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# THE DETECTION AND CHARACTERIZATION OF SOME VIRUSES INFECTING BLACKBERRY AND CHERRY IN SOUTH CAROLINA

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# THE DETECTION AND CHARACTERIZATION OF SOME VIRUSES INFECTING BLACKBERRY AND CHERRY IN SOUTH CAROLINA

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ABSTRACT

Three separate virus research projects were conducted. Blackberry yellow vein disease (BYVD), a disorder caused by virus complexes, has become a major threat to blackberry production in the United States, especially in the southeastern part of the country where blackberries are grown for the fresh market. More than 30 viruses have been found to be associated with the disease. Most of these induce no symptoms when infecting the plant alone. However, when more than a single virus is present in the host visible symptoms are displayed. The incidence of 6 different viruses (*Blackberry yellow vein-associated virus*, *Blackberry virus* Y, *Blackberry chlorotic ringspot virus*, Blackberry virus E, Blackberry virus Ω, and *Tobacco ringspot virus*) that have been commonly found in BYVD-infected plants was studied using sentinel plants dispersed in plantings of blackberry in the field. Experiments were completed at the two largest commercial blackberry farms in South Carolina using more than 1200 sentinel plants over the course of three years. The sentinel plants were tested for the presence of the 6 viruses before they were exposed in the field and were again tested for the presence of the 6 viruses after the plants had been recovered from the field and allowed to overwinter in the greenhouse. Both Blackberry virus E, and Blackberry virus Ω were found infecting blackberry in South Carolina for the first time. A potential new ilarvirus was identified in blackberry and veronica. Partial sequence information for the 3 genomic molecules has been obtained. The virus shows closest homology to the members of subgroup 1 of the genus *Ilarvirus*, but is unique. This subgroup includes BCRV, one of the viruses previously associated with the BYVD complex. Symptoms typical of virus infection were observed in the suckers/watersprouts growing from the 'Mazzard' rootstock of a flowering cherry tree growing at Musser Farm, Clemson University in 2011. However, the

scion of the tree, *Prunus serrulata* cv. Shirofugen, displayed no symptoms. Double-stranded RNA was isolated from the symptomatic tissues of the rootstock and used to provide templates for cDNA cloning and for nucleotide sequencing. Sequence data showed the virus to be most closely related to *Cherry rusty mottle-associated virus.*

DEDICATION

This dissertation is dedicated to my parents, Hem and Usha for not hesitating to use all their means and resources to bring me where I am today; to my husband, Sanjay, for being there in every moment; to my little love Nirvaan for giving me a different perspective on life as a mom; to my sisters, Bidhya and Biju for their love and support.

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# CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW

### Blackberry

Blackberry is a member of the genus *Rubus* in the family *Rosaceae*. The genus, which has 243 species and 263 accepted taxa, also includes raspberry together with dewberry and cloudberry. Although blackberry and raspberry plants look similar, the light green foliage of raspberry, and differences in their fruit architecture, easily distinguishes them from each other. When the fruit is picked, the blackberry fruit receptacle remains intact in the fruit whereas in raspberry the receptacle remains attached to the plant. Blackberry is native to several regions of the world including Europe, temperate west and central Asia, and North and South America.

The blackberry plant has been used for medicinal purposes, from ancient times. As early as the 16$^{th}$ century blackberry juice was used in Europe to treat mouth and eye infections. The powdered bark was used to treat toothaches and roots were used to treat dysentery:

*"Against dysentery, a bramble of which both ends are in the earth [tip layer!] take the newer root, delve it up, cut up nine chips with the left hand and sing three times the Miserere mei Deus and nine times the Mater Noster, then take mugwort and everlasting, boil these three worts and the chips in milk till they get red, then let the man sip at night fasting a pound dish full… let him rest himself soft and wrap himself up warm; if more need be let him do so again, if thou still need do it a third time thou wilt not need oftener." Leech Book* II 65 (Rohde, 1922).

Blackberry has become popular in modern times because of its nutritional value and the importance given to food antioxidants as a means to improve human health. Blackberry fruit contains large amounts of ellagic acids, tannins, cynadin glycosides, and antioxidant phenolics that have anti-carcinogenic, anti-inflammatory, and anti-neurodegenerative properties, as well as possessing the highest fiber content among edible fruits (Seeram et al., 2006). Halvorsen et al. (2006) reported that blackberry has the highest antioxidant content per serving size compared to other foods consumed on a regular basis in the United States. Blackberry extracts have also been shown to be effective against *Herpes simplex virus 1* in cell suspension cultures (Danaher et al., 2011).

Blackberry has a perennial crown and root and biennial canes. The vegetative shoots that grow during the first growing season are called primocanes and undergo dormancy during winter. The next season, the prior-season's primocanes become floricanes and produce flowers and fruits. Blackberries are classified according to their cane architecture into three types: erect, semi-erect, and trailing (Strik et al., 1992). The erect cultivars stand upright, the trailing types lie close to the ground, and the semi-erect cultivars are erect but require trellises for support. Erect-caned cultivars include the thorny 'Brazos', 'Tupy', and 'Cherokee' and the thornless 'Navaho' and 'Arapaho'. Semi-erect cultivars include 'Chester Thornless', 'Loch Ness', 'Thornfree', and 'Čačanska Bestrna'. Trailing cultivars include 'Marion', 'Silvan' and 'Thornless Evergreen' and the blackberry × raspberry hybrids 'Boysen' and 'Logan'. Two new cultivars with fruiting primocane 'Prime-Jim' and 'Prime-Jan', are erect, thorny types (Blackberry variety review, Cornell University, College of Agriculture and life science, Cornell cooperative extension, 2013).

Abaxial surfaces of blackberry leaves are mostly glabrate, green in summer, and darken to red-purple in the fall. The flowers are mostly white and have five petals with

multiple stamens. The fruit is an "aggregate" composed of many individual drupelets with seeds that surround the firm receptacle. The fruit changes from green to red, as it matures, eventually turning black at full maturity. Flowering and fruiting occurs in a racemose-cyme pattern, with primary fruit ripening prior to secondary, tertiary, and quaternary fruit (Clark et al., 2007).

There are more than 20,000 ha of blackberries planted and commercially cultivated worldwide, an increase of about 45% since 1995 (Strik et al., 2008). Blackberries grow best in warmer temperate regions with full sun and well-drained soil. Serbia was the largest producer worldwide in 2008 followed by the United States, which has been projected to be the world's largest producer by 2015 (Strik et al., 2008). Within the United States, Oregon is the largest producer followed by California, North Carolina, and Georgia. Twenty years ago, marketing of fresh blackberries was not feasible because of the fruit's short shelf-life; however, recent trends in blackberry breeding have placed fresh-market shipping as a priority (Clark and Finn, 2008). Also, the newly acquired knowledge on the role of antioxidants in prevention of several diseases has contributed to the increase in fresh blackberry acreage over the past two decades (Clark, 1999).

In the early 1990s, blackberry was not a common produce item on grocery store shelves in the eastern USA and was very rarely found in the western USA (Clark, 2005) but by late 1990s, 'Chester Thornless' had become a major fresh market blackberry with better shelf life because of its good fruit firmness. 'Navaho' which was developed by the University of Arkansas Blackberry Breeding Program is another cultivar with an extended shelf-life. The University of Arkansas Blackberry Breeding Program is the leading blackberry breeding program in the nation. The program has developed many blackberry cultivars that are suitable for fresh market. The cultivars 'Apache', 'Arapaho', 'Cherokee', 'Comanche',

'Cheyenne', 'Chickasaw', 'Choctaw', 'Kiowa', 'Ouachita', and 'Shawnee' are some of the fresh market blackberries developed by this program. The James Hutton Institute in Scotland has another leading blackberry breeding program. The program has developed some popular cultivars in Europe that includes 'Loch Ness', 'Loch Tay', and 'Loch Maree' that are grown in Europe as well as in North America.

With the increase in acreage and expansion to new areas of blackberry production, there have also been reports of new diseases and pathogens affecting these plants, one of the main diseases is Blackberry yellow vein disease (BYVD). The disease was first observed in 2000 in the Carolinas. Since then, it has become a serious threat in all southeastern US blackberry growing regions (Martin et al., 2004; Tzanetakis et al., 2008). For example, a few two-year-old 'Chicksaw' blackberry plants showed symptoms in a northwest Arkansas production field in 2003, but within two years, BYVD had spread throughout the field, reducing yield and plant vigor (Susaimuthu et al., 2008a). Typical symptoms include vein yellowing of primocane leaves, with new leaves usually being asymptomatic (Susaimuthu et al., 2007). Symptoms also include oak-leaf patterns, irregular chlorosis and line patterns (Susaimuthu et al., 2006). A mosaic on leaves of some infected plants has also been observed. Floricanes can be severely affected by the disease, resulting in dieback of canes during the fruiting season.

In these plants, BYVD was initially mistaken for infection by *Tobacco ringspot virus* (TRSV); however, grafting experiments proved that TRSV is asymptomatic in many blackberry cultivars (Gergerich, unpublished). Further studies carried out to determine the causal agent(s) of the disease reported a new crinivirus named *Blackberry yellow vein associated virus* (BYVaV) in asymptomatic samples (Martin et al., 2004). Although all symptomatic plants in this study were infected with BYVaV, further screening indicated that

BYVaV is latent in single infections (Susaimuthu et al., 2008a). It was thus speculated that BYVaV acts synergistically with other viruses to cause disease. Subsequently, several other viruses have been isolated from BYVD-infected plants, including *Blackberry virus Y* (Susaimuthu et al., 2008b), Blackberry virus X*, Impatiens necrotic spot virus* (Tzanetakis et al., 2009)*,* Rubus virus S*,* Blackberry virus E (Sabanadzovic et al., 2009; 2011) and *Blackberry chlorotic ringspot virus* (BCRV) (Tzanetakis et al., 2007). In addition an *Emaravirus*, and another *Ilarvirus* have been identified together with some other as yet completely uncharacterized viruses.

BYVD continues to be a serious threat in blackberry growing areas and new viruses associated with the disease are being discovered each year. It is a serious concern for the blackberry growers because these plant are clonally propagated and although there are a few schemes designed to provide virus-indexed material for planting it is possible that infected, but asymptomatic, plants may have been used to generate propagants. As these are planted in the field, infection of additional viruses may lead to synergism and cause BYVD. A normal blackberry planting can produce for 15 to 20 years depending on cultivars and cultivation practices. BYVD can reduce production to 5 to 7 years or less. Establishment of new plantings costs more than $10,000 per acre (Production and Marketing reports, NCSU) and because blackberry is a biennial crop, growers have to wait for at least two years to obtain a full crop, making BYVD a major threat for the viability of small farms. A survey was conducted by scientists at North Carolina State University and TRSV was found in a large number of samples that had been released from the University of Arkansas breeding program and entered nursery production. Currently, Dr. Zvezdana Pesic-VanEsbroeck from North Carolina State University and other scientists are involved in a blackberry certification

program which produces virus-tested material for nurseries which is in turn used to establish new plantings in the field.

As already stated BYDV is a complex of viruses. In studying the disease it is necessary to be aware of the characteristics of the viruses involved and, if offering suggestions on control measures, knowledge of the epidemiology of the individual viruses that might be members of the complex is needed. A brief introduction to the properties, characteristics, and epidemiology of the viruses that are examined in this work is presented here. The 6 viruses studied in examining BYDV complex using sentinel plants are shown in table 1.1.

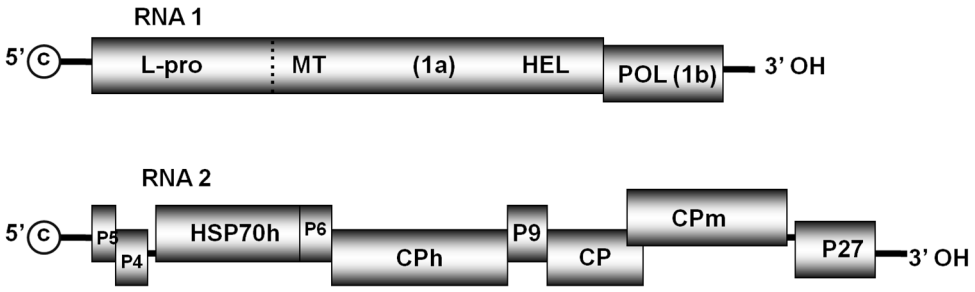
Table 1.1 List of viruses that were tested in sentinel blackberry plants experiment.

| Virus species | Acronym | Genus | Family |
|---|---|---|---|
| *Blackberry yellow vein-associated virus* | BYVaV | *Crinivirus* | *Closteroviridae* |
| *Blackberry chlorotic ringspot virus* | BCRV | *Ilarvirus* | *Bromoviridae* |
| *Blackberry virus Y* | BVY | *Brambyvirus* | *Potyviridae* |
| *Blackberry virus E* | BVE | Unassigned | *Flexiviridae* |
| *Blackberry virus Ω* | BVΩ | *Emaravirus* | *Unassigned* |
| *Tobacco ringspot virus* | TRSV | *Nepovirus* | *Secoviridae* sub family *Comovirinae* |

Blackberry yellow vein-associated virus, Genus *Crinivirus*, family *Closteroviridae*

The genus *Crinivirus* is included in the family *Closteroviridae* together with the genera *Closterovirus* and *Ampelovirus*. Closteroviruses are defined as plant viruses with thread-like particles having positive sense, single-stranded RNA genomes that are the largest among positive-stranded RNA plant viruses. All members of the *Closteroviridae* are also characterized by the possession of unique genes that code for a heat shock protein 70 homolog and two coat proteins (Tobias, 2002). Members of genus *Crinivirus* have genomes ranging from 15.3-19 kb, divided into two or three genomic molecules (Martelli et al., 2002) (Figure 1.1).

Figure 1.1 The bipartite genome of *Blackberry yellow vein associated virus*. Pro, papain-like protease; MT, methyltransferase; HEL, helicase; Pol, polymerase; HSP70h, heat shock protein homolog; CPh, coat protein homolog; CPm, coat protein minor (Tzanetakis, 2006).




RNA1 of the virus encodes a papain-like protease, methyltransferase, helicase and polymerase, all of which are involved in virus replication. The polymerase is expressed by a

+1 ribosomal frameshift typical for all closteroviruses. RNA2 encodes up to nine ORFs involved in virion assembly, vector transmission, movement, protection and other functions yet to be determined (German-Retana, 1999). CPm is the determinant of whitefly transmission. P5 is a part of hallmark closteroviruses gene array and P9 is a unique feature of the genus *Crinivirus* as it is not present in any other members of the family *Closteroviridae*. It has been shown that P5 and P9 can self-intereact with *Lettuce infectious yellows virus* (Stewart et al., 2009). No member of the genus *Crinivirus* can be transmitted by sap inoculation (Martelli et al., 2002).

Criniviruses have emerged as a threat to agricultural and horticultural production in the last three decades, *Beet pseudo yellows virus, Cucurbit yellow stunting disorder virus, Lettuce chlorosis virus, Lettuce infectious yellows virus, Strawberry pallidiosis associated virus, Tomato chlorosis virus,* and *Tomato infectious chlorosis virus* are some of the economically important viruses infecting agricultural crops (Celix et al., 1996; Duffus et al., 1996; Wisler et al., 2001; Tzanetakis et al., 2004a; Tzanetakis et al., 2006b). BYVaV (Tzanetakis et al., 2006b) and Beet pseudo yellows virus (BPYV) are the two criniviruses known to infect blackberry (Tzanetakis, 2004b. As a result of global warming, the whiteflies in the genera *Trialeurodes* and *Bemisia* that are extremely efficient vectors of these viruses, are now able to survive and increase in areas where they were not found previously and the incidence of crinivirus-associated diseases has increased dramatically (Wintermantel, 2004).

The large RNA genome, lack of mechanical transmissibility and association of criniviruses with the phloem tissue results in low titers in infected plants, and low yields during purifications, which make study of criniviruses difficult (Karasev, 2000). Criniviruses often induce symptoms that are mistaken for physiological or nutritional disorders or pesticide phytotoxicity. Criniviruses remain confined to phloem cells and the symptoms are

believed to be partially the result of phloem being plugged with large viral inclusion bodies possibly interfering with the normal vascular transport in plants (Wisler et al., 2001). Symptoms mostly include inter-veinal yellowing, reduction in photosynthetic capacity, early senescence, reduced plant vigor, and leaf brittleness. Nonetheless, symptoms differ among plants species and cultivars. The symptoms are generally more apparent on middle to lower parts of plants, but the new growth appears normal as criniviruses cannot invade immature phloem (Wintermantel, 2004). The most effective way to manage crinivirus infections in the field is by controlling the population of vectors.

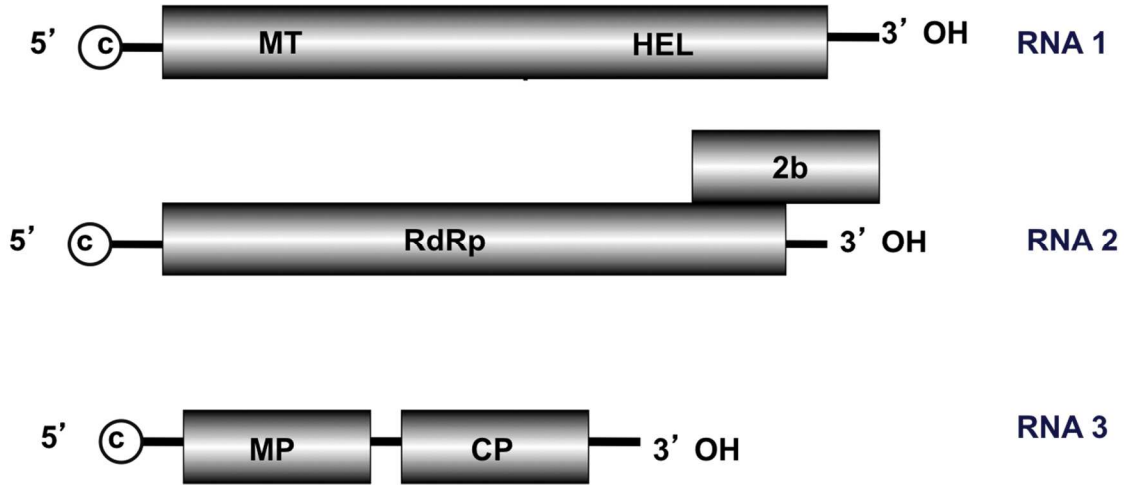
*Blackberry chlorotic ringspot virus*, Genus *Ilarvirus*, family *Bromoviridae*

The genus *Ilarvirus* is included in the family *Bromoviridae* together with five other genera: *Alfamovirus*, *Anulavirus*, *Bromovirus*, *Cucumovirus* and *Oleavirus.* With 19 species listed in the genus, and divided into six subgroups, ilarviruses constitute the largest genus in the family.

Ilarviruses are single stranded, positive-sense RNA viruses with a tripartite genome coding for four or five proteins (Figure 1.2). The virions are icosahedral or quasi-icosahedral, non-enveloped and range from 20 to 35 nm in diameter. The RNA1 and RNA2 of ilarviruses are involved in virus replication. RNA1 is monocistronic, encoding a protein with methyl-transferase and helicase motifs. RNA2 codes for an RNA-dependent RNA polymerase (RdRp). Members of the genus *Cucumovirus* and some members of the genus *Ilarvirus* possess an additional ORF located towards the 3' terminus of RNA2 that codes for a 2b protein which has been reported to be involved in suppression of RNA interference -RNA silencing (Shimura et al., 2013). It is also reported that the protein might be involved in cell-to-cell movement of the virus based on the similar gene function in *Cucumber mosaic virus* (Shi et

al., 2003). RNA3 codes for the movement protein and coat proteins at the proximal and distal halves of the molecule, respectively, and is involved in virus movement. The coat protein is expressed through a subgenomic RNA4 and is required for the activation of the genome by binding to structures near the 3'-termini of the viral RNAs, a characteristic shared between ilarviruses and *Alfalfa mosaic virus* (Jaspars, 1999). The CP of an ilarvirus can activate the genome of AMV and vice versa. This particular property of the coat proteins of Ilarviruses and *Alfalfa mosaic virus* (AMV) (genus *Alfamovirus*, family *Bromoviridae*), is unique among plant viruses. The coat protein of AMV is also required for virion formation, cell-to-cell movement and systemic spread of the virus (Tenllado and Bol, 2000). Mutations in the coat protein have been reported to be associated with altered symptom formation (Neeleman et al., 1991).

Figure 1.2 Schematic representation of the genome of Blackberry chlorotic ringspot virus, a typical subgroup 1 ilarvirus. RNA1: MT- methyl transferaese, HEL- Helicase, RNA2: RdRp-RNA dependent RNA polymerase, RNA3: MP- movement protein, CP- coat protein. Subgroup 1 and 2 viruses possess 2B ORFs but other ilarviruses do not.



Unlike many plant viruses that have vectors to move them from one plant to another, ilarviruses move by pollen and seeds. Even though very little is known about the mechanism of transmission and the vectors of ilarviruses, seven species have been reported to be pollen and seed transmissible (Card et al., 2007). The species include *Asparagus virus 2, Blueberry shock virus, Fragaria chiloensis latent virus, Prune dwarf virus, Prunus necrotic ringspot virus, Spinach latent virus*, and *Tobacco streak virus*. Some species like *Prunus necrotic ringspot virus* can invade pollen grains (Aparicio et al., 1999), giving ilarviruses the advantage of horizontal as well as vertical transmission. For many ilarviruses, insects that move infected pollen can easily disseminate the viruses to large numbers of plants and over large distances. Honeybees move *Blueberry shock virus* (Bristow and Martin 1999). *Prunus necrotic ringspot virus* and *Prunus dwarf virus* have been shown to be moved over considerable distances in the hives of bees that are moved northwards in California, Oregon and Washington to pollinate various crops (Mink, 1983). Thrips feeding on wind-blown pollen have been shown to inoculate plants (Greber et al., 1992) but although thrips are recognized as pollinators of some plant species videos of thrips feeding on and in cherry flowers have shown that they preen themselves to eliminate pollen grains before flying to another host (Mink, pers comm).

