Pathogen Ecology and Spread of Grapevine Red Blotch Virus in Napa Valley

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ABSTRACT

Understanding the ecology of grapevine red blotch virus is significant to the viticulture and wine making industries given this pathogen's recent emergence in 2008. Little research has been conducted around the virus, grape farmers and wine producers face issues in replanting costs of over $2.8 million statewide as a result of the red blotch virus. With only one known insect vector (three-cornered alfalfa hopper) this virus is spreading quickly through Napa Valley's vineyards, resembling symptoms similar to known pathogens such as leafroll disease. This study aims to provide relevant information on the pathogen ecology of the red blotch virus by examining its ability to spread across vineyards and denoting if infected vines exhibit one or both documented clades of the virus. The total amount sampled for this study was 863 vines spread throughout Napa Valley. Through DNA extractions followed by PCR amplification and Sanger sequencing, I was able to obtain relevant data surrounding the pathogen's infectivity. Genetic sequences were compared to reference sequences of the two known phylogenetic clades of the virus. Phylogenies were then composed to identify the similarities and relation between infected vines. Phylogenies also aided in providing distribution data correlated to geographic distance. Of the two known clades, clade II is most prevalent within vineyards and the least genetically variable compared to clade I. The virus has the ability to move across neighboring vineyards as sequencing data shows common linkage between infected vines from different vineyards.

KEY WORDS

infectivity, disease ecology, phylogenetic clade, genetic sequence, PCR amplification

INTRODUCTION

Napa Valley is home to California's wine industry, where grape vineyards stretch on for miles. However, new emerging pathogens have caused an increase in lost productivity for these grape farmers. These pathogens have rapidly growing infectivity rates, and cost farmers hundreds of millions of dollars statewide in replanting efforts (Trkulja et al. 2022). These newly emerging diseases cause damage ecologically and put the economic profitability of grape farmers at risk (Huang et al. 2020). For example, *Xylella fastidiosa* is a known pathogen in grapevines that causes severe onset of many other diseases such as Pierce's disease and the leafroll virus and is known as a quarantine pathogen (Sisterson et al. 2010). These diseases are primarily vector borne diseases, where insects cause quick infectivity within vineyards with their feeding patterns (Farigoule et al. 2022). New emerging pathogens are always on the rise, and with this comes little understanding around the ecology of these issues making it difficult to develop management strategies.

Understanding the genetic variability of plant pathogens and their host plants is critical in providing pathways in understanding how the pathogen moves between vineyards. In the case of *Xylella fastidiosa,* extensive phylogenies were constructed to be able to associate the pathogen to other known pathogens to help develop management strategies, predict vector movements, as well as host plant interactions (Kahn and Almeida 2022). This evolution and movement has not yet been mapped well enough for RBV (red blotch virus), however we know its emergence comes as recent as 2012 (Rwahnih et al. 2013). Studies today aim to provide researchers with more relevant data that is needed to develop a baseline understanding of this pathogen's movements (Bahder et al. 2016). The lack of ecological research surrounding the pathogen's spread and host plant interactions puts farmers at a disadvantage in managing and protecting their crops.

RBV, a recent and growing issue for Napa Valley grape farmers, is pushing researchers to better understand the movements of this pathogen. Symptoms of this pathogen have many similarities to many other known pathogens, making it difficult to identify in time for farmers to remove and prevent a potential spread (DeShields 2023, Cieniewicz et al. 2018). Red wine grapes experience differing symptoms than those of the white wine variety, however the common symptom between the two is the red blotching of the leaves. RBV has one established vector at

this time, the Three-Cornered Alfalfa Hopper (Flasco et al. 2023). Its growing infectivity rate raises the question as to how else it could be spreading between vineyards. It's possible that there are other insect vectors that are still yet to be identified, although, genotyping the virus within sites has shown to help understand its spread (Thompson 2022). It's still unclear as to how this virus is making its way through Napa Valley at such high rates, and given it has shown up in multiple sites, we're unsure as to whether the virus is spreading locally or from surrounding vineyards.

