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An Assessment of Seven New Grape Varieties and a Study of Grapevine Vein Clearing Virus in Native Vitaceae Plants

Kaylie A. Austin
Missouri State University, Kaylie1115@live.missouristate.edu

AN ASSESSMENT OF SEVEN NEW GRAPE VARIETIES AND A STUDY OF *GRAPEVINE VEIN CLEARING VIRUS* IN NATIVE VITACEAE PLANTS

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By

Kaylie Austin

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AN ASSESSMENT OF SEVEN NEW GRAPE VARIETIES AND A STUDY OF *GRAPEVINE VEIN CLEARING VIRUS* IN NATIVE VITACEAE PLANTS

William H. Darr College of Agriculture

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ABSTRACT

One of the most effective methods of reducing pesticide application in vineyards is breeding hybrid varieties for disease resistance. The new varieties must be assessed for disease resistance and viticultural traits. This thesis focuses on seven new varieties from a cross of 'Norton' and 'Cabernet Sauvignon'. 'Norton' was chosen because of its disease resistance and cold hardiness. 'Cabernet Sauvignon' was selected because of its high-quality wine making berries. The cross was made in 2005, seven were selected in 2011, and evaluation began in 2016. Using *Botrytis cinerea* as a model pathogen, resistance was measured with an incidence rate and severity scale. The varieties' berry chemistry, vine vigor, and growth stages were recorded, and three have been identified as promising varieties. 'Norton' is resistant to *Grapevine vein clearing virus* (GVCV), which became a second project for this thesis. GVCV infects vineyards and causes economic losses. Two symptomatic vines, a cultivated 'Chardonel' and a native vine, *Ampelopsis cordata,* were discovered 10 feet apart along a vineyard boundary. Both samples tested positive for GVCV using a polymerase chain reaction (PCR) assay. The sequences of the two isolates share a high percentage of nucleotide identity. This suggests the same GVCV isolate infects two genera. In addition, a survey of GVCV in native *Vitis* plants from the National Plant Germplasm Collection was also conducted. A triplex PCR assay was developed to test 380 samples. GVCV was not found in the survey indicating the spread of GVCV is likely a localized, recent event.

KEYWORDS: 'Norton', 'Cabernet Sauvignon', new variety, GVCV, grape, virus, *Vitis*

This abstract is approved as to form and content

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Wenping Qiu, PhD
Chairperson, Advisory Committee
Missouri State University

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Approved:

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Wenping Qiu, PhD

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Melissa Remley, PhD

_____
William McClain, PhD

_____
Julie Masterson, PhD: Dean, Graduate College

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INTRODUCTION

History of Missouri's Wine Industry

The wine industry in Missouri has a long and rich history. It began in 1837 when the town of Hermann was established by German settlers. The rocky soils were deemed unsuitable for farming and cropping, but ideal for vineyard production. By the 1880s, Missouri vineyards were producing two million gallons of wine per year with the addition of vineyards established by Italian immigrants in the St. James area (Missouriwine.org, 2015).

Complications began to arise in 1919 when prohibition was ratified and went into effect. The young Missouri wine industry was nearly destroyed. Even when the 18th amendment was repealed, several aftereffects set the industry back for decades (Missouriwine.org, 2015). Finally in the 1960s and 1970s the industry experienced a rebirth when several of the original wineries were restored and began expanding their wine production. In the 1980s an implemented tax on wine allowed the creation of the Missouri Wine and Grape Program (Missouriwine.org, 2015). This launched a new era in the Missouri wine industry. Important wine production areas were designated into official American Viticultural Areas (Barnard, 2009). The Missouri State Fruit Experiment Station built relationships with vineyard producers and started evaluating new grape varieties that could thrive in the Missouri climate.

The wine industry has continued to grow in the last five decades. 'Norton' was legally designated as the state grape in 2003 and the Missouri Wine and Grape Board was formed in 2005 (Missouriwine.org, 2015). By 2010 Missouri became the largest producer of 'Norton' with approximately 500 acres (Ambers & Ambers, 2004). In 2011, Missouri's 100th winery opened. This number has now grown to over 125 wineries that support an economically important industry in the Show-me state. The Missouri wine industry incorporates 425 grape growers that produce on 1,700 bearing acres and supports nearly 15,000 full-time jobs. Annually, it produces $1.76 billion, including $220 million in wine-related tourism and $52 million in the sale of retail wine (Frank R.C., 2015).

Even though it has continued to grow and prosper throughout the last few decades, the industry faces some difficult challenges. High disease pressure is the most challenging to vineyard managers. The three major diseases that affect grapes grown in Missouri are downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*), and *Botrytis* bunch rot (*Botrytis cinerea*) (Agriculture et al., 2006). Pesticides must be applied multiple times during each growing season to control pests and pathogens. Another challenge growers face is the unpredictable climate of Missouri. Late spring freezing can damage the primary buds and reduce a vine's yield significantly. It is vital to conduct research and develop new grape varieties that can provide solutions to solve these problems for sustaining the prosperous and economically important wine industry.

Comparison of 'Norton' and 'Cabernet Sauvignon'

One of the most effective methods of alleviating these problems is breeding new varieties. 'Norton' and 'Cabernet Sauvignon' were chosen to cross and an assessment of the resulting hybrids followed. 'Norton' is a native North American variety that is known as a cold-hardy, disease-resistant variety and is used to make dry red wine (Figure 1A) (Ambers & Ambers, 2004). However, the vigorous growth, and lower yield are not popular traits to vineyard managers. The berries can also be a challenge to wine makers because of their high pH and high malic acid contents. The cultivar's parentage is the native grape *Vitis aestivalis* and the cultivar 'Bland' which is a hybrid of *Vitis labrusca* and Chasselas (Ambers & Ambers, 2004). The disease resistance of 'Norton' is believed to be the result of high levels of secondary metabolites such as stilbenic compounds and constitutive expression of defense-related genes when compared to a more susceptible grape such as *Vitis vinifera* 'Cabernet Sauvignon' (Ali et al., 2011). The parentage of 'Cabernet Sauvignon' includes 'Cabernet Franc' and 'Sauvignon Blanc' which are both *Vitis vinifera* cultivars (Ambers & Ambers, 2004). 'Cabernet Sauvignon' creates a high quality red wine that is world-renowned (Figure 1B) (Lattey et al., 2010). The vine also produces a very manageable foliar growth when controlled by irrigation. However, it grows poorly and can be destroyed by unpredicted deep freezing temperatures under the variable Missouri climate. It is an early budding variety, but this is a weakness in Missouri. The climate has cold winters with warm snaps that can cause 'Cabernet Sauvignon' vines to initiate bud break which then experience freeze injury when the temperature decreases drastically. 'Norton' is a late budding variety which protects the vine from the variable winter and spring temperatures and delivers the buds safely into

spring without freeze injury. An ideal variety is a hybrid of these two that would be cold-hardy and disease resistant, that also produces a maintainable amount of foliage, and makes a new high quality wine.

Impact of *Botrytis cinerea* on Grape Production

Gray mold is a serious disease that can make the entire cluster unusable and is caused by the microorganism *Botrytis cinerea*. *B. cinerea* is a necrotrophic pathogen that grows on many fruits including strawberries, tomatoes, and grapes. The particular fungus that usually infects grapes is in the genus *Botrytis* and in the clade that attacks only Eudicot plants (Choquer et al., 2007). There are also two strains of *B. cinerea* that are unable to cross with each other: Group I (*Botrytis pseudocinera*) and Group II (*B. cinerea sensu stricto*). *Botrytis cinerea* can infect over 200 host plants and it kills live cells through producing toxins and reactive oxygen species, and inducing a plant-produced oxidative burst (Choquer et al., 2007). Once the host cells are dead, the fungus uses degrading enzymes to digest and receive energy and nutrients for reproduction from the degraded tissues. These stages are usually summarized by penetration, primary lesion formation, lesion expansion or tissue maceration, and sporulation (Choquer et al., 2007).

The grapevine and *B. cinerea* have been living with each other for centuries. The fungus uses its conidia to infect the leaves or inflorescences, and then develops into the reproductive stage in the fall on the ripe berries (Choquer et al., 2007). The initial entry into the host is not believed to be by physical pressure. Instead degrading plant cell walls with enzymes and causing an oxidative burst is more accepted (Choquer et al., 2007). Over time some plants have built up immunity or resistance to *Botrytis*. Some chemicals

that constitute resistance against gray mold in grapes include stilbenes and other secondary metabolites. The phytoalexin resveratrol has been found inside grapes that are more resistant to *B. cinerea* although levels do not usually reach a concentration that is lethal to the fungus. These sub-lethal concentrations allow the fungus to still live on the plant, but many of the clusters are still usable for wine making (Adrian & Jeandet, 2012).

Current Methods of Control

Current controls used in vineyards for *B. cinerea* are fungicides, cultural methods, and tolerant hybrids. There are also new control methods being studied such as different canopy management methods and elicitor chemicals which are compounds that activate defense reactions in plants (Adrian & Jeandet, 2012). The main goal of canopy management is to remove excess shoot and leaf growth to allow air movement into and through the canopy so there is less lingering moisture (Gubler et al., 1987). Fungi thrive in warm, moist environments so canopy maintenance tries to eliminate that type of environment through air flow. Leaf removal in some studies has reduced disease severity by 80-90% in unsprayed treatments (Gubler et al., 1987).

Parameters for Varietal Comparison

Growth Stages. Growth stage is an important factor to compare among grapes varieties. These can help determine where specific plants can be grown. The four main growing stages recorded in grape production are bud break, bloom, veraison, and harvest. Bud break occurs in the spring when the protective overwintering buds break open and a new green shoot begins growing rapidly (Lorenz et al., 1995). Bloom takes place in late

spring or early summer, just a few weeks after bud break. The developing inflorescences begin to swell, the flower caps come off, and the flowers open. Once the flowers are open, pollination and fruit set occur. Veraison takes place in late summer. Visual indicators of this stage are the berries on red varieties turn from green to red or purple and the berries on white varieties turn from green to a more translucent, lighter green. From this point on, accumulating sugars and organic acids change forms as the berries mature. The final stage recorded is the harvest date which is when the berries meet most of the chemistry requirements and have the correct levels of sugars and acids for wine making (Lorenz et al., 1995). The berries are then cut harvested from the vine to be used for wine making. These growing stages help a vineyard manager decide what varieties to plant in a particular location. If a plant has an early bud break, then it must be planted closer to the equator to ensure it doesn't get damaged by a late frost in the spring, but a plant that has a later bud break can be planted farther from the equator. Harvest dates are important also to avoid early frosts in the fall.

Berry Chemistry. There are three main berry chemistry measurements that vineyard managers rely upon to decide upon the optimal harvest of grapes. Therefore, when observing crosses, it is essential to record each variety's levels so buyers know what to expect if they choose that hybrid for a crop. The three parameters are the pH of the berry, the soluble solids (Brix), and the Total Titratable Acidity (TA). Berry juice pH is measured to gauge the hydrogen ion concentration in the berry which is related to the juice acidity (Dokoozlian, 2000). The Brix content of berry juice measures the amount of sugar. One degree Brix is converted to one gram of sucrose in 100 grams of solution. This is essentially a percentage of sugar by mass in the berry juice. However, if there are

other dissolved solids in the juice besides sucrose, the Brix can be inaccurate (Bates, 1942). The TA result includes the levels of tartaric and malic acids inside the berry. As the berries mature, the TA drops as malic acid is respired to form CO_2 and H_2O and the tartaric acid is diluted as the berry volume increases (Dokoozlian, 2000). This measurement is taken in grams of acid per milliliter.

Vine Balance. The two factors that determine the balance of the vine are pruning weight and yield. The pruning weight is collected in the winter in late February or early March. Each year, a vine produces new shoots which produce the berry clusters. Annually, the one-year-old growth is pruned off leaving short canes with a calculated number of nodes to produce the following year's clusters. The one-year-old growth that is pruned is weighed for each plant. The yield of the vine is measured when berries are mature and ready for harvest. The berries are collected and the rotten ones are removed. Then the official yield is measured. The weight of the one-year-old pruning should correlate to the amount of yield produced later that year according to the Ravaz index. This is used to determine if the vine is overproducing berries or vegetative growth.

Overall Objective of this Thesis

One of the most promising methods of controlling disease in a vineyard that can be incorporated to decrease disease incidence is growing resistant or less susceptible hybrids. This is the purpose of the current study. Many factors were judged in this variety trial. A disease resistance analysis on both leaves and berries was conducted in the laboratory using *Botrytis cinerea*. A viticultural evaluation was completed by recording growing stages and common berry chemistry parameters. The growing stages included

bud break, bloom, veraison, and harvest and the chemistry measurements included Total Titratable Acidity (TA), Brix, pH, and yield. Pruning weights were used for both comparison and to determine the nodes left during pruning for the next year's growth.

MATERIALS AND METHODS

Materials

A cross between 'Norton' and 'Cabernet Sauvignon' was made in May, 2005. A total of 100 progeny seedlings were planted in a vineyard in June, 2006. Preliminary evaluations on the progenies began in 2007 and continued to 2011. Seven promising varieties were selected based on the viticultural traits of vegetative vigor, cold and heat tolerance, yield, and berry chemistry measurements. The progenies were not sprayed with pesticides in order to determine preliminary levels of disease resistance and severity. Of the seven varieties chosen, four produce white berries and three produce red berries. The white varieties are named 'NC6', 'NC28', 'NC43', and 'CN21'. The red varieties are named 'NC17', 'NC60', and 'NC65'.

In 2011, three rows of grapes were planted in a vineyard in Mountain Grove, Missouri at the Missouri State Fruit Experiment Station. The three rows contain three replicate blocks of nine varieties: the seven selected varieties and the two parents. Each block contains four vines of each variety for a total of twelve vines per variety for evaluation (Figure 2). The baseline and control for this study was the two parental cultivars. They represented the extremes of the characteristics and the progeny were expected to fall between these extremes.

Botrytis cinerea Growth and Harvest

Two evaluations were completed for the resistance to disease. Both used the gray mold *Botrytis cinerea* as a model pathogen. An evaluation was completed on the leaves

of the nine total varieties and a second evaluation used the grape berries. *Botrytis cinerea* was collected from an infected cluster found in the greenhouse (Figure 3). New strains were purified and grown on potato dextrose media (Eddleman, 1998). A new plate was grown for 10 to 14 days before harvesting. When the strain had developed pure and strong growth over multiple generations, and leaves from the vines were collected, the spores were harvested. During harvest, a solution of deionized water was used to collect the spores. The spores along with mycelium were scraped from the media. The deionized water, spore, mycelium solution was filtered using two layers of miracloth. The miracloth was dipped in water to allow an easier flow through of the spore solution. This filtered out the *Botrytis* mycelium and left only the spores. A sample was then examined under 40X magnification. If some mycelium or other debris remained, another filtration was completed. Once the spore solution was clean, a hemocytometer was used to count the spores. 10µL of the spore solution was loaded into the chamber on the hemocytometer. Within the chamber, there was an etched grid that was visible under a microscope. The middle large square with the 25 small squares inside was screened. The four corner squares and the center square, for a total of five squares, were counted and averaged. The following calculation was used to determine the concentration of spores (Morris & Nicholls, 1978).

$$\frac{(\text{Square1} + \text{Square2} + \text{Square3} + \text{Square4} + \text{Square5})\ (25)\ (10)}{(5)} = \text{spores}/\mu\text{L}$$

Three samples of the solution were taken, counted, and calculated. The three results were averaged to get the concentration of spores in the solution. The solution was diluted to 1,500 spores/µL for the leaf evaluation or 200 spores/µL for the berry inoculation (Wan et al., 2015).

Evaluation of *Botrytis* Infection on Leaves

The evaluation of *Botrytis* infection on leaves was completed three times: the first with three leaves from each variety (one from each replicate block) and the second and third times with nine leaves from each variety (three from each replicate block). A total of 21 leaves per variety were evaluated.