Ilarviruses infect economically important plants that include many woody species and stone fruits and are found in very low titer in plants (Uyemoto and Scott, 1992). They have been reported to cause economic losses in *Citrus*, *Humulus*, *Malus*, *Prunus*, *Rosa* and *Rubus* spp due to the effect of virus on plant growth, fruit yield and maturity (Uyemoto and Scott 1992; Saade et al., 2000; Tzanetakis, 2007). Complete invasion of woody hosts by ilarviruses usually requires more than one year whereas the invasion of herbaceous host is more rapid. The most effective way to prevent the spread of ilarvirus is by quarantine and use of healthy plant stocks. Ilarviruses can be transmitted mechanically by sap inoculation,

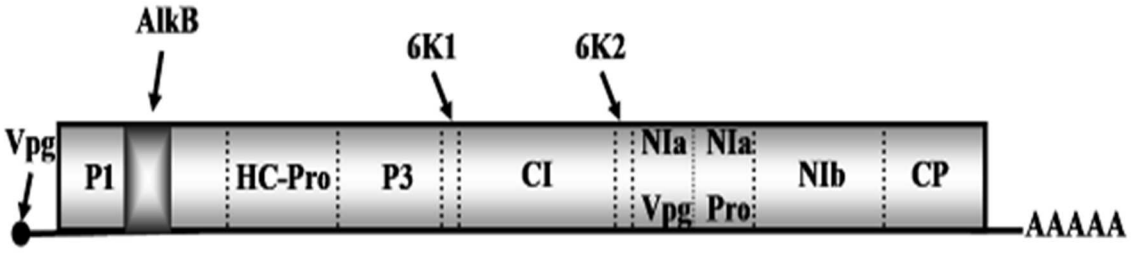
but this is of less concern in the field scenario where ilarviruses primarily infect woody perennial hosts. However, the practice of grafting and budding fruit trees can contribute to the rapid dispersal of an ilarvirus should a virus-infected host be chosen as a source of budwood by mistake. Use of virus-tested materials for establishment helps minimize the subsequent loss in orchards.

## Blackberry virus Y, Genus *Brambyvirus,* Family *Potyviridae*

Blackberry virus Y (BVY) is the sole member of the most recently created genus *( Brambyvirus*) in the family *Potyviridae* . Other genera in the family are: *Bymovirus, Ipomovirus, Macluravirus, Poacevirus, Rymovirus, Tritimovirus*. A few viruses with characteristics that resemble potyviruses have yet to be assigned to a genus. BVY is the largest potyvirus sequenced so far and the only potyvirus that encodes for an Alk B domain (Susaimuthu et al., 2008a). Both the viruses BYVaV and BVY are asymptomatic in single infection but cause disease symptoms when they co-infect blackberry plants (Susaimuthu et al., 2008b). The synergistic relationship between these two viruses results in a higher titer of BVY, which is different from typical potyvirus-crinivirus synergistic interaction. Usually the Crinivirus is the beneficiary (Want et al., 2009). The only other reported case in which the Potyvirus is the beneficiary instead of Crinivirus is between *Sweet potato chlorotic stunt virus* (*Crinivirus*) and *Sweet potato feathery mottle virus* (*Potyvirus*) (Karyeija et al., 2000). The genome of BVY is 10,851 nt in length excluding the polyA tail, making it the largest member of the family *Potyviridae* with a monopartite genome. Sites for post-translational cleavage have been identified, yielding the ten mature proteins of characteristic potyvirids

(Figure 1.3). But when the genomic organization is compared with other members of the family, BVY lacks the N-terminus of the HC-Pro Cistron that is involved in movement and transmission of virus. Another unusual feature of BVY that led to it being assigned to a new genus is the presence of AlkB domain within the P1 protein. The Alk B domain is found in bacteria and members of the *Flexiviridae* and *Closteroviridae* and plant genes. The Alk B domain reduces the effect of methylation and protects against nucleotide damage. It has been speculated that BVY might have acquired the domain during recombination in mixed infection with other viruses and bacteria or possibly by horizontal transfer from bacteria to viruses (vandern Born et al., 2008). BVY has been detected in both wild and cultivated plants and was been shown to spread in the field. However, transmission attempts with aphids and mites have been unsuccessful and the vector is still unknown.

Figure 1.3 Genomic organization of Blackberry virus Y, sole member of genus *Brambyvirus*. Vpg, Viral protein genome linked; P1, Polyprotein 1; Alk B, Alk B domain; HC-Pro, helper component protein; P3, Polyprotein 3; 6K1, 6 kDA protein; CI, Cylindrical Inclusion protein; NIa, Nla protease; Nlb, NlB protease, CP; Coat protein (Susaimuthu et al., 2008a).



Blackberry virus E, unassigned, potentially a member of the family *Aphaflexiviridae*

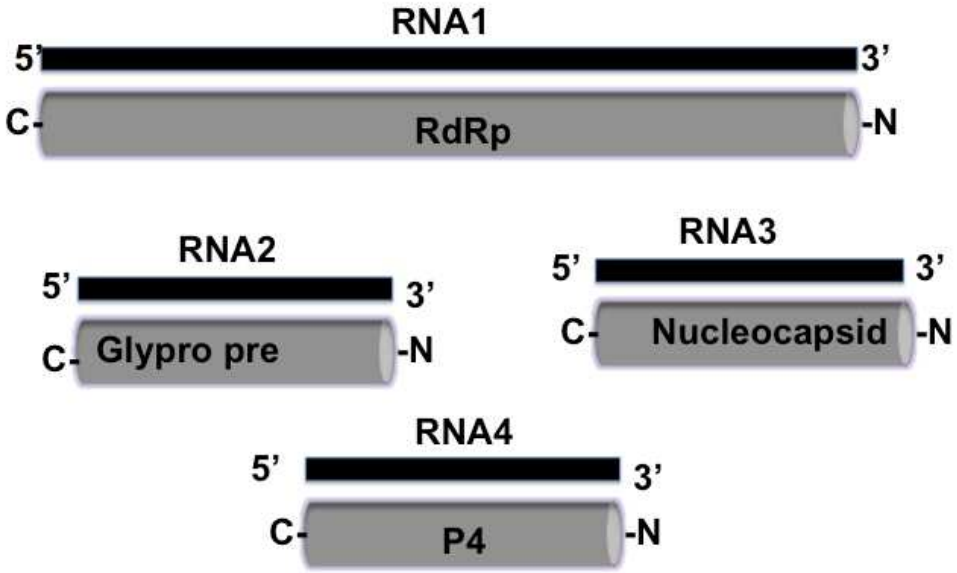
Blackberry virus E is a previously undescribed virus. It has not yet been assigned to one of the accepted genera. It is related to members of the genus *Allexivirus* in the family *Alphaflexiviridae* that infect species in the family *Alliaceae* and are transmitted by mites (Kang et al., 2007). The virus however, lacks the 3' ORF that encodes for the nucleotide-binding protein, a putative silencing suppressor in allexiviruses (Sabanadzovic, 2011), making it distinct from other members of genus. BVE has no known vector. See section on families *Alphaflexivirdae* and *Betaflexiviridae* for detailed information on molecular organization.

Black berry virus Ω, Genus Emaravirus

Emaravirus is one of the 14 genera not currently assigned to a virus family. The genus has six species of virus: *European mountain ash ringspot-associated virus* (type species), *Fig mosaic virus, Pigeonpea sterility mosaic virus*, *Raspberry leaf blotch virus*, *Rose rosette virus and Wheat mosaic virus*. All the viruses have genomes consisting of four or more negative sense RNAs (Figure 1.5) and virions are double membrane bound particles. Most species have been shown to be transmitted by mites (Mielke-Ehret and Muhlbach, 2012). After the genomic sequence of *European mountain ash ringspot-associated virus* (EMARaV) was obtained (Mielke & Muhlbach, 2007), a new genus, *Emaravirus* was established. Other previously uncharacterized viruses were identified that shared the characteristics of EMARaV and these were assigned to the genus *Emaravirus*. The RNA1 of the virus encodes a viral RNA-dependent RNA-polymerase (RdRp) that shows similarity to

RdRps of bunyaviruses and tenuiviruses (Mielke & Muhlbach, 2007; Benthack et al., 2005). RNA2 likely encodes a glycoprotein precursor, RNA3 possibly encodes N-protein, and RNA4 encodes protein P4, whose function has not yet been determined. Emaraviruses spread very quickly in the field and have been reported to be vectored by *Aceria* species of mites however the spread of Rose Rosette virus has been demonstrated for *Phyllocoptes fructiphilus* Keifer (Doudrick et al., 1986).

Figure 1.4 Genomic organization of Emaraviruses. Virus genome (minus strand) are represented by black lines and virus encoded proteins by the mRNAs are represented in grey box.RNA1: RdRp: RNa dependent RNA polymerase; RNA2: Glyco Pre: Glycoprotein Precursor; RNA4: P4, p4 protein.

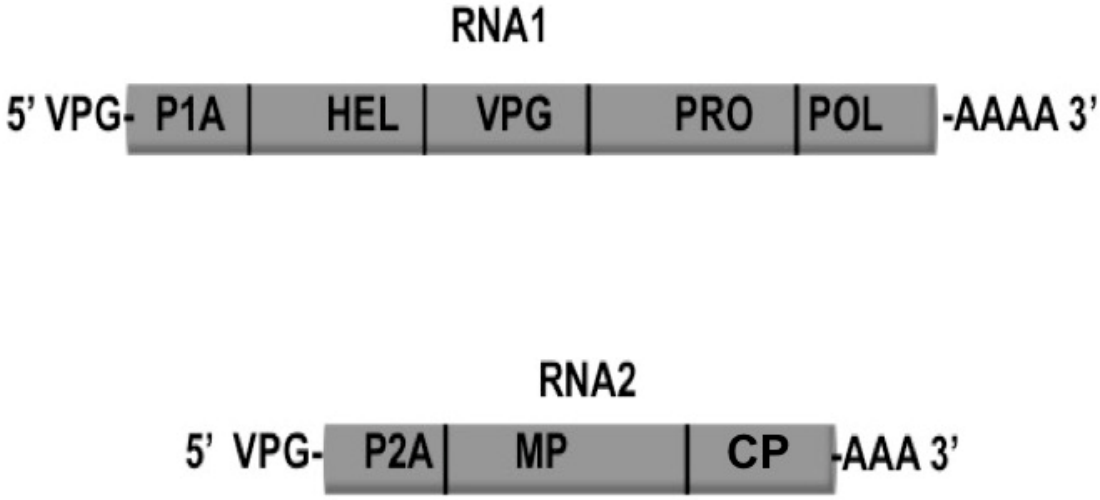



Tobacco ringspot virus, Genus *Nepovirus,* Subfamily *Comovirinae,* Family *Secoviridae*

The genus *Nepovirus,* together with the genera *Comovirus* and *Fabavirus,* comprises the family *Secoviridae* (King et al., 2012). It consists of 36 species with *Tobacco ringspot virus* as

the type virus. Nepoviruses are transmitted by nematodes belonging to the genera *Xiphinema* and *Longidorus*. This characteristic makes nepoviruses unique from other genera in the subfamily. Nepoviruses are linear, single-stranded, positive sense RNA viruses. Like other genera of the sub family, the members of the genus *Nepovirus* are characterized by a bipartite genome. Each genomic molecule is individually encapsidated in an icosahedral particle. Each genomic segment produces a polyprotein (Macfarlane et al., 1999; Mayo and Robinson 1996) that is post-translationally cleaved into functional proteins. RNA1 encodes proteins involved in virus replication whereas RNA2 encodes proteins that are involved in cell-to-cell movement and transmission (Figure 1.5).

Figure 1.5 Genomic organization of a typical Nepovirus. RNA1: P1A; RNA1 Polyprotein A; Hel, Helicase; VPG, virus protein genome-linked; Pro, Protease; Pol, polymerase; RNA2: P2A, RNA2 polyprotein A; MP, Movement protein; CP, Coat protein.




However, the 2A protein of RNA2 is required for the replication of RNA2 and also may be associated with RNA1-derived replication protein complex located close to the

nucleus (Gaire et al., 1999). In earlier studies, a correlation was found between nematode species and virus serotype in transmission studies. It was assumed that the coat protein was the determinant for virus transmission by nematodes as the virus had to "bind" to nematode mouthparts to be transmitted. Further research using pseudo recombinant virus isolates where the viral genomic RNAs were separated and recombined with viral RNAs from different isolates showed that for nepoviruses the CP is the sole determinant of transmission specificity, but the 9 C-terminal amino acids from the 2b protein are also critically involved in transmission (Belin et al., 1999; Belin et al., 2001).

## *Families Alphaflexiviridae* and *Betaflexiviridae, Order Tymovirales*

Both families, *Alphaflexiviridae* and *Betaflexiviridae,* had previously been grouped in a single family *Flexiviridae* with the subdivision into two distinct families occurring only recently. At the same time the two families, together with the *Gammaflexivirdae* and the *Tymoviridae* had been incorporated into a new order of viruses (*Tymovirales*).

The *Alphaflexiviridae* includes genera *Allexivirus*, *Botrexvirus*, *Lolavirus*, *Mandarivirus*, *Potexvirus*, *Sclerodarnavirus*, and a few unassigned members. The *Betalexiviridae* includes genera *Carlavirus, Foveavirus, Capillovirus, Vitivirus, Trichovirus*, *Tepovirus*, *Citrivirus* and a few unassigned members. Both families, contain single-stranded positive-sense RNA viruses (King et al., 2012) held in helical flexuous virions. This morphology is shared with the *Closteroviridae* (Dolja et al., 2006, Galiakparov et al., 2003) and the *Potyviridae* (Urcuqui-Inchima et al., 2001). The *Tymoviridae*, however, possess isometric particles but genetic evidence provides a convincing case for the viruses in *Tymovirales* sharing a common ancestor.

Flexiviruses possess monopartite, 3′-polyadenylated genomes that encode closely related methyl transferase, RNA helicase, and RNA polymerase domains in the viral replicase. In contrast, one or two proteases, and AlkB- homology domains are found in the replicases of some members (*Grapevine virus* A, genus *Vitivirus*, *Blueberry scorch virus,* genus *Carlavirus*,). There is also diversity in the number and nature of the 3′-proximal viral genes that are expressed via formation of subgenomic mRNAs. These genes code for viral movement proteins belonging to,either the"p30-like" superfamily of viral movement proteins, or to the triple gene block movement protein complex (Lazarovitz and Beachy, 1999; Morozovet et al, 2003). These genetic differences are important as they can be associated with variation in host ranges, pathogenicity level, and overall epidemiology of viruses within the family *Flexiviridae* (Martelli et al., 2007). Particles of the alpha and betaflexiviridae are formed by a single capsid protein subunit with a molecular weight ranging from 21 kDa in *Grapevine virus* D to 41 kDa in *Citrus leaf blotch virus*, and encapsidate a single RNA molecule of ~6 to ~9 kb that constitute ~5% of the particle weight (Adams et al., 2005). Studies on two other families of plant-infecting filamentous viruses, closteroviruses (Peremyslov et al., 2004, Satyanarayan et al., 2004) and potyviruses (Torrance et al., 2005), revealed that their virions possess terminal tail-like structures formed by additional viral proteins. As these proteins were also implicated in involvement in virus transport, the tails were proposed to represent specialized movement devices (Dolja et al., 2003). The ability of Potato virus X RNA and virions to associate with the viral 25-kDa movement protein of the triple gene block proteins *in vitro* (Karpova et al., 2006) suggests that the virions of other flexiviruses may also possess the tail-like appendages required for virus transport in infected plants.

Figure 1.6 Genome organization of a *Potato virus X,* a member of family *Alphaflexiviridae.* RdRp, RNA dependent RNA polymerase; TGB1, Triple gene block 1; TGB2, Triple gene block 2; TGB3, Triple gene block 3; CP, Coat protein.


Most members of the family *Betaflexiviridae* are transmissible from natural to experimental hosts by mechanical inoculation. The members of the genera *Potexvirus, Carlavirus, Allexivirus,* and *Trichovirus* can invade and multiply in parenchymatous tissue and are thus more readily transmissible mechanically than are the phloem-restricted viruses of either the genus *Foveavirus*, which are transmitted with difficulty, e.g. *Apple stem pitting virus* (ASPV) and *Apricot latent virus* (ApLV) or the genus *Vitivirus* which are not mechanically transmissible whatsoever e.g. *Grapevine rupestris stem pitting- associated virus* (GRSPaV). Thus, members of the family that do not have a vector depend on graft-transmission for their survival and dissemination. However, all flexiviruses infecting woody hosts, regardless of the genus, are very efficiently spread by nursery production/clonal propagation, the process that is largely responsible for the worldwide distribution of many fruit tree diseases. Although seeds represent important natural routes for virus dissemination, seed transmission is not of concern to the epidemiology of these two families. The few viruses transmitted by seeds include the citrus strain of *Apple stem grooving virus*

ASGV, potexviruses (*Potato virus X*, *Clover yellow mosaic virus, White clover mosaic virus, and Hosta virus X*), and carlaviruses (*Hop mosaic virus* , *Pea streak virus*, *Red clover vein mosaic virus* ) and some isolates of *Cowpea mild mottle virus* (CPMMV). Transmission rates of individual viruses are generally low and do not exceed 10% (Maury et al., 1998; Mink, 1993).

A number of members of the genus *Trichovirus* are transmitted by mites. *Peach mosaic virus* (PcMV) is transmitted by *Eriophyes insidiosus* (Keifer and Wilson 1955), *Cherry mottle leaf virus* (ChMLV) by *Eriophyes inequalis,* and *Grapevine berry inner necrosis virus* (GINV) by *Colomerus vitis* (Kunugi et al., 2000). However, no such transmission has been demonstrated for the type member of the genus (*Apple chlorotic leaf spot virus* (ACLSV)). Several capilloviruses, foveaviruses, and vitiviruses share the capacity to modify the host xylem known as stem-pitting or stem-grooving. The traits are characterized by the manner in which the woody cylinder is marked by localized, shallow surface indentations (pits), or by long narrow depressions (grooves). *Apple stem grooving virus* (ASGV), a capillovirus, and *Apple stem pitting virus* (ASPV), a foveavirus, causes abnormalities in some apple and pear cultivars, especially from far eastern countries, and can result in graft incompatibility such as the Japanese apple top working disease-Taka Tsugi Byo (Desvignes et al., 1999; Jelkman, W. 1997). *Apricot latent virus* (ApLV), a foveavirus causes symptomless infections in most apricot cultivars (Nemchinov et al., 2000) but possesses two molecular variants pathogenic to peach causing the foliar diseases peach asteroid mosaic and peach sooty ringspot, respectively (Desvignes et al., 1999, Gentit et al., 2001).

Trichoviruses are mainly pathogens of stone fruit trees (Almond, apricot, cherry, peach, and plum) and grapevine (*Grapevine berry inner necrosis virus,* GINV). These viruses produce a variety of symptoms, ranging from delayed bud break, stunted growth, mottling

and deformation of the leaves (*Peach mosaic virus*, *Cherry mottle leaf virus*), to severe damage of fruit such as false plum pox, plum bark split, fruit necrosis of cherry and apricot (*Apple chlorotic leafspot virus*), and necrosis of grape berries (GINV) (Desvignes et al., 1999, Larsen and Oldfield 1995,Terai and Yanase, 1992).

As has been stated, many of the *Betaflexiviridae* infect stone fruits. These crops are affected by a number of viruses in this family. The effects include reductions in tree growth, tree longevity, fruit size, fruit yield, and fruit quality. In addition to crops that yield fruit a number of *Prunus* species are popular ornamental species and are planted throughout the American landscape. Furthermore there are a number of wild cherry species found in the US and both flowering and wild cherries can act as alternate hosts for viruses that affect other prunus species (peach, apricot).

## Cherry

The sweet cherry (*Prunus avium* (L.) L. and sour cherries (*Prunus cerasus* L.) used for fruit production in the US are both members of the Prunus subgenus *cerasus*. The indigenous range of the sweet cherry extends through most of Europe, western Asia and parts of northern Africa, and the fruit has been consumed throughout its range since prehistoric times. Sweet cherry production in the US is located primarily on the west Coast: California, Oregon, and Washington states. This western production area extends into Canada in the Okanagan Valley. Total US production was 295,500 tons in 2014 (Non citrus fruit and nuts 20`14 preliminary summary United States Department of Agricutlure, National Agriculture statistics service, January, 2015). Sour cherry production is primarily

located in Michigan. Total US production was 288.8 million pounds in 2014. All cherry trees used in fruit production are either budded or grafted. The part above the graft/bud union is the scion and the part below the union is the rootstock. 'Mazzard' cherry (*Prunus avium*) also known as 'sweet cherry' has been used as a rootstock from ancient times. Mazzard' has been used to refer to a selected cultivar that comes true from seed, and which is used as a seedling root stock for fruiting cultivars. However, asexually propagated, virus-indexed sources of Mazzard (F12/1) are available. Mazzard cherries are graft-compatible to all sweet cherry scions. (Long & Kaiser, 2010.) Japanese flowering cherry trees (*Prunus serrulata* Lindl.) are widely grown in the urban landscape of the US. The most notable example being the flowering cherries located around the tidal basin in Washington DC. *P. serrulata* exists as a number of different varieties (Hortus III) Two of these varieties, var. Kwanzan and var. Shirofugen, have important roles as biological indicators for viruses of stone fruit (Kwanzan - Sour cherry green ring mottle virus, Shirofugen - *Prunus necrotic ringspot virus* – PNRSV;).