This study will aim to understand the disease ecology of the RBV in the Napa Valley. Through sampling multiple study sites, I aim to compile sequencing data of infected plants to understand how and if the virus is spreading within a vineyard or across multiple vineyards as well. Using known methods of testing for infectivity and using Sanger Sequencing, I will aim to provide relevant ecological information to provide better management strategies against RBV.

METHODS

Study site and sampling

The study sites were located across the greater Napa Valley area, and the sampling was completed by collaborators at the UC Cooperative Extension in Napa. Samples were selected based on symptom presentation on the leaves. Two sampling schemes were used. 'Neighbor sites,' or sites that were sampled over a large area of neighboring vineyard blocks, were sampled using an overlaid 12 cell grid where five symptomatic vines were selected from each grid. 'Non-neighbor sites' were sites that contained only a single block, scattered throughout the valley containing 24 sites. Twenty symptomatic vines were selected for each non-neighbor site (Figure 1). Samples were sent to the Almeida Lab on UC Berkeley's campus and stored at -20°C for preservation.

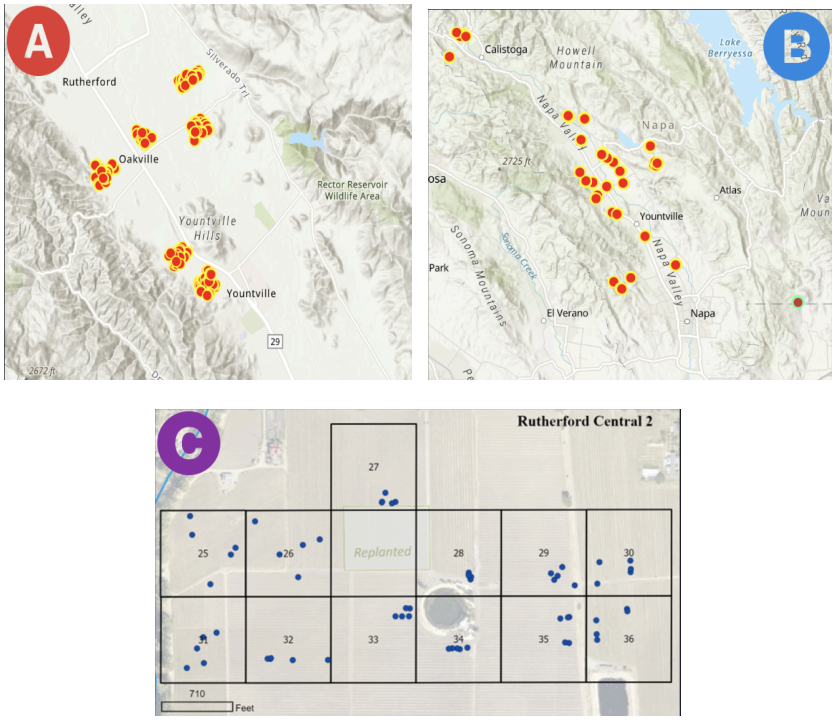

Figure 1. Neighbor sites (A) and one of 24 Non-neighbor sites (B). Neighbor sites and non-neighbor sites were scattered throughout the valley. Rutherford Central 2 (C) was one of six neighboring sites divided into grids to assess cross vineyard infectivity.

DNA Extractions

To determine whether a sample was positive or negative for the virus, I extracted DNA from our samples to be run for PCR amplification. Studies showed that the most robust way in determining whether a sample is positive or negative is through PCR amplification (Cieniewicz et al. 2019). To obtain a product for PCR amplification, I used the Qiagen company's DNA extraction kit (QIAGEN DNEasy Plant Kit). I selected three of the five petioles and chopped sections from each to weigh 0.1 grams. I then placed the material in a tube containing a metal bead and sealed it to be homogenized to break up the petioles further. This ensured that an adequate amount of plant material was exposed. Then I added 400uL of AP1 lysis buffer and 4uL of RNAse to further break down cell membranes to expose the DNA and eliminate the RNA.