During leaf collection, a random sampling of young, but mature leaves were collected one to three nodes from the end of the shoot. The leaves were stored in plastic bags on ice during collection. They were then brought into the laboratory and washed. Each side of the leaf was washed twice and rubbed by hand with tap water, then rinsed once with deionized water to remove all pesticide residues from the vineyard. The leaves were air dried and put into plastic containers with wet paper towels to contain a humid, moist environment for the fungus. Each leaf was scratched four times with a syringe needle to fill in a 1 cm x 1 cm square to wound the leaf (Figure 4). Each leaf was then sprayed with ~10 µL of spore solution for inoculation and the leaves were kept in the sealed boxes for six to seven days (Wan et al., 2015). Images of the leaves were taken and the affected areas were measured in square millimeters by the software Assess 2.0 from The American Phytopathological Society in Saint Paul, Minnesota.

Evaluation of *Botrytis* Infection on Berries

The *Botrytis* infection on berries evaluation was completed twice with 20 berries per variety for each replicate block for a total of 120 berries per variety. Healthy, disease-free, unwounded berries with the pedicel intact were collected from each plant

throughout the individual blocks. They were randomly sampled from the top, middle, and bottom of clusters.

Once the berries were brought into the lab, they were cut away from the cluster, keeping the pedicel intact so no wounding occurred. They were soaked in a ten percent bleach solution for ten minutes to kill any pathogens and remove any pesticides from the vineyard. Afterwards, they were rinsed with deionized water and allowed to air dry (Wan et al., 2015). They were kept in sealed plastic containers with wet paper towels on top of tube racks to keep them organized (Figure 5).

The treatments included wounded, unwounded, and control. Ten of the berries were left unwounded, sprayed with the spore solution, and kept in separate containers. Ten berries were wounded in the right side, away from the rachis with a syringe needle. They were also sprayed with the spore solution and kept in separate containers. The control had wounded and unwounded berries and were sprayed with the same deionized water used to make the spore solution. Each berry was kept for six to twelve days and evaluated on a scale of zero to five disease severity rating (Figure 6). An overall incidence percentage was also recorded along with the location(s) of infection. The locations included on the pedicel, from the wound (on wounded berries), and elsewhere on the berries.

The percentage of incidence of *Botrytis cinerea* was calculated by recording the number of berries out of ten that were displaying signs of fungal infection and averaging the three replicates. Both wounded and unwounded were included. The severity classification was determined by assigning each individual berry a rating of 0 to 5 (Table 1) (Liu et al., 2003). The ratings ranged from Resistant to Susceptible.

Recording of Developmental Stages

Four important stages in a vine's annual cycle were recorded throughout the growing season. When 80% of the 12 vines in each variety had reached the correct physical requirements for the next growth stage, the date was recorded. Vines were monitored for a few weeks in advance before each stage for signs of the physical changes. When the new buds of the vines became exposed from the nodes from the previous year, bud break was recorded. This took place in mid-April. The vines' blooming dates were recorded in late May and early June. Veraison occurs when the grape berries change color. The green berries change to a clearer, more translucent color for the white grape varieties and purple or blue for the red grape varieties (Nail, 2015). This stage occurred in mid-August. Harvest was judged by several berry parameters to determine if they were ripe and the berries were collected in late September until the middle of October.

Evaluation of Berry Chemistry Parameters

There are three main parameters that growers use to judge if a berry is ripe and ready to harvest. These can also determine characteristics of the resulting wine. Berries from each variety were collected weekly when the vines were getting close to harvest to check the levels of the parameters.

To measure the Brix of the berries, 3-4 berries were crushed and the juice was poured onto the glass slide of a refractometer. A Reichert ABBE Mark II digital refractometer in the laboratory was used. The target Brix level was >20. The pH of the berry juice was measured with a Corning 430 pH meter. The target was 3.3-3.4. To

measure the TA, a burette was filled with a 0.1N NaOH solution. Three drops of 1% phenolphthalein indicator solution was added to a beaker with 300 ml of distilled water. Increments of 0.1N NaOH from the burette were added to the distilled water until a faint pink color was observed. The solution was stirred after each addition. After the pink color remained after stirring, the level of the NaOH at the meniscus in the burette was recorded. Five milliliters of room temperature juice was added to the distilled water. Increments of 0.1N NaOH were then added to the juice and water solution until the color was correct for the variety. A white variety should reach a faint pink color. A red variety will reach a non-green, pinkish-brown color. The volume of 0.1N NaOH used to reach the end point was recorded. The following calculation was used to determine the %Total Acidity (Wilker, 2002).

$$\text{Tartaric acid, g/100ml} = \frac{(V)\,(N)\,(75)\,(100)}{(1000)\,(v)} = \%TA$$

Variables:

V = volume of NaOH used to titrate to the ending color

N = normality of the NaOH (0.1N)

V = volume of the juice sample (5ml)

The target TA was 0.65-0.75. In some varieties, these levels were not reached, but if some of the parameters were met or disease pressure on the vines was high, then the berries were harvested.

Statistical Analysis

The experiment was a randomized complete block design analyzed with a multi-factorial analysis of variance (ANOVA). This model was used to test for statistical

significance of varietal and replication effects on the pruning weight, yield, and *Botrytis* incidence using a general linear model in SAS version 9.4 (SAS Institute Inc., Cary, NC). Fixed variables included block and post inoculation time. All effects were considered significant when $P < 0.05$. When F test showed significance, means were separated using Tukey's pairwise comparison.

RESULTS

From June to December, 2016 an evaluation of resistance against *Botrytis cinerea* on the seven new varieties (Figure 7) and the two parents, 'Norton' and 'Cabernet Sauvignon' had been observed, recorded, and analyzed. An evaluation of viticultural traits and berry chemistry had also been completed.

Evaluation of Resistance Against *Botrytis cinerea*

Leaf Evaluation. No reliable data resulted from the leaf evaluation. 'Norton' leaves die quickly once separated from the vine causing *Botrytis cinerea*, a necrotrophic fungus, to thrive upon the dead tissue. This characteristic caused the spread of *Botrytis* to be more severe on the 'Norton' leaves than the 'Cabernet Sauvignon' leaves. Under vineyard conditions the fungus usually only affects the berries, so evaluations were re-directed to concentrate on the fruit.

Incidence. No statistical difference was detected between the three replicates in the vineyard. The results showed that 'Norton' berries have an average of 39.1% incidence of *Botrytis* infection while 82.5% of 'Cabernet Sauvignon' berries were infected with *Botrytis*. The seven varieties' incidences fell in between (Table 2). New variety 'NC6' had the highest incidence of *Botrytis* infection of the varieties (68.3%) still lower than the incidence on 'Cabernet Sauvignon'. Variety 'NC28' had the lowest incidence of infection (50%). There was a significant varietal effect on the *Botrytis* incidence. 'NC28', 'NC60', and 'NC65' levels of resistance were all significantly

different from ‘Cabernet Sauvignon’. ‘NC6’ was the only variety significantly different from ‘Norton’ (Table 1).

Severity. ‘Norton’ was classified as Resistant with a rate of 0.57 on average and ‘Cabernet Sauvignon’ was classified as Moderately Susceptible with an average of 3.07. Once again, the varieties fell into the range of ratings between the two parents. The most resistant variety was ‘NC17’ with 1.2 (Moderately Resistant) and ‘CN21’ was the most susceptible with 2.7 (Tolerant). The classifications for all seven varieties and the two parents are presented in Table 3.

When combining the two parameters of evaluating resistance against *Botrytis* infection, ‘Norton’ and ‘Cabernet Sauvignon’ resistances were at the two extremes. The more resistant varieties are ‘NC17’ and ‘NC28’. The more susceptible varieties are two of the white varieties: ‘NC6’ and ‘CN21’.

Evaluation of Viticultural Traits

Growth Stages. Bud break was recorded in April, 2016. The dates ranged from April 8th with ‘NC28’ to April 14th with ‘Cabernet Sauvignon’. Bloom was observed in late May to early June. The variety of earliest blooming was ‘NC28’ with May 29th and the variety for latest blooming was ‘NC6’ with June 6th. Veraison occurred in mid-August. The variety for earliest veraison was ‘Cabernet Sauvignon’ on August 13th and the variety of the latest veraison was ‘NC17’ on August 21st. Harvest was completed on a weekly basis. The harvest date was determined after berry chemistry was measured weekly and parameters were considered optimum. The dates ranged from late September into mid-October. The first variety to be harvested was ‘NC6’ on September 21st and the

last to be harvested was 'NC43' on October 17th. A full list of all the specific dates for each of the varieties is presented in Table 4.

Berry Weight and Chemistry. The average berry weights were taken by weighing berries from several full clusters and they all ranged between 0.81-1.58 g. 'Cabernet Sauvignon' had the lowest berry weight and the highest berry weight recorded was for 'NC43'. The pH of all the varieties were very close to the target range of 3.3 to 3.4. The measured range was 3.15-3.39 with 'Cabernet Sauvignon' at the lowest and 'NC17' with the highest. The target Total Titratable Acidity (TA) range was 0.65-0.75 g/100 ml. The range for the varieties and parents was 0.57-0.76 except for 'NC65', which was much higher with 0.99. 'NC6' and 'NC28' were low with 0.58 and 0.57, respectively. The target Brix was >20. They ranged from 19.4-24.4. The only one that was lower than the target was 'Cabernet Sauvignon'. Data of the measurements of the seven and the two parents are presented in Table 5.

Pruning Weight and Yield. There was a significant varietal effect upon both pruning weight and yield (Table 6). 'Cabernet Sauvignon' had the lowest pruning weight and the yield was not measured because there were not enough clusters. The pruning weights varied from 0.25 to 0.76 kg. The highest pruning weights were collected from 'CN21' and 'NC6' with 0.76 kg and 0.75 kg, respectively. The variety with the lowest pruning weight was 'NC17'. 'Norton' had the highest yield with 6.4 kg. The yields of the varieties ranged from 0.85 to 5.97 kg. The highest yielding variety was 'NC6' and the lowest was 'NC60'.

DISCUSSION

After five years of completing preliminary evaluations on 100 progenies of 'Norton' and 'Cabernet Sauvignon', seven varieties were chosen for further evaluation. The seven varieties were planted in 2011 and allowed to grow for five years before comprehensive evaluations were initiated. In 2016, a disease resistance evaluation was performed on the varieties using the fungal pathogen *Botrytis cinerea* as a model pathogen since it can be isolated to obtain cultures by selecting a single hyphae or spore on the media. A viticultural evaluation was also completed by measuring pruning weight, yield, and berry chemistry parameters.

A total of twelve factors were recorded for each variety and used for comparison. These were also recorded for the parents as control traits for comparison. Three traits (yield, pruning weight, and disease incidence) were compared using ANOVA statistical analysis. A significant effect of the variety was observed on all three traits. Differences between the varieties and the parents also occurred in the assessed traits, both in the vine and the fruit.

With all evaluated traits in consideration, three varieties stand out. The first is the white variety 'NC6'. It has a high pruning weight, and a high yield. 'NC6' was the first variety to be harvested, indicating it is an early-ripening variety and likely not have problems with damaged fruit because of an early frost. It is one of the more susceptible varieties to *Botrytis cinerea*, but incidence is still 14% lower than the highly susceptible 'Cabernet Sauvignon'. It was classified as Tolerant using the severity rating scale (Table 3).

The second promising variety is ‘NC65’ which produces red berries. It has one of the lower *Botrytis* incidence rates and is classified Moderately Resistant by the severity rating. Its berry parameters also reached the targets for the berry harvest standards. The vine produces one of the lower amounts of pruning weight and is in the middle of the range of yield with 2.53 kg amongst the varieties. ‘NC65’ also has one of the latest bud breaks in the season compared to the other varieties (Tables 4 and 6), a trait that may protect the vine against a late spring frost.

The final promising variety, ‘NC17’, also produces red berries. Its *Botrytis* incidence rate is 58.3% (Table 1), 24% lower than ‘Cabernet Sauvignon’ and 19% higher than ‘Norton’. Using the severity rating, ‘NC17’ was classified as Moderately Resistant. Its growth stage dates of bud break, bloom, and harvest dates are in the middle of the range among the seven varieties. The berry parameters also reached the target ranges for pH, TA, and Brix. However, ‘NC17’ produced the lowest pruning weight, even lower than the parent ‘Cabernet Sauvignon’. As a result, the yield was also lower even though the average berry weight was good and the clusters look like the parent ‘Norton’. A full summary of the favorable and unfavorable traits of the seven varieties and the two parents can be found in Table 7.

During the last five years of growing the varieties, some have acquired undesirable traits that have decreased interest in them. For example, ‘CN21’ has developed a problem with its fruit set. This resulted in uneven clusters. Many berries were pollinated, but stopped developing, causing lower yields. Another example is ‘NC60’, which developed a susceptibility to anthracnose early in the season. The vine did recover, but left the clusters stunted and smaller, also lowering the yield. There are

currently no plans to include these vines into new vineyards. However, they will most likely remain part of this vineyard for future study.

In the future, the three promising varieties will need to be expanded to new vineyards in new locations for further research and testing under different climates. Wine will also need to be made from them and evaluated by a panel of judges to determine the wine quality as it is the final product made from the vines and the economic resource to produce the vines. More comparison should also be carried out on the vines by tracking the changes in the levels of the berry chemistry parameters as they near harvest. This could provide another way to compare the varieties to investigate how they develop similarly or differently from each other and the parents. The main factor that needs to be considered is simply time. Every year is a different mix of temperatures and weather conditions. With only one year of data, it is unknown which variety will be a good contender to become an officially released cultivar. Several more years of data would greatly increase the amount of information about these cultivars and truly separate out the best varieties for different climates.

Table 1. Severity ratings (0-5) and classification system. This was used for *Botrytis cinerea* infection of berries after 6 days post inoculation.

Rating	Classification
0-1	Resistant
1.01-2	Moderately Resistant
2.01-3	Tolerant
3.01-4	Moderately Susceptible
4.01-5	Susceptible

Table 2. The incidence rate of infection of *Botrytis cinerea* by variety.

Variety		*Botrytis* Incidence Rate (%)
NC6		68.3 ± 0.068ab
NC28		50.0 ± 0.068bc
NC43		62.5 ± 0.072abc
CN21		58.3 ± 0.079abc
NC17		58.3 ± 0.06abc
NC60		52.5 ± 0.059bc
NC65		55.8 ± 0.055bc
Cabernet Sauvignon		82.5 ± 0.062a
Norton		39.1 ± 0.064c
	df	ANOVA F value (and probability)
Variety	8	4.81
		(0.0001)
Wound	1	50.14
		(0.0001)

Column means (±Standard Error), within the experiment, that are not followed by the same letter are significantly different ($p<0.05$, Tukey's pairwise comparisons).

Table 3. Seven varieties' severity ratings. The average rating and classification of *Botrytis* infection on the berries of the seven varieties and the two parents.

Variety	Average Rating	Classification
NC6	2.03	Tolerant
NC28	1.70	Moderately Resistant
NC43	1.30	Moderately Resistant
CN21	2.70	Tolerant
NC17	1.20	Moderately Resistant
NC60	1.77	Moderately Resistant
NC65	1.97	Moderately Resistant
Cabernet Sauvignon	3.07	Moderately Susceptible
Norton	0.57	Resistant

Table 4. Dates (month-day of 2016) of developmental stages. The date was recorded when 80% or more of the vines for each variety reached that developmental growth stage.

Variety	Bud Break	Bloom	Veraison	Harvest
NC6	4-11	6-6	8-15	9-21
NC28	4-8	5-29	8-18	9-27
NC43	4-11	6-2	8-15	10-17
CN21	4-12	6-3	8-15	9-27
NC17	4-11	6-4	8-21	10-3
NC60	4-9	6-2	8-17	10-10
NC65	4-13	6-3	8-18	10-10
Cabernet Sauvignon	4-14	6-4	8-13	10-3
Norton	4-13	6-3	8-15	10-3

Table 5. Berry characteristic measurements before harvest in 2016.

Variety	Average Berry Weight (g)	pH	Total Titratable Acidity (TA) (g/100 mL)	Brix (°Bx)
NC6	1.28	3.24	0.58	21.90
NC28	1.32	3.30	0.57	23.90
NC43	1.58	3.19	0.65	21.00
CN21	1.09	3.21	0.67	24.40
NC17	1.29	3.39	0.75	23.80
NC60	1.34	3.35	0.73	22.50
NC65	1.20	3.25	0.99	20.20
Cabernet Sauvignon	0.81	3.15	0.76	19.40
Norton	1.24	3.37	0.64	24.10

Table 6. Yield and pruning weight. Yield and pruning weights collected for the seven varieties and two parents in 2016.

