CHARACTERIZATION OF *XYLELLA FASTIDIOSA* STRAINS THAT CAUSE BACTERIAL LEAF SCORCH OF SOUTHERN HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM* INTERSPECIFIC HYBRIDS), AND DETECTION OF THE PATHOGEN IN PLANTS AND GLASSY-WINGED SHARPSHOOTERS [*HOMALODISCA VITRIPENNIS* (GERMAR)] (HEMIPTERA: CICADELLIDAE) IN SOUTH GEORGIA

by

LORNA DENISE NISSEN

(Under the Direction of Timothy P. Denny and Phillip M. Brannen)

ABSTRACT

Xylella fastidiosa is a genetically diverse species with a wide host range. This xylem-limited bacterium was recently proven to cause bacterial leaf scorch of blueberry (BLSB), a new disease of Georgia's most economically important fruit crop. Since little is known about the strains of *X. fastidiosa* affecting southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids), the objectives of this research were to characterize BLSB strains and develop assays to detect them in plants and the most probable insect vector, the glassy-winged sharpshooter [*Homalodisca vitripennis* (Germar)] (Hemiptera: Cicadellidae). Published, redesigned and novel primer pairs were used with the polymerase chain reaction (PCR) to analyze genomic DNA derived from pure cultures and DNA extracts of plant and insect tissue. Methods were developed to reduce PCR inhibition that occurred in extracts. BLSB strains in Georgia were found to be most similar to Dixon, an A-type strain of *X. fastidiosa* that causes almond leaf scorch.

INDEX WORDS: bacterial leaf scorch of blueberry, BLSB, *Xylella fastidiosa*, southern highbush blueberry, SHB, *Vaccinium corymbosum* interspecific hybrids, glassy-winged sharpshooter, GWSS, *Homalodisca vitripennis* (Germar), DNA extraction, PCR inhibition

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DEDICATION

To my family, who often had to fend for themselves, as an absent wife and mother continued a lifelong journey of taking the road less traveled. Especially to my children, I hope I have been an inspiration for pursuing your own dreams, and a model for self-discipline, steadfastness, fortitude, working hard and approaching everything with a spirit of excellence.

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CHAPTER 1

INTRODUCTION

Blueberries surpassed peaches in farm gate value in 2004 to become the most economically important fruit crop in Georgia (Boatright and McKissick 2010). Although blueberry production occurs throughout the state, it is concentrated in the southern coastal Flatwoods (Chang et al. 2009). The number of hectares planted in blueberry increased approximately 192% between the years 2000 and 2009, from 2,269 to 6,615 (Boatright and McKissick 2010). In comparison, the number of hectares planted in peach during the same period declined from 6,392 to 4,133. Blueberry production in Georgia has continued to increase in value and currently stands at more than $102.4 million. Southern highbush blueberries (SHB) (*Vaccinium corymbosum* interspecific hybrids) are generally more profitable than native rabbiteye (*Vaccinium virgatum* Aiton), as SHB are the earliest maturing blueberries in North America (April-May), and they mature during a market window that allows for high profitability (Scherm and Krewer 2003, Chang et al. 2009, Brannen et al. 2010).

However, a new disease, bacterial leaf scorch of blueberry (BLSB), threatens the blueberry industry in Georgia (Brannen et al. 2010). In 2006, Chang et al. (2009) completed Koch's postulates to demonstrate that *Xylella fastidiosa* is the causal agent of BLSB, particularly in SHB varieties. *X. fastidiosa* is a fastidious, gram-negative, xylem-limited plant pathogenic bacterium that is vectored primarily by xylem-feeding leafhoppers (Mizell et al. 2003, Costa et al. 2006). The glassy-winged sharpshooter (GWSS) [*Homalodisca vitripennis* (Germar)] (Hemiptera: Cicadellidae) is the largest leafhopper in Georgia, measuring between 1.5 and 2 cm