Cherry trees have been reported to be infected by virus and virus-like diseases since the early days of plant virology. In 1937, Cherry green ring mottle disease was reported from sour cherry and was confirmed as a viral disease in 1951 (Rasmussen et al., 1951). The virus was also reported to cause Cherry vein yellow spot disease (Milbrath, 1960) and infects several *Prunus* spp including sweet cherry, sour cherry, and oriental flowering cherry. *Cherry leaf roll virus* is a nepovirus that infects sweet cherry trees. Unlike other viruses of the genus *Nepovirus*, *Cherry leaf roll virus* is not transmitted by nematodes, and the mode of transmission is still unknown (von Bargen et al., 2009). *Cherry mottle leaf virus* (CMLV) was first reported in cherry in 1920 and causes chlorotic mottling and leaf distortion (Cheney and Parish, 1976). CMLV has genomic organization a similar to *Apple*

*chlorotic ringspot virus,* the type species of the genus *Trichovirus* (James et al., 2000). *Cherry rasp leaf virus* (a nepovirus), *Cherry virus A*, *Epirus Cherry virus*, *Little cherry virus 1*, and *Little cherry virus 2* are some of the other viruses that infect cherry trees. Little cherry virus devastated the cherry production in the Okanagan valley of British Columbia. Most recently Cherry Virus A is associated with infections in the US. This virus is believed to synergize with other viruses such as *Little cherry virus* (Komorowaka and Cielinska, 2004), or Mirabelle plum infected with Prune dwarf virus (Svanella-Dumas et al., 2005). In California Colt cherry were showing symptoms of leaf chlorotic rings and was found to be infected with *Cherry virus A*, *Plum bark necrosis and stem pitting associated virus* (Sabanadzovic et al., 2005).

Other important diseases of cherry associated with viruses include Cherry rusty mottle disease (CRMD) and Cherry necrotic rusty mottle disease (CNRMD). CRMD is graft-transmissible (Reeves, 1940). Affected leaves develop chlorotic mottling, leading to abscission. Though CRMD and CNRMD can cause similar symptoms in susceptible cherry cultivars, they can be easily distinguished by the distinct symptoms they produce in specific woody hosts. CNRMD is associated with large angular necrotic leaf spots, whereas CRMD induces yellow mottle symptoms with a bronze overtone on infected leave of the 'Sam' cherry biological indicator variety (Rott and Jelkman, 2011). The nucleotide sequence of Cherry necrotic rusty mottle virus (CNRMV), a virus associated with CNRMD has been determined (Rott and Jelkmann, 2001). CNRMV is an unassigned member of the family *Betaflexiviridae* (Adams et al., 2012). Cherry rusty mottle associated virus (CRMaV),is correlated with the appearance of cherry rusty mottle disease (CRMD) (Villamor et al. 2015) and has also been sequenced (Villamor et al., 2013).

The work presented in this thesis is designed to develop information on the

incidence of six viruses known to infect blackberry in the two largest production acreages in South Carolina. Both plantings were established with virus-indexed plants but have succumbed to virus infection within a year or two of establishment causing the growers to reevaluate the economic models on which they based their predicted involvement in growing blackberries. Blackberry yellow vein associated disease (BYDV) is a complex of viruses and the outcomes of infection have clearly been shown to be influenced by the composition of the viral population in a plant. The approach was to place sentinel plants within the blackberry plantings for a period of time during which they might become infected with viruses. The sentinel plants were then tested to detect the presence of 6 viruses for which sensitive and reliable RT-PCR detection systems exist. Characterization of the virus associated with the symptoms described in cherry was to provide information on this previously undescribed virus and determine if it posed a threat to the peach crop in the state of South Carolina. In completing this work the presence of a potentially new Ilarvirus infecting both Blackberry and Veronica was detected and *Cherry rusty mottle associated virus* was detected in cherry growing in South Carolina cherries for the first time.

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CHAPTER II

# INCIDENCE OF SIX VIRUSES IN TWO LARGE-SCALE PLANTINGS OF BLACKBERRY IN SOUTH CAROLINA.

## Introduction

Blackberry cultivation in the southeastern United States is flourishing with the release of new cultivars producing fruits suitable for the fresh market and the corresponding increase in demand by consumers for fresh fruit. With the increase in acreage and production, there have been increasing reports of diseases and insect pests on blackberry plants. One of the most important diseases in blackberry in southeastern United States is blackberry yellow vein disease (BYVD). The disease was first observed in 2000 in the Carolinas. Since then, it has become a serious threat to blackberry plants in many parts of the USA (Martin et al., 2004; Tzanetakis et al., 2008). For example, a few two-year-old 'Chickasaw' blackberries in a Northwest Arkansas production field showed symptoms in 2003, but within two years, BYVD had spread throughout the field, reducing yield and plant vigor (Susaimuthu et al., 2008a). Symptoms included vein yellowing of mature primocane leaves, with new leaves usually being asymptomatic (Susaimuthu et al., 2007). Symptoms may also include oak-leaf patterns, irregular chlorosis, and line patterns (Susaimuthu et al., 2006). A mosaic has also been observed on leaves of some infected plants. Floricanes can be severely affected by the disease, resulting in dieback of canes during the fruiting season.

BYVD was initially mistaken for infection by *Tobacco ringspot virus* (TRSV). However, grafting experiments proved that TRSV is asymptomatic in many blackberry

cultivars (Gergerich, unpublished) and was detected in only a small subset of BYVD-affected plants. Further studies carried out to determine the causal agent(s) of the disease reported the presence of a new crinivirus named *Blackberry yellow vein associated virus* (BYVaV) in symptomatic samples (Martin et al., 2004). Although BYVaV was detected in all symptomatic plants in this study further screening indicated that BYVaV alone produces latent infections (Susaimuthu et al., 2008a). It was speculated that BYVaV acts synergistically with other viruses to cause disease symptoms. Subsequently, several other viruses have been isolated from BYVD-infected plants including *Blackberry virus Y* (Susaimuthu et al., 2008b), Blackberry virus X*, Impatiens necrotic spot virus* (Tzanetakis et al., 2009)*,* Rubus virus S*,* Blackberry virus E (Sabanadzovic et al., 2009; 2011), and *Blackberry chlorotic ringspot virus* (BCRV) (Tzanetakis et al., 2007a). In addition, an *Emaravirus*, and another *Ilarvirus*, have been identified together with some other as yet incompletely characterized viruses.

BYVD is of serious concern for the blackberry growers, because as the plants are clonally propagated, it is possible that infected asymptomatic plants may have been used to generate propagules for the establishment of new plantings. As infected plants are planted in the field, infection with other viruses may lead to synergism and cause BYVD. A typical blackberry planting can produce for 15 to 20 years depending on the cultivars planted and cultivation practices. BYVD can reduce production to 5 to 7 years or less. Establishment of a new planting costs close to $10,000 per acre (Safley et al., 2006) and blackberry is a biennial crop, so growers have to wait for at least 2 years to obtain a full harvest. Therefore, BYVD is a major threat for the viability of blackberry production in the southeastern US.

## Sentinel plants

Sentinel plants is the term used to describe species that are sensitive to a specific substance, disease or pest and are used to detect the movement of these diseases and pests. They are either planted in the field to expose them to the agent of interest, or occur naturally in ecosystems. In both situations they are observed or tested to detect the presence of the specific agent to which they are sensitive. The movement of pest and diseases worldwide has increased the use of sentinel plants as a means of detecting invasive species. In the UK, plants susceptible to invading beetles, fungi, bacteria and viruses are being grown near places such as ports as part of an "early warning" system (http://www.independent.co.uk/environment/nature/network-of-plant-sentinels-on-the-lookout-for-pests-and-diseases-that-threaten-species-native-to-britain-10031424.html). In the US, the Sentinel Plant Network is a partnership with the National Plant Disease Network (NPDN) and the American Public Gardens Association that involves public garden professionals, volunteers, and visitors in the early detection of high-consequence plant pests and pathogens. This partnership merges the scientific and educational resources of the NPDN with the horticultural expertise and large public draw of the APGA to vastly expand the country's readiness to detect new plant pests and pathogens. In addition to detecting invasive species, statistical analysis can be applied to the data gathered and forecasts for the progress of "invasions" and or occurrence of the agent in field crops can be made (Vettraaino et al., 2015).

Sentinel plants help understand the temporal occurrence of the pathogen with in the field setting. Instead of sampling and surveying hundreds of acres of field, and sampling thousands of plants, a few hundred sentinel plants provide evidence of the type and severity

of virus infections in a particular field. Sentinel plants have been used as bio-monitors to understand herbicide drift and deposition and to detect herbicides with unique modes of action and characteristic injury patterns (Felsot et al., 1996). Sentinel plots have been used in soybean to understand the occurrence of viruses, fungal pathogens, and pests including soybean rust (Hobbs et al., 2010). The idea of using sentinel plants in virology is that the incidence of viruses in the field is related to the abundance of the vector in that field. For example *Prunus glandulosa* was identified as a sentinel plant for the early detection of *Plum pox virus* (Stobbs et al., 2005). In 2013, Wosula et al., conducted research on *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato virus G* (SPVG), and *Sweet potato virus* 2 (SPV2) on sweet potato (*Ipomoea batatas*). These 3 potyviruses are non-persistently transmitted by aphids. The research was focused on how aphid abundance, aphid species diversity, and virus titers relate to the spread of SPFMV, SPVG, and SPV2 in the field. Evidence of the temporal progression of virus incidence was observed. There is other ongoing research by USDA-ARS (Research Project #426054) that is looking into the spatial epidemiology of vector-borne plant viruses in potato, primarily *Potato virus* Y. After the onset of BYVD in blackberry in the Southeastern USA, Susaimuthu et al, 2007 used a small number of sentinel plants, to understand the movement of BYVaV and *Blackberry virus Y*. Here we used a large number of plants over 3 growing seasons and at 2 locations to obtain information on the presence of six viruses associated with BYVD in two commercial plantings of blackberry in South Carolina.

## Objective

The objective of this research was to determine the incidence of six viruses associated with the BYDV complex on sentinel plants located in two blackberry plantings. This information

could then be used to aid in the design of more extensive experiments that would examine the movement of the viruses into and within the crop, and allow strategies that minimize the economic impact of these viruses on the crop production to be proposed and tested.

## Material and Methods

### Field and cultivar selection

Two producers of blackberries in the upstate region of South Carolina were chosen as sites for conducting field trials. Cooley Farm is located in Chesnee, SC and has 40 acres of blackberries (35 acres of cv. 'Navaho' and 5 acres cv. 'Ouachita'). The other farm, Double J. Farm was located in Enoree, SC and has 7 acres of cv. 'Navaho' and 8 acres of cv. 'Prime Ark45' and 14 acres of cultivars 'Von', 'Osage', and 'Natchez' combined. As plants at Cooley's farm were already showing symptoms of viral infection, plants of the two cultivars Natchez and Ouachita were chosen for use as sentinel plants. Virus-indexed plants of these two cultivars are available and both are widely grown in the southeastern part of the US. The plants had been propagated in tissue culture from virus-indexed mother plants at North American Plants, McMinnville, Oregon. Prior to exposure in the field plants were maintained in a screened greenhouse (Figure 2.1) and tested by reverse transcriptase-polymerase chain reaction (RT-PCR) for the particular viruses being investigated in this study.

Figure 2.1 Blackberry plants grown in the screened greenhouse before they were taken to the field

List of viruses studied in sentinel plants experiment

Over 40 different viruses are known to infect blackberry plants. Some are newly described viruses, while others were previously described in other crops but are new to blackberry plants. In this work, the incidence of six viruses previously reported in blackberry was studied.

*Blackberry yellow vein-associated virus* (BYVaV)

BYVaV was the first virus to be identified in the BYVD complex (Susaimuthu et al., 2006). It was present in virtually every symptomatic sample tested when the disease was first reported. The virus was assumed to be the causal agent of the disease until it was also detected in samples collected from asymptomatic plants. BYVaV is still the most commonly occurring virus in the BYVD complex. The virus can interact synergistically with *Blackberry virus* Y, and cause symptoms of BYVD. It is possible to reproduce BYVD symptoms by graft-inoculating material from pure cultures of BYVaV and BVY into a single healthy blackberry plant (Susaimuthu et al., 2008a).

*Blackberry virus Y* (BVY)

Blackberry virus Y belongs to the family *Potyviridae* and the genus *Brambyvirus*. The majority of potyviruses are transmitted by aphids, but some are transmitted by whiteflies, eriophyid mites, and plasmidiophorid protists (Adret-Link & Fuchs, 2005). However,

transmission of BVY using aphids has not been successful. BVY was the second virus in the BYVD complex to be studied extensively (Susaimuthu et al., 2008b).

### *Blackberry chlorotic ringspot virus* (BCRV)

*Blackberry chlorotic ringspot virus* is another virus commonly found in the disease complex. BCRV is an ilarvirus. It can be transmitted by seeds (Poudel et al., 2014) and infects rose and apple in addition to blackberry thus suggesting that the epidemiology of the virus may be complex. Despite being transmissible by seeds, the virus was included in this study as the mechanical transmission by thrips feeding on virus-infected, wind-blown pollen, known to occur with other ilarviruses (Jones, 2005), might be a factor in the transmission of BCRV in field-grown blackberry.

### Blackberry virus E (BVE)

Blackberry virus E is a newly described virus in the family *Flexiviridae*. It is related to members of the genus *Allexivirus* that infect species in the family *Alliaceae* and are transmitted by mites (Kang et al., 2007). No known vector has yet to be identified for BVE but as related viruses are transmitted by mites they may be a candidate vector.

### *Tobacco ringspot virus* (TRSV)

*Tobacco ringspot virus* is a nepovirus and is transmitted by *Xiphinema americanum.* TRSV is one of the most frequently occurring viruses in the BYVD complex (Gergerich, unpublished).

TRSV and *X. americanum* have many alternate plant hosts. Thus infection can move from a weed species to the blackberry plants. Typically, sites in which blackberries are to be grown are preplant-fumigated, however this may only suppress nematode populations not eliminate them.

## Blackberry virus Ω (BVΩ)

Blackberry virus Ω is a putative new Emaravirus. BVΩ was first found in samples at University of Arkansas by Ilumina sequencing of sentinel plant samples. The tests have shown that the new virus is prevalent in blackberry fields and may be involved in the etiology of BYVD (Hassan, Unpublished).

## Sentinel plant experiments

Sentinel plant experiments were conducted at the two largest commercial blackberry farms in South Carolina. In the first year (2012), experiments were conducted only at Cooley's farm for three months, from June to August, using 100 (50 Natchez and 50 Ouachita) plants each month. Small plants received from North American Plants were established in 5" pots and maintained in a greenhouse screened with mesh capable of excluding aphids before being exposed in the field. Prior to exposure, the plants were indexed for the presence of the 6 viruses using RT-PCR (Appendix B and Appendix E) and the primers listed in Table 2.1. The plants were located in the field in groups of five along the existing rows of blackberry plants in order to use the planting's irrigation system to maintain the sentinel plants during

their month-long exposure in the field. After exposure, plants were returned to the greenhouse. A total of 10 different sites were chosen throughout the planting of each cultivar and a total of 300 plants were used.

In the second and third years, experiments were conducted at both Cooley Farm and the Double J. Farm. Sixty plants (30 Natchez and 30 Ouachita) were exposed for a month at each location during the period from May to August. Three plants of each cultivar (Figure 2.2) were put at 10 sites in each field. A total of 480 plants were used at each site for each year in 2013 and 2014. After exposure the plants were returned to the greenhouse and allowed to overwinter as dormant material (Figure 2.3). In all years, plants were treated with foliar (0.1 % Bifenthrin [Upstar gold]) as well as systemic ([1.25gms/gallon Merit 75 WP, Bayer]) insecticide before re-entry into the greenhouse.

Samples for virus-indexing against the 6 selected viruses were collected from the young, fully expanded leaves of new growth after overwintering. This particular stage of plant growth is optimal for the detection of the maximum number of plant viruses. Very young plant leaves will have a very low, but detectable, concentration of phloem-limited viruses such as BYVaV. Expanded mature leaves contain high concentrations of inhibitors that affect the quality of RNA and interfere with the PCR reactions.

Figure 2.2 Sentinel blackberry plants located in the field and linked to the in-field irrigation system.

Figure 2.3 Sentinel blackberry plants brought back from the field and placed in the green house allowing them to over-winter.

RNA extraction and RT-PCR for detection of viruses

Total nucleic acid extractions were performed as described in Appendix A. Reverse transcription (RT) was done using gene-specific reverse primers for each of the viruses (Table 2.1), following the protocol listed in Appendix B. Polymerase chain reaction (PCR) was done using specific primers pairs for the viruses (Table 2.1), and the protocol described in Appendix E. Primers for BVΩ, BVY and BVE were provided by Dr. I.E. Tzanetakis from University of Arkansas (Table 2.1). Products were visualized on 1% agarose gel.

Table 2.1 Primer pairs used to detect the viruses in sentinel blackberry plants. BVΩ: Blackberry virus Ω, BVE: Blackberry virus E, BYVaV: Blackberry yellow vein-associated virus, BCRV: *Blackberry chlorotic ringspot virus*, BVY: *Blackberry virus Y*, TRSV: *Tobacco ringspot virus.* F indicates forward or sense primer. R indicates a reverse or antisense primer.

| Primer name | Product size | Primer sequence 5'-3' |
|---|---|---|
| BVΩ P3F350 | ~ 330bp | CATAAAGGAATTCATACCCAGGAAC |
| BVΩ P3R680 | | AGTTGCATCTTACCTTTCGCGATC |
| BVER6270 | ~ 300bp | GCTCCACTGGAGGAGTTCTCCTG |
| BVEF6050 | | TGTGGACGATGCACGCCAGATCC |
| BYVaV4736F | ~ 300bp | TTGAAAGGAAACTTCACGGA |
| BYVaV5037R | | TAAGTTCATACGTTTCCTGCG |
| BCRVRNA31674F | ~ 450bp | ACCTGCTGATCAGCTWTCAGAGAA |
| BCRVRNA32237R | | TAGAACATCGACCCAAAGGT |
| BVYdet F | ~ 357bp | TCGTTGAGGGACCAGT |
| BVYdet R | | CTCGCTCTCCCCATTC |
| BVYRF | ~ 190 | GAATTTGATGCAGAGGCCATA |
| BVYRR | | TGCTTTAAGTGAGCCTTTCCA |
| TRSV F | ~450bp | TGACGTAGGGTTGGAGGTGC |
| TRSV R | | GGACATGGACTGTGCAACTGG |

## Results

Virus-like symptoms were seen in some plants as early as 4 weeks after being exposed in the field (Figure 2.4).

Figure 2.4 Virus-like symptoms observed in blackberry sentinel plants after being in a blackberry field for four weeks.


In 2012, (Table 2.2) viruses were detected in about 8% of the sentinel plants exposed during, June, July, and August. BYVaV was the most frequently detected virus, with most detections occurring in plants exposed in July. TRSV was not detected at any time during the season. BCRV was detected only in June. Fewer than 3 plants infected by BVE, BVY or BVΩ were detected during the entire growing season.

Table 2.2 Incidence of viruses in 300 sentinel blackberry plants from Cooley's farm in South Carolina over 3 summer months in 2012.

| Virus tested | June | July | August | Total |
|---|---|---|---|---|
| BYVaV | 2/100 | 11/100 | 0/100 | 13/300 |
| BVY | 0/100 | 0/100 | 1/100 | 1/300 |
| BCRV | 4/100 | 0/100 | 0/100 | 4/300 |
| BVE | 1/100 | 3/100 | 0/100 | 4/300 |
| TRSV | 0/100 | 0/100 | 0/100 | 0/300 |
| BVΩ | 2/100 | 1/100 | 0/100 | 3/300 |
| Total | 9/100 | 15/100 | 1/100 | 25/300 |

In 2013, the research was conducted at two different locations and over a 4 month period. Sixty plants exposed in each location each month. Viruses were detected in approximately 24% of the plants (Table 2.3). BVΩ was the virus most commonly detected in sentinel plants followed by BYVaV and BVE. The peak period for the detection of BYVaV was May at the Landa's farm unlike the peak occurrence of the virus in July 2012 at the Cooley Farm. BVΩ was detected at 3 out of 4 sampling times at both locations. Again there were peak periods for the detection of some viruses and there were notable differences in the incidence of the viruses at the two sites. Virus incidence was higher in Landa's farm in May whereas Cooley's farm had the higher incidence of viruses in the month of July. Only 4 of the six viruses tested were detected in growing season of 2013.

Table 2.3 Incidence of six viruses in 480 blackberry sentinel plants at two locations in South Carolina over four summer months in 2013.

| Virus tested | **May** | | **June** | | **July** | | **August** | | **Total** |
|---|---|---|---|---|---|---|---|---|---|
| | Cooley | Landa | Cooley | Landa | Cooley | Landa | Cooley | Landa | |
| BYVav | 0/60 | 19/60 | 0/60 | 0/60 | 4/60 | 2/60 | 0/60 | 1/60 | 26/480 |
| BVY | 2/60 | 0/60 | 0/60 | 3/60 | 0/60 | 0/60 | 0/60 | 0/60 | 5/480 |
| BCRV | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/480 |
| BVE | 1/60 | 0/60 | 1/60 | 0/60 | 0/60 | 0/60 | 8/60 | 11/60 | 21/480 |
| TRSV | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/480 |
| BVΩ | 6/60 | 9/60 | 3/60 | 0/60 | 16/60 | 14/60 | 0/60 | 6/60 | 54/480 |
| **Total** | 9/60 | 28/60 | 4/60 | 3/60 | 20/60 | 16/60 | 8/60 | 18/60 | 116/480 |

The experimental design for 2014 was same as 2013. Results similar to those in 2013 were obtained. Viruses were detected in about 21% of the sentinel plants. BVΩ was the virus most frequently detected in sentinel plants followed by BYVaV and. BCRV was detected in only a single sentinel plant (June –Landa) Unlike 2013, Cooley's farm had the highest incidence of viruses tested in the month of June and Landa's farm had the highest incidence in July.