Following this, the samples were incubated at 65°C in the dri-bath for ten minutes, vortexing every three minutes until the end of incubation. I added 130uL of neutralization buffer to eliminate the remaining lysis buffer, then incubated on ice for five minutes. During this time I

prepared our AW1 and AW2 wash buffers by adding the recommended volume of ethanol suggested per the Qiagen protocol. Once the samples were incubated on ice for five minutes and the wash buffers were prepared, I centrifuged the samples for five minutes at 20,000 RCF to separate the plant material from the supernatant. This supernatant is transferred to a QIShredder tube with a filter and I centrifuged the samples for two minutes at max speed.

A pellet formed at the bottom of the tube and all the supernatant that passes through was transferred and mixed with 600uL of AW1 wash buffer into a new spin column. This spin column contains a filter to entrap the DNA. I ran the mixed solution through the centrifuge at 7,000 RCF, discarding all flow through and changing tubes once switching to the AW2 wash buffer. I repeated this process for AW2, washing the spin column twice with 500uL of the buffer.

In the final step, I removed the filter off the spin column and placed this on top of a microcentrifuge collection tube. Then I added 100uL of AE elution buffer and waited five minutes to allow the buffer to bind to the DNA trapped within the filter. Then I centrifuged at 9,000 RCF allowing the DNA and buffer to pass through the filter. This step was done twice for a total volume of 200uL of DNA. Samples are stored at -20°C.

PCR Amplification & Gel Electrophoresis

PCR amplification is conducted upon DNA extraction to determine if a sample is positive or negative by amplifying any of the virus DNA that is present. I diluted the DNA in nuclease free water at a ratio of 80uL of water to 20uL of DNA. A complete PCR plate consists of 96 wells, allowing us to test up to 95 samples with one of the wells being for our non-template control. I combined 4uL of the diluted DNA and combined it with 16uL of a solution containing green-dyed GoTaq master mix, the forward and reverse primers, and nuclease free water (GoTaq LLC) . Using a thermocycler, I was able to amplify our DNA, having set the parameters as one cycle at 95°C for 3 minutes, 35 cycles of 95°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds. There was a final extension of 72 degrees celsius for 20 minutes. Upon completion of the PCR amplification, a positive or negative result was determined using gel electrophoresis. I made note of the positives and I took the remaining PCR product to be sent for sequencing at Barker Hall on UC Berkeley's campus.

Data analysis

The samples were analyzed using Geneious (Geneious Prime 20XX.x.x) and Rstudio (Version 2023.2) to create phylogenies as well as denote statistical similarities and differences across the phylogenetic clade distribution. Chromatograms were used to generate consensus sequences, which were compared across study sites. By implementing the two reference sequences of clades I and II, I was able to analyze distinguishable mutations in each sample. To determine whether the spread is localized or travels across vineyards in neighboring sites, phylogenies were created in Geneious to show if neighboring sites contained similar or the same sequences of the virus from adjacent sampled vineyards. Phylogenetic trees were also used to investigate whether one or both clades were present among the study site and how prevalent one was over the other. An isolation by distance (IBD) Mantel test was conducted by Oge Okpala to show whether there was a significance between genetic distance and geographic location.

RESULTS

DNA Sequencing

I found that our sampling method and study sites were well equipped in selecting and providing vines that were positive with the virus. Of the 863 samples selected and processed for PCR amplification, fewer than 30% tested negative for RBV. With the chromatogram data and consensus sequences, I observed that clades I and II were either both present in both neighbor and non-neighbor sites, as well as these same sites only containing one of the two clades. Clade I often contained more mutations than clade II. Sequencing data showed that in neighboring sites both phylogenetic clades were present among sites and were not evenly distributed (Figure 2). Consensus sequences were generated with the most common base pair found across all the sequences, while denoting which samples experienced either a missing base pair or mutation within the sequence. Clade two is less genetically variable than clade I and presents itself more across study sites. At study sites where both clades were evident, I found that clade II was often the more common clade (Figure 3).