in length at maturity (Conklin and Mizell 2002). Native to the southeastern United States, the GWSS feed on the xylem of more than 100 plant species and vectors *X. fastidiosa* (Turner and Pollard 1959, Conklin and Mizell 2002, Almeida and Purcell 2003, Costa et al. 2006). The GWSS is considered the most probable insect vector of BLSB in Georgia, as the leafhopper constituted 97% of insects sampled in 2009 from plants at two SHB fields in Berrien and Hoboken counties infected with *X. fastidiosa* (Brannen et al. 2010, Tertuliano et al. 2010, Nissen *unpublished data*). The GWSS can be an efficient vector of *X. fastidiosa*, as the bacterium colonizes the foregut of the insect and is persistent throughout the life of the insect when acquired by an adult (Severin 1949, Purcell and Finlay 1979, Brlansky et al. 1983, Hopkins 1989, Hill and Purcell 1995, Conklin and Mizell 2002, Mizell et al. 2003, Almeida and Purcell 2006, Costa et al. 2006). Nymphs are not efficient vectors because the acquired pathogen is lost during molting. *X. fastidiosa* also is known to spread in some hosts, such as alfalfa, citrus, grapevine and peach, through manual grafting and natural root grafts in the field (Weimer 1936, Hewitt 1939, He et al. 2000, Gould and Lashomb 2005).

Plant diseases caused by *X. fastidiosa* are most prevalent in the southeastern United States, but also occur in California, the lower midwestern states, Argentina, Brazil, Canada, Costa Rica, Paraguay, Taiwan and Venezuela (Smith et al. 1992, Mizell et al. 2003). Although not fully known, the host range of *X. fastidiosa* is diverse and vast, encompassing more than 30 families and 153 species of monocotyledonous and dicotyledonous plants (Freitag 1951, Hopkins 1989, Mizell et al. 2003, Costa et al. 2006). Pathotypes of *X. fastidiosa* are the causal agents of Pierce's disease of grapevine (Davis et al. 1978), citrus variegated chlorosis (Chang et al. 1993, Lee et al. 1993), phony peach disease (Hopkins et al. 1973, Hopkins and Mollenhauer 1973, Davis et al. 1981a, Davis et al. 1981b, Wells et al. 1981, Wells et al. 1983), plum leaf scald

(Kitajima et al. 1975, Davis et al. 1981a, Davis et al. 1981b, Wells et al. 1981, Raju et al. 1982), almond leaf scorch (Mircetich et al. 1976, Davis et al. 1980) and oleander leaf scorch (Purcell et al. 1999). *X. fastidiosa* multiplies and moves slowly up and down the xylem, restricting water and nutrient movement throughout the plant, which results in a number of characteristic symptoms (Purcell 1997, Mizell et al. 2003, Bextine et al. 2004).

Symptoms of disease vary depending on host, but generally involve a loss of plant vigor, branch dieback and eventual death of the plant (Mizell et al. 2003). Specific symptoms include delayed leafing in the spring, mottling and marginal scorching of leaves, interveinal chlorosis and necrosis, shoot dwarfing, wilting, premature coloring of fruit and root death. Initial symptoms of BLSB include chlorosis and subsequent necrosis of older leaves that begins at the margin and progresses throughout the entire leaf, resulting in a scorched appearance similar to that seen with extreme drought or fertilizer salt burn (Chang et al. 2009). New shoots often are abnormally thin, produce fewer flower buds and develop a yellow "skeleton-like" appearance once affected leaves drop. Death of the plant follows leaf drop, typically within two years after the onset of symptoms.

Detection of BLSB strains in plants and insect vectors is important to blueberry producers in terms of management of the disease. Eliminating sources of inoculum is critical for preventing or slowing the spread of disease. There are no strategies to prevent infection of plants by *X. fastidiosa* and no effective therapies for infected plants (Huang et al. 2006). Recommendations to manage BLSB are based on information derived from other plant systems affected by *X. fastidiosa* (Brannen et al. 2008). Control of *X. fastidiosa* is limited to pruning of infected shoots, removal of infected plants, use of healthy plants for planting, and identification and control of insect vectors (Huang et al. 2006). Chemical means exist to control insect vectors

but not the pathogen (Brannen et al. 2008). Identifying the time of *X. fastidiosa* acquisition would be valuable, as it would enable targeted applications of insecticide. But such information can only be derived through specific and sensitive detection methods such as PCR.