Variety		Yield per vine (kg)	Pruning Weight per vine (kg)
NC6		5.97 ± 1.72a	0.75 ± 0.22ab
NC28		2.65 ± 0.77bc	0.53 ± 0.15abcd
NC43		1.66 ± 0.26c	0.69 ± 0.21abc
CN21		3.68 ± 1.06b	0.76 ± 0.23a
NC17		1.90 ± 0.55bc	0.25 ± 0.07de
NC60		0.85 ± 0.25c	0.37 ± 0.11bcde
NC65		2.53 ± 0.39bc	0.37 ± 0.10cde
Cabernet Sauvignon		0*	0.32 ± 0.11e
Norton		6.40 ± 1.85a	0.54 ± 0.16abcd
	df	ANOVA F value (and probability	ANOVA F value (and probability)
Variety	8	17.76	6.81
		(0.0001)	(0.0001)
Block	2	1.92	1.2
		(0.1537)	(0.3063)

*Cabernet Sauvignon was not harvested and not included in the analysis.
Column means (±Standard Error), within the experiment, that are not followed by the same letter are significantly different ($p<0.05$, Tukey's pairwise comparisons).

Table 7. Summary of assessed traits for the seven varieties and two parents.

Variety	*Botrytis* Incidence	*Botrytis* Severity	pH	Total Titratable Acidity	Brix	Pruning Weight	Yield
NC6	-	+	+	+	+	-	+
NC65	+	+	+	+	+	+	-
NC17	+	+	+	+	+	-	-
NC28	+	+	+	+	+	+	-
NC43	-	+	+	+	-	+	-
CN21	+	-	+	+	+	-	+
NC60	+	+	+	+	+	+	-
Cabernet Sauvignon	-	-	+	+	-	+	-
Norton	+	+	+	+	+	+	+

+ Favorable Trait
- Unfavorable Trait

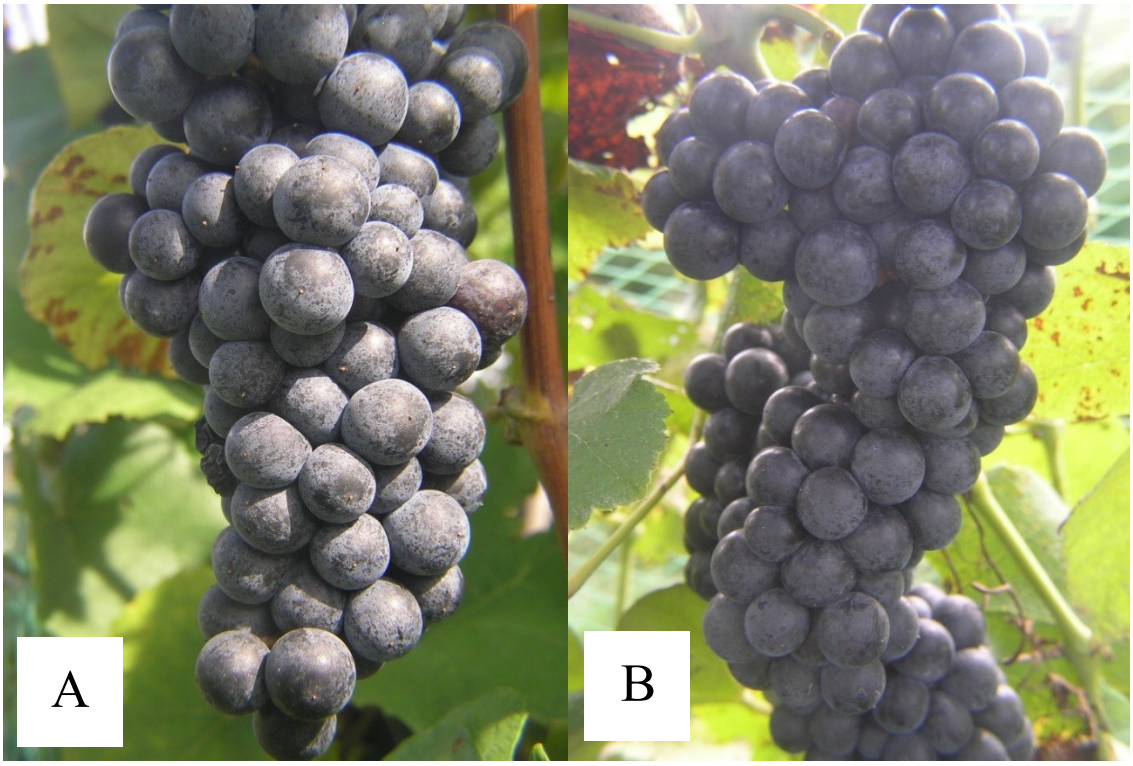


Figure 1. Parent clusters. Images of parents' clusters taken on September 8, 2016 in the test vineyard used for this study. (A) 'Norton'. (B) 'Cabernet Sauvignon' (Photos by Dr. Wenping Qiu).

Vine	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Row 5	NC28				Norton				NC65				Cabernet				NC60				NC6				CN21				NC28				NC43			
Row 6	NC17				NC60				NC43				Norton				NC65				CN21				NC60				NC65				NC6			
Row 7	NC6				CN21				Cabernet				NC28				NC17				NC17				Norton				NC17				Cabernet			

st plot vineyard layout. The three different colors represent the three randomly distributed replicate blocks. Each box name of the variety planted there and contains four vines of that variety.

Figure 3. *Botrytis* bunch rot infected ‘Chardonel’ cluster collected from the greenhouse. *Botrytis* was collected from this cluster, purified, and used for the inoculations.

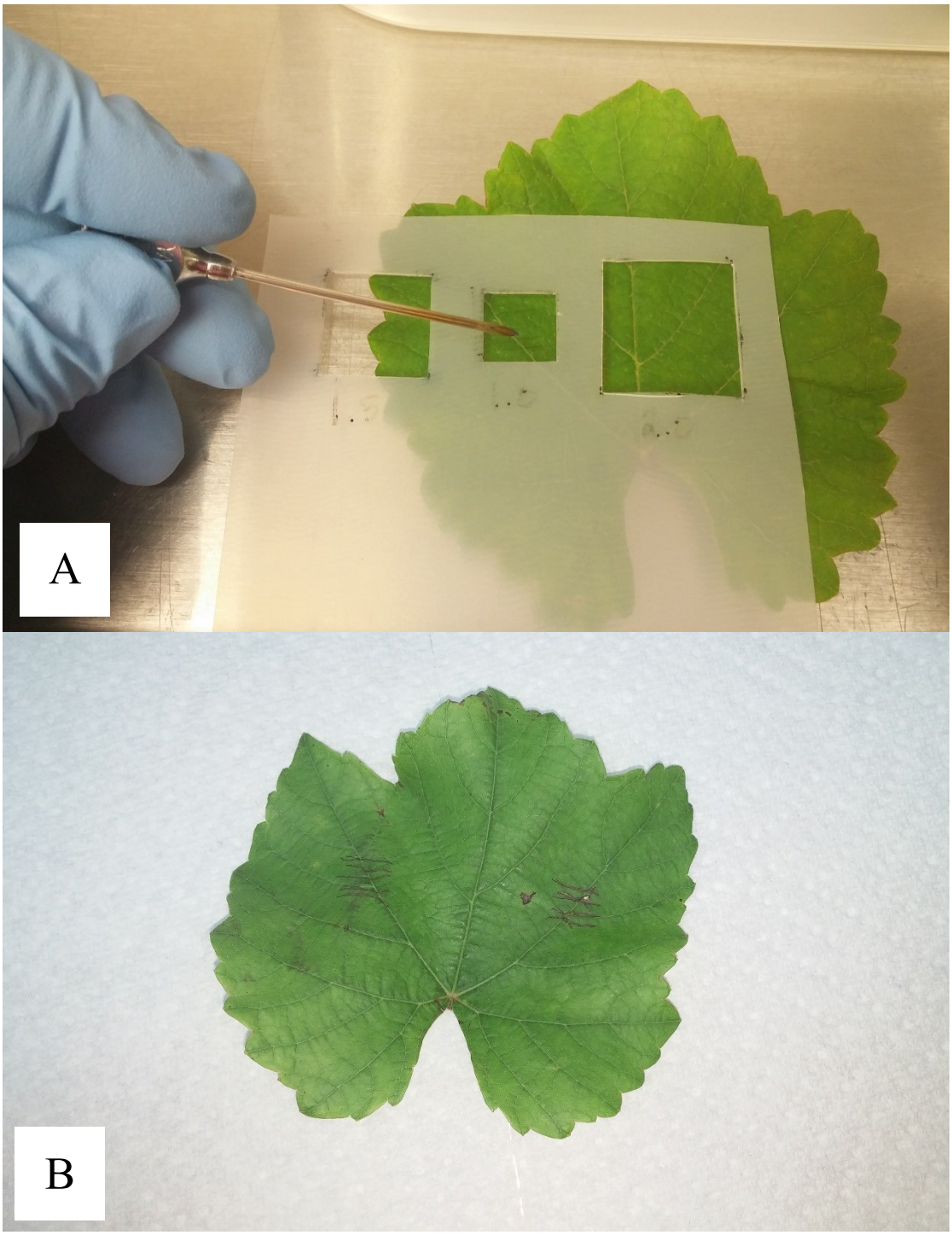


Figure 4. Leaf wounding. (A) Each leaf was wounded with four scratches to fill the middle square (1cm x 1cm). (B) This was done twice on each leaf, one wound on each half of the leaf.

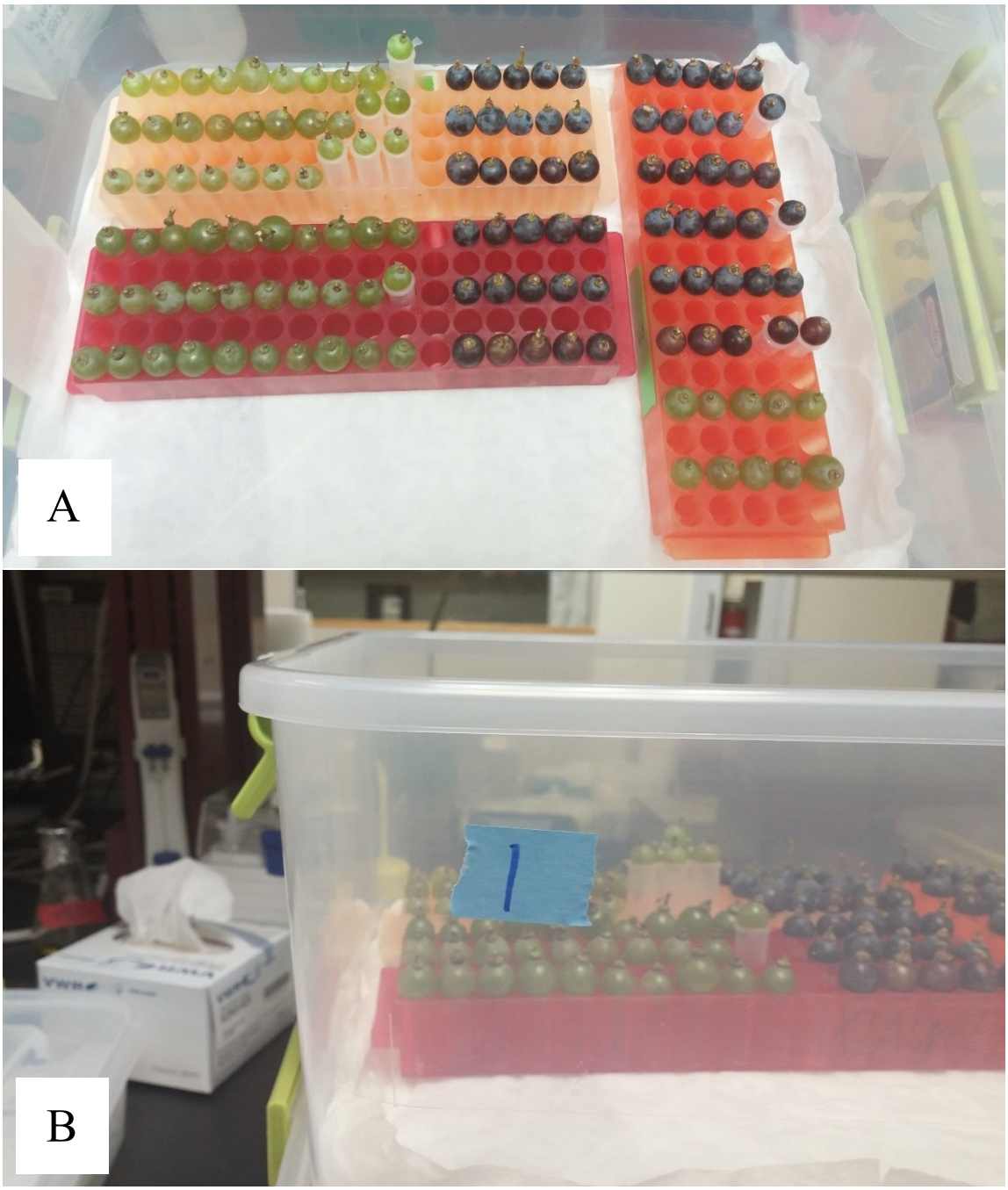
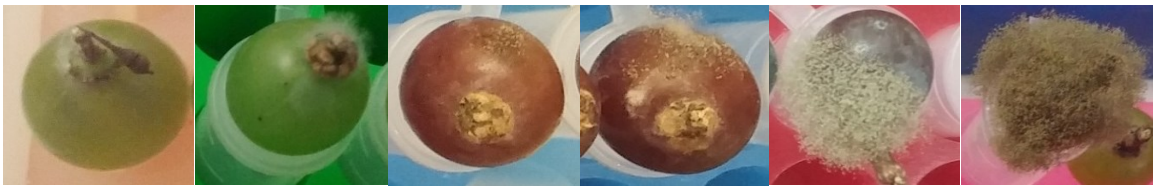


Figure 5. Assessment of berry resistance to *Botrytis cinerea*. (A) Tube racks were used to keep the berries organized for observation. (B) Wet paper towels were placed on the bottom and the boxes were sealed to maintain 100% humidity.



0 No infection	1 Infection contained to one area	2 Infection contained to one area on exterior, but berry changed color of interior flesh	3 Infection covers one-third of exterior of the berry	4 Infection covers about half of the exterior of the berry	5 Infection covers the entire berry

Figure 6. A rating index for *Botrytis cinerea* severity.