Table 2.4 Incidence of six viruses in 480 blackberry sentinel plants at two locations in South Carolina over four summer months in 2014.

| Virus tested | **May** | | **June** | | **July** | | **August** | | **Total** |
|---|---|---|---|---|---|---|---|---|---|
| | Cooley | Landa | Cooley | Landa | Cooley | Landa | Cooley | Landa | |
| BYVav | 0/60 | 0/60 | 5/60 | 0/60 | 12/60 | 0/60 | 4/60 | 0/60 | 21/480 |
| BVY | 1/60 | 5/60 | 4/60 | 0/60 | 2/60 | 4/60 | 0/60 | 6/60 | 22/480 |
| BCRV | 0/60 | 0/60 | 0/60 | 1/60 | 0/60 | 0/60 | 0/60 | 0/60 | 1/480 |
| BVE | 3/60 | 0/60 | 0/60 | 0/60 | 0/60 | 3/60 | 0/60 | 0/60 | 6/480 |
| TRSV | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/480 |
| BVΩ | 4/60 | 9/60 | 33/60 | 1/60 | 2/60 | 0/60 | 4/60 | 0/60 | 53/480 |
| **Total** | 8/60 | 14/60 | 42/60 | 2/60 | 16/60 | 7/60 | 8/60 | 6/60 | 103/480 |

When the virus incidence was compared yearwise between the two fields where experiments were conducted (Table 2.5). Landa's farm had higher incidence in 2013, and Cooley's farm had the highest incidence of the viruses tested in 2014. Co-infection, where a plant had at least two of the viruses tested was highest in 2013 (Table 2.5). When the two different cultivars were compared for the incidence of six viruses tested, 'Ouachita' was seen to have a greater incidence of the viruses than Natchez in year 2013 and 2014. In 2012, Natchez had the higher incidence of viruses, but the difference between the two cultivars was minimal compared to 2012 and 2013 (Table 2.5).

Table 2.5 Total number plants infected with at least one virus.

| Year | Plants infected with at least one virus | | | | |
|---|---|---|---|---|---|
| | Cooley's Farm | Landa's Farm | Cultivar Natchez | Cultivar Ouachita | Co-infection |
| 2012 | 8% | NA | 4.6% | 3.6% | 4% |
| 2013 | 17% | 27% | 13.3% | 30.8% | 14% |
| 2014 | 30% | 12% | 18.75 | 24.1% | 10.6% |

A comparison was made between the incidence of viruses in each year at each farm (table 2.6). The incidence of BVΩ was highest in Cooley's farm in 2014. BVE had the highest incidence in Landa's farm in 2013. TRSV was not detected in any sentinel plants throughout the study period. The incidence of BYVaV was highest in 2013 in Landa's farm. The highest number of BCRV positive sentinel plants were found in 2012 Cooley's farm. The highest incidence of BVY was found in 2014 in Cooley's farm (Table 2.5).

Table 2.6 Virus incidence in each farm.

| Virus tested | Particular virus incidence | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2012 | | 2013 | | 2014 | | |
| | Cooley's | Landa | Cooley's | Landa | Cooley's | Landa | Total |
| BVΩ | 3 | NA | 25 | 29 | 42 | 10 | 109 |
| BVE | 4 | NA | 10 | 11 | 3 | 3 | 31 |
| TRSV | 0 | NA | 0 | 0 | 0 | 0 | 0 |
| BYVaV | 13 | NA | 4 | 22 | 21 | 0 | 60 |
| BCRV | 4 | NA | 0 | 0 | 0 | 1 | 5 |
| BVY | 1 | NA | 2 | 3 | 8 | 15 | 29 |
| Total | 25/300 | | 41/240 | 65/240 | 74/240 | 29/240 | |

## Discussion

There is little information about the epidemiology of viruses in blackberry plantings. This research determined the incidence of six common viruses of blackberry in two conventional fields in South Carolina.

TRSV was not detected in any of the sentinel plants. The 4 week exposure of the plants may not have been sufficient for nematode transmission of the virus to occur even though assays of the soil around roots of the blackberries growing in the field showed *Xiphinema* spp populations to be present. TRSV is indigenous to the area in which the fields are located, having been detected in cucurbit species, and *Trifolium* species and isolated from Cheyenne Blackberry a number of years ago (Scott pers. Comm.). Also the sentinel plants were in pots, and although the pots had holes for drainage these could have limited the access of nematodes to the plant roots.

Although BCRV has been detected in rose, apple, and blackberry, and is seed transmissible at a very high rate (Poudel et al., 2014), It was only detected in 5 plants (4 plants at Colley's in 2012 and 1 plant at Landa's in 2014). This very low number of BCRV positives in our results in 2012 and 2014, and no positives in 2013, might be attributed to lack of a vector for the virus transmission. Ilarviruses are disseminated in pollen but then require either fertilization of the non-infected host to take place or feeding of thrips species on infected pollen and mechanical transmission of the virus to a non-infected host for transmission of the virus to occur. The majority of ilarviruses move relatively short distances in the crops that they infect (Howell and Mink, 1988), although if the planting had been established using propagants produced from an unknowingly infected source, levels of infection approaching 100% can be detected (Scott et al., 1989).

The other 4 viruses that were detected, BYVaV, BVY, BVE and BVΩ, all have known insect vectors, or by association with related viruses, may have an insect vector. BYVaV is a crinivirus. Other members of that genus are transmitted by whiteflies. BVY is a potyvirus and the majority of the members of that genus are transmitted by aphids. BVE is related to members of the genus *Allexivirus* that infect species in the family *Alliaceae* and are transmitted by mites (Kang et al., 2007).Emaraviruses (BVΩ) are transmitted by mites (Mielke-Ehret and Mühlbach, 2012, Tatineni et al., 2014).

The interaction of the viruses that we detected with insect vectors would be consistent with our data. For example, the large numbers of detections of BYVaV could be associated with the presence of a large population of whiteflies. This population might peak at different times at the two locations. Cooley's farm is at 911 feet above sea level and approx. 32 mile due north of Landa's Farm, which is at 700 feet above sea level. The variations in incidence for the other viruses might also be explained by variations in populations of aphids and or mites and the differential efficiency of species to transmit the viruses. Information on the pesticide spray schedules used at the two farms might allow peaks of insect vector flight to be more closely related to the incidence of the detected viruses, as might weather data for the 3 different growing seasons.

The new *Emaravirus,* BVΩ, has been found widely distributed in blackberry plants in Arkansas and in our study is the most frequently detected virus among the six tested. The incidence of this particular virus detected in sentinel plants suggests that the transmission by the vector is very efficient and rapid whatever species is involved and the virus may clearly be a threat to the industry throughout the southeast. Similarly, although vectors of BVY and BE are as yet unknown, observations elsewhere and in our research show them both to be moving in the field.

As might be expected we were able to identify a number of plants in which more than one virus was present Table 2.5). The most common co-infection was of BYVaV and BVΩ followed by co-infection between BYVaV and BVY (data not shown).

Thus our preliminary study on the incidence viruses in blackberry has achieved a number of milestones that can allow us to design additional research to increase the knowledge of the epidemiology of viruses in blackberry. This is the most extensive study involving sentinel plants for the detection of viruses in blackberry reported to date. It involved a total of 1260 sentinel plants exposed during growing seasons in the field over the course of 3 years. 4 weeks exposure was sufficient for insect-vectored viruses to be transmitted to the virus-tested healthy sentinel plants as evidenced by the development of symptoms on some of the plant (Figure 2.4). Records of the differences in the weather conditions from year to year would have undoubtedly added to information about peak vector flights and the incidence of virus transmission. Although weather data were lost for the period of our experiments they should certainly be recorded in future work. The populations of the whitefly vectors of criniviruses are adversely affected by heavy rainfall but increase dramatically in periods when the weather is dry and warm. Rainfall data could not be presented in this thesis due to loss of weather data in a local weather station close to the experiment fields.

We have PCR systems that allow us to detect these viruses. To obtain maximum information about the movement of these viruses in blackberry crops, it would be appropriate, in additional work, to collect weather data and also insect population data. This preliminary study has allowed us to detect BVΩ and BVE for the first time in South Carolina and provided a glimpse of the extent of the potential problem. The ideal experimental design would involve the scientists throughout the development of new

blackberry planting: collection of soil samples, collection of nematode samples, confirmation that the planting material is virus-indexed and in addition some entomological studies on the presence and movement of potential vectors in the crop would add greatly to our knowledge.

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# CHAPTER III

# A POTENTIAL NEW ILARVIRUS FROM SUBGROUP 1 INFECTING BLACKBERRY AND VERONICA

## Introduction

A number of ilarviruses have been reported to infect members of the genus *Rubus* over the years. Tobacco streak virus (TSV) was reported to infect black raspberry (Converse, 1972), red raspberry (Stace-Smith et al., 1982), and blackberry (Jones & Mayo, 1975). A virus initially named Black raspberry latent virus (Lister and Converse, 1972) was later accepted as being an isolate of TSV. Apple mosaic virus has been reported to infect red raspberry and tissue culture had to be implemented to obtain virus-free plants (Theiler-Hedtrich and Baumann, 1989).

In the past decade other ilarviruses have been reported to infect *Rubus* spp. In 2004, when Blackberry yellow vein disease (BYVD) was recognized as a major threat to fresh blackberry production in the southeastern United States, an extensive study confirmed that BYVD was a disease complex in which more than one virus is involved. In that research, Strawberry necrotic shock disease, previously associated with a strain of TSV, was recognized as being part of the BYVD complex (Tzanetakis et al., 2004) and the pathogen was recognized as a unique virus which was given the name *Strawberry necrotic shock virus* (SNSV). *Blackberry chlorotic ringspot virus* (BCRV) was another ilarvirus that was found in the BYVD complex (Tzanetakis et al., 2007). BCRV had first been detected in the United Kingdom 20 years earlier causing chlorotic symptoms in blackberries, but the virus was incompletely characterized and unnamed. After sequencing of the viral genome, the isolate

was recognized as a new virus (Jones et al., 2006). In the US, most ilarviruses reported to infect *Rubus* spp did not produce symptoms when occurring as a single infection but resulted in severe symptoms when plants were infected by a mixture of viruses (Tzanetakis et al., 2008).

In 2011, several virus-like symptoms were observed on sentinel blackberry plants collected in August at the University of Arkansas (AR). When dsRNA was extracted from these plants and subjected to electrophoresis, the banding pattern resembled those reported for viruses in the family *Bromoviridae*. Samples were deep sequenced using Illumina (Center of Genomic Research and Biocomputing, Oregon State University, Corvallis, OR) and the sequences obtained showed some degree of homology to sequences of the RNA 1 and RNA 3 of Ilarviruses subgroup 1. At the same time, the virus was detected in sentinel blackberry plants in North Carolina State University (NC) and blackberry plants in Mississippi State University (MS). The sequences obtained at all three locations were identical. Using degenerate ilarvirus primers, (Untiveros et al., 2010. Table 3.1), the presence of an Ilarvirus was confirmed in these the blackberry plants from all three states.

Also, in 2011, some plants of veronica (*Veronica verna*) 'Christy Speedwell' were received at Clemson University from Costa Farms, SC. The grower suspected a virus was responsible for the symptoms observed in these plants. RNA extracted from the veronica plants was tested by PCR using the universal ilarvirus primers (Untiveros et.al, 2010) and the amplicons produced were cloned and sequenced. The sequences obtained were identical to the sequences of the ilarvirus obtained from blackberries in AR, NC, and MS. Experiments were conducted to obtain the complete genomic sequences of both the viruses detected in blackberry and in veronica. Attempts to mechanically transfer the virus from either blackberry or veronica to an herbaceous host susceptible to many ilarviruses

(*Chenopodium quinoa* Willd.), so that purified virus might be obtained, were unsuccessful. Thus a molecular approach, extraction of dsRNA with which to obtain nucleic acid and RT-PCR using primers designed from the alignments of subgroup 1 ilarviruses to amplify other areas of the genomic sequences, was attempted.

## Objective

The objective of this study was to obtain sequence information of the potentially new ilarvirus detected in blackberry and veronica. This information could be used for molecular characterization of, and development of rapid detection techniques (PCR) for, the virus. This would allow the role of the virus in the BYDV complex, to be evaluated and an understanding of the evolutionary history and the etiology of the virus could be obtained.

## Material and Methods

Double-stranded RNA was extracted from veronica and blackberry plants as described in Appendix C. Reverse transcription (RT) was done using the purified dsRNA as a template (Appendix D). Polymerase chain reaction was done as described (Appendix E) using numerous pairs of primers that were designed to amplify fragments of the virus (Table 3.1). Primers for the virus were designed by aligning the sequences of closely related viruses from the National Center for Biotechnology Information (NCBI) using ClustalW (McWilliam et al., 2013) to find conserved regions. The viruses included in the alignments were *Strawberry necrotic shock virus*, *Tobacco streak virus*, *Parietaria mottle virus*, and *Blackberry*

*chlorotic ringspot virus,* all members of the subgroup 1 of the genus *Ilarvirus*. The 5' and 3' terminal sequences were obtained by using SMARTer RACE (Clonetech).

The PCR products were visualized in a 1% agarose gel buffered with 1X TBE stained with GelRed (Phenix Research, Candler, NC). The bands amplified by PCR were purified from the gel using a MinElute Gel Extraction (QIAGEN). Cloning and sequencing was done as described (Appendix H). Sequence fragments were analyzed with the CAP3 sequence assembly program (Huang and Madan, 1999) to obtain contiguous sequence. The phylogenetic trees were generated on MEGA 6, implementing two different methods: Maximum Likelihood and Neighbor Joining. A bootstrap value of 1000 replicates and the Kimura 2-parameter model (Kimura, 1980) were used in the construction of phylogenetic tree.

Table 3.1 List of primers used to obtain the sequence of the potential new ilarvirus from blackberry and veronica.

| Primer | Sequence 5'-3' |
|---|---|
| Ilarvirus specific primer | TCAGTATGAACGAGCAATGTCT |
| Ilarvirus specific primer | CCGAACATTGCAAAGATTC |
| NewilarRNA1-2225F -1320 | AAGTGGTACCCGGATTCACG |
| NewilarRNA1-2380F -1321 | GTTCAATTCTGGGCAACGCA |
| NewilarvirusRNA1-2606R -1322 | ACTTCATCGCGTCCGAACAT |
| NewilarvirusRNA1-2668R-1323 | AATCTGTCGGCAGCAAACCA |
| NewilarvirusRNA1-2580F -1324 | TCTTTGCAATGTTCGGACGC |
| NewilarvirusRNA1-2610F -1325 | CGAATCCGGACCGTCGATAG |
| NewilarRNA1-212F-1326 | GGTGATTTCCAGAAGTTAAATG |
| NewilarRNA1-735F-1327 | GGTCATATACCCTTCCTCAAT |
| NewilarRNA1-2120R-1328 | AGGTTGCGGGAATTGGTCAT |
| NewilarRNA1-2000R -1329 | GGCCTCTTGAGTCAAACCCA |
| NewilarRNA1-226R-1330 | CTTCTGGAAATCACCATTGCT |
| NewilarvirusRNA1-315R-1331 | GCAAAACTATGGGATGAATAACA |
| NewilarvirusRNA1-444R-1332 | CCTTGTTTTGCGTGAGTAACAT |
| NewilarvirusRNA1-857R-1333 | CATGAGAGTAACTTAAACCTGGTGC |
| New ilarvirusRNA2-2681R-1334 | CCAAATTTTGGAATATGGATGT |
| NewilarvirusRNA2-2478R -1335 | AACTTCGGTACCTTCACGGAGA |
| NewilarvirusRNA2-1353f-1336 | GGAAAATACCACCAGATATTTCA |
| NewilarvirusRNA2-1592R-1337 | CGTTGATAATCAACGGAAAA |
| NewilarvirusRNA2-282F-1338 | GAGATTGATCCCTTTTATCTTCCT |
| NewilarvirusRNA2-133F -1339 | GGGTTAGCATGTTTTTGTTCAAG |
| NewilarvirusRNA2-307R-1340 | TAAGGAAGATAAAAGGGATCAATC |
| NewilarvirsRNA2-740R-1341 | CCCACTCTTGGAAGAATTTATC |
| NewilarvirusRNA2-2494F-1342 | CCGAAGTTAAATATTGACTTCGA |
| NewIlarvirusRNA2-2665F-1344 | ATTCCAAAATTTGGTTGGAAAC |
| NewilarvirusRNA3-918F-1345 | AAGGACACAAGTGGTGGCAA |
| NewilarvirusRNA3-1514R=1346 | CCAGCGGACATTGCAACAAA |
| NewIlarvirusRNA3-1571R-1347 | ACAATTGGTGGATCGGGGAC |
| NewilarvirusRNA3-465R-1348 | TAGGCCAGGTCATCGTCAGA |
| NewilarvirusRNA3-407R-1349 | AAGCTCATCACCAGTTGCGA |
| New ilarvirusRNA3-141R-1350 | CCGCCTTCTCAAGACTGGAT |
| New Ilarvirus RNA3-1711F -1351 | GCTGACTATTGGGTCGCCAT |
| NewilarvirusRNA3-1805F-1352 | CCGGACGTGTGTCCGATTT |
| ilarRNA1F2deg452F[1] | AAYGTBCAYWSNTGYTGYCC |
| IlarRNA1-1700R[1] | GCCTTCATATGCGCAGGAA |
| ilar2f5deg[1] | TCRAYRTTYGAYAARTCNCA |
| Ilar2R9deg [1] | GGTTGRTTRTGHGGRAAYTT |
| Ilar1f5deg5[1] | GCNGGWTGYGGDAARWCNAC |

1: Primers from the study by Untiveros et al., 2010.

## Results

Only faint bands of dsRNA were observed when the end product of an extraction was examined in an agarose gel. (Figure 3.1). Partial sequences of the RNA1, RNA2, and RNA3 of the virus had been obtained as a result of the Illumina deep sequencing. This information has been supplemented and extended using the approach outlined above. Almost complete, contiguous sequence has been obtained for the RNA 1. Approximately 200 nt at the 5' terminus remains to be determined (Figure 3.2). The sequence obtained was aligned using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) provided by National Center of Biotechnology Information and shows highest similarity (75%) to *Ageratum latent virus*, a subgroup 1 ilarvirus.

The smallest amount of sequence of the three RNAs of the virus (220 nt) was obtained for the RNA 2 (Figure 3.3). When aligned using BLAST this sequence showed 76% identity to *Parietaria mottle virus*, a second subgroup 1 ilarvirus.

Approximately, 2kb of sequence for the RNA3 that encodes the movement protein and coat protein of the virus has been obtained, (Figure 3.4). Approx 200 nt at the 5' terminus remains to be determined. The sequence when aligned using BLAST is 74% identical to the RNA3 of *Strawberry necrotic shock virus*, another subgroup 1 ilarvirus.

When phylogenetic trees were constructed using sequences from the other species in the genus *Ilarvirus* using either Maximum Likelihood or Neighbor-joining methods, both generated almost identical trees. The Maximum Likelihood trees for RNA1 and RNA2 of the potential new ilarvirus along with other species of the genus have been presented in figure 3.5 and 3.6. The sequence obtained for RNA1 grouped together with *Parietaria mottle virus*

whereas the sequences of RNA3 grouped closest to *Blackberry chlorotic ringspot* virus, thus indicating that the new virus possibly belongs to the subgroup 1 of genus *Ilarvirus*.

Figure 3.1 Faint band of the potential new ilarvirus dsRNA extracted from veronica plant observed on Agarose gel. Lane 1: Blank, Lane 2: dsRNA of new ilarvirus, Lane 3: 1 Kb DNA marker.