X. fastidiosa has been well studied in a number of host plant systems, including almond, citrus, grapevine, oleander and peach. However, since little is known about *X. fastidiosa* in blueberry, the objectives of this research were to characterize BLSB strains and develop assays to detect them in plants and the most probable insect vector. Conventional polymerase chain reaction (PCR) was used with published, redesigned and novel oligonucleotide primers to analyze genomic DNA of *X. fastidiosa*. Methods also were developed to reduce PCR inhibition when using DNA extracts of plant and insect tissue as templates.

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CHAPTER 2

LITERATURE REVIEW

Blueberry production in Georgia. Blueberry production has grown considerably in Georgia since its inception in the mid-1950s (Scherm and Krewer 2003). Growth has been particularly rapid the past 10 years, as the number of planted hectares in the state increased 192% during that time, making blueberries the fastest growing fruit commodity in Georgia (Boatright and McKissick 2010b). Blueberries passed peaches in farm gate value in 2004 to become the most economically important fruit crop in the state. The value of blueberry production continues to increase and currently stands at more than $102.4 million. The number of harvested hectares in the state in 2008 nearly doubled from the previous year, allowing Georgia to pass New Jersey to garner a second-place national ranking behind Michigan (USDA NASS 2010). Although blueberry production can be found throughout Georgia, it is concentrated in the Flatwoods of southeastern and south-central Georgia, primarily in Appling, Bacon, Clinch and Ware counties, where the number of planted hectares in 2009 comprised nearly 75% of the state total (Scherm and Krewer 2003, Boatright and McKissick 2010a).

Approximately 83% of the 6,615 hectares of blueberry in Georgia are planted in rabbiteye varieties (*Vaccinium virgatum* Aiton), which are native to southern Georgia, northern Florida and southeastern Alabama, and have been in cultivation for more than 100 years (Scherm and Krewer 2003, Boatright and McKissick 2010b). The other 17% is planted in varieties of southern highbush blueberry (SHB) (*V. corymbosum* interspecific hybrids). Varieties of SHB have been cultivated in Georgia since the mid-1990s, as they are the earliest blueberries to ripen

in North America (April and May), allowing producers to garner a higher price in the fresh fruit market at a time when blueberries are in short supply (Krewer 2004, Williamson and Lyrene 2004, Schupska 2008). Conversely, rabbiteye varieties ripen in late May, June and July. However, growing SHB can be challenging, as varieties require acidic soils with good drainage and high organic matter. Blueberries are grown in a region of Georgia characterized by a high water table and soils that are sandy or sandy loam. Thus, SHB often is grown in raised beds consisting of pure milled pine bark, which provides acidity, good drainage and organic matter. SHB is susceptible to late winter and early spring freezes due to early flowering, and is more susceptible than rabbiteye to a number of plant pathogens, including stem blight (*Botryosphaeria* spp.) and root rot (*Phytophthora* spp.).

Bacterial leaf scorch of blueberry. Beginning in 2004, a disorder described as "yellow twig" or "yellow stem" was observed in the 'FL 86-19' (also known as 'V1') variety of SHB. Koch's postulates completed in 2006 by Chang et al. (2009) demonstrated that the disease, bacterial leaf scorch of blueberry (BLSB), is caused by *Xylella fastidiosa*. Initial symptoms in SHB include chlorosis and subsequent necrosis of older leaves that begins at the margin and progresses throughout the entire leaf, resulting in a scorched appearance similar to that seen with extreme drought or fertilizer salt burn (Chang et al. 2009). New shoots often are abnormally thin, produce fewer flower buds and develop a yellow "skeleton-like" appearance once affected leaves drop. Death of the plant follows leaf drop, typically within two years after the onset of symptoms.

Since its appearance, BLSB has become a major disease in SHB, affecting other varieties, such as 'O'Neal' and 'Star,' while 'V1' remains the most susceptible (Brannen et al. 2010). Based on a field disease survey and serological tests of sampled plants conducted in 2008,

varieties of SHB less susceptible to BLSB include ‘Emerald,’ ‘Jewel,’ ‘V5,’ ‘Millenia,’ ‘Southern Belle’ and ‘Windsor.’ Initial results of an ongoing greenhouse study at The University of Georgia also indicate susceptibility to BLSB depends on variety (H. Scherm and E. Gamliel Atinsky *unpublished data*). Rabbiteye varieties were thought to be resistant to BLSB; however, the bacterium has been found in several varieties, with some exhibiting symptoms in the field (Brannen et al. 2010). Although no specific yield loss data are available, survey data collected in 2008 indicate a near 100% loss for ‘V1’ and a 30% loss for ‘Star’ are projected to occur within 10 years of the onset of symptoms (P. Brannen, personal communication). Thus, BLSB is considered a threat to the blueberry industry in Georgia (Brannen et al. 2010).

