EPIDEMIOLOGY AND STRAIN IDENTIFICATION OF BLUEBERRY SCORCH VIRUS ON HIGHBUSH BLUEBERRY IN BRITISH COLUMBIA

by

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ABSTRACT

The epidemiology of *Blueberry scorch virus* (BlScV) in highbush blueberry (*Vaccinium corymbosum*) was studied over four consecutive years (2001 to 2004) in British Columbia. BlScV was first identified in British Columbia in 2000 and was identified in 40 commercial blueberry fields in 2001, and by 2004, it had spread to 122 fields. BlScV was also detected in cranberry (*V. macrocarpon*) and black huckleberry (*V. membranaceum*). The rate of BlScV spread in three commercial blueberry fields was determined by mapping diseased plants over three years. The percent increase in diseased plants ranged from 4.4% to 5.2% from 2001 to 2002, and from 4.2% to 9.6% from 2002 to 2004. Partial coat protein gene sequences of 12 BlScV isolates from BC shared 88-100% amino acid sequence identity with each other, and were more closely related to previously described strains BC-2 and NJ-2, than to strains BC-1, NJ-1 and WA-2.

Keywords: *Vaccinium corymbosum*, *Blueberry scorch virus*, *Carlavirus*, *Vaccinium macrocarpon*, epidemiology, coat protein.

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CHAPTER 1. INTRODUCTION

1.1 Blueberries

Blueberry is a member of the genus *Vaccinium*, which includes over 450 plant species. *Vaccinium* belongs to the family *Ericaceae*, which includes mainly woody shrubs such as rhododendrons, azaleas, heathers, heaths and mountain laurels. The *Vaccinium* genus became well established approximately 12,000 years ago following the glaciations of the Pleistocene period (Eck and Childers, 1989). Prior to the European colonization of North America, blueberries were referred to as "star berries" due to their star-shaped calyx, and according to legend, the Great Spirit sent star berries to relieve children's hunger during a famine (Gough, 1994). Native Americans gathered blueberries from the forests and bogs and consumed them fresh, or dried them in the sun for use in stews, cakes, breads, and meats, and in a pudding called *sautauthig* (Gough, 1994). Blueberry juice was used as a dye for baskets and cloth, and also to treat coughs (Anonymous, 2005a). A tea made from wild blueberry roots was used as a muscle relaxant during childbirth by the native settlers' wives (Anonymous, 2005b) and a tea made from the leaves and berries was used to relieve diarrhea and suppress menstruation (Russell, 1980).

In the 1890's, plant scientists in the eastern United States attempted to select and transplant superior wild blueberry bushes for commercial production. However, this was largely unsuccessful (Gough, 1994). In 1906, Dr. Frederick V. Coville, a botanist with

the United States Department of Agriculture (USDA), began researching methods for propagating blueberry bushes and discovered that blueberries require an acidic soil for growth (Eck and Childers, 1989). He collaborated with Elizabeth C. White, a commercial cranberry grower from New Jersey, USA. Mrs. White enlisted her pickers to search for exceptionally fine bushes in the wild pine barrens. Using plant breeding and cross-pollination techniques, Dr. Coville made crosses between the finest selections of *V. corymbosum* L. and swamp highbush blueberry (*V. australe*). This was the birth of the highbush blueberry industry. Prior to his untimely death in 1937, Dr. Coville had introduced the first 15 commercial varieties of blueberries, many of which are still grown today.

1.1.1 The commercial highbush blueberry industry

The three most important commercial blueberry species in North America are the northern highbush (*V. corymbosum* L.), lowbush or wild (*V. angustifolium* Ait.), and the southern rabbiteye (*V. ashei* Reade) (Anonymous 2005a). Over the past three decades, the demand for blueberries has risen dramatically, largely due to the health benefits they provide. Among other fruits and vegetables, blueberries have one of the highest levels of antioxidant activity (Anonymous 2005a). Antioxidants help to neutralize free radicals, which are linked to cancer, cardiovascular disease, cataracts and a number of other degenerative diseases.

North America is the largest commercial producer of highbush blueberries in the world, accounting for over 90% of world production (Anonymous 2005a). In 2004, an estimated 270 million pounds of highbush blueberries were produced in the United States

and Canada (primarily British Columbia). Approximately 60% were marketed as fresh blueberries and the remaining 40% were processed. More recently, New Zealand, Australia, Europe and South America have dedicated acreage to highbush blueberry production (Anonymous 2005a).

BC is the second largest producer of blueberries in the world, after first-ranked Michigan (Anonymous 2005a). Over 95% of highbush blueberry production in Canada occurs in BC. Most highbush blueberry production in BC occurs in the Fraser Valley – primarily in Abbotsford, Pitt Meadows, Surrey, and Richmond, and the remainder occurs in the province's interior and on Vancouver Island. The BC Blueberry Council, which is part of the larger North American Blueberry Council (NABC), represents blueberry growers in the province. The council promotes growth and development of the industry through its extensive marketing and research efforts.

Currently in BC, there are approximately 5,000 hectares of highbush blueberry in production, of which 600 hectares were established during the last three years (Geraldine Auston, pers. comm.). The 2004 crop produced over 30 million kg and had a retail value of over $90 million (Geraldine Auston, pers. comm.). Farms range in size from one ha to over 120 ha. The average yield in BC is 8,400 kg/ha; however, this yield can vary due to a number of factors, including losses due to pests and diseases, varying ages of plantings, cultivar spacing, method of harvesting, land type, and grower management practices. Prices have ranged widely over the last five years, from $0.88/kg to $2.65/kg, with fresh market sales generating the highest price.

1.1.2 Virus and virus-like diseases of highbush blueberry

Despite the rapid growth and value of the blueberry industry in BC, there are a number of challenges that continue to impede the potential growth of the industry. Among these, the most important are the management of diseases and pests. There are a number of virus and virus-like diseases of highbush blueberry. The Compendium of Blueberry and Cranberry Diseases (Caruso and Ramsdell, 1995) provides a review of these viruses. *Blueberry shoestring sobemovirus* is the main virus affecting highbush blueberry in the US, but not in Canada. There are four nepoviruses known to infect highbush blueberry: *Tobacco ringspot virus, Tomato ringspot virus* (ToRSV), *Blueberry leaf mottle virus, and Peach rosette mosaic virus*. Other viruses include *Blueberry scorch carlavirus*, *Blueberry red ringspot caulimovirus, Blueberry shock ilarvirus*, and Blueberry mosaic viroid-like RNA. There are two diseases of highbush blueberry caused by mycoplasma-like organisms; blueberry stunt and witches'-broom. Of these diseases, only *Blueberry shock virus, Blueberry scorch virus* and Blueberry mosaic viroid-like RNA have been detected in Canada.

1.2 *Blueberry scorch virus*

In 1970, a blighting disease was observed in the Sheep Pen Hill production area near Pemberton, NJ (Stretch 1983). The causal agent of this disease, known as Sheep Pen Hill Disease (SPHD), originally was unknown. A similar blighting disease was observed in a planting near Puyallup, Washington in 1980 (Martin and Bristow, 1988). Virus was purified from infected 'Pemberton' bushes from Puyallup and used as a source for transmission studies, electron microscope (EM) studies, and serology experiments (Martin and Bristow, 1988). The successful mechanical transmission of purified virus to

herbaceous hosts, the presence of bundles of rod-shaped particles typical of *Carlavirus*es in EM, and the serological reactivity of the virus with other *Carlavirus*es confirmed that the disease observed in Washington was caused by a *Carlavirus* (Martin and Bristow, 1988). The virus responsible for the blighting disease in Washington was named *Blueberry scorch virus* (Martin and Bristow, 1988). Preliminary EM studies of the Sheep Pen Hill associated virus (SPHAV) suggested that the particles causing SPHD were related to BlScV (Podleckis and Davis, 1988). SPHAV was purified and used to develop antibodies for immunosorbent electron microscopy (ISEM) (Podleckis and Davis, 1989). SPHAV and BlScV antibodies reacted with both of these viruses but not to other *Carlaviruses*, including *Potato virus M* and *Potato virus S,* confirming that SPHAV was a member of the *Carlavirus* group and was related serologically to BlScV.

In 1987, a survey for BlScV and ToRSV was conducted in BC, Oregon and Washington (Martin and MacDonald, 1988). Symptoms characteristic of BlScV were observed, with the exception that some plants recovered after showing symptoms for 1 to 4 years. When enzyme-linked immunosorbent assay (ELISA) failed to detect BlScV in these plants, the virus responsible for the symptoms similar to BlScV was characterized. A new virus with isometric particles was discovered, and temporarily named *Blueberry shock ilarvirus* (BSIV). Today, this *Ilarvirus* is known as *Blueberry shock virus*, BlShV (MacDonald, 1989). Neither virus was detected in wild *Vaccinium* or herbaceous weeds collected in and around infected blueberry plantings. Another survey was conducted in 1989, this time for BlScV and BlShV (MacDonald and Martin, 1990). BlScV was detected in two fields in the Willamette Valley in Oregon, and near Woodland and Puyallup, Washington and BlShV was detected in the Willamette Valley in Oregon and

along the I-5 corridor in Washington as far as Bellingham. However, neither virus was detected in the Fraser Valley of BC, despite the presence of characteristic symptoms (MacDonald and Martin, 1990). The rapid rate of spread of BlScV and BlShV suggested that blueberry plantings in BC were at high risk for infection (MacDonald and Martin, 1990). Subsequently, the Canadian Food Inspection Agency was requested to conduct a pest-risk analysis on BlScV. The analysis indicated a medium-high overall risk rating of BlScV for *Vaccinium* (AAFC Plant Health Risk Assessment, 1996).

Since both SPHAV and BlScV appeared at approximately the same time in widely separated areas, the fact that no other *Carlavirus*es have been reported on highbush blueberry, and as plant material was being exchanged between the two areas, it was assumed they were strains of the same virus (Martin et al., 1992). In addition, serological and hybridization data showed that BlScV and SPHAV were related more closely to each other than to other *Carlaviruses*, suggested the possibility that they were different strains of the same virus (Martin et al., 1992). To confirm the relationship between SPHAV and BlScV and to gain a better understanding of the *Carlavirus* genome, nucleotide sequencing was required. Two isolates of SPHAV (NJ-1 and NJ-2) and two isolates of BlScV (WA-1 and WA-2) were purified and the extracted RNA used for cloning and sequencing (Cavileer et al., 1994). The overall organization of the BlScV genome was very similar to that of other *Carlavirus*es for which sequence data was available. The 5' terminal region of one SPHAV isolate shared homology with two other *Carlavirus*es, *Potato virus M* and *Potato virus S.* Nucleotide differences between the 3' termini of BlScV and SPHAV revealed that they were distinct strains of the same virus. A comparison of the coat protein (CP) amino acid sequences revealed that the two

SPHAV isolates, NJ-1 and NJ-2, were distinct strains of BlScV. Thus, SPHAV is now referred to as *Blueberry scorch virus*. BlScV was identified for the first time in BC in June of 2000 in a mature 'Berkeley' planting near Abbotsford. This finding, along with numerous reports of symptoms resembling BlScV prompted a survey in 2000 during which the virus was detected in an additional 19 fields (Hudgins, 2000). BlScV was detected in a wide variety of cultivars and ages of plants throughout the Fraser Valley in BC (Hudgins, 2000). By the end of 2001, BlScV was confirmed in 60 fields throughout all blueberry production areas of BC (Wegener et al., 2002). In 2002, two BlScV-positive plants exhibiting different symptoms were collected from individual fields and sent to the Pacific Agriculture Research Centre in Summerland, BC. BlScV was transmitted by aphids from both plants to *Nicotiana occidentalis* Wheeler, an herbaceous host for BlScV (Bernardy et al., 2005). These isolates, identified as BC-1 and BC-2, were then sequenced and a phylogenetic analysis was performed based on a portion of the coat protein (CP) gene. The nucleotide sequence identity between strains BC-1 and BC-2 with NJ-2 was 83% and 77%, respectively. Phylogenetic analysis revealed that BC-1 and BC-2 are unique strains of BlScV.

