were labeled with the site name, row number, and vine number. For example, San Luis Obipso 1 9-2 was a sample taken from vineyard 1 in San Luis Obispo row 9 vine 2. The petiole samples were transported on ice and stored in 1.5 ml centrifuge tubes at -80°C. To detect the species present in each sample, also known as an isolate, I extracted RNA from each sample followed by RT-PCR and fragment analysis. Using the data from fragment analysis, I chose samples infected with *GLRaV-3* to sequence in order to determine which strain caused the disease. I compiled the consensus sequences from each isolate into a phylogenetic tree to determine the divergence between each strain.

High quality RNA extraction

I extracted high quality RNA from one petiole sample from all 548 samples using a similar protocol as described by Osman et. al. (2007) modified to fit this project. I cut 100 mg petioles into 3mm pieces and placed them into individual 2.0 ml microcentrifuge tubes with a pre sterilized $1/8^{th}$ inch chrome grade 5 ball bearing with 1.8 ml of extraction buffer (1.59g/l Na_2CO_3, 2.93 g/l $NaHCO_3$, pH 9.6 containing 2% PVP-40, 0.2% bovine serum albumin, 0.05% Tween 20 and 1% $Na_2S_2O_5$). I macerated the samples in the Precellys 24 Tissue Homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) run at 6500 Hz for two 10 second cycles with a 30 second break between cycles. Following maceration the samples were stored at -20°C.

Species level detection

To detect the species of *Grapevine leafroll-associated virus* in each sample I used RT-PCR designed to synthesize and analyze a 428bp fragment in the 3' terminal region of the genome from the RNA template. The samples were thawed and 125µl of GES denaturing buffer (0.1 M glycine, 0.05 M NaCl, 1mM EDTA and 0.5% Triton X-100) with betamercaptoethanol was added to 10µl of each sample and then heated at 95°C for ten minutes. Three reactions were prepared using nine sets of fluorescently labeled primers to test for each of *Grapevine leafroll-*

associated virus 1, 2, 3, 4, 5, 7, and 9, as well as a coat protein gene found in many strains of *GLRaV-3* (Supplementary Table 2). These primers were combined to be tested in three different master mix multiplexes. Multiplex 1 combined the *GLRaV-1, 2* and *3* primers, multiplex 2 combined the *GLRaV-4, 5* and *9* primers and multiplex 3 combined the *GLRaV-7*, and CP primers. The reaction for each multiplex was set up using a Qiagen OneStep RT PCR Kit (Qiagen, Germantown, MD, Catalog Number: 2012) using 5x buffer, Enzyme Mix, dNTP Mix, and RNase free water. I prepared the three master mixes, with a final primer concentration of 400nM per primer set per reaction. Wang, Sharma, Duff and Almeida (2010) designed the primers. PCR was set up using 0.8µl of template with 9.2µl of the multiplex. Samples were run using an initial reverse transcription step at 50°C for 30 minutes, followed by the PCR activation step at 95°C for 15 minutes followed by PCR. 35 cycles of PCR followed with denaturing at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute. PCR was followed by the final extension at 72°C or 74°C for ten minutes and finally the samples were held at 17°C and then stored at -20°C.

I prepared the samples for fragment analysis by adding 1.5µl of PCR product for each isolate to 10µl HiDi formamide and 0.3µl Genescan 500 LIZ size standard. Fragment analysis was performed at the Barker Hall Sequencing Facility located on the University of California, Berkeley campus using an Applied Biosystem's 96 capillary 3730xl DNA Analyzer. The results were analyzed with Applied Biosystems Peak Scanner version 1.0 software (Applied Biosystems, 2006). The florescent label and fragment size that occur following the denaturing and electrophoresis of fragment analysis allow for differentiation between each species of *GLRaV*. Each species has a unique florescent color at a specific fragment size base pair. To avoid false positives, caused by leakage that could occur between wells, a baseline height of 2000 for a florescent peak was established to be considered positive for each species of *GLRaV*. Samples with peaks occurring below a height of 2000 were considered negative.