Characteristics and culture of *X. fastidiosa*. *X. fastidiosa* is a gram-negative, xylem-limited bacterium that was first associated with a plant disease in the Western hemisphere by Goheen et al. (1973) and Hopkins and Mollenhauer (1973), and was later determined through Koch’s postulates by Davis et al. (1978) to be the cause of Pierce’s disease (PD) of wine grapes (*Vitis vinifera* L.) in California. Based on physiological and biochemical tests of 25 strains isolated from 10 plant hosts, Wells et al. (1987) proposed the bacterium be named *Xylella fastidiosa*, a new genus and species within the Gamma subgroup of Proteobacteria. They described cells of the bacterium as single, occasionally filamentous, non-motile, aflagellate, straight rods measuring 0.25 to 0.35 by 0.9 to 3.5 μm in size. The bacterium is gram-negative, catalase positive, oxidase negative and uses hippurate. It also produces gelatinase and often ß-lactamase but not ß-galactosidase, coagulase, lipase, amylase, phosphatase, indole or hydrogen sulfide. Wells et al. (1987) reported *X. fastidiosa* is a strict aerobe, with colony growth being optimum at 26-28°C and a pH of 6.5-6.9. Bacteria had a doubling time of 0.45 to 1.98 days in periwinkle wilt (PW) broth.

In determining that *X. fastidiosa* is the cause of PD of wine grapes in California, Davis et al. (1978) were the first to develop a specialized medium (JD-3) to cultivate the nutritionally fastidious prokaryote. During the subsequent 32 years, other specialized media have been developed to cultivate strains of *X. fastidiosa* from plant tissue, including PD2 and PD3 for PD strains (Davis et al. 1980a), buffered charcoal-yeast extract (BCYE) for strains causing phony peach disease (PPD) and plum leaf scald (Wells et al. 1981a), PW for strains causing periwinkle wilt, PPD and plum leaf scald (Davis et al. 1981a, Davis et al. 1981b, Davis et al. 1983), modified PW for oak leaf scorch strains (Kostka et al. 1984), PWG for PD and almond leaf scorch (ALS) strains (Hill and Purcell 1995), CS20 for strains causing PPD, periwinkle wilt and oak leaf scorch (Chang and Yonce 1987), and the chemically defined medium XF-26 for PD strains (Chang and Donaldson 1993). Growth and morphology of *X. fastidiosa* colonies reported by researchers was generally similar, varying somewhat according to the medium on which they were cultured. Colonies are opalescent, white or cream colored, circular in form, and either convex to pulvinate with a smooth surface and entire margin, or umbonate to flat with a rough surface and undulate margin. Colonies reached a diameter up to 1.0 mm and were visible without magnification within 7-14 d when incubated at 25-28°C.

All *X. fastidiosa* strains require selective media to grow but can be separated into two groups based on nutritional fastidiousness (Davis et al. 1983, Kamper et al. 1985, Hopkins and Adlerz 1988, Hopkins 1989). PD group strains grow on PD3, PW, BCYE and CS20, while PPD group strains grow on PW, BCYE and CS20 but not PD3. Wells et al. (1987) demonstrated that growth rates of the two groups of strains in PW broth was significantly different. Strains from grapevine, almond, elm and mulberry had higher growth rates, with doubling times ranging from

0.5 to 1.6 d, while strains from peach and plum had slower growth rates, with doubling times as long as 2 d.

