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Blackberry Virosome: A Micro and Macro Approach

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Blackberry Virosome:

A Micro and Macro Approach

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Abstract

Viruses pose a major concern for blackberry production around the world with more than 40 species known to infect the crop. Virus complexes have been identified recently as the major cause of plant decline with blackberry yellow vein disease (BYVD) being the most important disease of the crop in the Southern United States. The objective of this research was to study the blackberry virosome in both the macro and micro scale. The large scale approach involves identification of the major viruses known to be associated with BYVD in the Southern United States as well as the identification of other viruses whose prevalence is still unknown. RT-PCR was employed to detect the viruses present in wild, cultivated and sentinel blackberries from different states. In the micro approach, the virosome of a single field was studied using large scale sequencing. Understanding the virosome on a regional and local scale provides important information which could greatly enhance disease management. The ultimate goal of this research is to better understand virus distribution in nature and aid in the development of proper management strategies to control epidemics.

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1.1 Abstract

Viruses pose a major concern for blackberry production around the world with more than 40 species known to infect the crop. Virus complexes have been identified recently as the major cause of plant decline with blackberry yellow vein disease (BYVD) being the most important disease of the crop in the Southern United States. The objective of this research was to study the blackberry virosome in both the macro and micro scale. The large scale approach involves identification of the major viruses known to be associated with BYVD in the Southern United States as well as the identification of other viruses whose prevalence is still unknown. RT-PCR was employed to detect the viruses present in wild, cultivated and sentinel blackberries from different states. In the micro approach, the virosome of a single field was studied using large scale sequencing. Understanding the virosome on a regional and local scale provides important information which could greatly enhance disease management. The ultimate goal of this research is to better understand virus distribution in nature and aid in the development of proper management strategies to control epidemics.

1.2 Introduction

Blackberry belongs to the genus *Rubus* in the family Rosaceae which also includes strawberry, apple, rose, peach and plum among other species (Poling, 1997). Blackberries were harvested from the wild until recently when the crop was commercialized. Historically, efforts were made to develop cultivars in the late 1800s, and within the last 70-80 years blackberries have moved from being wild-harvested to a large scale commercial crop.

The genus *Rubus* is diverse as species range from tiny and prostrate plants to very large bushes (Clark 2007). Growth is herbaceous or semi-woody with biennial canes on a perennial crown and root system. As blackberries have flexible woody stems, they can be erect; growing without any support, upright and self-supporting; semi-trailing, free standing to the surface or base; or trailing, requiring trellises, poles or stakes to support the fruit load (Strik, 1992). Blackberry is adapted to temperate regions with well-drained, fertile soils. They can withstand high summer temperatures but not extreme cold regimes.

Wild blackberry species are perennial plants with biennial canes. During the first year, shoots grow vegetatively (-primocanes) and after the dormant period they start flowering, produce fruit and senesce (-floricanes). Flower and fruit emerge in a panicle-like or racemose-cymb pattern (Hummer and Janick, 2007). The receptacle contains multiple ovaries, styles and stigmas which upon fertilization leads to the production of the aggregate fruit which consists of a number of small fleshy fruit called drupes or drupelets. Blackberry can be distinguished from raspberry by the separation of the fruit from the receptacle. Blackberry has the receptacle attached to the fruit whereas in raspberry the receptacle stays with the plant. Blackberry is hence an ‘aggregate fruit’ with drupelets adhered to each other, each containing a small seed (pyrene) (Poling, 1997).

Blackberry is highly nutritious with 85% water, 10% carbohydrates, as well as macro- and microelements and vitamins. It has gained popularity among consumers, not only because of its taste, but also because of the high content of anthocyanins, phenolics and other compounds with antioxidant activity which act against free radicals and protect cells from oxidative damage (Dai *et al*., 2009; Huang *et al*., 2012; Wang and Lin, 2000). In addition, these compounds reduce the risk of coronary heart diseases (Renaud and Lorgeril, 1992), have anti-inflammatory and anti-carcinogenic activities, improve visual acquity and slow down aging (Hu *et al*., 2003; Seeram *et al*., 2006; Nichenametla *et al*., 2006; Dai *et al*., 2009).

1.3 Blackberry virus distribution in the southern United States

Viruses present a major concern for blackberry production today. *Rubus* species are propagated vegetatively in commercial settings and viruses may be introduced at any point during germplasm development, propagation or fruit production. Once infected with a virus, plants become less productive with both fruit quality and quantity being affected. A severe disorder referred to as blackberry yellow vein disease (BYVD) has emerged at the turn of the century in the southern United States (Martin *et al*., 2013). Several viruses associated with the disease have been reported, including blackberry yellow vein-associated virus (BYVaV), blackberry chlorotic ringspot virus (BCRV), beet pseudo-yellows virus (BPYV), blackberry virus S (BIVS) and blackberry virus Y (BVY). However, there are several *Rubus* viruses such as strawberry necrotic shock virus (SNSV), raspberry bushy dwarf virus (RBDV), rubus yellow net virus (RYNV), raspberry leaf mottle virus (RLMV) whose prevalence in the southern United States is still unknown.

Before the development of modern detection techniques virus characterization was based on the symptoms developed on indicator plants including *Rubus occidentalis* (black raspberry) and *R. henryii* (Stace-Smith, 1987). Since then there has been significant progress in the molecular characterization of *Rubus* viruses (Martin *et al*., 2013) including reverse transcription-polymerase chain reaction (RT-PCR) which is widely used for the detection of most blackberry viruses.

1.4 Blackberry yellow vein disease

Blackberry yellow vein disease (BYVD) is a devastating disorder that affects both cultivated and wild blackberries (Martin *et al*., 2013). Symptoms are observed mostly in a few, older primocane leaves and become more prominent as the season progresses. Typical symptoms include vein-yellowing, leaf mottling, ringspots, oak-leaf patterns, and may lead to die-back of the floricanes or even plant death (Susaimuthu *et al*., 2006; 2007; 2008a). Yet, the most severe effect of BYVD is the decline in the productivity that leads to replanting every 5-7 years compared to productivity of at least 20 years.

Initially, BYVD symptoms were thought to be caused by tobacco ringspot virus (TRSV), a prevalent virus in affected areas; however, experiments to ensure single infection that include nematode transmission of TRSV followed by grafting to multiple cultivars showed TRSV to be asymptomatic in modern blackberry cultivars (R. Gergerich, unpublished). Symptomatic plants were studied further and a new virus was identified in all plants used in the original study (Martin *et al*., 2004). The virus was named blackberry yellow vein associated virus (BYVaV), a crinivirus. Notwithstanding, Susaimuthu *et al*. (2008a) determined that BYVaV is latent in single infections on *Rubus occidentalis* 'Munger'. The hypothesis that additional viruses may infect

plants and synergistically cause disease symptoms was examined and verified as documented by the discovery and association of several additional viruses to BYVD. Susaimuthu *et al*. (2008b) determined that symptom severity were dependent on the number of viruses that infect plants. The viruses that have been associated with the disease are BYVaV (Martin *et al*., 2004), beet pseudo yellows virus (BPYV) (Tzanetakis and Martin, 2004), blackberry chlorotic ringspot virus (BCRV) (Tzanetakis *et al*., 2007), blackberry virus Y (BVY) (Susaimuthu *et al*., 2008b), blackberry virus S (BIVS) (Sabanadzovic *et al*., 2009), TRSV (Stace-Smith and Ramsdell, 1987), impatiens necrotic spot virus (INSV) (Tzanetakis *et al*., 2009), blackberry virus E (BVE) (Sabanadzovic *et al*., 2011) and blackberry vein banding associated virus (BVBaV) (Thekke-Veetil *et al*., 2013).

1.5 Blackberry viruses

1.5.1 Blackberry yellow vein-associated virus (BYVaV) and beet pseudo-yellows virus (BPYV)

BYVaV and BPYV are both members of the genus *Crinivirus,* family *Closteroviridae.* Closteroviruses are known to have highly diverse population structure because of the polymerase error rate, recombination and reassortment between variants or changes in the host range which may lead to genetic drift (Rubio *et al*., 2013a). Based on the genome size and organization, and epidemiology and biological properties, the family *Closteroviridae* is divided into four genera namely, the monopartite *Closterovirus*, *Ampelovirus* and *Velarivirus* and the bi- or tripartite *Crinivirus* (Martelli and Candresse, 2010; Martelli *et al*., 2012a; Martelli *et al*., 2012b). Criniviruses ranges in size from 13-19 kb (Martelli *et al*., 2012a) and their gene expression involves strategies common in closteroviruses, including translational frameshift, polyprotein processing, and the production of 3' co-terminal subgenomic RNAs (sgRNAs) (Dolja *et al*.,

2006). RNA 1 encodes for proteins with enzymatic motifs involved in replication including a papain-like protease, methyltransferase, helicase and RNA-dependent RNA polymerase. The RNA-dependent RNA polymerase is probably expressed via a +1 ribosomal frameshift typical of all closteroviruses. RNA 2 has several ORFs encoding proteins involved in movement, virus encapsidation and transmission including the heat shock protein 70 homolog (Hsp70h), the hallmark gene of the *Closteroviridae* (Tzanetakis *et al*., 2006a).

Criniviruses are recalcitrant to isolate and study because of the inability to transmit mechanically; they are phloem limited and yield few particles during purification (Karasev, 2000). When there is accumulation of viral inclusion bodies in the phloem, there is interference with the normal vascular transport (Wisler *et al*., 2001) and this is thought to be the reason behind symptoms such as interveinal yellowing, leaf brittleness, reduced photosynthesis and yield and early leaf senescence (Tzanetakis *et al*., 2013). Criniviruses are transmitted by whiteflies in the genus *Trialeurodes* and *Bemisia* in a semi-persistent manner. With the establishment and naturalization of the vectors, criniviruses have become a major agricultural threat across the temperate, subtropical and tropical areas of the world (Tzanetakis *et al*., 2013).

BYVaV RNA1 is 7.8 kb long and encodes the replication-associated polyprotein whereas RNA2 is 7.9 kb long and encodes eight ORFs similar in function to other criniviruses. However, BYVaV RNA2 contains an additional ORF at the 5' end of the genome that encodes for a second transmembrane protein which is not found in any other criniviruses (Tzanetakis *et al*., 2006a). Poudel *et al*., (2013) reported that the transmission of BYVaV from blackberry to blackberry is more efficient with *T. abutilonea* and less so with *T. vaporariorum*. More than twenty five plant species growing near blackberry fields having blackberries highly infected with BYVaV failed to identify any alternative host, even though BYVaV is graft transmissible to roses (Poudel *et al*.,

2013). Poudel *et al*., (2013) also reported the presence of BYVaV in both cultivated and wild blackberries in different states including Arkansas, Kentucky, Mississippi, North Carolina, South Carolina, and Tennessee but with low incidence in Georgia and Florida. BYVaV was also detected in California, Oklahoma, Illinois and West Virginia.

BPYV has similar genome structure to BYVaV but unlike the latter, has a natural host range including strawberry, vegetable crops, weeds and ornamentals (Duffus and Johnstone, 1981; Wisler *et al*., 1998; Tzanetakis *et al*., 2003). BPYV was first described in 1965 in California from sugar beet growing in a greenhouse and was the first crinivirus to be described (Duffus, 1965). In the latter years, BYVD infected plants were also found infected with BPYV among other viruses (Tzanetakis and Martin, 2004). *T. vaporariorum* (Westwood) is the only known vector of the virus. The wide host range of BPYV includes several weed species present in blackberry fields and this in combination to the naturalization of the greenhouse whitefly in blackberry fields may have led to the widespread distribution of the virus in the crop (Martin *et al*., 2013).

1.5.2 Blackberry chlorotic ringspot virus, strawberry necrotic shock virus and tobacco streak virus

BCRV, strawberry necrotic shock virus (SNSV) and tobacco streak virus (TSV) infect *Rubus* and *Fragaria* species alike. They are members of the family *Bromoviridae*. The *Bromoviridae* contains viruses with icosahedral or quasi-icosahedral virions encapsidating the positive sense, single stranded tripartite RNA genome encoding four or five proteins. BCRV, SNSV and TSV are member of subgroup 1 of the genus *Ilarvirus*; the largest genus of the family (ICTV Master Species List, 2014).

RNA 1 is monocistronic, and encodes for the viral replicase with the signature motifs for methyltransferase and helicase activity. RNA 2 encodes for an RNA-dependent RNA-polymerase (2a) and can be either monocistronic or bicistronic (Xin *et al*., 1998; Shiel and Berger, 2000). As is common in cucumoviruses, several ilarviruses including all members of subgroup 1, RNA 2 also codes for a gene involved in the suppression of RNA silencing (Shimura *et al*., 2013) and cell to cell movement (Xin *et al*., 1998). RNA 3 codes for the movement and coat proteins. The movement protein is expressed directly from the genomic RNA whereas the coat protein; required for virus movement and genome activation, is expressed through the sub-genomic RNA 4 (Jaspars, 1999, Neeleman *et al*., 2004). Based on the serological relationships, the species within the same genus is divided into subgroups (Fauquet *et al*., 2005). However, there are instances where serological relationships may be misleading (Scott *et al*., 2003; Tzanetakis and Martin, 2005). Today, ilarviruses are grouped more reliably based on genomic data (Scott *et al*., 2003). BCRV, SNSV and TSV have probably evolved from the same ancestral virus as they share conserved motifs in the viral polymerase and replicase (Tzanetakis *et al*., 2010).

BCRV is relatively a new member of the subgroup and was first discovered in blackberry in Scotland (Jones *et al*., 2006) and in rose in the United States (Tzanetakis *et al*., 2006a). Tzanetakis *et al*., (2007) also found BCRV infecting raspberry in the United States and in association with the BYVD, being one of the most widespread virus in diseased plants (Martin *et al*., 2013). In addition, BCRV is widely distributed in multiflora roses affected by rose rosette disease (Poudel *et al*., 2014). Apple has been verified as a host for the virus, expanding the host range of the virus and signifying the need for additional testing among members of the *Rosaceae* (Poudel *et al*., 2014).

TSV was first discovered in 1936 (Johnson, 1936). It is now known to infect more than 80 plant species belonging to the families *Asteraceae, Cucurbitaceae, Rosaceae, Brassicaceae, Solanaceae* and also some weeds (Fulton, 1948; Almeida *et al*., 2005). It is the type member of the genus and is transmitted in nature vertically through seed and horizontally by pollen and thrips (Sdoodee and Teakle, 1987). SNSV was first identified in 1956 in strawberry (Frazier *et al*., 1962). The virus can infect strawberry cultivars or *Rubus* species (Converse, 1972; Frazier, 1966). Similar to TSV, SNSV is spread by seed, pollen and by thrips (Johnson *et al*., 1984; Kaiser *et al*., 1982). Symptoms are rarely seen in either strawberry cultivars or *Rubus* species but the yield is compromised once plants are infected. The virus can reduce strawberry yield by more than 15% and runner production by 75% (Johnson *et al*., 1984). A similar symptomless virus was discovered in *Rubus* in mid-1960s and named as Black raspberry latent virus (BRLV) (Converse and Lister, 1969). Previously it was suggested that both BRLV and SNSV are the isolates of TSV as antisera made against one virus cross reacted very strongly with the other (Jones and Mayo, 1975). TSV was used to characterize these isolates as it was discovered first. Stenger *et al*., (1987) provided strong evidence that SNSV and TSV were significantly different as Northern hybridization using SNSV probes failed to detect the white clover or tobacco isolates of TSV. In 2004, several TSV isolates from *Fragaria* and *Rubus*, including some clones used in the original studies of SNSV and BLRV, were studied and determined that none was infected by the virus. Instead all were infected by a new virus and the SNSV name was revived (Tzanetakis *et al*., 2004). BRLV is now proven to be an isolate of SNSV. The virus has since been found in China and Australia (Li and Yang, 2011; Sharman *et al*., 2011). Hundreds of *Rubus* and *Fragaria* accessions have been tested for the presence of both SNSV and TSV (Tzanetakis, unpublished). More than a hundred plants were tested positive for SNSV while only two strawberry accessions

were tested positive for TSV (Tzanetakis *et al*., 2010). To this date and after the molecular characterization of SNSV no *Rubus* accessions have been identified as TSV positive.

1.5.3 Blackberry virus S and grapevine syrah virus 1

Tymovirus, Marafivirus and *Maculavirus* are the three genera that comprise the family *Tymoviridae* (Martelli *et al*., 2002; King *et al*., 2012). The number and cistron organization differ slightly between genera, but all viruses code for a large polyprotein necessary for viral replication (Dreher *et al*., 2005). The three genera have many traits in common which include their physicochemical properties, high cytidine content and peripheral vesiculation of mitochondria or chloroplasts in infected cells (i.e., alteration in the shape and structure of chloroplast and/or mitochondria) (Dreher *et al*., 2005; Martelli *et al*., 2002). The genome consists of a single molecule of single stranded positive sense RNA of ~6.0 to 7.5kb with high cytidine content (32-50%). The molecule is capped at the 5' end and contains a large ORF which encodes for replication associated polyproteins which is analogous to those encoded by other taxa of the 'alpha-like' super-group of ssRNA viruses (Goldbach *et al*., 1991). The signature amino acids motifs of the viral replicase include methyltransferase (MTR), endopeptidase/protease (PRO), helicase (HEL) and RNA-dependent RNA-polymerase (RdRp) (Goldbach *et al*., 1991). The genome is encapsidated into an isometric, non-enveloped virion that contains clusters of coat protein subunits arranged in pentamers and hexamers. The purified virus particles contain two components, one made up of non-infectious protein shells (T), which may contain small amounts of RNA, and the other made up of infectious nucleoproteins (B) which contain the virus genome (Boulila *et al*., 1990; Hirth and Givord, 1988). The expression of the genome is by post-translational autocatalytic cleavage of the largest ORF by the protease whereas the coat protein is expressed via sub-genomic RNA (Dreher *et al*., 2005; Edwards, 2000).

Blackberry virus S (BIVS) and grapevine syrah virus 1 (GSyV-1) are members of the genus *Marafivirus.* Marafiviruses are known to be transmitted by leafhoppers in a persistent manner. The genome of BIVS is polyadenylated and is phylogenetically related to oat blue dwarf virus and citrus sudden death-associated virus. This virus was reported as the first marafivirus to infect *Rubus* spp. in the 2009 (Sabanadzovic and Abou-Ghanem Sabanadzovic, 2009). The research focused mainly in the native blackberry germplasm in the Great Smoky Mountains National Park in Tennessee. Plants that showed BYVD symptoms were chosen for further analysis which led to the discovery of BIVS.

Grapevine syrah virus 1 was found to co-infect plants with other viruses in BYVD samples collected from Great Smoky National Park in Tennessee. GSyV-1 has a characteristic feature of circular permutation of RdRp motifs, which is not reported in other plant viruses to date (Sabanadzovic *et al*., 2009). The economic importance and distribution of this virus is yet to be understood. Partial data from on-going research indicate the presence of additional members of the family *Tymoviridae* in wild and cultivated blackberries (S. Sabanadzovic and Abou-Ghanem Sabanadzovic, unpublished)

1.5.4 Blackberry virus E

Blackberry virus E (BVE) is another recently discovered virus. The phylogenetic analyses revealed this virus to be close to the members of the genus *Allexivirus* and several other flexiviruses. However, the final taxonomic placement of the virus in the family *Alphaflexiviridae* is not yet determined because of genome discrepancies when compared with allexiviruses (Sabanadzovic *et al*., 2011). BVE contains an ORF which encodes a serine-rich protein and is regarded as the hallmark of all extant allexiviruses. However, unlike all other members in the

genus, BVE lack 3'-end proximal ORF which encodes for a nucleic acid-binding protein. Moreover, BVE infects a dicot unlike all allexiviruses which infect monocots. Based on these facts, BVE is considered as an unusual or atypical member of the family or the type member of yet to be established genus (Sabanadzovic *et al*., 2011).

1.5.5 Tobacco ringspot virus

TRSV was discovered in the 1920s and reported to infect wild blackberries in North Carolina (Rush *et al*., 1968). The virus has a wide host range including both monocots and dicots (Stace-Smith, 1985). TRSV is one of the most important viruses of blackberry in the United States. Initially, BYVD was thought to be caused by TRSV as the virus is widespread and has been found prevalently in affected areas. TRSV is a member of subgroup A of the genus *Nepovirus*, family *Secoviridae*. The genome is bipartite consisting of two polyadenylated positive-sense, single stranded RNA molecules; designated as RNA 1 and RNA 2. RNA 1 encodes for a polyprotein which is proteolytically processed to four mature non-structural proteins involved in virus replication whereas RNA 2 encodes for a polyprotein matures to the coat and movement proteins. The RNA molecules are encapsidated in spherical virions of 28 nm diameter (Rott *et al*., 1991 and Rott *et al*., 1995). TRSV is transmitted efficiently by seed (vertical), pollen (horizontal) and nematodes in the genus *Xiphinema*. The capsid plays a specific role in the interactions with the nematode, affecting virus transmission (Harrison *et al*., 1974).

1.5.6 Impatiens necrotic spot virus

Impatiens necrotic spot virus **(**INSV) belongs to the genus *Tospovirus*, family *Bunyaviridae*, members of which cause severe economic losses in a wide range of crops around the world (German *et al*., 1992; Pappu *et al*., 2009). Tospoviruses have enveloped, pleotropic

particles with a diameter of 80-120 nm. The genome is comprised of three, negative strand RNA segments: large (L), medium (M) and small (S) (Tsompana and Moyer, 2008). L RNA codes for the RNA-dependent RNA polymerase (de Haan *et al*., 1991; Adkins *et al*., 1995; van Knipperberg *et al*., 2002). M and S RNA segments use an ambisense expression strategy (de Haan *et al*., 1990; Kormelink *et al*., 1992). M RNA encodes for the movement protein (NSm) in the positive orientation and is known to affect disease development (Lewandowski and Adkins, 2005; Li *et al*., 2009). It also encodes the precursor of two glycoproteins (Gn and Gc) in the negative orientation which are integrated in the membrane that enclose the RNA segments and are needed for transmission (Whitfield *et al*., 2005; Kikkert *et al*., 2001; Ribeiro *et al*., 2008). The S RNA segment encodes for two ORFs. ORF1 codes for non-structural proteins (NSs) in the positive orientation which functions as suppressor of RNA silencing (Takeda *et al*., 2002; Bucher *et al*., 2003) whereas ORF2 codes for the nucleoprotein (NP) in the negative orientation. This protects the genomic RNA and is possibly involved in long distance movement (Bucher *et al*., 2003; Ribeiro *et al*., 2009).

Tospoviruses are known to be transmitted by thrips (-order Thysanoptera, family Thripidae). Thrips occur in large populations under wide climatic and geographic ranges and a diverse host ranges making them one of the most important agricultural pests which also serve as a virus vector (Pittman, 1927; Sakimura, 1962, 1963, 1969; German *et al*., 1992 and Iwaki *et al*., 1984). INSV has a broad host range including both monocots and dicots. The process of virus acquisition is life-stage-dependent as thrips can only acquire INSV in the first or second instars and then can transmit throughout the life in a persistent propagative manner (German *et al*., 1992; Ullman *et al*., 1992, 1995a, 1995b, 1996). Members of the genus *Frankliniella* namely, *F. occidentalis* (western flower thrips), *F. fusca* (tobacco thrips) and *F. intonsa* (flower thrips) have

been proven to be efficient vectors of the virus reaching 60% in the case of *F. occidentalis* (DeAngelis *et al*., 1994, Naidu *et al*., 2001). INSV was recently reported in blackberries (Tzanetakis *et al*., 2009). However, the transmission mode; whether done during pruning or by thrips, has not yet been determined. Enzyme-linked immunosorbent assay (ELISA), without the verification by an alternative detection method, detected more than 30% of the BYVD-affected plants from the southeastern United States to be infected with INSV (Guzman-Baeny, 2004).

1.5.7 Blackberry virus Y

BVY was identified in plants with BYVD symptoms when it was realized that BYVaV caused latent infections in sole infections. Investigations of the presence of additional agents involved in the symptomatology led to the observation of typical potyviral inclusion bodies and elongated particles under the electron microscope (Susaimuthu *et al*., 2007). BVY is the largest member of the family *Potyviridae* sequenced to date, the largest plant RNA virus family (Adams *et al*., 2011). Sequence comparison and phylogenetic analysis showed that BVY belongs to a new genus (*Brambyvirus*) as it shares less than 35% amino acid identity to any other member of the family (Susaimuthu *et al*., 2007). Potyviruses have a genome-linked protein (VPg) attached to the 5' end and a poly-adenosine tail at the 3' end of the genome which is expressed as a single polyprotein. The polyprotein is processed to 11 mature proteins: P1, HC-Pro, P3, P3N-PIPO, 6K1, CI, 6K2, Vpg, NIa-Pro, NIb and CP from the N to the C terminus of the polyprotein (Adams *et al*., 2005). P1 has a significant role in virus replication (Verchot and Carrington, 1995). HC-Pro is a multi-component proteinase involved in genome amplification, polyprotein processing, long distance transport, gene silencing and probably vector transmission (Revers *et al*., 1999; Stenger *et al*., 2006; Urcuqui-Inchima *et al*., 2001; Young *et al*., 2007). The P1 and HC-Pro are proteases with cis-cleavage activity releasing them from the polyprotein (Verchot *et al*., 1991). P3 is also

believed to be involved in virus replication and viral intercellular and intrcellular movement (Urcuqui-Inchima *et al*., 2001; Cui *et al*., 2010) as well host range and symptom development (Hjulsager *et al*., 2006; Suehiro *et al*., 2004). Recently, a small ORF termed PIPO was discovered to overlap with the P3 coding region in all members of the family. P3N-PIPO is thought to be translated by ribosomal frameshifting from the P3 coding region into the PIPO ORF (Chung *et al*., 2008). P3N-PIPO interacts with a host protein and helps in the cell-to-cell movement process of the potyviruses (Vijayapalani *et al*., 2012). 6K1 is one of the smallest proteins encoded by the potyviral genome. There have been no localization studies and no reported functions for the 6K1 protein. However, it was suggested that 6K1 together with P3 may play a role in virus replication and cell-to-cell movement (Hjulsager *et al*., 2006). CI is involved in cell to cell movement, RNA binding and genome amplification (Kadare and Haenni, 1997). 6K2 in potyviruses are believed to anchor the replication complex to ER membrane (Urcuqui-Inchima *et al*., 2001). VPg is required for the initial binding of the RNA and genome amplification. The NIa-Pro is involved in the cleavage of the remaining two-thirds of the polyprotein (Garcia *et al*., 1992a, Garcia *et al*., 1990). NIb is the RdRp and is required for genome replication. This protein is involved in RNA binding activities (Urcuqui-Inchima *et al*., 2001). The potyvirus coat protein in addition to encapsidation is also involved in movement and genome amplification (Urcuqui-Inchima *et al*., 2001).

BVY is the only potyvirus that has an AlkB domain embedded in the P1 coding area. AlkB orthologs are known to be present in prokaryotes, eukaryotes and viruses and are involved in the repair of nucleic acids after alkylation (Aas *et al*., 2003).

As with other blackberry viruses its significance in disease development is the result of its synergistic effects with other viruses. It has been proven that interactions between BYVaV and BVY lead to BYVD (Susaimuthu *et al*., 2008). Both viruses are latent in single infections but in

co-infections, they exhibit severe disease symptoms including plant death. The BVY vector is not yet known; however, phylogenetic analysis suggests that an eriophyid mite is involved in transmission (Susaimuthu *et al*., 2008).

1.5.8 Raspberry bushy dwarf virus

RBDV is known to occur in many *Rubus* species and cultivars including red raspberry, black raspberry, blackberry and blackberry-raspberry hybrid cultivars (Chamberlain *et al*., 2003). RBDV has been reported in blackberry in the United States, New Zealand, Europe and Chile (Jones and Wood, 1979; Matus *et al*., 2008). Infection may lead to leaf chlorosis and causes severe drupelet abortion (Strik and Martin, 2003). The name bushy dwarf is misleading and was adapted because of the symptoms of the plant where the virus was first identified. It is now known that the original raspberry clone was co-infected with RBDV and black raspberry necrosis virus (BRNV) (Jones *et al.,* 1979). RBDV is the only known member of the genus *Idaeovirus* (Jones *et al*., 1998) although during the process of identifying the causal agent of citrus blight Derrick *et al*. (2005) partially sequenced a virus with significant identities to the RNA 2 proteins of RBDV, indicating the possibility of expansion of the genus. The genome of the virus is comprised of two positive sense RNA molecules and is encapsidated in quasi-isometric particles of ~33 nm. RNA1 encodes a putative polymerase protein and RNA 2 encodes the MP and CP.