Strain level detection

Only samples that were identified as a single infection of *GLRaV-3* from the fragment analysis were sequenced. The method used to prepare crude extractions for PCR was the same as above. Primers used for PCR were nested primers for sequencing, CP210F and CP500R

(Appendix B), designed by Wang, Sharma, Duff and Almeida (2010). Three replicate reactions were run per sample to strengthen the accuracy of the results. After PCR is complete, Qintarabio lab (Albany, CA) preformed purification and sequencing. I determined which strain was present by comparing the sequence from a sample to sequences existing in GenBank, the National Institute of Health genetic sequence database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The genome of petioles that were identified to test positive with *GLRaV-3* were then compared with known genomes of each strain of species 3. The samples I sequenced had greater than 90% maximum identity, which is the maximal percent identity of the HSPs (high-scoring segment pairs), to samples with a known strain, and therefore, were determined to have the same strain. I assembled these sequences into a 428bp consensus sequence using Vector NTI version 11 applications Align X and Contig Express (Invitrogen, 2008) by overlapping all three reads per strand for each sample. This method identified the strains of *GLRaV* in each plant.

Phylogenetic analysis

I used AlignX, a module of Vector NTI version 11 (Invitrogen, 2008) to create a phylogenetic tree with the consensus sequences for the isolates found to be infected with *GLRaV-3* along with control sequences of known strains of *GLRaV-3a, b, c, d, e* and *f*. AlignX performs multiple sequence alignments using the Clustal W algorithm. Divergence between strains can be seen in the phylogenetic tree.

RESULTS

Species level detection

Of the 548 samples tested, 184 were found to be infected with a species of virus and 215 samples were found to be uninfected (Fig. 1). In each area studied entire vineyards tested negative for the virus (Fig. 2). Seven of the eleven vineyards tested in Amador and El Dorado had the virus present in some of the samples and in the other four all samples tested negative (Fig. 2). In San Luis Obispo only two of the five vineyards had the virus and in Lodi five of the eight vineyards had the virus.

I found species 1, 2, 3, 5, 7 and 9 to be present in the regions of California I sampled (Table 2 & Fig. 3). *GLRaV-4* was not present in any of the vineyards tested. Forty six percent of

all of the samples were positive for at least one species of *GLRaV*; the same proportion of samples that had a virus were infected with *GLRaV-3.* The second most common virus causing GLD in these regions was *GLRaV-2* at 34%. 11% of infected samples had *GLRaV-1,* 7% had *GLRaV-5,* about 2% had *GLRaV-9* and only one sample had *GLRaV-7.*

In Amador and El Dorado the majority of the samples had *GLRaV-2,* with almost 38% of the samples tested from the area infected with this virus (Fig. 3). Almost 30% of the samples tested were infected with *GLRaV-3.* Species 1, 5 and 9 were also present in the area. In Lodi the most common infection was species 3 with over 30% of the samples tested infected with *GLRaV-3. GLRaV-2* followed with almost 13% of the samples infected with this virus. *GLRaV-1, 5, 7* and *9* were present in a very small number of samples. In San Luis Obispo *GLRaV-3* was also the most common with almost 15% of the samples infected with the virus. The second most common was *GLRaV-1* at 4%. *GLRaV-2* at almost 3% was the only other species present in the county. Within all vineyards there were a total of six species of *GLRaV* present (Fig. 3).

In each region there were isolates infected with multiple species of the virus (Fig. 4). This was particularly common in Amador and El Dorado where over 30% of the samples had multiple species of the virus. It was much less common in Lodi and San Luis Obispo. In both of these areas less than 5% of all of the samples were infected with more than one virus.

Strain Level Detection

Forty five samples were sequenced to determine which strain of *GLRaV-3* was present. Within all vineyards I found five strains of *GLRaV-3* to be present (Fig. 5). *GLRaV-3a, b* and *c* are present in all of the regions (Fig. 5). In Amador and El Dorado only those three strains were found. In Lodi strain g is also present and in San Luis Obispo strain e is also present. I did not find strains d and f in any of the isolates that were sequenced.