A survey conducted in 2001 in Connecticut (CT) and Massachusetts (MA) revealed the presence of BlScV for the first time in New England (DeMarsay et al., 2004). BlScV was detected by an indirect ELISA protocol using antibodies developed at Rutgers University, and by reverse transcription polymerase chain reaction (RT-PCR) using primers which amplified a portion of the CP gene. BlScV was detected in the cultivars 'Berkeley' and 'Blueray' in both states, as well as in 'Elliott', 'Bluecrop', and 'Coville' in Massachusetts. BlScV was detected in both symptomatic and asymptomatic

bushes. Although BlScV was detected in limited areas of CT and MA, it was suspected that bushes in other areas were infected. Sequencing and phylogenetic analysis revealed that two of the MA bushes and two of the CT bushes were infected by closely related strains of BlScV. These strains were most similar to the NJ-2 strain, while the other strain from MA shared higher homology with NJ-1 (DeMarsay et al., 2004).

In New Jersey, Petrovic et al. (2004) evaluated variability within the BlScV population by comparing the CP sequences of 45 different isolates selected from various growing regions and blueberry cultivars. Based on CP sequence information, a strain distinct from the previously described NJ-1, NJ-2 and WA-1 was identified. This strain, known as NJ-3, had only 75% sequence identity to NJ-1 and NJ-2 at the amino acid level, with most of the variability around a proline-rich domain at the N-terminus of the CP.

BlScV recently was detected for the first time in Europe in 2005 (Ciuffo et al., 2005). Plants of the cultivars 'Blueray', 'Berkeley', and 'Bluecrop' in a field in southern Piedmont, Italy showed symptoms of BlScV, and were tested by ELISA using commercial antisera from Agdia (Elkhart, Indiana). Thirteen of the 23 leaf samples collected tested positive by ELISA, and flexuous rod-shaped particles characteristic of BlScV were observed by EM in negatively stained extracts. Efforts in Europe to mechanically transmit BlScV to herbaceous hosts were unsuccessful.

1.2.1 Biology

The *Carlavirus* genus belongs to the family *Flexiviridae*, which was created recently by the ICTV (Adams et al., 2004). *Carlavirus*es are flexuous, filamentous particles measuring 610-700 nm in length and 12-15 nm in diameter. The *Carlavirus*

genome is monopartite, positive-sense ssRNA, 7.4-7.9 kb in length. Most *Carlavirus*es are transmitted by aphids in a non-persistent manner, and have limited natural host ranges; however, most members are transmissible mechanically to a large number of hosts. Distinct *Carlavirus* species have <68% amino acid sequence identity in the core region of the coat protein (excluding the amino and carboxyl termini) while strains of individual viruses have 75-90% homology (Adams et al., 2004).

The particles of BlScV are flexuous and rod-shaped, measuring 690 ± 44 x 14 nm (Figure 1.1) (Martin and Bristow, 1988). The relative molecular mass of the coat protein was estimated to be 35,200 daltons ± 300, and the size of the nucleic acid 8,400 ± 200 bases (Martin and Bristow, 1988). The actual size of the genome of the strain of BlScV associated with Sheep Pen Hill disease is 8514 bases (Cavileer et al., 1994).

Figure 1.1 **Electron micrograph of negatively stained BlScV particles. The bar represents 100 nm (Martin and Bristow, 1988).**

1.2.2 Symptomatology

Biologically distinct strains of BlScV have been identified based on symptom variation on some blueberry cultivars and transmission properties (Bristow et al., 2000). Symptoms of BlScV in commercial plantings have only been reported on northern highbush blueberry. However, southern highbush and rabbiteye blueberry developed symptoms when graft inoculated with the WA-1 strain (Bristow et al., 2000). BlScV has a latent period of 1 to 2 years between infection and symptom expression (Bristow et al., 2000). Symptoms of BlScV can be confused easily with other diseases and conditions of highbush blueberry, including BlShV, frost damage, mummy berry caused by *Monilinia* spp., botrytis flower and leaf blight caused by *Botrytis cinerea* Pers., bacterial blight caused by *Pseudomonas syringae* pv. *syringae,* and frost damage (Martin and Bristow, 1988).

Stretch (1983) described the symptoms of SPHAV as a dieback of flowers and young vegetative shoots in the spring, resulting in a partial or total loss of fruit, followed by a flush of growth later in the summer. 'Weymouth' and 'Bluecrop' were particularly susceptible, and a necrotic line pattern was sometimes present on the foliage of infected plants (Stretch, 1983). Similar symptoms were reported in Washington for BlScV, with the exception that the autumn foliar line pattern that was common in New Jersey was only expressed on the cultivar 'Rubel' (Martin and Bristow, 1988). Infected plants exhibited blighting of flower clusters prior to the corolla opening, leaf necrosis, a 4-10 cm dieback of twigs bearing blighted blossoms, and marginal chlorosis of leaves produced on older wood. The cultivars 'Berkeley', 'Atlantic', 'Collins', 'Herbert', and 'Pemberton' were highly sensitive. In some cases, the entire bush was symptomatic,

while in other cases, only one or several branches were affected. Plants developed symptoms every year and sometimes died. In some cases, 'Berkeley' plants were killed within 3-6 years while 'Pemberton' plants exhibited a more gradual decline.

In 2000, Bristow et al. conducted a study on the transmission, field spread, cultivar response and impact of BlScV on the yield of highbush blueberry plants. Plants of 42 blueberry cultivars aged two-three years were graft inoculated with BlScV-infected cuttings, and maintained in a screenhouse until they were assessed for symptoms and tested by ELISA the following spring and every year for three years. Plants of various cultivars and selections were also assessed in a natural field setting. A range of symptoms was observed, including complete necrosis of leaves and blossoms as well as dieback. Leaves either turned black along the mid-rib and wilted, or turned tannish orange. Blighted blossoms eventually turned grey over time, and sometimes remained on the plant until the following season. In cases where blossom blighting was severe, the fruit load was reduced, causing infected plants to stand taller than healthy plants. Leaf margin chlorosis was observed in combination with leaf and blossom necrosis on the cultivars 'Atlantic', 'Berkeley', 'Bluejay', 'Blueray', 'Dixi', 'Herbert', 'Lateblue', 'Pemberton', 'Rubel', 'Spartan' and 'Weymouth'. The cultivars 'Bluecrop', 'Concord', 'Jersey', 'Washington' and U254 exhibited only marginal leaf chlorosis. 'Friendship' was the only grafted cultivar that remained uninfected for four years, but in subsequent studies it also became infected with BlScV. Cultivar reaction is likely strain dependent, since 'Bluecrop' plants develop symptoms when infected with the East Coast strains of BlScV (NJ-1, NJ-2, NJ-3) and remain asymptomatic when infected with the West Coast strains (WA-1, WA-2, BC-1, BC-2) (Bristow et al., 2000).

The productivity of BlScV infected plants declines as symptoms develop (Bristow et al., 2000). The yield of infected 'Pemberton' bushes was reduced by one-third in the first year of symptom expression, and by up to 85% within three years following symptom expression. The yield of asymptomatic BlScV-infected plants was unaffected. Yield losses have not been reported for the East Coast strains of BlScV (NJ-1, NJ-2, NJ-3), and no data on crop losses has been compiled in BC.

1.2.3 Methods of detection

Because the symptoms of BlScV are easily confused with other diseases and conditions of highbush blueberry, and because there is 1-2 year latent period between infection and symptom development, diagnostic testing is imperative for correct diagnosis and implementation of management strategies. BlScV can be identified morphologically by electron microscopy; however, the most economical and commonly used method of detection is the Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) using polyclonal antibodies. Martin and MacDonald (1988) tested various grinding buffers for use in DAS-ELISA, and determined an extraction buffer containing 0.5% nicotine was required in order to maintain the pH of blueberry leaf homogenates above 5.0, which is necessary for DAS-ELISA. BlScV is unevenly distributed in the plant, and the virus titer can fluctuate throughout the growing season. Older leaves proved to be a more reliable source for virus detection, and DAS-ELISA testing was reliable from June through September (Martin and Bristow, 1988). Blossoms have been shown to have a virus titer several times higher than leaf tissue (Cavileer et al., 1994). Samples are considered positive by

DAS-ELISA when the absorbance values at A_{405} values are greater than two times the mean of negative controls (MacDonald and Martin, 1990).

Variations of the ELISA test have been developed and used for detecting BlScV. ELISA and western blot detection systems for the NJ-2 strain of BlScV were developed using monoclonal and polyclonal antibodies produced against the NJ-2 strain (Petrovic et al., 2004). Virus concentrations as low as 1 ng/ml in plant extracts could be detected using ELISA. Agri-Check detection kits produced by Hydros, Inc. (Falmouth, MA) use an indirect ELISA protocol with antibodies developed against the NJ-1 and NJ-2 strains of BlScV. The simple protocol of this kit provides both field personnel and growers with the capability of rapidly detecting BlScV in field samples.

Since ELISA results were variable depending on the tissue source and the time of year in which samples were collected, and because low virus titers may escape detection, a more sensitive test was needed for the detection of BlScV. In 1995, a protocol for detecting the NJ-2 strain of BlScV by RT-PCR was developed (Halpern and Hillman, 1995). Due to the presence of phenolic compounds and other inhibitory substances in blueberry plants, nucleic acid extraction was difficult and only one out of four extraction methods tested yielded suitable levels of viral RNA for detection by RT-PCR. The combination of a lithium chloride (LiCl) or glycine/phenol extraction with the utilization of an Elutip-r minicolumn purification procedure(Schleiher and Schuel, Florham, NJ, USA) prior to reverse transcription consistently yielded viral RNA (vRNA) of adequate purity for RT-PCR detection from leaf and blossom tissue. Using primers developed in the putative 25-kDa helicase domain in the first open reading frame, the NJ-2 strain of

BlScV could be detected reliably by RT-PCR from field samples. RT-PCR was capable of detecting 10 fg of vRNA and 100 fg of plasmid DNA. RT-PCR was proven to be more sensitive than spot hybridization using a ^{32}P labelled probe (Halpern and Hillman, 1995).

