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Prevalence, Genetic Relationships, and Quantity of Grapevine Vein Clearing Virus in Aphis Illinoisenesis

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PREVALENCE, GENETIC RELATIONSHIPS, AND QUANTITY OF GRAPEVINE VEIN CLEARING VIRUS IN *APHIS ILLINOISENESIS*

A Master's Thesis

Presented to

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In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Plant Science

By

Adam Louis Uhls

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Agriculture

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ABSTRACT

Grapevine vein clearing virus (GVCV) is an emerging pathogen causing severe damage to cultivated grapevines in the Midwest area of the United States. The prevalence of GVCV has been reported in native *Vitis* spp. and *Ampelopsis cordata*, a close relative of *vitis* in the Vitaceae family, which act as a reservoir for the virus. GVCV can be transmitted from wild *A. cordata* to *Vitis* spp. by *Aphis illinoisensis* (grape aphids) under greenhouse conditions, but the prevalence and transmission in native populations remains unknown. Knowing the prevalence and diversity of GVCV variants in natural grape aphid populations would help monitor and mitigate its impact. In this study, grape aphids from native Vitaceae were collected across the state of Missouri in 2018 and 2019 and conducted diagnostic and genetic analyses. Ten aphids within each community were tested, and GVCV was detected in 91 of the 105 (87%) randomly sampled communities. GVCV was present in 212 of 525 single grape aphids (40%). GVCV variants in grape aphids are genetically diverse and are dispersed across the surveyed region. When comparing the DNA sequences from GVCV isolates from grape aphids and plants, it was found that the same GVCV variants in grape aphids were found in wild and cultivated Vitaceae. The GVCV genome number varied largely in the stylet and body of each individual aphid. These results show that grape aphids carry diverse GVCV variants and contribute to the epidemics of GVCV in wild Vitaceae and vineyards. Our study provides a snapshot of GVCV epidemics and genetic structure that can help implement disease management schemes. Furthermore, the native reservoir, grape aphids, and vineyards form an ideal agro-ecosystem for studying epidemiology, ecology, and the evolution of GVCV.

KEYWORDS: grapevine vein clearing virus, epidemiology, grape aphids, vector, virus transmission, virus, Vitis

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INTRODUCTION

In 2004, severe virus-like symptoms appeared on Chardonnay grapevines in the Midwest region of the United States (Qiu et al. 2007). These symptoms included small deformed leaves, zig-zag shoots, vigor decline, small berry clusters bearing few fruits, and vein clearing. These symptoms caused the quality and quantity of grapes to fall, and led to the removal of several vineyards in 2007 (Qiu and Schoelz 2017). Later, this disease was found to be associated with Grapevine vein clearing virus (GVCV), which infects grape varieties as well as several native vines in the Vitaceae family. Most GVCV infected vines die several years after the appearance of the typical symptoms associated with this disease.

GVCV genome has a double-stranded, circular, DNA of about 7,753 bp, belonging to the genus *Badnavirus* in the Caulimoviridae family (Zhang et al. 2011). There are three predicted open reading frames (ORFs) located on the plus strand of the genome, each encoding proteins whose functions have not fully been characterized (Zhang et al. 2011). After sequencing and analyzing regions in ORF III of GVCV, it was determined that GVCV existed as genetically diverse populations within an individual plant (Guo et al. 2014). Analyzing the sequences of three GVCV ORF II isolates found that ORF II was the most diverse region in the GVCV genome, and 90% or higher similarity of this region at the nucleotide level would be considered the same variant (Beach et al. 2017).

Previous studies have identified possible sources of GVCV (Beach et al. 2017; Petersen et al. 2019a). GVCV was found to infect several wild species of grapevine at 10% incidence. In wild *Vitis rupestris*, two isolates of GVCV, GVCV-VRU1 and GVCV-VRU2, had a 91% similar nucleotide identity to GVCV-CHA, an isolate of GVCV in a Chardonel cultivar in a commercial

vineyard while being only 100 yards apart (Beach et al. 2017). The native vine *Ampelopsis cordata*, a relative of grapevines in the Vitaceae family has been found to be infected with GVCV at a rate of 33% (Petersen et al. 2019a). Both wild *Vitis* and *A. cordata* are abundant across the Midwest with each species having its own distribution. These perennial species are a reservoir for GVCV (Beach et al. 2017; Petersen et al. 2019a); once infected, they remain as a constant inoculum source for the duration of their lives. This natural virus reservoir provides a constant source of GVCV for vineyards each season and with the aid of an insect vector allows GVCV to continually infect new hosts.

Typically, viruses in the *Badnavirus* genus infect perennial hosts, and are transmitted most often by vegetative propagation with the second most common mode of spread utilizing mealybugs and aphids for transmission (Bhat et al. 2016). Recently, it has been found that grape aphids can transmit GVCV from native *A. cordata* to cultivated Chardonel (Petersen et al. 2019a). Interestingly, Several other Badnaviruses including Rubus yellow net virus, Spiraea yellow leaf spot virus and Gooseberry vein banding associated virus (GBVaV) are transmitted by aphids in a semi-persistent manner (Bhat et al. 2016). However, the persistence of GVCV in grape aphids is currently unknown.

Several modes of virus transmission exist within aphids, which can be classified into three subgroups: non-persistent, semi-persistent and persistent transmission. In non-persistent and semi-persistent transmission, virions lack the ability to pass through membranes within the midgut into the haemocoel of the aphid and therefore are retained in the stylet and foregut (Whitfield et al. 2015). All non-persistent and semi-persistent viruses are therefore classified as non-circulative. In non-persistent transmission, aphids can acquire and transmit virions relatively quickly, but lose the ability to transmit the virus rapidly. Semi-persistent transmission has a

longer acquisition period, usually around 15 minutes, but aphids retain the virus transmission ability for up to two days (Van Emden and Harrington 2017).

Persistently transmitted plant viruses require a much longer acquisition period and an inoculation period, however, once this period has passed, the aphid can remain infective for life (Van Emden and Harrington 2017). Persistently transmitted plant viruses are circulative, meaning they can pass through midgut membranes within the aphid into the haemocoel and back to the salivary glands where they can be inoculated (Van Emden and Harrington 2017). Persistent plant viruses can be further classified into propagative and non-propagative based on if the virus can replicate inside the vector.

Aphids are well suited as vectors of plant viruses in part due to their complicated life cycle and feeding behavior. Aphids have been found to select a host primarily based on plant color with the preferred colors being green and yellow (Döring et al. 2009). As migrant aphids land on a host plant, they have been found to have a behavioral reflex to probe, even if the plant is not suitable (Powell et al. 1999). As the aphid probes into a plant, the stylet is able to pierce into individual plant cells without causing cell death (Tjallingii and Esch 1993). An aphid then samples the contents of the cell by first egesting salvia, potentially releasing virions into the cell and infecting the plant.

The life cycle and environmental conditions of an aphid also contributes to the timing and pattern of virus epidemics. Aphids undergo parthenogenic reproduction allowing them to reproduce large amounts of offspring which can be either alate (winged) or aptera (wingless), both contributing to virus spread. Since plant viruses are transmitted by aphids, factors that influence aphid movement also facilitate the spread of a virus. These factors can be intentional such as movement due to overcrowding, presence of predators, searching for a food source, or

chemical changes of host plant, or inadvertent movement caused by gravity, wind currents, or an impact (Van Emden and Harrington 2017). These movements can be from plant to plant, or by short- and long-range aerial flights of alate aphids. A model of aphid flight behavior showed that aphids within a convective boundary layer can travel as much as 6 km downwind, while aphids in a neutrally stable boundary layer can travel as far as 30 km/hour with some remaining in flight for over three hours after they stop flying (Reynolds and Reynolds 2009). Their ability to travel long distances and vector viruses is what allows aphids to devastate crops in a short period of time.

While the prevalence of GVCV in *A. cordata* and wild *Vitis* has been found to be 33% and 10%, it is currently unknown how wide spread this virus is in grape aphid populations. These findings will reveal the potentiality of aphid populations to infect new wild Vitaceae plants and cultivated grapevines, which further solidifies and expands the GVCV reservoir to commercial vineyards. Furthermore, the genetic relationship between GVCV variants in wild plants, commercialized vineyards and grape aphids remains unknown. This insight forms a link between the wild reservoir and the cultivated vineyard and unveils insights into the relationship of GVCV variants among the host plants and vector. Understanding these relationships are crucial in designing an effective disease management scheme for preventing incidences of GVCV.

In this study, the prevalence of GVCV in grape aphids on wild *Vitis* and *A. cordata* as well as their genetic relationship with cultivated and wild Vitaceae was investigated. The sequences of 25 grape aphids and nine of their infected host plants were compared in a nucleotide identity matrix and a phylogenetic tree using ORF II sequences of GVCV from the grape aphids as well as 139 sequences from wild *Vitis*, *A. cordata*, and cultivated *Vitis*. The number of GVCV DNA molecules acquired and retained in both the mouthparts as well as the

bodies of individual grape aphids was then quantified. This study presents a real-time status of GVCV in grape aphids and relationship of GVCV variants between a mobile vector and sessile hosts. Understanding this virus-vector-host interaction is vital in designing and implementing strategies to prevent GVCV incidences.

LITERATURE REVIEW

Aphids as vectors of plant viruses

Plants are sessile and as such infection by plant viruses occurs either mechanically or through a vector. Vectors can be fungi, mites, nematodes or insects, of these insects, aphids are by far the most frequent and effective transmitters of plant viruses (Brault et al. 2010; Ng and Perry 2004; Stevens and Lacomme 2017; Van Emden and Harrington 2017). This is due to several behavioral and physiological features of aphids. These features, outlined by Ng and Perry 2004, are: (1) the polyphagous behavior of aphids to feed on a wide range of plants, giving viruses the opportunity to infect new hosts. (2) Reproduction parthenogenically, allowing large quantities of aphids to build, and (3) the piercing and sucking mouthparts that allow the aphids to deliver virions directly into plant cells without causing irrevocable damage .

Many species of aphids feed on multiple hosts which aid in the transmission of plant viruses. This wide host range allows aphids to transmit viruses to more than one plant species. When making contact with a new plant, aphids make several brief probes, followed by rejection of the plant regardless of if the plant is a suitable host (Stevens and Lacomme 2017). The aphid will then travel to another plant and repeat this process allowing them to acquire or disperse a virus.