Figure 2. Chromatogram sequences display the base pairs of each sample developing an overall consensus sequence from the selected samples. Consensus sequence is developed based on the most occurring base pair or pattern within that line. Mutations are denoted by highlighting the different base pairs and as shown these mutations resemble a difference in clade I and II .

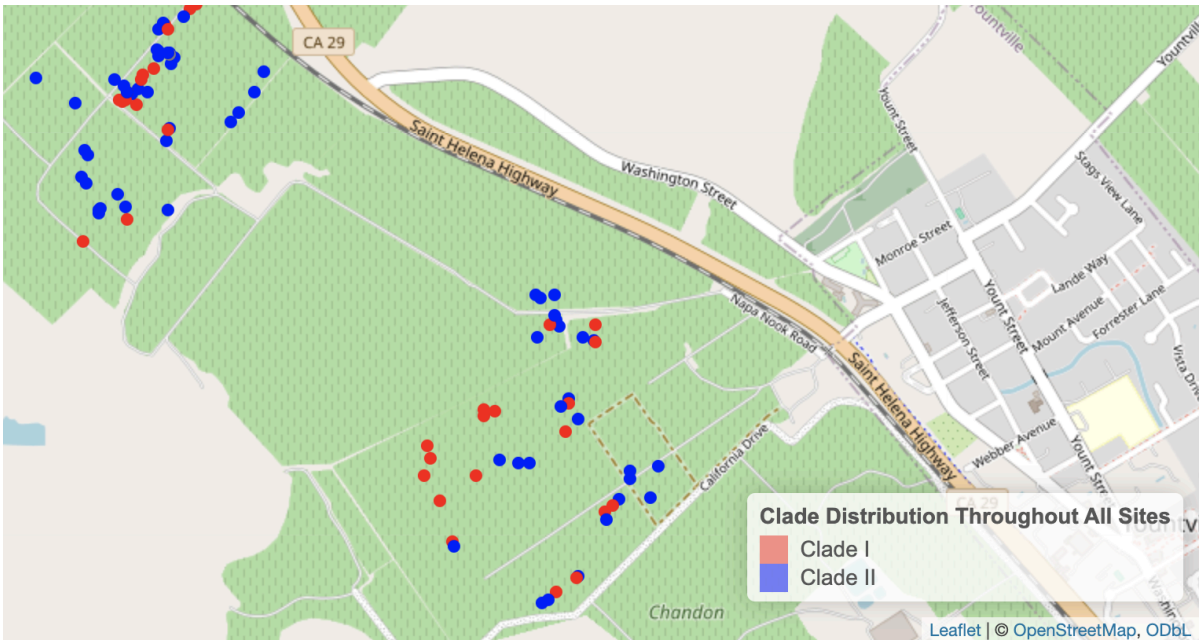

Figure 3. Clades I and II are distributed across these two study sites where clade two is more prevalent overall. Clade I is often grouped closely whereas clade II is distributed across the sites sporadically and does not follow a pattern.

Phylogenetic comparison

Clades I and II were both observed among all neighboring sites and differed in distribution. Neighboring sites showed evidence of the same sequence occurring from one site to the other (Figure 4). Neighboring sites ORW and OW2 contain genetic sequences of RBV that are identical or similar to one another from each respective site, in the case of both clades I and II. Samples 4.3, 2.5, and 23.2 were so significantly different from both clades I and II that they were shown to be separated into their own group outside of the known clades. Non-Neighbor sites such as RW2 contained only one of the two clades where site RC2 contained both (Figure 5). I observed that clade I and II were not distributed evenly across any of the study sites.