Phylogenetic analysis

I created a phylogenetic tree associating all of the isolates with identical genes and varying genes. The tree shows the divergence between the strains of *GLRaV-3* present in each isolate sequenced. Within the twenty-four vineyards I studied, I found five clades of *GLRaV-3* (Fig. 5). *GLRaV-3g* was vineyard specific but strains *GLRaV-3a, GLRaV-3b, GLRaV-3c,* and *GLRaV-3e* were present in multiple vineyards. Vineyard 1 in San Luis Obispo had strains

GLRaV-3a, b and *c* present. Vineyard 2 in Lodi offers a contrast with only *GLRaV-3g* present. These differences can be attributed to the number of infection events.

DISCUSSION

My study showed that *GLRaV-2* and *GLRaV*-3 were the most common species of virus causing GLD in vineyards in California. *GLRaV-1, 2, 3, 5, 7,* and *9* were found to be present in the region. *GLRaV-3* has been observed to be the most predominant species of the virus (Tsai, et al., 2008), and as I hypothesized, *GLRaV-3* is the most prevalent in the region. However, the difference in prevalence of *GLRaV-2* and *GLRaV-3* is relatively small with only about 5% more of the samples tested (30 samples) infected with species 3 than species 2. Also, *GLRaV-2* is the most prevalent in the Amador and El Dorado region. Within species 3 there are many strains present throughout these regions of California. The phylogenetic tree of the strains of *GLRaV-3* shows the genetic divergence of the strains present in all of the vineyards and provides insight into the spread of the virus through each vineyard.

Species level diversity in California

The diversity of species of *GLRaV* present throughout these areas of California was higher than expected. *GLRaV-3* was expected to be the highly dominant virus in all vineyards. Unlike other regions of California (Wang, Sharma, Duffy & Almeida, 2010), *GLRaV-2* is fairly widespread and many other species are present. *GLRaV-3* does not dominate every vineyard; three vineyards are primarily infected with *GLRaV-2. GLRaV-1* is also present in eight of the vineyards and *GLRaV*-5 was found in four vineyards. The presence of multiple and less common strains in this region suggest that the transmission of the virus throughout the vineyard was likely caused by multiple introduction events over time by either contaminated plant material or mealybugs. If the original vine was infected when it was planted (infected rootstock), the virus gets introduced to the vineyard, and then the virus can be propagated by re-planting material from an infected plant.

Understanding the symptoms caused by each species and strain of virus present in a vineyard is important for management strategies (Wang, Sharma & Almeida, 2010). Specifically,

mealybugs have been found to transmit *GLRaV-3*, but not other species (Daane et. al., 2009). Consequently, resources for virus control should only be used for mealybug pesticides in vineyards where mealybugs are the key vector for the transmitted species. If mealybugs are not the main vector, focus should be on removing contaminated plant material. Better management practices can help prevent propagation of the viruses through the vineyard and prevent economic losses (Habili et. al., 1995).

In vineyard 2 in Lodi, *GLRaV-3g* was detected for the first time in California. Although this strain has likely been present in the region for a while, it was only recently discovered and has not been detected in California until this study. The presence of this strain may be indicative of other strains or species that are currently unknown or untested that are being propagated throughout the state. Improving testing by improving primer sets and increasing the range of detection methods to pick up all viruses present in a vineyard should be an important priority for management. The sequences for strain g found in this study can now be used by testing companies including Foundation Plant Services at UC Davis which certifies rootstock sold throughout California and leads the California grapevine registration and certification program (Golino et. al., 2002). Improving testing and detection will help prevent the spread of this particular strain by allowing these facilities to use this information to test for the virus.

Phylogenetics & Divergence

The phylogenetic tree verifies that the strains of *GLRaV-3* found in this study have a large degree of genetic divergence (differences in genetic code indicating mutations and change over time). The presence of many strains and species can be very problematic for the wine industry, because each species can be spread throughout their vineyards and the plants can be infected with multiple species causing decreased crop yield, decreased sugar content in the fruit and plant death in their crops (Walker, Charles, Froud & Connolly, 2004). Frequently, when a virus diverges enough to be labeled as a new strain or species the current primers used by testing and certification facilities cannot detect the new virus. Unidentified species and strains can go undetected in current plant testing facilities and allows them to be propagated in the field.