Aphids reproduce rapidly through viviparous parthenogenic reproduction during spring and summer, with as many as five aphids being born per day (Hardie 2017). These aphids can be either apterous (wingless) or alate (winged), with each form having the capability of transmitting plant viruses. Apterous aphids travel locally and are responsible for most virus transmission, however, alate aphids are able to transmit viruses locally and long distance. During local

transmission alate aphids test taste multiple plants allowing viruses to be acquired and dispersed. During long distance transmission alate aphids are aided by air currents and are able to reach several different layers in the atmosphere allowing long distance dispersal (Fereres et al. 2017).

Aphids are equipped with a flexible stylet that allows them to probe into and out of individual cells. This flexible stylet is able to easily pass intercellularly and allows aphids to sample the contents within the epidermal and mesophyll cells (Tjallingii and Esch 1993). As their stylet enters the cell, aphids egest saliva, that if carrying virions, these virions will be down loaded into the cell where virus reproduces and will potentially cause an infection within the plant. After accepting the plant as a suitable host, the stylet is inserted deeply until reaching the sieve elements, and thus aphids are able to acquire viruses at every stage during probing (Ng and Perry 2004).

Grape aphids (*Aphis illinoisensis*)

Aphis illinoisensis (grape aphid) is an aphid that has several known hosts within the Vitaceae family and need more than one host to complete their life cycle. They reproduce both through sexual and asexual reproduction as well as having offspring that are both viviparous and oviparous. In the spring, grape aphids lay eggs on *Viburnum prunifolium* (Black haw) that hatch and begin feeding on Black haw. These newly hatched aphids (called stem mothers) begin feeding and birthing live, all-female offspring, that are genetically identical to the mothers. Alate aphids then search for Vitaceae plants which will be their hosts until fall, when alate male and female aphids form. These aphids will travel back to Black haw where the female will lay eggs that overwinter until spring.

Transmission modes of plant viruses

A critical component in the life cycle of plant viruses is their ability to infect new hosts. Since plants are sessile, and have strong cell walls, this interaction is usually facilitated by a vector, which can be either an insect, nematode, or fungi (Gray and Banerjee 1999). In order for a virus to infect a plant, the virus must first be transferred to the plant and then inside the plants cells without causing damage beyond repair. The majority of plant viruses are transmitted by aphids and white flies, and in most cases these vectors are capable of transmitting viruses in seconds, or as long as hours or days (Gergerich and Dolja 2006). Plant viruses form complex relationships with their vectors, and currently, there are several different types of plant viruses, each having one of three types of transmission mechanisms: nonpersistent, semipersistent, and persistent. Later on, the term circulative was used to describe persistent viruses, while noncirculative was used to describe nonpersistent and semipersistent viruses. With the introduction of electron microscopy, nonpersistent viruses were found to be associated with the cuticle and foregut of insects, while persistent viruses were found to be circulating across the salivary glands, hemolymph, and gut (Blanc et al. 2014).

Non-persistent and semi persistent viruses are able to be picked up and inoculated into another plant in seconds or hours, but the virus is not held in the vector for longer than a couple hours. Semi-persistent viruses are held in the vector for days to weeks, although in both cases the insects lose their ability to vector the viruses after shedding of their exoskeleton, as the virions bind to the cuticle lining in the stylets (Gray and Banerjee 1999). Both of these types of viruses can be classified as non-circulative viruses.

Persistent viruses enter the insect internally and pass multiple cell membranes making its way through the insect and eventually into the salivary canal where viruses are transmitted into a

plant cell. Persistent viruses stay with the insect for the remainder of its life. Persistent viruses require a long period of feeding by the vector, and have a latent period from one day to up to a week (Gray and Banerjee 1999). Persistent viruses can be further classed into propagative or non-propagative. Propagative viruses are able to replicate in the vector, while non-propagative viruses only circulate inside the vector, but do not replicate.

Most plant viruses are transmitted in a non-circulative manner and can be divided into semipersistent and nonpersistent. Semipersistent viruses build up in the vector as they feed until all the binding sites are filled, the virus can be bound in the vector for months or even years (Gray and Banerjee 1999). The virus is typically bound in the foregut in semipersistent viruses. In nonpersistent transmission, the virus binds to only the stylet. Typically, the longer an aphid feeds, the lower amount of virus particles bind. This is thought to occur because the virus binds weakly to the stylet, and as the vector continues to feed, the virus particles that have bound to the stylet may disassociate, these evacuated sites are not suitable for more viruses to attach (Gray and Banerjee 1999). Non-persistent viruses usually only last a few hours in the vector.

One accepted hypothesis of how viruses are transmitted by vectors, is called ingestion-salivation (Kennedy et al. 1962). In this theory, it is thought that the only transmissible virus is bound to the distal tip of the stylet, where the salivary gland and food canal meet. Virus particles bind to this tip as the vector feeds. When the insect moves or feeds on another plant the saliva that is secreted during feeding releases the virus into the plant.

Grapevine vein clearing virus

Zhang et al., 2011 first described a severe vein clearing and decline syndrome on hybrid grapevines in the Midwest region of the United States that began in 2004 (Citation). They

described the symptoms as leaves with translucent veins, short zigzagging internodes and decline of vine vigor, and they discovered that plants with these symptoms had one thing in common, they were all infected with a novel grapevine virus which is named grapevine vein clearing virus (GVCV), the first DNA virus discovered in grapevines.

Grapevine vein clearing virus is a Badnavirus in the Caulimoviridae family. Caulimoviridae are a family of pararetroviruses that replicate through an RNA intermediate (Bhat et al. 2016). GVCV has a circular double stranded genome of about 7,753 bp with three predicted open reading frames (ORFs) (Guo et al. 2014).

GVCV has been previously found to infect six grape cultivars(Zhang et al. 2011), multiple native species of *Vitis*, and native *A. cordata*. Previously it has been found that GVCV infects *A. cordata* at 31% (Petersen et al. 2019a) and wild *Vitis* spp. at 10% (Beach et al. 2017). These wild species are abundant across the Midwest and form a perennially reservoir of virus inoculum for GVCV transmission vectors. Identical GVCV variants have been found to infect both native and cultivated species (Petersen et al. 2019a), giving evidence that GVCV can be transmitted from wild to cultivated Vitaceae.

When sampling wild and cultivated Vitaceae, occasionally a community of grape aphids are present along newly formed shoots and leaves. Aphids are vectors of many viruses, and thus, grape aphids were collected and tested for GVCV. It was found that grape aphids collected on GVCV infected *A. cordata* contained the same GVCV variant as the host plant, and in greenhouse transmission experiments it was shown that grape aphids can transmit GVCV from native *A. cordata* to cultivated Chardonel (Petersen et al. 2019a).

Questions to be answered

The prevalence of GVCV in wild and cultivated Vitaceae is known, however, the prevalence of GVCV in grape aphids is unknown. What is the genetic structure and relationship of GVCV in natural grape aphid population, wild native plants, and cultivated grapevines? Knowing the prevalence of a virus in a vector is crucial in determining rate of spread and for managing viral disease (Stevens and Lacomme 2017). Identical GVCV variants exist in both cultivated and native Vitaceae, but this relationship is currently unknown within its mobile vector. Several different transmission modes exist within aphids, and currently not much is known about the quantities of virus present in aphids within their natural environment. This knowledge will reveal GVCV epidemics in native and cultivated plants by filling in a missing link between a virus reservoir and a commercial vineyard and is central to developing a disease management strategy.

MATERIALS AND METHODS

Collection of Grape Aphids and Plant Tissues

Grape aphids and their infested plant tissues were collected in their natural environment in May, June, and July 2018 from Springfield, Missouri (MO) and surrounding areas as well as areas near Festus in Jefferson county MO (Fig. 1). Multiple groups of grape aphids were found on each infested plant. Each group along a vine was collected individually along with the plant sample. In this project, a colony was defined as a collection of all grape aphids on an entire plant. A community was defined as a distinct group of aphids on one branch of the infested plant (Fig. 2). Samples of both aphids and vine branches were temporarily stored in one-gallon plastic bags containing a moist paper towel to prevent plant tissues from wilting and aphids from dying. Coordinates of each sample were recorded with the native iPhone app. Compass (Apple, Inc.) and later Gaia GPS (Trailbehind, Inc.) to improve accuracy. Samples were either transferred to the lab within two hours or stored in a cooler for up to two days before transfer. Aphids were collected from each sample and immersed in 80% ethanol in a 2 ml polypropylene tube for storage at -20°C until DNA extraction. Plant leaf samples were weighed to 5 ± 45 mg, wrapped in aluminum foil and placed in liquid nitrogen before they were stored at -80°C.

DNA extraction from grape aphids and plants

Grape aphids are very small, weighing up to 0.2 mg depending on their stage of development. Extracting enough detectable virus DNA from grape aphids poses a significant challenge. Therefore, GVCV was first tested on groups of ten grape aphids. After having success, individual grape aphids were tested for GVCV, as each viruliferous grape aphid has the

potential to transmit the virus, and groups of grape aphids contain potentially more than one virus variant. Randomly testing single grape aphids also reveals the percentage of the grape aphid population carrying the virus. Cochran's equation was used to determine the sample size needed to predict the occurrence of GVCV in single grape aphids with a 95% confidence level with a ± 5% margin of error.