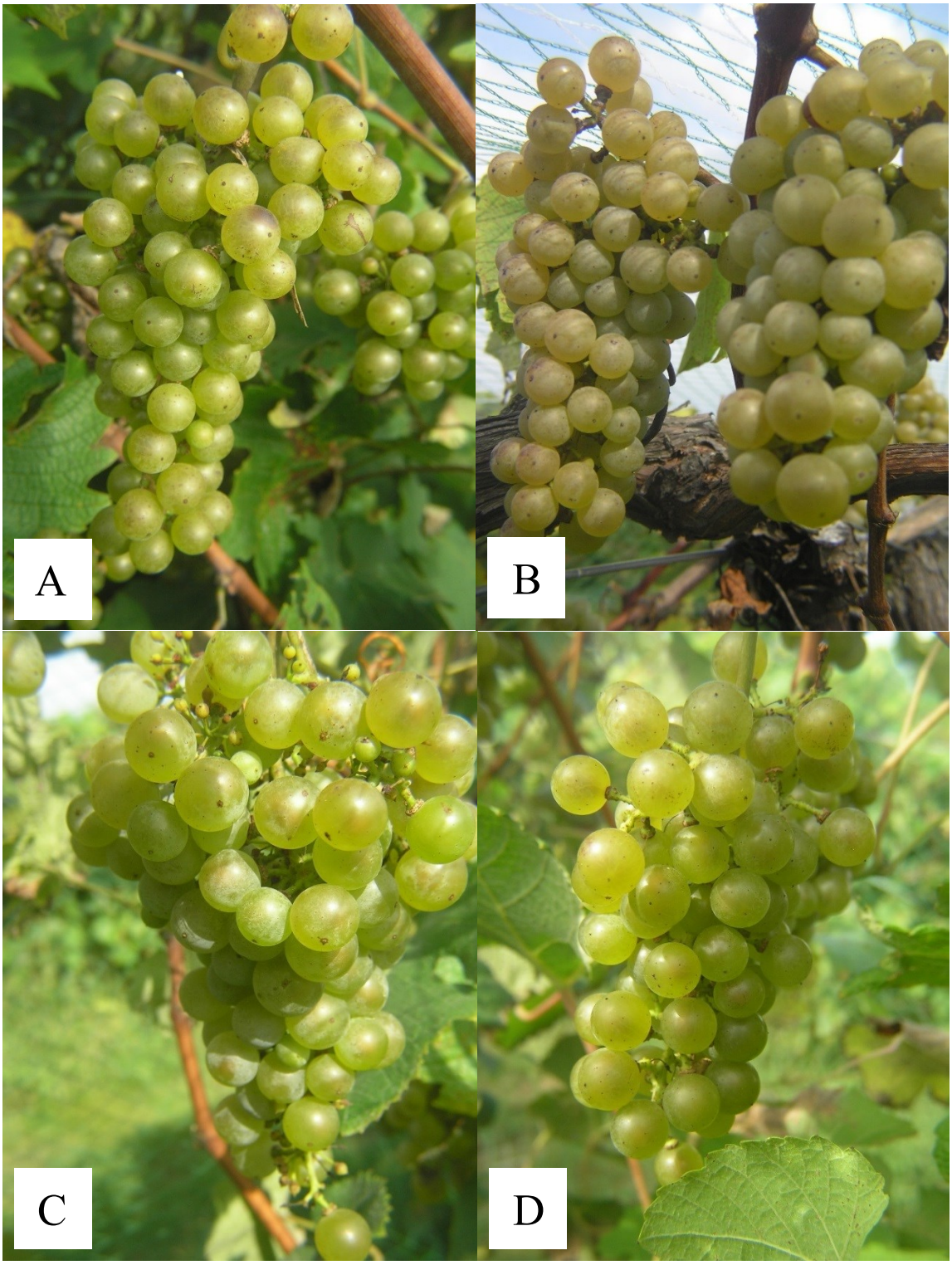


Figure 7. Clusters of the seven new varieties. (A) NC6, (B) NC28, (C) NC43, (D) CN21, (E) NC17, (F) NC60, (G) NC65 (Photos by Dr. Wenping Qiu). Continued on next page.

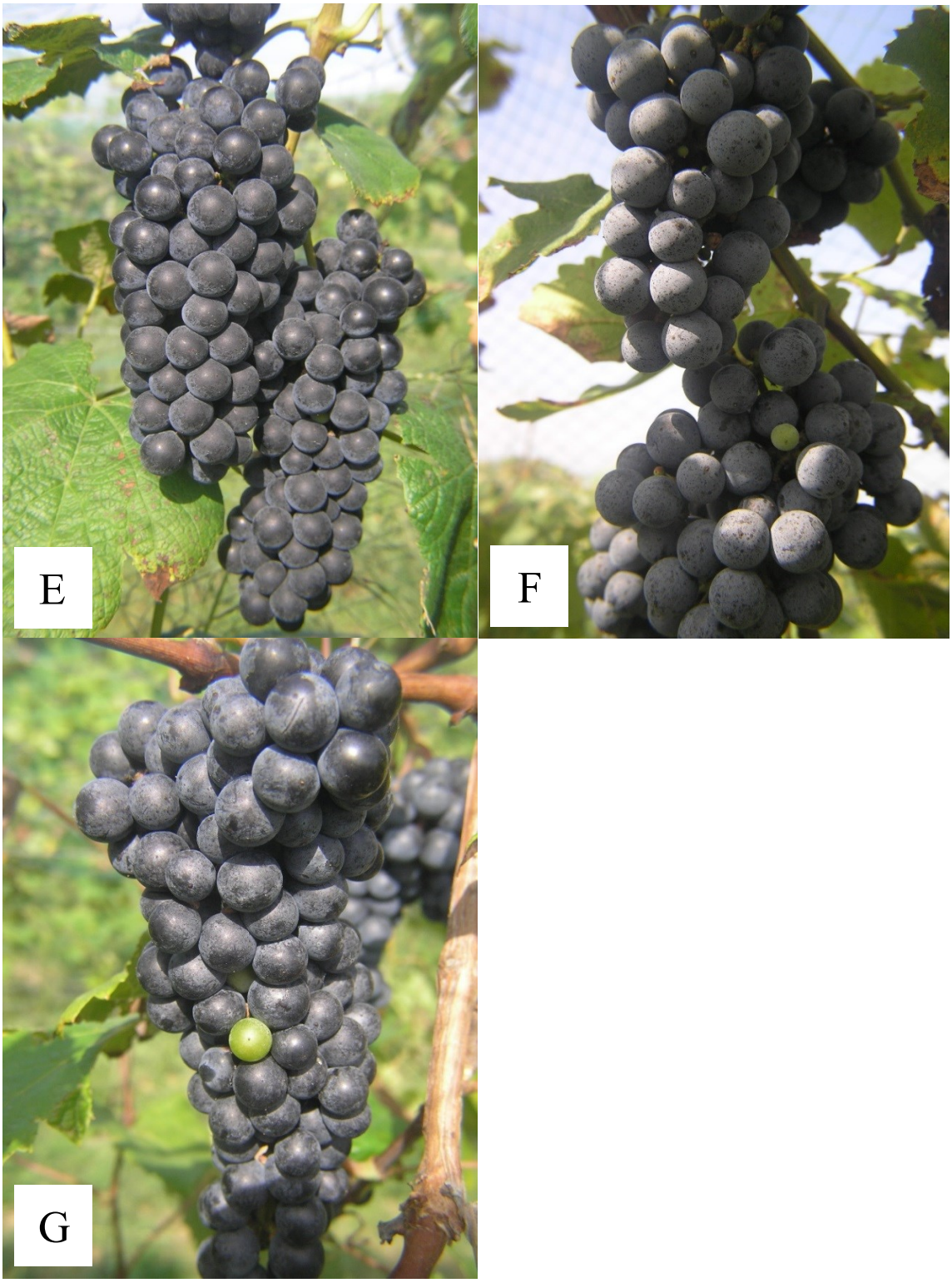


Figure 7 continued. Clusters of the seven new varieties. (A) NC6, (B) NC28, (C) NC43, (D) CN21, (E) NC17, (F) NC60, (G) NC65 (Photos by Dr. Wenping Qiu).

CHAPTER 2: A STUDY OF *GRAPEVINE VEIN CLEARING VIRUS* IN NATIVE VITACEAE PLANTS

INTRODUCTION

Characterization of an Unidentified Virus-like Disease

Beginning in 2004, vineyard managers began asking for help to find the cause of a severe disease with an unknown pathogen that was plaguing their vineyards. At first look, the disease seemed to be the result of a virus. Samples from infected vines were collected and tested for viruses that are known to infect grapevines. The samples were tested for *Grapevine leafroll associated virus 3* and *Tomato ringspot virus* by enzyme-linked immunosorbent assay (ELISA). The diseased vine tested negative for both viruses. A commercial testing also showed the symptomatic tissues were negative for *Arabis mosaic virus*, *Tobacco ringspot virus*, and *Peach rosette mosaic virus*. Reverse transcription polymerase chain reaction (RT-PCR) also verified the samples were negative for *Grapevine leafroll associated virus 3*, *Grapevine fanleaf virus*, *Tomato ringspot virus*, and *Arabis mosaic virus* (Qiu et al., 2007).

The disease symptoms included short and zig-zagged internodes, cupped and deformed leaves, small clusters, deformed berries, vigor decline of the vine, and chlorosis along leaf veins causing a translucent effect when leaves were held against sunlight (Figure 8) (Qiu et al., 2007). The chlorosis begins as a narrow strip of tissue along major and minor veins of the young leaves. As the leaves mature, the chlorosis develops into a mosaic pattern. This is the signature symptom of the disease (Zhang et al., 2011). Over

the years of observing infected vineyards, vines would decline and eventually die causing economic losses to vineyard owners. However, the pathogen remained elusive until a new technology for discovering viruses was applied in 2011.

Discovery of *Grapevine vein clearing virus*

In 2011, small RNAs from two vines were extracted and used to construct two cDNA libraries. One vine was symptomatic and the other was asymptomatic. Deep sequencing of these libraries identified small RNAs aligning with genomes of viruses in the genus *Badnavirus* and the family *Caulimoviridae*. However, complete genomes could not be assembled. Polymerase chain reaction (PCR) was used to amplify genomic fragments of the new viral DNA, which allowed the sequencing and assembly of the first known DNA virus to be found in grapevines. The new DNA virus was named *Grapevine vein clearing virus* (GVCV) because of its association with the signature vein-clearing symptom (Zhang et al., 2011). This genome became the reference genome known as GVCV-CHA for the future study of GVCV isolates. The genome-sense strand of the double-stranded DNA molecule contains three open reading frames (ORFs) with the most variable region within ORFII (Figure 9) (Guo et al., 2014, Beach et al., 2016). ORFIII encodes domains for a reverse transcriptase, RNase H, and DNA-binding zinc-finger protein (Guo et al., 2014). Transcription is initiated at nt 7,571 and terminated at nt 7,676 (Zhang et al., 2015).

In the last decade, GVCV has been tested and confirmed in multiple grape cultivars including ‘Chardonnay’, ‘Chardonel’, ‘Cabernet Sauvignon’, ‘Vidal Blanc’, ‘Cabernet Franc’, ‘Riesling’, ‘Traminette’, ‘Cayuga White’, and ‘Corot Noir’ (Zhang et

al., 2011, Guo et al., 2014). GVCV has only been found in vineyards in the Midwest region in Missouri, Arkansas, Illinois, and Indiana. Some cultivars have also been discovered to be resistant to GVCV including 'Chambourcin' and 'Norton' (Guo et al., 2014, Qiu, 2017).

Current Status of GVCV

During the last five years, the search for sources of GVCV has ensued. An interesting phenomenon was discovered in 2012 when wild native grapevines with mild symptoms were observed and tested positive for GVCV. The plants were identified as *Vitis rupestris* and became the next two GVCV isolates, GVCV-VRU1 and GVCV-VRU2, to be sequenced (Beach et al., 2016). Another native vine that was found to be infected with GVCV was *Ampelopsis cordata* in 2014 (Petersen, 2016). *V. rupestris* shares the same genus as cultivated varieties in vineyards and *A. cordata* shares the same family: Vitaceae. In 2016 two more GVCV genomes from separate *A. cordata* plants were sequenced and became isolates GVCV-AMP1 and GVCV-AMP2. All five isolates range from 7,726-7,765 bp in length and vary from 91.6-93.2 percent nucleotide identity (Petersen, 2016). Also GVCV-VRU1, GVCV-AMP1, and GVCV-AMP2 contain a 9bp insert in the ORFII region when compared to the GVCV-CHA reference genome and GVCV-VRU2. Both *V. rupestris* and *A. cordata* have a natural habitat range that overlaps with commercial vineyards in the Midwest.

In 2016, a survey of symptomatic and asymptomatic *A. cordata* in the wild throughout seven regions within three states was completed. Five of the regions are official American Viticultural Areas (AVAs) designated by the Alcohol and Tobacco Tax

and Trade Bureau (TTB). The regions were Ozark Mountain and Highlands AVA, Hermann AVA (MO), Augusta AVA (MO), Arkansas Mountain AVA (AR), and Altus AVA (AR). The other two regions were Little Rock, AR and Hinton, OK. 35 samples tested positive for GVCV from a total of 113 samples. The survey indicates a 31% incidence rate of GVCV in native wild *A. cordata* species that are growing in proximity to cultivated grapevines of Midwest vineyards (Qiu, 2017). The wild native grapevines could be a reservoir of GVCV.

Following the survey, the 35 positive samples plus the previously sequenced genomes, for a total of 48 ORFII regions, were combined into another study to determine the phylogeny and to discover a geographic pattern of GVCV's spread (Qiu, 2017).

Continued Study of Native Species and GVCV Relationship

To continue the study of the relationship between wild native species and GVCV, I completed a survey of GVCV in native *Vitis* species in the National Plant Germplasm Collection. This collection contained 380 samples representing 31 wild native species from regions around the globe. The collection contained DNA samples taken from live plants transplanted in Geneva, NY and Davis, CA.

In a related study, a cultivated symptomatic 'Chardonel' vine was sampled along with an *A. cordata* vine located about ten feet away along a fence surrounding the vineyard. Both plants tested positive for GVCV and the ORFII regions were sequenced (Qiu, 2017). The nucleotide sequences in the ORFII of the GVCV isolates are 100% identical. Therefore, I sequenced the genomes of both isolates to investigate the

relationship between them to discover a potential route of spread from a wild plant to a cultivated grapevine or vice versa.

MATERIALS AND METHODS

Materials

In a survey of the National Plant Germplasm Collection, 380 samples were tested for *Grapevine vein clearing virus*. The samples were collected from around the world and included 31 species from the *Vitis* genus (Table 8). Three primer sets were used to perform a triplex polymerase chain reaction (PCR) to test each sample. One set generated an 835 bp amplicon, one generated a 442 bp amplicon, and one generated a 105 bp amplicon that was used to determine the presence of grapevine DNA.

For sequencing the genomes of Vit16-25IIIa and Amp16-3IIIa isolates, the original plant tissues were collected from a cultivated 'Chardonel' vine and a wild *Ampelopsis cordata* vine in a vineyard in Coffman, MO. These were also tested using the triplex PCR primer sets.

Development of a Triplex Polymerase Chain Reaction Assay

The DNA of 380 samples of native *Vitis* species was sent to the laboratory by Dr. Jason Londo from the USDA-Genetics Unit in Geneva, New York. A PCR assay was conducted on each sample. In the past, various primer sets were used to test samples for GVCV. The most common was a duplex PCR using a primer set designed for the GVCV genome that would create an 835 bp amplicon and another primer set that identified 16S ribosomal RNA genes (Petersen, 2016). The 835 primers were 1101F (5'-CTGAAAGGTAGATGTCCACG-3') and 1935R (5'-TCGGTGTAGCACTTGTATTCT-3'). The 16S forward (5'-TGCTTAACACATGCAAGTCGGA-3') and reverse (5'-

AGCCGTTTCCAGCTGTTGTTC-3') primer set was used to test the quality of DNA extracted from each sample (Krenz et al., 2014). Another PCR assay used was a triplex reaction using the 835 bp amplicon primers, the 16S ribosomal RNA primers, and a set of primers used to generate an amplicon of 246 bp length. The 246 primers were 4142F (5'-GTAAACCTCATGACTCTCATG-3') and 4387R (5'-CTTCTCCTTCAGAAATTGAGCAGAT-3'). However, this triplex would produce inconsistent results.

Several combinations of primers at different temperatures were tested. A satisfactory PCR triplex was developed by adding a primer set that generated an amplicon of 442 bp to the duplex PCR that produced GVCV-specific 835 bp and grapevine 16S rRNA DNA fragments. The primers used for the 442 bp amplicon were 4363F (5'-ATCTGCTCAATTTCTGAAGGAGAAG-3') and 4804R (5'-GGAATGCATTGTGCTCGTAG-3'). A Veriflux PCR protocol was used to discover the optimum annealing temperature for the three sets of primers. The PCR master mix consisted of all necessary reagents shown in Table 9. A total of twelve samples were set up: 6 with positive DNA controls and 6 with no DNA added to the master mix (negative controls). An Applied Biosystems Veriti 96 well thermocycler was used to complete the temperature cycles. The thermocycler was set on a veriflux option to find the best annealing temperature for the combined three sets of primers for the PCR triplex (Table 10). The annealing temperatures used were 53°C, 55°C, 57°C, 59°C, 61°C, and 63°C. After the thermocycler program was completed, 10µL of each reaction were loaded into a GelRed-stained 1% agarose gel. The gel was electrophoresed at 100 volts in a 1X Tris-

borate EDTA buffer for 40 minutes. After viewing the gel under UV light, 55°C was decided the optimum annealing temperature for the new PCR triplex.

Once the new PCR was developed, the 380 germplasm samples were subjected to this assay. Each sample's DNA concentration was also recorded using a Thermo Scientific NanoDrop 2000 spectrophotometer and the quality was assessed by measuring DNA at 260/280 nm. The minimum testable DNA concentration was 10 ng/µL. Once the DNA quality was recorded, the new PCR triplex assay was used to test the 380 samples for GVCV-specific fragments. A total of 40 samples were tested each assay. The samples were loaded into two GelRed-stained 1% agarose gels, electrophoresed, viewed under UV light, and individual samples' results were recorded.