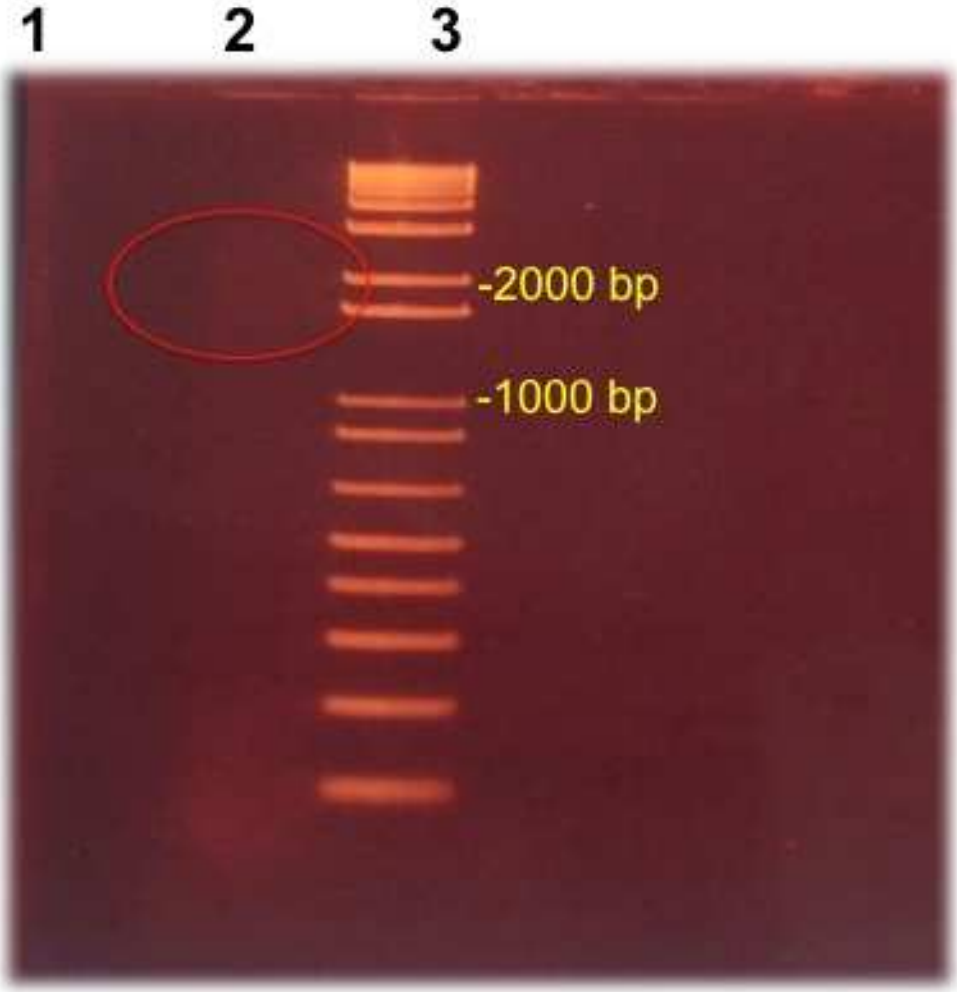


Figure 3.2. Nucleotide sequence of RNA1 from the potential new ilarvirus. From approximate position: 5'-214 nt to 3358 nt-3'. The underlined sequences indicate the nucleotides that translate to a putative aa sequence that aligns with corresponding regions of other subgroup 1 ilarviruses.

```
TGGTGATTTC CAGAAGTTAA ATGTCGGTTT CCGATTAACC CCTGATGAAA AGAACGCATT        60
GAAAACGAGC TTCCCTGGTT TGGAGATCGT TTTCAAGGAT TCTTGTCAAT CTTCACACAG       120
TTTTGCTGCT GCGCATAGAG TGTGTGAGAC CTTAGACATA TACAAGCGCT TCAACACAAA       180
GACTGAAAAA ATAATCGACC TTGGTGGTAA CTATGTCACA CATGCTAAGC ATGGTAGGTC       240
GAATGTTCAT TCTTGTTGTC CAATCCTTGA TGTTCGAGAC GGTGCTCGAC ACACGGATAG       300
GTATATGTCT CTAGCTGCCG CTGTGGAAAA ACATCACAAG GATCTTCCAG TAGATTTTTG       360
TTGCCACAGA TTCGAAGACT GTAACGTACA GGCACCGTAT GCCATGGCTA TTCATTCCAT       420
CAGTGACATT CCAATTGCAA CCGTCGCAAC GCATTGCGTG AGACGTGGAG TGAGAAAATT       480
GATCGCTTCG GTGATGATGG ATCCGGCCAT GATGCTTTAT GACAAAGGTC ATATCCCTTT       540
ACTCAACATT GATTGGGAAA AGGAAGATGT TAGTGTCGAT GGAAAATTTA AGACACTCAT       600
ACATTTCCAC TTTGTTGATG CCCCAGGGTT GAGTTATTCC CACGATTTCT CAGTGTTGTC       660
GCAATACATG GTGACTAACC AGGTGATAGT TGGTGATGGC TACTCTTATA GAGTTGAGCG       720
TACCGCCGAC TTAAATGGTG TGTTCATAGT TGAGATGACT CTGAGTATGA CCGATGGAGA       780
GACCCTAAAC CACATGAAAC CGCTCACGGA TATTTCGTGT GCTTGGCTAT CGAAGTTGAG       840
AAAGAAAGTT TTCGTGAAAC TTGCTGTACC AATTTCAGAC CCATCATGGT ATACCGAATC       900
GTTTGAGATT CGATGGGCTC TGATGGATGA ATCCTGGGTG AGATACGTCT CAGAAGCTGC       960
CTTCCGCCAG TTTTCGAAAA CCAAGGATCC AGAGACTGTG GTGCAGTACA TCGCAACCAT      1020
GTTGTCGTCT TCTTCGAATC ATGTGGTCAT AAATGGGATT ACCATGAGGA ACGGGAGTCC      1080
CATAGCCATT GATGAGTACG TTCCTTTGGC TGTTACCTTT TACGCCATGG CTGCTTGGCG      1140
ATATAAGATG ATTTCCCCTG GATTAAGTGC AGTCACCACC TCTGCGAGAA AGAATATTGA      1200
TGATTATCAT TCAAGTCGAG GTGAAGAAAC TCTATCTGAT GTCCTTCGGG AGGCTCAAAT      1260
GAATATCCTT CCGGATGATG ATTTTGGTCT GAAGAACTGT GAAAGGATAC CAGACTTCAT      1320
CAAATCCGTT GGATTAAGGT GTGTCAAGGG TAAATCGGTA GAGAAGATCA GGGATGATGT      1380
TTCCTTGTTG AAATCTCGAA GCTACTTCTT AGAGACTGTG CAAGAGATTA AACAGTTTTT      1440
CGGTTTGACC GTAACTGGTT CCGACTTCAA TTTTGTTGAT GGTACTCCCT CATGTTTGAA      1500
GTCAACCACA GTCTGGAAAG TCTTCACGGA AAATGTAAAA TTTCCCTCAT GTCTGAACGT      1560
CTCTGAATGT TCATATGACT TGATGAACAA GCATCTTGTT CAAAAGATTG AGGATGAACG      1620
TGAAGAGAAG AGGAGAAGAG ATTTCCTAGA TGCCAGAGAT AAAGCTCTGA TTTCGATCGC      1680
TAAGGTACTT GAGAAACCTC AGGTCCCAGA CGGATTGTTA CCAATTCTAG ACTTATGTCA      1740
AGTCAGAGAT GAATTGATTG CTGCCACGAA TTCCTTGGGT TTGACTCAAG AGGCCAAAGA      1800
GGCCATACAC AGTTCTGACA CGAGGTTACC AGTCACTTCA GCGACTGAGG TTAATCCATA      1860
TGCCGATTCA ATTAAAGAGG CCATAAGCTA CTTTAATGAA ATCGAGATGA CCAATTCCCG      1920
CAACCTCAAA GCACTTGGGT GCTATTTGAA TTGGAAAGCC GGAAATTCAT GGATGTACTC      1980
TGCCCTTAGG GGCCGCAATG AAAATGTCAG AGTGTATGTA CCATTTGAAC GTAAGTGGTA      2040
CCCGGATTCA CGTGATCTAC CTCAGTATGA ACGAGCAATG TCTGAGGATG GATACGTTTC      2100
GCTCCACTGG AATGGCAATG AAATTTCTGC CAACTGCCAA AATATCATTG GAAAGTACCA      2160
TGTGTTGGTG GTTGATGAAT CATGTGTGTT CAATTCTGGG CAACGCATGA TACCAGCGTT      2220
GGAATCCGCG CTGAAATTAA AACCAAATTT CAAAGTAACG ATCATCGACG GTGTGGCTGG      2280
TTGTGGTTGT GGGAAGACGA CCCATCTAAA GAAAATTGCG AGGTTAGATT CAAATCCAGA      2340
TGTTGTTCTC ACTAGTAATA GGAGCTCGTC TGATGAACTG AAAGAATCTT TGCAATGTTC      2400
GGACGCGATG AAGTACCGAA TCCGGACCGT CGATAGTTAC TTAATGTTAA AGTCATGGTT      2460
TGCTGCCGAC AGATTATTGT TCGATGAATG CTTCTTGACG CGTGCTGGTT GCATATATGC      2520
GGCTGCGACC TTAGCTCAGG TTAAGGAGGT TATTGCTCTA GGCGATACCG AGCAAGTTCC      2580
CTTCATAGCT CGATTACCTG AGTTTCGCAT GCAACATCAT AAGATCAGTG GTAAGATTGA      2640
AACACAGACC ACGACTTATC GTTGTCCAAG AGATGCCACG TATTGTTTGA AGACGTTGTT      2700
CTACAAAAAC AAGACCGTGA AGACCGCGAG CTGCGTTGAG CGCTCGCTGA ATTATGTCCA      2760
ATCAGTAGTC CAGTCCAGAT ACCGTGTGAA AACGACACAT TATATGTAAC ACACACGAGG      2820
TCTGATAAAG ATGCCCTTCT TAGAATACCA GGATTCAGGA AGGAGAATAT AAAAACCACA      2880
CATGAATCTC AAGGTGACAC TTGGGATAAG GTGGTTTTGT TCCGTTTATC AAAAACGACG      2940
AATTTGCTGC ATTCTGGAAA AGGACCTGAT TTAGGCCCAT GTCATAACTT GGTTGCCCTG      3000
TCAAGACATC GTAAATCTTT GCGGTATTAT ACCGTGGCTC CAAATGATTT AGATGATCAA      3060
ATTGTCAGGA GTATAAATTT GAGCAAGACA TTGGCTATCA ATGATCTTGA CTCTGTGAGG      3120
CGATTGCCCT CACCAACTTG ATTGATTTCA AGTCCACATA GTGATGCCCC ATTTGGGAAG      3180
A                                                                      3181
```

Figure 3.3 Nucleotide sequence of RNA2 from the potential new ilarvirus, from approximate position: 5'-117 nt to 347 nt-3'

```
GAGGATTACA CAGAGTGGGT TAAGCTCTGG TTATTTCAAA ATGTCGTAGA GCATACTGCT        60
ATTCACGACT TTCCTTTGTT TGTTACGCTT GTCATGGTAT TATGCCGTCT CGGTGCTGAA       120
TATTATCCCG AGGTAGAAGA GGAAGAATGG CAGTCTCCAC CGATTGATCC GTTCTATCTA       180
CCTTACGATG ATTTAGACAC CGATTACACC ATGCTGAGTC A                           221
```

Figure 3.4 Nucleotide sequence of RNA3 from the potential new ilarvirus. From approximate position: 5'-164 nt to 2053 nt-3'. The underlined nucleotides indicate the ORF of the movement protein (nt 72- 953) and the coat protein (nt 1127 – 1786).

```
CGGTTGTTGA TTTTCTCAGA TCACGGTTGC TGTTGAGATC AAGTGATTAT TTTCCATTGC        60
GAATTGGTGC GATGGCTTTG TCCCCGTCCT ATAAAGCGCT TACTTTCTCT GCTGATGATG       120
AATCCAGTCT TGAGAAGGCG GTTCTTGAGG CTTTATCTGG ATCTGTCGAT CTAAACATGG       180
GCATTCGAAG ATGTGCAGCA TTCCCGGCCA CCAACACGGA GGCATTTCTG TGTGAATTGA       240
CGTCAAAGGA AACGAAATCA TTCGTCGGAA AATTTACCGA CAAGGTCAGA GGACGCGTTT       300
TCATTGACCA CGCTGTCATT CACATGATGT ATATACCCGT GATTTTGAAT ACCACATTTG       360
CGGTTTCTGA ATTGAAGATA AAGAATCTCG CAACTGGTGA TGAGCTTTAT GGTGGTACGA       420
AGGTTAATCT CAATGAAGCA TTTGTTCTGA CGATGACCTG GCCTAGGTCA CTTTTTGCAG       480
ACGCAGTCAA CAACCACAGA GGTTTGTTCC TGGGTGGTAC TGTGTCTTGT GCCTCATCAG       540
TTCCCAAAGG TGCCAAAATA GGGATGTGGT ACCCCTTATG GACCGAAAAA GTGTCGAACA       600
AGCAGTTGTA TCAGAAAACG ACTGACATTG TTAACACTAG AGCTCTTGAG ACGTTTACCA       660
GAACAATGAT CTCCAATGAT AAAGAGATGA GAAGTCTGTT GAGAAGTCGT GCCTCAATAG       720
ACATTGCTGC AAAGGATCCC GAGAGACCCG TTGTCTGTTC TTCAAGTGTG AATCTTTTAG       780
ACAGATCGAC GACAGGTATT GATTTTACCA CTAAGATCGT TAGTGAAGCG CCGCCCTCAC       840
TTACCAGCGG AAGTTCCATT CTTGTGAATA AGTTAGTTCC CGCTGAGGAG AGTTCAGATT       900
CTAAGGAGCA GGAAGCGAAG GACACAAGTG GTGGCAACCA CTTGACTGCT TGATTTGTTG       960
TTAGCAACAT GGGGGGGTTT TTGGAAGAGG GGGATGTACC CTAGATTGGG TTCAATATTT      1020
CGGTGTGTGA ATTCTTTCAT TAGATAAGCC ACTGATTGGA CTTACCTGGG ATGTACGGAG      1080
ACTTCGACCA CTTATCCAGC TTCACCAACC AACGCCATGT CTGCTAGGGG AAATAACAAC      1140
AATCACGTCT GCCAACATCG TTTCGATGAG CTTGATGCCG CATCTTCTAG ATGTGAGATA      1200
TGTCATCCTG TGTCAAATAG ACAACGTCGC AATCAGCGCC GTGCAGCTGC ATTTCGTAAT      1260
GCAATTACGA ATAATAATGC CTCACAGAAT GTGAGAAGAC CTGTGCCGGT CATACCTGTT      1320
GGAAACTCAA GACCAACTTT TAGGTTACCA GGGAATCAGG TTTGGATTCG ACTGACTGCC      1380
AGTTCTTGGG CAGCAAAGAC AGTCGATACT AACGATGTAC TTCCGTTAAA GAATATCTTC      1440
AATGGTATTA ACGAAATTGA TTCTGAGACG AAGATTTTCC GTCTTTTGAT CGGTTTTGTT      1500
GCAATGTCCG CTGGGACATT TGGACTTGTC GATGGCGTCA CGTCTACCTC GGTCCCCGAT      1560
CCACCAATTG TTGGTAGGGT GGGATTCGAG AAGAATACGT ATCGCAGCCG AGACTTTGAT      1620
CTCGGTGGCA AGACTCCGCT ACAGCTTGAT GGTAAAGCTG TAGTTTGGTG CCTCGAAGAA      1680
CATCGTCGAG ATGAAAAGCG TGTGCAGTTG GCTGACTATT GGGTCGCCAT TTCACGACCG      1740
CAACCGCTTA TGCCACCAGA GGATTTCCTG GTGAACTCTC AATGACTAGA TGGTCTAGTC      1800
ACTTCCGGAC GTGTGTCCGA TTTGCCTCAC ATAGATGGAA ATTCCTGTGT GAGATGTGGA      1860
TATGCTCCCC GTTACATTGT AGATCAATGT TATTGGGAAT GCACTCTTTG TTCGTATGAA      1920
CAACGGGCGT AGCAACCTCG CATCCGGGTT TAAGACTACG TATTCTACTA TTATTAATTT      1980
CTTAATAATA GTATTGCCAC CAAAG                                           2005
```

Figure 3.5. Phylogenetic analysis of RNA1 of potential new ilarvirus. The phylogenetic analysis was done using MEGA 6. The evolutionary history was inferred with the Maximum Likelihood method based on the Kimura 2-parameter model with 1000 bootstrap value. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. A phylogenetic tree constructed using the sequences of RNA1 other species in the family *Bromoviridae* and the sequence of the new ilarvirus. BCRV: *Blackberry chlorotic ringspot virus* (GenBank DQ091193.1), SNSV: *Strawberry necrotic shock virus* (GenBank: DQ318818.3), TSV: *Tobacco streak virus (*GenBank: KM504246.1), PMoV: *Parietaria mottle virus* (GenBank: FJ858202.1), newilar: Potential new ilarvirus, AV-2: *Asparagus virus* 2 (GenBank: EU919666.1), ApMV: *Apple mosaic virus* (GenBank: NC_003464.1), APLPV: *American plum line pattern virus* (GenBank: AF235033.1), PDV: *Prune dwarf virus* (GenBank: U57648.1) FCiLV: *Fragaria chiloensis latent virus* (GenBank: AY682102.1).

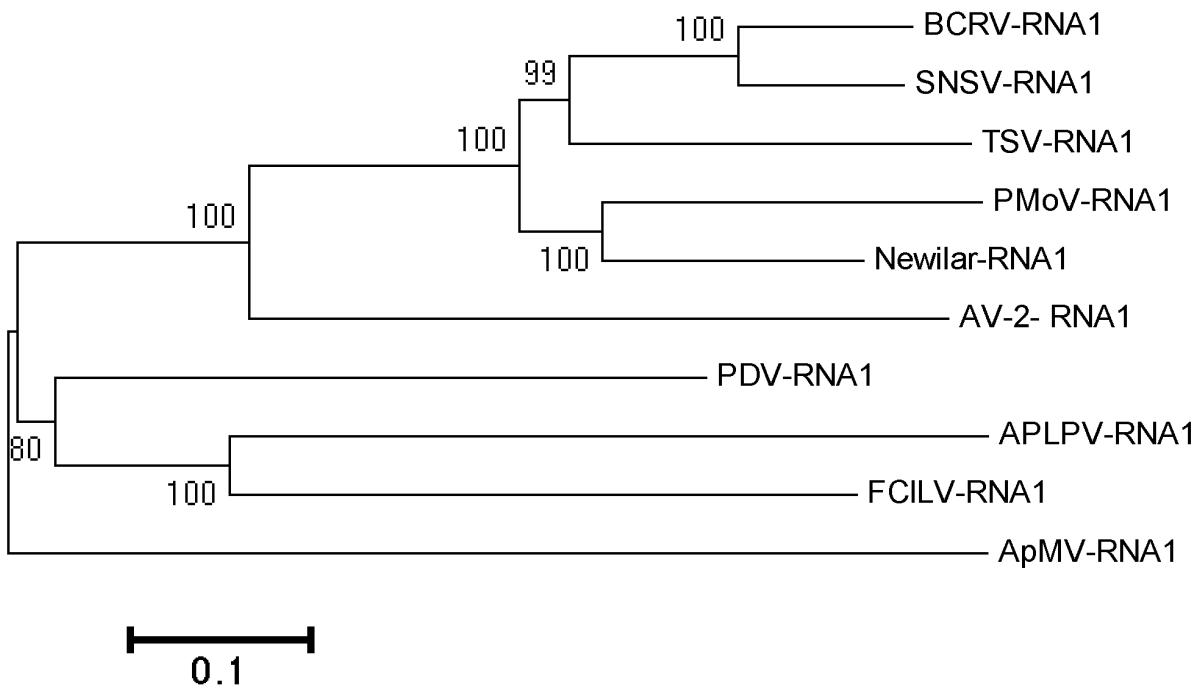
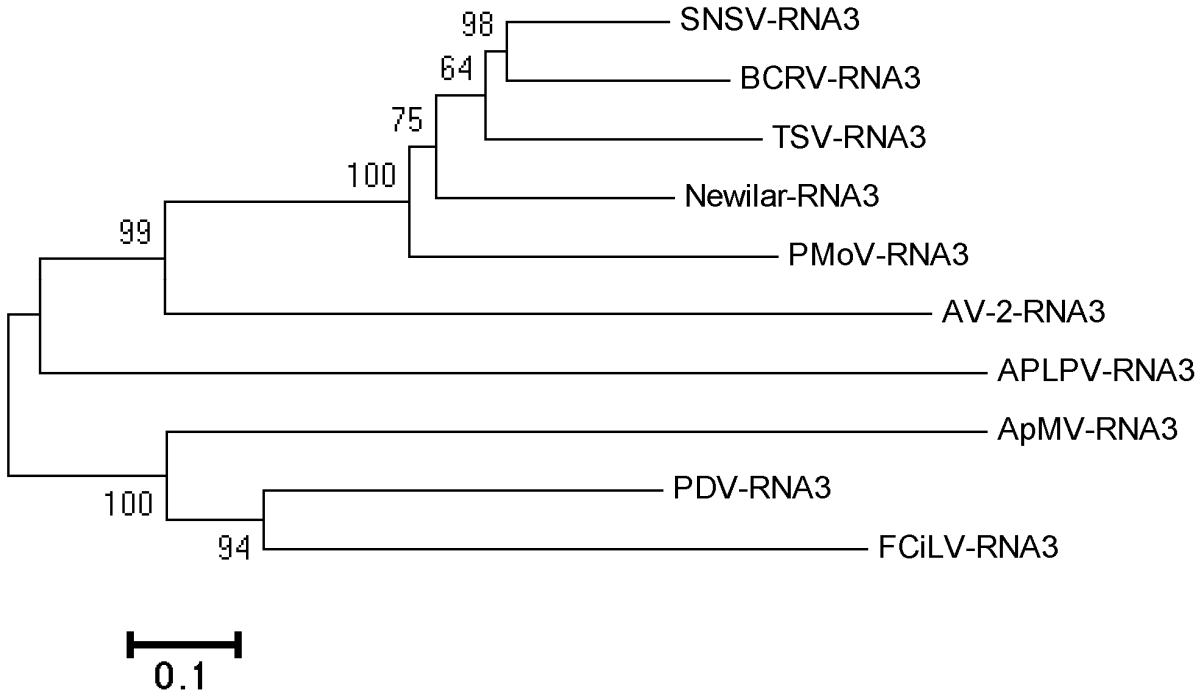


Figure 3.6. Phylogenetic analysis of RNA3 of potential new ilarvirus. The phylogenetic analysis was done using MEGA 6. The evolutionary history was inferred with the Maximum Likelihood method based on the Kimura 2-parameter model with 1000 bootstrap value. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. A phylogenetic tree constructed using the sequences of the RNA 3 of other species in the family *Bromoviridae* and the sequence of the new ilarvirus. BCRV: *Blackberry chlorotic ringspot virus* (GenBank: DQ091195.2), SNSV: *Strawberry necrotic shock virus* (GenBank: AY363228.2), TSV: *Tobacco streak virus* (GenBank: FJ561301.1), PMoV: *Parietaria mottle virus* (GenBank: U35145.1), newilar: Potential new ilarvirus, AV-2: *Asparagus virus* 2 (GenBank: X86352.1), ApMV: *Apple mosaic virus* (GenBank: AM490197.2), APLPV: *American plum line pattern virus* (GenBank: AF235166.1), PDV: *Prune dwarf virus* (GenBank: L28145.1), FCiLV: *Fragaria chiloensis latent virus* (GenBank: AY707772.1).