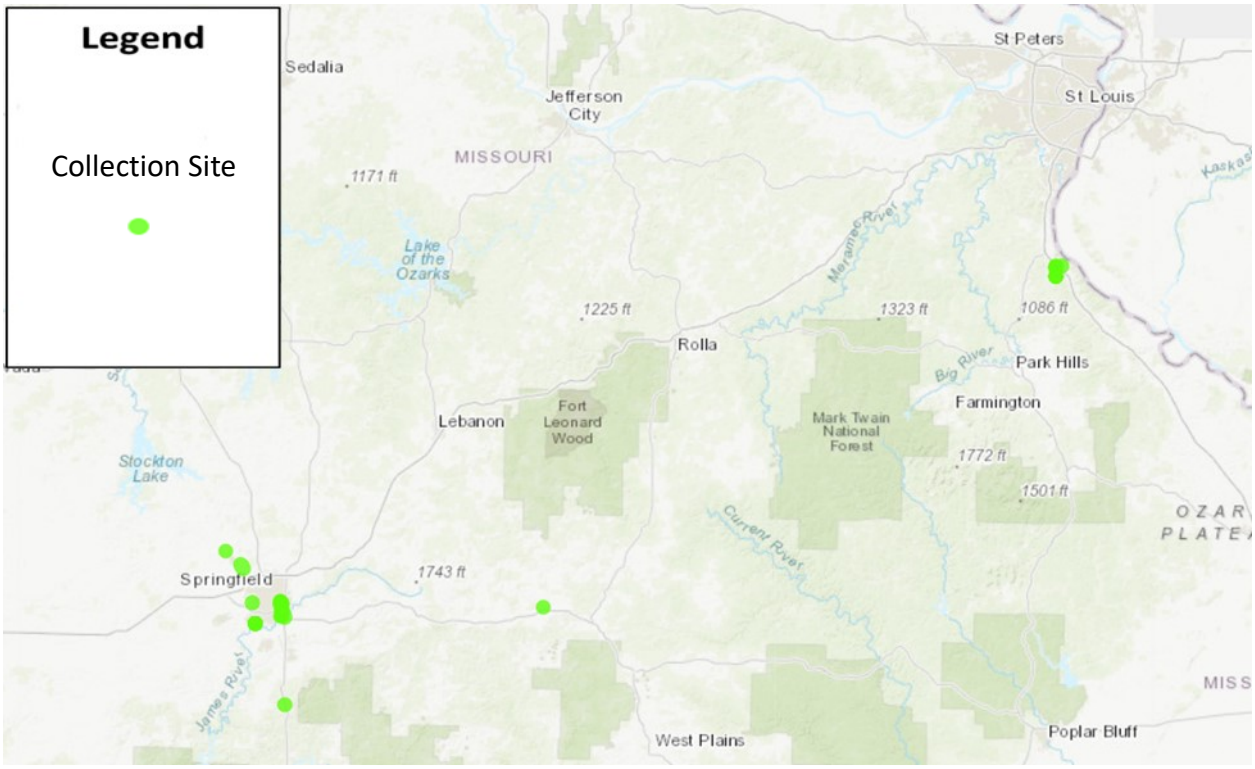

Fig. 1. Collection sites of grape aphids and host plants in Missouri, 2018.

In the first phase, DNA was extracted collectively from a group of 10 aphids from each of the 105 communities using an Insect DNA E.Z.N.A Kit (Omega Bio-tek, Inc.) according to the protocol with two modifications. During the first step of the protocol, aphids were crushed with 1000 µl sealed pipette tips in CTL buffer provided by the kit instead of ground in liquid nitrogen. During the elution step, 30 µl of ddH_20 was used for elution instead of 50 µl ddH_20.

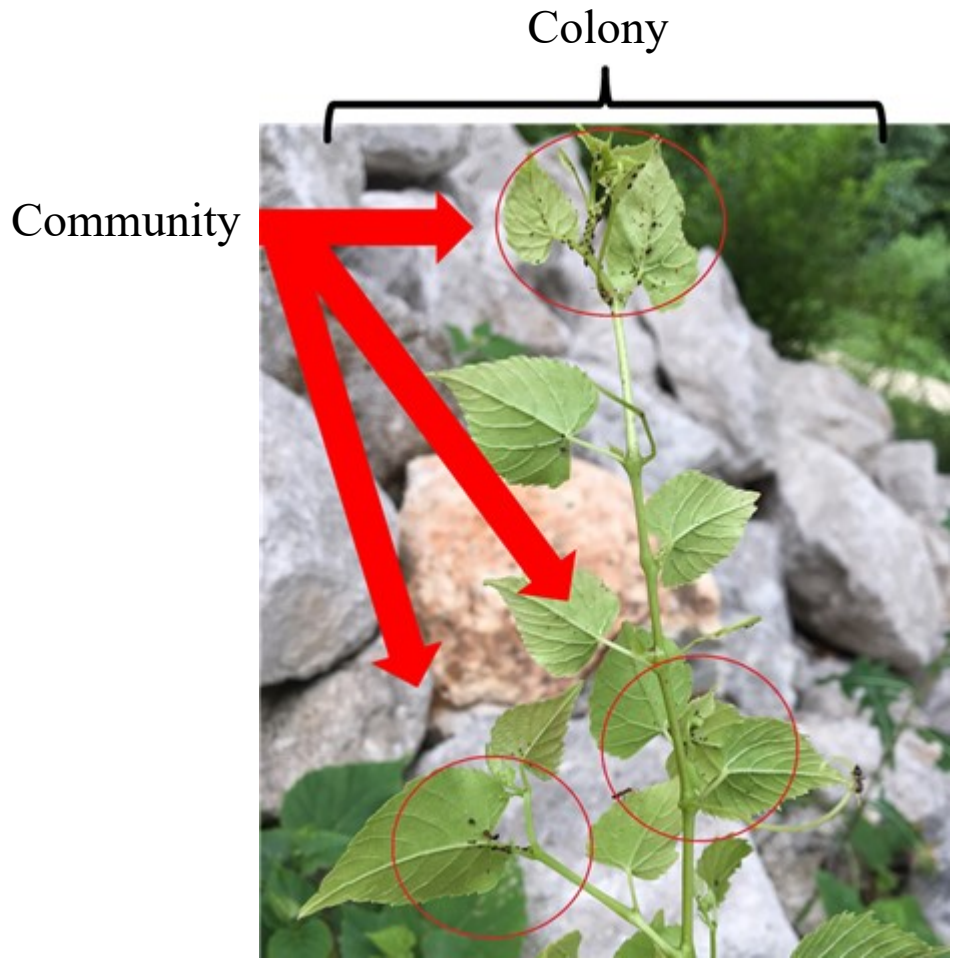

Fig. 2. An image depicting the difference between a colony and a community of grape aphids on *Ampelopsis cordata.*

In the second phase, DNA was extracted from 512 single aphids using a 10% chelex-100 system in STE buffer (0.1 mM NaCl, 1mM EDTA pH 8, 10 mM Tris-HCL pH 8) according to a modified protocol from Wang and Wang (2012). Grape aphids were removed from storage and examined under a microscope prior to extraction to record size, color, and life stage of each aphid. A single grape aphid was ground in 100 µl of the chelex DNA extraction solution in a sterile tube. Ground aphid tissues were incubated at 65°C for 20 minutes, and then boiled at 100°C for 10 minutes. Afterwards, the samples were centrifuged at 15,700 g for five minutes and

the supernatant containing the DNA was transferred into new sterile tubes. The extracted DNA was analyzed on a Nanodrop 1000 instrument (Thermofisher Scientific). Final DNA concentration was adjusted to 50 ng/µl.

Grape aphid DNA extraction for real-time quantitative PCR. The number of GVCV genomes in the stylet and body of single grape aphids were also measured. Twenty single aphids were selected from a GVCV infested community. Grape aphids were examined under a microscope and the stylets of single aphids were severed from their bodies using a razorblade. To prevent cross contamination, razorblades were dipped in a 10% bleach solution and wiped dry. This step was repeated three times in between samples. The stylet and the body were then placed into separate tubes and labeled accordingly. DNA was extracted from the stylet and body parts by the Insect DNA E.Z.N.A kit (Omega Bio-tek, Inc.) with two modifications: stylets from the aphids were not ground to prevent accidental removal, and DNA was eluted in 40 µl nuclease-free water.

Plant DNA extraction. Forty-five mg of leaf tissue from each aphid infested plant was processed using a SYNERGY 2.0 Plant DNA Extraction Kit (OPS DIAGNOSTICS) according to the protocol. The DNA was eluted in 100 µl and the concentration and purity was measured using a Nanodrop 1000 instrument (Thermo Fisher Scientific). The eluted DNA was diluted to 10 ng/µl and stored at -20°C.

PCR detection of GVCV

To check for GVCV in grape aphid communities and single aphids, and to ensure that high quality DNA was extracted, a duplex polymerase chain reaction (PCR) was used. The first set of primers, 5044F and 5387R (Table 1A) amplified a 344-bp fragment within the ORF III

region of GVCV. The second set of primers, EFF and EFR (Table 1A) amplified a 200-bp fragment within the grape aphid elongation Factor 1-α (EF1) (Accession KC897260) gene to ensure the quality of the extracted DNA. When testing grape aphids using PCR, one µl of undiluted DNA from communities, and 50 ng/µl DNA from single grape aphids was used as template in a total volume of 25 µl. The thermocycling conditions for this duplex PCR were: an initial denaturation at 95°C for 1 minute followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s and finally 72°C for 10 mins.

Plant samples were tested for GVCV with primers 5044F and 5387R. To ensure quality DNA from the plant samples, primers 16sF and 16sR (Table 1A) amplifying a 105-bp fragment of the 16S ribosomal RNA gene, were used. The thermocycling conditions were: a one minute initial denaturation at 95°C followed by 35 cycles of 95°C for 15 s, 57°C for 15 s and 72°C for 25 s, and then a final extension of 72°C for five mins.

To prevent false negatives due to the variability within the GVCV genome, a second set of primers, 963F and 1634R (Table 1A), was used to amplify the ORF II region of the virus in both plant and grape aphid samples. Polymerase chain reaction using primers 963F and 1634R were ran using an initial 95°C for one minute denaturation, 45 cycles of 95°C for 15s, 61°C for 30 s, 72°C for one min, and 72°C for seven mins.

Sequencing of GVCV ORF II

Grape aphid and plant tissues positive for GVCV were amplified with primers overlapping the ORF II region (963F and 1634R). The amplified products were gel-purified from a 1% agarose gel and purified through a Wizard SV Gel and PCR Clean-Up System (Promega Corporation). The resulting DNA purity and concentration was measured using a Nanodrop 1000

instrument (Thermo Fisher Scientific). DNA samples with low concentrations were re-amplified in a second PCR and extracted using the same method. Nested PCR, as exemplified in Fig. 3,

Table 1. Oligonucleotides used in this study **A,** A list of primers designed to amplify *Aphis illinoisensis*, *Vitis* spp. or GVCV specific DNA fragments **B,** Oligonucleotide designed for generation of the standard curve in a qPCR assay.