Sequencing the ORFII Region of GVCV in *A. cordata* and 'Chardonel'

Collection and Transport of Samples. Samples were collected for a survey of GVCV in the native species *A. cordata* in Coffman, MO in June of 2016. A cultivated Chardonel grapevine with symptoms of GVCV was noticed in close proximity to a symptomatic *A. cordata* vine. Both plants were sampled by collecting 3-4 young leaves. The leaves were wrapped in wet paper towels and placed in labeled plastic bags on ice inside a cooler until they were brought back to the laboratory. The two samples were labeled Vit16-25IIIa and Amp16-3IIIa. Once in the laboratory, three samples were taken of the leaves, each weighing 130 mg. The samples were wrapped in foil, labeled, and frozen in liquid nitrogen and stored in a -80°C freezer.

Extraction of DNA and GVCV Triplex PCR. The samples were taken out of the -80°C freezer and immediately dropped into liquid nitrogen. The sample was

unwrapped and dropped directly into a mortar with liquid nitrogen and ground with a pestle. The fine powder was scraped and put into a 2.0 ml tube for DNA extraction. A Qiagen DNeasy Plant Mini Kit was used to extract the DNA from the samples by following the supplier's protocol. 15 µL of autoclaved deionized water was used to elute the DNA from the silica filter. The DNA was then tested for quality and quantified by a Thermo Scientific NanoDrop 2000 spectrophotometer. The concentration was adjusted to be 10 ng/µL for each sample. The triplex PCR was used to test the two samples for GVCV. Both samples tested positive for GVCV.

Isolation, Sequencing, and Comparison of ORFII Region. As part of the survey, all positive samples' ORFIIs were isolated and amplified. High-fidelity PCR using Platinum Taq DNA Polymerase was implemented to extract the ORFII region of the GVCV isolate of each sample. A 672 bp amplicon containing the ORFII of both samples was generated with a PCR assay using primers 963F (5'-TCCATCACAGATCTAACGGCA-3') and 1634R (5'-CAAGGTAGCGGGCACGAG-3'). The master mix reagents and concentrations are shown in Table 11 and the thermal cycler protocol is shown in Table 12.

Once the thermal cycler program was completed, 5 µL of 4X loading dye was added to each 20 µL reaction and mixed by pipetting. 15 µL of the mixture was then loaded into a GelRed-stained 1% agarose gel. The gel was electrophoresed at 100 volts for 40 minutes in a 1X Tris-borate EDTA buffer. The gel was then viewed under UV light and the fragment sizes were verified using a 1KB Plus Ladder. The bands in the gel were cut out, placed in new 2.0 ml tubes, and the GVCV amplicons were extracted and purified from the gel (Vogelstein & Gillespie, 1979). To extract the DNA from the gel, a

Qiagen MinElute Gel Extraction kit was used following the Qiagen protocol. 15 µL of autoclaved deionized water was used to elute the purified DNA. The DNA concentration and quality was measured using a Thermo Scientific NanoDrop 2000 spectrophotometer.

The PCR-amplified DNA fragments were sequenced using the enzymatic dideoxy chain-termination method called Sanger sequencing (Sanger & Coulson, 1975) at the Nevada Genomics Center, University of Nevada in Reno, Nevada. Each sample had at least 5 µL of total volume and contained at least 250 ng of DNA mixed with 1 µL of 10 µM primer and autoclaved deionized water. The same forward and reverse primers used in the high-fidelity PCR were used in sequencing and two tubes were prepared for each sample: one with the 963F forward primer and the other with the 1634R reverse primer.

When the results of the sequencing were available on-line, they were downloaded as chromatographs and viewed using the software Codon Code Aligner. Each nucleotide shows a Phred score. This is a quality measurement that assigns an error probability to each base as it is called by its peak during the sequencing process. In Codon Code Aligner, if a nucleotide is below a 30 Phred score, it is colored a light lime green and if one is scored below 20, a darker lime green color. Nucleotides with a Phred score of less than 20 were trimmed, leaving only high quality reads for the final assembly of the ORFII region of each sample. The score 20 was chosen because it indicates 99% probability that the base was called correctly.

Sequencing of GVCV Genome in *A. cordata* and 'Chardonel' Isolates

Separation of Genome into Three Overlapping Fragments. The genome of a GVCV isolate is usually ~7,750 bp in length. To increase the speed and accuracy of sequencing, the genome is broken into three overlapping fragments (Chao et al., 1983). The same procedure used for isolating ORFII was used for the three fragments. PCR with High Fidelity PlatinumTaq polymerase was used with a different thermocycler program (Tables 13 and 14) to allow time for the long length of the fragments. The primers for the Fragment I amplicon were 988F (5'-ACCTAAGCCGATTGAAGCAG-3') and 4387R (5'-CTTCTCCTTCAGAAATTGAGCAGAT-3'). The primers for the Fragment II amplicon were 4142F (5'-GTAAACCTCATGACTCTCATG-3') and 6795R (5'-GCTGGCGTAAGCACAGATTC-3'). The primers for the Fragment III amplicon were 6666F (5'-ACTTCCTCCACCCCACGCAGTTATC-3') and 1935R (5'-TCGGTGTAGCACTTGTATTCT-3'). After the thermocycler protocol was completed, the samples and their resulting fragment sizes were verified on an agarose gel as described in the previous section. Fragment I had ~3,400 bp, Fragment II had ~2,650 bp, and Fragment III had ~3,000 bp (Figure 10). Once the sizes were confirmed, the DNA was extracted from the gel as previously described.

Inserting Fragments into TOPO Vector. The insertion of the fragments into a vector plasmid was completed in the same day as the high-fidelity PCR in order to use fresh DNA for increasing the efficiency of cloning a DNA fragment. The Invitrogen pCR8/GW/TOPO TA Cloning kit was used to insert each fragment into separate TOPO vectors. These vectors were used because they accept a DNA fragment that has an A-overhang added during PCR with the Platinum Taq polymerase. This is because of an A-

overhang on the amplified product and overhanging T nucleotides in the vector on the TOPO cloning site. The vector also contains GW1 and GW2 priming sites that flank the DNA fragment insertion site and a spectinomycin resistance gene. The vector with the DNA fragment inside creates a recombinant plasmid that can be transformed into *E. coli* bacteria for cloning (Imanaka & Aiba, 1981).

Bacterial Transformation and Cloning. The recombinant plasmid, including one of the three fragments, was transformed into One Shot Competent *E. coli* bacteria for cloning. The transformation was completed by heat-shocking the bacteria that were then grown in super optimal broth with catabolite repression (SOC) for one hour. The bacteria were spread onto two room temperature Luria-Bertani agar plates containing 100 µg/mL spectinomycin in a sterile environment. One plate was spread with 40 µL of bacteria and the other with 90 µL. The plates were kept in a 37°C incubator for 24 hours. The spectinomycin was used to select for transformed bacteria. If the bacteria were not successfully transformed, they would not contain the spectinomycin resistance gene inside the TOPO vector and would die. This allowed the growth of only the bacteria containing the recombinant plasmid. As the bacteria grew over a 24-hour incubation period, the viral DNA fragments inside were multiplied exponentially.

After the 24 hours, two colonies of transformed bacteria were chosen and labeled on the plate. Half of the chosen colonies were taken and transferred to a tube of Luria-Bertani broth and grown in the 37°C incubator for 14 hours. After the broth turned cloudy, 2ml were collected and centrifuged to separate the bacteria from the broth. Plasmid DNA was extracted from the bacteria using the Qiagen QIAprep Spin Miniprep kit. The DNA was eluted using 50 µL of autoclaved deionized water. The quality and

concentration was analyzed using a Thermo Scientific NanoDrop 2000 spectrophotometer.

Following extraction, the recombinant plasmid DNA was tested for the presence of the viral DNA fragments using PCR with GoTaq polymerase. The reagents used are shown in Table 15 and the thermal cycler program was the same as the high fidelity PCR because of the long fragment length. The initial primer sets for generating the corresponding DNA fragments were used for testing the presence of the target DNA fragments in the recombinant plasmid. The lengths of the fragments were verified with UV light on a GelRed-stained 1% agarose gel.

Sequencing and Primer Walking. The recombinant DNA plasmid samples were sent to the Nevada Genomics Center following the same protocol as previously described. The primers used for the first sequencing were GW1 and GW2 whose sequences are on the TOPO vector that flank the inserted viral DNA fragment. Two samples were sent for each fragment, one for each of the two primers. Each time a sample is sequenced, an average of 500 nucleotides are sequenced. Since each fragment is 2,600-3,400 nucleotides long, this primer set was only the first of many. About 70 GVCV-specific primers have already been designed to sequence GVCV isolates. These primers covered most of the genome. The remaining gaps were sequenced by designing primers complementary to sequences close to the ends of the sequenced reads. This process is called primer walking (Kieleczawa et al., 1992). The primers used for sequencing are listed in Tables 16 and 17.

RESULTS

Survey of *Vitis* Species in the National Plant Germplasm Collection

When the triplex PCR assay was applied to the 380 *Vitis* species' DNA samples and the PCR product was visualized on an agarose gel, the images were all the same (Figure 11). All 380 samples tested negative for GVCV-specific viral DNA.

Sequence Analysis of Two ORFII Regions

When the resulting sequences from samples Vit16-25IIIa ('Chardonel') and Amp16-3IIIa (*A. cordata*) were received from Nevada Genomics center, they were compared and trimmed using Codon Code Aligner. This analysis showed the ORFII regions of the two GVCV isolates were a 100% match (Figure 12). This result led to the sequencing of the entire GVCV genome of both isolates for further study.

Sequence Analysis of GVCV Genomes in *A. cordata* and 'Chardonel' Isolates

An attempt was made to separate each genome into three overlapping fragments and acquire each fragment by using a high-fidelity PCR assay. The gel result is presented in Figure 13. Fragments II for both isolates had the correct sizes when compared with the 1KB Plus ladder. Fragment III from sample Amp16-3IIIa was also acquired. Fragment III from sample Vit16-25IIIa, however, was not successful on this gel, but was captured in a later gel. Fragments I for both isolates were thought to be successful on this gel, however, after sequencing part of both, many errors occurred so these were unsuccessful as well. After gel extraction, cloning, and plasmid DNA extraction as previously described in

Materials and Methods, the plasmid DNA samples were sent to Nevada Genomics Center for Sanger sequencing.

From December, 2016 to March, 2017 samples of the plasmid DNA from the two GVCV isolates were sent for sequencing, trimmed upon their return, and compared. The returned samples were aligned and new primers were chosen to sequence areas of the three fragments that contained gaps (Tables 16 and 17). The Vit16-25IIIa isolate is currently 60.2% sequenced, mainly in Fragments II and III along with the ORFII region in the overlapping area of Fragments I and III. The Amp16-3IIIa isolate is currently 66.2% sequenced in similar areas as Vit16-25IIIa.

A total of 4,694 nucleotides have been sequenced and compared between the two isolates as these areas have been successfully sequenced in both samples (Figures 14-18). Of the nearly 4,700 nucleotides, only twelve nucleotides differ from each other. Of the twelve nucleotide changes, only four cause amino acid changes. The other eight still code for the same amino acid as the other isolate. With the twelve differing nucleotides, the areas of the genome that are currently sequenced match with 99.7% identity. The amino acid sequences of the two isolates match 99.9%.

DISCUSSION

The first study in this chapter allowed us to understand GVCV from a variety of angles. First, the National Plant Germplasm survey provided us with new insights about the origin of GVCV. The 380 native *Vitis* samples were collected world-wide for over a century, yet none of them contained any GVCV-specific DNA. This suggests that the spread of this virus is likely a recent, localized event taking place in the Midwest region of the United States. This information assures us that our germplasm repositories have not been infected with the virus and can safely be used for research or breeding purposes without spreading GVCV.

In the second study, we learned more about how GVCV spreads. In the previous analysis of ORFII regions, the highest percentage identity matching between an *A. cordata* isolate and a *Vitis* isolate was 97% (Qiu, 2017). Comparing the five GVCV genomes that have been completely sequenced, the highest similarity was 93.9% (Petersen, 2016). The two isolates in the current study, Vit16-25IIIa and Amp16-3IIIa were the first to be extracted from two plant genera and share 100% identity in the ORFII region, indicating that they are likely the same isolate.

We then formulated the hypothesis that since the ORFII region is identical, then the entire genome of the two isolates shall be identical. The previously sequenced isolates of GVCV share at the most, 93.2% of their identity (Petersen, 2016). The 60% of the two genomes sequenced at this point currently have a matching identity of 99.7%. This indicates most likely the same isolate is infecting two plants of *Vitis* and *Ampelopsis* species that are in the same family. The result provides crucial evidence to support the

conclusion that GVCV spreads from the wild *A. cordata* plant to a cultivated grapevine in a vineyard or vice versa.

By identifying the route a virus spreads, we can find ways to stop the transmission from plant to plant and contain the virus to limited regions. If wild, native *A. cordata* plants are a reservoir for this virus, this could be how the virus comes into close proximity of cultivated vineyards. Vineyard managers could spray the edges of their vineyards to keep *A. cordata* from growing there, and reduce the infection of cultivated vines by GVCV.

In the future, these two isolates will be completely sequenced and compared to the entire genomes of the previously sequenced five isolates. They can also be analyzed phylogenically to generate information about the spread of and mutations of GVCV. From a broader perspective, the two new *Badnavirus* isolates are new members of the *Caulimoviridae* family.

Table 8. *Vitis* species in germplasm survey. *Vitis* species included in the germplasm survey from the National Plant Germplasm Collection in Geneva, NY and Davis, CA including the number of samples and native region for each species.

Vitis Species	Number of Samples	Native Region (collected from 1893 to 2000)
V. acerifolia	21	South-Central Great Plains, USA
V. aestivalis	21	Eastern North America, USA
V. afghanistan	1	Afghanistan
V. amurensis	21	Far East Amur region
V. arizonica	4	Western USA
V. biformis	7	Mexico
V. blancoii	1	Western Mexico
V. bloodworthiana	5	N/A
V. bourgaena	1	N/A
V. cinerea	40	OK, TX, USA
V. coignetiae	5	Russian, Korea, Japan
V. flexuosa	1	Asian tropical and temperate zones
V. heyeana	2	Asian
V. hybrid	2	Eastern North America
V. labrusca	45	Eastern North America
V. lanata	1	Sub-Himalayan Tract
V. monticola	11	TX, USA
V. mustangensis	7	Southern USA (MS, AL, LA, TX, OK)
V. nesbittiana	5	Central Mexico (Veracruz)
V. palmata	11	South, Central & Southeastern USA
V. peninsularis	1	N/A
V. piasezki	11	N/A
V. popenoei	1	N/A
V. riparia	89	Eastern Canada, Central & Northeastern USA
V. romanetii	1	China
V. rotundifolia	13	South Eastern, & South-Central USA
V. rupestris	31	USA
V. shuttleworthii	5	FL, AL, USA
V. treleasei	2	Western USA
V. vinifera	4	European
V. vulpina	10	Eastern & Central USA, Ontario Canada

Table 9. Reagents for triplex PCR assay.

Reagent	Concentration
Autoclaved dH_20	To 25 µL
5X Buffer with contains $MgCl_2$	1X
dNTPs	0.28 mM
GVCV-1101F primer	0.4 µM
GVCV-1935R primer	0.4 µM
GVCV-4363F primer	0.24 µM
GVCV-4804R primer	0.24 µM
rRNA-16SF primer	0.14 µM
rRNA-16SR primer	0.14 µM
GoTaq polymerase	1.25 units
DNA	0.4 ng/µL

Table 10. Thermocycler program for Veriflux Triplex PCR assay to find the optimum annealing temperature.

Cycle	Temperature	Time	Repetitions
Initial denature	94°C	1 minute	1
Denature	94°C	30 seconds	35
Anneal (Veriflux settings)	53°C, 55°C, 57°C, 59°C, 61°C, 63°C	30 seconds	35
Extension	72°C	1 minute	35
Final extension	72°C	10 minutes	1

Table 11. Reagents for high fidelity PCR assay for amplification of open reading frame II.