1.5.9 Black raspberry necrosis virus (BRNV), raspberry leaf mottle virus (RLMV), rubus yellow net virus (RYNV)

BRNV, RLMV and RYNV are the major viruses involved in raspberry mosaic disease (RMD) in North America and Europe (Converse, 1987; Tzanetakis *et al*., 2007). All three viruses are transmitted readily by both the small and large raspberry aphids. The symptoms caused by the

virus complex differ depending on the identity of the viruses present in the plant and the genotype. Black raspberry is known to show severe symptoms whereas red raspberry shows intermediate and blackberry shows the mildest of symptoms. Both wild and cultivated blackberries are infected with the viruses but are generally considered tolerant. The fruit quality and yield of the plant may be reduced even though they do not show any visual symptoms (Stace-Smith, 1987). When the plant is infected with RBDV and one or more RMD viruses there are severe drupelet abortion and/or chlorosis in some cultivars (Martin *et al*., 2013).

BRNV was originally described in 1955 as the causal agent of tip necrosis in infected black raspberry plants (Stace-Smith, 1955), a symptom that was later determined to be caused by RLMV. It is known to be widespread in areas with raspberry growing history (Jones and Wood, 1979). The virus belongs to the family *Secoviridae* (genus unassigned) and has a bipartite RNA genome encapsidated in 30 nm spherical particles. RNA 1 encodes for a polyprotein that is proteolytically processed to five mature proteins involved in replication: a putative protease cofactor (Pro-C), helicase (Hel), viral genome-linked protein (VPg), protease (Pro) and RNA dependent RNA polymerase (RdRp). RNA 2 encodes for polyprotein that is hydrolyzed to three mature proteins: movement protein (MP) and the large and small coat proteins (CPl and CPs, respectively) (Halgren *et al*., 2007).

RLMV is widespread in the UK and the Pacific Northwest (Martin *et al*., 2013). It is latent in many cultivars but some may develop symptoms. The virus cause tip necrosis in black raspberry, a pathognomonic symptom (Jones and McGavin, 1998; Murant, 1974). RLMV is different from BRNV because it is aphid- but not mechanically transmissible.

RYNV belongs to the genus *Badnavirus* in the family *Caulimoviridae*. The particles of RYNV are bacilliform in shaped and measure 80-150 X 25-30 nm. Like banana streak virus and other badnaviruses, the virus can integrate into the plant genome (Geering *et al*., 2001; Ndowora *et al*., 1999). The virus is transmitted by aphids in a semi-persistent manner and by grafting when episomal (Stace-Smith and Jones, 1978). RYNV is reported to infect all red raspberry cultivars tested and most blackberry and hybrid berry cultivars. Most of the infections are latent or can develop very faint vein netting symptoms on leaves (Stace-Smith and Jones, 1987; Jones, 1991; Jones and McGavin, 1998).

1.5.10 Blackberry leaf mottle associated virus

An emaravirus named blackberry leaf mottle associated virus was recently identified to be associated with blackberry yellow vein disease (Martin *et al*., 2013). Emaraviruses have segmented genomes consisting of four or more negative sense RNA and transmitted by eriophyid mites (Amrine *et al*., 1988; Mielke *et al*., 2007; Elbeaino *et al*., 2009; McGavin *et al*., 2010). Their putative virions are double membrane-bound particles and the genus consist of five recognized members including european ash ringspot associated virus (EMARAV), fig mosaic virus (FMV), pigeon pea sterility mosaic virus, rose rosette virus (RRV) and raspberry leaf blotch virus (RLBV) and three recently identified viruses including redbud yellow ringspot virus (RYRSV), wheat mosaic virus (WMV) and BLMaV (Laney, 2010; McGavin *et al* 2012; Hassan *et al*., 2011). BLMaV has four RNAs identified to date. Predicted translation products of these RNAs shared similarities with FMV and RRV.

1.6 Field Virosome - Understanding the virus movement in the field scale

Blackberry production has increased significantly since the 1990s (Clark, 2005; Strik, *et al*., 2007). Production in the U.S. is increasing constantly because of the nutritional value and consumer preference for blackberries. There is production in many states in both the eastern and western US with Oregon, Washington and California accounting for most of the U.S. production for both fresh market and processed fruit. In recent years, demand for fresh fruit has led to the increased cultivation of blackberries in the southern U.S.; primarily North Carolina, Florida, South Carolina and Georgia. As described earlier, viruses affect both blackberry yield and quality and several studies have been conducted to identify viruses that may be involved in the virus complexes that cause disease. *Rubus* species are propagated vegetatively and are subjected to infection by viruses at any point of propagation. As cultivation and nursery production has become more widespread, there has been a significant increase in the number of viruses that infect this crop (Martin *et al*., 2013). These viruses are transmitted by a number of vectors found in nature, from aphids, whiteflies, nematodes, mites to fungi. Most of the viruses are latent as single infection, but still can be widespread and destructive. As several regulatory agencies function on the basis that viruses cause visual symptoms, it is challenging to limit virus diseases. This has led to the movement of the virus infected material both nationally and globally through the propagation pipeline. It is now understood that a combination of two or more viruses are required for the diseases in blackberry and other berry crops (Martin *et al*., 2013).

A plethora of new viruses are being identified since the turn of the century leading to the in-depth study of their biology and epidemiology. Detection tests are potentially unreliable as they are based on the few known isolates. Development of novel technologies and methods for the detection and discovery of numerous viruses has brought a drastic change in the field of virology (Martin *et al*., 2013; Ho and Tzanetakis 2014). One of the best technologies or methods

that helped in the detection and discovery of many viruses is the use of large scale sequencing (LSS) together with the application of bioinformatics analyses. LSS, a sequence neutral tool is able to detect any isolate of a particular virus and also help in the discovery of new viruses (Ho and Tzanetakis, 2014; Parkinsons *et al*., 2012). Pyrosequencing (454 Life Sciences, Brandford, CT) and illumina dye sequencing (Illumina, San Diego, CA) are the popular platforms for LSS (Al Rwahnih *et al*., 2011; Quito- Avila *et al*., 2013; Al Rwahnih *et al*., 2013; Thekke-Veetil *et al*., 2013; Vives *et al*., 2013).

The regional distribution is important for understanding disease epidemics, but also understanding virus distribution at the field level is of paramount importance for disease management. Knowledge of arthropod movement at a seasonal timeframe and major viruses moving within the field can lead to identification of potential vectors and custom-made control approaches for vectors and viruses alike. Efficient measures can be taken to control the vector population as it moves in the field minimizing replication and virus transmission, minimizing the risk for large scale epidemics. (Koenig et al., 1988)

1.7 References

Aas, P.A., Otterlei, M., Falnes, P.Ø., Vågbø, C.B., Skorpen, F., Akbari, M., Sundheim, O., Bjørås, M., Slupphaug, G., Seeberg, E., Krokan H. E., 2003. Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA. Nature 421, 859–863.

Adams, M.J., Antoniw, J.F., Beaudoin, F., 2005. Overview and analysis of the polyprotein cleavage sites in the family Potyviridae. Mol. Plant Pathol. 6, 471–487.

Adams, M.J., Zerbini, F.M., French, R., Rabenstein, F., Stenger, D.C., Valkonen, J.P.T., 2011. Family potyviridae. Virus Taxon. Ninth Rep. Int. Comm. Taxon. Viruses (AMQ King, MJ Adams, EB Carstens EJ Lefkowitz, eds) 1069–1089.

Adkins, S., Richmond, K., German, T.L., 1995. Enzyme activities associated with virions of tomato spotted wilt virus. Tospoviruses Thrips Flor. Veg. Crop. 431 209–218.

Almeida, Á.M.R., Sakai, J., Hanada, K., Oliveira, T.G., Belintani, P., Kitajima, E.W., Souto, E.R., Novaes, T.G. de, Nora, P.S., 2005. Biological and molecular characterization of an isolate of Tobacco streak virus obtained from soybeans in Brazil. Fitopatol. Bras. 30, 366–373.

Al Rwahnih, M., Daubert, S., Urbez-Torres, J.R., Cordero, F., Rowhani, A., 2011. Deep sequencing evidence from single grapevine plants reveals a virome dominated by mycoviruses. Arch. Virol. 156, 397–403.

Al Rwahnih, M., Dave, A., Anderson, M.M., Rowhani, A., Uyemoto, J.K., Sudarshana, M.R., 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. Phytopathology 103, 1069–1076.

Amrine Jr, J.W., Hindal, D.F., Stasny, T.A., Williams, R.L., Coffman, C.C., 1988. Transmission of the rose rosette disease agent to Rosa multiflora by Phyllocoptes fructiphilus (Acari: Eriophyidae). Entomological news 99, 239-252.

Boulila, M., Boscia, D., Di Terlizzi, B., Castellano, M.A., Minafra, A., Savino, V., Martelli, G.P., 1990. Some Properties of a Phloem-Limited Non Mechanically-Transmissible Grapevine Virus. J. Phytopathol. 129, 151–158.

Bucher, E., Sijen, T., de Haan, P., Goldbach, R., Prins, M., 2003. Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. J. Virol. 77, 1329–1336.

Chamberlain, C.J., Kraus, J., Kohnen, P.D., Finn, C.E., Martin, R.R., 2003. First report of Raspberry bushy dwarf virus in Rubus multibracteatus from China. Plant Dis. 87, 603.

Chung, B.Y.-W., Miller, W.A., Atkins, J.F., Firth, A.E., 2008. An overlapping essential gene in the Potyviridae. Proc. Natl. Acad. Sci. 105, 5897–5902.

Clark, J.R., 2005. Changing times for eastern United States blackberries. Horttechnology 15, 491–494.

Clark, J.R., 2007. Toward the perfect blackberry in cultivar development. HortScience 42, 442-442.

Converse, R.H., 1972. Tobacco streak virus in black raspberry. Phytopathology 62, 1001–1004.

Converse, R.H., Lister, R.M., 1969. The occurrence and some properties of Black Raspberry latent virus. Phytopathology 59, 325–333.

Converse, R.H., 1987. Virus diseases of small fruits. Agric. Handbook, USDA. 631.

Cui, X., Wei, T., Chowda-Reddy, R. V., Sun, G., Wang, A., 2010. The Tobacco etch virus P3 protein forms mobile inclusions via the early secretory pathway and traffics along actin microfilaments. Virology 397, 56–63. doi:10.1016/j.virol.2009.11.015.

Dai, J., Gupte, A., Gates, L., Mumper, R.J., 2009. A comprehensive study of anthocyanin-containing extracts from selected blackberry cultivars: extraction methods, stability, anticancer properties and mechanisms. Food Chem. Toxicol. 47, 837–847.

DeAngelis, J.D., Sether, D.M., Rossignol, P.A., 1994. Transmission of impatiens necrotic spot virus in peppermint by western flower thrips (Thysanoptera: Thripidae). J. Econ. Entomol. 87, 197–201.

De Haan, P., Kormelink, R., Resende, R. de O., Van Poelwijk, F., Peters, D., Goldbach, R., 1991. Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. J Gen Virol 72, 2207–2216.

De Haan, P., Wagemakers, L., Peters, D., Goldbach, R., 1990. The S RNA segment of tomato spotted wilt virus has an ambisense character. J. Gen. Virol. 71, 1001–1007.

Derrick, K., Beretta, M., Barthe, G., 2005. Detection of an Idaeovirus in citrus by subtraction hybridization. Phytopathology 95.

Dolja, V. V, Kreuze, J.F., Valkonen, J.P.T., 2006. Comparative and functional genomics of closteroviruses. Virus Res. 117, 38–51.

Dreher, T.W., Edwards, M.C., Gibbs, A.J., Haenni, A.L., Hammond, R.W., Jupin, I., Koenig, R., Sabanadzovic, S., Abou Ghanem-Sabanadzovic, N., Martelli, G.P., 2005. Family Tymoviridae (Book Chapter In: Virus Taxonomy: Viith Report of the International Committee on Taxonomy of viruses.) Academic Press, San Diego, 969–973.

Duffus, J.E., Johnstone, G.R., 1981. Beet pseudo-yellows virus in tasmania - the first report of a whitefly transmitted virus in Australasia. Australas. Plant Pathol. 10, 68–69.

Duffus, J.E., 1965. Beet pseudo-yellows virus, transmitted by the greenhouse whitefly (Trialeurodes vaporariorum). Phytopathology 55, 450–453.

Edwards M.C., 2000. Genus Marafivirus. Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, 969–973.

Elbeaino, T., Digiaro, M., Alabdullah, A., De Stradis, A., Minafra, A., Mielke, N., Castellano, M.A., Martelli, G.P., 2009. A multipartite single-stranded negative-sense RNA virus is the putative agent of fig mosaic disease. J. Gen. Virol. 90, 1281–1288.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., 2005. Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses. Academic Press.

Frazier, N.W., 1966. Non-retention of two semipersistent strawberry viruses through ecdysis by their aphid vector. Phytopathology 56, 1318-1319.

Frazier, N.W., Jorgensen, P.S., Thomas, H.E., Johnson Jr, H.A., 1962. Necrotic shock--A virus disease of strawberries. Plant Dis. Rep 46, 547–550.

Fulton, R.W., 1948. Hosts of the tobacco streak virus. Phytopathology 38, 421–428.

García, J.A., Laín, S., Cervera, M.T., Riechmann, J.L., Martín, M.T., 1990. Mutational analysis of plum pox potyvirus polyprotein processing by the NIa protease in Escherichia coli. J. Gen. Virol. 71, 2773–2779.

García, J.A., Martín, M.T., Cervera, M.T., Riechmann, J., 1992. Proteolytic processing of the plum pox potyvirus polyprotein by the Nla protease at a novel cleavage site. Virology 188, 697–703.

Garcia, J. A., Riechmann, J. L., Lain, S., 1989. Proteolytic activity of the plum pox potyvirus-nia-like protein in Escherichia coli. Virology 170,362-369.

Geering, A.D.W., Olszewski, N.E., Dahal, G., Thomas, J.E., Lockhart, B.E.L., 2001. Analysis of the distribution and structure of integrated Banana streak virus DNA in a range of Musa cultivars. Mol. Plant Pathol. 2, 207–213.

German, T.L., Ullman, D.E., Moyer, J.W., 1992. Tospoviruses: diagnosis, molecular biology, phylogeny, and vector relationships. Annu. Rev. Phytopathol. 30, 315–348.

Goldbach, R.W., Le Gall, O., Wellink, J., 1991. Alpha-like viruses in plants. Semin. Virol. 2, 19-25.

Guzman-Baeny, T.L., 2004. Incidence, Distribution, and Symptom Description of Viruses in Cultivated Blackberry (Rubus subgenus Eubatus) in the Southestern United States. M.Sc. thesis. North Carolina State University, Raleigh.

Halgren, A., Tzanetakis, I.E., Martin, R.R., 2007. Identification, Characterization, and Detection of Black raspberry necrosis virus. Phytopathology 97, 44–50. doi:10.1094/PHYTO-97-0044.

Harrison, B.D., Robertson, W.M., Taylor, C.E., 1974. Specificity of retention and transmission of viruses by nematodes. J. Nematol. 6, 155-164.

Hassan, M., Keller, K.K., Martin, R.R., Sabanadzovic, S., Tzanetakis, I.E., 2011. Characterization of a novel Emaravirus infecting blackberry, in: Phytopathology. 101, S70.

Hjulsager, C.K., Olsen, B.S., Jensen, D.M.K., Cordea, M.I., Krath, B.N., Johansen, I.E., Lund, O.S., 2006. Multiple determinants in the coding region of Pea seed-borne mosaic virus P3 are involved in virulence against sbm-2 resistance. Virology 355, 52–61.

Ho, T., Tzanetakis, I.E., 2014. Development of a virus detection and discovery pipeline using next generation sequencing. Virology 471, 54–60.

Huang, W., Zhang, H., Liu, W., Li, C., 2012. Survey of antioxidant capacity and phenolic composition of blueberry, blackberry, and strawberry in Nanjing. J. Zhejiang Univ. Sci. B 13, 94–102.

Hummer, K.E., Janick, J., 2006. Rubus iconography: antiquity to the Renaissance. Acta Hortic. 759, 89-106.

Hu, C., Zawistowski, J., Ling, W., Kitts, D.D., 2003. Black rice (Oryza sativa L. indica) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems. J. Agric. Food Chem. 51, 5271–5277.

Iwaki, M., Honda, Y., Hanada, K., Tochihara, H., Yonaha, T., Hokama, K., Yokoyama, T., 1984. Silver mottle disease of watermelon caused by Tomato spotted wilt virus. Plant Dis. 68, 1006–1008.

Jaspars, E.M.J., 1999. Genome activation in alfamo-and ilarviruses. Arch. Virol. 144, 843–863.

Johnson, H.A., Converse, R.H., Amorao, A., Espejo, J.I., Frazier, N.W., 1984. Seed transmission of tobacco streak virus in strawberry. Plant Dis. 68, 390–392.

Johnson, J., 1936. Tobacco streak, a virus disease. Phytopathology 26, 285–291.

Jones, A.T., 1991. Rubus host range of rubus yellow net virus and its involvement with other aphid-borne latent viruses in inducing raspberry veinbanding mosaic disease. Ann. Appl. Biol. 118, 331–338.

Jones, A.T., Mayo, M.A., 1975. Further properties of black raspberry latent virus, and evidence for its relationship to tobacco streak virus. Ann. Appl. Biol. 79, 297–306.

Jones, A.T., McGavin, W.J., 1998. Infectibility and sensitivity of UK raspberry, blackberry and hybrid berry cultivars to Rubus viruses. Ann. Appl. Biol. 132, 239–251.

Jones, A.T., Wood, G.A., 1979. The virus status of raspberries (Rubus idaeus L.) in New Zealand. New Zeal. J. Agric. Res. 22, 173–182.

Jones, A.T., McGavin, W.J., Gepp, V., Zimmerman, M.T., Scott, S.W., 2006. Purification and properties of blackberry chlorotic ringspot, a new virus species in subgroup 1 of the genus Ilarvirus found naturally infecting blackberry in the UK. Ann. Appl. Biol. 149, 125–135.

Jones, A.T., McGavin, W.J., Mayo, M.A., Graham, J., 1998. Natural infection with raspberry bushy dwarf virus (RBDV) of the putatively RBDV-resistant red raspberry cultivar Glen Moy, and the demonstration that it does not contain the RBDV resistance gene, Bu. Ann. Appl. Biol. 133, 403–414.

Jones, A.T., 1979. The effects of black raspberry necrosis and raspberry bushy dwarf viruses in Lloyd George raspberry and their involvement in raspberry bushy dwarf disease. J. Hortic. Sci. 54, 267–272.

Kadaré, G., Haenni, A.L., 1997. Virus-encoded RNA helicases. J. Virol. 71, 2583-2590.

Kaiser, W.J., Wyatt, S.D., Pesho, G.R., 1982. Natural hosts and vectors of tobacco streak virus in eastern Washington. Phytopathology 72, 1508–1512.

Karasev, A. V, 2000. Genetic diversity and evolution of closteroviruses. Annu. Rev. Phytopathol. 38, 293–324.

Kikkert, M., Verschoor, A.D., Kormelink, R., Rottier, P., Goldbach, R., 2001. Tomato spotted wilt virus glycoproteins exhibit trafficking and localization signals that are functional in mammalian cells. J. Virol. 75, 1004–1012.

King A.M., Lefkowitz E., Adams M.J., Carstens E.B., 2012. Virus taxonomy: classification and nomenclature of viruses. Ninth report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego, CA.

Koenig, R., Hirth, L., and Givord, L. 1988. Tymoviruses. Pages 163-212 in: The Plant Viruses, Springer US.

Kormelink, R., de Haan, P., Meurs, C., Peters, D., Goldbach, R., 1992. The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments. J. Gen. Virol. 73, 2795.

Laney, A.G., Keller, K.E., Martin, R.R., Tzanetakis, I.E., 2011. A discovery 70 years in the making: characterization of the Rose rosette virus. J. Gen. Virol. 92, 1727–1732.

Lewandowski, D.J., Adkins, S., 2005. The tubule-forming NSm protein from Tomato spotted wilt virus complements cell-to-cell and long-distance movement of Tobacco mosaic virus hybrids. Virology 342, 26–37.

Li, W., Lewandowski, D.J., Hilf, M.E., Adkins, S., 2009. Identification of domains of the Tomato spotted wilt virus NSm protein involved in tubule formation, movement and symptomatology. Virology 390, 110–121.

Li, L., Yang, H., 2011. First Report of Strawberry necrotic shock virus in China. Plant Dis. 95, 1198.

Martelli, G.P., Candresse, T., 2010. Closteroviridae. In: Encyclopedia of Life Sciences (ELS). John Wiley & Sons Ltd., Chichester 1–9.

Martelli, G.P., Agranovsky, A.A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R.H.A., Dolja, V.V., Hu, J.S., Jelkmann, W., Karasev, A.V., Martin, R.R., Minafra, A., Namba, S., Vetten, H.J., 2012b. Family Closteroviridae. In: King, A., Adams, M.J., Carstens, E.B., Lefkowitz, E. (Eds.), Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier-Academic Press, San Diego, pp. 987–1001.

Martelli, G.P., Abou Ghanem-Sabanadzovic, N., Agranovsky, A.A., Al Rwahnih, M., Dolja, V. V, Dovas, C.I., Fuchs, M., Gugerli, P., Hu, J.S., Jelkmann, W., others, 2012. Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated members of the genus Ampelovirus and the putative species unassigned to the family. J. Plant Pathol. 94, 7–19.

Martelli, G.P., Agranovsky, A.A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R.H.A., Dolja, V. V, Falk, B.W., Gonsalves, D., Jelkmann, W., others, 2002. The family Closteroviridae revised. Arch. Virol. 147, 2039–2044.

Martin, R.R., MacFarlane, S., Sabanadzovic, S., Quito, D., Poudel, B., Tzanetakis, I.E., 2013. Viruses and virus diseases of Rubus. Plant Dis. 97, 168–182.

Martin, R.R., Tzanetakis, I.E., Gergerich, R., Fernandez, G., Pesic, Z., 2004. Blackberry yellow vein associated virus: A new crinivirus found in blackberry, in: X International Symposium on Small Fruit Virus Diseases 656. pp. 137–142.

Matus, J. T., Medina, C., Arce-Johnson, P., 2008. Virus incidence in raspberries, blackberries and red currant commercial plantings of central and south Chile. Acta Hortic. 777, 361-366.

McGavin, W.J., MacFarlane, S.A., 2010. Sequence similarities between Raspberry leaf mottle virus, Raspberry leaf spot virus and the closterovirus Raspberry mottle virus. Ann. Appl. Biol. 156, 439–448.

McGavin, W.J., Mitchell, C., Cock, P.J.A., Wright, K.M., MacFarlane, S.A., 2012. Raspberry leaf blotch virus, a putative new member of the genus Emaravirus, encodes a novel genomic RNA. J. Gen. Virol. 93, 430–437.

Mielke, N., Muehlbach, H.-P., 2007. A novel, multipartite, negative-strand RNA virus is associated with the ringspot disease of European mountain ash (Sorbus aucuparia L.). J. Gen. Virol. 88, 1337–1346.

Murant, A.F., 1974. Viruses affecting raspberry in Scotland. Bull. Scottish Hortic. Res. Inst. Assoc.

Naidu, R.A., Deom, C.M., Sherwood, J.L., 2001. First report of Frankliniella fusca as a vector of Impatiens necrotic spot tospovirus. Plant Dis. 85, 1211.

Ndowora, T., Dahal, G., LaFleur, D., Harper, G., Hull, R., Olszewski, N.E., Lockhart, B., 1999. Evidence That Badnavirus Infection inMusaCan Originate from Integrated Pararetroviral Sequences. Virology 255, 214–220.

Neeleman, L., Linthorst, H.J.M., Bol, J.F., 2004. Efficient translation of alfamovirus RNAs requires the binding of coat protein dimers to the 3' termini of the viral RNAs. J. Gen. Virol. 85, 231–240.

Nichenametla, S.N., Taruscio, T.G., Barney, D.L., Exon, J.H., 2006. A review of the effects and mechanisms of polyphenolics in cancer. Crit. Rev. Food Sci. Nutr. 46, 161–183.

Pappu, H.R., Jones, R.A.C., Jain, R.K., 2009. Global status of tospovirus epidemics in diverse cropping systems: successes achieved and challenges ahead. Virus Res. 141, 219–236.

Parkinson, N.J., Maslau, S., Ferneyhough, B., Zhang, G., Gregory, L., Buck, D., Ragoussis, J., Ponting, C.P., Fischer, M.D., 2012. Preparation of high-quality next-generation sequencing libraries from picogram quantities of target DNA. Genome Res. 22, 125–133.

Poling, E.B., 1997. Blackberries. J. Small Fruit Vitic. 4, 33–69.

Poudel, B., Wintermantel, W.M., Cortez, A.A., Ho, T., Khadgi, A., Tzanetakis, I.E., 2013. Epidemiology of Blackberry yellow vein assosciated virus. Plant Dis. 97, 1641–1644. doi:10.3201/eid1510.090115.

Poudel, B., Ho, T., Laney, A., Khadgi, A., Tzanetakis, I.E., 2014. Epidemiology of Blackberry chlorotic ringspot virus. Plant Dis. 98, 547–550.

Pittman, H.A., 1927. Spotted wilt of tomatoes. Prelim. note Concern. Transm. spotted wilt tomatoes by an insect vector (Thrips tabaci Lind.). Aust. Counc. Sci. Ind. Res. Bull. 1, 74–77.

Quito-Avila, D.F., Brannen, P.M., Cline, W.O., Harmon, P.F., Martin, R.R., 2013. Genetic characterization of Blueberry necrotic ring blotch virus, a novel RNA virus with unique genetic features. J. Gen. Virol. 94, 1426–1434.

Renaud, S. de, de Lorgeril, M., 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339, 1523–1526.

Revers, F., Le Gall, O., Candresse, T., Maule, A.J., 1999. New Advances in Understanding the Molecular Biology of Plant/Potyvirus Interactions. Mol. Plant-Microbe Interact. 12, 367–376. doi:10.1094/MPMI.1999.12.5.367.

Ribeiro, D., Borst, J.W., Goldbach, R., Kormelink, R., 2009. Tomato spotted wilt virus nucleocapsid protein interacts with both viral glycoproteins Gn and Gc in planta. Virology 383, 121–130.

Ribeiro, D., Foresti, O., Denecke, J., Wellink, J., Goldbach, R., Kormelink, R.J.M., 2008. Tomato spotted wilt virus glycoproteins induce the formation of endoplasmic reticulum-and Golgi-derived pleomorphic membrane structures in plant cells. J. Gen. Virol. 89, 1811–1818.

Rott, M.E., Gilchrist, A., Lee, L., Rochon, D., 1995. Nucleotide sequence of tomato ringspot virus RNA1. J. Gen. Virol. 76, 465–473.

Rott, M.E., Tremaine, J.H., Rochon, D.M., 1991. Nucleotide sequence of tomato ringspot virus RNA-2. J. Gen. Virol 72.

Rubio, L., Guerri, J., Moreno, P., 2013a. Genetic variability and evolutionary dynamics of viruses of the family Closteroviridae. Front. Microbiol. 4, 1–15. doi:10.3389/fmicb.2013.00151.

Rush, M.C., Gooding, G. V, Ellis, D.E., 1968. Wild *Rubus* spp as natural hosts of Tobacco ringspot virus, Phytopathology. p. 1065.

Sabanadzovic, S., Ghanem-Sabanadzovic, N.A., 2009. Identification and molecular characterization of a marafivirus in Rubus spp. Arch. Virol. 154, 1729–1735.

Sabanadzovic, S., Ghanem-Sabanadzovic, N.A., Tzanetakis, I.E., 2011. Blackberry virus E: an unusual flexivirus. Arch. Virol. 156, 1665–1669.

Sabanadzovic, S., and Abou Ghanem-Sabanadzovic, N. 2009. Identification and molecular characterization of a marafivirus in Rubus spp. Archives of Virology 154:1729-1735.

Sakimura, K., 1969. A comment on the color forms of Frankliniella schultzei (Thysanoptera: Thripidae) in relation to transmission of the tomato spotted wilt virus. Pacific Insects, 11. 761-762.

Sakimura, K., 1963. *Frankliniella fusca*, an additional vector for Tomato spotted wilt virus, with notes on Thrips tabaci, another vector. Phytopathology 53, 412.

Sakimura, K., 1962. The present status of thrips-borne viruses. Biol. Transm. Dis. agents 33–40.

Scott, S.W., Zimmerman, M.T., Ge, X., 2003. Viruses in subgroup 2 of the genus Ilarvirus share both serological relationships and characteristics at the molecular level. Arch. Virol. 148, 2063–2075.