A

Organism	Primer name	Sequence 5'-3'
A. Illinoisensis	EFF	GGCTCTCCGTCTCCCACTCC
A. Illinoisensis	EFR	TGGTGATGTTGGCAGGTGCG
Vitis spp.	16s F	TGCTTAACACATGCAAGTCGGA
Vitis spp.	16s R	AGCCGTTTCCAGCTGTTGTTC
GVCV	5044F	ATTCCAGCCTCTTGCGCAG
GVCV	5387R	TCATTCCCTGCGAGGATCAT
GVCV	963F	TCCATCACAGATCTAACGGCA
GVCV	1634R	CAAGGTAGCGGGCACGAG
GVCV	402F	AGTAGGAGAGGACGGACAACT
GVCV	560R	GGGTGTGCGTTCAGATCTCT
GVCV	877F	ACCAGATCGAGCTCCTTCG
GVCV	1866R	TCTTGCTGCCGGTCTATGAC

B

Organism	Oligonucleotide name	Sequence 5'-3'
GVCV	GVCV-155	TTCAAGGGAACGGAACCTGGAG ACACCGGCCGAGTTCTTAGTAA GCGGTTCAAGAAGGAGACTGAT GCAAAGAATAGAACAACAAAAG TTTGAGGAGGAGATAGAATCTT

was later implemented using primers 877F and 1866R (Table 1A) initially and in the second round of PCR, 963F and 1866R. The thermocycling conditions for the nested PCR reaction were: an initial denaturation of 94°C for 1 min, 40 cycles of 94°C for 15 s, 61°C for 30 s and 68°C for 1 min, and then 7 mins at 68°C.

DNA samples were prepared for sequencing by following the directions provided by Nevada Genomic's Sanger sequencing sample submission guidelines. Two picomoles of either 963F or 1634R were added to 20 ng of DNA and sent to Nevada Genomics. The resulting sequencing chromatograms were loaded into Codoncode Aligner (Codeoncode Corporation), and aligned using the LBC0903 reference genome. Only sequences containing nucleotides at least 20 or higher using the Phred quality score were used. Sequences were exported as a Fasta file.

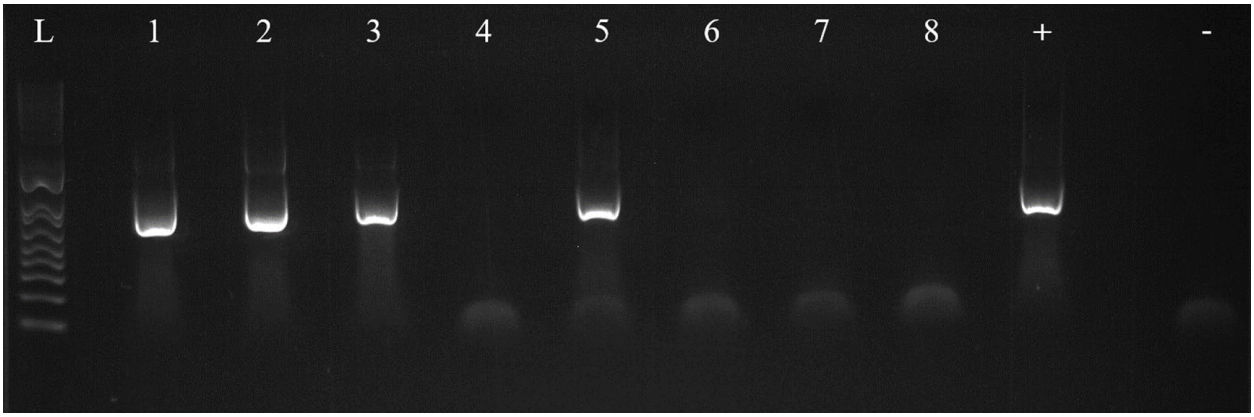

Fig. 3. Nested PCR on eight individual grape aphid samples. From left to right: DNA ladder L, nested PCR samples 1-8, positive control +, and negative control -.

Sequence analysis

GVCV ORF II sequences derived from both *Vitis* spp. and grape aphids were loaded into MEGA X (Kumar et al. 2018) software and aligned using the default settings in the ClustalW algorithm (Thompson et al. 1994). A phylogenetic analysis of 174 sequences using the Maximum Likelyhood method was created from the aligned sequences.

Using 25 GVCV ORF II sequences from grape aphids, and nine sequences from their infected host plants, a percent identity matrix was created using Clustal2.1. Groups of GVCV isolates ≥99% similar were then assigned a color and plotted geographically using ArcGis.

Quantitative PCR optimization using SYBR green

Real-time quantitative PCR (qPCR) using a fluorescent double-stranded DNA (dsDNA) binding dye such as SYBR Green requires several considerations when designing a robust assay. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) was proposed to establish a set of minimum guidelines to standardize and evaluate qPCR experiments (Bustin et al. 2009). Within these guidelines, several essential parameters that need to be addressed during the optimization of the assay are: PCR efficiency, linear dynamic range, limit of detection (LOD) and precision.

Seven GVCV genomes from the NCBI database were aligned using Geneious Prime software (Geneious Prime 2019.2.3). Two primer sites that are conserved among all seven genomes overlapping part of the intergenic region and ORF I were chosen (Fig. 4). The forward and reverse primers 402F and 560R (qPCR primers) (Table 1A) were designed to amplify a 155-bp fragment. To maximize PCR efficiency, the annealing temperature of the qPCR primers was determined by veriflex PCR. A temperature gradient from 58 to 68°C increasing by intervals of 3°C was used for the annealing temperature step in a qPCR reaction. The SYBR normalized to ROX fluorescence (dRn) was measured on the 33rd cycle and plotted in Excel (Fig. 5).

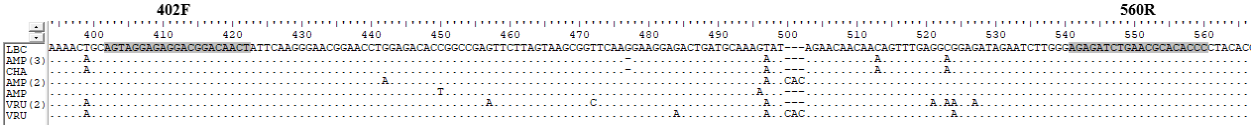

Fig. 4. Multiple sequence alignment of seven Grapevine vein clearing virus (GVCV) genomes. The comparison was created using sequences from GenBank. Dots represent nucleotides that are identical to the GVCV-CHA reference sequence, and letters represent nucleotides that differ from the GVCV-CHA sequence. Forward primer 402F and reverse primer 560R are highlighted in gray, and GVCV variants are show on the left. The alignment was created using MEGA X and the Muscle algorithm, the sequence is displayed using BioEdit.

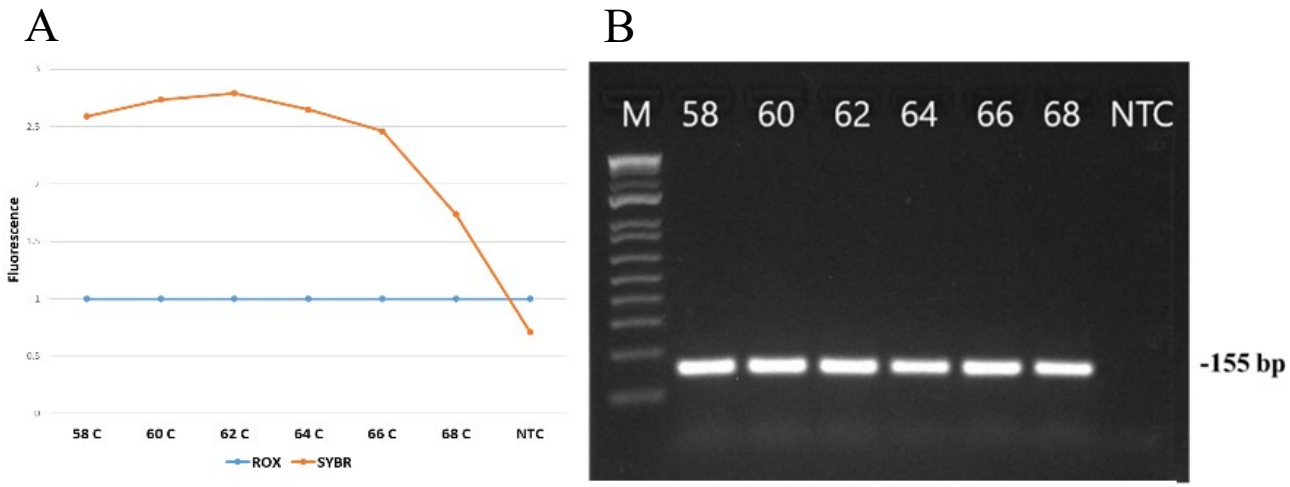

Fig. 5. Efficiency testing of primers 402F and 560R at an increasing annealing temperature. The annealing step in a qPCR assay was increased in intervals of 3°C from 58 C to 68°C. The fluorescence level was measured at the 33rd cycle and **A,** Fluorescence density was plotted into a line graph. **B,** PCR products were analyzed on an agarose gel.

Differing concentrations of the forward and reverse primers can affect the efficiency of a PCR assay, therefore, the optimal forward and reverse primer concentration was determined by a primer optimization matrix. Incrementally increasing concentrations of both the forward and reverse qPCR primers were added to the X and Y-axis in duplicate qPCRs from 100 – 800 nm in 100 nm increments. Cq values from each point in the primer matrix were plotted in Excel (Microsoft) and used to create a heat map (Fig. 6).

During DNA extraction, inhibitors of PCR could be purified along with the DNA. These inhibitors reduce the efficiency of the PCR reaction and increase the Cq value reflecting an inaccurate amount of initial template quantities. To address this, sample DNA can be diluted to reduce the level of inhibitors present until optimal efficiency is obtained. To check the amount of inhibitor present in DNA samples, an equalized pool of samples reflecting the average PCR inhibitor level was created. The pooled sample was serially diluted 1:2 in five points in duplicate

(serial 1 and serial 2, Fig. 7) and ran through a qPCR reaction. The Cq values were plotted in Excel (Microsoft) to create a standard curve (Fig. 7).