The large phylogenetic divergence between the strains of species 3 is apparent in the horizontal distance between them (Figure 5). The genetic distances shown in the phylogenetic

tree of the isolates from this study (Figure 5) are larger than the distances between different species of the virus seen in phylogenetic analysis by Martelli et. al. (2002) suggesting that the distinction between naming a virus a new strain verse a new species is not well defined. A system for determining what makes two viruses unique species or unique strains of the same species needs to be established to prevent this confusion (A. Sharma, personal communication, April 2011).

Distribution

The distribution of species of the virus throughout each vineyard helps to discern how the virus is being spread. Based on the quantity of strains of *GLRaV-3* in a vineyard the number of virus introduction events can be estimated. In vineyards with multiple species and strains the virus likely had multiple introduction events by both mealybugs and contaminated plant material. However, if only one species or strain of *GLRaV-3* is present it is indicative of a single introduction event, likely by propagating contaminated plant material from a single infected plant. In vineyard 1 in San Luis Obispo strains a, b and c were present, indicative of multiple introduction events. It is likely that either contaminated plant material was brought in to the vineyard at multiple times or mealybugs brought in new strains of the virus, or both occured. In vineyard 2 in Lodi only strain *GLRaV-3g* was found suggesting a single introduction event. It is most likely that contaminated rootstock was brought in to the vineyard and that plant was propagated throughout the entire vineyard such that each vine has the exact same strain of the virus.

Many isolates were infected with multiple species of the virus resulting in overlap between the areas covered by each species. The presence of multiple species in one plant has been found to be a common occurrence (Golino & Almeida, 2008). A plant can be infected multiple times by grafting two plants with different species or by mealybugs infecting a plant that already has one species of the virus with a different species of the virus (Golino et. al., 2002). Multiple infections can be economically devastating for the vineyard because it greatly reduces the crop yield and ultimately requires a number of different treatment approaches (Golino et. al., 2002).

Limitations

Discerning the source and method of spread of the virus can be aided by having complete historical records of the rootstock for the vineyard and information on the plants used for propagation. The spread of the virus in these vineyards was inferred based on the location of the virus in the vineyard and using the phylogenetic tree. Some vineyards I studied lacked information about the source of their rootstock. Without this information it is not possible to trace the movement of the rootstock to see if the virus follows the same pattern. In addition, the methods used to study the virus were limited by the primer sets used to detect the virus. The primers are designed using highly conserved regions of genetic material based on a compilation of genetic information of the virus from previously studied isolates. These primers can have problems detecting new strains of the virus if there is any mutation in this conserved region. The detection of the strain of *GLRaV-3g* was a chance occurrence from primers detecting a different strain.

Future directions

Continued research and similar studies to determine which viruses cause GLD in grape growing regions would greatly benefit the grape industry by improving virus management and control strategies. More research is needed on the vectors that spread each of the species of the virus. Further investigation into the presence of the strain *GLRaV-3g* is needed to determine whether it is present in more of the vineyards I studied. In addition, primers that can detect this strain need to be designed and used so that rootstock certification can include this strain in their testing methods. This study should be continued throughout other regions of California, and other grape growing regions of the world, to learn more about the virus, how to manage it, and how to eliminate its propagation and diversification.

Broader implications & conclusions

The genetic information identified in this study will help isolate the conserved viral sequences for *GLRaV,* which can be used for future studies of the virus that will improve virus

control strategies. Conserved genetic material is used to create the primers that test for the species and strains of the virus in a plant. Rootstock testing and certification companies can use this information to improve their testing methods and thus stop the spread of the virus.

The results of this study will help managers understand the propagation of the virus and adds to the genetic database for *GLRaV*. Vineyard managers now have much more information about the species of *GLRaV* and strains of *GLRaV-3* in their vineyards and can improve their management protocol based on this knowledge. The genetic sequences from each sample has been added to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and can now be used researchers around the world.

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Table 1: Sample collection. 548 samples were collected from visibly infected vines at three different sites. Amador and El Dorado were grouped together because of proximity and possible spread of virus through both counties.