Sdoodee, R., Teakle, D.S., 1987. Transmission of tobacco streak virus by Thrips tabach a new method of plant virus transmission. Plant Pathol. 36, 377–380.

Seeram, N.P., Adams, L.S., Zhang, Y., Lee, R., Sand, D., Scheuller, H.S., Heber, D., 2006. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. J. Agric. Food Chem. 54, 9329–9339.

Sharman, M., Constable, F., Perera, R., Thomas, J.E., 2011. First report of Strawberry necrotic shock virus infecting strawberry (Fragaria vesca) from Australia. Australas. Plant Dis. Notes 6, 54–56.

Shimura, H., Masuta, C., Yoshida, N., Sueda, K., Suzuki, M., 2013. The 2b protein of Asparagus virus 2 functions as an RNA silencing suppressor against systemic silencing to prove functional synteny with related cucumoviruses. Virology 442, 180–188. doi:10.1016/j.virol.2013.04.015

Shiel, P.J., Berger, P.H., 2000. The complete nucleotide sequence of apple mosaic virus (ApMV) RNA 1 and RNA 2 ApMV is more closely related to alfalfa mosaic virus than to other Ilarviruses. J. Gen. Virol. 81, 273–278.

Stace-Smith, R., 1987. Virus and viruslike diseases of Rubus (raspberry and blackberry). Agric. handbook-United States Dep. Agric. Comb. For. Pest Res. Dev. Progr.

Stace-Smith, R., 1955. Studies on rubus virus diseases in British Columbia: II. Black raspberry necrosis. Can. J. Bot. 33, 314–322.

Stace-Smith, R., Ramsdell, D.C., 1987. Nepoviruses of the Americas, in: Current Topics in Vector Research. Springer, pp. 131–166.

Stace-Smith, R., 1985. Tobacco ringspot virus. Commonw. Mycol. Inst./Assoc. Appl. Biol. Kew, Surrey, Engl. Description of Plant Viruses no. 309.

Stace-Smith, R., Jones, A. T., 1978. Rubus yellow net virus. Commonwealth Mycological Institute/Association of Applied Biologists. Description of Plant Viruses 188.

Stenger, D.C., Hein, G.L., French, R., 2006. Nested deletion analysis of Wheat streak mosaic virus HC-Pro: Mapping of domains affecting polyprotein processing and eriophyid mite transmission. Virology 350, 465–474. doi:10.1016/j.virol.2006.02.015

Stenger, D.C., Mullin, R.H., Morris, T.J., 1987. Characterization and detection of the Strawberry necrotic shock isolate of Tobacco streak virus. Phytopathology 77, 1330–1337.

Strik, B., Martin, R.R., 2003. Impact of Raspberry bushy dwarf virus on'Marion'blackberry. Plant Dis. 87, 294–296.

Strik, B., Martin, R.R., 1997. Impact of Raspberry bushy dwarf virus on " Marion " Blackberry. Plant Dis. 87, 1993–1995. doi:10.1094/PDIS.2003.87.3.294.

Strik, B.C., Clark, J.R., Finn, C.E., Bañados, M.P., 2007. Worldwide blackberry production. Horttechnology 17, 205–213.

Strik, B.C., 1992. Blackberry cultivars and production trends in the Pacific Northwest. Fruit Var. J. 46, 202-206.

Suehiro, N., Natsuaki, T., Watanabe, T., Okuda, S., 2004. An important determinant of the ability of Turnip mosaic virus to infect Brassica spp. and/or Raphanus sativus is in its P3 protein. J. Gen. Virol. 85, 2087–2098. doi:10.1099/vir.0.79825-0

Susaimuthu, J., 2006. Identification and characterization of two new viruses associated with blackberry yellow vein disease. ProQuest.

Susaimuthu, J., Gergerich, R.C., Bray, M.M., Clay, K.A., Clark, J.R., Tzanetakis, I.E., Martin, R.R., 2007. Incidence and ecology of Blackberry yellow vein associated virus. Plant Dis. 91, 809–813.

Susaimuthu, J., Tzanetakis, I.E., Gergerich, R.C., Kim, K.S., Martin, R.R., 2008a. Viral interactions lead to decline of blackberry plants. Plant Dis. 92, 1288–1292.

Susaimuthu, J., Tzanetakis, I.E., Gergerich, R.C., Martin, R.R., 2008b. A member of a new genus in the Potyviridae infects Rubus. Virus Res. 131, 145–151.

Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S., Okuno, T., 2002. Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. Febs Lett. 532, 75–79.

Thekke-Veetil, T., Aboughanem-Sabanadzovic, N., Keller, K.E., Martin, R.R., Sabanadzovic, S., Tzanetakis, I.E., 2013. Molecular characterization and population structure of Blackberry vein banding associated virus, new ampelovirus associated with yellow vein disease. Virus Res. 178, 234–240.

Tsompana, M., Moyer, J. W., 2008. Encylopedia of Virology (3rd edition). Mahy, B. W. J. and Regenmortel M. van. (eds). Elsevier Academic Press. London. 157-163.

Tzanetakis, I.E., Postman, J.D., Martin, R.R., 2007. First report of Blackberry chlorotic ringspot virus in Rubus sp. in the United States. Plant Dis. 91, 463.

Tzanetakis, I.E., Guzmán-Baeny, T.L., VanEsbroeck, Z.P., Fernandez, G.E., Martin, R.R., 2009. First Report of Impatiens necrotic spot virus in Blackberry in the Southeastern United States. Plant Dis. 93, 432.

Tzanetakis, I.E., Mackey, I.C., Martin, R.R., 2004. Strawberry necrotic shock virus is a distinct virus and not a strain of Tobacco streak virus. Arch. Virol. 149, 2001–2011.

Tzanetakis, I.E., Martin, R.R., 2005. New features in the genus Ilarvirus revealed by the nucleotide sequence of Fragaria chiloensis latent virus. Virus Res. 112, 32–37.

Tzanetakis, I.E., Martin, R.R., 2004. Complete nucleotide sequence of a strawberry isolate of Beet pseudoyellows virus. Virus Genes 28, 239–246.

Tzanetakis, I.E., Martin, R.R., Scott, S.W., 2010. Genomic sequences of blackberry chlorotic ringspot virus and strawberry necrotic shock virus and the phylogeny of viruses in subgroup 1 of the genus Ilarvirus. Arch. Virol. 155, 557–561.

Tzanetakis, I.E., Martin, R.R., Wintermantel, W.M., 2013. Epidemiology of criniviruses: an emerging problem in world agriculture. Front. Microbiol. 4.

Tzanetakis, I.E., Susaimuthu, J., Gergerich, R.C., Martin, R.R., 2006a. Nucleotide sequence of Blackberry yellow vein associated virus, a novel member of the Closteroviridae. Virus Res. 116, 196–200.

Tzanetakis, I.E., Wintermantel, W.M., Cortez, A.A., Barnes, J.E., Barrett, S.M., Bolda, M.P., Martin, R.R., 2006b. Epidemiology of Strawberry pallidosis-associated virus and occurrence of pallidosis disease in North America. Plant Dis. 90, 1343–1346.

Tzanetakis, I.E., Wintermantel, W.M., Martin, R.R., 2003. First report of Beet pseudo yellows virus in strawberry in the United States: A second crinivirus able to cause pallidosis disease. Plant Dis. 87, 1398.

Ullman, D.E., 1996. Thrips and Tospoviruses□:Advances and Future Directions.

Ullman, D.E., Cho, J.J., Mau, R.F.L., Hunter, W.B., Westcot, D.M., Custer, D.M., 1992. Thrips-tomato spotted wilt virus interactions: morphological, behavioral and cellular components influencing thrips transmission, in: Advances in Disease Vector Research. Springer, pp. 195–240.

Ullman, D.E., German, T.L., Sherwood, J.L., Westcot, D.M., 1995a. Thrips transmission of tospoviruses: future possibilities for management, in: Thrips Biology and Management. Springer, pp. 135–151.

Ullman, D.E., Westcot, D.M., Chenault, K.D., Sherwood, J.L., German, T.L., Bandla, M.D., Cantone, F.A., Duer, H.L., 1995b. Compartmentalization, intracellular transport, and autophagy of tomato spotted wilt tospovirus proteins in infected thrips cells. Phytopathology 85, 644–654.

Urcuqui-Inchima, S., Haenni, A.-L., Bernardi, F., 2001. Potyvirus proteins: a wealth of functions. Virus Res. 74, 157–175.

Van Knippenberg, I., Goldbach, R., Kormelink, R., 2002. Purified Tomato spotted wilt virus particles support both genome replication and transcription in vitro. Virology 303, 278–286.

Verchot, J., Carrington, J.C., 1995. Evidence that the potyvirus P1 proteinase functions in trans as an accessory factor for genome amplification. J. Virol. 69, 3668–3674.

Verchot, J., Koonin, E. V, Carrington, J.C., 1991. The 35-kDa protein from the N-terminus of the potyviral polyprotein functions as a third virus-encoded proteinase. Virology 185, 527–535.

Vijayapalani, P., Maeshima, M., Nagasaki-Takekuchi, N., Miller, W.A., 2012. Interaction of the trans-frame potyvirus protein P3N-PIPO with host protein PCaP1 facilitates potyvirus movement. PLoS Pathog. 8. doi:10.1371/journal.ppat.1002639.

Vives, M.C., Velázquez, K., Pina, J.A., Moreno, P., Guerri, J., Navarro, L., 2013. Identification of a new enamovirus associated with citrus vein enation disease by deep sequencing of small RNAs. Phytopathology 103, 1077–1086.

Wang, S.Y., Lin, H.-S., 2000. Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. J. Agric. Food Chem. 48, 140–146.

Whitfield, A.E., Ullman, D.E., German, T.L., 2005. Tospovirus-thrips interactions. Annu. Rev. Phytopathol. 43, 459–489.

Wisler, G.C., Duffus, J.E., 2001. Transmission properties of whitefly-borne criniviruses and their impact on virus epidemiology. Virus-Insect-Plant Interact. KF Harris, OP Smith, JE Duffus, eds. Acad. Press. San Diego, CA 293–308.

Wisler, G.C., Li, R.H., Liu, H.-Y., Lowry, D.S., Duffus, J.E., 1998. Tomato chlorosis virus: a new whitefly-transmitted, phloem-limited, bipartite closterovirus of tomato. Phytopathology 88, 402–409.

Xin, H.-W., Ji, L.-H., Scott, S.W., Symons, R.H., Ding, S.-W., 1998. Ilarviruses encode a cucumovirus-like 2b gene that is absent in other genera within the Bromoviridae. J. Virol. 72, 6956–6959.

Young, B.A., Hein, G.L., French, R., Stenger, D.C., 2007. Substitution of conserved cysteine residues in wheat streak mosaic virus HC-Pro abolishes virus transmission by the wheat curl mite. Arch. Virol. 152, 2107–2111.

Chapter II

Blackberry virus distribution in the Southern United States

2.1 Abstract

Blackberry production has increased dramatically around the world in recent years. With the increase in acreage, there has been an emergence of several new diseases including blackberry yellow vein disease (BYVD), a disorder caused by virus complexes. This chapter focuses on the occurrence of viruses known to be associated with the disease the southern United States as well as other viruses whose prevalence has not been studied yet. Wild, cultivated and sentinel blackberries, grown in fields with high BYVD incidence, were collected from different states and tested by RT-PCR. Viruses previously known to be associated with BYVD were found to be more prevalent in the Southern United States compared to other viruses tested. This chapter provides an understanding of the virus flow in nature, knowledge which could be used for the development of virus management strategies.

2.2 Introduction

Blackberry, also known as bramble or caneberry is a highly nourishing fruit that has been consumed from ancient times. Starting in the late 1800s, commercial cultivars were developed. In the past 70-80 years, development of improved cultivars has moved blackberry to commercial production leading to dramatic expansion in production in the past 15-20 years (Strik *et al*., 2007). Traditionally, pests and diseases did not have a major impact on this crop. However, with production expansion and climate change, an increase in pest and disease incidence has been reported. There have been several studies on the impact of viruses on raspberry production (Converse, 1987; Jennings *et al*., 1992; Quito-Avila *et al*., 2014); however such studies are lacking for blackberry.

Viruses have a major impact on blackberry production, affecting both yield and vigor. Viruses may be introduced at any point during cultivar development, propagation and fruit production. In the past decade, there has been significant progress in the molecular characterization of many viruses that infect *Rubus spp*. There are now over 40 viruses known to affect the crop with reverse transcription-polymerase chain reaction (RT-PCR) being the most widely used method for the detection.

The main objective of this research is to understand the distribution of major blackberry viruses in the southern United States. In recent years acreage has increased dramatically with expansion in areas where the crop was never grown before. This has led to the emergence of several new diseases including Blackberry yellow vein disease (BYVD). The disease became more prominent at the turn of the century in the Carolinas. Since then, BYVD has become a serious threat to blackberry production (Martin *et al*., 2004; Tzanetakis *et al*., 2007; Martin *et al*.,

2013). Disease symptoms include vein yellowing of primocane leaves with new leaves usually being asymptomatic (Susaimuthu *et al*., 2007). Other symptoms include irregular chlorosis and line patterns, oak-leaf patterns (Susaimuthu, 2006). The most severe effect of BYVD is the decline in the productivity leading to the need to replant every 5-7 years compared to sustained production for at least 20 years historically.

BYVD is caused by virus complexes with blackberry yellow vein associated virus (BYVaV) being the most prominent virus (Poudel *et al*., 2013). BYVaV is latent in single infection and symptoms develop only when the virus is found in mixed infections with other viruses (Susaimuthu *et al*., 2008a). Several other viruses have been discovered in BYVD-infected plants, including beet pseudo-yellows virus (BPYV) (Tzanetakis *et al*., 2004), blackberry chlorotic ringspot virus (BCRV) (Tzanetakis *et al*., 2007), blackberry virus Y (BVY) (Susaimuthu *et al*., 2008b), impatiens necrotic spot virus (INSV) (Tzanetakis *et al*., 2009), blackberry virus S (BIVS) (Sabanadzovic and Ghanem-Sabanadzovic, 2009), tobacco ringspot virus (TRSV) (Stace-Smith *et al*., 1987), blackberry virus E (BVE) (Sabanadzovic *et al*., 2011) and blackberry vein banding associated virus (BVBaV) (Thekke-Veetil *et al*., 2013).

Given the economic importance of BYVD and its distribution over a wide area, the research presented here targets the viruses known to be associated with the disease. The goal of the study is to determine their incidence not only in wild and cultivated blackberries but also in sentinel plants used to determine virus movement in areas with high disease incidence. Potted sentinel plants were replaced monthly during the blackberry growing season (April-September) along with a yellow sticky insect trap to allow evaluation of the seasonal movement of the viruses examined. After removal from the field plants were maintained in an insect-free greenhouse. After overwintering, plants were tested for several viruses. In addition to the survey on viruses

associated with BYVD, this research also targets viruses whose prevalence in the southern United States is still unknown.

The sentinel plant could assist in the identification of virus vectors based on their prevalence in the field during the time of infection. The ultimate goal is to identify virus vectors which in turn will provide the important information on controlling vectors, viruses and eventually disease.

2.3 Materials and methods

2.3.1 Sample Collection

Fully expanded but relatively young leaves from Arkansas, Illinois, Florida, Georgia, North Carolina, South Carolina and West Virginia were collected from cultivated and wild blackberries between 2008 and 2012. Sentinel plants were placed in fields with high disease incidence in Arkansas and North Carolina between 2010 and 2012. The sentinel plants were set as follows: 24 or 30 plants were placed in the field in Arkansas and North Carolina respectively and were rotated with a new set of plants every month for a total of 144 (AR) or 180 (NC) plants per field season. The first set of plants were labeled as AR 1-24 or NC 1-30, the second as AR 25-48 or NC 31-60 and so on (Table. 2.1).

2.3.2 Total nucleic acid isolation

Total nucleic acid isolations were performed as described by Poudel *et al*., 2013. Briefly, leaf tissue was homogenized in 1 ml of extraction buffer (200 mM Tris-HCL, pH 8.5, 300 mM lithium chloride, 1.5% lithium dodecylsulphate, 10 nM ethylene diamine tetra-acetic acid (EDTA), 1% sodium deoxycholate, 1%NP-40 and 1% 14M β-mercaptoethanol solution (vol/vol) (added right

before use). Six hundred microliters (600 µl) of 5.8 M potassium acetate (3.8 M potassium, 5.8 M acetate) was added to 600 µl of supernatant collected from the homogenized tissue. The tubes were mixed well and subjected to centrifugation at 20,000 g for 10 min. Seven hundred and fifty (750) µl of the supernatant was collected and mixed with an equal volume of 100% isopropanol. Tubes were chilled at -20^{o}C for at least 30 min and centrifuged for 20 min. The pellet was resuspended in 500 µl wash buffer (10 mM Tris-HCL, pH 7.5, 0.5 mM EDTA, 50 mM NaCl, 50% ethanol). Twenty (20) µl of silica/glass milk was added to the tube, mixed and pulse centrifuged for 10 sec at 10,000 g. The pellet was washed again with 500 µl wash buffer to eliminate inhibitors and centrifuged for 2 min at 20,000 g. The pellet was dried in a speedvac (Thermo Fisher Scientific) and suspended in 150 µl TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0). The tubes were left at room temperature for 5 min and centrifuged at 20,000 g for 2 min. One hundred (100) µl supernatant was transferred to a new tube without touching the silica (which binds proteins and inhibits downstream reactions) and stored at -80^{o}C till further use.

2.3.3 Reverse transcription

Reverse transcription was performed using 5 µl of total nucleic acids as template. The reaction was primed with 0.5 µl of 0.3 µg/µl random hexameric primers, 100nM OligodT and 10nM Crini-end and consisted of 80 units of SuperScript® III Reverse Transcriptase (Invitrogen), 8 units of RiboLock RNase Inhibitor (Invitrogen), 0.4 mM DNTPs, 5X reverse transcriptase buffer (250 mM Tris-HCL, pH 8.3 at 25^{o}C, 375 mM KCL, 15 mM $MgCl_2$, 50 mM DTT) and water to 50µl. The reaction was incubated at room temperature for 5 min and then 85min at 50^{o}C followed by denaturation for 5 min at 85^{o}C to inactivate the enzyme. The cDNA produced was diluted 1:4 in water to reduce potential problem with PCR inhibitors.

2.3.4 Virus detection by polymerase chain reaction

Amplification of NADH dehydrogenase gene (internal control) was carried out prior to virus detection to evaluate nucleic acid quality (Tzanetakis *et al*., 2007). List of all the viruses tested is given in Table 2.2. The PCR reaction was carried out using previously diluted 2.5 µl cDNA, 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 µM primers, 0.2 mM DNTPs, 1.25 units of *Taq* Polymerase (Genescript) and water to 25µl. Oligonucleotide primers used in the detection are listed in Table 2.3. The PCR program differed based on the virus specific primers used. The overall program consisted of initial denaturation at 94^{o}C for 3 min, followed by denaturation at 94^{o}C for 30-45 sec, annealing at 50-55^{o}C for 15-35 sec and extension of 72^{o}C for 30 sec, repeated for 35-40 cycles and a final extension of 72^{o}C for 10 min. Five µl of the PCR product was mixed with 2 µl of the loading dye and subjected to gel electrophoresis in a 1.5% TBE- agarose gel and visualized after staining for 20 min with GelRed® (Biotium).

Table 2.1 List of samples used for study

Wild Blackberries	**State**	**Year**	**Number**
	AR	2010	67
	WV	2010	9
	IL	2010	7
Total			83
Cultivated Blackberries	State	Year	Number
	AR	2008	37
	NC	2008	9
	GA	2008	26
Total			72
Cultivated Blackberries	State	Year	Number
	NC	2009	37
	SC	2009	10
Total			47
Cultivated Blackberries	State	Year	Number
	GA	2011	19
	FL	2011	26
Total			45
Sentinel Blackberries	State	Year	Number

Table 2.1 List of samples used for study (Cont.)

	NC	2010	158 (22 missing/dead)
	NC	2011	160(20 missing/dead)
Sentinel Blackberries	NC	2012	112(12 missing/dead)
Total			430
Sentinel Blackberries	State	Year	Number
	AR	2010	150
	AR	2011	144
	AR	2012	144
Total			438
Grand Total			1,115

Table 2.2 List of all the viruses detected

S. No.	Virus Name	Acronym	Mode of Transmission	Genus
1	Blackberry yellow vein associated virus	BYVaV	Whitefly	*Criniviurs*
2	Beet pseudo yellows virus	BPYV	Whitefly	*Crinivirus*
3	Blackberry chlorotic ringspot virus	BCRV	Pollen, seed	*Ilarvirus*
4	Tobacco streak virus	TSV	---	*Ilarvirus*
5	Strawberry necrotic shock virus	SNSV	Thrips, pollen, seed	*Ilarvirus*
6	Blackberry virus S	BIVS	---	*Marafivirus*
7	Grapevine syrah virus 1	GSyV-1	---	*Marafivirus*
8	Blackberry virus E	BVE	---	Unassigned
9	Tobacco ringspot virus	TRSV	Nematode, pollen, seed	*Nepovirus*
10	Impatiens necrotic shock virus	INSV	Thrips	*Tospovirus*
11	Blackberry leaf mottle associated virus	BLMaV	---	*Emaravirus*
12	Raspberry bushy dwarf virus	RBDV	Pollen, seed	*Idaeovirus*
13	Blackberry virus Y	BVY	---	*Brambyvirus*
14	Rubus yellow net virus	RYNV	Aphids	*Badnavirus*
15	Raspberry leaf mottle virus	RLMV	Aphids	*Closterovirus*
16	Black raspberry necrosis virus	BRNV	Aphids	Unassigned

Table 2.3 List of oligonucleotide primers used in the detection

S. No.	Primer Name	Sequences
1	NADH-F	5’-GGACTCCTGACGTATACGAAGGATC-3’
	NADH-R	5’-AGTAGATGCTATCACACATACAAT-3’
2	BCRV1836F	5’-ACCTGCTGATCAGCTWTCAGAGAA-3’
	BCRV2237R	5’-TAGAACATCGACCCAAAGGT-3’
3	BYVaVF	5’-TTGAAAGGAAACTTCACGGA-3’
	BYVaVR	5’-TAAGTTCATACGTTTCCTGCG-3’
4	BPYVCPmF	5’-TTCATATTAAGGATGCGCAGA-3’
	BPYVCPmR334	5’-TGAAAGATGTCCRCTAATGATA-3’
5	SNSVCPbegF	5’-GAGTATTTCTGTAGTGAATTCTTGGA-3’
	SNSVCPendR800	5’- ATTATTCTTAATGTGAGGCAACTCG-3’
6	TSV CP F	5’- ACGAGTATTAAGTGGATGAATTCT-3’
	TSV CP R	5’-ACTTACAATACGTCGAGGTGTG-3’
7	MF05-21-R(TRSV)	5’- CAATACGGTAAGTGCACACCCCG -3’
	MF05-22-F(TRSV)	5’- CAGGGGCGTGAGTGGGGGCTC -3’
8	INSV2F	5’-GATCTGTCCTGGGATTGTTC-3’
	INSV2R	5’-GTCTCCTTCTGGTTCTATAATCAT-3’
9	BVY312F	5’- CTGTGGGGAGATTTGGAGAA -3’
	BVY695R	5’- TCATTCCATGGGTGTGTC -3’
10	RYNVFor	5’-CGTGATAACGGCTTGGTTTT-3’

Table 2.3 List of oligonucleotide primers used in the detection (Cont.)

S. No.	Primer Name	Sequences
	RYNVRev-463	5’-CGTAAGCGCAGATTTCTTCC-3’
11	RLMVF	5’- CGAAACTTYTACGGGGAAC -3’
	RLMVR	5’- CCTTTGAAYTCTTTAACATCGT -3’
12	BIVS-CPF	5’-AATGTCACCTCCCAGGTCGG-3’
	BIVS-CPR	5’-ATGCGGCTCACGTCAAGAGG-3’
13	GSyV-1F	5’- CAAGCCATCCGTGCATCTGG-3’
	GSyV-1R	5’- GCCGATTTGGAACCCGATGG -3’
14	BVE-F	5’-CTACCACAACGGACTCCTCC-3’
	BVE-R	5’-GCATGGCGAGCATGTTTC-3’
15	P3-F (BLMaV)	5’-AGTTCCCGATGTTCCTGATAAC-3’
	P3-R (BLMaV)	5’-GCTGGCGATCGTTCAATTTC-3’
16	RBDV-F	5’-TTCATCCTCCAAATCTCAGCAAC-3’
	RBDV-R	5’-CGTCGACGGCACCGCCCACCACA-3’
17	BRNV-F	5’- TAGATGAGTGCGTCCAAGTTTGGTCCAC -3’
	BRNV-R	5’- CCGATACAACGGCCCTCGTCCCAAG -3’

2.4 Results

2.4.1 Virus incidence in cultivated and wild blackberries

Two hundred forty seven blackberry yellow vein disease affected blackberry plants collected from seven states; Arkansas, Georgia, North Carolina, South Carolina, West Virginia, Illinois, Florida passed the internal control test (NADH) and were further assayed for the presence of sixteen viruses using RT-PCR (Table 2.3). Results on the presence of individual virus can be seen in Tables 2.4 to 2.17.

BYVaV was detected in approximately 43% and 54% of cultivated and wild blackberry samples, respectively (Table 2.4). BCRV was detected in approximately 5 and 72% of cultivated and wild blackberry samples, respectively (Table 2.5). BPYV was detected in approximately 5 and 12% of cultivated and wild blackberry samples, respectively (Table 2.6). BVY was detected in approximately 9 and 21% of cultivated and wild blackberry samples, respectively (Table 2.7). BIVS was detected in approximately 5 and 20% of cultivated and wild blackberry samples, respectively (Table 2.8). BVE was detected in approximately 9 and 3% of cultivated and wild blackberry samples, respectively (Table 2.9). BLMaV was detected in approximately 41 and 80% of cultivated and wild blackberry samples, respectively (Table 2.10). INSV was detected in approximately 13 and 18% of cultivated and wild blackberry samples, respectively (Table 2.11). TRSV was detected in approximately 14 and 25% of cultivated and wild blackberry samples, respectively (Table 2.12). SNSV was detected in approximately 15 and 38% of cultivated and wild blackberry samples, respectively (Table 2.13). TSV was not detected in any of the samples from both cultivated and wild blackberry samples congruent with the idea that TSV may not infect *Rubus* (Martin et al., 2013). GSyV-1 was detected in approximately 2 and 6% of cultivated and wild blackberry samples, respectively (Table2.14).

Similarly, all the samples were also tested for other aphid borne viruses like BRNV, RYNV, and RLMV and pollen and seed borne virus RBDV using RT PCR. RYNV was not detected in cultivated and wild blackberry samples. RBDV was detected in approximately 6 and 14% of cultivated and wild blackberry samples, respectively (Table 2.15). RLMV was detected in approximately 1.5 and 2.5% of cultivated and wild blackberry samples, respectively (Table 2.16). BRNV was not detected in cultivated blackberry samples while it was detected in approximately 3% of wild blackberry samples (Table 2.17). RYNV was not detected in any of the samples.

2.4.2 Virus incidence in sentinel blackberries

Sentinel plants were used to evaluate virus movement. A subset of those plants (~24) were randomly selected before placement in the field and subjected to dsRNA extraction (Tzanetakis *et al*., 2004) to determine whether there were any bands present, indicative of virus infection. No plant was found to contain any bands confirming plant quality before planting. Sentinel plants were placed in areas with high disease incidence in Arkansas and North Carolina between 2010 and 2012. Incidence of 16 different viruses was studied in a total of 438 plants from Arkansas and 430 from North Carolina. Figures 2.4.1 to 2.4.13 represents the gel electrophoresis image few samples among all the viruses that were tested positive.

Plants were left in the field one month at a time. However, even during such a short period of time there were several viruses introduced to the plants. Few viruses were present throughout the blackberry growing season whereas others were absent (Table 2.18 to 2.24). Figures 2.4.14 to 2.4.19 show the distribution of viruses in the field both in Arkansas and North Carolina. BYVaV was transmitted in almost all months in sentinel plants from both Arkansas and North Carolina. The incidence of BYVaV peaked in mid-summer (June/July) in Arkansas whereas it was found in

both early and late summer in North Carolina. BCRV is the other major virus that has been found infecting sentinel plants in both the states. Like BYVaV, BCRV was also detected in almost all the months excluding September in sentinel plants from Arkansas and July in sentinel plants from North Carolina. This virus might have been introduced from the arthropods carrying infected pollen from surrounding plants. Other than BYVaV and BCRV, BPYV, BVY, BIVS, SNSV and INSV were also detected in a few plants from both states. Given the small number of infected plants identified, the distribution of these viruses during the season cannot be reliably predicted.