## Discussion

Ilarviruses are regarded as recalcitrant viruses with which to work when using the classical methods. Mechanical inoculation to an alternate herbaceous host and purification of virus preparations is difficult. The viruses occur in very low titer and as the sigla that composes their name indicates (***i***sometric ***la***bile ***r***ingspot virus) they are readily labile and easily disrupted during purification. Blackberries have high amount of antioxidants and phenolic acids (Huang et al., 2012) which have health benefits. But this also means that blackberry is not an ideal host from which to extract nucleic acids for work in molecular biology as the antioxidants and phenolic compounds, and other inhibitors present in these plants interfere in the process of nucleic acid extraction, resulting in poor quality RNA. We designed a large number of primers based on some other viruses of Ilarvirus Subgroup 1 in our attempts to obtain sequence of the virus. Although the epidemiology of the virus is completely unknown, the sequences developed in this work will allow the rapid detection of viruses by PCR, using gene specific primers. As sequence for the RNA3 includes information for the complete coat protein of the virus, antibodies for the virus can be developed for Enzyme-Linked Immunosorbent Assay (ELISA) using an expression vector to provide antigen of the CP.

The virus is latent in both blackberry and veronica (Figures 3.7 and 3.8). Another ilarvirus, *Blackberry chlorotic ringspot virus* (BCRV), is also asymptomatic in many blackberry cultivars, although it is one of the viruses detected most frequently in the BYVD complex (Ioannis E. Tzanetakis, Personal communication). Also, BCRV has now been found to be naturally infecting apples, and has a high seed transmission rate (Poudel et al., 2014). These new findings imply that each virus detected in the disease complex should be studied

in depth. This research made an effort to provide some basic information on the viruses: sequence information and transmissibility to herbaceous hosts. The phylogenetic analysis of the sequences obtained allowed the associations with currently accepted ilarviruses at the molecular level to be determined. However, additional work that includes, transmitting the virus to herbaceous hosts as part of completion of Koch's postulates, and study on transmission of the virus within a field and between the fields is needed. Most ilarviruses are transmitted by pollen, and although transmission may occur through fertilization with infected pollen, the role of thrips in the "mechanical transmission" of the viruses while feeding on infected pollen needs to be evaluated. Since the virus is asymptomatic, an infected mother plant could be used for clonal propagation and this spreads virus to many other plants. This requires the need for proper detection technique, so the virus can be detected in mother plants before they are used for propagation in nursery.

Figure 3.7 Blackberry plant infected with the potential new ilarvirus. The plant has been asymptomatic for last three years (2012-2015).

Figure 3.8. Veronica plants infected with new ilarvirus. This plant has remained asymptomatic for last three years.

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CHAPTER IV

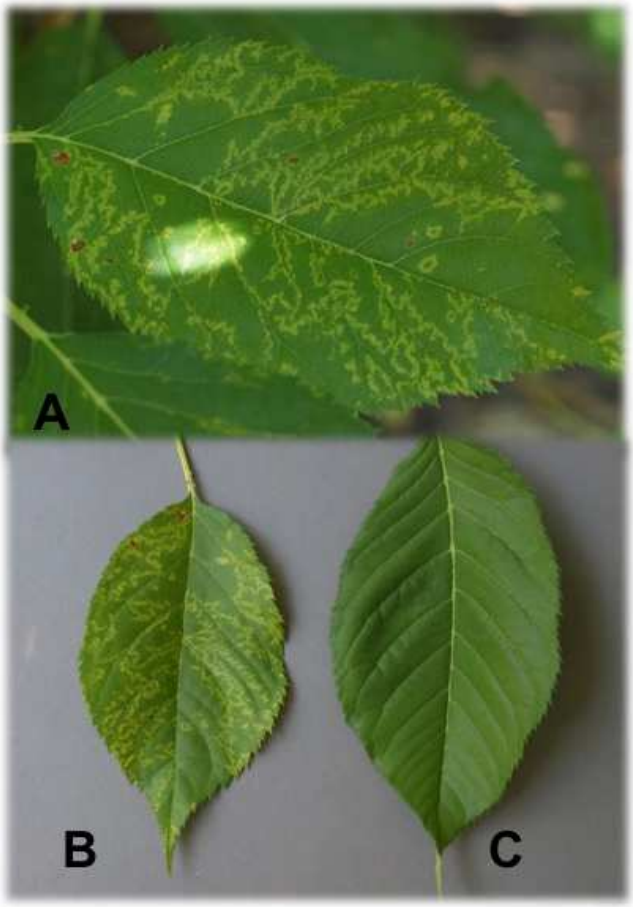
# DETECTION AND CHARACTERIZATION OF *CHERRY NECROTIC MOTTLE-ASSOCIATED VIRUS* FROM 'MAZZARD' ROOTSTOCK CHERRY

## Introduction

In summer and fall of 2011, a cherry tree at Musser Farm, part of Clemson University at South Carolina, showed symptoms that resembled a mixture of mottling and vein clearing and line patterns (Figure 4.1). The scion of the tree was *Prunus serrulata* Lindl. cv. 'Shirofugen', and was not displaying any symptoms, but suckers growing from the 'Mazzard' cherry F12/1 rootstock (*Prunus avium* (L.) L). were displaying virus-like symptoms. The symptoms re-appeared in 2012 (Figure 4.1). The reoccurrence of symptoms indicated a systemic, established infection. The symptoms exhibited by the leaves in the rootstock suckers were typical of viral infection, but similar symptoms had not been observed previously on cherry growing in South Carolina. Therefore, various experiments were performed to determine the identity of the virus. This tree had been used as a bioassay host for detecting two ilarviruses: *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV). Material from a 'Yoshino' cherry (*Prunus* × *yedoensis* Matsum*)* growing on campus at Clemson University and known to be infected with PNRSV and PDV, had been inoculated onto the tree as part of a demonstration of the 'Shirofugen' bioassay (Mink & Parsons, 1965) used to detect PNRSV and PDV. Chip bud inoculation with PNRSV induces a hypersensitive reaction whereas chip bud inoculation with PDV produces a slow-moving systemic reaction. Thus the limbs of the tree used for testing are removed after 6 weeks. Molecular tests of the

scion and rootstock did not detect either PNRSV or PDV. Therefore, TriFoCap- PCR, a system that can detect many of the other viruses reported to infect cherry was conducted.

Figure 4.1. Virus-like symptoms on the leaves of suckers from a 'Mazzard F12/1' cherry rootstock in 2011 and 2012 growing at the Clemson University Musser Farm. A: symptoms observed in summer and fall of 2011 B: Symptomatic leaves from the sucker of rootstock C: Asymptomatic leaves from the *Prunus serrulata* Lindl. 'Shirofugen' scion D: Symptoms reappearing in leaves of 'Mazzard' rootstock sucker in 2012.




Objective

The objective of this study was to identify the previously undocumented virus in 'Mazzard' cherry, obtain the sequence information, predict the introduction of the virus in that particular cherry tree and to the incidence of the virus in other, nearby cherry trees.

## Material and Methods

Double-stranded RNA was extracted from the symptomatic leaf tissue of the Mazzard cherry rootstock using the method described in Appendix C. Reverse transcription (RT) and polymerase chain reaction (PCR) using the TriFoCap method was completed as in Appendix F.

A high molecular weight band of dsRNA visible on a 1% agarose gel (Figure 4.2), similar in size to the genomes of viruses in the genera *Trichovirus*, *Foveavirus* or *Capillovirus*, was used in a nested RT-PCR (TRIFOCAP) assay to amplify the 362 bp conserved region of the polyprotein of the above mentioned three families as described in Foissac et al., 2005 (Appendix F). The final product of about 362 bp was visualized (Figure 4.3) by electrophoresis on a 1% agarose, 1× TBE (90mM Tris-HCl, 90 mM boric acid, 2mM EDTA, pH 8.0) gel stained with Gelred (Phenix Research).

Figure 4.2 dsRNA extracted from symptomatic leaves of suckers on a 'Mazzard' cherry rootstock visualized on 1% agarose gel. Lane 1: 1 kb DNA marker, lane 2: dsRNA extracted from symptomatic cherry leaves.

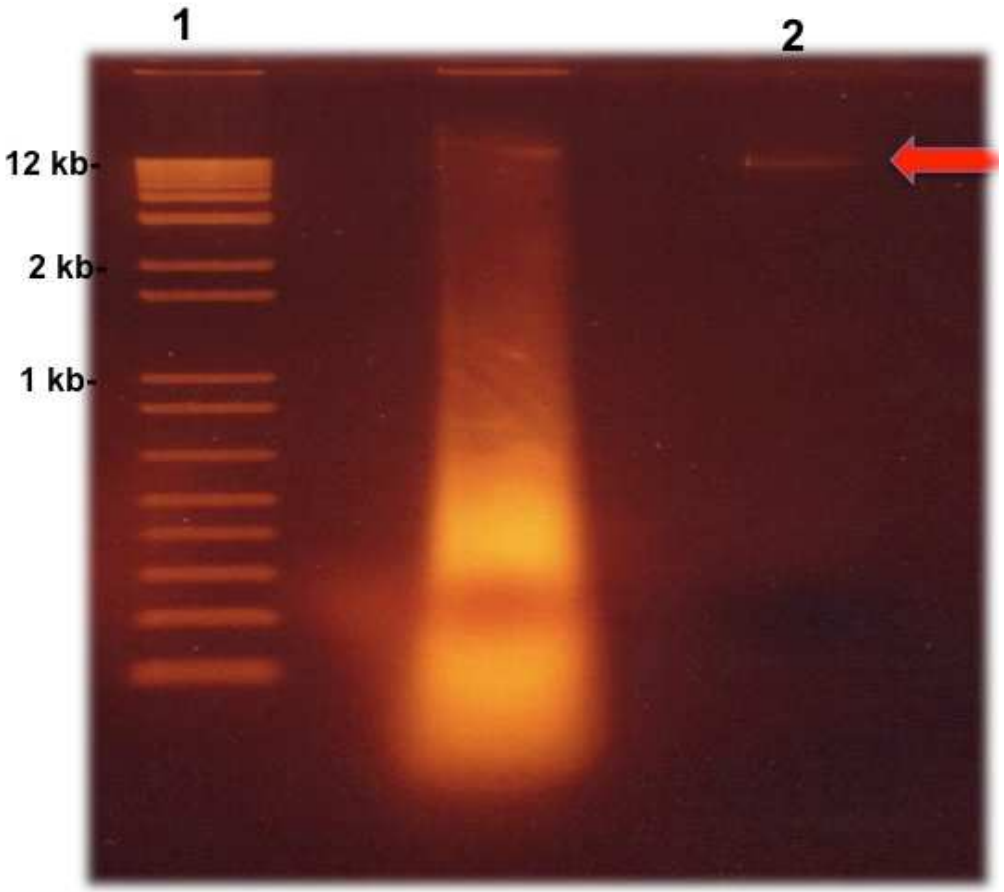
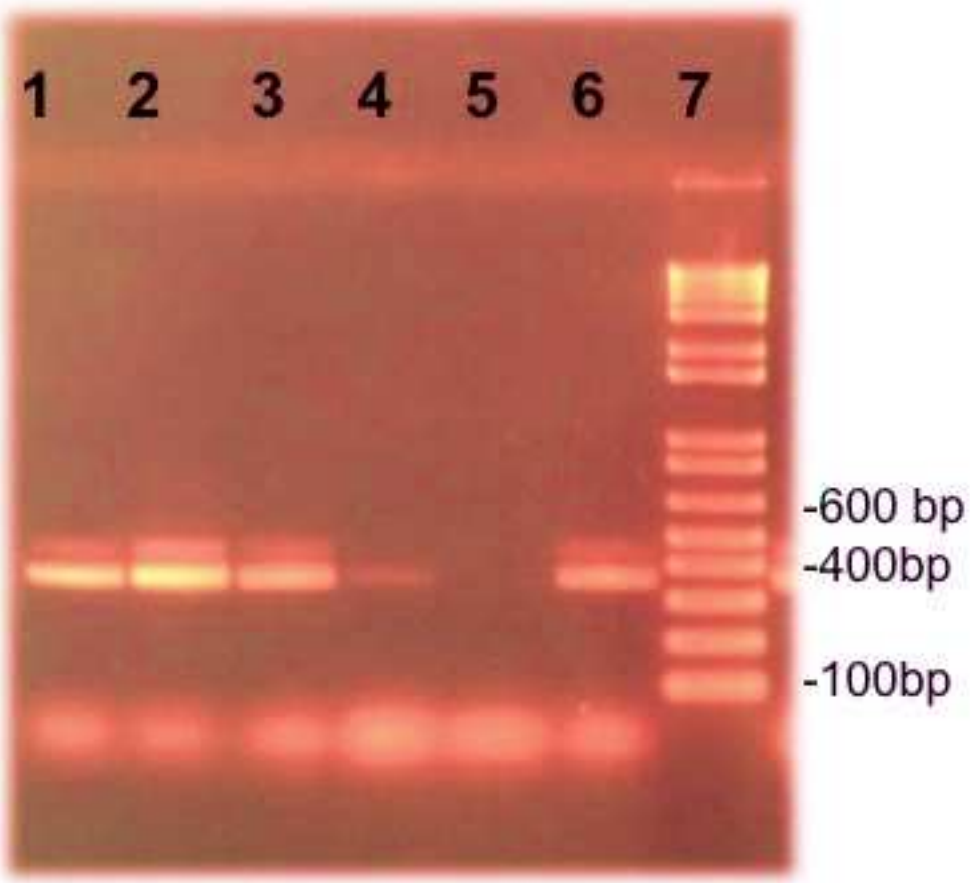


Figure 4.3. The end products of TriFoCap RT-PCR visualized on a 1% agarose gel. Lanes 1, 2, 3, 4: samples of dsRNA from symptomatic cherry leaves subjected to RT-PCR, lane 5: negative control, lane 6: positive control, lane 7: 100 bp DNA marker.



## Obtaining sequence information of the virus

The 360 bp fragment obtained from TriFoCap RT-PCR confirmed that the cherry tree had at least one virus present. Cloning and sequencing was completed as described in Appendix H. The sequences of the amplicon were aligned using the search tool Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) website and relationships to viruses already in GenBank were identified and used to suggest the identity of the unknown virus. The sequence resembled those of *Cherry necrotic rusty mottle virus*. Numerous sets of primers were designed to amplify the fragments of the virus (Table 4.1). As the viral genera detected by TriFoCap all have a poly- A tail at the 3' end, oligoDT was used in a reverse transcription reaction. A primer designed from the sequence of the TriFoCap amplicon (primer 1269) and primer 930 (Table 4.1) were used in a PCR reaction to amplify the sequence between the poly A-tail and the TriFoCap amplicon. A series of RT-PCR experiments were done to amplify the fragments of virus genome extending towards the 5' terminus using pairs of primers designed from the sequences of related viruses present in GenBank. Reverse transcription (RT) was done using purified dsRNA as a template (see Appendix D). TriFoCap polymerase chain reaction (PCR) was done as explained in Appendix E. The sequence at the 5' terminus of the genomic molecule was obtained by using SMARTer RACE 5' (Clonetech). Sequences were analyzed with the CAP3 sequence assembly program (Huang and Madan, 1999). Phylogenetic analysis was conducted on MEGA 6 (Tamura et al., 2013). The phylogenetic trees were generated on MEGA 6, implementing two different methods that included Maximum Likelihood method and Neighbor Joining method. A bootstrap value of 1000 replicates and the Kimura 2-parameter model (Kimura, 1980) were used in the construction of phylogenetic tree.

Table 4.1 List of primers used to obtain sequences of the virus detected in cherry.

| Primer name | Primer sequence 5-3’ |
|---|---|
| 929 | GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV |
| 930 | GACCACGCGTATCGATGTCGAC |
| 1268 CNRMV5439 | CTGGGCAAGGCCTTGAAC |
| 1269CNRMV5810R | CGCACATATCATCACCAGC |
| 1270CNRMV26467F | GCCTAAGGATTCCAGAGGG |
| 1271CNRMV3327 F | TGCATGAATTAATGAGCGG |
| 1272 CNRMV3698F | GGTCAGTATGACTGGTTGGC |
| 1273CNRMVSWSF | AATYTACAACCCTGTAATGG |
| 1274CNRMVSWSR, | CCCTTATCAAAAAGAATTCTTGG |
| 1278CNRMV, 2948F | GGCATGCAGTCTCTTCCAATA |
| 1279CNRMV3309F | GCAGAATCCAACAAAATTCTG |
| 1280CNRMV4703R, | CCCTATCAACTTCATCCTTGC |
| 1281CNRMV4799R, | GCCCTTCATCATTAACACTGG |
| 1282CNRMV4953R | CCCTTACCTTTGT GAGTTTCTG |
| 1283CNRMV4367R | CCCATGTTGGCCTTGACCAC |
| 1284CNRMV4323R | GGGGATAGATAATCAGCCTT |
| 1285CNRMV2928R | TATGGAAGAGACTGCATGCC |
| 1286CNRMV3289R | CAGAATTTTTGTTGGATTCTGC |
| 1287CNRMV1623F | GGGTTGGCAAAACATCTGA |
| 1288CNRMV1941F | GCCTACCCGTGCTCACTT |
| 1289CNRMV1642R | TCAGATGTTTTGCCAACCC |
| 1290CNRMV1959R | AAGTGAGCACGGGTAGGC |
| 1291CNRMV149F | GTTCTTGCTCAATTCTCCTCT |
| 1292CNRMV109F | CTACAACCCTATAATGGCCTTG |
| 1293CMRMV42F | GCCCTAGCGCATAGGCTT |
| 1294CNRMV3685R | GGAATTGATAACACCAGTTTG |
| 1295CNRMV3712R | CCAGTCATACTGACCTGCCA |
| 1296CNRMV131R | CAAGGCCATTATAGGGTTGTAG |
| 1297CNRMV170R | AGAGGAGAATTGAGCAAGAAC |
| 1298CNRMV2910F | GGCATGCAGTCTCTTCCAT |
| 1299CNRMV1800F | GGAGGCTAAGGCTTAGAAAT |
| 1300CNRMV2898R | CCATCAGCCTTCACAGGGA |
| 1301CNRMV2936R | GCCCAAATATGGAAGAGACT |
| 1304CNRMV 3130F | CTGGGTTGTTGTTGAACCAT |
| 1305CNRMV 337R | CAGGATGACTGTGAGTCTCAT |

Survey of ornamental cherry trees on the campus of Clemson University for detection of the virus

In December 2013, dormant twigs from 15 'Yoshino' cherry trees on the campus of Clemson University (Table 4.2) were collected and stored at $4^0$ C to break dormancy. In the spring of 2014, virus-tested 'Mazzard' cherry seedlings were grafted with the buds from the twigs collected in December. Cherry plants were allowed to grow for about 2 months and then were tested for the presence of the unknown virus.

## Results

### dsRNA extraction and TriFoCap RT-PCR

dsRNA extracted from symptomatic cherry leaves displayed bands of high molecular weight (~8kb) when visualized on an agarose gel (Figure 4.2), suggesting that the virus may belong to one of the TriFoCap (*Trichovirus, Foveavirus, Capillovirus*) genera of viruses. The 362 bp (Figure 4.3) product obtained from TriFoCap RT-PCR had the highest similarity with *Cherry necrotic rusty mottle virus* (GenBank Accesssion EU188438). However, as larger fragments of genomic sequence were obtained and assembled the virus was shown to be more similar to *Cherry rusty mottle-associated virus* (GenBank Accession KF356396.2), another unassigned species in the family *Betaflexiviridae* (Adams et al., 2012). An almost complete genomic sequence (~ 8 kb) except the first 106 bp of 5' end of the virus has been obtained. The virus is 97% identical to the sequences of *Cherry rusty mottle-associated virus* presently held in GenBank (Accession KF356396.2), and it is a previously undocumented virus in cherry trees in South Carolina.

## Sequencing

The almost complete sequence of the virus detected in 'Mazzard' cherry growing at Musser Farm at Clemson University aligns with nucleotides 106 -8,400 (Figure 4.3) of the sequence of *Cherry rusty mottle-associated virus* (CRmaV) presently held in GenBank (accession: KF356396.2.). The sequence was 97% identical to this particular GenBank accession and less than 85% identical to any other isolates of the *Cherry rusty mottle-associated virus* (CNRMV) currently available in GenBank. When a phylogenetic tree was constructed using sequences from species in other genera of the family *Betaflexiviridae*, and the Maxiumum Likelihood method and Neighbor-Joining method, the isolate we have sequenced grouped closest to CRMaV, followed by CNRMV. Both the methods yielded the result in figure 4.5.

Figure 4.4 Sequence of *Cherry rusty mottle-associated virus* isolated from symptomatic leaves from the 'Mazzard' rootstock at Clemson.