1.2.4 Epidemiology

Research on the aphid transmission of *Carlavirus*es has been limited, and in particular, the transmission of BlScV has only been extensively studied in the last four years. A study by Bristow et al. (2000) provided the first direct evidence that BlScV is transmitted in a non-persistent manner by the resident blueberry aphid, *Ericaphis fimbriata* Richards (Figure 1.2). BlScV was transmitted from early-May to mid-August, during which time the plants are colonized by aphids, mostly *E. fimbriata*. Subsequently, two periods of transmission were shown to take place in commercial blueberry fields in BC: an early phase in early May and a later phase from mid-June to early September (French et al., 2003). BlScV spreads radially from a point source, and a sigmoidal curve results when disease incidence is plotted over time (Bristow et al., 2000). The number of infected plants nearly doubled each year for the first 4-5 years in an infected 'Pemberton' field (Bristow et al., 2000). Similar results were noted in a 'Berkeley' planting, where the disease incidence went from 0% to approximately 80% within four years (Bristow et al., 2000).

Figure 1.2 **Blueberry aphid (*Ericaphis fimbriata*) winged form (Photo courtesy of Dr. David Raworth, Agriculture and Agri-Food Canada).**

Field-to-field spread was limited to less than 1 km and BlScV spread readily between fields located 5-10 meters apart (Bristow et al., 2000). Mechanical harvesters may assist in the long distance transmission of BlScV by transporting aphids (Bristow et al., 2000).

While conducting aphid transmission studies, Bristow et al. (2000) discovered that transmission of BlScV was inefficient when single viruliferous aphids (*E. fimbriata)* were used, indicating that this aphid is not an efficient vector of BlScV. However, when aphid populations were not controlled, the number of infected plants in a commercial field nearly doubled each year.

Since nonpersistent viruses are characterized by short acquisition and retention times, migrant (non-colonizing) aphids are capable of virus transmission. Typically, non-persistent viruses are transmitted by a large number of aphid species (Raccah, 1986). A study was conducted from 2001 through 2003 to determine which aphid species are present in highbush blueberry fields in BC and to gain an understanding of the life history of *E. fimbriata* (Raworth, 2004). Emergence of *E. fimbriata* from eggs occurred in March, and by May, high numbers of winged aphids were present on blueberry plants. The population of non-winged *E. fimbriata* increased until early July, reaching a population of 300 to 9000 aphids per plant, depending on the field.

Over 50 species of aphids originating from a wide range of hosts were collected, and the composition of species varied among fields (Raworth and Robertson, 2003). High numbers of *Euceraphis betulae* (Koch) and *Rhopalosiphum padi* (L.) were found. Aphid species that were found in high numbers were selected for use in transmission

studies. *Myzus persicae* Sulzer, the green peach aphid, was capable of transmitting BlScV from highbush blueberry to *Beta vulgaris* L. under greenhouse conditions (French et al., 2003). BlScV can also be transmitted by grafting and through infected cuttings used for propagation (Bristow et al. 2000).

1.2.5 Host range

The natural host range of BlScV is restricted to northern highbush blueberry (Martin and Bristow, 1988). However, southern and half-highbush types have been infected with BlScV by aphids and through grafting under experimental conditions (Bristow et al., 2000). MacDonald and Martin (1990) tested wild *Vaccinium* spp. and weeds in and around blueberry fields in Washington, Oregon and British Columbia, and no BlScV-positive plants were detected.

BlScV has been transmitted successfully to a number of other hosts under experimental conditions. Strains NJ-1 and NJ-2 were transmitted to *Chenopodium quinoa* Willd and *C. amaranticolor* (Cavileer et al., 1994). However, attempts to mechanically inoculate herbaceous hosts with the West Coast strains of BlScV were unsuccessful (Martin and Bristow, 1988). Lowery et al. (2005) screened 12 herbaceous hosts for aphid (*E. fimbriata*) and mechanical transmission. BlScV was transmitted mechanically under greenhouse conditions to *Nicotiana occidentalis* L., *Solanum tuberosum* L., *Gomphrena globosa* L., and *Chenopodium amaranticolor* L., and by aphids and mechanical transmission to *Nicotiana occidentalis* (Wheeler). The infection rates and virus titre were notably higher for *N. occidentalis* than blueberry, and as a result

this herbaceous host proved to be useful for aphid transmission studies and molecular characterization of BlScV (Lowery et al., 2005).

1.2.6 Control

The recommendations that have been made for the control of BlScV include removal of infected plants, application of integrated pest management (IPM) strategies to control aphids, and planting virus-free planting stock (Martin et al., 2001). Plant removal alone will not effectively control BlScV in the presence of asymptomatic cultivars and the potential of alternate hosts of BlScV must be considered in order to develop effective control strategies (Bristow et al., 2000). Since *E. fimbriata* has proven to be an inefficient vector of BlScV, and migrant aphids are capable of transmitting viruses after feeding for a short period of time (seconds to minutes), the use of insecticides may not provide adequate control of BlScV (Lowery et al., 2004). However, when Malathion [1,2-di(ethoxycarbonyl)ethyl O, O-dimethyl phosphorodithioate] was applied to a commercial field, the aphid population declined dramatically, and was followed by a decrease in aphid transmission, indicating that the use of insecticides is an effective means of control for BlScV (Bristow et al., 2000).

Following the discovery of BlScV in BC in 2000, an emergency registration was obtained for imidacloprid (Admire) ([1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine). The efficacy of Imidacloprid for the control of aphids on highbush blueberry in BC is variable (Raworth, 2004). Following application, aphid populations either decline and then rebound later in the season, decline slowly, or are unaffected (Raworth, 2004). Malathion was shown to have better efficacy, confirming previous

results obtained by Bristow et al. (2000). As an alternative to chemical control, the efficacy of dormant oil has been tested. Dormant oil applications had no effect on aphid emergence (Raworth, 2004). The poor efficacy of post-bloom applications of imidacloprid and the failure to prevent aphid emergence with dormant oil suggested a potential solution is for a pre-bloom insecticide to be applied in April immediately following egg hatch but prior to the production of winged aphids (Raworth, 2004).

In addition to plant removal and aphid control, certification programs should be implemented to prevent the spread of BlScV to new areas (Martin and Bristow, 1995). Currently, there is no certification program in place for blueberry planting stock in BC. Quarantines have been established in Oregon, Washington and Michigan. Propagation material shipped into these States must be part of a certification or testing program.

1.3 Objectives of research

The increased incidence of BlScV in BC and the likelihood of different virus strains indicated that additional research was needed on this potentially devastating disease. A continuation of the BlScV survey that was conducted in 2000 was required in order to provide important epidemiological information, including disease incidence, geographical distribution, rate of spread, cultivar reaction and strain diversity. Epidemiological information is critical for the development of effective virus control strategies. Therefore, the objectives of this study were to:

1. Establish the distribution of BlScV in blueberry fields in the Fraser Valley of BC through sampling conducted in 2001-2004.
2. Determine the rate of virus spread within three diseased blueberry fields.

3. Describe the symptomatic responses of specific blueberry cultivars to infection.
4. Characterize the strains of BlScV present in BC.

CHAPTER 2. MATERIALS AND METHODS

2.1 *Blueberry scorch virus* survey

To facilitate a widespread survey of blueberry farms in the Fraser Valley of BC for BlScV, a voluntary plant testing program was conducted from 2001 to 2004.

2.1.1 Submission of samples

Growers provided samples of leaf tissue from symptomatic blueberry plants (with necrosis of blossoms and young leaves, leaf chlorosis, or shoot blight) (Figure 3.1) to the BC Ministry of Agriculture and Lands, (BCMAL) Plant Diagnostic Laboratory in Abbotsford, BC. Information on blueberry cultivar, age of planting, and symptoms accompanied tissue samples. The total number of samples submitted increased with each year of the study. This was likely the result of enhanced awareness of BlScV, which resulted in more growers and field personnel collecting samples and submitting them for testing.

Samples from a total of 163 blueberry fields (defined as a distinct growing area separated by borders) were tested in 2001, 174 fields in 2002, 175 fields in 2003 and 227 fields in 2004. Some of the same fields were sampled in consecutive years. In addition, cranberry fields in close proximity to BlScV-infected blueberry fields were randomly sampled and tested by DAS-ELISA in 2003 and 2004, and wild *Vaccinium* species and annual or perennial herbaceous hosts in and around blueberry plantings were sampled and tested in 2004.

2.1.2 Enzyme-Linked Immunosorbent Assay testing

Each sample consisted of 1-10 mature leaves taken from different branches of one bush (individual plant sample), or 2-3 leaves per plant from up to five bushes (pooled sample). For each sample, the tips of the leaves were torn off and homogenized (1:10 w/v) in 0.1 M borate buffer (pH 8.0) containing 0.5% Tween-20 (v/v), 2% polyvinylpyrrolidone-44 (w/v), 0.5% nicotine (v/v), and 0.1% dried skim milk powder (w/v) (Martin and Bristow, 1988), using an automated tissue grinder (Dalichow, Am Bahndamm 51-3000, Hannover 91). Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) testing was performed according to an established protocol (Clark and Adams, 1977), with the exception that the borate grinding buffer was used. All reagents for ELISA testing were used at a total volume of 100 µL/well in Nunc-Immuno Maxisorp™ 96-well plates (VWR International, Mississauga, ON). Leaves from previously tested healthy or infected blueberry bushes were included as negative and positive controls, respectively, in each test. Microtiter plates were coated (1 µg/mL) with polyclonal antiserum specific for BlScV (Martin and Bristow 1988), followed by washing with 0.5 X PBST (phosphate-buffered saline + 0.05% Tween-20), then incubated overnight with leaf sap. Plates were washed again and BlScV antibody conjugated with alkaline phosphatase (Martin and Bristow 1988) was added, followed by incubation at room temperature for 2 hr. Plates were washed a final time and p-nitrophenolphosphate (1 mg/mL) was added. After 30 min, absorbance in each well was determined at 405nm (A_{405}) using an automated plate reader. Samples were recorded as positive for BlScV if they had an absorbance value greater than or equal to two times the absorbance value of the negative control.

2.1.3 Alternate host testing

Cranberry fields in close proximity (within 100m) to BlScV-infected blueberry fields were sampled randomly and tested by DAS-ELISA in 2003 and 2004. Wild *Vaccinium* species and annual or perennial herbaceous hosts in and around blueberry plantings were sampled and tested in 2004. The methodology used to detect BlScV in highbush blueberry was applied to alternate hosts (Boivin, 2004).

2.2 Extent of disease increase

To determine the rate of spread of BlScV, three diseased blueberry fields on three different farms in the Fraser Valley of BC were chosen based on disease symptomatology observed in 2000, anticipated grower management practices and location. Each field was sampled systematically in 2001, 2002 and 2004 (Figure 3.2). Data were collected for only one of the three fields in 2003. Management practices were different for each of the fields sampled. In Field 1, only infected 'Berkeley' plants were rogued and Imidacloprid was applied according to label recommendations. The grower did not rogue infected 'Bluecrop' plants in this field because they were asymptomatic. Management practices in Field 2 included rouging of only a few infected plants and Imidacloprid was only applied in 2001 and 2002. In Field 3, all infected plants were rogued immediately following their detection, and aphicide applications were made repeatedly.