Reagents	Concentration
Autoclaved dH_2O	To 25 µL
10X High Fidelity Buffer	1X
50mM $MgSO_4$	2 mM
dNTPs	0.2 mM
GVCV-963F primer	0.2 µM
GVCV-1634R primer	0.2 µM
High Fidelity PlatinumTaq polymerase	1 unit
DNA	0.4 ng/µL

Table 12. Thermocycler program for high fidelity PCR.

Cycle	Temperature	Time	Repetitions
Initial Denature	94°C	2 minutes	1
Denature	94°C	30 seconds	35
Anneal	55°C	40 seconds	35
Extension	68°C	4 minutes	35
Final Extension	68°C	10 minutes	1

Table 13. High Fidelity PCR master mixes for each of the three fragments. A different primer set was used for each fragment.

Fragment I Reagents	Concentration
Autoclaved dH_2O	To 25 µL
10X High Fidelity Buffer	1X
50mM $MgSO_4$	2 mM
dNTPs	0.2 mM
GVCV-988F primer	0.2 µM
GVCV-4387R primer	0.2 µM
High Fidelity PlatinumTaq polymerase	1 unit
DNA	0.4 ng/µL
Fragment II Reagents	
Autoclaved dH_2O	To 25 µL
10X High Fidelity Buffer	1X
50mM $MgSO_4$	2 mM
dNTPs	0.2 mM
GVCV-4142F primer	0.2 µM
GVCV-6795R primer	0.2 µM
High Fidelity PlatinumTaq polymerase	1 unit
DNA	0.4 ng/µL
Fragment III Reagents	
Autoclaved dH_2O	To 25 µL
10X High Fidelity Buffer	1X
50mM $MgSO_4$	2 mM
dNTPs	0.2 mM
GVCV-6666F primer	0.2 µM
GVCV-1935R primer	0.2 µM
High Fidelity PlatinumTaq polymerase	1 unit
DNA	0.4 ng/µL

Table 14. Fragment amplification thermocycler program. Thermocycler program for amplifying three separate fragments of the GVCV genome.

Cycle	Temperature	Time	Repetitions
Initial Denature	94°C	2 minutes	1
Denature	94°C	30 seconds	35
Anneal	55°C	40 seconds	35
Extension	68°C	4 minutes	35
Final Extension	68°C	10 minutes	1

Table 15. Reagents used for fragment size verification PCR with GoTaq polymerase.

Fragment I Reagents	Concentration
Autoclaved dH_2O	To 25 µL
5X Buffer (contains Mg)	1X
dNTPs	0.2 mM
GVCV-988F primer	0.2 µM
GVCV-4387R primer	0.2 µM
GoTaq polymerase	1.25 units
DNA	0.4 ng/µL
Fragment II Reagents	
Autoclaved dH_2O	To 25µL
5X Buffer (contains Mg)	1X
dNTPs	0.2 mM
GVCV-4142F primer	0.2 µM
GVCV-6795R primer	0.2 µM
GoTaq polymerase	1.25 units
DNA	0.4 ng/µL
Fragment III Reagents	
Autoclaved dH_2O	To 25 µL
5X Buffer (contains Mg)	1X
dNTPs	0.2 mM
GVCV-6666F primer	0.2 µM
GVCV-1935R primer	0.2 µM
GoTaq polymerase	1.25 units
DNA	0.4 ng/µL

Table 16. List of primers used for sequencing isolate Vit16-25IIIa.

Primer	Sequence (5'-3')	T_m°C	Fragment
81F	AATCGTGTAGGGAATCGTTA	56.3	III
217R	TCTCACAACGGGCTACTACC	62.4	III
1179R	GCCACGTGGACATCTACCTT	62.4	I, III
1915F	AGAATACAAGTGCTACACCGA	58.7	I, III
2460F	AGACACAGGAGAAAGGGTAACT	60.8	I
3163F	AGGGTAAAAACTGCGACGGCTA	62.7	I
4828F	AAACAGGAACTCCAAGCTGC	60.4	II
5242R	TGCAGCCAGTGTCTATGATG	60.4	II
5405F	CAGCCTTCGAAATGAACATGC	60.6	II
5755F	GATATCACCATTGAGGCAAAGC	60.8	II
7068F	AAGGCTTGCCCAGAATGT	57.6	III
7635F	CCAGTTCCAGTTCCAGTGTTCTTAATGC	66.1	III

Table 17. List of primers used to sequence isolate Amp16-3IIIa.

Primer	Sequence (5'-3')	T_m°C	Fragment
81F	AATCGTGTAGGGAATCGTTA	56.3	III
217R	TCTCACAACGGGCTACTACC	62.4	III
1179R	GCCACGTGGACATCTACCTT	62.4	I, III
1915F	AGAATACAAGTGCTACACCGA	58.7	I, III
3468F	ATCCTCCCTCCTGAAGTAGC	62.4	I
3615R	TTCTCTTTCCCTTGGTCC	57.6	I
4142F	GTAAACCTCATGACTCTCATG	58.7	I, II
4828F	AAACAGGAACTCCAAGCTGC	60.4	II
5242R	TGCAGCCAGTGTCTATGATG	60.4	II
5405F	CAGCCTTCGAAATGAACATGC	60.6	II
5755F	GATATCACCATTGAGGCAAAGC	60.8	II
6004F	AGTCTGCCTGGAATCACCTC	62.4	II
7068F	AAGGCTTGCCCAGAATGT	57.6	III
7635F	CCAGTTCCAGTTCCAGTGTTCTTAATGC	66.1	III

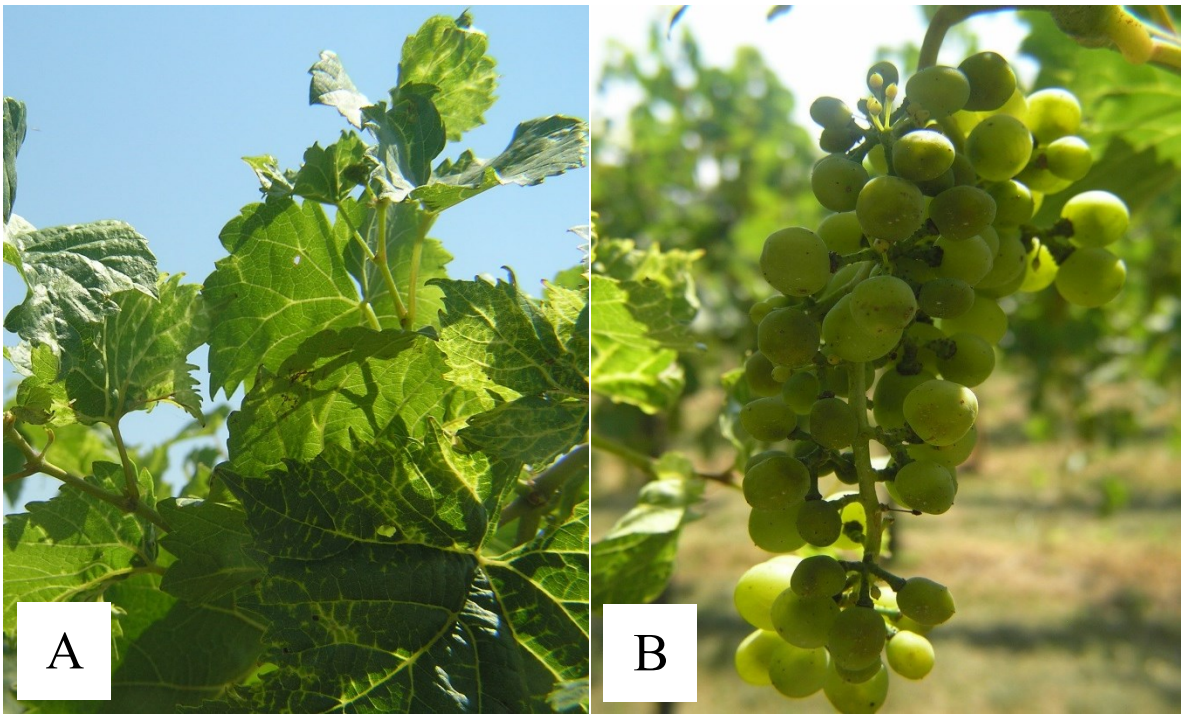


Figure 8. Symptoms of GVCV: (A) translucent veins and (B) deformed berry clusters. (Photos by Dr. Wenping Qiu).

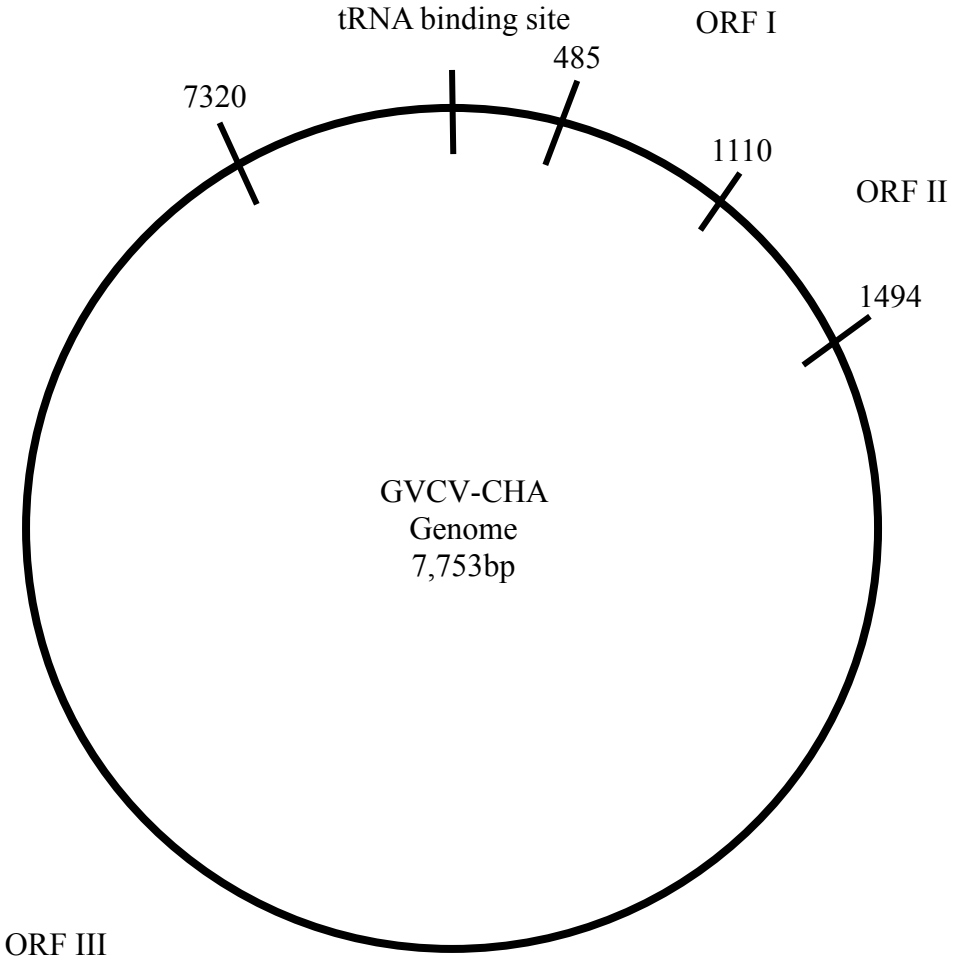


Figure 9. GVCV-CHA reference genome demonstrating the three open reading frames and the tRNA binding site.

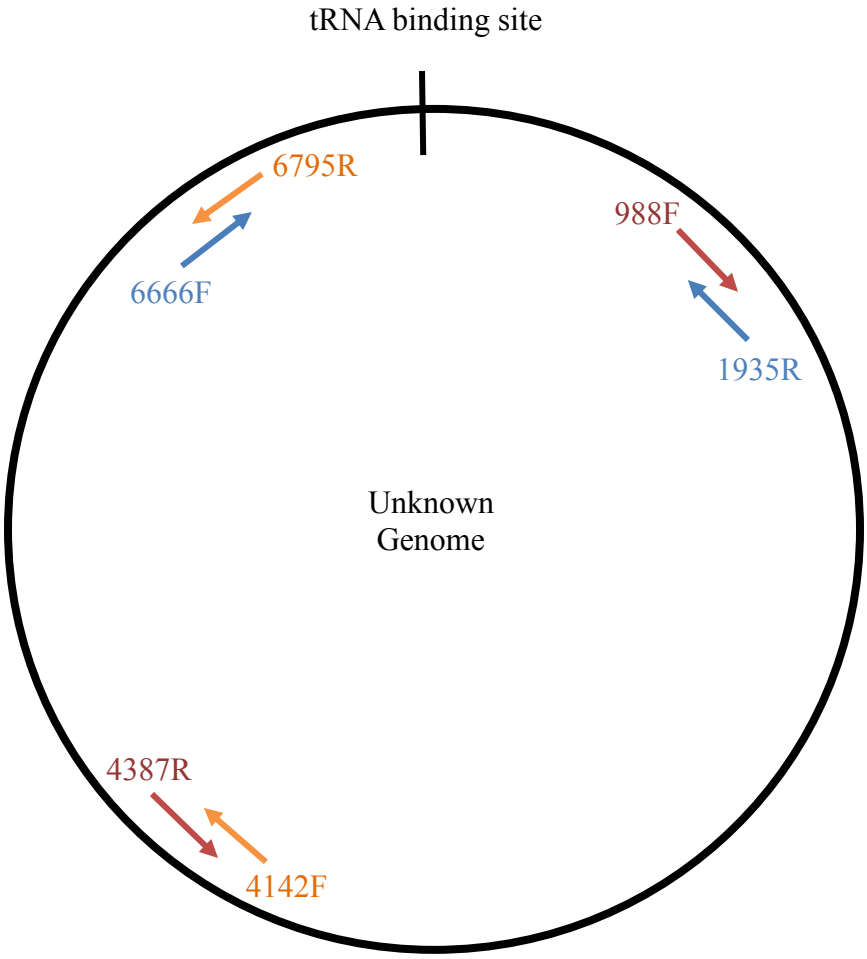


Figure 10. Genome map of the unsequenced isolates of GVCV demonstrating the overlapping fragment method and the primer sets used.