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CAACCCTATA ATGGCCTTGC ACACAATCAC TCCAGCTGAA GGTGTTCTTG CTCAATTCTC        60
CTCTGAGGAG GCCAGTCGAA TTGGAGCTTC TGCTATCTCC AATTTTTCAA AACTTGAGTC       120
AGAATACCAC TCCCTCTTTC ATTTCCACCT CCCTGCTTAT GCGAAAAGTA AACTCTCCAA       180
CAGGGGTTTT TACCTTTCAC CCTTTTCTTA TGAGACTCAC AGTCATCCTG TCAGTAAAAC       240
CATTGAGTCT CATTTAATAA ATGTGAAGTC ACCCAATTAT ATTAATGAGG ATTTTTTAAT       300
TGTAGGAATA AAAGAAAATA AATTAAGTGT ACTCAGAAAA GACAAAAAAA TGAGATTTCT       360
TGAAGCTCTT AATCGCTGCG TGACGTCTCA CGATGTCCAA AGGTATGGAC CGAGCTTTCA       420
CTTTGAAAAA GCCAAATCCA ACTGGAGGAG TGACTTCTCA GGGGTCAATT TATCTGTTGG       480
TGTGCAAAGT TTGTTGCCAA GAGTGCTGTT CGACAAAGGG AAAATGTTTG ACTCTCAGAT       540
CTTTCTGTAT GATGAGCTCC ACTACTGGAG TATGAAGGAC ATAGTGGATT TCCTTGAAAT       600
TTCAAGAGCG AAGACCATTA TAGGATCCTT TGTTTTTCCT TCTGAGATAT TGGCTGGTGC       660
ACGGACCAGT TTAAATCCCT GGGCTTATGA GTTCAAAATC AAAGGGGATA AGATGATATA       720
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Figure 4.4 (continued)

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TGCCCCGGAT GGTGTCTGGT CGGAATCTTA TGAACAACCT TTATCCGCTG GACAACTGTT       780
GAAGTTTAAC AAGATAATGA CCAGAAATGG CAGCTACTCA GTTCAGGTAA GAGATTCCAT       840
TTACAGTCAT TGCCTGGTCA TTATCAACAG GGATGAACTG CTCTGCGAGG AATTCCGGGT       900
GTTCAGCGAC TTTGACGCAA TTTCCATTCG CAGAATTGGT TATTTGGGGG GTAATGCTGA       960
TGATGTTATT CCAGTTAGAC ACGAGGTTAT TTTGTCCATC TTCAAGTACA TCAGAACCTT      1020
GAAGAAACCT GACCTCCAGT CTGGAATAGC CAAACATAGG CAATTGGTCG ACAACCCAAC      1080
TGGCTTCGAG ATACGGTTTG TGGAGGATTT TGTGCAATTC ATACTAGAGC ATCATGAGAG      1140
GTTCAACCTC ATTGAGCAGA AATTTTCAAA TTTCTTCAGT TCTGCCTGCA TAAGTCTGCT      1200
CCCAAGATAC ATGCAGCGAT TCTTCAACAG CTTCAAGGGC TACAGTTTAG GTAAGTTCAT      1260
TGAGGAGATA GAGCCCTTCA CGTTCACTTT GAAATGCCAG ACTTACTCAC GGTTTGGCTT      1320
CAGGACTAGT TTCACTGATG AAGAGGATGA AATAGTTGCC GCCACAGACC CAATGTGTTT      1380
AACCATCAAG CAGTCCAATA GCAAGTTAAT TTGCTTCAAT GACTATCCTG ATTTGATCTT      1440
TAATGCACAC ATTTCAGTTT TTGCCAACCC CCACCCGAGT ATTACCCTGA TGTTGGTTAA      1500
AATGTTTATC AATGTGTGGG TTGGCAAAAC ATCTGATGGT TATTACCAAT CACTCATTGC      1560
CCTCAAACAA GCACTCAATC AAAAAAGTTC AAAGCTGTTC ATGCTTCACA ATGAAAACTA      1620
CAATTCATTG GTGATTTTTG CCAACCTTGT TGATTCATAT TTGTTCAAGA ACTTACTGAG      1680
GAATGAGATC AGAAGGAGGT TAAGGCTTAG GAATTGCGTG AGAGGGCTTT TAAGGAATGA      1740
TTTGCCACCA AGTCACCCAG ACGCCAAGAG AGAAGTCAGA TTCATTTCAA GTTACAAATC      1800
TTTGCTGGCT GACTTTAAAA AGATGAATGA GGAATGCCTA CCCGCGTTCA CTTTGATTAA      1860
AACATGTGGT CTCGATGATC AAATTTATGC CATGAAAAAA AATTTTGTTG TTGATCAGCC      1920
ACTGATCAAG AATACGCAAA AAAAGAAAGA GCACAAGCCA GAATCATCTG ACACCAATCT      1980
GGGCTACACT GAAGAGCACC ACCTGGAGGC CCCAATTAAT GGTATGACAA ACGGTGAGAC      2040
CAGTCAAGCA GTCAGTAAGG AAAATACCAC ATCTGCATTG GCATCAGAGG AAACCAAGGT      2100
TGGTAGCAGT GATCTGTTCA TATCAAGCAT AATAAAAGTT GGACCTTTTA AGGATTCCCA      2160
AACTATATCT TTTGTTGAGG GCCTTGATTT CTCAAAGGGG CACAACCACA ATGGGAGAAA      2220
GTCACTTTTC TTTTCAGATG GAGGTTTTTC ATATGGATTC AACAATACTG TGTACCAGTC      2280
ACAAGGTTGG CCCAATGTCT TCAAGGAGCT GTATGGATCC AGATTTAATT CCTGTCTCAT      2340
TCAAAGGTAC AGCAGTGGTG CCACCCTCGG TTTCCATGCT GATGATGAGA AATGCTATGA      2400
CCAAGATCAT GAGGTTTTAA CTGTTAATCT CTTTGGATCA GCCACCATTG CTTTTTCGAA      2460
GGGTAACTTT GCATCCTCAA ATGTGAGTAA TCCTGAACTG TATTTGGAGA TGAATCTTGA      2520
TCATTGTGAC TGGCTTTTAA TGCCTAAAGG ATTCCAGAGG GGGTACAAGC ATAGTGTCAG      2580
GGATACCACT GAAGGCAGAA TCTCTCTCAC ATTTAGAAAG CAAAGCCGCA CTCTAGAGGG      2640
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Figure 4.4 (continued)

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AACCTCAATC CAAAGTGGAA CCACAAGTGA TAATTCAGAT GCTGACAACC ATGATGGCGG      2700
GTTTTACTTT GAGGAAATCA ATAAGTGTTC GATCACCTCC GCCCCTGATT CTGTGAAGTG      2760
CAGCTTATCA ATATTCCCTG TGAAGGCTGA TGGTGATTGT TTCTGGCATG CAGTCTCTTC      2820
CATATTTGGG CTTGAAGCTA AGGAATTAAA GCAACTGGTT CATGACAGAG CTATAGCTGA      2880
AAATTGCATT GATCAATGTC ACATGAAGGA TTTCCTGCAT GAGATGGAAC CTAAGGTGTA      2940
TGCCAGCAAC GCATCATTAG CTGCCACATG CTATCTTATG AACCTCAAGC TCATAATCAA      3000
GCTCACCGGC CTTGAAGATG ATAGCTGGGT TGTTGTTGAA CCATTGGCCC TATCTAATGA      3060
AAAAGCTTCC ATCGGTTACT TGGTGTTGAA CCAAAAATGC CATCATTTTG ACCTAGCTGT      3120
GCCAAAGGAG GGCTGCGTCG TTAGAGCAGT CAGTGAGTTT TTGAAGCAGA ATCCAACAAA      3180
AATTCTGAGT GTGCTGAGTG CCAATTGTTC AAAGGAATTG CTGCATGAAC TAATGAGCGG      3240
GCTGGGTATT CAGGAATTTC ATCTGGAAGA GATCTTTTCC ATCTTCGATA TTTGTGCCGA      3300
GGTTAGCGAT GGAGTTAGTT CAAGAGTGCT AAATAAAAAG GGTTCTAGAG CAGCAAAATT      3360
CATTGTCGAT AAGGACCACT TTTCCTTCTG TCCTGGCACA AAGGCTTCCA CCAATCTAGG      3420
AGCTTTCAAA TCCCCAAACG GTAGCTCCAT GATTGCAATT GAAAAGTATG ATGAGTTTTT      3480
GAAGTCCAAT GCCAATGTTG TCCCTTTCAC TCCATCTCTG CCCCTAGCTA AAAAACTTGC      3540
AGATTCTTTC TTAAGCGGTC AAACTGGTGT TATCAATTCC AAAATTGTGG CAGGTCAGTA      3600
TGACTGGCTG GCTAATACTA ATAAGCTTTG CTTCGATGAG AGAAGAGTGG GGGCCATTGT      3660
TGGAACATTT GGGTCCGGCA AAAGTCACAA TGTAATTGAG TTGATAAGGC ACAATTTGGG      3720
ATACCAGAAT TTGATCATCT CCCCAAGAAG GAGCCTGAAG GATCAATTCA TAAGCACGCT      3780
GGATTTGGTG AATGCTAGGA GTAAAGGAAA GAAAACTTCC ACTGATGTGG TCACATTTGA      3840
GGTTGCATTG AAGAAAAATG GACTCCTCAA GAAAGCTAGA ATCTTCATTG ACGAAGCTCA      3900
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CGAGTCAAAA CGTTTTAGGA ATCCTATGTT TGTTGGAAGA TTGCCCTGCA CGTTTGACTC      4140
CAGCAGGTTA ACCCTTGAGA AAGAGGAGTA CGCTGTTTTT GACTCCTTCA AAGCTTTTAA      4200
GGCTGATTAT CTATCCCCAA AGATCAAAAC TTTCCTTGTG AGCTCATTTA CTGAAAAAAA      4260
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TATGAATTTT GATTATGTCT GTGTTCTCCT GACTCAAGAT AGCATGCTTG TTGATGAACG      4380
AAGATGGGTG GTAGCACTTT CCAGAGCAAA AATCAACATT TCTTTTATCA ATTTGTCTGG      4440
TTTGTCACTC CCTGAATTCT GCACTCAGAT GATGGGTGGA GTTGTGCATA AGTTCTTCAC      4500
```

Figure 4.4 (continued)

```
ATCCACGGCA ACCTTTAATG ACCTGAGAGA GCTCCTTCCT GGTGACCCTA TTTTTTCAAA      4560
AAAATTCCAG CGACTTGGCA AGGATGAAGT TGATAGGGAG GGTAGACTGT CCGGCGATCC      4620
TTGGTTGAAA ACAAAGGTGT TCCTCGGGCA GAGAGAAGAA AGAATTGAGG AAACCAGTGT      4680
TAATGATGAA GGGCTAAAAG ACGTAAAGGT TAAAGTTCAC TGCCCAGTTG GTTCAATTGG      4740
TTCAACATTG GCAGACATTC AAGCTGGGGT AAGGGTCAAA GAAGCAAGGG AATTCAGGGT      4800
TGAAAATCTG GTCACTGAAC AATTCTCAGA AACTCACAAA GGTAAGGGTA AAGTGCTCAC      4860
TGCTGCCCCT GATAATTTTG AAGCTATTTA CCCAAGACAT AAAGCCGGTG ACACTGCAAC      4920
CTTTGTAATG GCTGCAAGAA AGAGATTGAA GTTTTCGTTC CCAGCAAGAG AGAGGCAGAA      4980
GTACATGGCT GCGATCCCTT ATGGAGAGAG CATGTTGCAG GTATTTCTTA AAAGAGTTAA      5040
GCTTCAGCCC AATTTTGATC ATAGGTTGTT TGAGGAATCA AGGGCTGACT TTGAAGAGAA      5100
GAAACTTCAG AAATCCATGG CCACATTGGA AAATCATAGT GGGAGATCAG ACCCTGATTG      5160
GAGTGTCGAG AAGGCACTGA TTTTCATGAA GAGCCAGCTG TGCACAAAAT TTGACAACCG      5220
ATTCCGGAAT GCAAAGGCTG GACAAACTTT GGCATGTTTT CATCATGATG TACTCTGCCG      5280
CCTTGCTCCC TATATCCGTT ACATTGAAAA GAAAGTATTC AAGGCCTTGC CCAGTAATCT      5340
TTACATCCAT TCTGCACGCA ATTTTGAAGA ACAAAGGGAT TGGGTGATTA AGAATAACTT      5400
TACTGGAGTT TGCACTGAAT CTGACTATGA GGCGTTTGAC TCTTCACAAG ATGCAAATAT      5460
TCTGGCTTTT GAGGTGAGTT TGATGGAACA TTTAAGGTTG CCAAGGGATC TGATTGAGGA      5520
CTATAAATAC TTGAAATTCC ATACTCATTC AAAGCTTGGT CAGTTTGCTG TGATGAGATT      5580
CACTGGTGAG GCTGGAACCT TTTTGTTCAA TACTTTAGCT AACATGGTTT TCACATTTAT      5640
GAGGTATGAA ATCAATGGAA AGGAAGCCAT ATGTTTTGCT GGTGATGATA TGTGCGCAAA      5700
TAAGCTTCTG AGGAAGAAAA GTGAATTTGA ACACATCCTT GACAGGATGA CCTTGAAAGC      5760
AAAAGTTCAG CACACCACTG AACCAACTTT TTGTGGATGG CGCTTAGGGA ATTTTGGCAT      5820
TGTGAAGAGG CCCCAACTCG TGCAGGAGAG AATTCTCATT GCTTTGGAGA AAGGAAACTT      5880
TCATGAATGT ATTGATAATT ATGCAATTGA GGTTTCCTAT GCCTATAATT TAGGTGAGAG      5940
GCTGATCTCC ATAATGTCTG AAAAAGAATT GGATGCGCAT TACTTTTGTG TTAGAACTTT      6000
CTTACAAAAT AAAAAATTGT TCAGTTCTAA CGCATTGGAA TTTTTCTCTG AAAGTGAAGG      6060
TTGTTTGAGT CCTGAGAGGA ACTTTGGTTG ATGGAAGTTG TCCACAATTA TCTGCTTGAT      6120
GCTAACTTTA CACGGACTGA ATTTTCACTT AGTTTTCCTA TTGTTGTGCA TGGTGTGCCT      6180
GGTTGCGGGA AATCAACTTT TGTCAAGCGT TTACTGGATT GTGAAGACTT TCACGCTCAG      6240
TCTTACGGTG TTGTTAAGCC CACAAATTTG GCTGGGCGTG GCGTTGAAAA AGCTTTACAG      6300
CCTTTACAAT CCGGATTTAA CGTTCTTGAC GAGTATCTGT CTGGACCTTC TTACGAGGGT      6360
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Figure 4.4 (continued)

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TTTGATTTGT TGCTTTCCGA TCCTTATCAG AATTTCCGCA AACCACTTAC TGCTCATTTC      6420
ATCAATAGTT TTACTTATAG GTTTGGCCAT TCGGTTTGCA AATACCTTAA CCTATTAGGA      6480
TTTGAGATTA GTTCCAAAAA GGAGGAAGAC ACTGAACTTA TTCTTGGTAA AATCTTTGAA      6540
GGGGAGATTA GAGGGGAAAT CATTTGCTTT GAGAAGGAGG TTCAGGAGCT TCTGGACAAT      6600
CACTCTGCAA AACACCACCA TCCGTGCAAT TTGAGGGGAG CTGAATTTGA GCACGTCACT      6660
TTCATATCAG CTCATTCAGA CCTTCAGGAG ATAGTTGGAC CTGACCTTTA TGTGGCTCTC      6720
ACACGAGCTT CAAAAAGTTT AACTATTCTT ACCCCATAAA TGAGCCTCAA ACCACCAACT      6780
GACTGGTCCA AACCGATCTT ATTTGCTTCA GTTGGAATTG CAGTTTCTCT GGTTTGTTTC      6840
GTGTTCAAGG CTGATTACTT ACCAAAGGTG GGAGATAACA TTCACTCTCT CCCCCATGGT      6900
GGGTCTTATC GGGATGGTAC CAAGTCAATC AACTACAACG GTTTGAGATG TGCGGAGAAT      6960
TCTAGTGTCG ACCCTTTTCA TCAGTCAGGA AAGTTTTTAG CCTTTTGCTC TGTTGTCGTA      7020
CTTAGTGTGT TAATATATGT CTGTAGTAAA TGTAATGATA GGTCTAGTCG CATTCATCAT      7080
TTCTGCGTGC ATCATCACAA TAATTAGTAG TCACAGTAGT AATGTGTGTA CCATAATTGT      7140
CACTGGTGAA AGAGCCGTTG TGTCCGGCTG TGAAATAACT CCAGAGCTGA GCAATCTGCT      7200
CTCCCACTTG AAGCCTCATA CACATAGCTT AGGTTTTTAA TCACCAGTTT TTTGAAATTG      7260
TAGTGATAGT GTTAGAGGAT AATTGTAATG GCAGACGCAG TTGAGTACGA GCAGAACGAG      7320
GATGGTACTT TCAAGTTGGA CTCAGCAGGG CAGAAGATCC AAAAGAAAAA GACGTCCGGG      7380
CCCGACCCTG TCATACCTGG AACTGGGGGC CAGCAATCCA AGAAATCGGA CCTTGAAATC      7440
CTTAGAGCAA GAAGAAGAAG AGTCACCTTC GATCCAAAAA ATCCCACCTC TTGTCCTGGC      7500
AGAGACTTCA TCAGCAGTAT TCAAGATGCA GACCCAACTA CGCTTAACAT TGCCTCCGAC      7560
GACTCCGTCA AGGCAATTGC GGCTGATTGG GTCGAGCATC TTAAGATTCC AGAAGCAGAA      7620
GTATTTAATT GCATCTTTGA TATTGTCTGG TACTGTTATC ATAACAGCTC CAGTGACAAA      7680
ACGAAGTTTG TTGGTAGAGC AAAGTGTGGA GTTGAACTTG AAAGTCTTGC TAGTACTGTT      7740
AGGAGCTACT GCTCTTTACG CAGTTTCTGC TCGAAATATG CTCCAATAGT CTGGAATCAT      7800
GGAATCAGCA AGGACATACC ACCCGCTAAT TGGCAGAGGA GGAAGGTTAT AGAAAGCGCC      7860
AAATTTGCAT CATTTGACTT CTTCGAGGCA GTAACCAGTG CTGCTGCTCT TCAGCCCATT      7920
GATGGACTCG TGAGGTACCC AACAGATAAA GAGATGACTG CCGGAGCATC TCTTAAAGAA      7980
ATCAGTCTTA TCAGAGACGA AATCCGGAGA GGAACCAGTG CCACATTGAT GACTGAGGTT      8040
ACTGGAGGCA GGACTGGCCA AGTTCAACCA ATCAAGAAAA TCGGTTCGGA TGAATGATAA      8100
ACCCCTGCAA ACCCAACTTT ACAGTTGGCC CGTTTTAGTG ATACGGGGCG AAGCTCAAAT      8160
CACTTACCTA TCTTTACAGT TTTAATTAAT TTTCTGTATT TCCAAGTTTT AAATAAACTT      8220
```

Figure 4.4 (continued)

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AAAAAGCTCC TTAACCTAAT TAGGAGCTGG CTGTAGGGTT TTAATATATT TTCCTTTAGT      8280
TT                                                                     8282
```
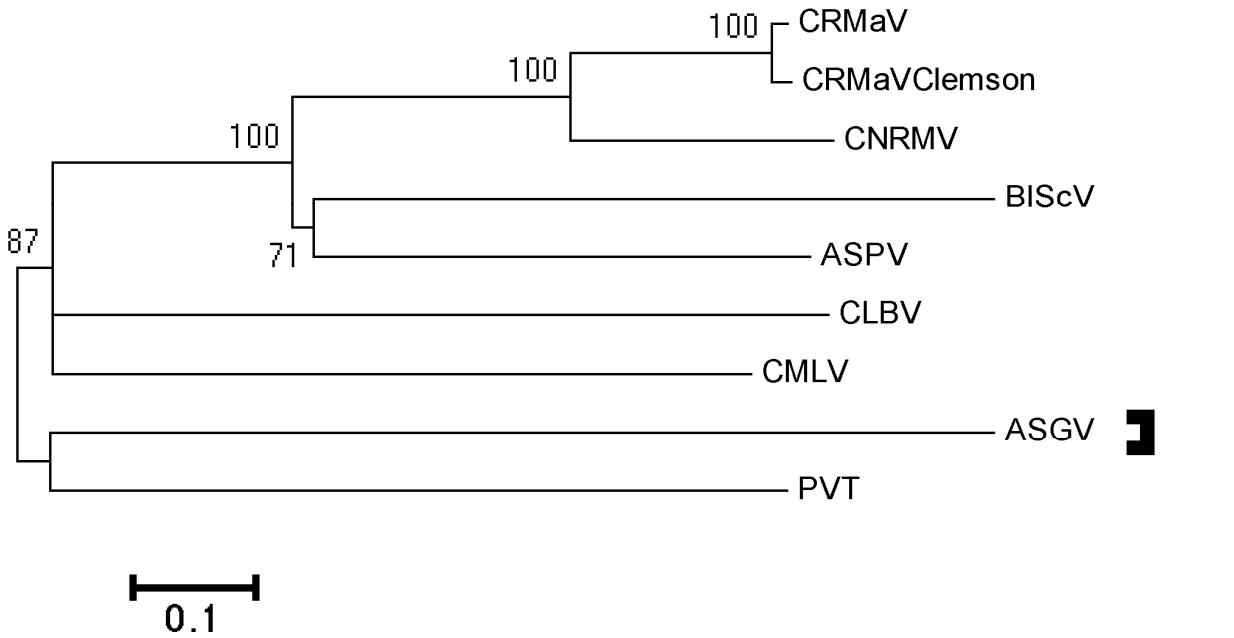

Figure 4.5 The phylogenetic analysis was done using MEGA 6 .The evolutionary history was inferred with the Maximum Likelihood method based on the Kimura 2-parameter model with 1000 bootstrap value. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. A phylogenetic tree was constructed using the sequences of other species in the family *Betaflexiviridae* and the sequence of *Cherry rusty mottle-associated virus*, Clemson isolate. CLBV: *Citrus leaf blotch virus*, CMLV: *Cherry mottle leaf virus*, CNRMV: *Cherry necrotic rusty mottle virus*, CRMaV: *Cherry rusty mottle-associated virus*, CRMaVclemson: *Cherry rusty mottle-associated virus*-Clemson isolate, BlScV: *Blueberry scorch virus*, ASPV: *Apple stem pitting virus* ASGV: *Apple stem grooving virus*, PVT: *Potato virus T*



On campus cherry trees survey

Buds from each of the 15 'Yoshino' trees (Table 4.2) included in the survey, were grafted to five certified virus-tested 'Mazzard' cherry seedlings (Lawyers Nursery, Plains, Montana). The seedlings were maintained in the greenhouse and then tested for the presence of the virus that had been detected in the tree at Musser Farm by using RT-PCR and specific primers developed from the sequence information that had been generated. Amplicons were detected in four of the seedlings. Each had been grafted from a different sample (Table 4.2). Virus-like symptoms were also observed in the leaves of these seedlings.