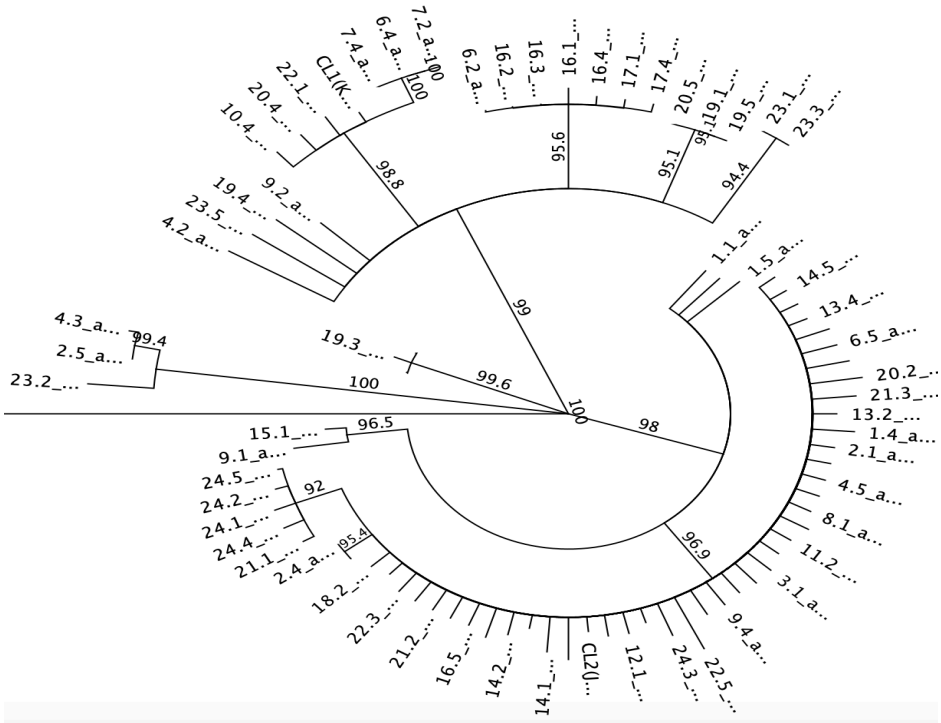

Figure 4. Sites ORW (1-12) and OW2 (13-24) show cross site infection with their genetic sequences occurring in both sites. Groupings resemble how closely samples are related to one another and indicate cross vineyard spread. Samples 4.3, 2.5, and 23.2 did not belong to either clade I or II according to sequence assemblies.