Site	# of Vineyards	Total # of Samples
San Luis Obispo	5	149
Lodi	8	167
Amador & El Dorado	11	232
All Sites	24	548

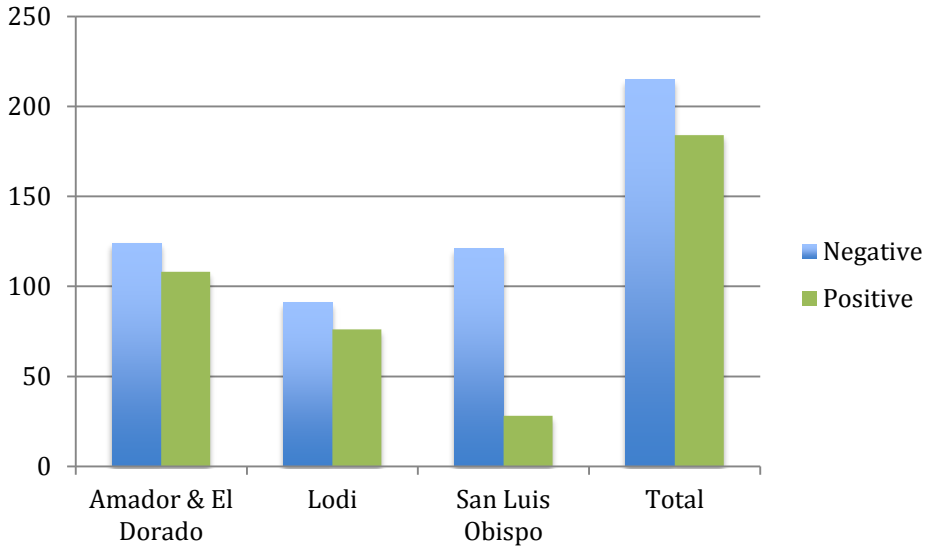

Figure 1. Number of negative and positive samples. A greater number of negative samples were found all regions.

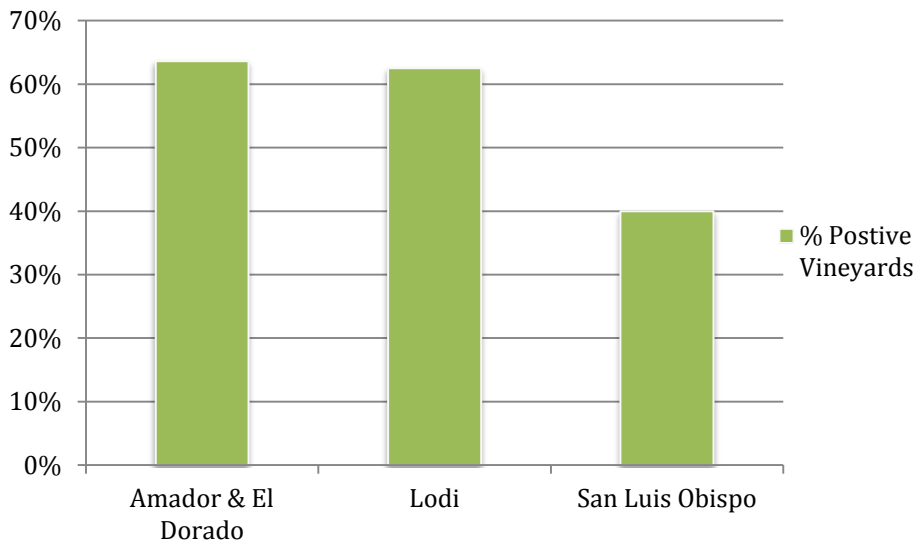

Figure 2. Percent of positive vineyards in each region. In each region entire vineyards tested negative. 4 of 11 vineyards did not have any infected samples in Amador and El Dorado, 3 of 8 vineyards did not have any infected samples in Lodi, and 3 of 5 vineyards in San Luis Obispo did not have any infected samples.

Table 2. Percent positive samples in each region. Percent of samples infected with each species of the virus in each region and the total number of infected samples as well as the total number of samples tested in each region.