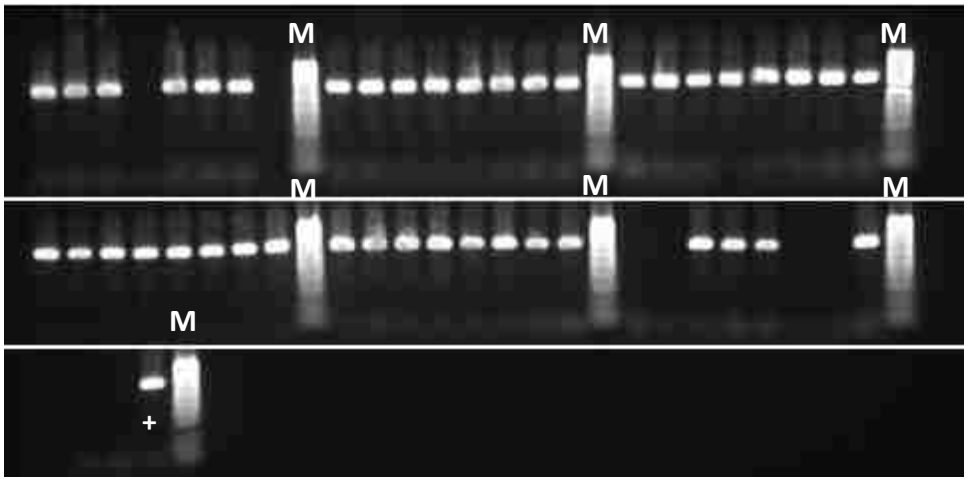

Figure 2.4.1 Agarose gel electrophoresis of PCR confirming the presence of NADH, M: Hyperladder IV molecular weight marker. + indicates positive. Size of PCR product ~ 700bp

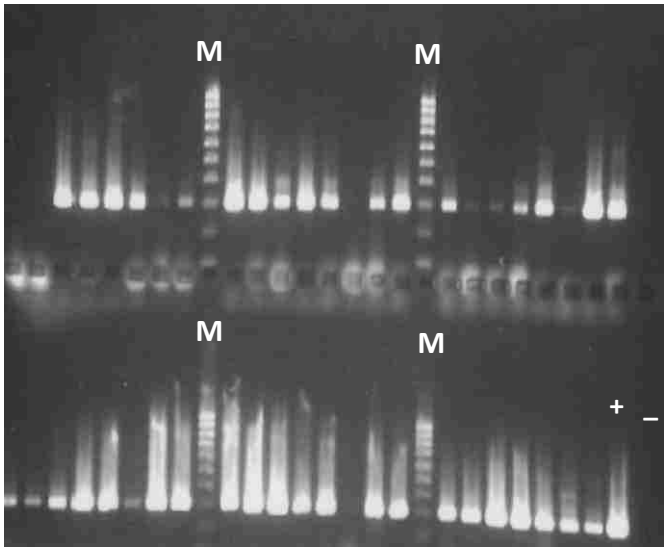

Figure 2.4.2 Agarose gel electrophoresis of PCR confirming the presence of BYVaV, M: Hyperladder IV molecular weight marker. + indicates positive control, - indicates negative control. Size of PCR product ~300

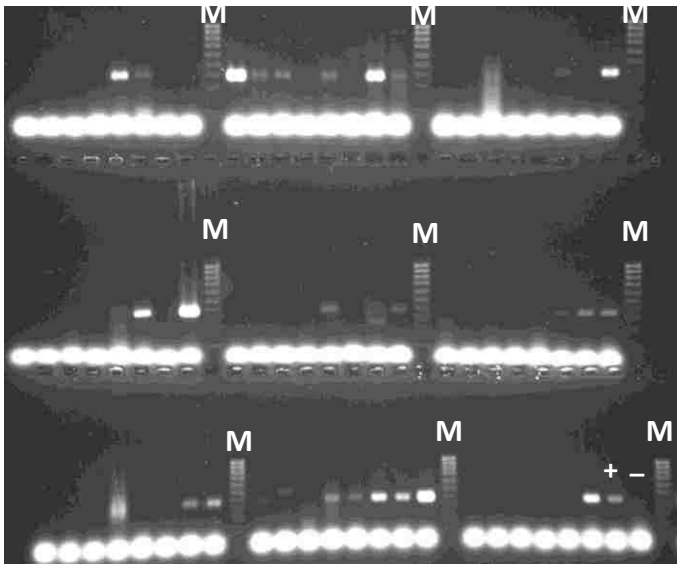

Figure 2.4.3 Agarose gel electrophoresis of PCR confirming the presence of BCRV, M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~ 400 bp.

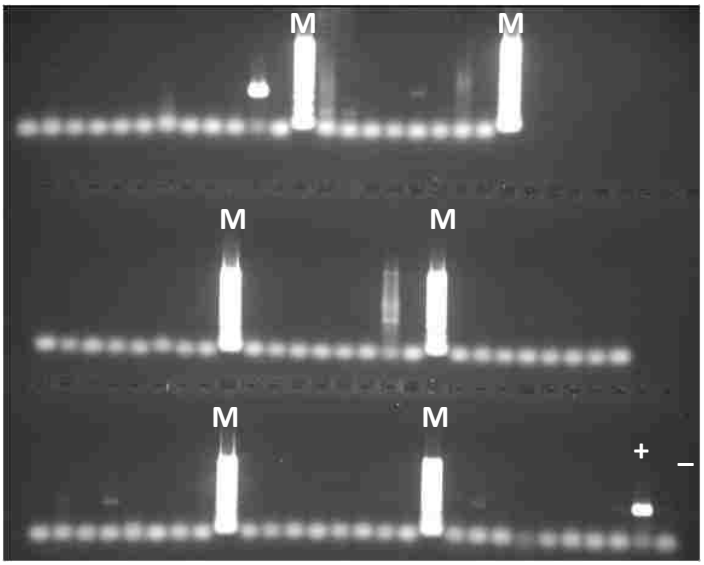

Figure2.4.4 Agarose gel electrophoresis of PCR confirming the presence of BPYV, M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~ 334 bp.

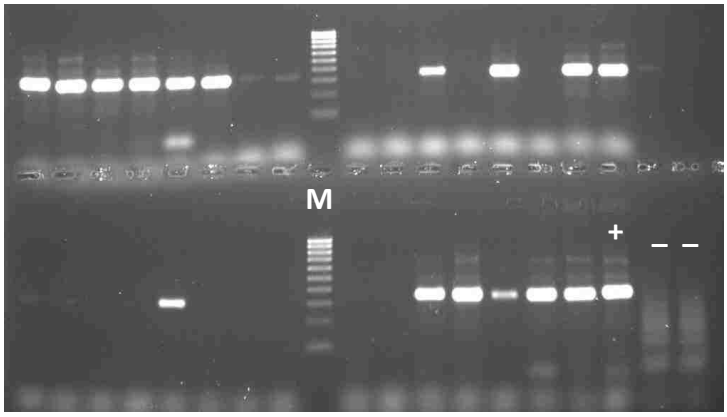

Figure 2.4.5 Agarose gel electrophoresis of PCR confirming the presence of BVY,
M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~ 383 bp.

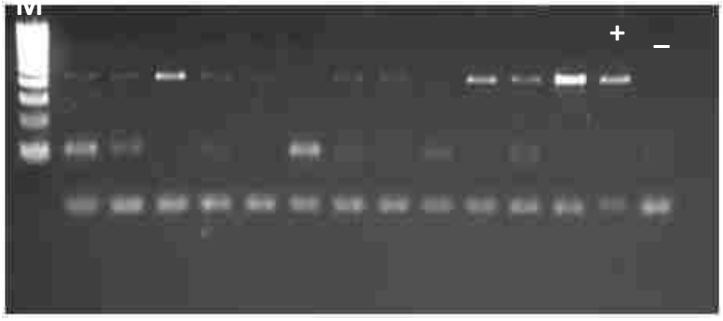

Figure2.4.6 Agarose gel electrophoresis of PCR confirming the presence of INSV,
M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~ 460 bp.

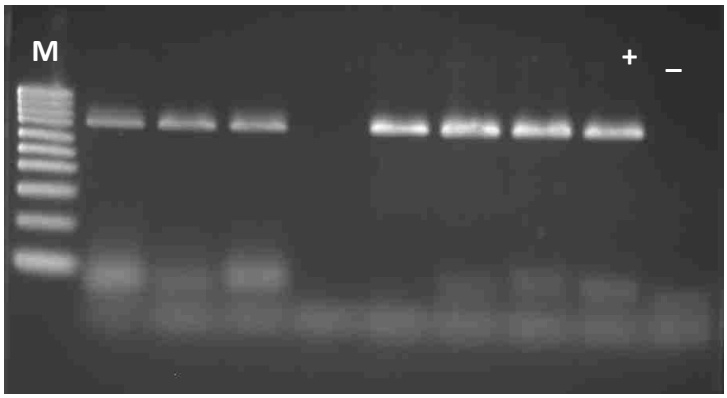

Figure 2.4.7 Agarose gel electrophoresis of PCR confirming the presence of SNSV,

M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~ 824 bp.

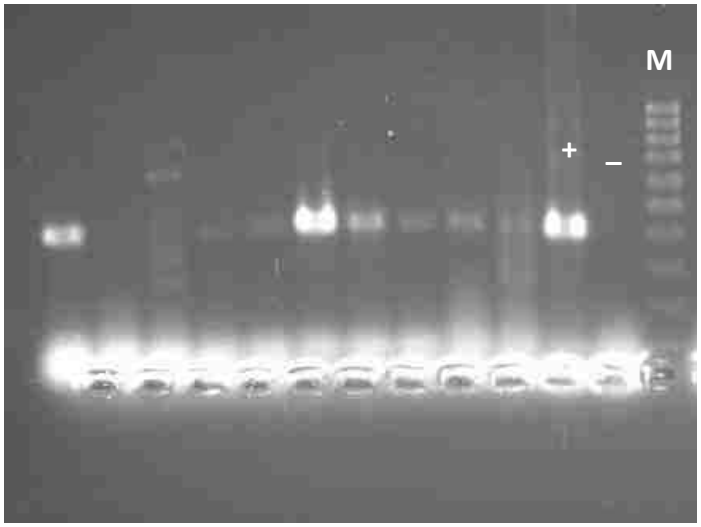

Figure 2.4.8 Agarose gel electrophoresis of PCR confirming the presence of BIVS,
M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~ 434 bp.

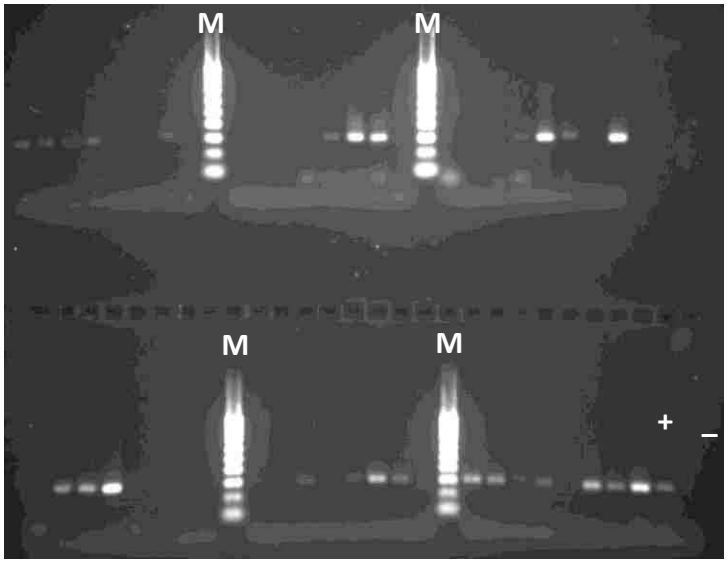

Figure 2.4.9 Agarose gel electrophoresis of PCR confirming the presence of TRSV, M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~ 330 bp.

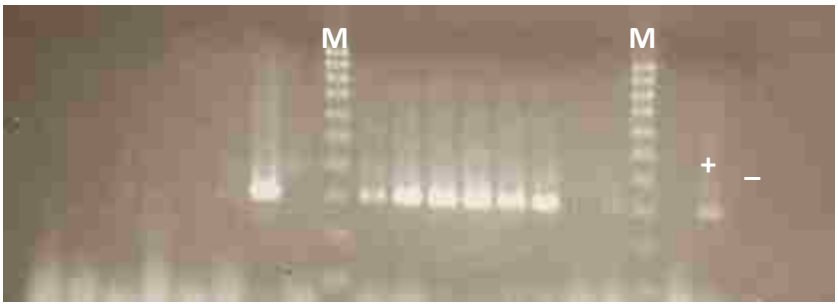

Figure 2.4.10 Agarose gel electrophoresis of PCR confirming the presence of GSyV-1, M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~ 300 bp.

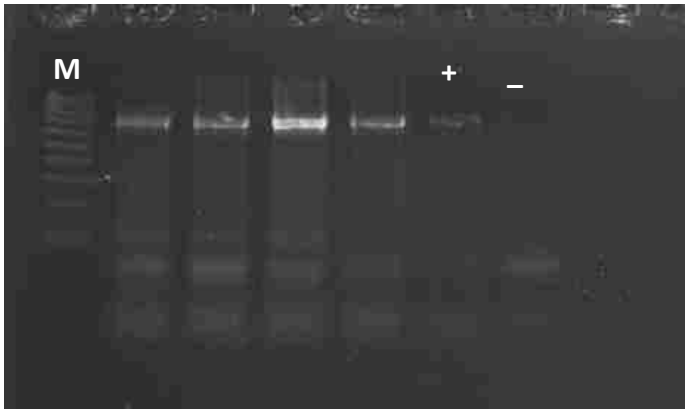

Figure 2.4.11 Agarose gel electrophoresis of PCR confirming the presence of BRNV, M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~ 790 bp.

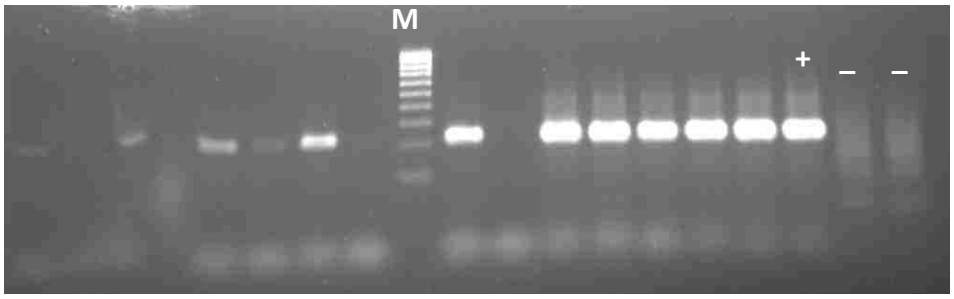

Figure 2.4.12 Agarose gel electrophoresis of PCR confirming the presence of RBDV, M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~ 245 bp.

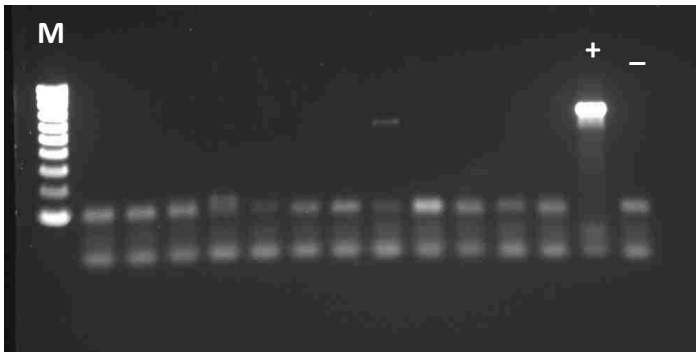

Figure 2.4.13 Agarose gel electrophoresis of PCR confirming the presence of RLMV, M: Hyperladder IV molecular weight marker, + indicates positive control, - indicates negative control. Size of PCR product ~470 bp.

Table 2.4 Geographical incidence of blackberry yellow vein associated virus in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	18/37	N/A	18/37
2008	North Carolina	9/9	N/A	9/9
2008	Georgia	3/26	N/A	3/26
2009	North Carolina	30/37	N/A	30/37
2009	South Carolina	8/10	N/A	8/10
2010	Illinois	N/A	6/7	6/7
2010	West Virginia	N/A	7/9	7/9
2010	Arkansas	N/A	32/67	32/67
2011	Florida	1/26	N/A	1/26
2011	Georgia	0/19	N/A	0/19
Total	**……**	**69/164**	**45/83**	**114/247**

Table 2.5 Geographical incidence of blackberry chlorotic ringspot virus in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	1/37	N/A	1/37
2008	North Carolina	0/9	N/A	0/9
2008	Georgia	0/26	N/A	0/26
2009	North Carolina	2/37	N/A	2/37
2009	South Carolina	1/10	N/A	1/10
2010	Illinois	N/A	7/7	7/7
2010	West Virginia	N/A	6/9	6/9
2010	Arkansas	N/A	47/67	47/67
2011	Florida	1/26	N/A	1/26
2011	Georgia	3/19	N/A	3/19
Total	**……**	**8/164**	**60/83**	**68/247**

Table 2.6 Geographical incidence of beet pseudo-yellows virus in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	3/37	N/A	3/37
2008	North Carolina	0/9	N/A	0/9
2008	Georgia	1/26	N/A	1/26
2009	North Carolina	3/37	N/A	3/37
2009	South Carolina	0/10	N/A	0/10
2010	Illinois	N/A	1/7	1/7
2010	West Virginia	N/A	2/9	2/9
2010	Arkansas	N/A	7/67	7/67
2011	Florida	0/26	N/A	0/26
2011	Georgia	0/19	N/A	0/19
Total	**……**	**7/164**	**10/83**	**17/247**

Table 2.7 Geographical incidence of blackberry virus Y in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	3/37	N/A	3/37
2008	North Carolina	2/9	N/A	2/9
2008	Georgia	2/26	N/A	2/26
2009	North Carolina	4/37	N/A	4/37
2009	South Carolina	2/10	N/A	2/10
2010	Illinois	N/A	1/7	1/7
2010	West Virginia	N/A	1/9	1/9
2010	Arkansas	N/A	16/67	16/67
2011	Florida	1/26	N/A	1/26
2011	Georgia	2/19	N/A	2/19
Total	**……**	**16/164**	**18/83**	**34/247**

Table 2.8 Geographical incidence of blackberry virus S in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	2/37	N/A	2/37
2008	North Carolina	1/9	N/A	1/9
2008	Georgia	1/26	N/A	1/26
2009	North Carolina	2/37	N/A	2/37
2009	South Carolina	2/10	N/A	2/10
2010	Illinois	N/A	0/7	0/7
2010	West Virginia	N/A	0/9	0/9
2010	Arkansas	N/A	17/67	17/67
2011	Florida	1/26	N/A	1/26
2011	Georgia	0/19	N/A	0/19
Total	**……**	**9/164**	**17/83**	**26/247**

Table 2.9 Geographical incidence of blackberry virus E in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	3/37	N/A	3/37
2008	North Carolina	2/9	N/A	2/9
2008	Georgia	1/26	N/A	1/26
2009	North Carolina	0/37	N/A	0/37
2009	South Carolina	1/10	N/A	1/10
2010	Illinois	N/A	0/7	0/7
2010	West Virginia	N/A	0/9	0/9
2010	Arkansas	N/A	2/67	2/67
2011	Florida	0/26	N/A	0/26
2011	Georgia	7/19	N/A	7/19
Total	**……**	**14/164**	**2/83**	**16/247**

Table 2.10 Geographical incidence of blackberry leaf mottle associated virus in plants showing virus-like symptoms.

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	15/37	N/A	15/37
2008	North Carolina	12/9	N/A	12/9
2008	Georgia	5/26	N/A	5/26
2009	North Carolina	27/37	N/A	27/37
2009	South Carolina	0/10	N/A	0/10
2010	Illinois	N/A	0/7	0/7
2010	West Virginia	N/A	0/9	0/9
2010	Arkansas	N/A	67/67	67/67
2011	Florida	2/26	N/A	2/26
2011	Georgia	7/19	N/A	7/19
Total	**……**	**68/164**	**67/83**	**135/247**

Table 2.11 Geographical incidence of impatiens necrotic spot virus in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	5/37	N/A	5/37
2008	North Carolina	2/9	N/A	2/9
2008	Georgia	4/26	N/A	4/26
2009	North Carolina	5/37	N/A	5/37
2009	South Carolina	2/10	N/A	2/10
2010	Illinois	N/A	1/7	1/7
2010	West Virginia	N/A	1/9	1/9
2010	Arkansas	N/A	13/67	13/67
2011	Florida	1/26	N/A	1/26
2011	Georgia	3/19	N/A	3/19
Total	**……**	**22/164**	**15/83**	**37/247**

Table 2.12 Geographical incidence of tobacco ringspot virus in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	0/37	N/A	0/37
2008	North Carolina	9/9	N/A	9/9
2008	Georgia	0/26	N/A	0/26
2009	North Carolina	8/37	N/A	8/37
2009	South Carolina	3/10	N/A	3/10
2010	Illinois	N/A	2/7	2/7
2010	West Virginia	N/A	4/9	4/9
2010	Arkansas	N/A	15/67	15/67
2011	Florida	1/26	N/A	1/26
2011	Georgia	2/19	N/A	2/19
Total	**……**	**23/164**	**21/83**	**44/247**

Table 2.13 Geographical incidence of strawberry necrotic shock virus in plants showing virus like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	0/37	N/A	0/37
2008	North Carolina	0/9	N/A	0/9
2008	Georgia	25/26	N/A	25/26
2009	North Carolina	1/37	N/A	1/37
2009	South Carolina	0/10	N/A	0/10
2010	Illinois	N/A	0/7	0/7
2010	West Virginia	N/A	3/9	3/9
2010	Arkansas	N/A	29/67	29/67
2011	Florida	0/26	N/A	0/26
2011	Georgia	0/19	N/A	0/19
Total	**……**	**26/164**	**32/83**	**58/247**

Table 2.14 Geographical incidence of grapevine syrah virus-1 in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	1/37	N/A	1/37
2008	North Carolina	1/9	N/A	1/9
2008	Georgia	0/26	N/A	0/26
2009	North Carolina	1/37	N/A	1/37
2009	South Carolina	1/10	N/A	1/10
2010	Illinois	N/A	0/7	0/7
2010	West Virginia	N/A	0/9	0/9
2010	Arkansas	N/A	5/67	4/67
2011	Florida	0/26	N/A	0/26
2011	Georgia	0/19	N/A	0/19
Total	**……**	**4/164**	**5/83**	**9/247**

Table 2.15 Geographical incidence of raspberry bushy dwarf virus in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	1/37	N/A	1/37
2008	North Carolina	0/9	N/A	0/9
2008	Georgia	9/26	N/A	9/26
2009	North Carolina	1/37	N/A	1/37
2009	South Carolina	0/10	N/A	0/10
2010	Illinois	N/A	0/7	0/7
2010	West Virginia	N/A	0/9	0/9
2010	Arkansas	N/A	12/67	12/67
2011	Florida	0/26	N/A	0/26
2011	Georgia	0/19	N/A	0/19
Total	**……**	**11/164**	**12/83**	**23/247**

Table 2.16 Geographical incidence of raspberry leaf mottle virus in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	1/37	N/A	1/37
2008	North Carolina	0/9	N/A	0/9
2008	Georgia	0/26	N/A	0/26
2009	North Carolina	1/37	N/A	1/37
2009	South Carolina	0/10	N/A	0/10
2010	Illinois	N/A	0/7	0/7
2010	West Virginia	N/A	0/9	0/9
2010	Arkansas	N/A	2/67	2/67
2011	Florida	0/26	N/A	0/26
2011	Georgia	0/19	N/A	0/19
Total	**……**	**2/164**	**2/83**	**4/247**

Table 2.17 Geographical incidence of black raspberry necrosis virus in plants showing virus-like symptoms

		Positive/Total		
Year	**State**	**Cultivated blackberry**	**Wild blackberry**	**Positive**
2008	Arkansas	0/37	N/A	0/37
2008	North Carolina	0/9	N/A	0/9
2008	Georgia	0/26	N/A	0/26
2009	North Carolina	0/37	N/A	0/37
2009	South Carolina	0/10	N/A	0/10
2010	Illinois	N/A	0/7	0/7
2010	West Virginia	N/A	0/9	0/9
2010	Arkansas	N/A	3/67	3/67
2011	Florida	0/26	N/A	0/26
2011	Georgia	0/19	N/A	0/19
Total	**……**	**0/164**	**3/83**	**3/247**

Table 2.18 Incidence of different viruses in sentinel plants for Arkansas and North Carolina between 2010 and 2012

S.N.	Virus	Arkansas 2010	Arkansas 2011	Arkansas 2012	North Carolina 2010	North Carolina 2011	North Carolina 2012	Total Count
1.	**BYVaV**	3/150	2/144	3/144	3/158	8/160	3/112	**22/868**
2.	**BCRV**	6/150	1/144	2/144	2/158	3/160	1/112	**15/868**
3.	**BPYV**	0/150	2/144	2/144	1/158	0/160	1/112	**6/868**
4.	**BVY**	1/150	1/144	0/144	0/158	0/160	1/112	**3/868**
5.	**BIVS**	3/150	1/144	0/144	0/158	0/160	0/112	**4/868**
6.	**BVE**	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7.	**BLMaV**	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8.	**INSV**	1/150	0/144	0/144	0/158	0/160	0/112	**1/868**
9.	**TRSV**	0/150	0/144	0/144	0/158	0/160	0/112	**0/868**
10.	**SNSV**	0/150	1/144	2/144	1/158	1/154	0/112	**5/868**
11.	**TSV**	0/150	0/144	0/144	0/158	0/160	0/112	**0/868**
12.	**GSyV-1**	0/150	0/144	0/144	0/158	0/160	0/112	**0/868**
13.	**RBDV**	0/150	0/144	0/144	0/158	0/160	0/112	**0/868**
14.	**RLMV**	0/150	0/144	0/144	0/158	0/160	0/112	**0/868**
15.	**RYNV**	0/150	0/144	0/144	0/158	0/160	0/112	**0/868**
16.	**BRNV**	0/150	0/144	0/144	0/158	0/160	0/112	**0/868**

Table 2.19 Number of viruses found every month in sentinel plants from Arkansas 2010

Virus	April	May	June	July	August	September
BYVaV	--	---	1	2	---	---
BCRV	1	2	---	2	1	---
BPYV	---	---	---	---	---	---
BVY	---	---	---	---	1	---
BIVS	---	---	1	1	1	---
BVE	---	---	---	---	---	---
BLMaV	---	---	---	---	---	---
INSV	---	1	---	---	---	---
TRSV	---	---	---	---	---	---
SNSV	---	---	---	---	---	---
TSV	---	---	---	---	---	---
GSyV-1	---	---	---	---	---	---
RBDV	---	---	---	---	---	---
RLMV	---	---	---	---	---	---
RYNV	---	---	---	---	---	---
BRNV	---	---	---	---	---	---

Table 2.20 Number of viruses found every month in sentinel plants from Arkansas 2011

Virus	April	May	June	July	August	September
BYVaV	---	1	---	1	---	---
BCRV	---	1	---	---	---	---
BPYV	---	---	1	---	1	---
BVY	---	---	---	---	---	1
BIVS	---	---	---	---	1	---
BVE	---	---	---	---	---	---
BLMaV	---	---	---	---	---	---
INSV	---	---	---	---	---	---
TRSV	---	---	---	---	---	---
SNSV	---	---	---	---	---	1
TSV	---	---	---	---	---	---
GSyV-1	---	---	---	---	---	---
RBDV	---	---	---	---	---	---
RLMV	---	---	---	---	---	---
RYNV	---	---	---	---	---	---
BRNV	---	---	---	---	---	---

Table 2.21 Number of viruses found every month in sentinel plants from Arkansas 2012

Virus	April	May	June	July	August	September
BYVaV	---	---	1	---	1	1
BCRV	---	---	1	1	---	---
BPYV	1	---	---	1	---	---
BVY	---	---	---	---	---	---
BIVS	---	---	---	---	---	---
BVE	---	---	---	---	---	---
BLMaV	---	---	---	---	---	---
INSV	---	---	---	---	---	---
TRSV	---	---	---	---	---	---
SNSV	---	1	---	1	---	---
TSV	---	---	---	---	---	---
GSyV-1	---	---	---	---	---	---
RBDV	---	---	---	---	---	---
RLMV	---	---	---	---	---	---
RYNV	---	---	---	---	---	---
BRNV	---	---	---	---	---	---

Table 2.22 Number of viruses found every month in sentinel plants from North Carolina 2010