560R in nM \ 402F in nM	100	100	200	200	400	400	600	600	800	800
100	29.97	29.89	29.19	29.46	28.93	28.91	NA	29.04	28.79	29
200	29.76	29.48	28.8	28.64	28.72	28.79	28.61	28.78	28.69	28.8
400	29.39	29.36	28.83	28.71	28.66	28.74	28.83	28.96	28.77	28.94
600	29.58	29.61	28.8	28.53	28.67	28.6	28.96	28.93	28.76	28.86
800	29.28	29.05	28.81	28.8	28.84	28.69	28.73	28.7	28.65	28.4

Fig. 6. Heat map of a qPCR assay primer optimization matrix. Primers 402F and 560R were used in increasing concentrations from 100 to 800 nm. Cq values of each reaction were then imported into Microsoft Excel and mapped from lowest Cq (green) to highest Cq (red).

During DNA extraction, inhibitors of PCR could be purified along with the DNA. These inhibitors reduce the efficiency of the PCR reaction and increase the Cq value reflecting an inaccurate amount of initial template quantities. To address this, sample DNA can be diluted to reduce the level of inhibitors present until optimal efficiency is obtained. To check the amount of inhibitor present in DNA samples, an equalized pool of samples reflecting the average PCR inhibitor level was created. The pooled sample was serially diluted 1:2 in five points in duplicate (serial 1 and serial 2, Fig. 7) and ran through a qPCR reaction. The Cq values were plotted in Excel (Microsoft) to create a standard curve (Fig. 7).

The absolute quantitative method of qPCR requires that you run a standard of known copies alongside unknown samples. Using the GVCV genome from the Amp3 isolate (Accession

MH319694) from the NCBI database, the 155-bp theoretical dsDNA fragment (GVCV-155) (Table 1B) generated during PCR amplification using the qPCR primers was ordered from Eurofins Genomics Gene Fragments. GVCV-155 was rehydrated in nuclease-free water according to the manufacturer's recommendations, measured with a spectrophotometer and stored at -20°C. The DNA concentration of GVCV-155 was then diluted from ng/µl to DNA copy number/µl.

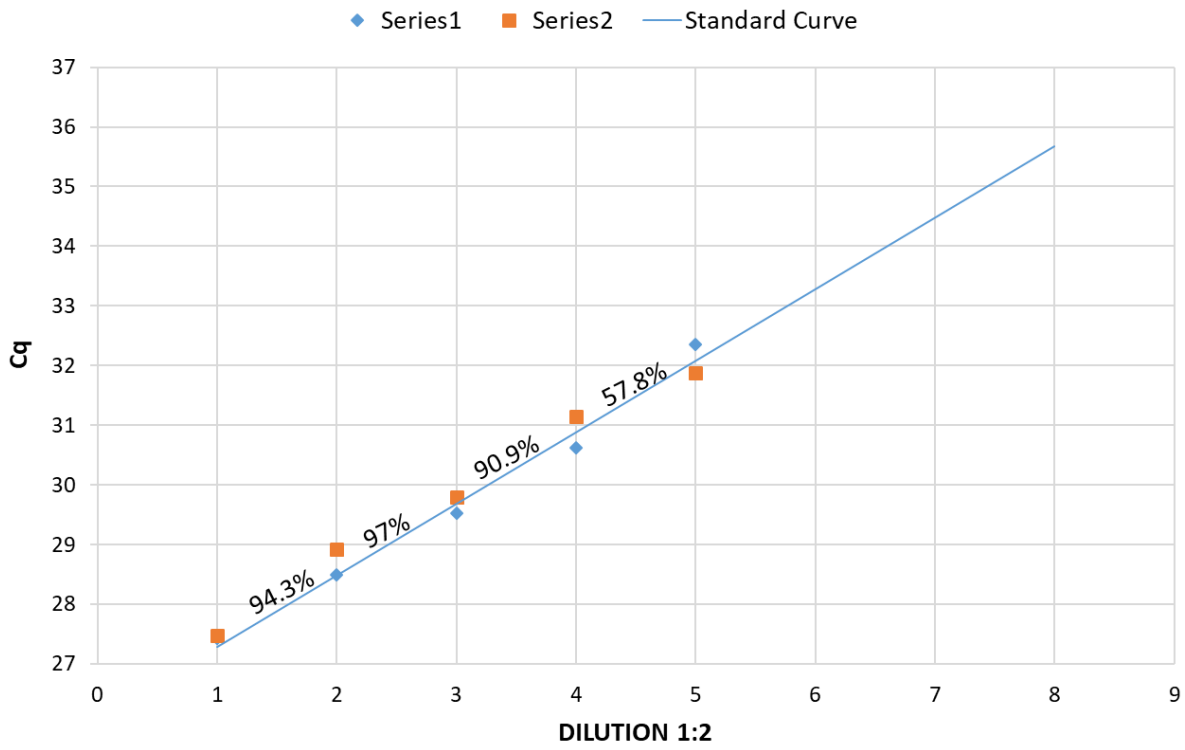

Fig. 7. Optimal DNA sample dilution level using the Omega E.Z.N.A Insect DNA extraction kit. Ten microliters from random samples extracted from the Omega E.Z.N.A Insect DNA extraction kit were used to create a representative sample of the average amount of PCR inhibitors present. DNA was diluted 1:2 at each point and tested in a quantitative PCR (qPCR) reaction in duplicate (Series1 and Series2). Quantitative cycle (Cq) values were used to create a best fit line and the efficiency between each point is displayed.

RESULTS

GVCV Detection in Grape Aphids

One hundred and five grape aphid communities were tested using PCR and 91 (87%) tested positive for GVCV (Table 2). Positive and negative communities were plotted on a map, and each location sampled was found to have at least one positive community (Fig. 8). A DNA extraction method modified from Wang and Wang 2012 was used to detect GVCV in single grape aphids. GVCV was detected in single grape aphids (Fig. 9). To investigate the prevalence of GVCV among native grape aphid populations, aphids were collected from 9 locations in the state of Missouri. Cochran's equation was used to determine that 385 samples were needed given a large unknown population size, an unknown population proportion and a 95% confidence level with a ±5% margin of error (Israel 1992). GVCV was detected in 212 of 525 single aphids, a 40% prevalence (Table 3). Therefore, the 40% prevalence of GVCV in sampled 525 grape aphids in the state of Missouri is at a 95% confidence level with a 4.1% margin of error.

Table 2. Prevalence of grapevine vein clearing virus (GVCV) in 105 communities of grape aphids (*Aphis illinoisensis*) that were collected from native hosts in six locations across the state of Missouri, USA in 2018.

Location of Samples (Town, MO)	Number of samples	GVCV Positive	Percentage
Springfield	52	47	90%
Battlefield	26	23	88%
Willard	2	2	100%
Saddlebrook	2	2	100%
Mountain Grove	1	1	100%
Plattin	22	16	73%
Total	105	91	87%

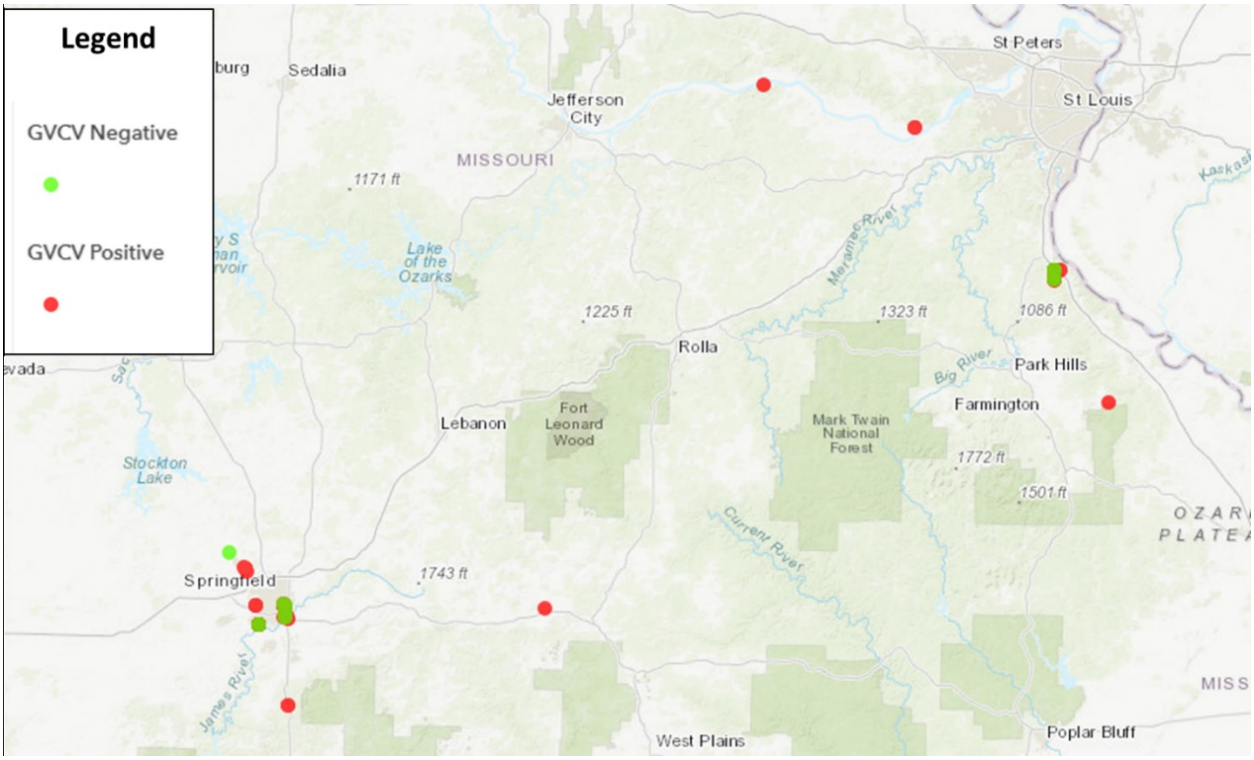

Fig. 8. GVCV positive (red) and negative (green) grape aphid community collection sites in Missouri in 2018 and 2019. Each collection site was plotted at 25% transparency using each collection site's unique GPS coordinates.