Species	**Amador & El Dorado**	**Lodi**	**San Luis Obispo**
GLRaV-1	11.64%	0.60%	4.03%
GLRaV-2	37.50%	12.57%	2.68%
GLRaV-3	29.31%	31.14%	14.77%
GLRaV-5	9.05%	1.20%	0.00%
GLRaV-7	0.00%	0.60%	0.00%
GLRaV-9	2.16%	1.20%	0.00%
GLRaV-4	0.00%	0.00%	0.00%
Total # Infected Samples	108	76	28
Total # Samples	232	167	149

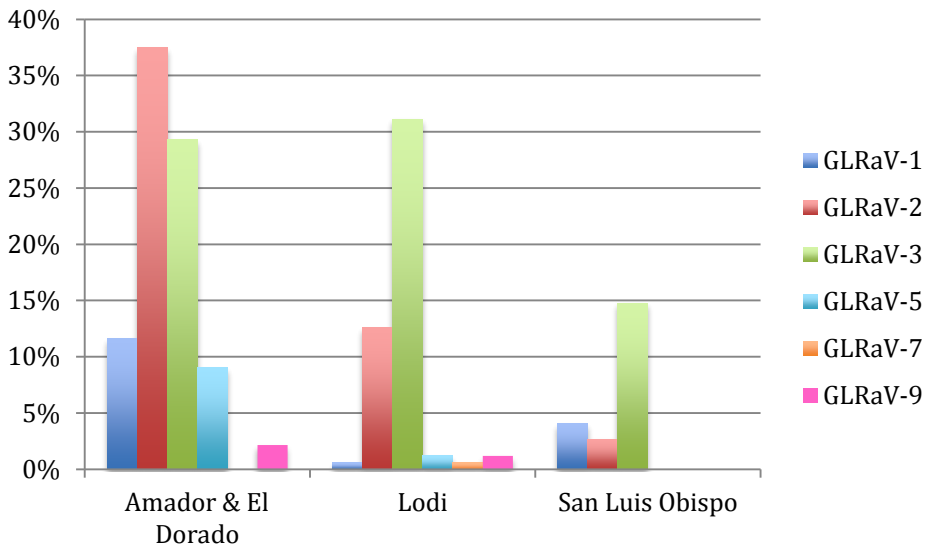

Figure 3. Species of *GLRaV* in each region. Percent of total samples infected with each species.

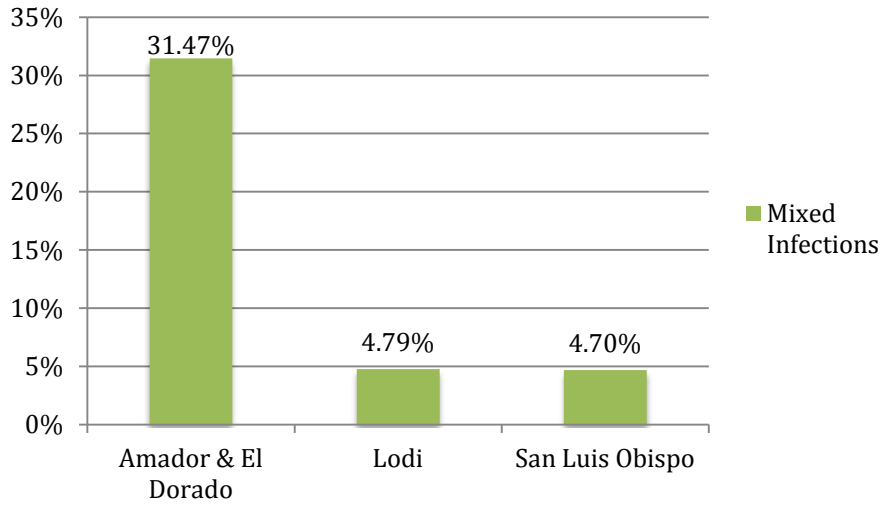

Figure 4. Percentage of isolates with multiple infections in each region. Percent of total samples that are infected with more than one species of the virus.