Virus	May	June	July	August	September	October
BYVaV	2	---	---	1	---	
BCRV	1	---	---	---	---	1
BPYV	---	---	---	1	---	---
BVY	---	---	---	---	---	---
BIVS	---	---	---	---	---	---
BVE	---	---	---	---	---	---
BLMaV	---	---	---	---	---	---
INSV	---	---	---	---	---	---
TRSV	---	---	---	---	---	---
SNSV	---	---	1	---	---	---
TSV	---	---	---	---	---	---
GSyV-1	---	---	---	---	---	---
RBDV	---	---	---	---	---	---
RLMV	---	---	---	---	---	---
RYNV	---	---	---	---	---	---
BRNV	---	---	---	---	---	---

Table 2.23 Number of viruses found every month in sentinel plants from North Carolina 2011

Virus	May	June	July	August	September	October
BYVaV	2	1	---	---	1	4
BCRV	---	---	---	2	1	---
BPYV	---	---	---	---	---	---
BVY	---	---	---	---	---	---
BIVS	---	---	---	---	---	---
BVE	---	---	---	---	---	---
BLMaV	---	---	---	---	---	---
INSV	---	---	---	---	---	---
TRSV	---	---	---	---	---	---
SNSV	1	---	---	---	---	---
TSV	---	---	---	---	---	---
GSyV-1	---	---	---	---	---	---
RBDV	---	---	---	---	---	---
RLMV	---	---	---	---	---	---
RYNV	---	---	---	---	---	---
BRNV	---	---	---	---	---	---

Table 2.24 Number of viruses found every month in sentinel plants from North Carolina 2012

Virus	May	June	July	August	September	October
BYVaV	---	1	1	---	---	1
BCRV	---	1	---	---	---	---
BPYV	---	---	---	1	---	---
BVY	---	1	---	---	---	---
BIVS	---	---	---	---	---	---
BVE	---	---	---	---	---	---
BLMaV	---	---	---	---	---	---
INSV	---	---	---	---	---	---
TRSV	---	---	---	---	---	---
SNSV	---	---	---	---	---	---
TSV	---	---	---	---	---	---
GSyV-1	---	---	---	---	---	---
RBDV	---	---	---	---	---	---
RLMV	---	---	---	---	---	---
RYNV	---	---	---	---	---	---
BRNV	---	---	---	---	---	---

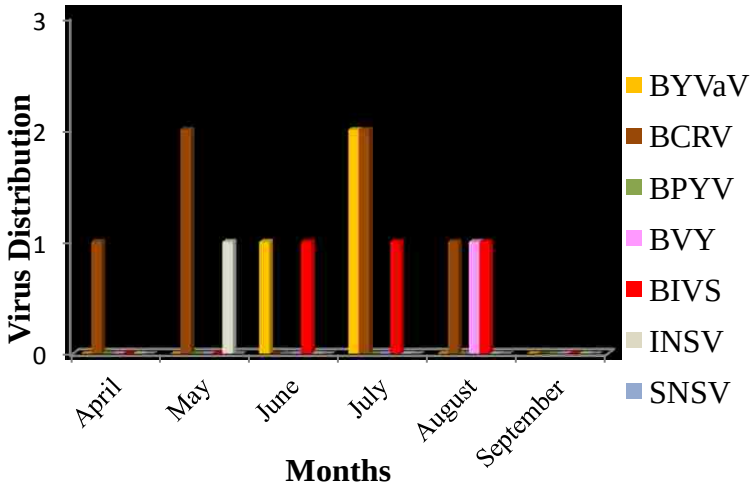

Figure 2.4.14 Major virus distributions for sentinel plants from Arkansas 2010

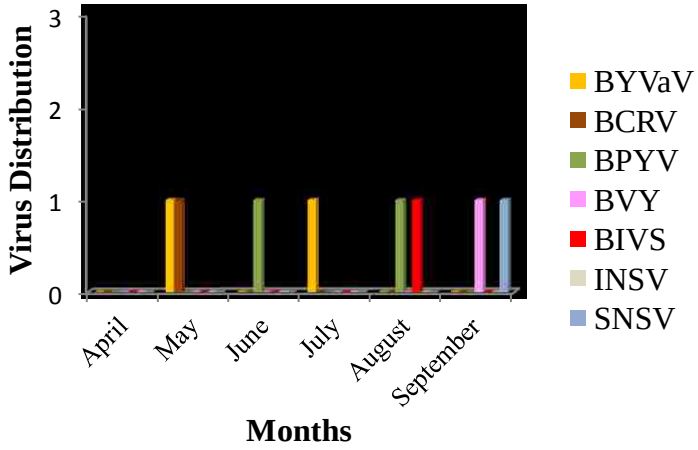

Figure 2.4.15 Major virus distributions for sentinel plants from Arkansas 2011

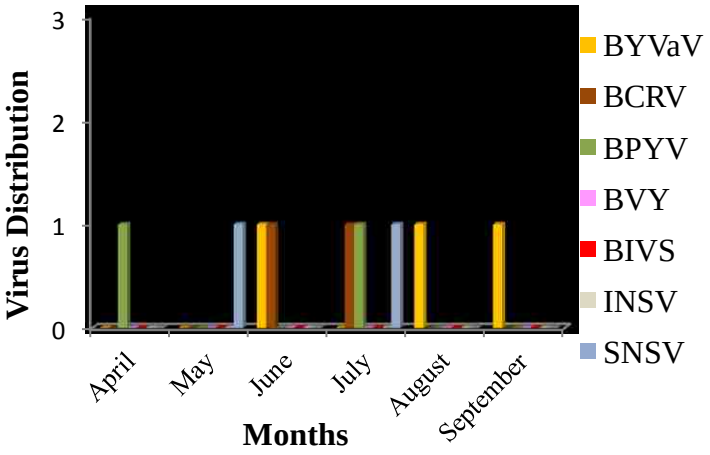

Figure 2.4.16 Major virus distributions for sentinel plants from Arkansas 2012

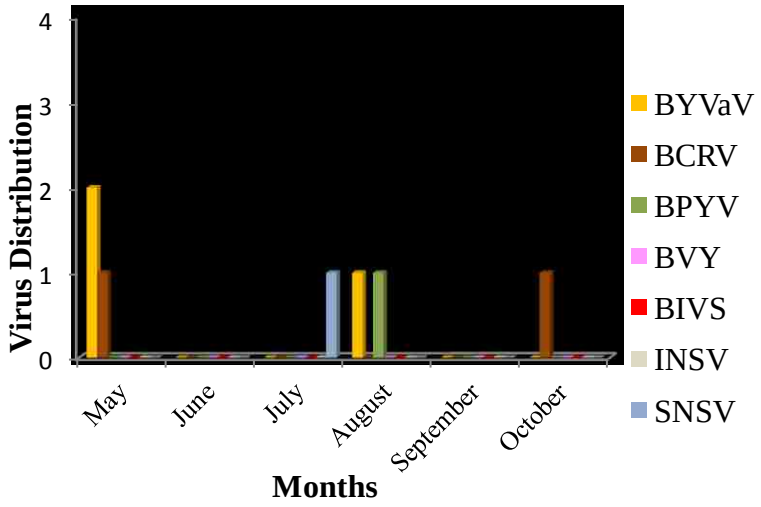

Figure 2.4.17 Major virus distributions for sentinel plants from North Carolina 2010

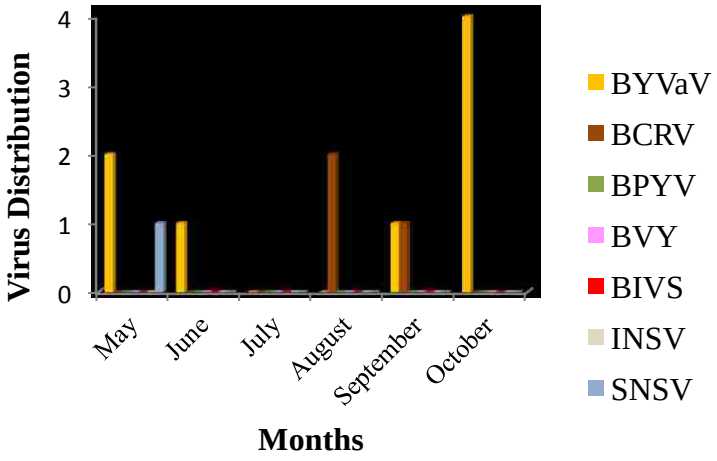

Figure 2.4.18 Major virus distributions for sentinel plants from North Carolina 2011

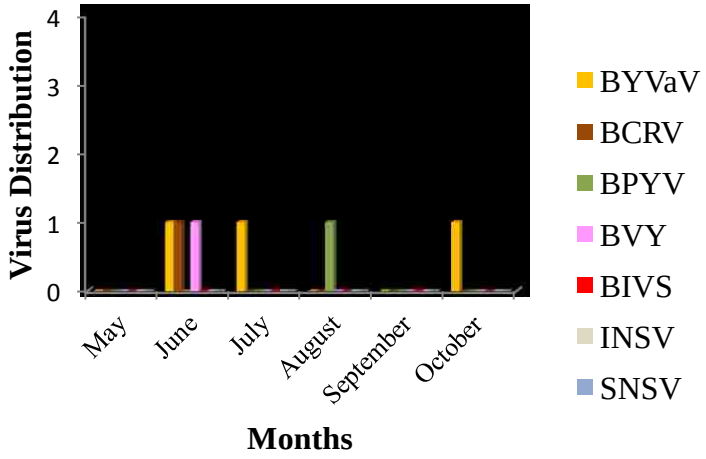

Figure 2.4.19 Major virus distributions for sentinel plants from North Carolina 2012

2.5 Discussion

It is now understood that the majority of virus diseases in berry crops are caused by the combination of two or more viruses. Most of the viruses are latent as single infection. Being obligate parasites, viruses have co-evolved with the host to sustain by having minimal impact on their hosts. Many new viruses have been discovered recently on blackberries indicating that there might be more yet to be identified.

This chapter focused on understanding the distribution of major blackberry viruses in the Southern United States. This chapter also studied the prevalence of other viruses whose distribution in the Southern United States was unknown. The presence of viruses associated with BYVD was verified in most states surveyed. BYVaV and BCRV were prevalent viruses in wild plants with incidence of 54% and 72% respectively. The presence of other viruses ranged from 12% to 20% with the exception of BLMaV which had the highest incidence of all with 81% in wild and 41% in cultivated blackberries. BLMaV hence seems to be an important virus considering its incidence. Wild blackberries may serve as an inoculum source for nearby plantings and hence a consideration when establishing or growing blackberries should be taken. In all the other cases, viruses were detected at lower levels in cultivated plants. The virus flow in cultivated blackberries is most probably coming from the wild plants versus the potential movement through nursery propagation material. This is an important point to consider when developing disease management strategies.

Moreover, the distribution of the viruses whose prevalence was still unknown in blackberries in the southern United States (SNSV, TSV, RBDV, RYNV, BRNV and RLMV) provided a much needed insight in the quest to develop disease control strategies. BCRV, SNSV

and TSV belong to the same subgroup in the genus *Ilarvirus*. SNSV was detected in the highest percentage (~38%) in wild plants (eight random positive samples were sequenced and verified) compared to other viruses, TSV was not detected in any of the samples supporting the previous statement that TSV may not infect plants in the genus *Rubus* (Tzanetakis *et al*., 2010). In case of the aphid borne RMD associated viruses, RLMV and BRNV were detected in very low percentages in both the wild and cultivated plants, whereas RYNV was not detected in any sample. RBDV was also detected at a low percentage in both wild and cultivated blackberries. Thus, the viruses previously known to be associated with BYVD are more prevalent in the Southern United States compared to the RMD associated viruses, RBDV and TSV.

Virus control is based on the use of clean propagation material, control of vectors and resistance. This communication provides evidence that wild plants may serve as virus inoculum to the commercial fields. In addition, although in low percentages, viruses were also present in cultivated plants. Propagation material may not be free of viruses but no universal infections in individual fields were observed, indicative that virus movement in propagation material is not as prevalent now as at the beginning of the BYVD epidemic (Susaimuthu *et al*., 2007).

Virus management strategies based on resistance is challenging in the case of BYVD as the disease is caused by the synergistic effects of multiple viruses. The easiest and most effective way for disease control is the use of clean propagative material and vector control, a feasible approach for many growers, who in the past have been propagating their own planting stock. Establishing fields with virus-tested plants allow fields to stay productive for longer periods of time; yielding better and providing producers with better quantity and quality product.

Given that the majority of virus diseases in the berry crops are caused by the combination of two or more viruses, it is often impossible to eliminate all viruses from the system. Efforts to

identify the weakest link, the easiest virus/vector combination to eliminate, in a particular environment is the better approach to minimize disease impact. Vector control has a prerequisite knowledge on the epidemiology and transmission properties of viruses. This approach will minimize disease impact and prolong field longevity, even though some plants may be infected with viruses, yet symptoms are not devastating.

Detection of BYVaV in sentinel plants from the sites where whiteflies are scarce suggests that the virus can move very efficiently. Whiteflies are regarded as the emerging pests globally and particularly in North America since the turn of the century. The increasing population of whiteflies and their spread into new geographic regions is a proposed threat to the global agriculture. Similarly, BCRV was detected in sentinel plants grown only for a month in the field. BCRV is a seed and potentially pollen borne virus (Poudel *et al*., 2014) and therefore it might be introduced from arthropods carrying infected pollen during the flowering season. Moreover, apple has been confirmed as an alternative host for the virus, suggesting that there might be a wider host range among rosaceous hosts (Poudel *et al*., 2014). Hence the flora surrounding commercial production should be taken into consideration when considering planting sites.

As in the case of BYVaV, there is potential for BPYV spread because of the naturalization of the vectoring whitefly species, the greenhouse whitefly. Moreover, BPYV is known to have a wide host range and thus additional reservoir species around blackberry fields. The presence of other viruses is sparse in commercial fields and no meaningful predictions could be drawn. Study of viruses present in sentinel plants could provide a significant benefit to producers as it provides information on how viruses move in the field. Based on paired entomological studies on the presence of potential vectors at each time point we can predict the virus-vector relationships

and thus produce models on vector movement. Controlling this part on the disease triangle could control the disease itself.

2.6 References

Converse, R.H., 1987. Virus and virus-like diseases of Rubus (Raspberry and Blackberry). Virus Dis. Small Fruits. USDA ARS Agric. Handb. 167–168.

Jennings, D.L., Daubeny H.A., Moore J.N., 1992. Blackberries and raspberries (Rubus). Acta Horticulturae 290, 331-389.

Martin, R.R., MacFarlane, S., Sabanadzovic, S., Quito, D., Poudel, B., Tzanetakis, I.E., 2013. Viruses and virus diseases of Rubus. Plant Dis. 97, 168–182.

Martin, R.R., Tzanetakis, I.E., Gergerich, R., Fernandez, G., Pesic, Z., 2004. Blackberry yellow vein associated virus: A new crinivirus found in blackberry, in: X International Symposium on Small Fruit Virus Diseases 656. pp. 137–142.

Poudel, B., Ho, T., Laney, A., Khadgi, A., Tzanetakis, I.E., 2014. Epidemiology of Blackberry chlorotic ringspot virus. Plant Dis. 98, 547–550.

Poudel, B., Wintermantel, W.M., Cortez, A.A., Ho, T., Khadgi, A., Tzanetakis, I.E., 2013. Epidemiology of Blackberry yellow vein associated virus. Plant Dis. 97, 1352–1357.

Quito-Avila, D.F., Ibarra, M.A., Alvarez, R., Peralta, E.L., Martin, R.R., 2014. A raspberry bushy dwarf virus isolate from Ecuadorean Rubus glaucus contains an additional RNA that is a rearrangement of RNA-2. Arch. Virol. 159, 2519–2521.

Sabanadzovic, S., Ghanem-Sabanadzovic, N.A., 2009. Identification and molecular characterization of a marafivirus in Rubus spp. Arch. Virol. 154, 1729–1735.

Sabanadzovic, S., Ghanem-Sabanadzovic, N.A., Tzanetakis, I.E., 2011. Blackberry virus E: an unusual flexivirus. Arch. Virol. 156, 1665–1669.

Stace-Smith, R., Ramsdell, D.C., 1987. Nepoviruses of the Americas, in: Current Topics in Vector Research. Springer, pp. 131–166.

Strik, B.C., Clark, J.R., Finn, C.E., Bañados, M.P., 2007. Worldwide blackberry production. Horttechnology 17, 205–213.

Susaimuthu, J., 2006. Identification and characterization of two new viruses associated with blackberry yellow vein disease. ProQuest.

Susaimuthu, J., Gergerich, R.C., Bray, M.M., Clay, K.A., Clark, J.R., Tzanetakis, I.E., Martin, R.R., 2007. Incidence and ecology of Blackberry yellow vein associated virus. Plant Dis. 91, 809–813.

Susaimuthu, J., Tzanetakis, I.E., Gergerich, R.C., Kim, K.S., Martin, R.R., 2008a. Viral interactions lead to decline of blackberry plants. Plant Dis. 92, 1288–1292.

Susaimuthu, J., Tzanetakis, I.E., Gergerich, R.C., Martin, R.R., 2008b. A member of a new genus in the Potyviridae infects Rubus. Virus Res. 131, 145–151.

Thekke-Veetil, T., Aboughanem-Sabanadzovic, N., Keller, K.E., Martin, R.R., Sabanadzovic, S., Tzanetakis, I.E., 2013. Molecular characterization and population structure of Blackberry vein banding associated virus, new ampelovirus associated with yellow vein disease. Virus Res. 178, 234–240.

Tzanetakis, I.E., Martin, R.R., 2004. Complete nucleotide sequence of a strawberry isolate of Beet pseudoyellows virus. Virus Genes 28, 239–246.

Tzanetakis, I.E., Martin, R.R., Scott, S.W., 2010. Genomic sequences of blackberry chlorotic ringspot virus and strawberry necrotic shock virus and the phylogeny of viruses in subgroup 1 of the genus Ilarvirus. Arch. Virol. 155, 557–561.

Tzanetakis, I.E., Postman, J.D., Martin, R.R., 2007. First report of Blackberry chlorotic ringspot virus in Rubus sp. in the United States. Plant Dis. 91, 463.

Tzanetakis, I.E., Guzmán-Baeny, T.L., VanEsbroeck, Z.P., Fernandez, G.E., Martin, R.R., 2009. First Report of Impatiens necrotic spot virus in Blackberry in the Southeastern United States. Plant Dis. 93, 432.

Chapter III

Field Virosome- Understanding virus movement in the field scale

3.1 Abstract

Viruses and virus-like diseases pose major issues for blackberry production as they cause significant losses and affect plant longevity. More than 40 viruses are known to infect Rubus and new viruses are discovered frequently. Most of the virus diseases of blackberry and berry crops are caused by the combination of two or more viruses, posing a challenge in virus disease management. The goal of this chapter is to understand the virosome of a blackberry field i.e. to identify all viruses infecting plants in the field scale. Large scale sequencing was employed and results were analyzed using an automated bioinformatics pipeline. Many previously known viruses were detected whereas potentially new viruses were discovered. This chapter adds to our understanding on how viruses are moving in the field; providing much needed information on disease management strategies.

3.2 Introduction

Blackberry popularity has increased due to the demand for fresh fruit, release of improved cultivars, and relative profitability of the crop (Clark, J. R. 1992; Susaimuthu et al., 2007). It was not until the late 1990s that fresh blackberries became readily available in retail markets in the United States (Clark, 2005; Strik, et al., 2007). Since then, blackberries have established a prominent place in the marketplace due to prolonged shelf life and off-season availability (Clark, 2005; Strik et al., 2007). Although the vast majority of cultivated blackberry production in the U.S. is concentrated in the Pacific Northwest, production for the fresh market has increased during the last decade in the Southeastern United States.

Even though the outlook for blackberry production is encouraging, viruses and virus-like diseases can cause significant losses and affect the longevity of blackberry plantings (Ellis et al., 1997). Not all viruses cause severe symptoms; still some are widespread and destructive. It is now understood that most of the viral diseases in blackberry and berry crops in general are caused by the combination of two or more viruses making disease management a challenge (Martin et al., 2013). Knowledge of virus distribution and epidemiology are important factors to consider when establishing blackberries. There has been a dramatic increase in the number of viruses affecting blackberries, primarily because of novel technologies and methods (Martin et al., 2013; Ho et al., 2015; Ho and Tzanetakis 2014). Control is challenging because of the complex mode of transmission and activity of blackberry virus vectors. Several blackberry viruses are seed and pollen-transmitted whereas the majorities are vector-transmitted by aphids, hoppers, whiteflies, thrips, mealybugs, nematodes or mites.

In the last decade there have been a number of new viruses identified in blackberry, many of which have not been studied in great detail when it comes to their biology and epidemiology. Detection methods are often based on a single isolate and therefore may not identify all isolates of the viruses. Large scale sequencing (LSS) together with bioinformatics analyses has brought a radical change in the field of virology by enabling scientists to detect all known viruses but also discover novel ones. Prior knowledge of viral sequences or their genetic makeup is not necessary allowing for the detection of any virus isolate or novel species per se. Popular platforms for LSS includes pyrosequencing (454 Life Sciences, Brandford, CT) and Illumina dye sequencing (Illumina, San Diego, CA) (Al Rwahnih et al., 2011; Quito- Avila et al., 2013; Al Rwahnih et al., 2013; Thekke-Veetil et al., 2013; Vives et al., 2013). Bioinformatics analyses are of utmost importance for correct virus identification. For this reason a novel automated pipeline, VirFind, was developed and specifically used for virus detection and discovery (Ho and Tzanetakis, 2014). This is the tool used in the analyses of the data collected during this study.

Studies have been conducted to comprehend disease epidemics at regional levels (Chapter 2 of this Thesis) whereas this work aims to understand virus distribution at the field level, an important factor for disease control.

Understanding the small scale movement could assist with the management of disease complexes and eliminate large scale disease epidemics. The identification of the major viruses present in the field and movement of potential vectors in a seasonal timeframe could lead to the identification of vectors and development of custom-made control strategies based on virosome of the field and the region alike.

3.3 Materials and Methods

3.3.1 Sample Collection

Samples for the study were collected from the University of Arkansas System Division of Agriculture (UASDOA) Fruit Research Station, Clarksville, Arkansas. Blackberry breeding program was started in this station in 1964 by James N. Moore. Primocane leaf samples from the same plants were collected at two different times, in May and September. Twenty-four samples from each season were pulverized in liquid nitrogen right after the collection and stored at -80^{o}C till further use.

3.3.2 Double stranded RNA enrichment

Double stranded RNA (dsRNA) enriched total nucleic acid isolations were performed as described by Poudel et al. (2013) with minor modifications. Briefly, 0.5 gram leaf tissue was homogenized in 2 ml of extraction buffer (200 mM Tris-HCL, pH 8.5, 300 mM lithium chloride, 1.5% lithium dodecylsulphate, 10 nM ethylene diamine tetra-acetic acid (EDTA), 1% sodium deoxycholate, 1% NP-40 and 1% of 14M β-mercaptoethanol solution (vol/vol) added right before use). One ml of 5.8 M potassium acetate (3.8 M potassium, 5.8 M acetate) was added to one ml of supernatant collected from the homogenized tissue. The tubes were mixed well and subjected to centrifugation at 20,000 g for 10 min. One ml of the supernatant was collected and mixed with the equal volume of 100% isopropanol. The tubes were then mixed well and chilled at -20^{o}C for at least 30 min before being centrifuged for 20 min at 20,000 g. The pellet was resuspended in one ml wash buffer (10 mM Tris-HCL, pH 7.5, 0.5 mM EDTA, 50 mM NaCl, and 50% ethanol) and 50 µl of silica/glass milk was added to the tube and mixed well. The suspension was then pulse centrifuged for 10 sec at 12,000 g. The pellet was washed again with one ml wash buffer to eliminate inhibitors and centrifuged for 2 min at 20,000 g. Pellet was dried in speedvac (Thermo

Fisher Scientific) and suspended in 150 µl water. Tubes were left at room temperature for 5 min and centrifuged at 20,000 g for 2 min. Twenty five µl of supernatant was transferred to a new tube for DNAse and RNase digestion. Tubes containing the remaining supernatant and silica were stored at -80^{o}C for future use.

For nuclease digestion nucleic acids where brought to 200 µl using 2X Sodium Tris EDTA (0.2M NaCl, 0.04 M of Tris-HCl pH 7.5, 2mM EDTA) before adding 8 unit of T1 RNase, 20 µl of 1 M $MgCl_2$ and 1 unit of DNaseI. Material was digested at 37^{o}C for 1 h before termination of the reaction using 500 µl of 0.5 M EDTA, pH 8. One µl of glycogen (20 mg/ml) and 30 µl of 3 M Sodium Acetate were added to the mix and volume was brought to 1 mL by adding ice-cold 100% ethanol. The tubes were then vortexed and incubated at -20^{o}C overnight at which point they were centrifuged at 10,000 g in a microcentrifuge for 30 minutes. The supernatant was carefully removed and discarded. The pellet was washed three times with ice-cold 70% ethanol and centrifuged at 10,000 g for 5 minutes. The pellet was allowed to air dry at room temperature for 5 minutes and was then dissolved in 25 µl of RNase-free water. RNA was quantified using NanoDrop^{TM} and 4 µl of dsRNA enriched preparation was taken for further analyses. Approximately 20 ng (+/- 3 ng) RNA per reaction was used.

3.3.3 Degenerate Oligo-Primed Reverse Transcription Polymerase Chain Reaction (DOP-RT PCR)

DsRNA denaturation was done using 0.04 M methylmercury hydroxide (CH_4HgO). Four µl of dsRNA enriched preparation was mixed well with 4 µl of CH_4HgO. The mixture was incubated in the fume hood for 10 min. Reverse transcription was carried out by mixing the denatured dsRNA with the mastermix that consisted of 10 µl of 5X reverse transcription buffer

(250 mM Tris-HCL, pH 8.3 at 25^{o}C, 375 mM KCL, 15 mM $MgCl_2$, 50 mM DTT), 2 µl of 0.4 mM each dNPT, 2 µl of 20 µM KpnI-RT primer (Table3.1), 6 Unit of RiboLock RNase Inhibitor (Thermo Scientific), 50 units of Maxima^{TM} reverse transcriptase (Thermo Scientific) and water to 50µl. The mixture was incubated at room temperature for 10 min followed by reactions at 50^{o}C for 60 min, and then at 85^{o}C for 5 min to deactivate the enzyme.

PCR was set up as follows: 5 µl of 10 X PCR reaction buffer (GenScript) (500 mM KCL, 100 mM Tris-HCL, pH 9.0 at 25^{o}C, 1% Triton X-100, 15 mM $MgCl_{2)}$, 2 µl of 20 nM KpnI-PCR primer depending on the RT primer used with appropriate barcodes for multiplexing (Table 3.1), 2 µl of dNTPs of 0.2 mM each, 2 µl of cDNA, 2 U of Taq DNA polymerase (GenScript) and water to 50µl. The program consisted of 2 min denaturation at 94^{o}C followed by 35 cycles of 20 s at 94^{o}C, 20 s at 50^{o}C, and 30 s at 72^{o}C, with a final extension of 10 min at 72^{o}C.

Five µl of the product was then mixed with 2 µl of the loading dye and subjected to gel electrophoresis in a 1.5% TBE- agarose gel and visualized after staining for 20 min with GelRed® (Biotium) according to manufacturer's recommendation. Hyperladder 100 bp (Bioline) was used as a molecular size marker. The remaining product was purified using the GeneJET PCR Purification Kit (Thermo Scientific) following manufacturer's recommendations with DNA eluted in 30 µl water.

DNA quality and quantity were measured using NanoDrop^{TM} 1000 spectrophotometer (Thermo Scientific) according to manufacturer's recommendation. The purity ($A_{260/280}$) of DNA was higher than 1.75 and at least 2.5 µg of DNA was sent for LSS.

3.3.4 Large Scale Sequencing and Bioinformatics Analysis

DNA was sequenced using the 454 junior platform at the Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK. A total of 48 samples were divided into eight sets of six samples. The subsets were named as A1 to A6 (May), B1 to B6 (September), C1 to C6 (May), D1 to D6 (September), E1 to E6 (May), F1 to F6 (September), G1 to G6 (May) and H1 to H6 (September). The primer set was comprised of an RT primer (with a random hexamer at the 3' end) and 48 barcoded PCR primers (Table 3.1), facilitating multiplexed LSS runs without the need of further barcoding by the sequencing service provider. Table 3.2 illustrates the grouping of the samples with the primer used. For each set, three LSS were run multiplexing equimolar amount of samples; six samples from May in first run; six samples from September in second and all the 12 from May and September combined together in the third for a total of 12 runs. VirFind.org was used to analyze the raw LSS output. VirFind is an automated online tool used specifically for virus detection and discovery (Ho and Tzanetakis, 2014). The program uses raw LSS data in sff format to identify known and unknown viruses. A detailed flowchart of the steps performed by VirFind is illustrated in Figure 3.1.