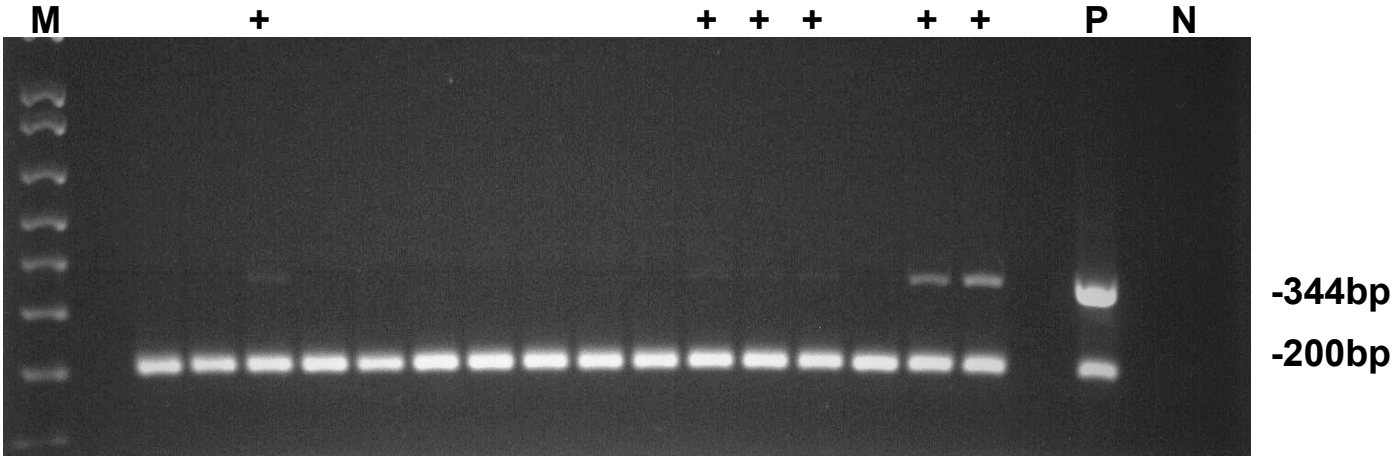

Fig. 9. Electrophoresis image of grapevine vein clearing virus in single grape aphids**.** A set of primers was designed to amplify a 344 bp fragment of GVCV by polymerase chain reaction (PCR), its presence is shown by a '+' sign. Another pair of primers were designed to amplify a 200 bp fragment of the elongation factor (EF) gene in the grape aphid genome. P: GVCV-positive aphid DNA used as a positive control; N: No DNA template as a negative control. M: markers of DNA molecule size in base pairs.

Table 3. Prevalence of grapevine vein clearing virus (GVCV) in single grape aphids (*Aphis illinoisensis*) on native and cultivated Vitaceae that were collected across the state of Missouri, USA, in 2018 and 2019.

Location of samples (Town, MO)	Number of samples	GVCV Positive	Percentage
Springfield	192	80	42%
Battlefield	54	20	37%
Willard	10	3	30%
Saddlebrook	1	1	100%
Mountain Grove	6	3	50%
Plattin	107	39	36%
Hermann[1]	14	7	50%
Augusta	30	10	33%
Coffman	111	49	44%
Total	525[2]	212	40%

[1] Bold identifies locations where aphids were collected within 500 meters of a vineyard.
[2] Total consists of 493 aphids from native *Vitis* and *Ampelopsis* plants and 32 aphids from cultivated grapevines.

Comparison of GVCV ORF II sequences in grape aphids and hosts

Nucleotide sequences of ORF II are the most variable among GVCV genomes, and are thus used as criteria for delineating GVCV variants (Beach et al. 2017). If the ORF II nucleotides are identical, it is likely that the entire genome of the two GVCV isolates are identical (Petersen et al. 2019b). Therefore, ORF II is a good candidate for differentiating GVCV variants. Twenty five GVCV ORF II sequences from grape aphids and nine sequences from host plants were acquired. A percent identity matrix was then created to compare the 34 ORF II sequences.

Identity of the GVCV ORF II sequences ranged from 85.42 to 100% (Fig. 10). GVCV ORF II sequences from grape aphids with 99% or greater identity are shown in green in Fig. 10. Fifteen GVCV isolates from grape aphids are arranged into five distinct groups (numbered, Fig. 10; Fig. 11; Table 4). In group 1, four aphids and their host *A. cordata* all contained the same GVCV variant (Fig. 10, lines 3-7; Fig. 11, red). Group 2 displays the same GVCV variant in two

aphids (Fig. 10, lines 9, 10), one from a GVCV-positive *A. cordata*, and the other from a GVCV-negative *Vitis* host, 288 km apart (Fig. 11, orange; Table 4). The GVCV variant in group 3 was found in aphids colonizing both *Vitis* and *A. cordata*. Four were collected proximally to each other (Fig. 10, lines 11-14); however, one was found 277 km away (Fig. 9, line 17; Fig. 11, purple; Table 4). In group 4, the same GVCV variant is from two grape aphids collected from GVCV-positive *A. cordata* 280 km apart (Fig. 10, lines 20 and 22; Fig. 11, green; Table 4). The GVCV sequence from one of the *A. cordata* plants matched the GVCV isolated from the two aphids (Fig. 10, line 21), whereas the GVCV from the second *A. cordata* had only 90-91% identity with GVCV from the two aphid samples (Fig. 10, line 7, 19Amp006). In group 5, the two aphids carry the same variant that were collected from GVCV-negative *A. cordata* and *Vitis* plants separated by 280 km (Fig. 10, lines 27, 28; Fig. 11, black; Table 4).

In 11 cases, the ORF IIs of GVCV isolates in grape aphids were ≥99% identical to those of GVCV isolates in plants from which the aphids were *not* collected (italicized, Fig. 10). The GVCV-infected Vitaceae are 18Vit054, 18Vit053, 18Amp01, and 18Amp031, and each of these plants are more than 270 km from aphids carrying the same variant.

This detailed analysis shows that aphids carry a diverse array of GVCV variants. Aphids feeding side-by-side on host plants carry the same variant in few instances, but different variants in most cases. These GVCV-bearing aphids may migrate long distances and feed on different species of hosts that are both GVCV-positive and GVCV-negative.

Phylogenetic analysis

A phylogenetic tree was constructed by using all currently known GVCV ORF II sequences, which amount to a total of 174 sequences. Twenty five sequences were from grape

aphids and 149 were from wild *Vitis, A. cordata*, and cultivated grapevines (Fig. 12). Within the phylogenetic tree, there are 12 clades that contained GVCV ORF II sequences from grape aphids (Fig. 12A). Three well-supported clades (bootstrap values over 0.5) include aphid GVCV isolates in the same lineage with both cultivated grapevines and wild Vitaceae (Fig. 12B, C, D).

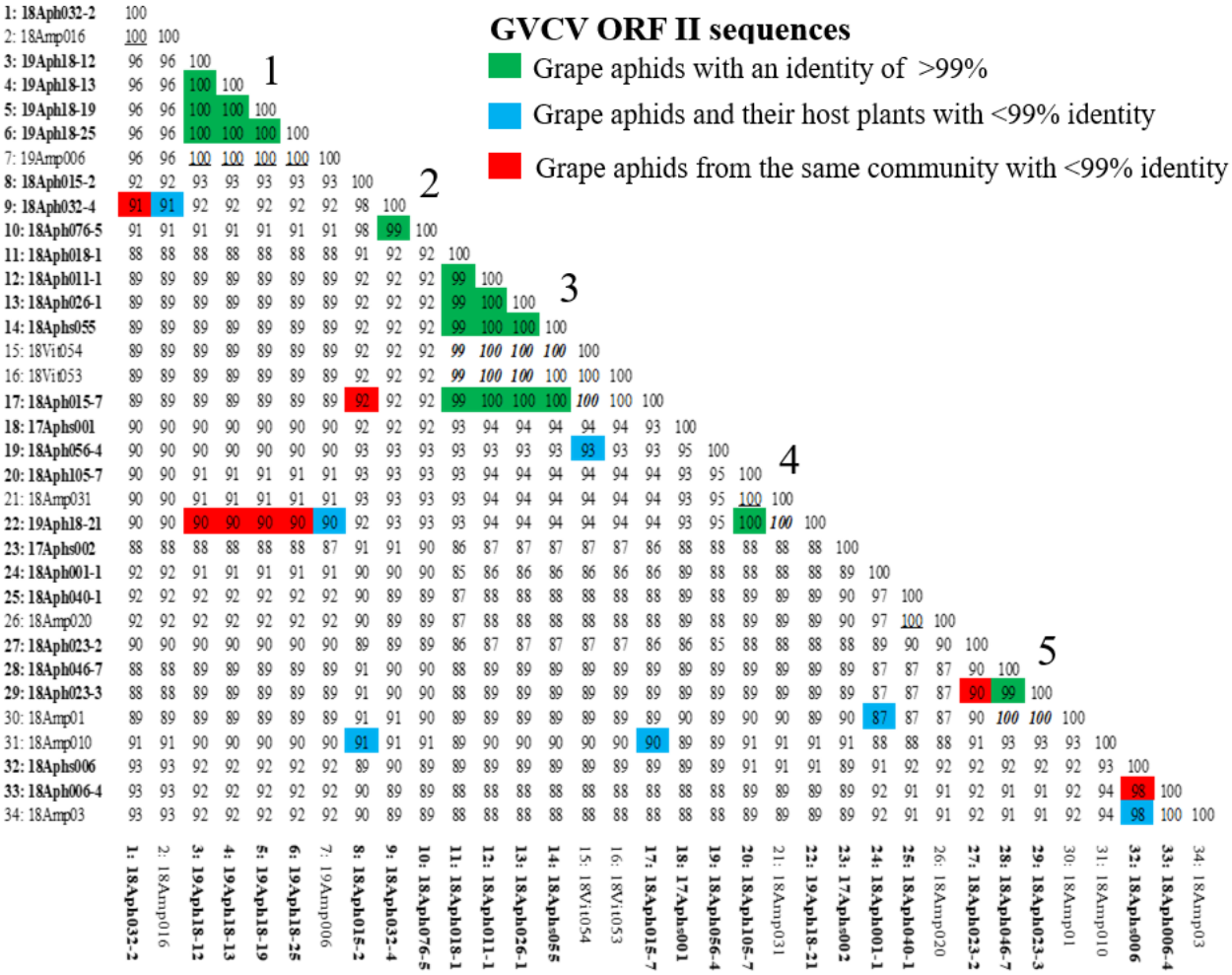

Fig. 10. Percent identity matrix of 25 GVCV ORF II sequences from grape aphids and nine sequences from their host plants**.** Sequences from grape aphids with ≥99% identity are green. Blue indicates GVCV isolates from grape aphids and host plants sharing <99% identity in ORF II. Sequences from grape aphids of the same community with <99% identity are red. GVCV isolates whose ORF II sequences are ≥99% identical to a Vitaceae from which they were not collected are bold and italicized. Underlined indicates that GVCV isolates from grape aphids and their host plants are identical. Grape aphid samples are bold.