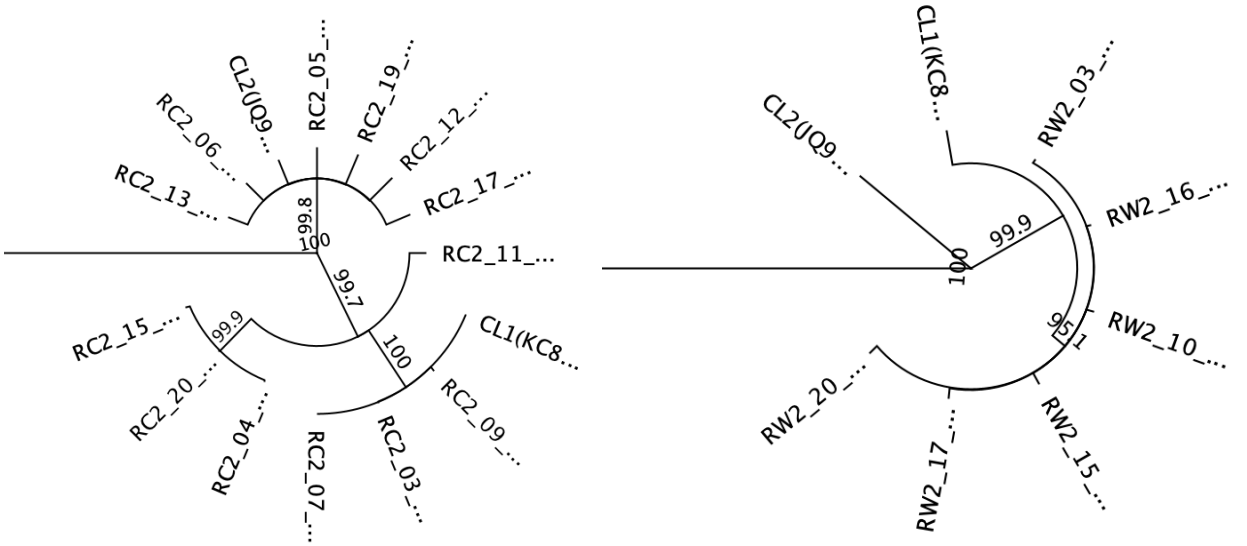

Figure 5. Non-Neighbor sites RC2 and RW2. Clade I and II were observed in site RC2, but only clade I was observed in site RW2. Phylogeny shows how closely related samples are to each respective clade. Selected samples exhibit close relation with reference sequences of clades I and II.

Genetic and geographical distance

Clades I and II did not follow a specific pattern in their distribution among the vineyards. The IBD Mantel test provided a value of a Pearson correlation of r=0.07 with a p-value of 0.05 for clade I and clade II had a Pearson correlation of r=-0.107 with a p-value of 0.997. Clade I has a weak positive relationship between genetic and geographic distance where clade II did not show a relationship (Figure 6).

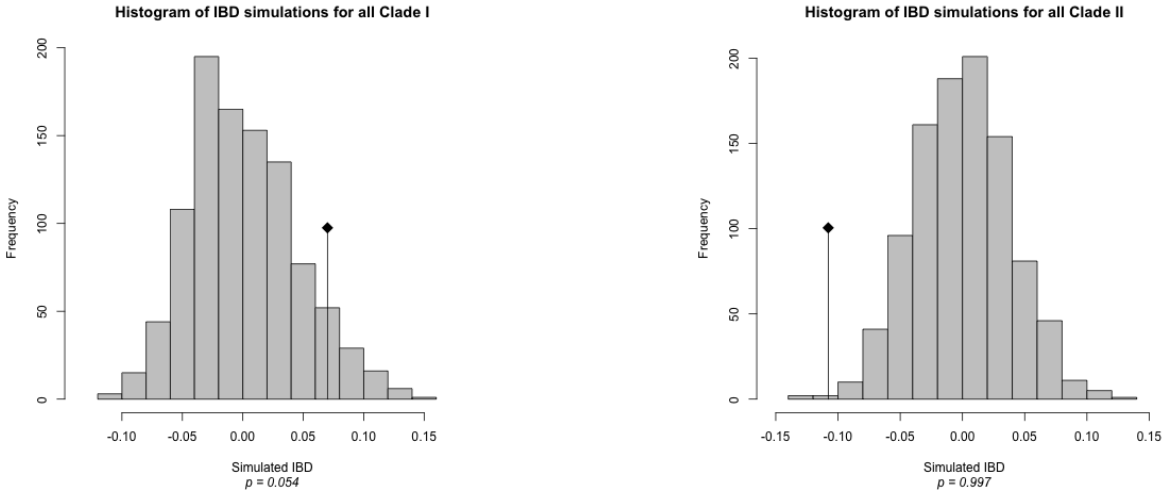

Figure 6. Clade I exhibits a weak relationship between genetic and geographic distance where Clade II does not show any relationship through a Mantel test. Statistical test completed by Oge Okpala denoting the relationship between the genetic and geographical distance of the two clades. Clade I presented a p-value of 0.054 and clade II presented a p-value of 0.997.