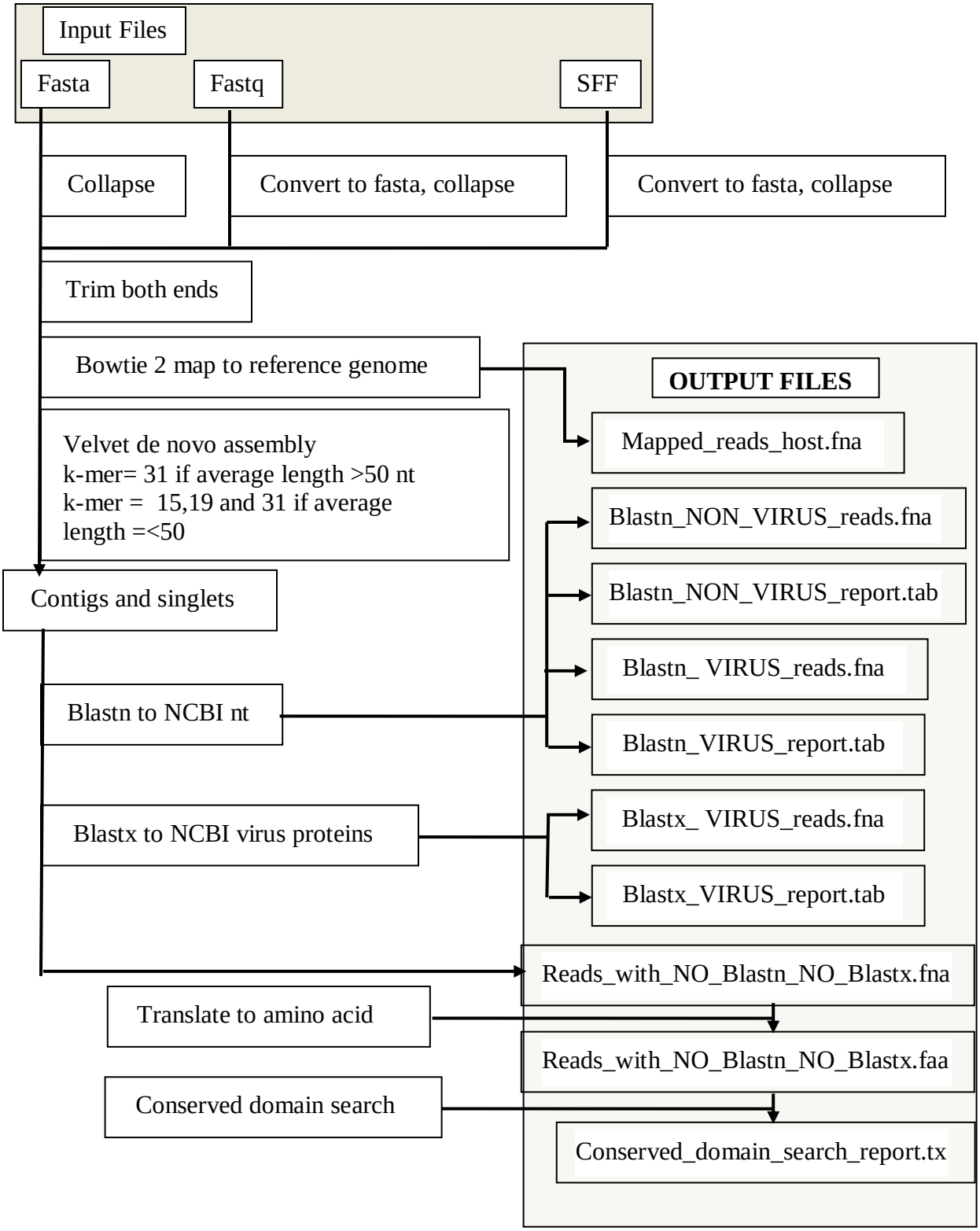

Figure 3.1 VirFind flowcharts for virus detection and discovery using next generation sequencing data. Stars indicate steps where users can set their own parameters (adapted from Ho

Table 3.1 List of primers used in DOP-RT PCR

Primer Name	Sequences
KpnI-RT[a]	TGGTAGCTCTTGATCANNNNNN
KpnI-RPI1-PCR[b]	CGTGATAGAGTTGGTAGCTCTTGATC
KpnI-RPI2-PCR[b]	ACATCGAGAGTTGGTAGCTCTTGATC
KpnI-RPI3-PCR[b]	GCCTAAAGAGTTGGTAGCTCTTGATC
KpnI-RPI4-PCR[b]	TGGTCAAGAGTTGGTAGCTCTTGATC
KpnI-RPI5-PCR[b]	CACTGTAGAGTTGGTAGCTCTTGATC
KpnI-RPI6-PCR[b]	ATTGGCAGAGTTGGTAGCTCTTGATC
KpnI-RPI7-PCR[b]	GATCTGAGAGTTGGTAGCTCTTGATC
KpnI-RPI8-PCR[b]	TCAAGTAGAGTTGGTAGCTCTTGATC
KpnI-RPI9-PCR[b]	CTGATCAGAGTTGGTAGCTCTTGATC
KpnI-RPI10-PCR[b]	AAGCTAAGAGTTGGTAGCTCTTGATC
KpnI-RPI11-PCR[b]	GTAGCCAGAGTTGGTAGCTCTTGATC
KpnI-RPI12-PCR[b]	TACAAGAGAGTTGGTAGCTCTTGATC
KpnI-RPI13-PCR[b]	TTGACTAGAGTTGGTAGCTCTTGATC
KpnI-RPI14-PCR[b]	GGAACTAGAGTTGGTAGCTCTTGATC
KpnI-RPI15-PCR[b]	TGACATAGAGTTGGTAGCTCTTGATC
KpnI-RPI16-PCR[b]	GGACGGAGAGTTGGTAGCTCTTGATC
KpnI-RPI21-PCR[b]	CGAAACAGAGTTGGTAGCTCTTGATC
KpnI-RPI22-PCR[b]	CGTACGAGAGTTGGTAGCTCTTGATC
KpnI-RPI23-PCR[b]	CCACTCAGAGTTGGTAGCTCTTGATC
KpnI-RPI24-PCR[b]	GCTACCAGAGTTGGTAGCTCTTGATC
KpnI-RPI27-PCR[b]	AGGAATAGAGTTGGTAGCTCTTGATC
KpnI-RPI28-PCR[b]	CTTTTGAGAGTTGGTAGCTCTTGATC
KpnI-RPI29-PCR[b]	TAGTTGAGAGTTGGTAGCTCTTGATC
KpnI-RPI30-PCR[b]	CCGGTGAGAGTTGGTAGCTCTTGATC
KpnI-RPI31-PCR[b]	ATCGTGAGAGTTGGTAGCTCTTGATC
KpnI-RPI32-PCR[b]	TGAGTGAGAGTTGGTAGCTCTTGATC
KpnI-RPI33-PCR[b]	CGCCTGAGAGTTGGTAGCTCTTGATC
KpnI-RPI34-PCR[b]	GCCATGAGAGTTGGTAGCTCTTGATC

Table 3.1 List of primers used in DOP-RT PCR (Cont.)

Primer Name	Sequences
KpnI-RPI35-PCR[b]	AAAATGAGAGTTGGTAGCTCTTGATC
KpnI-RPI36-PCR[b]	TGTTGGAGAGTTGGTAGCTCTTGATC
KpnI-RPI37-PCR[b]	ATTCCGAGAGTTGGTAGCTCTTGATC
KpnI-RPI38-PCR[b]	AGCTAGAGAGTTGGTAGCTCTTGATC
KpnI-RPI41-PCR[b]	GTCGTCAGAGTTGGTAGCTCTTGATC
KpnI-RPI42-PCR[b]	CGATTAAGAGTTGGTAGCTCTTGATC
KpnI-RPI45-PCR[b]	GAATGAAGAGTTGGTAGCTCTTGATC
KpnI-RPI46-PCR[b]	TCGGGAAGAGTTGGTAGCTCTTGATC
KpnI-RPI47-PCR[b]	CTTCGAAGAGTTGGTAGCTCTTGATC
KpnI-RPI48-PCR[b]	TGCCGAAGAGTTGGTAGCTCTTGATC

[a] RT primer used for DOP-PCR with KpnI-PCR primers.

[b] DOP-PCR primers. Underlined portion indicates barcode region.

Table 3.2 List of samples and primers used in the experiment

S.N.	Set	Sample	Name	Primer
1	A1	25M	Comanche	Kpn1
2	A2	25S		Kpn11
3	A3	27M	153	Kpn5
4	A4	27S		Kpn13
5	A5	29M	Cheyenne	Kpn9
6	A6	29S		Kpn23
7	B1	30M	Choctaw	Kpn2
8	B2	30S		Kpn12
9	B3	31M	Tupy	Kpn6
10	B4	31S		Kpn14
11	B5	48M	Y12-185B	Kpn10
12	B6	48S		Kpn24
13	C1	47M	Y11-185	Kpn3
14	C2	47S		Kpn9
15	C3	2M	Osage	Kpn5
16	C4	2S		Kpn11
17	C5	6M	A-2416T	Kpn10
18	C6	6S		Kpn14
19	D1	7M	A-2427T	Kpn15
20	D2	7S		Kpn16
21	D3	5M	A-2418T	Kpn12
22	D4	5S		Kpn24
23	D5	14M	A-2453T	Kpn27
24	D6	14S		Kpn28
25	E1	15M	A-2454T	Kpn29
26	E2	15S		Kpn30
27	E3	16M	A-2450T	Kpn31
28	E4	16S		Kpn32

Table 3.2 List of samples and primers used in the experiment (Cont.)

S.N.	Set	Sample	Name	Primer
29	E5	17M	Natchez	Kpn33
30	E6	17S		Kpn34
31	F1	18M	A-2491T	Kpn35
32	F2	18S		Kpn36
33	F3	19M	A-2473T	Kpn37
34	F4	19S		Kpn38
35	F5	21M	156B	Kpn41
36	F6	21S		Kpn42
37	G1	23M	Arapaho	Kpn45
38	G2	23S		Kpn35
39	G3	26M	153B	Kpn3
40	G4	26S		Kpn4
41	G5	32M	ORUS	Kpn15
42	G6	32S		Kpn16
43	H1	38M	Y2-190B	Kpn21
44	H2	38S		Kpn22
45	H3	43M	Y7-205B	Kpn1
46	H4	43S		Kpn7
47	H5	45M	Y9-219B	Kpn2
48	H6	45S		Kpn8

M indicates samples collected in May

S indicates samples collected in September

Map showing all the samples used in this study is shown in supplementary figure S1 and S2.

3.3.5 Verification

Two additional sets of nucleic acid extractions were carried out in order to verify results and eliminate the possibility of cross-contamination during the LSS sample preparation. Individual samples were pulverized in liquid nitrogen right after the collection and stored at -80^{o}C for further use. Total nucleic acid isolations, RT and evaluation of nucleic acid quality were performed as described by Poudel et al., 2013 and presented in Chapter 2 of this Thesis.

For previously known viruses, published primers (Table 3.3) were used whereas for potential new viruses, three different sets of primers were developed for each virus based on the sequences obtained from LSS (Table 3.4). All 48 samples were tested against these primers for verification. The PCR program differed based on the virus specific primers used. The overall program consisted of initial denaturation at 94^{o}C for 3 min, followed by denaturation at 94^{o}C for 30-45 sec, annealing at 52-57^{o}C for 15-35 sec and extension of 72^{o}C for 30 sec, repeated for 35-40 cycles and a final extension of 72^{o}C for 10 min. Five µl of the PCR product was mixed with 2 µl of the loading dye and subjected to gel electrophoresis in a 1.5% TBE- agarose gel and visualized after staining for 20 min with GelRed® (Biotium).

Table 3.3 List of detection primers designed for the known virus hits

Virus	Primer sequences
blackberry yellow vein associated virus	
Forward	TTGAAAGGAAACTTCACGGA
Reverse	TAAGTTCATACGTTTCCTGCG
blackberry virus Y	
Forward	CTGTGGGGAGATTTGGAGAA
Reverse	TCATTCCATGGGTGTGTC
blackberry virus X	
Forward	CACCTAGCAGCCTTGA
Reverse	TGGTTTGACCAGCGAT
blackberry vein banding associated virus	
Forward	CCGACCTTTCATCCTCACTAC
Reverse	TGGGCTCTGCGTTGTTTA

Table 3.4 List of detection primers designed for verification of potential new virus hits

Virus	Primer sequences
Caulimovirus (PCSV-like)	
PCSV223	
Forward	TCTTGATGTTCCAACAAATTGGG
Reverse	GCAAAGCCAGCATCTACATTTC
PCSV299	
Forward	CGATTTGTTGGAACAACGAGAA
Reverse	TTTCTGAGGACATTCATTTGCATAG
PCSV249	
Forward	GTTGGAACAACGAGAAT
Reverse	GCCAGCATCTACATTTC
Iflavirus (TMaV-like)	
TMaV446	
Forward	CGAACTATCGCGACCAGAAA
Reverse	CGAACTGACCTGCTACATACTC
TMaV285	
Forward	TGGAGTTAGTGCTTCAGGATTG
Reverse	CACAATGGTTCAGAGAGGTAGG
TMaV231	
Forward	CCTACCTCTCTGAACCATTGTG
Reverse	CCTGCTACATACTCCTGAAACTC
Rhabdovirus (SCNaV-like)	
SCNaV317	
Forward	CCATCTCTGGAAGAATTGAGAGC
Reverse	TAGACCTGGAGTTGGGACAAT
SCNaV291	
Forward	GCTTGTTCTCCATCTCTGGAAG
Reverse	CTGGGATCAAGAGCTACCAATC

Table 3.4 List of detection primers designed for verification of potential new virus hits (Cont.)

Virus	Primer sequences
SCNaV238	
Forward	GCTCATAGGGCTTGCTAAGAA
Reverse	GAAGAAGGTGACGGGTGAAG
Soymovirus (BRRV-like)	
BRRV397	
Forward	TCCCTTACAACAACCTGAAGAG
Reverse	GGTTGTCTGGAAGATAATTCTTGTT
BRRV379	
Forward	CAACCTGAAGAGAATGACGAAATC
Reverse	GGAAGATAATTCTTGTTACCTGCAA
BRRV331	
Forward	TCTTCCTCCCTTACAACAACC
Reverse	GCCAGTTTAATAATCTTCCTCTATCAG
Pararetrovirus (RFDV-like)	
RFDV340	
Forward	TGCAAAGCAGAAGGGCATTA
Reverse	GGCATTGGCAATAGTCACAAAC
RFDV329	
Forward	TATGCAAACAAGTGTCCTCAGA
Reverse	GTCTCTAGGCATTGGCAATAGT
RFDV257	
Forward	CATAATGCAGATACTGGCTTTGC
Reverse	GACCTCTCTTTGGTATTCTTCTTCT
Caulimovirus (SPV-like)	
SPV309	
Forward	TTAGCATCAGGAAATCTATCTGGAA
Reverse	AAAGCAGGCTCCATCAATACT

Table 3.4 List of detection primers designed for verification of potential new virus hits (Cont.)

Virus	Sequences
SPV258	
Forward	GGATAACATTGCCGTTAACCTTG
Reverse	TGAGGTTGCAAAGCTGATAGT
SPV206	
Forward	CTAGGATTATTCCGTGCTGAACT
Reverse	CATTATGATGGTTAGTCATGCCTTT
Caulimovirus (FMV-like)	
FMV241	
Forward	CCCTGTGGGATAATTCTGTTCT
FMV219	
Forward	GATGTTAGTGTTTGGAGTTCTTG
Reverse	CAGGATTAATAGCAATGTTATCTCC
FMV218	
Forward	AAAGGCTGGAGCATTCAAA
Reverse	CCCTTACAACAACCTGAAGAG
Nanovirus (FBNSV-like)	
FBNSV369	
Forward	GTATCGATTAGGATCCGGCAAG
Reverse	GTGACTATACTGGGCTTCATGG
FBNSV349	
Forward	GATCCGGCAAGAGCCATAAT
Reverse	CTGGGCTTCATGGAGTTCTT
FBNSV330	
Forward	GACAGGCAAAGGCGAGTATAA
Reverse	CACCGGTCACAATCCTTCTT
Trichovirus (GPGV-like)	
GPGV301	
Forward	GTGGTGAAGAAAGGCTCAAAC

Table 3.4 List of detection primers designed for verification of potential new virus hits (Cont.)

Virus	Sequences
Reverse	GCCAGTAAAGTTGCGATCAAG
GPGV266	
Forward	GGAACTTTCTGGGACAAACAAC
Reverse	CTGCAACGAAGATCAACTTCAC
GPGV212	
Forward	TCACTCAAGAAAGTGGTGAAGAA
Reverse	CAGAGCACCATGACCATTGA
Fijivirus (OSDV-like)	
OSDV344	
Forward	CAGACTGGCCTATTCACTAGTTT
Reverse	TTGGCCATATGCTTCAGTCA
OSDV273	
Forward	GCATTGATCAGACTGGCCTATT
Reverse	GTGGTCAAATCGTTTGGTAGGA
OSDV302	
Forward	AGGGTGCTTCTCAATCAGTTC
Reverse	TCAACCCGGTGGTCAAATC
Carlavirus (PMV-like)	
PMV297	
Forward	AGGTAACCATTGGCGATCTG
Reverse	CCCGGTGTAGAGAACTTTGATAC
PMV225	
Forward	CTAACAGAGAAGCCACCTAAGA
Reverse	CCCTCAACCTCCAGTAATAAGA
PMV204	
Forward	CTGCTGGTTATAAGCCTCACT
Reverse	CACCACTGGAACAAGGAGAA

Table 3.4 List of detection primers designed for verification of potential new virus hits (Cont.)

Virus	Sequences
Badnavirus (citrus yellow mosaic virus-like)	
Forward	AGTAAGACTGTTGGTAATGCCA
Reverse	TTTCTCCATGTAGGCTTTGA
Alphacryptoviruses (RCCV-like)	
RCCV261	
Forward	CATCGAAGTGTTCGACGATGA
Reverse	GCTCTGACAACCACGACAA
Virus	Primer sequences
RCCV217	
Forward	ATGAATCGGGTGTCGGAAG
Reverse	GGTTCACCGCCGTCAATA
RCCV209	
Forward	CGACGACCGATCTGAGTTTC
Reverse	CACGACAAATATGACTGGTTCAC
Marafivirus (MRFV-like)	
MRFV284	
Forward	CGAACTGGGTGGAAATGGA
Reverse	CCAGAGTTGGTAGCTCTTGAT
MRFV225	
Forward	GTGGAAATGGAGGTCCTGAG
Reverse	AGCTCTTGATCACATCTACATCC
MRFV217	
Forward	ATAGGTGCCCGGCTCTC
Reverse	CGCCTCTCACCTAACCAAC
Totivirus (BVF-like)	
BVF263	

Table 3.4 List of detection primers designed for verification of potential new virus hits (Cont.)

Virus	Sequences
Forward	TGCATCGAGTTTGTTACGTTCTA
Reverse	TAGGAGAGATAAGCTGGCAGAG
BVF237	
Forward	AGTCCTATACCTATGCGCTCTAT
Reverse	CACTGGGAGTTTGTGAGTACC
BVF216	
Forward	GCGTGAACAGTCCTATACCTATG
Reverse	CGCAAAGCAGGTCAAAGAAAG
Iflavirus (SV-like)	
SV400	
Forward	AAAGGCACCCACCGATTT
Reverse	GAAGAGGTTAGAGAGCGAGAAAC
SV329	
Forward	GCACCCACCGATTTGTTAATG
Reverse	GGCACCCAAATCAACTGTAATG
SV251	
Forward	AACATAATCGCCGCCTCATC
Reverse	ATCCTCAAGGCACCCAAATC

3.4 Results

3.4.1 DOP-RT-PCR assay for multiplexed LSS

Total nucleic acids (TNA) extractions yielded genomic DNA and ribosomal RNAs as expected (Figure 3.2). Nuclease digestions removed all material other than dsRNA which is resistant to nuclease degradation (Fig. 3.3). After clean up and glycogen precipitation, internal control PCR was performed to verify there was no undigested genomic RNA in the sample (Figure 3.4). The digested product was subjected to DOP RT-PCR and a homogeneous smear between 200 to 800 bp (Figure 3.5) were purified, quantified, normalized to the same amount for each sample, multiplexed as shown in Table 3.2 and sequenced.

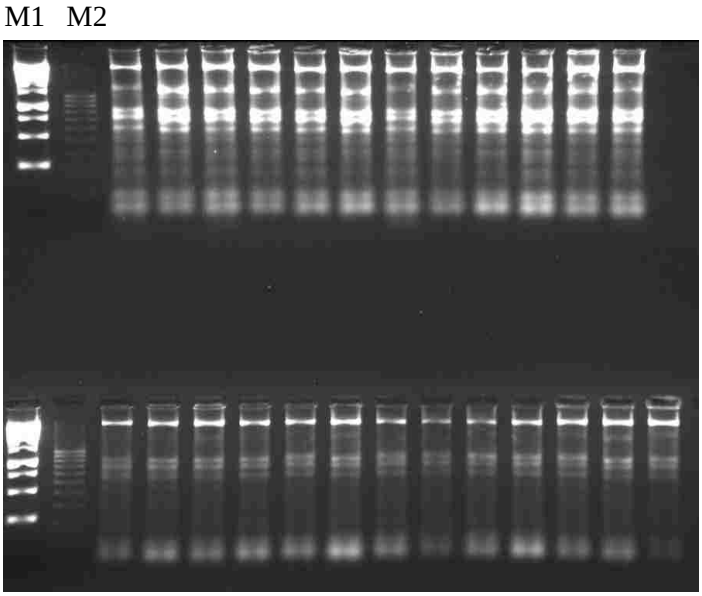

Figure 3.2 Agarose gel electrophoresis of total nucleic acid extraction. M1: 1KB ladder; M2: Hyperladder IV molecular weight marker.

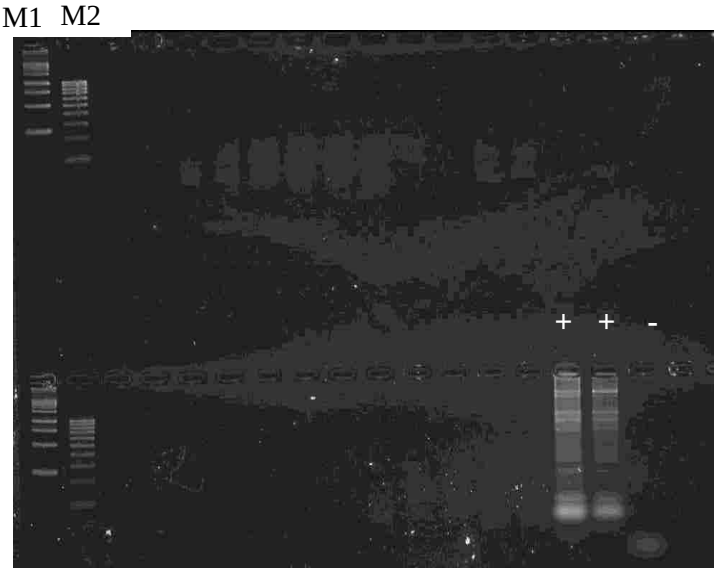

Figure 3.3 Agarose gel electrophoresis of total nucleic acid extraction after DNase and RNase treatment and glycogen precipitation. M1: 1KB ladder; M2: Hyperladder IV molecular weight marker. + denotes total nucleic acid control without DNase and RNase digestion and – denotes water control.

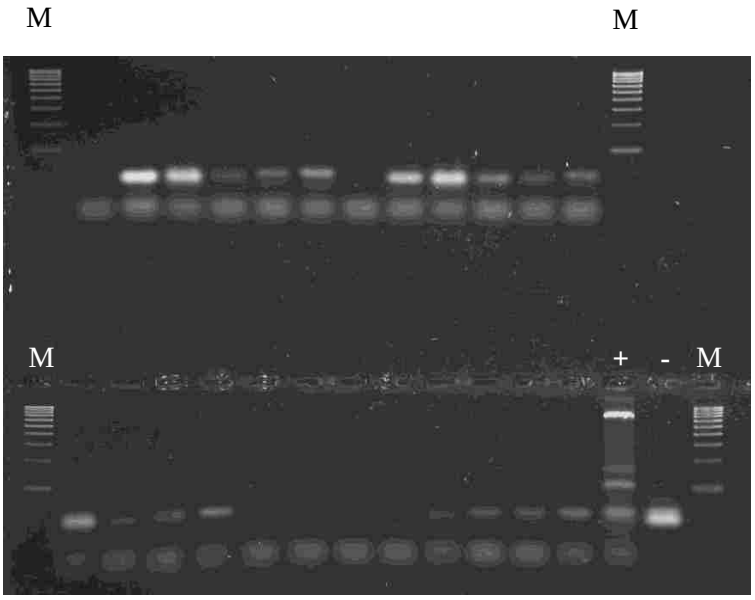

Figure 3.4 Agarose gel electrophoresis of NADH PCR run on DNase and RNase digested product. Hyperladder IV molecular weight marker. + denotes positive control, while – denotes negative control.

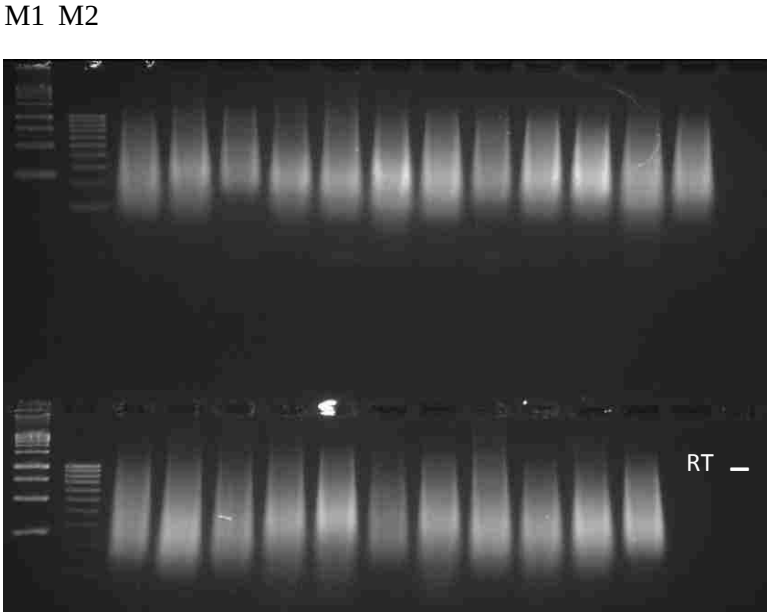

Figure 3.5 Agarose gel electrophoresis of DOP-RT-PCR. M1: 1 KB ladder M2: Hyperladder IV molecular weight marker RT denotes the RT control, while - denotes the water control.

3.4.2 Bioinformatics analysis

LSS generated between 43,380 to 145,575 raw reads per run. Bioinformatics analyses identified several known and potentially new viruses in all runs (Table 3.5). The total counts for each set is illustrated in supplementary tables (Table S.1 to Table S.4). Known virus hits included blackberry yellow vein associated virus, blackberry virus y, blackberry vein banding associated virus, blackberry virus x and potential new virus hits included two new iflavirus-like virus similar to tomato matilda virus and sacbrood virus, a new marafivirus similar to maize raydo fino virus, a new carlavirus similar to poplar mosaic virus, two alphacryptoviruses similar to red clover cryptic virus 1 and fig cryptic virus and, few pararetroviruses similar to soymovirus, caulimovirus, badnavirus, a nanovirus similar to faba bean necrotic stunt virus, a new trichovirus similar to grapevine pinot gris virus, a new fijivirus similar to oat sterile dwarf virus, and a cytorhabdovirus similar to soybean cyst nematode associated northern cereal mosaic virus.

3.4.3 Verification

Three different sets of primers developed for each potentially new virus were employed to verify the results. The primers were tested against two separately extracted TNA from all samples as well as the original dsRNA enriched material used for DOP RT-PCR. In total, the verification was done using three different sets of nucleic acid extractions. For viruses that are already known to infect blackberries, previously published PCR detection primers were employed. Blackberry yellow vein associated virus, blackberry virus y, blackberry virus x, and blackberry vein banding associated virus were detected both in LSS and verification PCR (Fig.3.6-3.9). Table 3.7 illustrates all the known and potential new virus detection using specific primers. BYVaV and BVY are the major viruses found in both techniques. PCR could amplify additional BYVaV samples absent in LSS results. BYVaV was detected as the most prominent virus with 21 samples

found infected. BVY and BVX followed with 10 samples. BVBaV was detected in 3 samples. One new infection (infection only in September) in case of BYVaV, four new infections in case of BVX and one new infection in case of BVBaV were also observed (Table. 3.7).