Colonization of *X. fastidiosa* in plants and insects. *X. fastidiosa* migrates slowly up and down xylem vessels through twitching motility using type IV pili (Merz et al. 2000, Skerker and Berg 2001, Mattick 2002, Meng et al. 2005, Galvani et al. 2007, Li et al. 2007). Twitching motility allows bacteria without flagella, such as *X. fastidiosa*, to move long distances over moist surfaces, and occurs through extension, attachment and then retraction of type IV pili at cell poles that allow bacteria to move forward (Merz et al. 2000, Skerker and Berg 2001, Mattick 2002, Meng et al 2005, Galvani et al. 2007). Also present at the same cell pole as type IV pili are type I pili (Meng et al. 2005), which are thought to be involved in cell aggregation and biofilm development along with type IV pili (Li et al. 2007). Cell aggregation and biofilm development allow *X. fastidiosa* to adhere to and block water and nutrient conducting xylem vessels in roots, stems and leaves of plants. Adhesion to xylem vessels by *X. fastidiosa* is necessary for colonization in plants and can also occur through afimbrial adhesins produced by the bacterium (Lindow 2005). Newman et al. (2004) demonstrated that cell-cell signaling, or quorum sensing, controls *X. fastidiosa* interactions with both plants and insects. Killiny and Almeida (2009b) demonstrated that afimbrial adhesins are controlled by a cell-cell signaling regulatory system (Chatterjee et al. 2008), and are also necessary for *X. fastidiosa* colonization in insect vectors and subsequent transmission to host plants.

X. fastidiosa attaches to and colonizes the foregut of insect vectors (Severin 1949, Frazier 1965, Purcell and Finlay 1979, Purcell et al. 1979, Brlansky et al. 1983, Hopkins 1989, Hill and Purcell 1995, Conklin and Mizell 2002, Almeida and Purcell 2003b, Mizell et al. 2003, Almeida and Purcell 2006, Costa et al. 2006). The foregut is part of the exoskeleton and is composed

primarily of chitin and chitin-like polysaccharides (Almeida and Purcell 2006, Killiny and Almeida 2009a). Xylem vessels also are composed of structural carbohydrates, including pectin, cellulose and hemicellulose. Roper et al. (2007) demonstrated that degradation of pectin, which is a major component of xylem pit membranes, is required for *X. fastidiosa* pathogenicity and movement in plants. Killiny and Almeida (2009a) compared growth of *X. fastidiosa* on PWG, a unique defined medium (*X. fastidiosa* medium or XFM) and XFM supplemented with either glucan or pectin. They observed phenotypic changes in the bacterium based on medium composition, with pectin inducing greater changes than glucan. They also used gene expression profiling to explore these phenotypic changes and found differences among *X. fastidiosa* cells grown in the four media tested, with XFM supplemented with pectin inducing the largest number of changes in gene transcription levels. Killiny and Almeida (2009a) also found that pectin and glucan regulated gene expression, which resulted in changes in *X. fastidiosa* phenotype required for vector transmission of the bacterium.

Diseases caused by *X. fastidiosa*. Bacterial colonization of xylem vessels results in blockages and formation by the plant of tyloses and gums, which combine to restrict water and nutrient movement and likely are the mechanisms that result in characteristic symptoms of disease (Hopkins 1989, Mizell et al. 2003, Li et al. 2007). Most hosts of *X. fastidiosa* are symptomless (Hopkins 1989). When symptoms do occur, they vary according to host and stress factors such as drought, but generally involve a loss of plant vigor, branch dieback and eventual death of the plant (Hopkins 1989, Mizell et al. 2003). Symptoms in most hosts are not visible until fruit maturation or during senescence. Specific symptoms may include delayed leafing in the spring, mottling and marginal scorching of leaves, interveinal chlorosis and necrosis, shoot dwarfing, wilting and premature coloring of fruit, and root death.