DISCUSSION

This project aimed to distinguish infectivity patterns and prevalence of the two clades between study sites. The two known clades of this virus have been well established across multiple studies and are noted to be the two most common sequences between infected samples with notable mutations within clade I and few mutations within clade II (Kren et al. 2014). The study revealed that the management surrounding this virus has to be aggressive and the infectivity of this virus has continued to accelerate faster. My individual and collaborative findings indicate that this virus is able to spread both locally as well as across vineyards, posing threats to surrounding vineyards that are adjacent to infected areas.

Infectivity Across Neighboring and Non-Neighboring Sites

Insect vectors are one of the leading causes of plant pathogenesis because their feeding patterns allow these diseases to make their way into vineyards and acres. Napa Valley's study included both neighboring and non-neighboring vineyards to encompass a larger area for this study (Figure 1). My data suggests that the distribution of clade I and clade II did not follow a recognizable relationship or pattern between the sites. Throughout neighboring sites where both clades were present simultaneously or independently, the dispersal of clade I and II were scattered throughout with some clustering of clades in small areas of the site. Previous research confined much of their studies to smaller areas, rendering their conclusions to be limited in terms of applying it to a large scale (DeShields et al. 2021). For the 863 samples tested, the management team at Napa Valley was responsible for the sample collection and samples were chosen based upon their presented symptoms aligning with the virus, asymptomatic plants often do not yield positives as studied by (Yepes et al. 2018). Data suggests that both variants were present among most neighbor study sites and specific sequences present in one site appear in other sites as well, indicating cross vineyard spread of the virus. Sequences indicate that the same variant was apparent across adjacent study sites and were able to move from one vineyard to the other.

It's important to consider as well that infection can occur from infected planting material, so similar sequences can arise across the county if growers use contaminated material. Sequencing data allowed me to denote the visible mutations as well what specific base pairs were indicative of each of the two clades (Bahder et al. 2016). The three cornered alfalfa hopper is noted to be the only known vector of the virus and studies reveal it's responsible for the growing infectivity of this virus (Flasco et al. 2023). The movements of this vector across vineyards allow for the pathogen to spread both locally and between adjacent vineyards. In some cases, the distance between infectivity can be large depending on the overall dispersal of these vectors within a vineyard.

With sequencing data revealing that specific sequences were present within multiple vineyards leads to a greater issue surrounding this pathogen. Most studies surrounding this virus were completed on a smaller scale to produce real-time data to help manage for this virus (Cieniewicz et al. 2019). Our study aimed to produce a large-scale investigation into how this

pathogen moves and behaves in open conditions. Other vectors with similar feeding and anatomical characteristics to the three cornered alfalfa hopper could be secondary vectors of this virus. A study by Kron and Sisterson (2020) was conducted in a greenhouse to control and affirm that the three cornered alfalfa hopper is a vector. Napa Valley contains this vector throughout its vineyards and is noted to be the leading cause of this pathogen's movement (Kc et al. 2022). Host-virus interactions are important aspects to consider when establishing a vector of a disease, and further studies should be done to investigate other potential vectors of RBV.

Phylogenetic comparison and genetic distance

The two known variants were evident across most neighboring study sites and multiple non-neighboring sites. Noticeable mutations had occurred in their sequences as well, mostly in clade I which is known to be more genetically variable in its transmission. Mutations in genetic sequence can indicate and pave the way for many possibilities surrounding this virus' evolution. In sites ORW and OW2 I observed three samples that did not belong to either clade I or II and were genetically similar to each other. Their major difference between the reference clades and respective samples within those clades could suggest that there is potential for a third clade to be established. As observed in Figure 4, how closely samples were related, denoted by the phylogenetic distance between them, revealed which sample belonged to which clade and how close their relation is to one another. I observed that sequences from neighboring blocks were present between adjacent sites, showing their ancestry and relation was very similar or identical to one another. I was not able to establish a solidified relationship between the two phylogenetic clades and the grapevine variety as Cieniewicz et al. (2018) did with denoting specific symptoms to specific genetic sequences experienced by infected vines. Instead I was able to denote whether there was a relationship between the genetic and geographic distance of clades I and II.