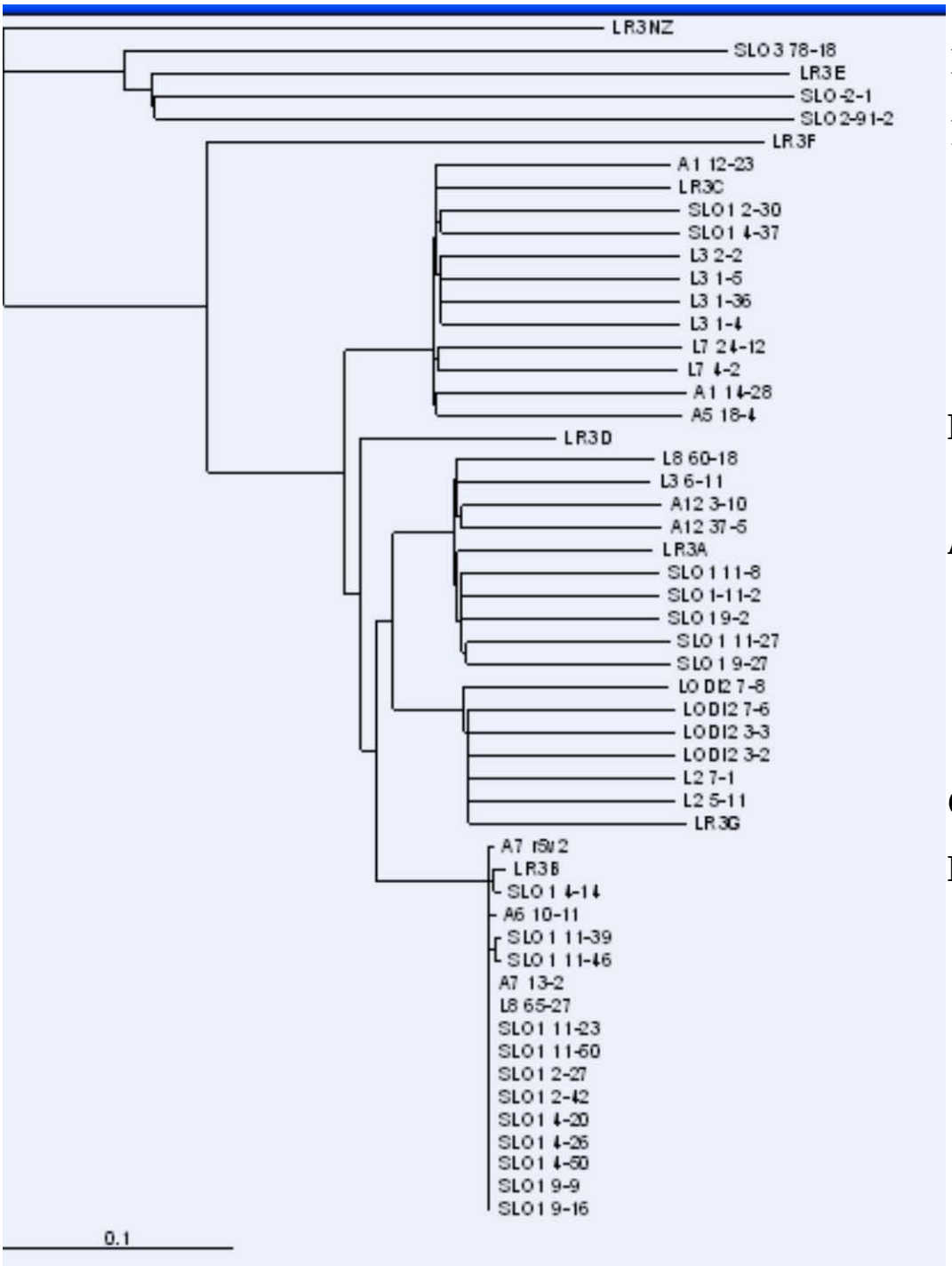

Figure 5. Phylogenetic tree of sequences isolates. Phylogenetic tree of 428 base pair consensus sequences of the 3' end of *GLRaV-3's* genome, from isolates in vineyards in Amador, El Dorado County, San Louis Obispo and Lodi California. Each clade is considered to be a strain of *GLRaV-3* and is labeled with a letter, "a" through "g."