In the United States, plant diseases caused by *X. fastidiosa* are prevalent in Florida, Georgia, Mississippi and Texas, but also occur in Alabama, Arizona, California, District of Columbia, Indiana, Kentucky, Louisiana, Montana, New Jersey, New York, North and South Carolina, Tennessee and West Virginia (Smith et al. 1992). Outside the United States, plant diseases caused by *X. fastidiosa* occur in Argentina, Brazil, Canada, Costa Rica, Paraguay, Taiwan and Venezuela. Although not fully known, the host range of *X. fastidiosa* is diverse and vast, encompassing more than 30 families and 153 species of monocotyledonous and dicotyledonous plants (Freitag 1951, Hopkins 1989, Mizell et al. 2003, Costa et al. 2006). The principal economic host of *X. fastidiosa* in North America is grapevine (Hopkins 1989, Smith et al. 1992). Other less important economic hosts include peach (*Prunus persica*), plum (*Prunus domestica*), almond (*Prunus dulcis*), sweet orange (*Citrus sinensis*), alfalfa (*Medicago sativa*), American elm (*Ulmus americana*), American sycamore (*Platanus occidentalis*), red oak (*Quercus rubra*), red mulberry (*Morus rubra*) and red maple (*Acer rubrum*). Although they may not develop symptoms, various trees, shrubs, plants and weeds are natural hosts of *X. fastidiosa* and may act as reservoirs for the pathogen. These include but are not limited to American beautyberry, American elder, Bermudagrass, blackberry, blue elder, Boston ivy, buckeye, coyote brush, dallisgrass, Eastern Baccharis, English ivy, goldenrod, hairy crabgrass, Japanese beech bonsai, Johnsongrass, ladino clover, lillies, miner's lettuce, mugwort, Oriental bittersweet, peppervine, periwinkle, poison hemlock, porcelain berry, sumac, umbrella sedge, Virginia creeper, wild grape and wild strawberry (Freitag 1951, Wells et al. 1980, Raju et al. 1983, Yonce and Chang 1987, Hopkins and Adlerz 1988, Hopkins 1989, Sherald and Kostka 1992, Smith et al. 1992, Gould and Lashomb 2005).

In addition to PD, plant diseases found to be caused by *X. fastidiosa* include PPD (Hopkins et al. 1973, Hopkins and Mollenhauer 1973, Davis et al. 1981a, Davis et al. 1981b, Wells et al. 1981a, Wells et al. 1983), citrus variegated chlorosis (CVC) (Chang et al. 1993, Lee et al. 1993), alfalfa dwarf (Goheen et al. 1973, Hopkins and Mollenhauer 1973), periwinkle wilt (McCoy et al. 1978) and stunting of ragweed (Timmer et al. 1983). *X. fastidiosa* also causes leaf scorch of almond (Mircetich et al. 1976, Davis et al. 1980b), plum (Kitajima et al, 1975, Davis et al. 1981a, Davis et al. 1981b, Wells et al. 1981a, Raju et al. 1982), sycamore (Hearon et al. 1980, Sherald et al. 1983, Sherald et al. 1985), elm (Hearon et al. 1980, Kostka et al. 1981, Sherald 1990, Sherald 1993), oak (Hearon et al. 1980, Chang and Walker 1988), oleander (Purcell et al. 1999), maple (Sherald et al. 1987), mulberry (Kostka et al. 1986, Hernandez-Martinez 2006c), pear (Leu and Seu 1993), pecan (Sanderlin and Heyderich-Alger 2000), coffee (DeLima et al. 1998, Li et al. 2001), blueberry (Chang et al. 2009), chitalpa (Randall et al. 2007, Randall et al. 2009) and of various landscape species, including crape myrtle, olive, daylily and southern magnolia (Hernandez-Martinez et al. 2006a).

Wine grape and peach production affected by *X. fastidiosa*. PD and PPD are geographically limited to tropical or subtropical climates with mild winters and were both first observed in the 1880s in California and Georgia, respectively (Hutchins 1933, Cochran and Hutchins 1974, Hopkins 1977, Sherald and Kostka 1992). Although first described in California, PD is endemic to the southeastern United States and is the principal factor limiting wine grape production in that region (Hopkins 1977, Adlerz and Hopkins 1979, Hopkins 1989, Sherald and Kostka 1992, Blua et al. 1999). PPD also is endemic to the Southeast, but has been reported as far north as southern Illinois and Missouri (Hutchins 1933, Hopkins et al. 1973, Hopkins 1989). Not as economically important as PD in grapevine, PPD in peach often is the principal factor

limiting orchard life and thus stone fruit production in the Southeast (Horton and Mizell http://www.ent.uga/peach/peachhbk/insects/leafhoppers.pdf, Mizell http://www.ent.uga/peach/peachhbk/bacterial/phonypeach/pdf).