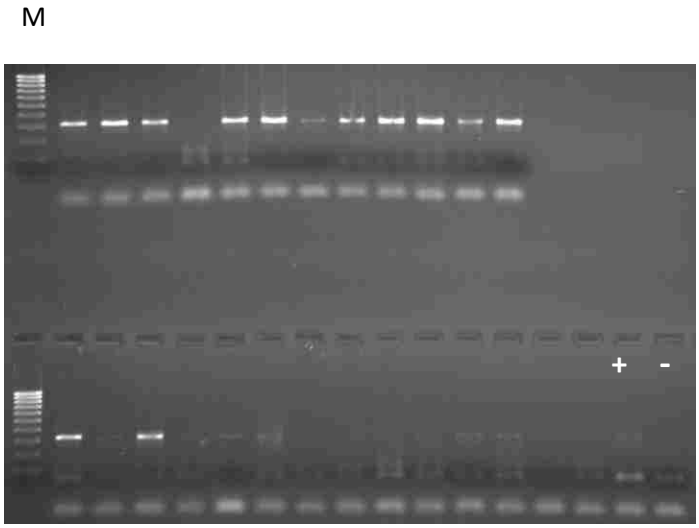

Figure 3.6 Agarose gel electrophoresis of PCR confirming the presence of BYVaV identified using VirFind. Row 1: Sample collected May; Row 2: Sample collected in September. M: Hyperladder IV molecular weight marker. RT: RT control, +: positive control and -: negative control. Sanger sequencing confirmed virus identities.

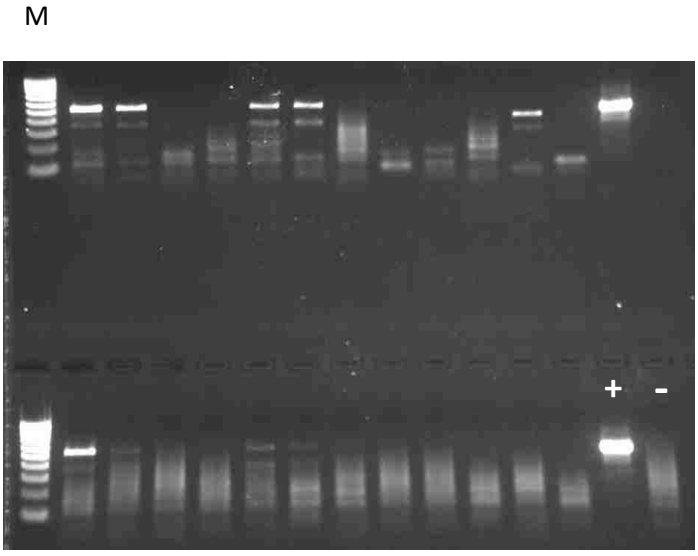

Figure 3.7 Agarose gel electrophoresis of PCR confirming the presence of BVY identified using VirFind. M: Hyperladder IV molecular weight marker. + denotes the positive control, while - denotes the negative control. Sanger sequencing confirmed virus identities.

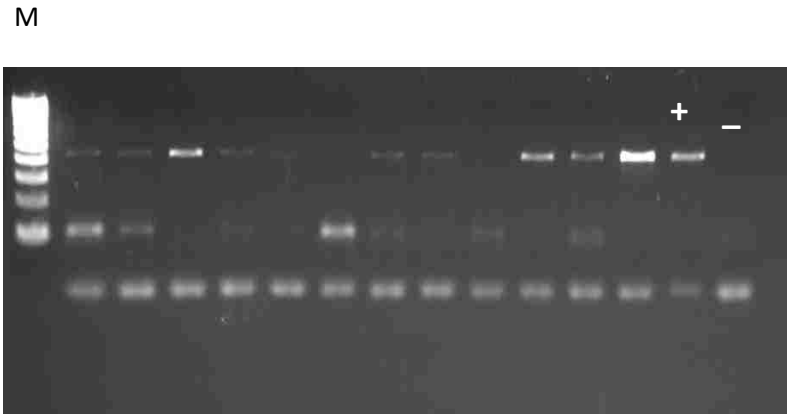

Figure 3.8 Agarose gel electrophoresis of PCR confirming the presence of BVX identified using VirFind. M: Hyperladder IV molecular weight marker. + denotes the positive control, while - denotes the negative control. Sanger sequencing confirmed virus identities.

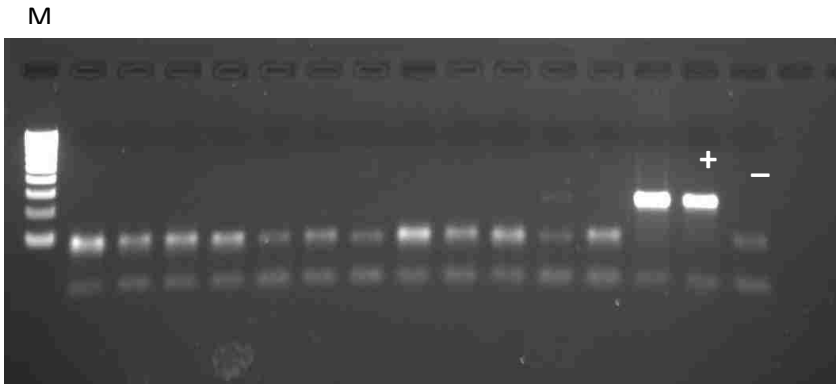

Figure 3.9 Agarose gel electrophoresis of PCR confirming the presence of BVBaV identified using VirFind. M: Hyperladder IV molecular weight marker. + denotes the positive control, while - denotes the negative control. Sanger sequencing confirmed virus identities.

3.4.4 Virus discovery

Presence of all potential new viruses was verified by three different PCR amplifications using detection primers designed from the assembled contigs followed by Sanger sequencing (Figure 3.10 to 3.14). Two new iflavirus-like viruses, similar to tomato matilda virus (GenBank accession numbers KU258125 to KU258134) and sacbrood virus (GenBank accession numbers KU258135 to KU258144) (Figure 3.10; 3.11) were found infecting blackberries. Other viruses include a new carlavirus (GenBank accession numbers KU258117 AND KU258118), similar to poplar mosaic virus (Figure 3.12) a marafivirus (GenBank accession numbers KU258119 to KU258124) similar to maize raydo fino virus (Figure 3.13) and a fijivirus (GenBank accession numbers KU258091 to KU258116) similar to oat sterile dwarf virus (Figure 3.14) A list of all the GenBank accession numbers is provided in supplementary table 5.

Results from all three PCR reactions were consistent in many cases with few not being amplified or faintly amplified. However, two out of three PCR reactions giving consistent positive amplicons were considered as positive for every virus, as primers were not extensively optimized given the relative small number of samples found infected with individual viruses. Out of 48 samples ten were found to be positive to the iflaviruses with two new infections in September. The marafivirus had three new infections in September. There were few positive amplicons for the trichovirus, however, they were present in different samples and all three PCR could not confirm their consistency. Hence they were not counted as positive. The fijivirus was present in thirteen samples including one new infection in September. One new infection was found for the carlavirus with two consistent PCRs. Several pararetroviruses were identified in the LSS results, hitting the RT/RNAseH motifs, an area with high homology to retrotransposons. Primers were designed and tested verifying that they were indeed retrotransposons. Seven samples were found

to be free of any virus tested both in May and September by RT-PCR whereas LSS resulted in 24 samples that were not infected by any of the virus tested. Table 3.6 and 3.7 illustrates the results in detail.

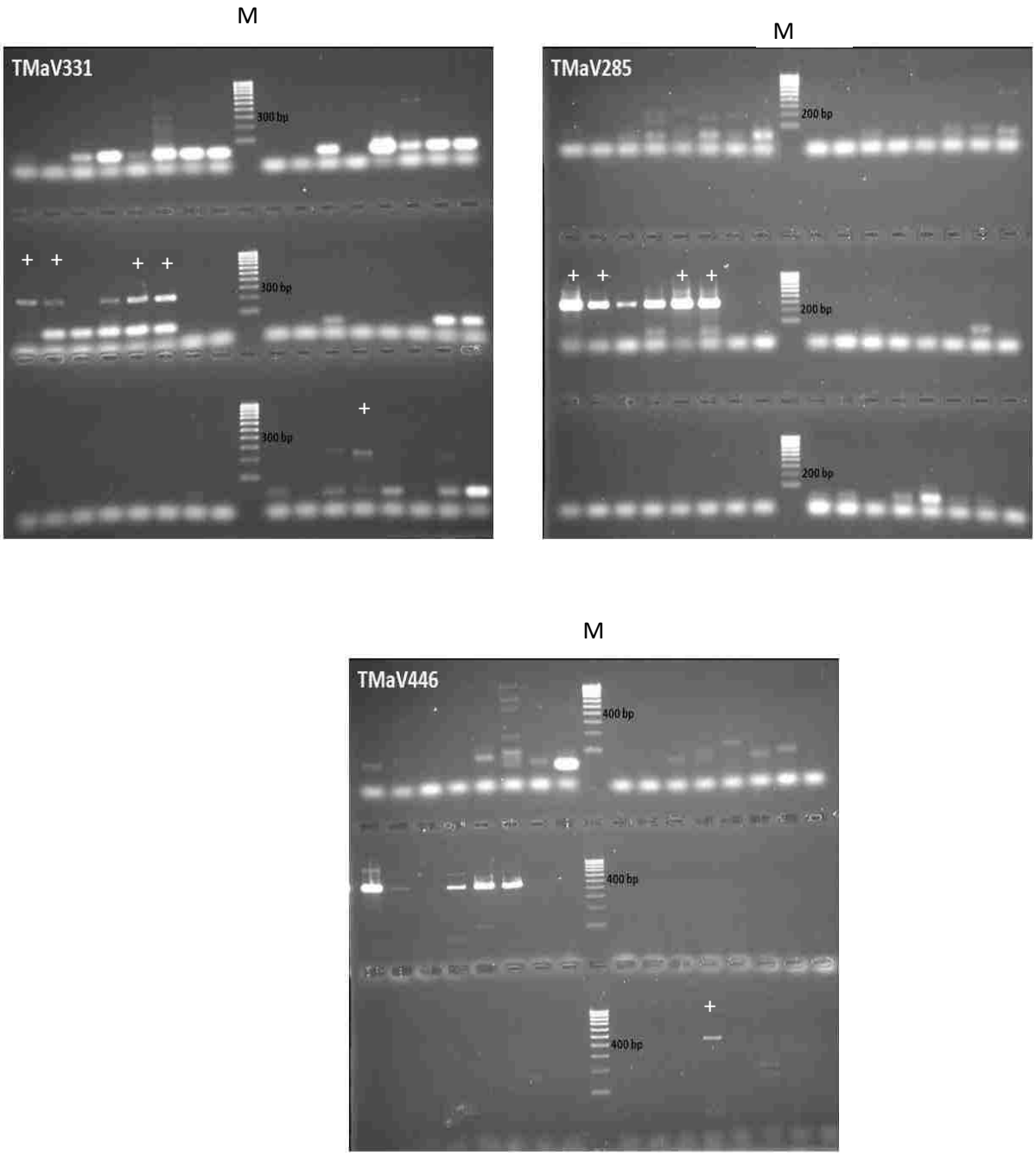

Figure 3.10 Agarose gel electrophoresis of PCR confirming the presence of *Iflaviruss* identified using VirFind. M: Hyperladder IV molecular weight marker. Sanger sequencing confirmed virus identities of all the positive amplicons. + indicates amplicons that were found to be consistently positive for two different primer sets.

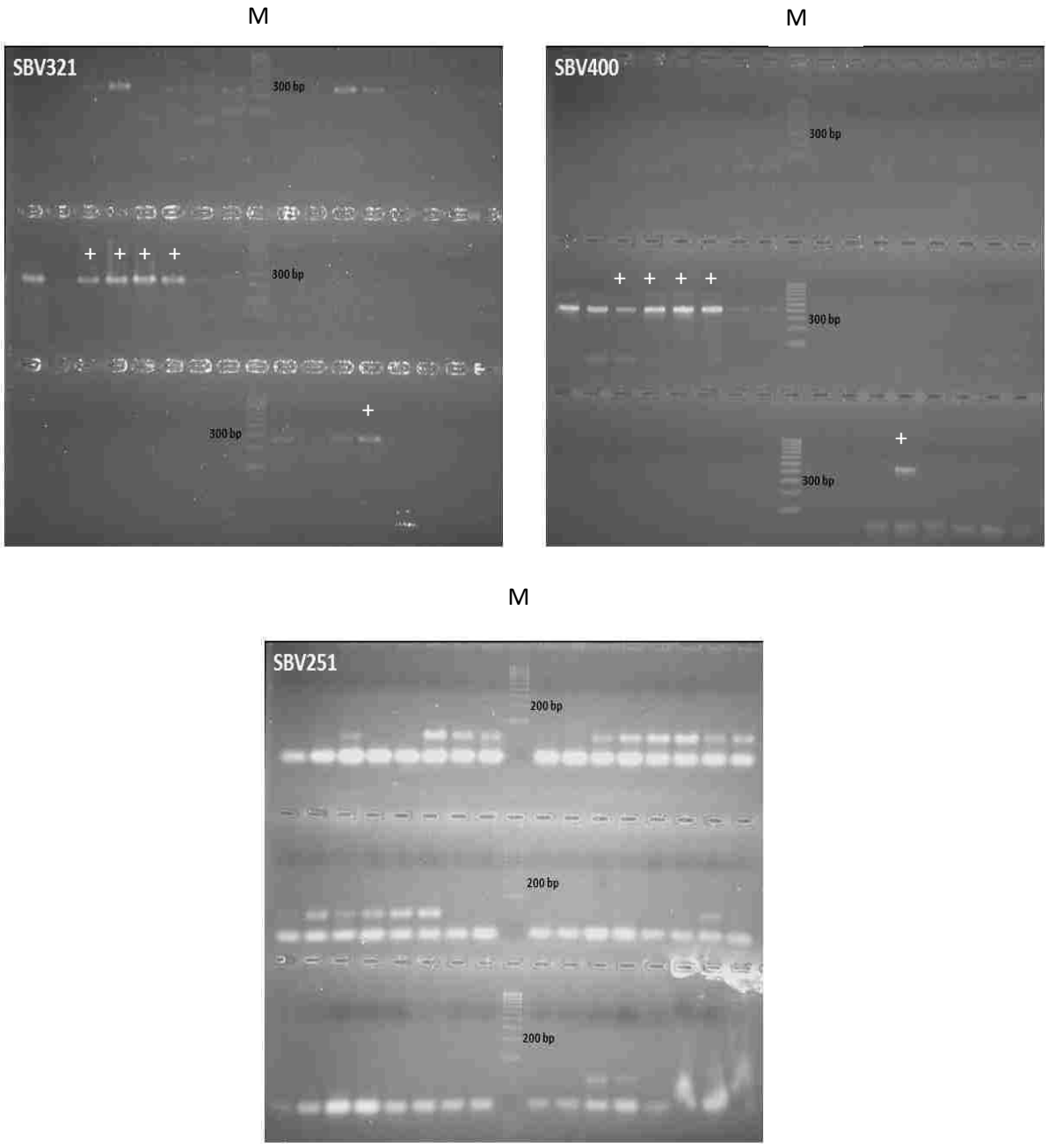

Figure 3.11 Agarose gel electrophoresis of PCR confirming the presence of SBV identified using VirFind. M: Hyperladder IV molecular weight marker. Sanger sequencing confirmed virus identities of all the positive amplicons. + indicates amplicons that were found to be consistently positive for two different primer sets.

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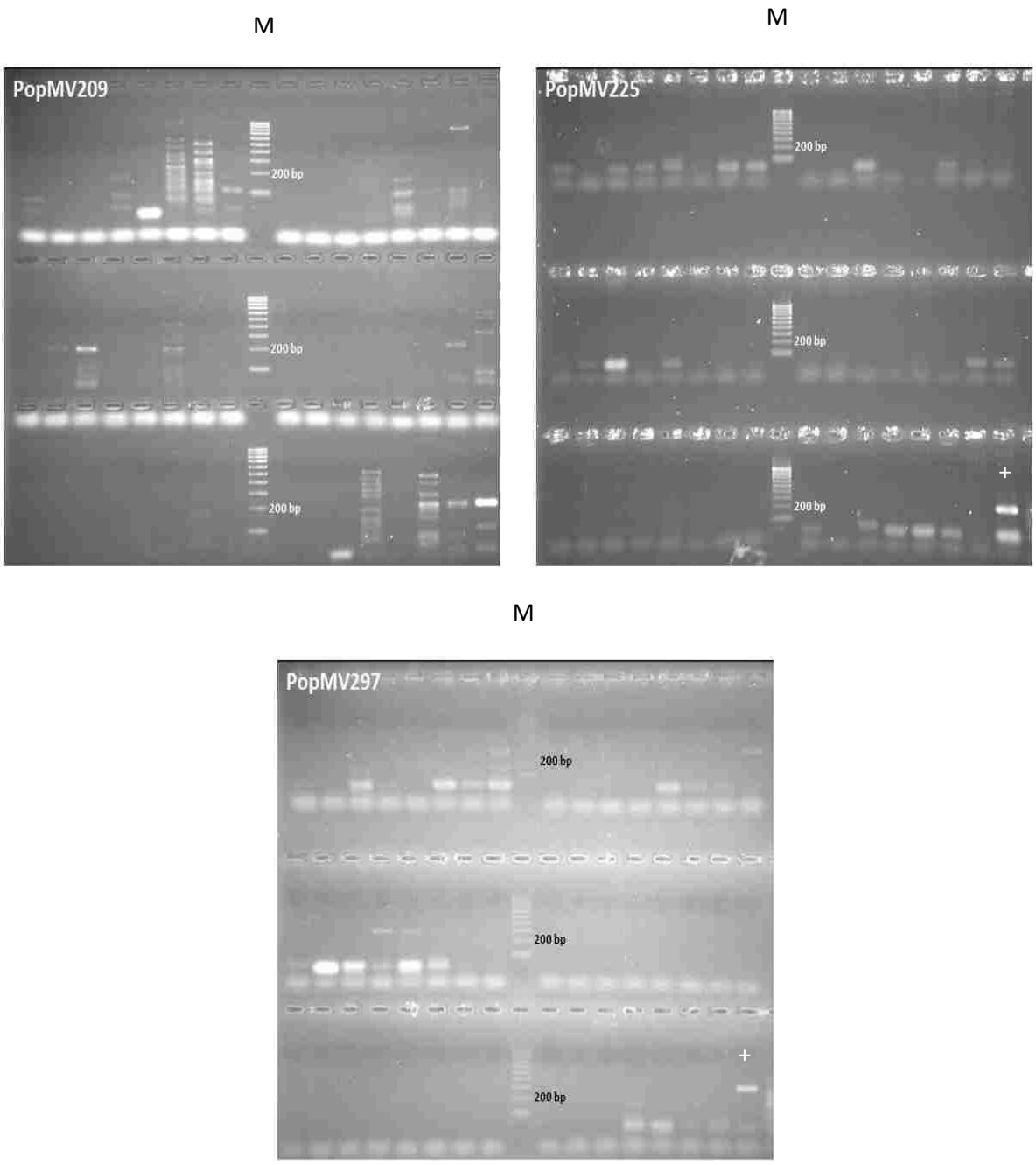

Figure 3.12 Agarose gel electrophoresis of PCR confirming the presence of PopMV identified using VirFind. M: Hyperladder IV molecular weight marker. Sanger sequencing confirmed virus identity of the single positive amplicon. + indicates amplicons that were found to be consistently positive for two different primer sets.

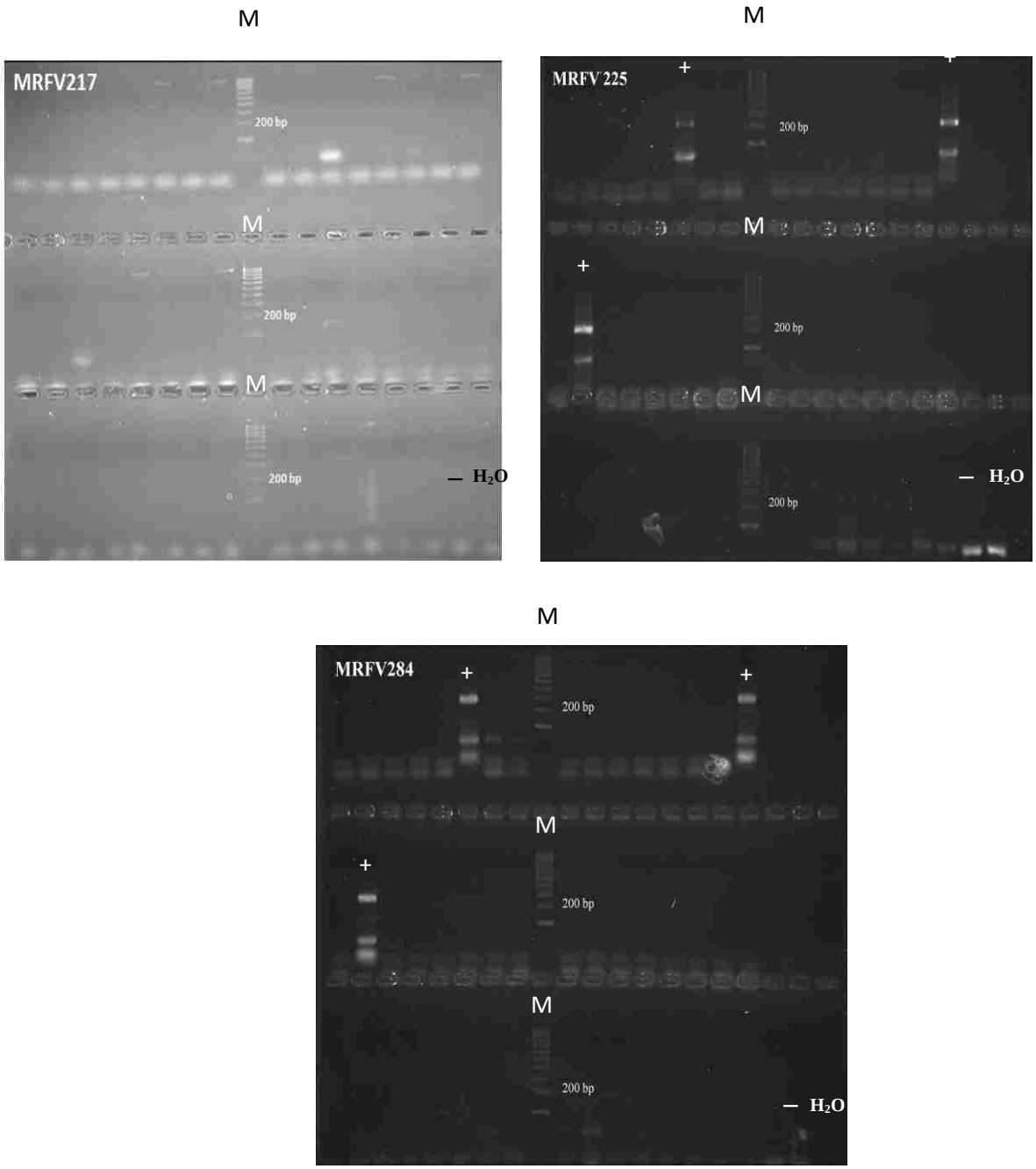

Figure 3.13 Agarose gel electrophoresis of PCR confirming the presence of MRFV identified using VirFind. M: Hyperladder IV molecular weight marker. Sanger sequencing confirmed virus identities. – denotes negative control and H_2O denotes water control. + indicates amplicons that were found to be consistently positive for two different primer sets.

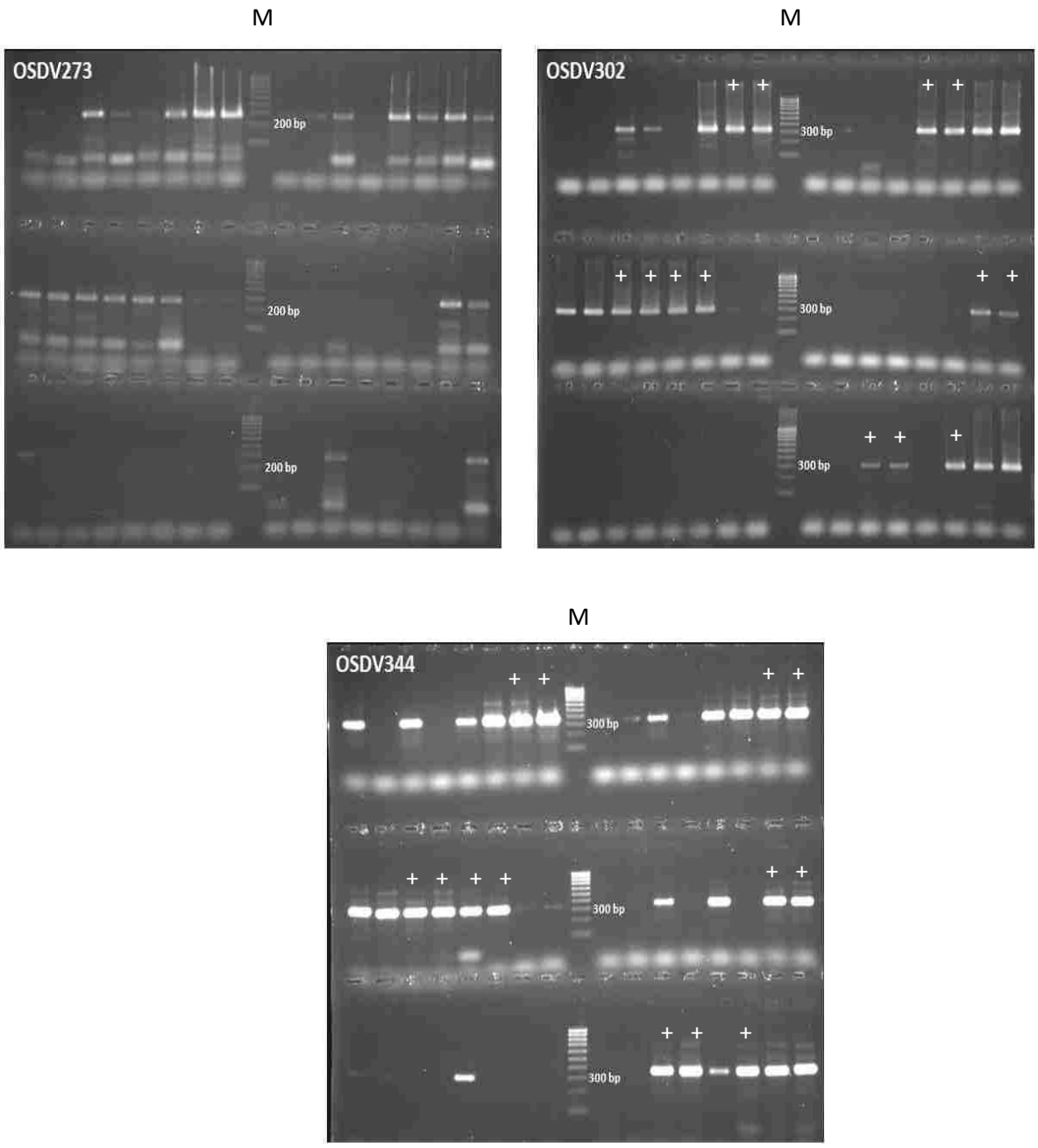

Figure 3.14 Agarose gel electrophoresis of PCR confirming the presence of OSDV identified using VirFind. M: Hyperladder IV molecular weight marker. Sanger sequencing confirmed virus identities of 13 positive amplicons. + indicates amplicons that were found to be consistently positive for two different primer sets.