Table 4.2 Cherry trees located on campus and used to graft into virus-indexed cherry seedlings. The names in bold indicate the location of trees, from where the virus transmission to virus indexed material was a success using bud grafting.

| Cherry tree | Location | Number of seedlings with virus/ total number of seedlings |
|---|---|---|
| C1 | Behind life science building close to perimeter road | 0/5 |
| C2 | Behind life science building close to perimeter road | 0/5 |
| C3 | Behind life science building close to perimeter road | 0/5 |
| **C4** | **C11 parking lot adjacent to first three/cherry road** | **1/5** |
| C5 | C11 parking lot adjacent to first three /cherry road | 0/5 |
| **C6** | **Behind Lehotsky building** | **1/5** |
| **C7** | **Behind Lehotsky building** | **1/5** |
| C8 | In front of library | 0/5 |
| **C9** | **In front of library** | **1/5** |
| C10 | In front of library | 0/5 |
| C11 | In front of library | 0/5 |
| C12 | In front of library | 0/5 |
| C13 | In front of library | 0/5 |
| C14 | In front of library | 0/5 |
| C15 | In front of library | 0/5 |

## Discussion

Cherry trees have been reported since the 19th century to show symptoms of what is now known to be a virus and virus-like disease. In 1937, Cherry green ring mottle virus was reported to be infecting sour cherry and was described as viral disease in 1951 (Rasmussen et al., 1951). The virus was reported to cause Cherry vein yellow spot disease (Milbrath, 1960) and infects several *Prunus* spp including sweet cherry, sour cherry, and oriental flowering cherry. *Cherry leaf roll virus* is a nepovirus that infects sweet cherry trees. Unlike other viruses of the genus *Nepovirus*, *Cherry leaf roll virus* is not transmitted by nematodes, and the mode of transmission is still unknown (von Bargen et al., 2009). *Cherry mottle leaf virus* was first reported in cherry in 1920, causes chlorotic mottling and leaf distortion (Cheney and Parish, 1976), and bears genomic resemblance to *Apple chlorotic ringspot virus,* a Trichovirus (James et al., 2000). *Cherry rasp leaf virus*, *Cherry virus A*, *Epirus Cherry virus*, *Little cherry virus 1*, and *Little cherry virus 2* are some other viruses that infect cherry trees.

However, the symptoms that were observed in the cherry tree at Musser Farm had never previously been reported in South Carolina. The development of virus-like symptoms in a cherry that was grown from virus-indexed material, needed research to provide answers as to the identity of the virus, the origins of the virus and possible control measures that might be need to prevent the virus infecting commercially significant crops like peach. The survey and grafting experiments, confirmed that the virus detected in the cherry at Musser Farm is graft-transmissible and also occurs in some 'Yoshino' cherry (*Prunus* × *yedoensis* Matsum*)* trees growing on the campus of Clemson University in South Carolina. These data provide clues with regard to the presence of the virus in the cherry

tree at Musser Farm. The scion of the tree at Musser was *Prunus serrulata* Lindl. cv. Shirofugen, and was not showing any symptoms, but suckers growing from the certified virus indexed (CVI) Mazzard cherry F12/1 rootstock (*Prunus avium* (L.) L. displayed the symptoms. This tree had been used as a bioassay host for detecting two ilarviruses: *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV). Infection with CRMaV might have occurred when material from a 'Yoshino' Cherry growing on campus at Clemson University, and known to be infected with PNRSV and PDV was inoculated to the tree as part of a demonstration of the 'Shirofugen" bioassay. . Chip bud inoculation with PNRSV induces a hypersensitive reaction whereas chip bud inoculation with PDV produces a slow-moving systemic reaction and the limbs of the tree used for testing are removed after 6 weeks to avoid any transmission of the viruses. This was supported by molecular tests of the scion and rootstock where neither PNRSV nor PDV was detected. Thus the "new virus" most probably entered the tree from the buds used in the 'Shirofugen' assay and established a systemic infection in the rootstock although the scion appears to be resistant to virus infection. This is the first report of *Cherry rusty mottle-associated virus*, in South Carolina. As the virus was detected in other 'Yoshino' cherry trees at widely separated locations on campus the most likely explanation for the presence of the virus is that it was present in the trees when they were planted. Japanese flowering cherry has a long history of the presence of viruses and, as there are no schemes that test nursery sources of flowering cherry prior to the use of budwood in propagation, it is probable that the trees were propagated from an infected source. The only other identifications of CRMaV have been from the west coast of the US where the virus was detected in Portuguese laurel (*Prunus lusitanica*) which is native to southwestern France, Spain, Portugal, Morocco, and Macaronesia (the Azores,

Canary Islands and Madeira). On the west coast of the US it is used to produce an attractive hedge and is widely propagated in nurseries.

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# CHAPTER V

## CONCLUSIONS AND FUTURE DIRECTIONS

Plant viruses cause billions of dollars in losses in agriculture worldwide and are reported to be the second leading cause of plant disease loss after fungi. Scholthof et al., 2011 gave list of viruses that were important in molecular biology and included *Tobacco mosaic virus*, *Tomato spotted wilt virus*, *Tomato yellow leaf curl virus, Cucumber mosaic virus, Potato virus Y, Cauliflower mosaic virus, African cassava mosaic virus, Plum pox virus, Brome mosaic virus, Potato virus X, Citrus tristeza virus, Barley yellow dwarf virus, Potato leafroll virus*, and *Tomato bushy stunt virus*. The top 10 list of economically important plant viruses in 2015 (Rybicki) consisted of the African cassava mosaic disease begomovirus complex, *Banana bunchy top nanovirus*, *Banana streak virus*, Barley yellow dwarf disease luteovirus complex, *Cucumber mosaic virus*, Maize streak mastrevirus (MSV), *Maize dwarf mosaic virus /Sugarcane mosaic virus*, Rice tungro disease complex, *Rice yellow mottle virus*, *Sweet potato feathery mottle virus*.

Even though these lists of well-studied viruses of great importance exist, new plant viruses are continually identified each year. Although annual crops may be infected with one virus, perennial, long-lived crops have the opportunity to accumulate viruses during each subsequent growing season. This can result in a plant being infected with a complex of viruses, the constituents of which may be added to with time resulting in changes in symptoms and adverse effects on the plant. Some important virus disease complexes that have caused huge economic loss include *Grapevine leaf roll disease* (Naidu et al., 2014), *Cotton leaf curl disease* (Sattar et al., 2013), African cassava mosaic disease (Alabi et al., 2011), Rice tungro disease

(Bunawam et al., 2014). Virus complexes exist in many crops particularly woody perennial fruit crops. Blackberry yellow vein disease is a complex of viruses affecting blackberry and is known to reduce both plant vigor and the longevity of plantings (Martin et al., 2014).

The first project described in this thesis 'Incidence of known viruses in conventional South Carolina farms using sentinel plants' was done with the objective of detecting six of the many viruses reported to infect blackberry that might occur in South Carolina. The six viruses in this study, *Blackberry yellow vein-associated virus* (BYVaV), *Blackberry virus Y* (BVY), *Blackberry chlorotic ringspot virus* (BCRV), Blackberry virus E (BVE), *Tobacco ringspot virus* (TRSV), and Blackberry virus Ω (BVΩ) are some of the most frequently occurring viruses in the blackberry yellow vein disease (BYVD) complex (Tzanetakis I.E, personal observation). To our knowledge, this is the first intensive study done on blackberry viruses in South Carolina. Virus symptoms have been observed on blackberry plantings in the state for at least two decades.*Tobacco ringspot virus* was isolated from a plant of Cheyenne blackberry showing distinct symptoms typically associated with infection by a virus in 1993 and a preliminary survey of a number of small plantings detected the presence of BYVaV in 2004. (Scott pers.comm). Other samples collected in South Carolina have been tested for the presence of BYVaV, and BVY at the University of Arkansas but, there has not been any work completed in commercial plantings of blackberry inSouth Carolina.

In this study, we gathered information about the incidence of these viruses over three growing season at two different locations. The locations for the sentinel plants were maintained from season to season to see if there is any correlation between location and virus incidence, for example virus incidence in the middle of field vs edges of field. A few locations were chosen close to naturally growing wild blackberries. Wild blackberries can be the source of initial

infection in the field where virus-tested plants have been used. Also, wild blackberries can play a role in producing recombinant isolates, thus affecting the population structure of the virus (Poudel et al., 2012).

The use of sentinel plants in the study helped to understand virus behavior in a field setting. Our results, showed that 4 weeks exposure was sufficient for the virus to transmit itself to the virus-tested healthy plant, and reproduce symptoms on the plant. The differences in the weather conditions from year to year provide support for the possible influence of vector populations on virus transmission. In 2013 the incidence of BYVaV was low and during the same period an unusual amount of rain fell and reduced the whitefly population which transmits BYVaV (Poudel et al., 2013).

Fruit production of cherry trees does not occur in South Carolina although there are significant plantings of ornamental flowering cherries in the urban landscape. Thus the development of virus-like symptoms in a cherry that was grown from virus-indexed material, needed research to provide answers as to the identity of the virus, the origins of the virus and possible control measures that might be need to prevent the virus infecting commercially significant crops like Peach. A small survey was conducted on the campus of Clemson University to determine if other Yoshino cherries were infected with the virus and revealed that approximately 26% of the trees sampled were infected with the virus. This is perhaps not surprising based on the history of the Yoshino cherry and its widespread cultivation in the US (Cheong et al., in press).

Since there are many viruses in the family *Betaflexiviridae* that have not been assigned to existing genera, it is essential to have the maximum amount of sequence information before assigning a name to the virus that we isolated. Initial sequences showed homology with *Cherry*

*necrotic rusty mottle virus* (GenBank: EU188438.1) but as the sequence information was extended the greatest homology was with *Cherry rusty mottle-associated virus* (GenBank: KF356396.2).

Work on the potential new ilarvirus from subgroup 1 infecting blackberry and veronica has been difficult. Attempts to transfer the viruses to a herbaceous host, *C. quinoa*, were unsuccessful. Transfer to this host would have allowed attempts at purification of the virus from a source that typically develops concentrations of ilarviruses that are higher than most naturally infected hosts and that contains many fewer polyphenolic compounds to inhibit purification and nucleic acid extraction. However, the sequence data generated thus far will allow comparison with sequences from known ilarviruses and allow a decision to be made as to whether this a previously undocumented member of the genus ilarvirus or whether it is a strain of an existing species within the genus. Once this decision has been made, research on the epidemiology of the virus, appropriate sampling and detection techniques, and also understanding the population structure of virus can be completed. That this virus has been recorded from multiple states and in two widely divergent hosts, blackberry (Rosaceae) and veronica (Scrophulariaceae), offers the opportunity for some intriguing research to determine how both species were infected and how the virus became so widely distributed. In conclusion, the research presented in this thesis is a gateway to many follow-up projects to understand the epidemiology, importance, and life cycle of these viruses.

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APPENDIX INDEX

APPENDICES

## Appendix A

### Total Nucleic Acid Extraction

Leaf tissue (50 mg) was ground briefly in 1 ml of extraction buffer (200 mM Tris-HCl, pH 8.5, 300 mM lithium chloride, 1.5% lithium dodecylsulfate, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium deoxycholate, 1% NP-40 and 1% 14M β-mercaptoethanol solution v/v (added just before use). An equal amount of 5.8 M potassium acetate (3.8 M potassium, 5.8 M acetate) was added to the mixture (600 µl) and centrifuged at 16,000 **g** for 10 min. Seven-hundred-fifty microliters of the supernatant was mixed with an equal volume of 100% isopropanol and chilled at -20˚C for at least 30 m. The mixture was then centrifuged for 20 min at 16,000 **g** and the resulting pellet was resuspended in 500 µl wash buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 50 mM NaCl, 50% ethanol) and 20 µl of silica/glass milk was added. The mixture was centrifuged briefly for 10 sec at 9,400 **g** and washed twice to eliminate inhibitors. After the final wash, the mixture was centrifuged for 2 min at 16,000 **g** the supernatant was removed and the pellet was dried by turning the tubes upside down and incubating at 37˚C for 2 m. The pellet was resuspended in 150 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and centrifuged for 2 min at 13,500 **g**. The purified nucleic acids contained in the supernatant were used in reverse transcription-polymerase chain reaction (RT-PCR) amplification.

## Appendix B

### Reverse Transcription RT using Total Nucleic Acid

Purified nucleic acids (2.5 µl) prepared as in appendix A were used in reverse transcription (RT) reactions containing 0.5 µl of 10 µm gene specific reverse primers. Maxima$^{TM}$ reverse transcriptase (Fermentas) (50 U) was used with 6 U of RiboLock RNase Inhibitor (Fermentas), 0.4 mM DNTPs, 5µl 5x reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3 at 25˚C, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT) and water to a final volume 25 µl. The RT mix was incubated at 50˚C for 75 min, followed by denaturation for 5 min at 85°C.

## Appendix C

### Double Stranded RNA Extraction

STE (25mM Tris-HCl, 50 mM NaCl, 0.5 mM EDTA, pH 7.0) buffer was used throughout this procedure. Approximately 20 g of tissue was ground in liquid $N_2$ until completely pulverized. The powder was mixed with 50 ml of 2× STE, 30 ml STE-saturated phenol, 10 ml 10% (w/v) sodium dodecyl sulfate, and 1 ml of 14 M β-mercaptoethanol. The mixture was shaken for 3 h at room temperature and then centrifuged at 15,000 **g** for 10 m. Ethanol was added to the supernatant to a final concentration of 18%, using 1×STE to adjust the volume to 100 ml. Whatman CF 11 cellulose (1g) was added and the mixture was shaken at room temperature for 10 m to bind nucleic acid onto the cellulose matrix. The mixture was then centrifuged for 10 m at 15,000 **g** and the cellulose was washed an additional five times with wash buffer (STE/18% Ethanol) before being packed into a 100-ml chromatography column (VWR). The cellulose was

washed two more times in the column. The column was allowed to drain and eluted with 15 ml STE. The eluted nucleic acids were digested for 1 h at 37˚C after addition of $MgCl_2$ and $CaCl_2$ to final concentrations of 100 and 10mM respectively, as well as 40 U DNAseI (Sigma-Aldrich), and 250 U T1 RNAse (Sigma-Aldrich). Two-hundred-fifty microliters of 0.5M EDTA (pH 8.0) was added to stop the reaction, and the volume was brought to 30 ml with 95% EtOH. Silica milk (25 µl) was added, and the mixture was centrifuged at 850 **g** for 5 m. The pellet was washed with 500 µl wash buffer (Tzanetakis et al., 2007) centrifuged for 1 m and the silica pellet was dried by inverting the tubes at 37˚C. Then, the pellet was resuspended in 100 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and centrifuged for 2 m at 13,500 **g**. Approx 25 µl of the supernatant was combined with 5 µl of 6x loading buffer and visualized by electrophoresis on a 1% agarose, 1× TBE (90mM Tris-HCl, 90 mM boric acid, 2mM EDTA, pH 8.0) gel stained with Gelred (Phenix Research).

## Appendix D

## Reverse Transcription using ds RNA

Template dsRNA (5 µl) was mixed with 4 µl of each reverse primer (20µM, Table1), 4 µl of 40 mM methyl-mercury hydroxide for each 25 µl reaction and incubated at room temperature for 20 m. The Maxima$^{TM}$ Reverse Transciptase (Fermentas) (50 U) was used in reverse transcription with 6 U of RiboLock RNase Inhibitor (Fermentas), 0.4 mM DNTPs, 5µl 5x reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3 at 25˚C, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT) and water to a final volume 25 µl. The enzyme mix was added until the solution was no longer opaque. The RT mix was incubated at 50˚C for 75 min, followed by denaturation for 5 min at 85˚C.

## Appendix E

### Polymerase Chain Reaction

The cDNA obtained by reverse transcription was used in PCR to detect the presence of the viruses. For a 25 µl PCR reaction, 1 µl cDNA was used and the reaction consisted of 2.5 µl of 10x PCR reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1% Triton X-100) 2 mM $MgCl_2$, 0.4 mM primers, 0.2 mM DNTPs and 1.25 units of *Taq* Polymerase (Genescript) and water to a final volume of 25 µl. The PCR program consisted of initial denaturation at 94˚C for 2 min, followed by denaturation at 94˚C for 30 s, annealing at 55˚C for 15 s and extension at 72˚C for 30 s, repeated for 40 cycles and final extension of 72˚C for 10 m. The samples were visualized in 1.5% TBE-agarose gel stained with GelRed (Phenix research).

## Appendix F

### Trifocap: Polyvalent Degenerate Oligonucleotides (PDO) Nested RT- PCR Amplification

Purified nucleic acids (2.5 µl) were used in reverse transcription (RT) reactions containing 0.5 µl of 10µm of primer PDO-R3i and PDO-R4i. Maxima™ Reverse Transcriptase (Fermentas) (50 u) was used with 6 u of RiboLock RNase Inhibitor (Fermentas), 0.4 mM DNTPs, 5µl 5x reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3 at 25˚C, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT) and water to a final volume 25 µl. The RT mix was incubated at 42˚C for 45 min, followed by denaturation for 3 min at 95˚C.

The cDNA obtained by reverse transcription was used in PCR reactions to detect the presence of the viruses of genus *Trichovirus, Foveavirus,* and *Capillovirus*. For a 25 µl PCR

reaction, 2 µl cDNA was used and the reaction consisted of 2.5 µl of 10x PCR reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1% Triton X-100) 2 mM $MgCl_2$, 0.4 mM primers (PDO-F1i, PDO-R3i and PDO-R4i), 0.2 mM DNTPs and 1.25 units of *Taq* Polymerase (Genescript) and water to a final volume on 25 µl. The PCR program consisted of initial denaturation at 95°C for 3 min followed by 35 cycles (30 s at 95°C, 30 s at 42°C, and 30 s at 72°C) and final denaturation at 72°C for 10 m. For the second nested PCR 1µl of final product from the first PCR reaction was used as template. For second nested PCR 1µl final product from first PCR was used and the reaction consisted of 2.5 µl of 10x PCR reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1% Triton X-100) 2 mM $MgCl_2$, 0.4 mM primers (PDO-F2i, PDO-R1i), 0.2 mM DNTPs and 1.25 units of *Taq* Polymerase (Genescript) and water to a final volule on 25 µl. The second PCR program consisted of initial denaturation at 95°C for 3 min followed by 30 cycles (30 s at 95°C, 30 s at 42°C, and 30 s at 72°C) and final denaturation at 72°C for 10 mins. The final product of 362bp was visualized in 1.5% TBE- agarose gel stained with GelRed (Phenix research).

## Appendix G

## Silica Milk Preparation

Silica milk was prepared according to Rott and Jelkman (European Journal of Plant Pathology 107:411-420, 2001). Silica particles (60 g, Sigma S5631) were added to 500 ml of distilled $H_2O$ in a 500 ml measuring cylinder. The mixture was mixed well and allowed to settle for 24 hours. The upper 470 mls were discarded. Distilled water adjusted to pH 2.0 using hydrochloric acid was added to 500 ml and the mixture was allowed to sit for 5 hours or overnight. The upper 440 ml was decanted. The pH of the slurry was checked to confirm a pH of 2.0. The silica milk thus

prepared was autoclaved and stored in dark bottle at room temperature. Alternatively, the silica milk was aliquoted into 2.0 ml tubes and stored at 4°C for several months.

## Appendix H

## Gel Purification, Cloning and Sequencing

The PCR products were visualized in a 1% TBE- agarose gel stained with GelRed® (Phenix Research). Samples containing the anticipated amplicon were gel purified using Qiaquick Minelute gel extraction kit (Qiagen). Purified product (3 µl) was cloned into 1 µl pGEM plasmid vector (Promega) with 5µl ligation butter and 1 µl of DNA ligase. The ligation was incubated either at room temperature for 1 h or overnight at 4°C. The ligation (5µl) was transformed in 50 µl JM109 Competent cells (Promega). The transformation reactions were incubated on ice for 20 m and subjected to heat shock (42°C) for 45 s and incubated in ice for 2 m. S.O.C (500 µl) (2% tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the transformed cells and the mixture was shaken at 37°C for 1 hr. The bacterial culture was plated onto two (150 µl culture each plate) LB Agar plates (1% tryptone, 0.5 % yeast extract, 10 mM NaCl, 1.5 % agar) containing 100µg/µl Ampicillin and 40µg/µl bromo-chloro-indolyl-galactopyranoside (X-gal) and 40µg/µl isopropyl thiogalactoside (IPTG). The plates were incubated at 37°C for 20 h and the recombinant colonies were sequenced at either CUGI, Clemson University or Functional Biosciences Inc. (Madison, WI) with the M13 forward and reverse primers.