Fig. 11. Location of grape aphids collected from the five groups with ≥99% similarity (green, numbered, Fig. 10).

In clade B, the ORF IIs of two GVCV isolates '18Aph040-1' and its host plant 18Amp020 align within the same clade, as well as two GVCV isolates from vineyards in close proximity (Fig. 12B). This clade also contains two GVCV isolates that are separated by a distance of almost 300 km, one from a grape aphid and the other from a native *A. cordata*. Two highly similar GVCV isolates, '18Aph001-1' and 'AMP1' sharing 99.5% nucleotides in ORF II, were not only separated spatially, but also temporally and were collected three years apart.

Clade C contains nine GVCV isolates from grape aphids, wild *Vitis* and cultivated grapevines. In one lineage of five isolates that share identical ORF II sequences, two are from grape aphids and two from *Vitis* plants in the same location, however, one is from a grape aphid that was sampled almost 300 km away. Two identical GVCV isolates are from cultivated grapevines at a distance of over 200 km. The very first GVCV-CHA variant is also present in

this clade and shares more than 99.47% identical nucleotides to isolates from grape aphids, cultivated grapevines, and wild *Vitis*.

Table 4. Five groups of grapevine vein clearing virus (GVCV) variants in *Aphis illinoisensis* (grape aphids) that share 99% or more identical nucleotides in ORF II.

Groups	GVCV isolates	Town of Missouri	Distance (km)	Host plant	GVCV in host plant
1	19Aph018-12	Coffman	0	19Amp006[1]	+
	19Aph018-13	Coffman		19Amp006	+
	19Aph018-19	Coffman		19Amp006	+
	19Aph018-25	Coffman		19Amp006	+
2	18Aph032-4	Plattin	288.8	18Amp016	+
	18Aph076-5	Battlefield		18Vit056	-
3	18Aph011-1	Springfield	277.8	18Vit023	-
	18Aph018-1	Springfield		18Vit030	-
	18Aphs055*	Springfield		18Vit053	+
	18Aph015-7	Springfield		18Amp010	+
	18Aph026-1	Plattin		18Vit038	-
4	19Aph018-21	Coffman	280.7	19Amp006	+
	18Aph105-7	Springfield		18Amp031	+
5	18Aph46-7	Battlefield	283.1	18Amp21-5	-
	18Aph23-3	Plattin		18Vit035	-

[1] Sample Code "19Amp006", 19: year of 2019; Amp: host plant *Ampelopsis cordata*; Vit: *Vitis* spp; 006: sample number.
* indicates the ORF II sequence was acquired from a community of aphids.

Clade D contains 14 GVCV isolates that are from all four sources: grape aphids, cultivated grapevines, wild *Vitis*, and *A. cordata*. The distance at which these samples were collected ranges from less than 5 km to almost 300 km.

This analysis shows that grape aphids carry a diverse range of GVCV variants that are highly related to the variants found in plants. These variants are transmitted by aphids throughout

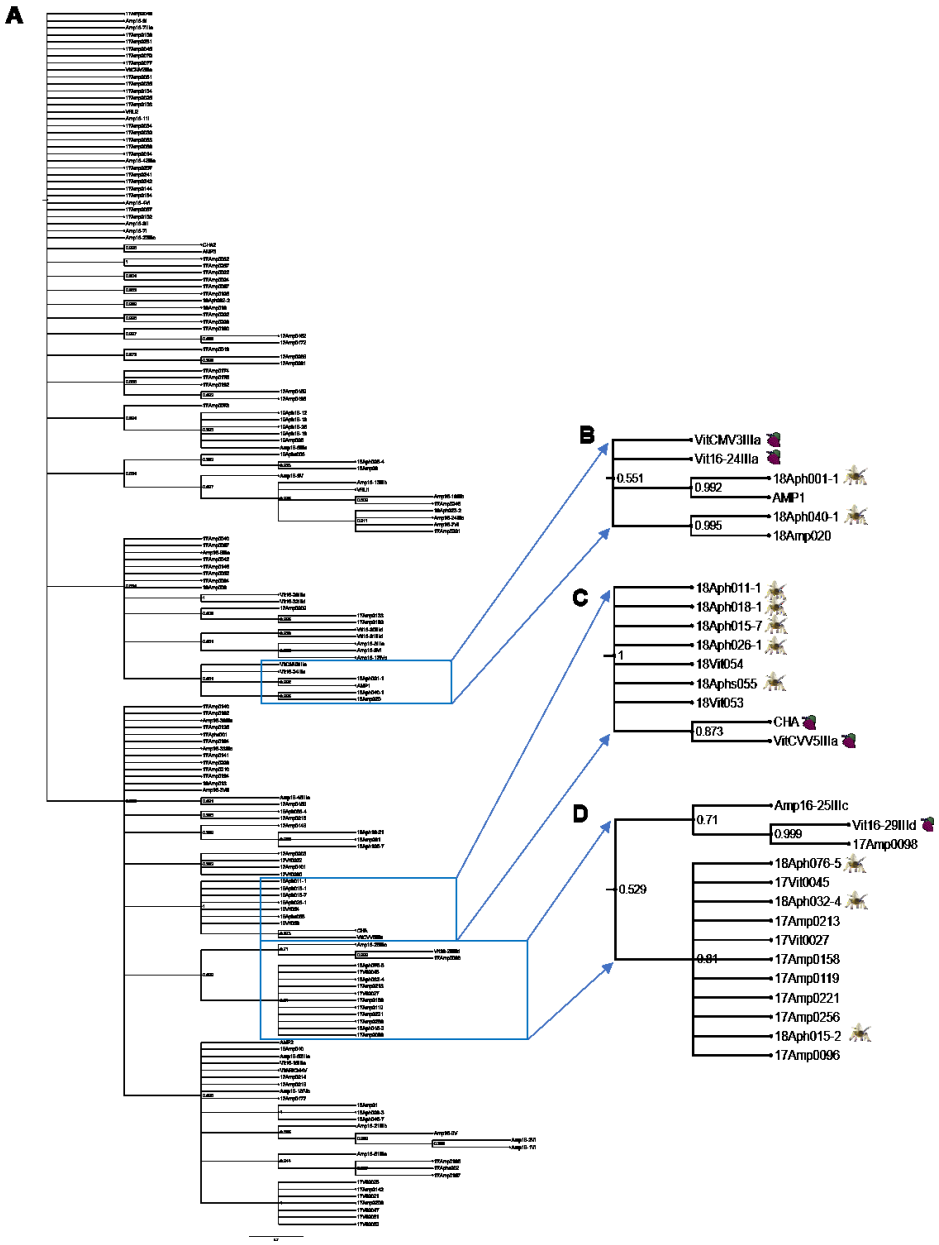

Fig. 12. Phylogenetic placement of GVCV isolates from grape aphids and Vitaceae. **A,** Phylogenetic tree of 174 GVCV ORF II sequences from grape aphids, cultivated grapevines and wild Vitaceae. The tree was created using the Maximum Likelihood method and Tamura-Nei model with 1000 bootstrap replicates. Clades produced in less than 50% of replicates are collapsed. The tree with the highest log likelihood (-7115.64) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X.
B, C, D, Highlighted clades showing the close relationship of GVCV isolates from grape aphids as indicated by an aphid illustration, cultivated grapevines in vineyards indicated by a grape cluster, and wild Vitaceae from native habitats. Code of a sample "19Amp006", 19: year of 2019; Amp: host plant *Ampelopsis cordata*; Vit: *Vitis* spp.; 006: series of sample n

wild plants and cultivated grapevines at different locations across the state of Missouri.

Real-Time Quantitative PCR on Grape Aphid Stylets and Bodies

While tools for in silico analysis are important in the validation of qPCR assays, it is essential that specificity be measured empirically (Bustin et al. 2009). Several methods can be used to determine the specificity of a qPCR assay such as DNA sequencing, dissociation curve profile, and gel electrophoresis. Using melt curve analysis, only one product was observed when testing the primers at concentrations of both the forward and reverse primer ranging from 100 – 800 nm of each (Fig. 13). Furthermore, the PCR product was sequenced and found to be identical to the delimited region on the GVCV reference genome (GenBank accession number: JF301669.2)

Sensitivity of the qPCR assay. To assess the sensitivity of the real-time qPCR assay,10 fold serial dilutions were made from 5 x 10^8 to 5 copies from the standard stock solution, and each dilution was tested in triplicate. Analysis of the standard curve showed that a high level of efficiency was obtained (Eff. = 95.7), within the range of 5 x 10^8 to 5 copies. Within our settings, a high level of efficiency was achieved making it possible to estimate the initial copy number reliably.

Quantification of GVCV in aphids. Quantity of virions in aphid vectors has been measured in groups and individual aphids by qPCR (Khelifa 2019a; Liu et al. 2019), but those experiments were done under greenhouse conditions. Quantitative PCR was applied to measure GVCV genomes in the stylet and body of 20 individual aphids from natural populations on Vitaceae in their native habitats. GVCV-specific fragments were amplified from all 20 grape aphid stylets and their bodies. The total volume of the extracted DNA in conjunction with the

estimated efficiency of the DNA extraction kit was used to determine the number of viral genome copies. The stylets contained 14 to 260,571 GVCV viral genomes while the bodies contained from 136 to 1,713,143 genome copies (Table 5).