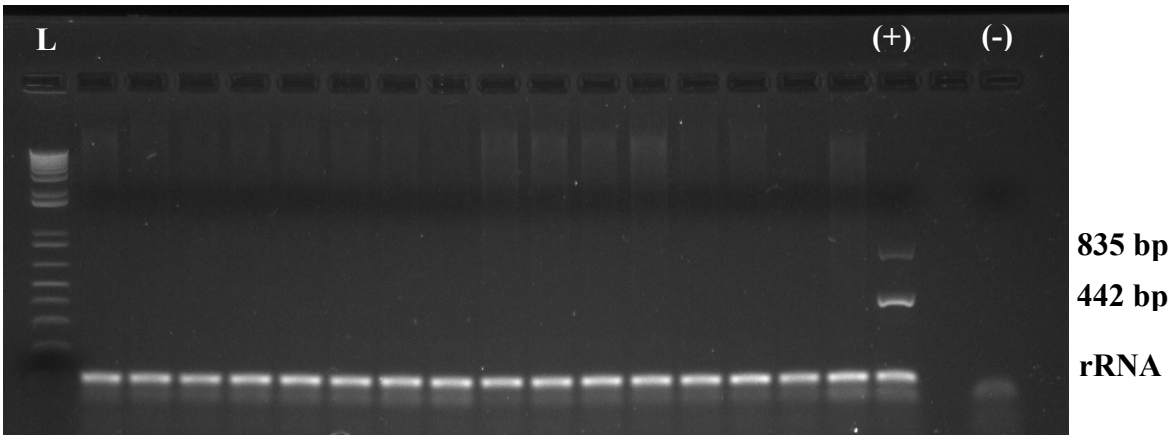


Figure 11. Germplasm survey agarose gel. Gel electrophoresis image showing two DNA fragments (835bp and 442bp) of GVCV in the infected grapevine (positive control, +) and a DNA fragment of grapevine rRNA gene. A total of 16 samples were tested in this assay and none contained GVCV-specific fragments.

```
Vit16-25IIIa       1 ATGTCCACGTGGCAAATTGCTGCTGCCACAGAAGAATACAAGAACGCCAT     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa        1 ATGTCCACGTGGCAAATTGCTGCTGCCACAGAAGAATACAAGAACGCCAT     50

Vit16-25IIIa      51 AAAAGCGACTGCAACCCTCACCAAGGACGAAAGAGCAGTTGGCTTTGTCA    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa       51 AAAAGCGACTGCAACCCTCACCAAGGACGAAAGAGCAGTTGGCTTTGTCA    100

Vit16-25IIIa     101 AGCCCCACGAGTTCGAACCAAATTTCAGTGACACCAACATCCAAAGGCAA    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      101 AGCCCCACGAGTTCGAACCAAATTTCAGTGACACCAACATCCAAAGGCAA    150

Vit16-25IIIa     151 AACAATACTTTGATCCATCTGTTGATACAGAACCTTGAGGAAATCAAAGA    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      151 AACAATACTTTGATCCATCTGTTGATACAGAACCTTGAGGAAATCAAAGA    200

Vit16-25IIIa     201 GCTCCGTGCTCAGGTTCAGACCCTCAACGATCGTATTGTAACCTTGGAAA    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      201 GCTCCGTGCTCAGGTTCAGACCCTCAACGATCGTATTGTAACCTTGGAAA    250

Vit16-25IIIa     251 AGGGGAAGTCAGCTGTCACCCTTCCTGATAACGTGGTAGAACAAATCTCC    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      251 AGGGGAAGTCAGCTGTCACCCTTCCTGATAACGTGGTAGAACAAATCTCC    300

Vit16-25IIIa     301 ACCCAACTAAAGGAAGCAAAGTTTGGAACTCAGAAGGAAGGCTTGGTGAA    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      301 ACCCAACTAAAGGAAGCAAAGTTTGGAACTCAGAAGGAAGGCTTGGTGAA    350

Vit16-25IIIa     351 AGGGACAAAAGGCACCTTCCGGGTCTGGAAGTGA    384
                     ||||||||||||||||||||||||||||||||
Amp16-3IIIa      351 AGGGACAAAAGGCACCTTCCGGGTCTGGAAGTGA    384
```

Figure 12. Clustal comparison of ORFII regions of two GVCV isolates from Vit16-25IIIa and Amp16-3IIIa. Each vertical line indicates matching nucleotides between the two isolates.

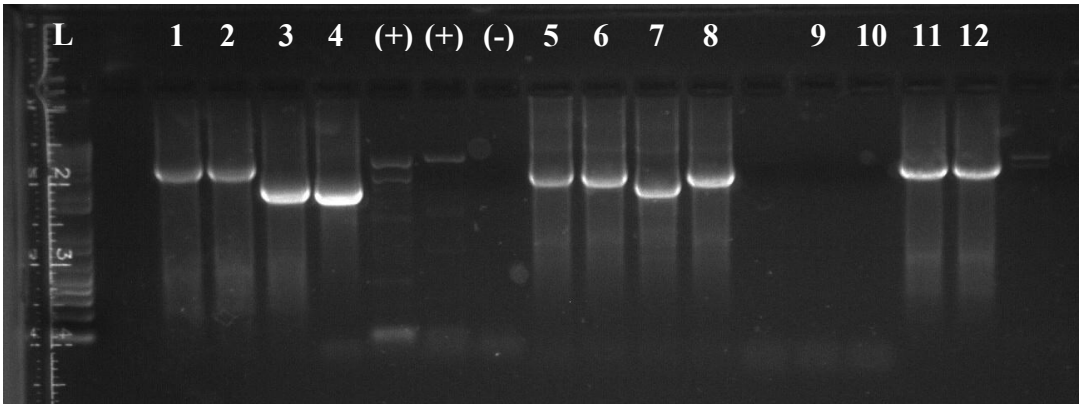


Figure 13. Fragment amplification agarose gel. Electrophoresis image showing the results of high fidelity PCR to amplify GVCV-specific fragments I, II, and III for sequencing. Lane 1&2: Vit16-25IIIa Fragment I; 3&4: Amp16-3IIIa Fragment I; 5&6: Vit16-25IIIa Fragment II; 7&8: Amp16-3IIIa Fragment II; 9&10: Vit16-25IIIa Fragment III; 11&12: Amp16-3IIIa Fragment III. The positive and negative controls are between lanes 4 and 5. Two positives were used (GVCV-CHA and GVCV-AMP1) because of uncertainty of comparable GVCV isolate.

```
Vit16-25IIIa       1 AATCTGGGAATTTCTACAATTATTCCTTCAAGATTATGATGAGGAACTAA     50
                     |||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa        1 AATCTGGGAATTTCTACAATTATTCCTTCAAGATTATGATGAGGAACTAA     50

Vit16-25IIIa      51 CTCTCATAATCGTGTAGGGAATCGTTAGTAGGATCTCAGAACAAGGTTCT    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||.|
Amp16-3IIIa       51 CTCTCATAATCGTGTAGGGAATCGTTAGTAGGATCTCAGAACAAGGTTTT    100

Vit16-25IIIa     101 TATCCCCTCATACTACTGATTTTTGGTATATAGGCTGGAAACACGACACT    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      101 TATCCCCTCATACTACTGATTTTTGGTATATAGGCTGGAAACACGACACT    150

Vit16-25IIIa     151 GTTACGATCCCACTTCTGTTGGAGTGGTAGTAGCCCGTTGTGAGACAACG    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      151 GTTACGATCCCACTTCTGTTGGAGTGGTAGTAGCCCGTTGTGAGACAACG    200

Vit16-25IIIa     201 CCACGTACCATTTTCAGTCTTCCTAGCCTAAATCCCCATGAACAGAACTC    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      201 CCACGTACCATTTTCAGTCTTCCTAGCCTAAATCCCCATGAACAGAACTC    250

Vit16-25IIIa     251 CCACGGTCAATAAGCTTCAACAGGATCCCTAGCCCAACAATACTGAAAGT    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      251 CCACGGTCAATAAGCTTCAACAGGATCCCTAGCCCAACAATACTGAAAGT    300

Vit16-25IIIa     301 CCTAGGACAGGCTGCGACGCGAAGTATCACTAGTTCAGGCGATGCTGTTC    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      301 CCTAGGACAGGCTGCGACGCGAAGTATCACTAGTTCAGGCGATGCTGTTC    350

Vit16-25IIIa     351 CGCCGACTATTTGTGAGAAAACAGCAGTAGGAGAGGACGGACAACTATTC    400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      351 CGCCGACTATTTGTGAGAAAACAGCAGTAGGAGAGGACGGACAACTATTC    400

Vit16-25IIIa     401 AAGGGAACGGAACCTGGAGACACCGGCCGAGTTCTTAGTAAGCGGTTCAA    450
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      401 AAGGGAACGGAACCTGGAGACACCGGCCGAGTTCTTAGTAAGCGGTTCAA    450

Vit16-25IIIa     451 GAAGGAGACTGATGCAAAGAATAGAACAACAAAAGTTTGAGGAGGAGATA    500
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      451 GAAGGAGACTGATGCAAAGAATAGAACAACAAAAGTTTGAGGAGGAGATA    500

Vit16-25IIIa     501 GAATCTTGGGAGAGATCTGAACGCACACCCCTACACGGTTACCGTGATCT    550
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      501 GAATCTTGGGAGAGATCTGAACGCACACCCCTACACGGTTACCGTGATCT    550

Vit16-25IIIa     551 TGTGGAATACCCCCGTTACGAAAGAAATCAGCATTTCCCATCTGCAAAGT    600
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      551 TGTGGAATACCCCCGTTACGAAAGAAATCAGCATTTCCCATCTGCAAAGT    600

Vit16-25IIIa     601 TCCC    604
                     ||||
Amp16-3IIIa      601 TCCC    604
```

Figure 14. Clustal comparison of nt 23 to 628. Comparison of nt 23 to628 in GVCV genome from Vit16-25IIIa and Amp16-3IIIa. Each vertical line indicates matching nucleotides between the two isolates.

```
Vit16-25IIIa       1 ATGTCCACGTGGCAAATTGCTGCTGCCACAGAAGAATACAAGAACGCCAT     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa        1 ATGTCCACGTGGCAAATTGCTGCTGCCACAGAAGAATACAAGAACGCCAT     50

Vit16-25IIIa      51 AAAAGCGACTGCAACCCTCACCAAGGACGAAAGAGCAGTTGGCTTTGTCA    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa       51 AAAAGCGACTGCAACCCTCACCAAGGACGAAAGAGCAGTTGGCTTTGTCA    100

Vit16-25IIIa     101 AGCCCCACGAGTTCGAACCAAATTTCAGTGACACCAACATCCAAAGGCAA    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      101 AGCCCCACGAGTTCGAACCAAATTTCAGTGACACCAACATCCAAAGGCAA    150

Vit16-25IIIa     151 AACAATACTTTGATCCATCTGTTGATACAGAACCTTGAGGAAATCAAAGA    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      151 AACAATACTTTGATCCATCTGTTGATACAGAACCTTGAGGAAATCAAAGA    200

Vit16-25IIIa     201 GCTCCGTGCTCAGGTTCAGACCCTCAACGATCGTATTGTAACCTTGGAAA    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      201 GCTCCGTGCTCAGGTTCAGACCCTCAACGATCGTATTGTAACCTTGGAAA    250

Vit16-25IIIa     251 AGGGGAAGTCAGCTGTCACCCTTCCTGATAACGTGGTAGAACAAATCTCC    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      251 AGGGGAAGTCAGCTGTCACCCTTCCTGATAACGTGGTAGAACAAATCTCC    300

Vit16-25IIIa     301 ACCCAACTAAAGGAAGCAAAGTTTGGAACTCAGAAGGAAGGCTTGGTGAA    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      301 ACCCAACTAAAGGAAGCAAAGTTTGGAACTCAGAAGGAAGGCTTGGTGAA    350

Vit16-25IIIa     351 AGGGACAAAAGGCACCTTCCGGGTCTGGAAGTGATGTCTCGGTCCAGAAC    400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      351 AGGGACAAAAGGCACCTTCCGGGTCTGGAAGTGATGTCTCGGTCCAGAAC    400

Vit16-25IIIa     401 TCAGACCACTGAGTTGCCTCGCGCAACCAGAAGATCGACTAGCCCAGTCG    450
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      401 TCAGACCACTGAGTTGCCTCGCGCAACCAGAAGATCGACTAGCCCAGTCG    450

Vit16-25IIIa     451 AAAGGCTAGATGATCAGATCCGCGGCTACAGGCGGATGGCTCGTGCCCGC    500
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      451 AAAGGCTAGATGATCAGATCCGCGGCTACAGGCGGATGGCTCGTGCCCGC    500

Vit16-25IIIa     501 TACCTTGCGGAGCAACGAATACGTAGGTCCTTCTCAAGGAACTACAGGGA    550
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      501 TACCTTGCGGAGCAACGAATACGTAGGTCCTTCTCAAGGAACTACAGGGA    550

Vit16-25IIIa     551 AACTCTGGAAAGACGCCTAGACCCAGAGGCTGAATTACAGCTCAGTCGAA    600
                     ||||||||||||||||||||.||||||||||||||||||||||||||||||
Amp16-3IIIa      551 AACTCTGGAAAGACGCCTAGATCCAGAGGCTGAATTACAGCTCAGTCGAA    600

Vit16-25IIIa     601 GAAGAAGAGCTAACTTAGTACCAGCGGAAGTACTATACTCCCTCAACTAC    650
                     ||.||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      601 GACGAAGAGCTAACTTAGTACCAGCGGAAGTACTATACTCCCTCAACTAC    650

Vit16-25IIIa     651 AATGAACCCCAGAATAGGGTTTATCAACACTATGAAGAGGTGAGATCCCA    700
                     ||||||||||||||||||||||||||||||||||||||||||||||||||.||
Amp16-3IIIa      651 AATGAACCCCAGAATAGGGTTTATCAACACTATGAAGAGGTGAGATCTCA    700

Vit16-25IIIa     701 TGTCATAGACCGGCAGCAAGATTTCCGGTTTATCGAAGAACAGTCCTACC    750
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      701 TGTCATAGACCGGCAGCAAGATTTCCGGTTTATCGAAGAACAGTCCTACC    750
```

Figure 15. Clustal comparison of nt 1113 to 1902. Comparison of nt 1113 to 1902 in GVCV genome from Vit16-25IIIa and Amp16-3IIIa. Each vertical line indicates matching nucleotides between the two isolates.

```
Vit16-25IIIa       1 AATGCTACCTCTGTGGCATTGAAGGCCACTATGCTCGTGAATGCCCAAAG     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa        1 AATGCTACCTCTGTGGCATTGAAGGCCACTATGCTCGTGAATGCCCAAAG     50

Vit16-25IIIa      51 AAGCATGTCAGGCCTGAAAGAGCAGCCTACTTCGAAGGCATGGGCTTAGA    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa       51 AAGCATGTCAGGCCTGAAAGAGCAGCCTACTTCGAAGGCATGGGCTTAGA    100

Vit16-25IIIa     101 TGTCAACTGGGATGTGATAAGTGTTGACCCAGGAGATCAAGATGGATCGG    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      101 TGTCAACTGGGATGTGATAAGTGTTGACCCAGGAGATCAAGATGGATCGG    150

Vit16-25IIIa     151 ACATCTGCTCAATCTCCGAAGGAGAAGCCCAACATGGGATGGAAGACCTA    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      151 ACATCTGCTCAATCTCCGAAGGAGAAGCCCAACATGGGATGGAAGACCTA    200

Vit16-25IIIa     201 GCTGCTTTCAAAGCCCAACTTCCATATCCAGTGGAAGCCCAATATGAGCA    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      201 GCTGCTTTCAAAGCCCAACTTCCATATCCAGTGGAAGCCCAATATGAGCA    250

Vit16-25IIIa     251 GCACCAGGCCTTTGTGGTTATCCAGACAACTTTTAAAAAGGAGGATAAGC    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      251 GCACCAGGCCTTTGTGGTTATCCAGACAACTTTTAAAAAGGAGGATAAGC    300

Vit16-25IIIa     301 CCCAAGGCTCTTGGCGTATGTCAAAGCCCATCCCAGAAGCCCAACAGCAA    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      301 CCCAAGGCTCTTGGCGTATGTCAAAGCCCATCCCAGAAGCCCAACAGCAA    350

Vit16-25IIIa     351 TGCCAGCATACATGGGATGATATGTATGCCCTAGCAGAAGGCCAGCAAGC    400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      351 TGCCAGCATACATGGGATGATATGTATGCCCTAGCAGAAGGCCAGCAAGC    400

Vit16-25IIIa     401 ATGCAGCACTTGCCAGACCATCACTGTACTTGGTCGCCGTACCACATGCA    450
                     ||||||||||||||||||||||||||||||||||||||.|||||||||
Amp16-3IIIa      401 ATGCAGCACTTGCCAGACCATCACTGTACTTGGTCGCCGTGCCACATGCA    450

Vit16-25IIIa     451 CCCTTTGCCTACTCAACCTCTGCTCACTCTGCGCTGGTTTAGACTTCGGT    500
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      451 CCCTTTGCCTACTCAACCTCTGCTCACTCTGCGCTGGTTTAGACTTCGGT    500

Vit16-25IIIa     501 CTCAAAATAGTTCCTAAAACTGCTACACGTGCTGACTGGAAATTCCAGGA    550
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      501 CTCAAAATAGTTCCTAAAACTGCTACACGTGCTGACTGGAAATTCCAGGA    550

Vit16-25IIIa     551 TCGTGATACCCTTATCGCCTCCCTATATGAGCACAATGCATTCCTTCTTC    600
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      551 TCGTGATACCCTTATCGCCTCCCTATATGAGCACAATGCATTCCTTCTTC    600

Vit16-25IIIa     601 GACAAGTTGAAGGACTGAAACAGGAACTCCAAGCTGCCAAGGAACAGCTT    650
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      601 GACAAGTTGAAGGACTGAAACAGGAACTCCAAGCTGCCAAGGAACAGCTT    650

Vit16-25IIIa     651 CAACTGCTACACTCGGTTGATATGATCAACCTCTCTGATGACGGATTAGA    700
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      651 CAACTGCTACACTCGGTTGATATGATCAACCTCTCTGATGACGGATTAGA    700
```