An area within each field was selected for mapping (location was determined by the area of infection, and size was dependent on the initial level of infection). Each plant within the selected area was identified by coordinates of row number and position within the row. The cultivar and presence or absence of visual symptoms (flower and leaf

necrosis, leaf chlorosis and shoot blight) were recorded in each year. In each field, every plant was tested in each year of this study. Three leaves were taken from each of three adjacent plants to produce a bulked sample. Leaf samples were stored at 4°C until ELISA testing was conducted (within 1 week). When a positive ELISA result was obtained, each of the three plants comprising the collective sample was re-sampled and re-tested individually. Diseased plants were marked on a schematic grid to indicate their location in the field and disease gradient maps were generated.

2.3 Reverse Transcription Polymerase Chain Reaction

2.3.1 Selection of virus isolates

Leaf samples were collected from blueberry plants that showed symptoms of BlScV infection and tested positive by DAS-ELISA in each of the three fields selected for mapping and from seven additional fields that tested positive for BlScV by DAS-ELISA (Figure 2.1). In two of the fields selected, two samples were collected for sequencing, either each from a different year, or from a different cultivar. The 10 fields from which the samples were selected represented all geographical areas in the Fraser Valley of BC where highbush blueberries are grown and were chosen to include a range of symptoms typically observed on BlScV-infected plants in BC.

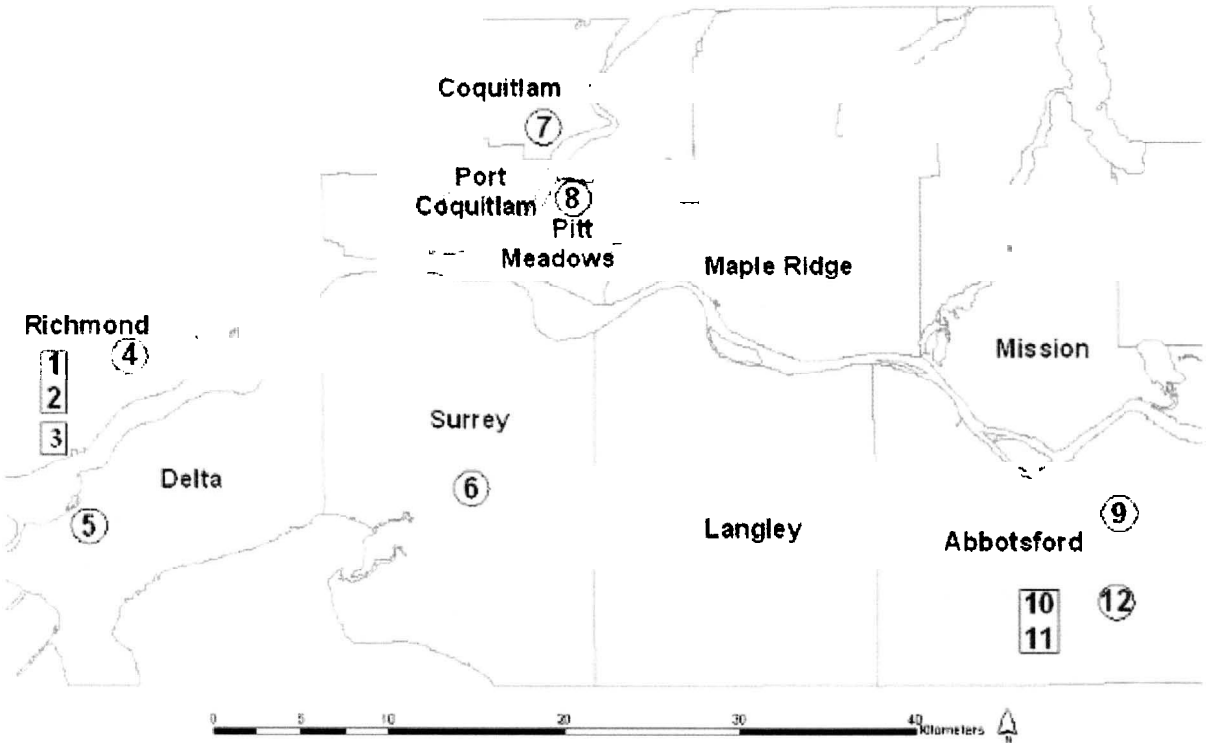

Figure 2.1 **Map of the Fraser Valley of British Columbia showing the approximate locations of commercial blueberry fields selected for sampling (1-12). Fields selected for detailed mapping are represented by square boxes. (1) Strain BC2-5 on 'Stanley', (2) Strain BC2-6 on 'Bluecrop', (3) Strain BC3-3 on 'Bluecrop', (4) Strain BC2-7 on 'Bluecrop', (5) Strain BC3-1 on 'Duke', (6) Strain BC-5 on 'Bluecrop', (7) Strain BC3-2 on 'Bluecrop', (8) Strain BC2-4 on 'Bluecrop', (9) Strain BC-4 on 'Bluejay', (10) Strain BC2-1 on 'Berkeley', (11) Strain BC2-2 on 'Bluecrop', (12) Strain BC2-3 on 'Bluecrop'.**

2.3.2 RNA extraction

Leaf tissue was stored at -70°C until RNA was extracted. Total nucleic acids were extracted from each isolate as described by Rowhani et al. (2000), and extracts were either used immediately for RT-PCR or aliquoted and stored at -70°C for future use.

2.3.3 RT-PCR and gel electrophoresis

First-strand cDNA synthesis and PCR were performed using Ready-To-Go™ RT-PCR Beads (Amersham Biosciences, NJ, USA). For reverse transcription, each reaction contained 4 µL of total RNA, 0.5 µg of $pd(T)_{12-18}$ random primer (Amersham Biosciences), 43 µL of nuclease-free water and one Ready-To-Go™ RT-PCR bead. Reverse transcription was performed at 42°C for 30 min, followed by incubation at 95°C for 5 min to denature the reverse transcriptase. Following reverse transcription, 100 pmoles of each forward and reverse primer were added to the tubes. Six sets of primers which produced overlapping fragments were designed from the published NJ-2 sequence of BlScV (GenBank Accession No. NC_003499) (Table 2.1). Primers BlScV 1F and BlScV 1R successfully amplified a 928 bp region of the coat protein gene in all samples (Figure 2.3). In 8 of 12 isolates, primers BlScV 2F and BlScV 2R successfully amplified a 1131 bp fragment encompassing a portion of the coat protein gene and the 16 KDa cysteine-rich protein (p16), which overlapped the coat protein (Figure 2.3). Primers BlScV 3F and BlScV 2R amplified a 558 bp fragment encompassing the 3' end of the coat protein region and part of the p16 of the four isolates not amplified with 2F and 2R (Figure 2.3). Positive ELISA results for cranberry and black huckleberry samples were confirmed by RT-PCR using primers which amplified a portion of the methyl transferase

and helicase domains, and/or a portion of the RNA-dependent RNA polymerase region (RdRp) of BlScV. PCR amplifications were carried out using a Peltier PTC-200 Thermal Cycler (MJ Research, Watertown, MA). Thermal cycling consisted of an initial denaturation at 95°C for 2 min followed by 35 cycles at 95°C for 1 min, 50°C for 1 min and 72°C for 2 min, and a final extension step at 72°C for 10 min.

PCR products were analyzed by electrophoresis on a 2% agarose gel in 0.5 X TBE buffer at 100 volts for 1 hr. Gels were stained with ethidium bromide and photographed on a UV-transilluminator. A 100 bp ladder (Invitrogen) was used to estimate sizes of PCR products.

Table 2.1 DNA primers used for reverse transcription polymerase chain reaction (RT-PCR) amplification of BlScV isolates in this study.

Primer	Position	Sequence	Amplicon Size (bp)	Amplified Isolates
1F	7188a	5'-CAGTTATGCCTCCGAAAG-3'	928	All
1R	8116a	5'-CCCGCATTTCGATGATTGCG-3'		
2F	7381a	5'-GAGACTCATGAACCTCATCG-3'	1131	BC2-1, BC2-2,
2R	8512a	5'-CTAAAATAGTTTAAAAACAAA TTATTTTTATAGTTTCGCCC-3'		BC2-3, BC2-4, BC2-5, BC2-6, BC2-7, BC-5
3F	7954a	5'-GCTAATTCGCGGACCGACTC-3'	558	BC-4, BC3-1, BC3-2, BC3-3
4F	4586b	5'-CTTCAGGATGAGTGGTGCAA -3'	482	cranberry
4R	5164b	5'-CGCGTGCTGGAAGCATACAA -3'		
5F	276c	5'-CCGTCTGCAAGACATTAGAG-3'	467	cranberry
5R	743c	5'-TCTTCTTCACCTCGTACTCG-3'		
6F	708c	5'-TCAATCCGTGGTGCTACGAG-3'	480	black huckleberry
6R	1188c	5'-ACAGTGCGCAATGTTCCAGT-3'		

[a] Coat protein gene
[b] RdRp region
[c] Methyl transferase and helicase genes

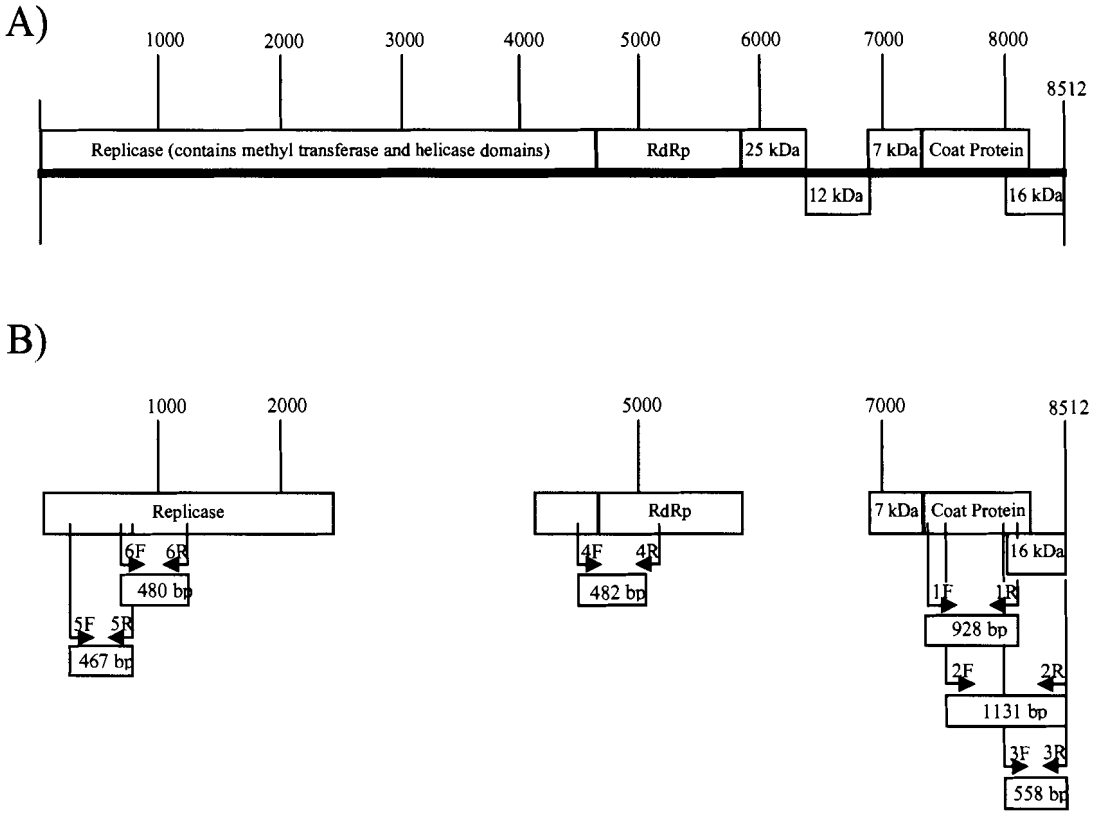

Figure 2.2 **A) Genome structure of *Blueberry scorch virus* (Bernardy et al., 2005), B) partial genome map showing the positions of primers used for RT-PCR within the methyl-transferase and helicase genes, the RdRp region and within the coat protein region and the 16 KDa putative nucleic acid binding protein.**

2.4 Coat protein sequencing

RT-PCR products were purified using the "Montage PCR Centrifugal Filter Device" (Millipore) and sequenced in both directions at Macrogen (Seoul, South Korea) using the dideoxy chain termination method (Sanger et al., 1977) in an ABI3730 XL automatic DNA sequencer. Sequences were edited using the Seqman II tool from the Lasergene 6 software (DNASTAR, Madison, WI, USA) and aligned using ClustalX, version 1.83 (Thompson et al., 1997).