Appendix A. Location and variety of samples. Information on vineyards surveyed in San Luis Obispo, Lodi, Amador and El Dorado, California.

Region		Vineyard	Varietal	Date Sampled
San Luis	SLO-1	Laetitia	Pinot Noir	10/20/10
Obispo	SLO-2	Pac. Vineyard Co.	Syrah	10/20/10
	SlO-3	Pac. Vineyard Co.	Pinot Noir	10/20/10
	SLO-4	Pac. Vineyard Co.	Syrah	10/20/10
	SLO-5	Pac. Vineyard Co.	Syrah	10/20/10
Lodi	LODI-1	Miller	Merlot	10/29/10
	LODI-2	Stanton Lange	Cab Sauv	10/29/10
	LODI-3	Lange Twins 80	Petite Syr.	10/29/10
	LODI-4	Lange Twins JW2	Zn (Primotivo)	10/29/10
	LODI-5	Lange Twins 100	Cab Sauv	10/29/10
	LODI-6	Wells Ln. (WL) ??	Zinfindel	11/10/10
	LODI-7	Quail Run	Zinfindel	11/10/10
	LODI-8	Aberle Acres (AA)	Pinot Noir on Syrah	11/10/10
	LODI-9	Dr. Gill	Pinot Noir	11/10/10
Amador/	AmEl-1	Shakeridge Ranch	Zn (Hearts Desire)	10/29/10
El Dorado	AmEl-2	Shakeridge Ranch	Zn (Monte Rosso)	10/29/10
	AmEl-3	Alviso	Zn	10/29/10
	AmEl-4	Deaver	Zn	10/29/10
	AmEl-5	Latchum	Cab Franc	10/29/10
	AmEl-6	Naylor	Petite Syr.	10/29/10
	AmEl-7	DK Cellars	Merlot	10/29/10
	AmEl-8	Standeven (St)	Monte Vina Barberra	11/10/10
	AmEl-9	Standeven Primo (St-P)	Primotivo	11/10/10
	AmEl-10	Witters (W)	Gamay	11/10/10
	AmEl-11	Cardanini (C)	Barbera	11/10/10
	AmEl12	Grace (G)	Pinot Noir	11/10/10

Appendix B. Primer sets. Primer sets and multiplexes for species level detection of *Grapevine leafroll-associated viruses*.

GLRaV	Probe-Sequence	Probe Color	Size (bp)	Plex
1	F: VIC-ACCTGGTTGAACGAGATCGCTT	Green	144	1
	R: GTAAACGGGTGTTCTTCAATTCTCT			
2	F: 6FAM-CATTATATTCTTCATGCCTCTCAGGAT	Blue	90	1
	R:GATGACAACTTCTGTCCGCTATAGC			
3	F:NED-AAGTGCTCTAGTTAAGGTCAGGAGTGA	Yellow	231	1
	R:GTATTGGACTACCTTTCGGGAAAAT			
4	F:6FAM-ATATACATACCAACCGTTGTGGGTATAA	Blue	93	2
	R:CCCTATAAACTAGCACATCCTTCTCTAGT			
5	F;VIC-AACACTCTGCCTTTTCTGCTGGC	Green	162	2
	R:CTTTTTATGTCCCATAAACGAGTACA			
9	F:NED-CGGGCATAAGAAAAGATGGCAC	Yellow	82	2
	R:TCTTTATGTCTACGGTAGAACCAACAC			
7	F:6FAM-AATGACTGTGATGTCGCTTTTAC	Blue	190	3
	R:TACCACTACCAGGAGGTTTATTCA			
CP	F: FAM-GAACTGAAATTAGGGCAGATATA	Blue	320	3
	R: GCC CAT AAC CTT CTT ACA CA			

Appendix C. Sequencing Primers. Primers used for sequencing for strain level detection of *Grapevine leafroll-associated virus-3.*

Primer	Probe-Sequence
CP 210F	**TAGTAAGGCGAGTTTCTTA**
CP 500F	**GGCTCGTTAATAACTTTCGGT**