PD occurs in vineyards in California near alfalfa hay fields and irrigated pastures (central region), riparian woodlands (coastal region) and citrus groves (Temecula Valley in the south-central region), which serve as sources for breeding and feeding for insect vectors and thus as reservoirs for the bacterium (Raju et al. 1980, Raju et al. 1983, Hopkins 1989, Purcell and Saunders 1999). *X. fastidiosa* is vectored primarily by xylem-feeding leafhoppers (Mizell et al. 2003, Costa et al. 2006). The blue-green sharpshooter (BGSS) [*Graphocephala atropunctata* (Signoret)] (Hemiptera: Cicadellidae) is the most important vector of *X. fastidiosa* in coastal California (Redak et al. 2004). It is an efficient vector of the bacterium when compared with other sharpshooters, and is the most-studied vector of *X. fastidiosa* in relation to its transmission biology (Severin 1949, Purcell and Finlay 1979, Almeida and Purcell 2003b, Almeida and Purcell 2006). The glassy-winged sharpshooter (GWSS) [*Homalodisca vitripennis* (Germar)] (Hemiptera: Cicadellidae), which is believed to have been introduced to California as eggs on nursery plants from the southeastern United States or northern Mexico and was first observed in Orange and Ventura counties in 1989, is the primary leafhopper vector of PD in grapevine in the Temecula Valley (Sorensen and Gill 1996, Blua et al. 1999, Purcell and Saunders 1999, Perring et al. 2001). The insect has been found in large numbers in citrus groves adjacent to vineyards, which has influenced the incidence, severity and disease dynamics of PD in grapevine (Perring et al. 2001, Hopkins and Purcell 2002).

The occurrence of PD in the Southeast results primarily from secondary (vine to vine) transmission by leafhopper vectors reproducing and feeding within vineyards (Adlerz and

Hopkins 1979, Hopkins 1989). In its native habitat in the Southeast, the GWSS also is the primary vector of PD in grapevine (Frazier and Freitag 1946, Turner and Pollard 1949, Turner and Pollard 1959, Kaloostian et al. 1962, Adlerz and Hopkins 1979, Purcell and Saunders 1999, Krewer et al. 2002). Other key vectors of PD in the Southeast are *Draeculacephala* spp., *Graphocephala* spp., *Homalodisca insolita* and *Oncometopia* spp. (Mizell et al. 2003, Myers et al. 2007).

Symptoms of PD in grapevine usually appear first on the oldest leaves and develop in an acropetal direction (Hopkins 1989). Symptoms include necrosis of leaf margins, with reddening or yellowing of adjacent tissue (Smith et al. 1992). The entire leaf may eventually turn brown, shrivel and drop, leaving the petiole attached to the vine. Diseased vines often mature irregularly, with patches of brown and green tissue. Infected plants in later years develop late and produce chlorotic shoots. Death of the plant occurs within one to two years of the onset of symptoms.

Severe winter climates may limit the geographical range of PD (Hopkins 1989). Controlled experiments in which PD-infected grapevines were exposed to -8°C to -12°C for various periods of time were found by Purcell (1977) to result in either temporary remission of symptoms or recovery in some test plants. However, environmental experiments in which PD-infected grapevines were exposed to various winter climates did not always result in more recovery of plants exposed to colder climates (Purcell 1980). Wine grape production is concentrated in the southern foothills of the Appalachian Mountains in the Blue Ridge region of northern Georgia, where PD is limited due to cooler temperatures as a result of higher elevations (Brannen 2007). Based on vineyard surveys throughout the state, it is recommended that wine grapes not be planted at elevations less than 1300 feet above sea level. However, warmer winters can shift the PD “safe” zone to yet higher elevations.

PPD occurs from North Carolina to Texas, wherever the average minimum temperature is warmer than -10.5°C (Mizell http://www.ent.uga/peach/peachhbk/bacterial/phonypeach/pdf). PPD is commercially important in Georgia south of a line from LaGrange to Augusta. Major epidemics occurred in 1929, 1951 and 1976. The disease was particularly important in the 1940s, when orchards 5 years and older were often found to be 50-100% affected (Smith et al. 1992). The primary insect vectors of PPD are the GWSS and *Oncometopia orbona*, which are particularly active in May and June (Turner and Pollard 1959, Wells et al. 1983, Yonce 1983, Horton and Mizell http://www.ent.uga/peach/peachhbk/insects/leafhoppers.pdf). *Graphocephala versuta* also is a key insect vector. Considered less important as vectors are *H. insolita* and *Cuerna costalis*. Leafhopper abundance fluctuates with climate. High populations are common in summers that follow warm winters. Leafhopper abundance and species also are influenced by vegetation in and around orchards. Wild cherry, wild and cultivated plums, and various perennial weeds are known reservoirs for *X. fastidiosa* strains that cause PPD (Yonce and Chang 1987). PPD also is transmittable in the field by root grafts, as the bacterium is plentiful in roots (Mizell http://www.ent.uga/peach/peachhbk/bacterial/phonypeach/pdf). Grafting experiments and microscopic examination indicated few bacteria are present in stems during much of the year.