2.5 Phylogenetic analysis

The 12 blueberry isolates selected from BC were compared to each other and to five previously described BlScV strains: NJ-1, NJ-2, WA-2, BC-1 (GenBank Accession No. AY941198) and BC-2 (GenBank Accession No. AY941199) (Bernardy et al., 2005). Sequence data for the coat protein regions in strains NJ-1 and WA-2 are unpublished and were obtained from Dr. Brad Hillman (Department of Plant Biology and Pathology, Rutgers University, New Brunswick, NJ, *personal communication*); however, the WA-1 sequence was unavailable for comparison. The cranberry and black huckleberry isolates were compared to strains BC-1, BC-2 and NJ-2. The black huckleberry isolate was also compared to an additional isolate collected from a blueberry field, for which sequence data was also available for the methyl transferase and helicase genes. Sequence data outside the coat protein gene was unavailable for strains NJ-1 and WA-2.

The partial nucleotide sequences of the coat protein gene of the 12 blueberry isolates, the partial RdRp and replication associated genes of the cranberry isolate, and

the partial replication associated genes and domains of the black huckleberry isolate were deposited in the GenBank database and assigned accession numbers (Table 2.2).

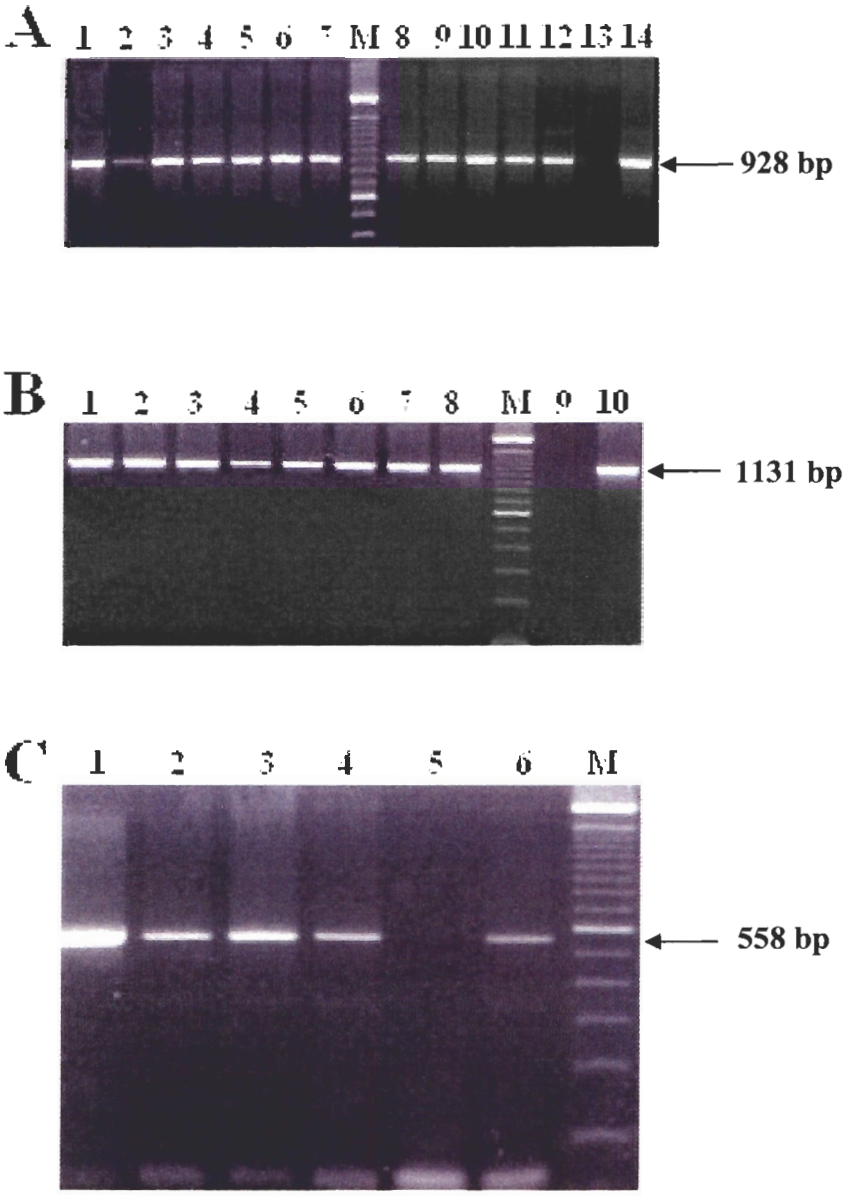

Figure2.3 **Agarose gels showing RT-PCR products from highbush blueberry with different primer combinations; A) primers 1F and 1R. Lanes 1-12: BlScV isolates; lane M: 100 bp ladder; lane 13: negative control; lane 14: positive control; B) primers 2F and 2R. Lanes 1-8: BlScV isolates; lane M: 100 bp ladder; lane 9: negative control; lane 10: positive control; C) primers 3F and 2R. Lanes 1-4: BlScV isolates; M: 100 bp ladder; lane 5: negative control; lane 6: positive control.**

Table 2.2 Genbank accession numbers for BlScV isolates.

BlScV Isolate	GenBank Accession Number
BC2-1	DQ266458
BC2-2	DQ266457
BC2-3	DQ266460
BC2-4	DQ266461
BC2-5	DQ266466
BC2-6	DQ266465
BC2-7	DQ266459
BC3-1	DQ266462
BC3-2	DQ266463
BC3-3	DQ266464
BC-4	DQ266467
BC-5	DQ266456
Cranberry methyl transferase and helicase genes	DQ370051
Cranberry RdRp	DQ370052
Black huckleberry methyl transferase and helicase genes	DQ370050

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Symptomology

In each year of the study, initial symptoms on blueberry bushes were observed during the second week of May. Disease symptoms were similar to those described earlier in the USA (Bristow et al., 2000; Martin and Bristow, 1988; Stretch, 1983). BlScV-infected 'Bluecrop' plants showed chlorosis of young leaves (Figure 3.1a) in addition to aborted flowers in June. Blighted flower blossoms were observed on 'Berkeley' (Figure 3.1b), 'Bluecrop', 'Duke', 'Bluejay', 'Concord', 'Dixi', 'Pemberton', 'Rancocas', 'Rubel', 'Spartan', 'Stanley', 'Weymouth' and 'Hardiblue' (1613-A). Infected cultivars 'Berkeley', 'Concord', 'June', 'Stanley' and 'Weymouth' retained necrotic blossoms from the previous growing season on the bush over the winter (Figure 3.1c). Leaf mottling was observed on the cultivar 'Bluejay' (Figure 3.1d). Shoot and leaf blight developed over the entire bush in severe cases (Figure 3.1f). BlScV-infected 'Bluecrop', 'Stanley', 'Duke', 'June', and 'Weymouth' developed foliar red line patterns in late fall (Figure 3.1g). Bristow et al. (2000) reported that 'Rubel' developed foliar oak-leaf line patterns when infected with the Northwest strain of BlScV. The oak-leaf symptom was also observed in our study. Leaves of 'Rubel' observed in a field suspected to be infected by the East Coast strain due to the presence of asymptomatic 'Jersey' and symptomatic 'Bluecrop', developed an oak-leaf line pattern in October (Figure 3.1h).

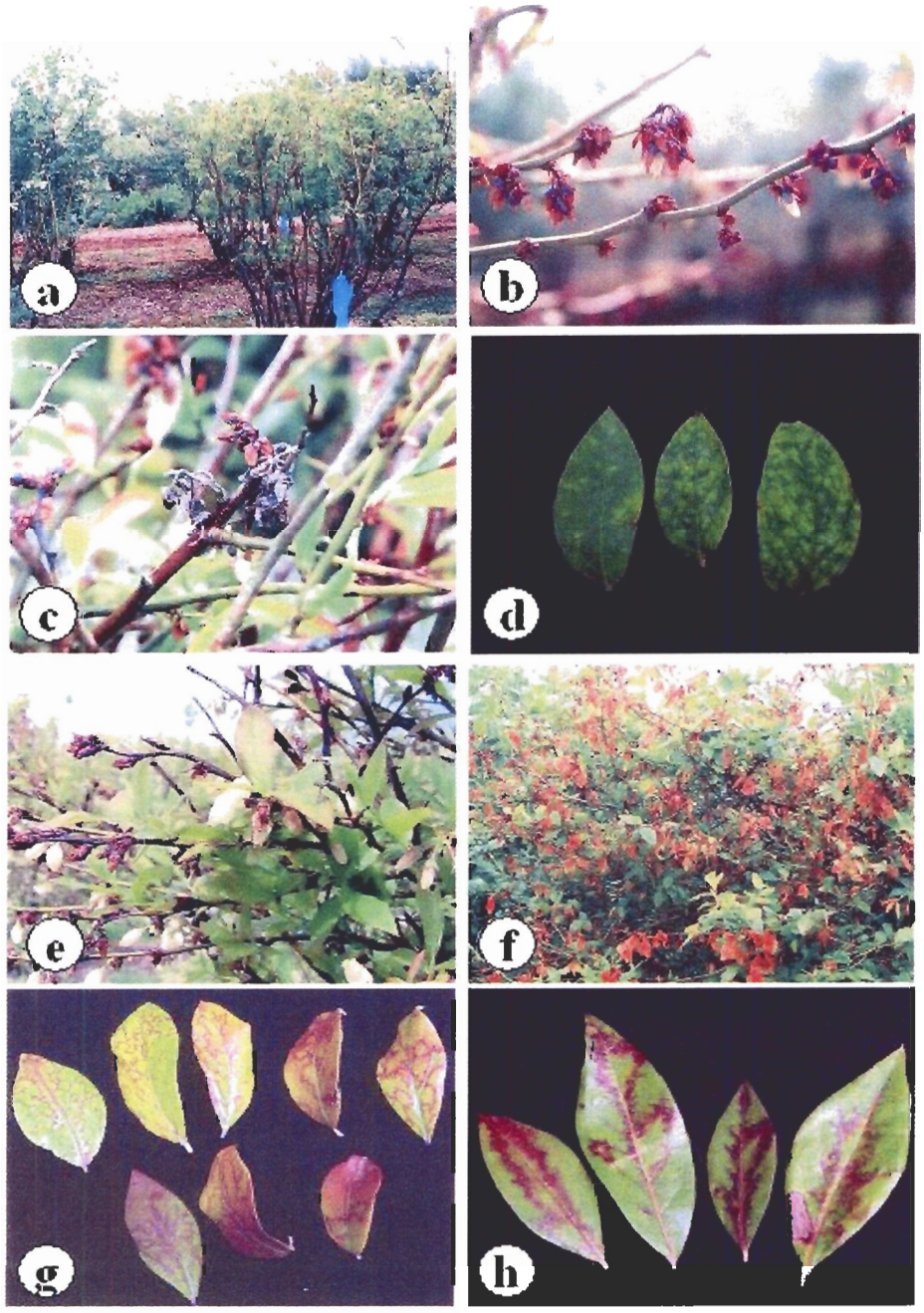

Figure 3.1 **Symptoms of Blueberry scorch virus on diseased highbush blueberry cultivars (a-h); a) leaf chlorosis on 'Bluecrop' bush in June, b) aborted flower blossoms on 'Berkeley' bush in June, c) necrotic flower blossoms retained over the winter on infected 'Berkeley' bush, d) leaf mottling on 'Bluejay' bush in May, e) dieback on 'Bluecrop' bush in July, f) leaf blight on 'Northland' bush in August, g) line pattern symptom on 'Bluecrop' leaves in October, h) oak-leaf line pattern on an 'Rubel' leaves in October. The presence of BlScV was confirmed in all diseased bushes by DAS-ELISA using a polyclonal antibody to BlScV.**