Figure 16. Clustal comparison of nt 4212 to 5431. Comparison of nt 4212 to 5431 in GVCV genome from Vit16-25IIIa and Amp16-3IIIa. Each vertical line indicates matching nucleotides between the two isolates. Continued on next page.

```
Vit16-25IIIa     701 GAATTTTTCTGTTGAGGAAAAATCCTTTTTAAGAGGGGGAGGGGGAACCA    750
                     |||||||||.|||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      701 GAATTTTTCCGTTGAGGAAAAATCCTTTTTAAGAGGGGGAGGGGGAACCA    750

Vit16-25IIIa     751 GTAGCAGTTCAATCAAAATCTCATCAACAACAACACCCCCTGGTTTTCCT    800
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      751 GTAGCAGTTCAATCAAAATCTCATCAACAACAACACCCCCTGGTTTTCCT    800

Vit16-25IIIa     801 ACAACACCCAATAGATTCCAGCCTCTTGCGCAGGAAAAACTTAAAGGAAT    850
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      801 ACAACACCCAATAGATTCCAGCCTCTTGCGCAGGAAAAACTTAAAGGAAT    850

Vit16-25IIIa     851 ACAGGAAGACCTATCTCTGGCAGTACAGTTTGATGATGTCAGACAACAAG    900
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      851 ACAGGAAGACCTATCTCTGGCAGTACAGTTTGATGATGTCAGACAACAAG    900

Vit16-25IIIa     901 AACAGGCGTATACTGAAATGCCTCGAGGAGCTCACAACAAACTATACCAC    950
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      901 AACAGGCGTATACTGAAATGCCTCGAGGAGCTCACAACAAACTATACCAC    950

Vit16-25IIIa     951 GTAGTGGTAACTTTCAGAATCCCTAACGATAAGGGACAGCTCCTTGAATT   1000
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      951 GTAGTGGTAACTTTCAGAATCCCTAACGATAAGGGACAGCTCCTTGAATT   1000

Vit16-25IIIa    1001 TGATATCAACGCCATCATAGACACTGGCTGTACATGCTGCTGCATCAACC   1050
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1001 TGATATCAACGCCATCATAGACACTGGCTGTACATGCTGCTGCATCAACC   1050

Vit16-25IIIa    1051 TCACAAAGGTGCCTGATGGAGCAATAGAAAATGCCTCCATAATCCAAGAA   1100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1051 TCACAAAGGTGCCTGATGGAGCAATAGAAAATGCCTCCATAATCCAAGAA   1100

Vit16-25IIIa    1101 GTCTCTGGGATTAACAGCAAGACAGTAGTCACCAAGAAACTCAGACAAGG   1150
                     ||||||||||||||||||||||||||||||||||||||||||||.|||||
Amp16-3IIIa     1101 GTCTCTGGGATTAACAGCAAGACAGTAGTCACCAAGAAACTCAGGCAAGG   1150

Vit16-25IIIa    1151 CAAGATGATCCTCGCAGGGAATGATTTCTACATTCCTTATGTCTCAGCCT   1200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1151 CAAGATGATCCTCGCAGGGAATGATTTCTACATTCCTTATGTCTCAGCCT   1200

Vit16-25IIIa    1201 TTGAGATGAACATGCCTGGG   1220
                     ||||||||||||||||||||
Amp16-3IIIa     1201 TTGAGATGAACATGCCTGGG   1220
```

Figure 16 cont. Clustal comparison of nt 4212 to 5431. Comparison of nt 4212 to 5431 in GVCV genome from Vit16-25IIIa and Amp16-3IIIa. Each vertical line indicates matching nucleotides between the two isolates.

```
Vit16-25IIIa       1 TTGGAAGGAACTGAGGTCACCTTCTACAAAACCATCACCAGGATTCAAAC     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa        1 TTGGAAGGAACTGAGGTCACCTTCTACAAAACCATCACCAGGATTCAAAC     50

Vit16-25IIIa      51 TACCCTGGAACCTCAAAAGATAGCGTACTTGGAAGAGCTGGTAGAAGCTG    100
                     ||||||.||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa       51 TACCCTAGAACCTCAAAAGATAGCGTACTTGGAAGAGCTGGTAGAAGCTG    100

Vit16-25IIIa     101 AAGATCTACACTATGAGCTCGCAGCTGCAAGTATGCCTGAGCCCACTGCT    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      101 AAGATCTACACTATGAGCTCGCAGCTGCAAGTATGCCTGAGCCCACTGCT    150

Vit16-25IIIa     151 GAAGGACTCAGAAACACTAAGCTCCTAGCTGAGCTAAAAGAACAAGGCTA    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      151 GAAGGACTCAGAAACACTAAGCTCCTAGCTGAGCTAAAAGAACAAGGCTA    200

Vit16-25IIIa     201 CATTGGTGAAGAACCCCTGAAGCACTGGTCAAAGAATAGGGTTCGTTGCA    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      201 CATTGGTGAAGAACCCCTGAAGCACTGGTCAAAGAATAGGGTTCGTTGCA    250

Vit16-25IIIa     251 AATTGGATATCATAAATCCAGACATCACCATTGAAGCAAAGCCACCTGGA    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      251 AATTGGATATCATAAATCCAGACATCACCATTGAAGCAAAGCCACCTGGA    300

Vit16-25IIIa     301 CACCTGACTCTGGAGGACAAGGTCAAGTATCAGAAGCACATTGACGCCCT    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      301 CACCTGACTCTGGAGGACAAGGTCAAGTATCAGAAGCACATTGACGCCCT    350

Vit16-25IIIa     351 CCTAGATCTTGGAGTCATCAGACCCAGCAAGAGCAGACACAGGTCCGCAG    400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      351 CCTAGATCTTGGAGTCATCAGACCCAGCAAGAGCAGACACAGGTCCGCAG    400

Vit16-25IIIa     401 CTTTCATAGTTGCCTCTGGGACCTCTGTAGATCCTAAAACTGGCAAGGAA    450
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      401 CTTTCATAGTTGCCTCTGGGACCTCTGTAGATCCTAAAACTGGCAAGGAA    450

Vit16-25IIIa     451 ACTCGCGGTAAAGAAAGAATGGTGATCGATTACCGCATGCTTAACGACAA    500
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      451 ACTCGCGGTAAAGAAAGAATGGTGATCGATTACCGCATGCTTAACGACAA    500

Vit16-25IIIa     501 CTGCTATAAGGATCAATACAGTCTGCCTGGAATCACCTCCATCATTAAAT    550
                     ||||||||||||||||||||||||||||||||||||||||||||||.||||
Amp16-3IIIa      501 CTGCTATAAGGATCAATACAGTCTGCCTGGAATCACCTCCATCATCAAAT    550

Vit16-25IIIa     551 CTCTTGGACAGGCTAAAATCTTCAGCAAATTTGACCTGAAGTCTGGCTTT    600
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      551 CTCTTGGACAGGCTAAAATCTTCAGCAAATTTGACCTGAAGTCTGGCTTT    600

Vit16-25IIIa     601 CACCAAGTCATGATGGAAGAAGAAAGCATCCCCTGGACTGCTTTCATCAG    650
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      601 CACCAAGTCATGATGGAAGAAGAAAGCATCCCCTGGACTGCTTTCATCAG    650

Vit16-25IIIa     651 CCCCGCAGGCTTGTATGAATGGCTAGTTATGCCATTTGGGATTCAAAATG    700
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      651 CCCCGCAGGCTTGTATGAATGGCTAGTTATGCCATTTGGGATTCAAAATG    700

Vit16-25IIIa     701 CGCCTGCAATATTCCAAAGAAAGATGGATGAATGCTTCAAAGGAACCGAG    750
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      701 CGCCTGCAATATTCCAAAGAAAGATGGATGAATGCTTCAAAGGAACCGAG    750
```

Figure 17. Clustal comparison of nt 5486 to 7222. Comparison of nt 5486 to 7222 in GVCV genome from Vit16-25IIIa and Amp16-3IIIa. Each vertical line indicates matching nucleotides between the two isolates. Continued on next page.

```
Vit16-25IIIa     751 GATTTCATCGCTGTTTATATCGATGATATTCTGGTATTCTCCAACTCCAT    800
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      751 GATTTCATCGCTGTTTATATCGATGATATTCTGGTATTCTCCAACTCCAT    800

Vit16-25IIIa     801 CAAAGAGCATGAAAAGCACCTGCAGAGAATGCTGAGTATCTGCAAGGAAC    850
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      801 CAAAGAGCATGAAAAGCACCTGCAGAGAATGCTGAGTATCTGCAAGGAAC    850

Vit16-25IIIa     851 ATGGGCTCGTCCTTAGCCCAACAAAAATGAAGATCGCTGTCCCAGGAATT    900
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      851 ATGGGCTCGTCCTTAGCCCAACAAAAATGAAGATCGCTGTCCCAGGAATT    900

Vit16-25IIIa     901 GATTTCCTTGGTGCCCATATCAGAAACAGCAGAGTAAGTCTGCAACCGCA    950
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      901 GATTTCCTTGGTGCCCATATCAGAAACAGCAGAGTAAGTCTGCAACCGCA    950

Vit16-25IIIa     951 CATCATCAAGAAGATTGCTGACAAGAAAGATGATGAGCTGATGACCCTTA   1000
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      951 CATCATCAAGAAGATTGCTGACAAGAAAGATGATGAGCTGATGACCCTTA   1000

Vit16-25IIIa    1001 AAGGCCTCAGAAGCTGGCTTGGGGTAATCAACTATGTCAGGCAGTACATT   1050
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1001 AAGGCCTCAGAAGCTGGCTTGGGGTAATCAACTATGTCAGGCAGTACATT   1050

Vit16-25IIIa    1051 CCTAAGTGCGGAACACTTCTAGGTCCCCTCTATGCTAAAACATCTGAGCA   1100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1051 CCTAAGTGCGGAACACTTCTAGGTCCCCTCTATGCTAAAACATCTGAGCA   1100

Vit16-25IIIa    1101 TGGTGATCGAAGATGGCACCCCAAAGACTGGGAAATAGTGAGACAGATCA   1150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1101 TGGTGATCGAAGATGGCACCCCAAAGACTGGGAAATAGTGAGACAGATCA   1150

Vit16-25IIIa    1151 AGAAGATGGTTCAATCCCTTCCTGATCTAGAACTTCCTCCACCCCACGCA   1200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1151 AGAAGATGGTTCAATCCCTTCCTGATCTAGAACTTCCTCCACCCCACGCA   1200

Vit16-25IIIa    1201 GTTATCATCATTGAATCTGATGGATGCATGGAAGGATGGGGAGGAATCTG   1250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1201 GTTATCATCATTGAATCTGATGGATGCATGGAAGGATGGGGAGGAATCTG   1250

Vit16-25IIIa    1251 CAAATGGAAAAACTCAAAAGGGGAATCTAAAGGCAAAGAGCGAATCTGTG   1300
                     ||||||||||||||||||||||||||||||||.||||||||||||||||||
Amp16-3IIIa     1251 CAAATGGAAAAACTCAAAAGGGGAATCTAAAGACAAAGAGCGAATCTGTG   1300

Vit16-25IIIa    1301 CTTATGCCAGTGGAAAATTCCCAACAGTCAAATCCACCATAGATGCTGAA   1350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1301 CTTATGCCAGTGGAAAATTCCCAACAGTCAAATCCACCATAGATGCTGAA   1350

Vit16-25IIIa    1351 ATCTATGCAGTCATGGCATCCCTGGAAAACTTCAAGATTTACTATCTTGA   1400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1351 ATCTATGCAGTCATGGCATCCCTGGAAAACTTCAAGATTTACTATCTTGA   1400

Vit16-25IIIa    1401 TAAACGGGAAATCACTATCAGAACGGACTGCCAAGCCATAATCAGCTTCT   1450
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1401 TAAACGGGAAATCACTATCAGAACGGACTGCCAAGCCATAATCAGCTTCT   1450

Vit16-25IIIa    1451 ATGATAAAATGGCTGTCAAGAAACCCAGCAGAGTTCGCTGGATTAATTTC   1500
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1451 ATGATAAAATGGCTGTCAAGAAACCCAGCAGAGTTCGCTGGATTAATTTC   1500
```

Figure 17 cont. Clustal comparison of nt 5486 to 7222. Comparison of nt 5486 to 7222 in GVCV genome from Vit16-25IIIa and Amp16-3IIIa. Each vertical line indicates matching nucleotides between the two isolates. Continued on next page.

```
Vit16-25IIIa    1501 TGTGATTATATCACTAACACAGGAATCAAAGTCCAGTTCGAACATATAAA   1550
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1501 TGTGATTATATCACTAACACAGGAATCAAAGTCCAGTTCGAACATATAAA   1550

Vit16-25IIIa    1551 GGGCCAAGATAACCAGCTAGCAGACCAGCTCTCAAGGCTAGCCCAAGGAC   1600
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1551 GGGCCAAGATAACCAGCTAGCAGACCAGCTCTCAAGGCTAGCCCAAGGAC   1600

Vit16-25IIIa    1601 TTTGCAGCATTCAAGTCATCCCTGAAGCAGCCCACGAAGCTCTCACCATC   1650
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1601 TTTGCAGCATTCAAGTCATCCCTGAAGCAGCCCACGAAGCTCTCACCATC   1650

Vit16-25IIIa    1651 ATCCTTGAACAGGATTGCACAGCCCAAGAGCTCATGGCCCAGTTCAACTC   1700
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1651 ATCCTTGAACAGGATTGCACAGCCCAAGAGCTCATGGCCCAGTTCAACTC   1700

Vit16-25IIIa    1701 CATGCTGCAAGCAAACCTCAGGCTTAACCAAGGAAGG   1737
                     ||||||||||||||||||||||||||||||||||||
Amp16-3IIIa     1701 CATGCTGCAAGCAAACCTCAGGCTTAACCAAGGAAGG   1737
```

Figure 17 cont. Clustal comparison of nt 5486 to 7222. Comparison of nt 5486 to 7222 in GVCV genome from Vit16-25IIIa and Amp16-3IIIa. Each vertical line indicates matching nucleotides between the two isolates.

```
Vit16-25IIIa       1 TGAGCTGTCGATGGGGCCCAATGAGTACCCGAGCTCCAAAAGTAACTTAC     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa        1 TGAGCTGTCGATGGGGCCCAATGAGTACCCGAGCTCCAAAAGTAACTTAC     50

Vit16-25IIIa      51 CTCTGGTTGCTTTTGTAAACCTTAGTTAGGTTTGTTTGCTTTTCTCCCCT    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa       51 CTCTGGTTGCTTTTGTAAACCTTAGTTAGGTTTGTTTGCTTTTCTCCCCT    100

Vit16-25IIIa     101 ATATAAGGGAGCCTCTCTTTTGTAAGAAGGCACCGAACAGAGCAATATCT    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      101 ATATAAGGGAGCCTCTCTTTTGTAAGAAGGCACCGAACAGAGCAATATCT    150

Vit16-25IIIa     151 CTGAGCGCTCCTTCTCTCTAGTTTTCCTGTGTGCTTGTATCTTTCCAGTT    200
                     ||||||||||||||||||||||||||.||||||||||||.||||||||||
Amp16-3IIIa      151 CTGAGCGCTCCTTCTCTCTAGTTTTCTTGTGTGCTTGTACCTTTCCAGTT    200

Vit16-25IIIa     201 CCAGTGTTCTTAATGCAACTTGAAGTTTTCTTACTCTATGTTATTCTGTT    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      201 CCAGTGTTCTTAATGCAACTTGAAGTTTTCTTACTCTATGTTATTCTGTT    250

Vit16-25IIIa     251 CATAGTTCTTTTCCGCTACTTATACTCTGTGATCCAAGTTTTTAAATTGT    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Amp16-3IIIa      251 CATAGTTCTTTTCCGCTACTTATACTCTGTGATCCAAGTTTTTAAATTGT    300

Vit16-25IIIa     301 GATCTGTTTACT    312
                     ||||||||||||
Amp16-3IIIa      301 GATCTGTTTACT    312
```

Figure 18. Clustal comparison of nt 7439 to 7753. Comparison of nt 7439 to 7753 in GVCV genome from Vit16-25IIIa and Amp16-3IIIa. Each vertical line indicates matching nucleotides between the two isolates.

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