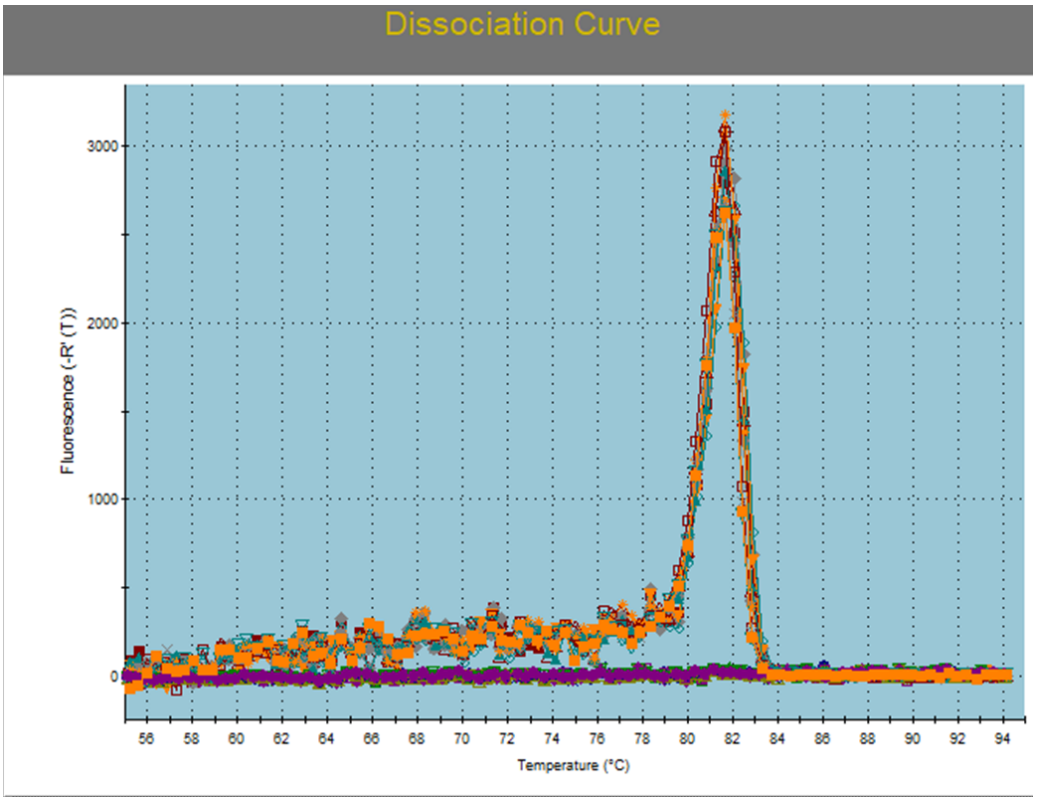

Fig. 13. Dissociation curve of the primer matrix assay showing the presence of only one product. A primer matrix was created using the forward and reverse quantitative polymerase chain reaction (qPCR) primers ranging from concentrations of 100 – 800 nm of each primer (Fig. 6). The matrix was used as template in a qPCR reaction followed by melt curve analysis.

Two of these aphids contained very high GVCV genome numbers in their stylets, ranging from 51,388 in aphid sample 9 to 260,571 in sample 10. Interestingly, both aphids were wingless. In contrast, the number of GVCV genomes is low in two of the three stylets of the winged aphids. In the majority of aphids (16 of 20), the body contained more GVCV genomes than the stylet.

Table 5. Number of grapevine vein clearing virus (GVCV) genomes in the stylet and body of 20 single aphids measured by quantitative polymerase chain reaction.

Sample Number	Genome Number			
	Stylet		Body	
	Actual	Extrapolated[1]	Actual	Extrapolated
1	7	43	107	609
2	23	131	124	707
3[w]	74	422	89	510
4	29	163	137	783
5	*2.46	*14.06	97	557
6[w]	7	41	383	2,188
7 [w]	11	61	234	1,338
8	127	723	23,380	133,600
9	8,993	51,389	299,800	1,713,143
10	45,600	260,571	85,620	489,257
11	13	134	71	705
12	25	245	225	2,245
13	76	762	26	258
14	8	80	37	372
15	47	467	83	825
16	14	141	*1.05	*10.5
17	67	666	113	1,127
18	66	664	23	233
19	7	74	32	323
20	20	197	14	136
Average	2,906	16,683	21,610	123,627

[w] indicates winged aphids.

* indicates GVCV genome number is below the threshold of detection.

[1] The average of three replicated reactions from each sample was used to determine initial template quantity. The initial quantity was transformed to the extrapolated GVCV genome number by accounting for the total amount of DNA extracted and the efficiency of the DNA extraction kit. Bold denotes samples in which the head was removed from the body.

DISCUSSION

In this study, GVCV was detected in 87 of 105 (87%) communities of grape aphids using conventional PCR (Table 2). Ten aphids from each community were tested giving us a total of 1,050 aphids tested. In every location tested, at least one community was found to be positive (Fig. 8). Later, 212 of 525 (40%) single grape aphids were tested for GVCV, showing us the natural prevalence of GVCV in native grape aphid populations (Table 3). The prevalence of GVCV was found to be much higher when groups of ten aphids were tested. This is logical because if even one grape aphid of the ten was positive it would deem the whole community as positive. The high prevalence of GVCV in grape aphids does not necessarily mean that every aphid has the capability to transfer this virus, as there are many factors that indicate the transmissibility of a virus, however, the high incidence does indicate the potential risk of spread. Similar incidence has been found in two species of aphids carrying Citrus tristeza virus (CTV), where *Aphis spiraecola* and *Aphis gossypii* were found to carry the virus at 35.4 and 28.8%, respectively (Elhaddad et al. 2016).

Most of the GVCV positive aphids were collected from plants that were not infected by GVCV. After observing these GVCV infected aphids' sequences, it was found that more diverse GVCV isolates from grape aphids existed on negative plants. In five separate communities, the GVCV ORF II sequences ranged from 90 to 98% between individual grape aphids. This evidence suggests that the grape aphids first acquired GVCV from multiple infected sources before migrating to these uninfected plants. One explanation could be viral manipulation of the infected host plant and the virus-bearing vector. It has been found that plant viruses can influence their host plants, and the vectors that disperse them (Ingwell et al. 2012; Roosien et al. 2013). In

the case of Barley yellow dwarf virus (BYDV), the aphid *Rhopalosiphum padi* was found to prefer plants infected with BYDV. However, when they had acquired the virus, they shifted preference to uninfected plants (Ingwell et al. 2012). This tactic by plant viruses of attracting native vectors on virus-infected hosts and propelling viruliferous vectors to seek uninfected plants promotes virus spread. In addition, it has been found through modeling that this behavior promotes virus spread when virus sources are limited in a population (Sisterson 2008). This conforms with what has been found with the prevalence of GVCV in infected Vitaceae, as the rate of infection has been found to be relatively low, 34% in *A. cordata* (Peterson et al., 2019 and this study), 10% in native *Vitis* spp. (Beach et al., 2017 and this study), and 8% in cultivated *Vitis* (J. Schoelz, personal communication).

Among the 25 GVCV isolates from grape aphids and host Vitaceae, there were five groups that had two or more aphids with ≥99% identical nucleotides (Table 4). In each of these groups at least one GVCV isolate was found to be more than 270 km away from another, suggesting that these variants could have been translocated by aphids through long distant aerial flight. Native *A. cordata* and *Vitis* spp. vines are densely growing among native flora, and several of these native and cultivated Vitaceae species have been found to harbor the same variants of GVCV. Therefore, it is also possible that viruliferous grape aphids carried these variants from local sources. Phylogenetic analysis of 174 GVCV ORF II sequences showed three clades containing closely related sequences from grape aphids, native Vitaceae and cultivated grapevines (Fig. 12), indicating that grape aphids are capable of transmitting GVCV from native hosts to cultivated grapevines. This analysis allows us to infer genetic structure at a spatial scale, further sampling and sequencing of grape aphids over multiple years will reveal temporal relationships.

GVCV was detected in the stylet and body of single grape aphids. Overall, more GVCV genomes were detected in the body than in the stylet. In samples where the head was included with the body there was a 53% increase in the average number of GVCV genomes after outliers were removed (Table 5). It is still unclear if GVCV virions concentrate in the distal tip of the stylet, and of the biological significance of GVCV virions in the head. Although GVCV is present in the body, it cannot be discerned if GVCV only exists in the gut and/or in the hemolymph. Many Badnaviruses transmitted by aphids are transmitted in a semi-persistent manner (Bhat et al. 2016). This is consistent with our results, but more research is required to distinguish the transmission mode.

The number of GVCV genomes varies largely in single grape aphids, similar results were also found between non-persistently transmitted Potato virus Y and aphids (Khelifa 2019b) in which PVY genome number differed largely among individual aphids even under experimental conditions. It appears that the number of viral particles that are acquired by aphid vectors differ among individuals in nature and under experimental conditions. This large variation can be attributed to the following factors: aphids acquire GVCV from plants with differing titers of GVCV, feeding periods differ among individual aphids, and the developmental stage of the aphid and virus retention. The transmission efficiency and epidemiological significance of this large variation in viral titers warrants further study. Furthermore, qPCR is more sensitive at detecting GVCV in single aphids than conventional PCR. One study found that qPCR was 100 times more sensitive than tradition PCR (Ratti et al. 2004). Therefore, the prevalence of GVCV in aphids assessed using conventional PCR may appear lower than the actual prevalence.

Across the Midwest, native *A. cordata* and *Vitis* spp. grow ubiquitously along the edge of vineyards, and throughout the natural environment. These native species form a vast perennial

reservoir of GVCV that create large corridors of inoculum extending to and connecting vineyards. Each spring, a new generation of grape aphids hatch and migrate to these infected plants. They feed and acquire GVCV forming enormous populations with highly diverse GVCV isolates. The high prevalence of GVCV in these aphids form a massive, constant supply of genetically diverse isolates. These isolates are dispersed from plant to plant through short walks, locally through short distance flights, and at great distances through long distance aerially migration aided by the jet stream (Van Emden and Harrington 2017). This behavior creates constant opportunities for devastating GVCV outbreaks within vineyards.

This study reveals insights into the dynamics of GVCV within its vector. The knowledge and evaluation of the impact of virus factors such as dispersal, virus mutability, natural sources, weather and cultural practices allow for the most effective design against a specific virus (Van Emden and Harrington 2017). Long distance migration by aphids presents a challenge in reducing incidences of GVCV. Preventative strategies are the most effective techniques in preventing these incidences. One of the most effective strategies is by planting virus-tested clean grapevines or virus-resistant varieties. Removing nearby sources of inoculum and rouging infected plants can also reduce incidences caused by local aphid migration. These methods can help reduce spread of GVCV from infected reservoirs to cultivated vineyards.

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