The genetic and geographic distance did not exhibit a strong relationship to one another and instead my findings suggest that the dispersal of the clades occurs through random dispersal events and long pathways. My findings here suggest that the distribution of the virus is likely occurring as a result of vector movements throughout the valley and potentially infected planting material. Clade I and II are dispersed sporadically throughout the sites and small clusters of clade I suggest that there is a weak relationship to how far this clade can travel. The mutations that

clade I often experience can be contributions to how this virus will progress among other vectors or plant species (Sudarshana et al. 2015). Evolution of this virus has already allowed it to make its way from Europe to America as noted by Thompson (2022) and as most viruses, it will continue to evolve to survive and better spread. Work conducted around *X. fastidiosa,* a plant bacterium that infects an array of staple crops by Almeida and Khan (2022) indicates how evolutionary mutations have allowed this disease to infect more plant species. It's unclear as to if RBV will be able to infect other plants, but it's important to consider the study of these other disease systems to produce the necessary knowledge surrounding this pathogen. RBV is still in its early emergence period and these mutations are indications that this disease will further progress in either its infectivity, intensity, and overall threat level to the yield produced by infected vines.

Limitations & Future Directions

My study was designed to investigate the pathogen ecology and spread of the RBV in Napa Valley's vineyards. In this study we learned that the virus does not follow a specific distribution pattern. The genetic and geographical distances do not exhibit much of a relationship, instead the dispersal is random and is likely associated with vector movements. The infectivity of this virus has the ability to cross over to adjacent vineyards and often clade I is observed to be slightly clustered in local vineyards where clade II is scattered throughout the vineyard. Although clade I clusters in local areas, it's able to move across sites and can be transmitted to neighboring vineyards in either short or long distances, similar to clade II. The study was conducted by myself, Iona McCabe, and Oge Okpala working collaboratively and independently to investigate multiple aspects of this virus. Error is possible with poor sample preparation and the selection of petioles to test for infectivity, allowing samples to render a negative result if areas were chosen where the virus did not exist. This can contribute to loss of samples needed to compare across one another, where some sites contained an abundance of positive results and others yielded fewer numbers. Although the study area was much larger compared to other studies, this prevented the control of certain real world elements that could ultimately influence the pathogen and vector movements.

Future research on this virus should still be conducted to better understand the potential movements and advancements associated with this virus. The next steps should encapsulate studying if there is a relationship between the phylogenetic clade and the grapevine variety. By examining how the phylogenetic clade is dispersed along with which varieties it does or does not appear in will provide the needed information to correlate a relationship. Further studies should be conducted to determine whether other vectors can exist by examining insects closely related to the three cornered alfalfa hopper, as well as non relatives that could be potential carriers of the pathogen. These future studies should aim to compile more data surrounding the overall infectivity and characteristics of infectivity as done by my project to help farmers and industries manage their vineyards.

IMPLICATIONS

RBV is a newly emerging pathogen that is only continuing to advance in its rate of spread and infectivity. Symptoms often do not present until the vine is too far along with the infection for it to be saved and now is a host for potential other infections. This study revealed how far this virus can spread and indicates that this pathogen is only going to evolve towards infecting masses of vineyards and potentially other crops that are prominent to the world. This study also showed that this virus is capable of moving great distances across vineyards and the geographic distance does not significantly affect the pathogens movements. Management for this pathogen should encompass routine symptom checks as well as removal of positively infected vines as well as surrounding vines (Cieniewicz et al. 2019). Positively infected plants are detrimental to the vineyards as their presence only allows for further potential infections of the remaining vineyard and the remaining plant material left in the soil can contribute to new infections of newly planted vines. It's important as well to manage for these insects that can carry and spread the virus because their movements dictate the spread of the virus as much as the presence of positively infected plants (Kc et al 2022). All points considered, research on this pathogen is still in its early stages and further studies should be conducted to better understand this pathogen and prevent it from advancing further into viticulture and horticulture worlds.

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