3.2 BlScV survey

BlScV was detected in all blueberry production areas of the Fraser Valley of BC and on Vancouver Island. In 2001, BlScV was detected by DAS-ELISA in 40 previously uninfected fields, bringing the total number of BlScV-infected fields in BC to 60. In 2002, 19 new BlScV-infected fields were identified. In 2003, 49 additional BlScV-infected fields were identified, and in 2004, BlScV was detected in 12 additional fields, bringing the total number of confirmed BlScV-infected fields in BC to 140 at the end of the 2004 growing season. This represents approximately one-third of the current number of highbush blueberry fields in the Fraser Valley of BC.

The DAS-ELISA test used in this study was shown to detect BlScV in all positive controls (blueberry bushes with characteristic symptoms and previous positive ELISA tests) collected from early June through to the end of August. Prior to bloom, detection of BlScV was inconsistent using DAS-ELISA (unpublished observations). Additionally, the production of unknown chemicals (likely polyphenols) in blueberry plants after September increased the background A_{405} reading, making the distinction between positive and negative samples difficult. Leaves collected from early June through the end of July, and blossoms or leaves sampled from branches with typical symptoms, had the highest A_{405} values.

There are several possible explanations for the widespread distribution of BlScV reported in this study. First, many blueberry growers produce propagative material either for their own use or for local sale. Young propagated plants (<2 years old) are often symptomless when infected with BlScV. Since a blueberry certification program does not

currently exist in BC, the dissemination of potentially diseased asymptomatic propagation materials by growers and propagators could have spread the virus. Second, the aphid, *Ericaphis fimbriata* (Richards) is present in most blueberry fields in BC (Raworth, 2004) and is capable of transmitting BlScV (Bristow et al., 2000). Aphid populations in blueberry fields were reported to be exceptionally high in the spring of 1999, which may have contributed to subsequent virus spread.

Following the discovery of BlScV in BC in 2000, an emergency registration was obtained for imidacloprid. However, the efficacy of imidacloprid for the control of aphids on highbush blueberry in BC is variable (Raworth, 2004). At present, growers are advised to apply aphicides and to identify and remove BlScV-infected plants from blueberry fields to minimize virus spread. However, the effect of aphicides on virus spread has not been determined, and the retention time of BlScV in aphids is unknown. Research is currently underway to determine which additional species of aphids (migrant species) are associated with highbush blueberry in BC and whether they are potential virus vectors. If migratory aphids, such as the green peach aphid (*Myzus persicae* Sulzer), are also shown to be important vectors of BlScV, aphicide applications may not adequately prevent virus spread since they do not have a sufficiently rapid mode of action to kill non-persistent vectors before acquisition and inoculation occurs (Raccah, 1986).

The majority of infected plant samples (>60%) submitted by growers were from symptomatic 'Bluecrop' and 'Duke' plants, suggesting that many fields were infected with a putative East Coast-like strain, since previous studies determined that these cultivars remain symptomless when infected with the Northwest strain (Bristow et al.,

2000). The majority of blueberry plants which tested positive for BlScV using DAS-ELISA were more than 10 years old. BlScV is reported to have a latent period of one to two years in blueberry plants prior to symptom appearance (Bristow et al., 2000). Most growers noted in the survey forms that no visible symptoms of blueberry scorch were apparent in their field prior to 2001, the year this study was initiated. However, symptoms of virus infection may have been attributed to other diseases and stresses of highbush blueberry, such as bacterial blight caused by *Pseudomonas syringae* pv. *syringae*, *Blueberry shock virus*, winter moth feeding damage, botrytis flower and leaf blight caused by *Botrytis cinerea* Pers., mummy berry caused by *Monilinia vaccinii-corymbosi* (Reade), or frost damage. Since the symptoms observed in most blueberry fields in 2001 were very severe, it is likely that BlScV had been present in BC for a number of years prior to the onset of this study and was either overlooked or was latent. Exceptionally high aphid populations reported in 1999 (Mark Sweeney, pers. comm.), may have contributed to the outbreak of BlScV in 2001.

3.3 Detection of BlScV in alternate hosts

The widespread occurrence of BlScV on highbush blueberries in BC suggested that additional *Vaccinium* hosts of the virus may exist. In 2003, BlScV was detected for the first time on cranberry plants in Abbotsford, BC, in a field adjacent to a BlScV-infected blueberry planting (Wegener et al., 2004). A total of 10 uprights were collected from this field and tested for BlScV by DAS-ELISA, 7 of which tested positive for BlScV. The high incidence of BlScV in this cranberry field prompted a random survey of cranberry fields in BC. BlScV was detected in 7 out of 42 bogs surveyed. The following year, BlScV was detected by ELISA and RT-PCR in black huckleberry in the

Kootenay region of BC (located over 500 km from the Fraser Valley), and in the Lynn Valley area in North Vancouver (located at least 30 km from the nearest blueberry field in the Fraser Valley). The BlScV-infected cranberry and black huckleberry were symptomless. BlScV was not detected in any other *Vaccinium* spp. herbaceous or non-herbaceous hosts collected in and around blueberry fields in this study (Appendix A).

3.4 **RT-PCR detection**

The electrophoretic analysis of RT-PCR amplicons showed the expected size products of 928 bp, 1131 bp, or 558 bp (depending on the primer combinations used) in leaf tissues from each of the three diseased blueberry fields selected for mapping and from the seven additional fields selected for coat protein sequencing (Figure 2.3). No PCR products were obtained from healthy blueberry bushes. Results from nucleotide sequencing of the RT-PCR products confirmed the presence of BlScV in each of the three fields mapped and in each of the seven additional fields selected for coat protein sequencing. Positive ELISA results for cranberry and black huckleberry samples were confirmed by RT-PCR and the amplicons of expected size were sequenced.

3.5 Extent of disease increase

An increase in diseased plants was observed annually in each of the three mapped fields (Table 3.2, Figure 3.2). The increases ranged from 4.4% to 5.2% between 2001 and 2002, and from 4.2% to 9.6% between 2002 and 2004, depending on the field. Field 1 had the lowest initial level of infection; however, the extent of spread between 2001 and 2004 for this field was the highest (14%) among all three fields. Diseased plants were clustered at one edge of the field, and disease spread occurred in an eastern direction. Focal points of infection from which BlScV spread radially outward were observed in Fields 2 and 3. This latter pattern is characteristic of non-persistently vectored virus diseases (Bos, 1999). Individual diseased plants were frequently found at a distance away from the disease focus in Field 3. In Field 1 and Field 2, the level of infection increased over each year of the study, whereas in Field 3 the rate of spread declined each year. Among the three fields, the rate of spread was the lowest in Field 3 (2.3% between 2002 and 2003 and 1.9% between 2003 and 2004). This is possibly the result of the stringent management practices implemented in Field 3. The blueberry cultivar, aphid species populations and use of management practices such as roguing of diseased bushes and applying aphicides, can potentially affect the rate of virus spread. The declining spread in Field 3 suggests that either the removal of diseased bushes and/or the application of aphicides had a negative impact on BlScV spread.

Graft transmission experiments have been conducted to determine if there are blueberry cultivars with resistance to BlScV, or cultivars which remain asymptomatic when infected with either the 'East Coast strain' (Bristow et al., unpublished data), or the 'West Coast strain' (Bristow et al., 2000). The cultivar 'Friendship' initially showed

promise when Bristow et al. (2000) reported that it could not be infected through graft-transmission with the West Coast strain of BlScV. Following additional grafting studies with the 'East Coast strain', it was reported that 'Friendship' became infected by BlScV (Bristow et al., unpublished data). At present, there are no known cultivars with resistance to BlScV.