Table.3.5 Number of raw reads in each set from LSS

Set	Number of raw reads
A	59,783
B	69,718
AB	70,113
C	136,250
D	141,220
CD	136,063
E	145,575
F	57,508
EF	10,3158
G	124,489
H	104,479
GH	112,024

Table 3.6 List of samples and viruses detected/discovered using LSS in May Vs September

Set		A	B	C	D	E	F	G	H	I	J	K	L	M	N
A	25M	--	Y	--	Y	--	--	--	--	Y	--	--	--	--	--
A	25S	Y	Y	--	--	--	--	--	--	--	--	--	--	--	--
A	27M	Y	Y	--	--	Y	--	Y	--	--	--	--	--	--	--
A	27S	--	Y	Y	--	--	Y	--	--	--	--	--	--	--	--
A	29M	--	Y	--	--	--	--	--	--	--	Y	--	--	--	--
A	29S	--	Y	--	--	--	--	--	--	--	Y	--	--	--	--
Unmatched A		--	Y	--	Y	--	--	--	--	--	--	--	--	--	--
B	30M	Y	--	--	--	--	--	--	--	--	--	--	--	--	--
B	30S	--	--	--	--	--	Y	--	--	--	--	--	--	--	--
B	31M	Y	--	--	--	--	--	--	--	--	--	--	--	--	--
B	31S	--	--	Y	--	--	--	--	--	Y	--	--	--	--	--
B	48M	--	Y	--	--	--	--	--	--	--	--	--	--	--	--
B	48S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Unmatched B		Y	Y	--	--	--	Y	--	--	--	--	--	--	--	--
AB	25M	--	Y	--	Y	--	--	--	--	--	--	--	--	--	--
AB	25S	--	Y	--	--	--	--	--	--	--	--	--	--	--	--
AB	27M	--	Y	Y	--	--	--	--	--	--	--	--	--	--	--
AB	27S	--	Y	Y	--	--	--	--	--	--	--	--	--	--	--
AB	29M	--	Y	--	--	--	--	--	--	--	--	Y	--	--	--

Table 3.6 List of samples and viruses detected/discovered using LSS in May Vs September (Cont.)

Set		A	B	C	D	E	F	G	H	I	J	K	L	M	N
AB	29S	--	Y	--	--	--	Y	--	--	--	--	--	--	--	--
AB	30M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
AB	30S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
AB	31M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
AB	31S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
AB	48M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
AB	48S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
Unmatched AB		--	Y	--	--	--	Y	--	--	--	--	Y	--	--	--
C	47M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
C	47S	Y	--	--	--	--	Y	--	--	--	--	--	--	--	--
C	2M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
C	2S	--	--	Y	--	--	--	--	--	--	--	Y	--	--	--
C	6M	--	--	Y	--	--	Y	--	--	--	--	Y	--	--	--
C	6S	--	--	--	Y	--	Y	--	--	--	--	--	--	--	--
Unmatched C		Y	--	Y	--	--	Y	--	--	--	--	Y	--	--	--

Table 3.6 List of samples and viruses detected/discovered using LSS in May Vs September (Cont.)

Set		A	B	C	D	E	F	G	H	I	J	K	L	M	N
D	7M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
D	7S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
D	5M	Y	--	--	--	--	Y	--	--	--	--	--	--	--	--
D	5S	Y	--	--	--	--	--	--	--	--	--	Y	--	--	--
D	14M	--	--	Y	--	--	--	--	--	--	--	Y	--	--	--
D	14S	--	--	Y	--	--	--	--	--	--	--	Y	--	--	--
Unmatched D		--	--	--	Y	--	--	--	--	--	--	Y	--	--	--
CD	47M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
CD	47S	--	--	--	Y	--	Y	--	--	--	--	Y	--	--	--
CD	2M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
CD	2S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
CD	6M	--	--	Y	--	--	Y	--	--	--	--	--	--	--	--
CD	6S	--	--	--	--	--	Y	--	--	--	--	Y	--	--	--
CD	7M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
CD	7S	--	--	--	--	--	Y	--	--	--	--	--	--	--	--
CD	5M	--	--	--	--	--	Y	--	--	--	--	Y	--	--	--
CD	5S	--	--	--	--	--	Y	--	--	--	--	--	--	--	--
CD	14M	Y	--	Y	--	--	--	--	--	Y	--	Y	--	--	--
CD	14S	Y	--	--	--	--	--	--	--	--	--	--	--	--	--

Table 3.6 List of samples and viruses detected/discovered using LSS in May Vs September (Cont.)

Set		A	B	C	D	E	F	G	H	I	J	K	L	M	N
Unmatched CD		Y	--	Y	Y	--	Y	--	--	--	--	--	--	--	--
E	15M	--	--	--	--	--	--	--	--	--	--	Y	Y	--	--
E	15S	--	--	Y	--	--	--	--	--	--	--	--	--	Y	--
E	16M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
E	16S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
E	17M	--	--	Y	--	--	--	--	--	--	--	--	--	--	--
E	17S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Unmatched E		Y	--	--	--	--	--	--	--	--	--	--	--	--	--
F	18M	--	--	--	--	--	--	Y	--	--	--	--	--	--	--
F	18S	Y	--	--	--	--	--	--	--	--	--	--	--	--	--
F	19M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
F	19S	Y	--	--	--	--	--	--	--	--	--	--	--	--	--
F	21M	--	--	--	--	--	--	--	Y	--	--	--	--	--	--
F	21S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Unmatched F		Y	--	--	--	--	--	--	--	--	--	--	--	--	--
EF	15M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
EF	15S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
EF	16M	--	--	--	--	--	--	--	--	--	--	--	--	--	--

Table 3.6 List of samples and viruses detected/discovered using LSS in May Vs September (Cont.)

Set		A	B	C	D	E	F	G	H	I	J	K	L	M	N
EF	16S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
EF	17M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
EF	17S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
EF	18M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
EF	18S	--	--	--	--	--	--	--	--	Y	--	--	--	--	--
EF	19M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
EF	19S	Y	--	--	--	--	Y	--	--	--	--	--	--	--	--
EF	21M	--	--	Y	--	--	--	--	--	Y	--	--	--	--	--
EF	21S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Unmatched EF		--	--	--	--	--	Y	--	--	--	--	Y	--	--	--
G	23M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
G	23S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
G	26M	--	--	--	--	--	Y	--	--	--	--	--	--	--	Y
G	26S	Y	--	--	--	--	--	--	--	--	--	--	--	--	--
G	32M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
G	32S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
Unmatched G		--	--	Y	--	--	Y	--	--	--	--	Y	--	--	--
H	38M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--

Table 3.6 List of samples and viruses detected/discovered using LSS in May Vs September (Cont.)

Set		A	B	C	D	E	F	G	H	I	J	K	L	M	N
H	38S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
H	43M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
H	43S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
H	45M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
H	45S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
Unmatched H		--	--	--	Y	--	--	--	--	--	--	Y	--	--	--
GH	23M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
GH	23S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
GH	26M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
GH	26S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
GH	32M	--	Y	--	--	--	--	--	--	--	--	--	--	--	--
GH	32S	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
GH	38M	--	--	--	--	--	--	--	--	--	--	--	--	--	--
GH	38S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
GH	43M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
GH	43S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
GH	45M	--	--	--	--	--	--	--	--	--	--	Y	--	--	--
GH	45S	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Unmatched GH		--	Y	--	Y	--	--	--	--	--	--	Y	--	--	--

Table 3.6 List of samples and viruses detected/discovered using LSS in May Vs September (Cont.)

A indicates blackberry yellow vein associated virus

B indicates blackberry virus Y

C indicates blackberry virus X

D indicates blackberry vein-banding associated virus

E indicates marafivirus

F indicates iflavirus

G indicates carlavirus

H indicates fijivirus

I indicates alphacryptovirus

J indicates totivirus

K indicates pararetrovirus

L indicates nanovirus

M indicates trichovirus

N indicates rhabdovirus

Table 3.7 List of samples and viruses detected/discovered using RT-PCR in May vs. September

Sample	A	B	C	D	E	F	G	H
25M	--	Y	--	Y	--	--	--	--
25S	Y*	Y	--	Y	--	--	--	--
27M	Y	Y	--	--	--	--	--	--
27S	Y	Y	Y*	--	--	--	--	--
29M	--	Y	--	--	--	--	--	--
29S	--	Y	--	--	Y*	--	--	--
30M	Y	--	--	--	--	--	--	Y
30S	Y	--	--	--	--	--	--	Y
31M	Y	--	--	--	--	--	--	--
31S	Y	--	Y*	--	--	--	--	--
48M	--	Y	--	--	--	--	--	--
48S	--	Y	--	--	--	--	--	--
47M	Y	--	--	--	--	--	--	Y
47S	Y	--	--	--	--	--	--	Y
2M	--	--	--	--	--	--	--	--
2S	--	--	Y*	--	Y*	--	--	--
6M	--	--	Y	--	--	Y (T)	--	--
6S	--	--	Y	Y*	Y*	Y (T)	--	--
7M	--	--	--	--	--	Y (S)	--	Y
7S	--	--	--	--	--	Y (S)	--	Y
5M	Y	--	--	--	--	Y (T,S)	--	Y

Table 3.7 List of samples and viruses detected/discovered using RT-PCR in May vs. September (Cont.)

Sample	A	B	C	D	E	F	G	H
5S	Y	--	--	--	--	Y (T,S)	--	Y
14M	--	--	Y	--	--	--	--	--
14S	--	--	Y	--	--	--	--	--
15M	--	--	--	--	--	--	--	--
15S	--	--	Y*	--	--	--	--	--
16M	Y	--	--	--	--	--	--	--
16S	Y	--	--	--	--	--	--	--
17M	--	--	--	--	--	--	--	--
17S	--	--	--	--	--	--	--	--
18M	Y	--	--	--	--	--	--	Y
18S	Y	--	--	--	--	--	--	Y
19M	Y	Y	--	--	--	--	--	--
19S	Y	Y	--	--	--	--	--	--
21M	--	--	--	--	--	--	--	--
21S	--	--	--	--	--	--	--	--
23M	--	--	--	--	--	--	--	--
23S	--	--	--	--	--	--	--	--
26M	Y	--	--	--	--	--	--	--
26S	Y	--	--	--	--	--	--	--
32M	--	--	Y	--	--	--	--	--

Table 3.7 List of samples and viruses detected/discovered using RT-PCR in May vs. September (Cont.)

Sample	A	B	C	D	E	F	G	H
32S	--	--	Y	--	--	--	--	--
38M	Y	--	--	--	--	--	--	Y
38S	Y	--	--	--	--	Y*(T,S)	--	Y
43M	--	--	--	--	--	--	--	--
43S	--	--	--	--	--	--	--	Y*
45M	--	--	--	--	--	--	--	--
45S	--	--	--	--	--	--	Y*	--

* indicates new infection in September

A indicates blackberry yellow vein associated virus

B indicates blackberry virus Y

C indicates blackberry virus X

D indicates blackberry vein-banding associated virus

E indicates marafivirus

F indicates iflavirus (T-tomato matilda virus/ S-sacbrood virus)

G indicates carlavirus

H indicates fijivirus

3.4.4 Discussion

LSS and bioinformatics analyses identified several known and unknown viruses infecting blackberry. Different number of samples were multiplexed (6-6-12) in a total 12 LSS runs. The genome of the host plant used in this study i.e. *Rubus* or blackberry is still unavailable on the GenBank, hence the filtering steps removed a subset of host sequences, leaving a number of non-hit sequences. Identifying a virus hit to GenBank nucleotide or virus protein database is relatively simple in the case of long contigs with high sequence identity to known species. Still, it can be a challenging task in the case of short contigs and high e-values because of the possibility of false positives.

Viruses detected in LSS result did not completely match with viruses detected by PCR in all 48 samples but it provided a good prediction on what and how many viruses may be present in a field. Out of 48 samples, seven samples were found to be free of viruses tested in RT-PCR. Whereas in LSS, 24 samples were found to be uninfected by viruses tested. LSS was performed by multiplexing six samples in each set (e.g. A1-A2, B1-B6, etc.) and 12 samples together (e.g. A1-A6 + B1-B6, C1-C6 + D1-D6, etc.). This could be the major issue in the identification of viruses as they may have different titers and detection of low titer viruses may be challenging with multiplexing. Out of 24 uninfected samples, 15 belonged to the set of 12 samples multiplexed. Multiplexing too many samples into one reaction could have hindered or overwhelmed the sequencing process. Among known viruses, BYVaV was the most prominent virus followed by BVY and BVX. Again, multiplexing could be the reason behind detection of BYVaV by PCR but not by LSS in some samples. BYVaV is a low titer virus and hence when multiplexing with five other samples, there is the possibility that detection is affected. Moreover, co-infection with BVY infects the virus titer. Susaimuthu et al., 2008 stated that the presence of

BVY represses the titer of BYVaV sometimes to the level undetectable by RT-PCR. Five out of 48 plants were detected to be co-infected with BYVaV and BVY. It appears that seasonal changes may play a role in virus titer. Samples collected in September showed faint BYVaV amplicons while ones in May showed strong ones.

In addition, LSS results showed a lot of unmatched sequences to any samples/barcodes. Each sample had its own barcode for multiplexing. However, we observed a lot of mismatches in the barcode regions and hence a lot of sequences were not matched to any of the used barcodes. In all sets (set A to set H), a number of viruses have been identified but put under the unmatched category. In most of the cases, viruses have been observed in samples collected in May but the same viruses are missing from September collection. However, those viruses were detected in unmatched or mismatched category. Hence, this could be a potential reason behind finding many viruses in May and not in September and vice-versa.

Several potential new viruses were discovered and three sets of primers were designed for each new virus. As described above three different PCRs were run and results were analyzed. Viruses belonging to the genera *Iflavirus, Marafivirus, Carlavirus, and Fijivirus* gave consistent result in at least two PCRs followed by Sanger sequencing. *Iflavirus* (tomato matilda virus) is a recently identified *iflavirus*-like virus infecting tomato (Saqib et al., 2015). This is the first report of a plant-infecting virus resembling members of the *Iflaviridae* and a new genus Tomavirus (*Iflavirus*) is proposed to be created within the family *Iflaviridae*. Another *Iflavirus* (sacbrood virus) is an *Iflavirus* known to infect bee larvae. RT-PCR results for both iflaviruses gave 10 positive amplicons. Alignment of TMaV with SBV using ClustalW gave a score of 60% and blastx of TMaV performed against all sequences in NCBI database gave a number of hits to the polyprotein of SBV.

The carlavirus was detected in 2 and 1 sample by LSS and RT-PCR amplifications respectively. The LSS generated sequence was around 500 bp. The marafivirus was detected in 1 and 3 samples by LSS and RT-PCR amplifications, respectively. RT-PCR showed three new infections i.e. the virus was present only in September and not in May. Blastx of the virus performed against all sequences in NCBI database gave a number of hits to different viruses with 75% identity to RdRp of grapevine fleck virus and with 76% identity to the polyprotein of blackberry virus S (BIVS).

The fijivirus was detected in one sample by LSS. Surprisingly, 13 out of 48 samples were RT-PCR positive followed by Sanger sequencing confirmation. Out of the 13 samples, one sample was found only in September which is potentially a new infection. Based on the results from RT-PCR, this could potentially be a virus of importance given its high incidence within a small number of samples. However, further testing and study is required in order to verify the infection. LSS results showed a number of DNA viruses but further analyses proved them to be retrotransposons and not viruses. Few samples with viruses in the genera *Caulimovirus, Soymovirus, Nanovirus* in LSS outcome however were not confirmed to be retrotransposons and were further analyzed starting with RT-PCR. In some samples, faint bands around the expected size were seen but Sanger sequencing could not be confirmed. Hence, they were not taken into further consideration. In this chapter, LSS depended on use of completely random primers. DsRNA enriched extraction followed by DOP-RT-PCR generated amplicons that were randomly amplified hence giving a homogeneous smear. These randomly amplified products were then sequenced to obtain the idea on viruses present in the samples. As all the steps followed were based on completely random events, separate verification tests were very important. In this chapter, three separate PCRs have been carried out using virus specific primers for verification

purposes. This could possibly explain the reason behind the inconsistent results seen between two tests in the experiment. Amplification using random primers for LSS could have been compromised because of the titer of different viruses. As the event is completely random, viruses having high titers might have hindered amplification of the rest with low titers. Whereas, verification using specific RT-PCR primers could amplify viruses present even in low titers. This can explain why many viruses were detected in RT-PCR test while not in LSS.

This chapter provides a valuable insight on the virosome of a blackberry field. Randomly selected forty eight samples from two different seasons give insight on how viruses are moving in a small scale. Although LSS and verification by RT-PCR did not give consistent results, the overall outcome of two different tests are useful providing insights of what might be happening in a field at a micro level. Based on the results, in addition to viruses previously known to infect blackberries, some potential new viruses were also detected by both methods. Moreover, verification with separate PCRs helped in confirmation as well as detection of those viruses in other samples. A number of viruses are being discovered rapidly complicating the detailed study of their biology and epidemiology. Development of reliable detection tests is therefore of utmost importance. LSS does not require prior knowledge on the genetic composition of the virus, hence helping in the detection of any isolate of a virus and discovery of new viruses.

3.5 References

Al Rwahnih, M., Daubert, S., Urbez-Torres, J.R., Cordero, F., Rowhani, A., 2011. Deep sequencing evidence from single grapevine plants reveals a virome dominated by mycoviruses. Arch. Virol. 156, 397–403.

Al Rwahnih, M., Dave, A., Anderson, M.M., Rowhani, A., Uyemoto, J.K., Sudarshana, M.R., 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. Phytopathology 103, 1069–1076.

Clark, J.R., 1992. Blackberry production and cultivars in North America east of the Rocky Mountains. Fruit Varieties Journal 46, 217-222.

Clark, J.R., 2005. Changing times for eastern United States blackberries. HortTechnology 15, 491-494.

Ellis, M., R.H. Converse, R.N. Williams, and B. Williamson. 1997. Diseases caused by viruses and virus like agents. In: Ellis M. et al, ed. Compendium of raspberry and blackberry diseases and insects. St. Paul, Minnesota, USA: APS Press,The American Phytopathological Society: 42-62.

Ho, T., Martin, R.R., Tzanetakis, I.E., 2015. Next-Generation Sequencing of Elite Berry Germplasm and Data Analysis Using a Bioinformatics Pipeline for Virus Detection and Discovery. Plant Pathol. Tech. Protoc. 301–313.

Ho, T., Tzanetakis, I.E., 2014. Development of a virus detection and discovery pipeline using next generation sequencing. Virology 471, 54–60.

Martin, R.R., MacFarlane, S., Sabanadzovic, S., Quito, D., Poudel, B., Tzanetakis, I.E., 2013. Viruses and virus diseases of Rubus. Plant Dis. 97, 168–182.

Poudel, B., Wintermantel, W.M., Cortez, A.A., Ho, T., Khadgi, A., Tzanetakis, I.E., 2013. Epidemiology of Blackberry yellow vein associated virus. Plant Dis. 97, 1352–1357.

Quito-Avila, D.F., Brannen, P.M., Cline, W.O., Harmon, P.F., Martin, R.R., 2013. Genetic characterization of Blueberry necrotic ring blotch virus, a novel RNA virus with unique genetic features. J. Gen. Virol. 94, 1426–1434.

Saqib, M., Wylie, S.J., Jones, M.G.K., 2015. Serendipitous identification of a new Iflavirus-like virus infecting tomato and its subsequent characterization. Plant Pathol. 64, 519–527.

Strik, B.C., Clark, J.R., Finn, C.E., Bañados, M.P., 2007. Worldwide blackberry production. Horttechnology 17, 205–213.

Susaimuthu, J., Gergerich, R.C., Bray, M.M., Clay, K.A., Clark, J.R., Tzanetakis, I.E., Martin, R.R., 2007. Incidence and ecology of Blackberry yellow vein associated virus. Plant Dis. 91, 809–813.

Susaimuthu, J., Tzanetakis, I.E., Gergerich, R.C., Martin, R.R., 2008. A member of a new genus in the Potyviridae infects Rubus. Virus Res. 131, 145–151.

Thekke-Veetil, T., Aboughanem-Sabanadzovic, N., Keller, K.E., Martin, R.R., Sabanadzovic, S., Tzanetakis, I.E., 2013. Molecular characterization and population structure of Blackberry vein banding associated virus, new ampelovirus associated with yellow vein disease. Virus Res. 178, 234–240.

Vives, M.C., Velázquez, K., Pina, J.A., Moreno, P., Guerri, J., Navarro, L., 2013. Identification of a new enamovirus associated with citrus vein enation disease by deep sequencing of small RNAs. Phytopathology 103, 1077–1086.

Chapter IV

Conclusions

4.1 Abstract

Blackberry production around the world is greatly affected by the presence of viruses that are known to infect the crop. Till date more than 40 virus species is known to infect the crop. Virus complexes have been identified recently as the major cause of plant decline with blackberry yellow vein disease (BYVD) being the most important disease of the crop in the Southern United States. The objective of this research was to study the blackberry virosome in both macro and micro scale. The macro approach, which involved identification of viruses present in the Southern United States, identified major viruses known to be associated with BYVD as well as other viruses whose prevalence was still unknown. RT-PCR was employed to detect sixteen different viruses in wild, cultivated and sentinel blackberries collected from six different states. In addition to the identification of viruses associated with BYVD, this experiment allowed us to identify viruses that were not associated with this disease and whose prevalence is still unknown. In the micro approach, the virosome of a single field was studied using large scale sequencing. By studying a field virosome, we were able to identify five potential new viruses in addition to few other viruses previously known to infect blackberries. Understanding the virosome on a regional and local scale provided us important information which could greatly enhance disease management. The ultimate goal of this research was to better understand virus distribution in nature and aid in the development of proper management strategies to control epidemics.

4.2 Significance of studying Blackberry virus distribution in the Southern United States

With the recent increase in acreage for blackberry, there has been an emergence of several new diseases including Blackberry yellow vein disease (BYVD). The disease became more prominent at the turn of the century in the Carolinas. Since then, BYVD has become a serious threat to blackberry production (Martin *et al*., 2004; Tzanetakis *et al*., 2007; Martin *et al*., 2013). It is now understood that the majority of virus diseases in berry crops are caused by the combination of two or more viruses. Most of the viruses are latent as single infection. Being obligate parasites, viruses have co-evolved with the host to sustain by having minimal impact on their hosts. Many new viruses have been discovered recently on blackberries indicating that there might be more yet to be identified.

The main objective of this research was to understand the distribution of major blackberry viruses in the southern United States. In addition to the identification of viruses that are associated with BYVD, several other viruses were identified in a significant number whose prevalence was previously unknown. Understanding distribution of viruses at a regional level is very important for the control and management of viral diseases. Virus control is based on the use of clean propagation material, control of vectors and resistance. This communication provided evidence that wild plants may serve as virus inoculum to the commercial fields. In addition, although in low percentages, viruses were also present in cultivated plants. Propagation material may not be free of viruses but no universal infections in individual fields were observed, indicative that virus movement in propagation material is not as prevalent now as at the beginning of the BYVD epidemic (Susaimuthu *et al*., 2007).

Virus management strategies based on resistance is challenging in case of BYVD as the disease is caused by the synergistic effects of multiple viruses. The easiest and most effective way

for disease control is the use of clean propagative material and vector control, a feasible approach for many growers, who in the past have been propagating their own planting stock. Establishing fields with virus-tested plants allow fields to stay productive for longer periods of time; yielding better and providing producers with better quantity and quality product.

Given that the majority of virus diseases in the berry crops are caused by the combination of two or more viruses, it is often impossible to eliminate all viruses from the system. Efforts to identify the weakest link, the easiest virus/vector combination to eliminate, in a particular environment is the better approach to minimize disease impact. Vector control has a prerequisite knowledge on the epidemiology and transmission properties of viruses. This approach will minimize disease impact and prolong field longevity, even though some plants may be infected with viruses, yet symptoms are not devastating.

Study of viruses present in sentinel plants provided a significant benefit as it provided information on how viruses move in the field. Based on paired entomological studies on the presence of potential vectors at each time point we can now predict the virus-vector relationships and thus produce models on vector movement. Controlling this part on the disease triangle could control the spread of the disease.

4.3 Significance of studying field virosome to understand virus movement in the field scale

Viruses and virus-like diseases can cause significant losses and affect the longevity of blackberry plantings, even though the outlook for blackberry production is very encouraging (Ellis et al., 1997). Some viruses are widespread and destructive which can adversely affect the production. It is now understood that most of the viral diseases in blackberry and berry crops in general are caused by the combination of two or more viruses making disease management a challenge (Martin et al., 2013). There has been a dramatic increase in the identification of number of viruses affecting blackberries, primarily because of novel technologies and methods (Martin et al., 2013; Ho et al., 2015; Ho and Tzanetakis 2014). Control is challenging because of the complex mode of transmission and activity of blackberry virus vectors. In the last decade there have been a number of new viruses identified in blackberry, many of which have not been studied in great detail when it comes to their biology and epidemiology. Knowledge of virus distribution and epidemiology are important factors to consider when establishing blackberries.

Large scale sequencing (LSS) together with bioinformatics analyses has brought a drastic change in the field of virology by enabling scientists to detect all known viruses but also discover novel ones (Al Rwahnih et al., 2011; Quito- Avila et al., 2013; Al Rwahnih et al., 2013; Thekke-Veetil et al., 2013; Vives et al., 2013; Ho and Tzanetakis, 2014). Prior knowledge of viral sequences or their genetic makeup is not necessary allowing for the detection of any virus isolate or novel species per se.

Chapter 2 of this thesis provided us with the idea about disease epidemics at regional levels whereas this work aimed to understand virus distribution at the field level, an important factor for disease control. Understanding the small scale movement could assist with the

management of disease complexes and eliminate large scale disease epidemics. The identification of the major viruses present in the field and movement of potential vectors in a seasonal timeframe could lead to the identification of vectors and development of custom-made control strategies based on virosome of the field and the region alike.

This chapter provided a valuable insight on the virosome of a blackberry field. It gave us an insight on how viruses are moving in a small scale. The overall outcome of this research provided us with insights of what might be happening in a field at a micro level. Based on the results, in addition to viruses previously known to infect blackberries, some potential new viruses were also detected. A number of viruses are being discovered rapidly complicating the detailed study of their biology and epidemiology. Development of reliable detection tests is therefore of utmost importance. LSS does not require prior knowledge on the genetic composition of the virus, hence helping in the detection of any isolate of a virus and discovery of new viruses.

4.4 2.6 References

Al Rwahnih, M., Daubert, S., Urbez-Torres, J.R., Cordero, F., Rowhani, A., 2011. Deep sequencing evidence from single grapevine plants reveals a virome dominated by mycoviruses. Arch. Virol. 156, 397–403.

Al Rwahnih, M., Dave, A., Anderson, M.M., Rowhani, A., Uyemoto, J.K., Sudarshana, M.R., 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. Phytopathology 103, 1069–1076.

Ellis, M., R.H. Converse, R.N. Williams, and B. Williamson. 1997. Diseases caused by viruses and virus like agents. In: Ellis M. et al, ed. Compendium of raspberry and blackberry diseases and insects. St. Paul, Minnesota, USA: APS Press,The American Phytopathological Society: 42-62.

Ho, T., Tzanetakis, I.E., 2014. Development of a virus detection and discovery pipeline using next generation sequencing. Virology 471, 54–60.

Ho, T., Martin, R.R., Tzanetakis, I.E., 2015. Next-Generation Sequencing of Elite Berry Germplasm and Data Analysis Using a Bioinformatics Pipeline for Virus Detection and Discovery. Plant Pathol. Tech. Protoc. 301–313.

Martin, R.R., Tzanetakis, I.E., Gergerich, R., Fernandez, G., Pesic, Z., 2004. Blackberry yellow vein associated virus: A new crinivirus found in blackberry, in: X International Symposium on Small Fruit Virus Diseases 656. pp. 137–142.

Martin, R.R., MacFarlane, S., Sabanadzovic, S., Quito, D., Poudel, B., Tzanetakis, I.E., 2013. Viruses and virus diseases of Rubus. Plant Dis. 97, 168–182.

Quito-Avila, D.F., Brannen, P.M., Cline, W.O., Harmon, P.F., Martin, R.R., 2013. Genetic characterization of Blueberry necrotic ring blotch virus, a novel RNA virus with unique genetic features. J. Gen. Virol. 94, 1426–1434.

Susaimuthu, J., Gergerich, R.C., Bray, M.M., Clay, K.A., Clark, J.R., Tzanetakis, I.E., Martin, R.R., 2007. Incidence and ecology of Blackberry yellow vein associated virus. Plant Dis. 91, 809–813.

Thekke-Veetil, T., Aboughanem-Sabanadzovic, N., Keller, K.E., Martin, R.R., Sabanadzovic, S., Tzanetakis, I.E., 2013. Molecular characterization and population structure of Blackberry vein banding associated virus, new ampelovirus associated with yellow vein disease. Virus Res. 178, 234–240.

Tzanetakis, I.E., Postman, J.D., Martin, R.R., 2007. First report of Blackberry chlorotic ringspot virus in Rubus sp. in the United States. Plant Dis. 91, 463.

Vives, M.C., Velázquez, K., Pina, J.A., Moreno, P., Guerri, J., Navarro, L., 2013. Identification of a new enamovirus associated with citrus vein enation disease by deep sequencing of small RNAs. Phytopathology 103, 1077–1086.