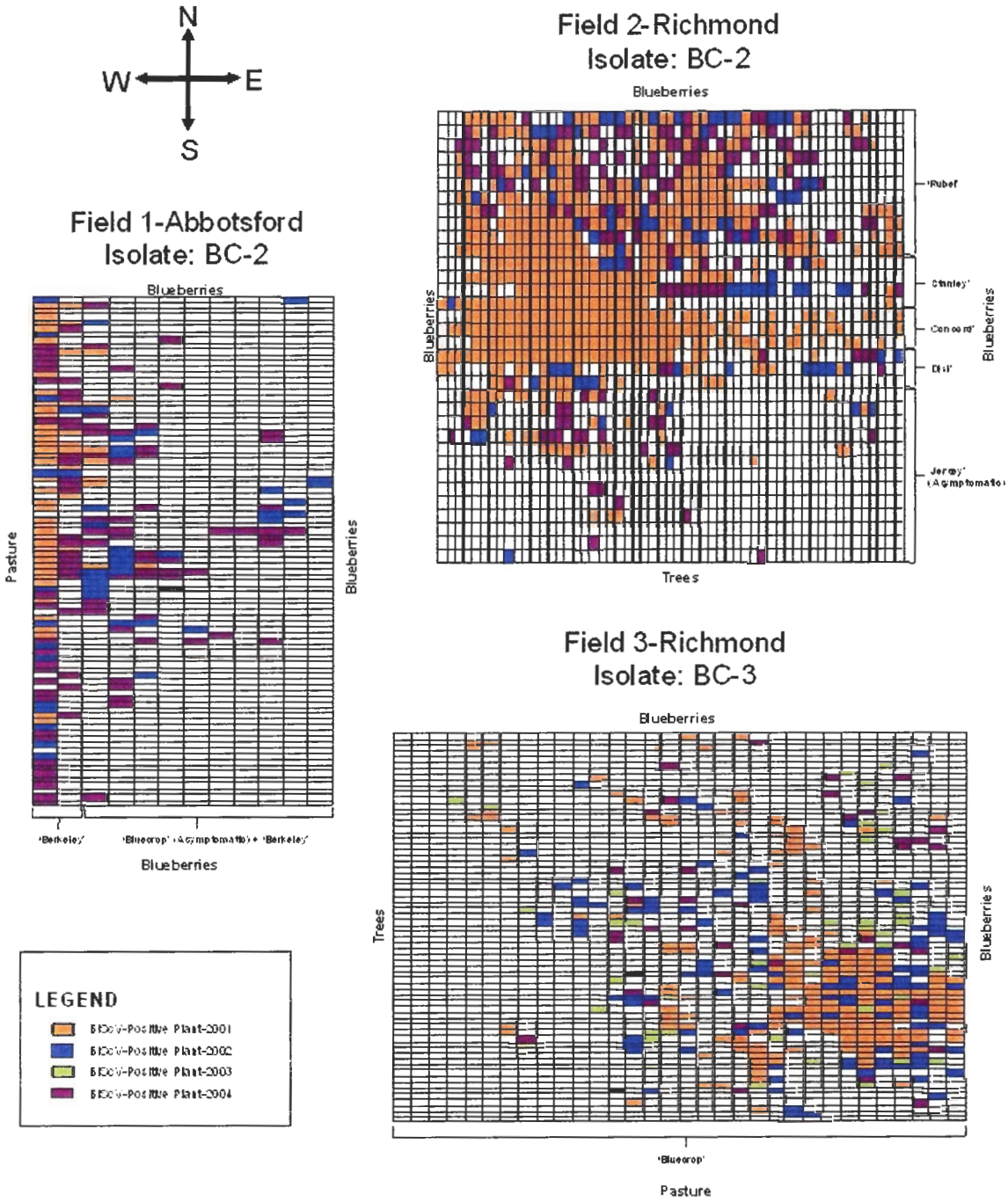

Figure 3.2 **Maps of three BlScV-infected blueberry fields showing location of plants that tested positive for BlScV by DAS-ELISA in 2001, 2002, 2003 and 2004.**

Table 3.1 **Disease increase over the period 2001 to 2004 due to BlScV infection in three mapped blueberry fields.**

Field	*BlScV-infected plants (%)*[a]			
	2001	2002	2003	2004
1	5.7	10.1	/[b]	19.7
2	29.4	34.0	/[b]	41.7
3	10.2	15.4	17.7	19.6

[a] Infection was determined by DAS-ELISA testing
[b] Data not collected

3.6 Phylogenetic analysis

Nucleotide sequencing of a portion of the coat protein gene of 12 BlScV isolates revealed a degree of divergence from the previously published sequences of strains NJ-1, NJ-2 and WA-2 (Cavileer et al., 1994), and from the recently described strain BC-1 (Figure 3.3). Seven of the twelve BC isolates had >98% sequence identity at the amino acid level with the recently described strain BC-2, and as a result were classified as BC-2 (Table 3.2, Figure 3.3). The remaining five BC isolates (BC3-1, BC3-2, BC3-3, BC-4 and BC-5) were classified as new strains of BlScV based on divergent coat protein amino acid sequences. The three BC-3 strains, when collectively compared to strains NJ-2, NJ-1, WA-2, BC-1 and BC-2 shared 87-88%, 88%, 77%, 87%, and 93% amino acid sequence identity, respectively. Strains BC-4 and BC-5 were more closely related to the NJ-2 strain than to any other strain, each sharing 96% amino acid sequence identity with strain NJ-2. The 12 BC isolates were very divergent from the WA-2 sequence, sharing only 74-77% amino acid identity. Strain BC-3 was the most distantly related to strain WA-2. For all isolates, more sequence variability was observed at the N- terminus region of the coat protein gene. The origins of the diverse strains of BlScV in BC are unknown, but they may have evolved from the transition to and from alternate cultivars or more distantly related hosts (i.e. cranberry and huckleberry), through adaptation to specific vectors, or long term vegetative propagation.

Although the proposed strain classification in this study is based primarily on the coat protein sequence information, some additional differences were observed in the symptomology caused by certain isolates. The BC-3 isolates collectively gave low A_{405}

values in ELISA tests, and the symptoms observed in infected fields were unique. Symptoms on 'Bluecrop' in the fields infected with strains BC3-2 and BC3-3 were variable from year to year, ranging from barely visible to very severe. The titer of BlScV in these fields also appeared to fluctuate, as determined by variable ELISA reactions from year to year. The leaves of 'Bluejay' plants infected with strain BC-4 of BlScV were very mottled and distorted. This symptom was not observed in any other blueberry fields during this study. The symptoms observed in the field in which strain BC-5 was detected, were similar to that in fields where strain BC-2 was present. 'Bluecrop' plants in these fields were chlorotic and had markedly reduced fruit production.

Wide geographic distribution was observed for BlScV strains sharing 100% amino acid sequence identity (Figure 2.1). Strains BC3-1, BC3-2 and BC3-3 were separated from each other by distances in the range of 14-50 km, ruling out the possibility of aphid transmission between these fields since non-persistent aphid transmission is unlikely to occur over such a great distance and other modes of transmission have not been reported. These observations indicate that BlScV was likely spread by the distribution of propagation material within BC, The primers which successfully amplified a portion of the coat protein gene of 12 highbush blueberry BlScV isolates from BC as well as other primers specific to the NJ-2 strain, with the exception of 4F/4R, 5F/5R and 6F/6R, failed to amplify the isolates originating from cranberry and black huckleberry. As a result, coat protein sequence data could not be obtained for these isolates for comparison. Positive controls were successfully amplified in these experiments, ruling out the possibility that the failed amplification was the result of experimental conditions. The cranberry field from which BlScV was detected was located several metres from the

blueberry field infected with strain BC-4, although this strain was not detected in cranberry. The RdRp and methyl transferase and helicase primers used to detect BlScV in cranberry and black huckleberry were capable of amplifying BlScV strains in highbush blueberry, allowing for sequence comparisons to be made.

The BlScV isolate from cranberry had 82%, 80% and 99% nucleotide sequence identity and 93%, 95% and 100% amino acid sequence identity to the published RdRp sequences of strains NJ-2, BC-1 and BC-2, respectively. For the methyl transferase and helicase genes, the cranberry isolate had 76% nucleotide sequence identity with strains NJ-2, BC-1 and BC-2, and 94% amino acid sequence identity with strains NJ-2 and BC-1, and 99% homology with strain BC-2, respectively. The black huckleberry amplicon shared 90%, 84% and 77% nucleotide homology and 97%, 96% and 88% amino acid sequence identity, respectively, with strains NJ-2, BC-1 and BC-2. The origins of these putative strains in cranberry and black huckleberry remain undetermined.

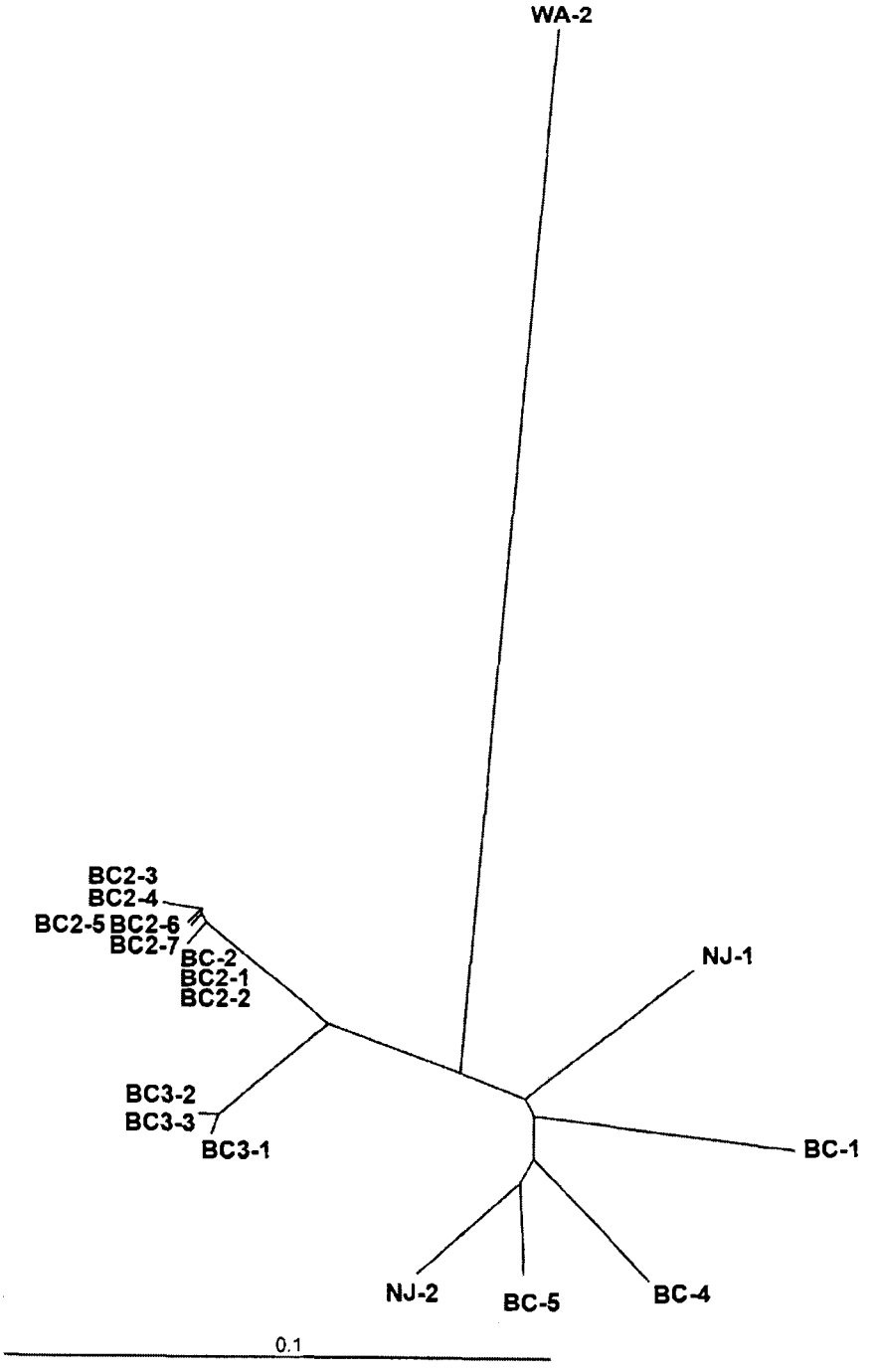

Figure 3.3 Phylogenetic relationship of twelve selected BlScV isolates from BC and strains NJ-1, NJ-2, WA-2, BC-1 and BC-2 using the coat protein gene sequences. Multiple sequence alignment was generated using Clustal X and the phylogenetic tree was created using the neighbour-joining algorhythm. The data set was subjected to 1000 bootstrap replicates.

Table 3.2 **Percentage coat protein sequence identities at the nucleotide level (upper half) and amino acid level (lower half) between British Columbia highbush blueberry BlScV isolates and other highbush blueberry isolates of BlScV. Alignments were performed using Clustal X (version 1.83).**

	BC 2-1	BC 2-2	BC 2-3	BC 2-4	BC 2-5	BC 2-6	BC 2-7	BC 3-1	BC 3-2	BC 3-3	BC 4	BC 5	BC 1	BC 2	NJ 1	NJ 2	WA 2
BC 2-1	**100**	99	99	99	99	99	99	90	90	90	79	77	74	99	81	79	70
BC 2-2	100	**100**	99	99	99	99	99	90	90	90	79	77	74	100	81	79	70
BC 2-3	99	99	**100**	99	99	99	99	90	90	90	79	78	74	99	81	79	70
BC 2-4	98	98	99	**100**	99	99	99	90	90	90	79	79	74	99	81	79	70
BC 2-5	98	98	99	98	**100**	99	99	90	90	90	80	79	74	99	81	79	70
BC 2-6	98	98	99	98	100	**100**	99	90	90	90	79	79	74	99	81	79	70
BC 2-7	98	98	99	98	99	99	**100**	90	90	90	79	78	74	99	80	79	70
BC 3-1	93	93	94	93	93	93	94	**100**	99	99	79	79	74	90	80	79	71
BC 3-2	93	93	94	93	93	93	94	99	**100**	99	80	79	74	90	80	80	72
BC 3-3	93	93	94	93	93	93	94	99	100	**100**	80	79	74	90	81	80	72
BC 4	90	90	90	89	89	89	90	89	89	89	**100**	88	82	79	90	89	64
BC 5	90	90	90	89	89	89	90	89	89	89	95	**100**	81	77	85	85	67
BC 1	88	88	88	87	87	87	88	87	87	87	92	92	**100**	74	83	83	65
BC 2	100	100	99	98	98	98	98	93	93	93	90	90	88	**100**	81	79	70
NJ 1	89	89	89	89	89	89	89	88	88	88	94	94	90	89	**100**	89	68
NJ 2	89	89	89	88	88	88	89	87	88	88	96	96	91	89	94	**100**	67
WA 2	75	75	75	74	75	75	75	77	77	77	77	77	77	75	75	76	**100**

CHAPTER 4. SUMMARY AND FUTURE RESEARCH

4.1 Application of research results

Prior to this study, epidemiological information regarding *Blueberry scorch virus* in British Columbia was limited. The information obtained from this study should provide blueberry producers with more information to minimize virus spread and avoid spread of the disease to new areas, and for researchers to potentially develop more sensitive diagnostic tests to reveal the presence of the virus in field plantings and during propagation. This research will contribute significantly to the long-term viability of the blueberry industry in Canada.

BlScV was detected in all blueberry production areas in BC, and the severity of infection varied between fields. Aphid control coupled with roguing infected plants will likely reduce the rate of BlScV spread within infected fields. With this information, growers can now implement more effective disease management strategies. The use of virus-free propagation material is critical for the control of insect-borne virus diseases. Presently, blueberry propagation in BC is unregulated and there is no formal certification program in place. However, a few large commercial propagators are involved in the voluntary certification of propagation material to access export markets. The results from this study can be used to assist in the implementation of virus testing and virus prevention protocols required for a certification program.

Since there are certain cultivars in BC which remained asymptomatic when infected with strains of BlScV, and there is a latent period between infection and symptom development, reliable diagnostic testing methods are critical. As for any plant virus, early detection of BlScV aids in control. Furthermore, to certify planting material, there must be the assurance that all strains of the virus are being detected. The high genetic variability among the BlScV isolates revealed in this study must be taken into consideration when selecting virus detection methods, as it is possible that some BlScV isolates may escape detection. The sequence information generated from this study will assist in the development of more reliable detection methods for BlScV.

4.2 Directions for future research

In order to control the spread of Blueberry scorch disease, an understanding of how BlScV spreads both temporally and spatially is essential. The widespread distribution and rapid rate of spread of BlScV in BC warrants the need for a continuation of the BlScV testing program. Industry-wide testing will provide important surveillance information on disease incidence, distribution and spread. Furthermore, survey information will be useful for selecting additional BlScV isolates for sequencing, as it is likely that there are strains of BlScV present in BC which have not yet been identified.

To facilitate the continuation of the virus-testing program, improved diagnostic methods are required. Presently, ELISA testing using a polyclonal antibody is the primary method for BlScV detection. However, ambiguous results were frequently encountered during this study. 'False-negative' results with ELISA are of particular concern, especially when viruses occur at a low concentration or are unevenly distributed

within a plant, as is the case with BlScV. The sequence information obtained from this study will assist in the development of new polyclonal and monoclonal antisera for a more reliable ELISA test.

The strains of BlScV detected in BC exhibited variable reactions with the primers currently available. The development of a diagnostic PCR test capable of detecting all strains of BlScV would be useful for early detection of BlScV when the titer is too low for ELISA detection, and for checking ambiguous ELISA results. A sensitive PCR test would also be extremely useful for aphid transmission studies.

An accurate understanding of the variability and the relationships between the strains of BlScV is essential for disease management. Field isolates often consist of a mixture of several strains, and mixed infections of BlScV within fields and within individual plants are highly likely. In this study, PCR products were not cloned, but rather were directly sequenced. There are distinct advantages to using this approach. However, the target species most homologous to the primers used may have been amplified, and this may not be the dominant species present in the field sample. Detailed surveys should be conducted in the fields from which the five strains of BlScV were isolated, since different strains of a given virus species may induce very different symptoms and or levels of infection. Since the strains of BlScV identified in BC are divergent from previously characterized strains in the USA, their impact on highbush blueberry has not been evaluated. The prevalence of each strain in BC, as well as the cultivar responses to these strains, especially to BC-2 require further investigation. Crop

loss data for BlScV strains in BC should be compiled, as it would be very useful for the BC blueberry industry.

The presence of BlScV in alternate hosts has implications for its control, and it must be established whether transmission occurs between alternate hosts and highbush blueberry. Although aphids have not been reported to be a pest of cranberry, the possibility of migrant aphids transmitting BlScV from cranberry to highbush blueberry or vice versa exists and warrants investigation. Full-length sequencing of the cranberry BlScV isolates will assist in determining whether aphid transmission of BlScV has occurred between cranberry and highbush blueberry. A detailed survey of alternate *Vaccinium* hosts is warranted in BC, WA and OR, and the potential of these hosts to act as BlScV inoculum sources should be investigated, since this would greatly influence the control of BlScV.

Presently, there are no known cultivars with resistance to BlScV. However, some cultivars are tolerant to certain strains of BlScV and remain symptomless when infected. Since highbush blueberry is a long-lived perennial crop, host resistance is a very attractive approach for the control of BlScV. It would be useful to examine the BlScV-tolerant highbush blueberry cultivars as well as cranberry and black huckleberry for possible resistance mechanisms.

With recent expansion of acreage, there are now over 550 blueberry growers in the Fraser Valley. Many of the newer growers do not understand the importance of a virus management program. The progress made in this study has provided clearer direction for blueberry growers; however this is just one step towards managing BlScV.

It is now essential that this new information be communicated to all growers through an effective awareness program in order to sustain a profitable highbush blueberry industry in BC.

APPENDIX: PLANT ACCESSIONS TESTED

Plant accessions tested for presence of BlScV in 2003 by DAS-ELISA (Boivin, 2004)

Latin name	Common name	Samples tested	Number of ELISA positives
Acer sp.	maple	4	0
Amaranthus	pigweed	10	0
Anethum graveolens	dill	1	0
Anthriscus sylvestris	wild chervil	1	0
Arctium	common burdock	1	0
Betula sp.	birch	6	0
Sinapsis arvensis	wild mustard	1	0
Capsella bursa-pastoris	shepherd's purse	1	0
Cardamine oligosperma	bitter cress	3	0
Chenopodium album	lamb's quarters	14	0
Cirsium arvense	Canada thistle	11	0
Cirsium vulgare	bull thistle	6	0
Convolvulus arvensis	field morning glory	3	0
Digitalis purpurea	foxglove	11	0
Epilobium angustifolium	yukon fireweed	19	0
Epilobium ciliatum	willowherb	79	0
Equisetum arvense	field horsetail	1	0
Erigeron canadensis	canada fleabane	2	0
Erodium cicutarium	stork's bill	1	0
Galeopsis tetrahit	hemp nettle	11	0
Galium aparine	cleavers	1	0
Gaultheria shallon	salal	7	0
Geranium bicknelli	Bicknell's geranium	2	0
Gnaphalium uliginosum	cudweed	6	0
Hibiscus sp.	n/a	1	0
Hypericum perforatum	wild St. John's wort	1	0
Hypochaeris & Leontodon	Dandelion – Other	23	0
Ilex aquifolium	English holly	2	0
Lactuca serriola	prickly lettuce	4	0
Laurus sp.	laurel hedge	1	0

Plant accessions tested for presence of BlScV in 2003 by DAS-ELISA (Boivin, 2004)			
Latin name	Common name	Samples tested	Number of ELISA positives
Ledum groenlandicum	Labrador tea	7	0
Lotus conriculatus	bird's foot trefoil	1	0
Maianthemum dilatatum	Ffalse lily of the valley	3	0
Malus sp.	crab apple	6	0
Malva neglecta	round-leaved mallow	1	0
Matricaria matricariodes	pineappleweed	6	0
Medicago Sativa	alfalfa	1	0
Melilotus alba	white sweet clover	1	0
Mycelis muralis	wall lettuce	2	0
Myosotis scorpioides	forget-me-not	3	0
Plantago sp.	plantain	6	0
Polygonum sp.	smartweed	34	0
Polygonum convolvulus	wild buckwheat	4	0
Portulaca oleracea	purslane	3	0
Potentilla norvegica	rough cinquefoil	3	0
Ranunculus repens	buttercup	4	0
Rorippa sylvestris	yellow cress	7	0
Rosa sp.	n/a	7	0
Rosa nutkana	Nootka rose	3	0
Rubus sp.	n/a	2	0
Rubus discolor	Himalayan blackberry	19	0
Rubus ideaus	raspberry	6	0
Rubus laciniatus	evergreen blackberry	19	0
Rubus parviflorus	thimbleberry	3	0
Rumex acetosella	sheep sorrel	18	0
Rumex crispus	curled dock	1	0
Sambucus racemosa	red elderberry	5	0
Senecio vulgaris	groundsel	16	0
Silene pratensis	white cockel, campion	1	0
Sisymbrium officinale	hedge mustard	1	0
Solanum nigrum	black nightshade	1	0
Solidago canadensis	goldenrod	1	0
Sonchus	sow thistle	21	0
Sorbus	mountain ash	5	0
Spiraea douglassi	hardhack	27	0
Stachys lanata	lamb's ear	2	0

Plant accessions tested for presence of BlScV in 2003 by DAS-ELISA (Boivin, 2004)			
Latin name	Common name	Samples tested	Number of ELISA positives
Stellaria media	chickweed	8	0
Symphoricarpus albus	common snowberry	2	0
Tanacetum vulgare	common tansy	1	0
Taraxacum officinale	dandelion	19	0
Trientalis	star flower	6	0
Trifolium repens	clover	16	0
Urtica dioica	stinging nettle	1	0
Vaccinium sp.	n/a	4	0
Vaccinium myrtilloides	velvet-leaved blueberry	10	0
Vaccinium ovatum	evergreen huckleberry	1	0
Vaccinium parvifolium	red huckleberry	27	0
Viburnum sp.	n/a	4	0
Vicia cracca	tufted vetch	6	0
Unidentified		28	0

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