Symptoms of PPD in peach are different from other *X. fastidiosa* diseases. PPD does not cause foliar wilting, chlorosis or necrosis, dieback of branches or tree death (Hopkins 1989, Mizell http://www.ent.uga/peach/peachhbk/bacterial/phonypeach/pdf, Smith et al. 1992). Disease symptoms typically occur 18 or more months after infection (Davis et al. 1981a) and may develop in one limb or over the entire tree at the same time. Infected trees bloom and leaf earlier, and leaves senesce later in the fall (Hopkins 1989, Smith et al. 1992). Beginning in July, foliage of diseased trees appears darker green, flatter and more compact, due to stunted young

shoots, shortened leaf internodes and profuse lateral branching (Hopkins 1989, Sherald and Kostka 1992, Smith et al. 1992. Quantity, size and quality of fruit are increasingly reduced until fruit becomes unmarketable after 3-5 years (Cochran and Hutchins 1974, Hopkins 1989, Sherald and Kostka 1992, Smith et al. 1992). PPD also may cause trees to be more susceptible to other pathogens.

Epidemiology of *X. fastidiosa*. Known to vector *X. fastidiosa*, the GWSS is native to the southeastern United States and is a piercing-sucking insect that feeds on the xylem of more than 100 plant species (Turner and Pollard 1959, Blua et al. 1999, Purcell and Saunders 1999, Conklin and Mizell 2002, Almeida and Purcell 2003b, Hoddle et al. 2003, Costa et al. 2006). The GWSS is the largest leafhopper in Georgia, measuring between 1.5 and 2 cm in length at maturity (Conklin and Mizell 2002), and is considered the most probable insect vector of BLSB in the state, as the leafhopper constituted 97.22% of leafhoppers sampled in 2009 from plants at two SHB fields in Berrien and Hoboken counties infected with *X. fastidiosa* (Brannen et al. 2010, Tertuliano et al. 2010, Nissen *unpublished data*). Other leafhoppers sampled at the two fields were *Draeculacephala* spp. (1%), *Graphocephala versuta* (0.51%), *H. insolita* (0.25%), *Oncometopia nigricans* (0.25%) and *Paraulacizes irrorata* (0.77%) (Brannen et al. 2010, Tertuliano et al. 2010). Leafhoppers were first observed in May and were most abundant from June through early September (Tertuliano et al. 2010). Choice of plant host of the GWSS observed in caged greenhouse studies conducted by Tertuliano et al. (2010) was similar to that observed in the field, with 'Emerald' (55%) preferred to 'Star' (27%) and 'V1' (17%) varieties of SHB.

Leafhoppers can be efficient vectors of *X. fastidiosa*, as the bacterium can be transmitted without a latent period after being acquired (Severin 1949, Frazier 1965, Purcell and Finlay

1979, Purcell et al. 1979). *X. fastidiosa* attaches to and colonizes the precibarium, cibarium and lining of the esophagus in the foregut of the insect and is persistent throughout the life of the insect (several months) when it is acquired by an adult (Severin 1949, Frazier 1965, Purcell and Finlay 1979, Purcell et al. 1979, Brlansky et al. 1983, Hopkins 1989, Hill and Purcell 1995, Conklin and Mizell 2002, Almeida and Purcell 2003b, Mizell et al. 2003, Almeida and Purcell 2006, Costa et al. 2006). Nymphs are not efficient vectors because the acquired pathogen is lost when the lining of the foregut is shed during molting (Purcell and Finlay 1979, Purcell et al. 1979, Hopkins 1989).

Studies by Hill and Purcell (1995) and Almeida and Purcell (2006) indicated small numbers (< 100) of cultivable *X. fastidiosa* cells in the foregut of the BGSS are sufficient for transmission of the bacterium to grapevine. The BGSS is native to California and is the most important vector of *X. fastidiosa* in coastal regions of the state (Redak et al. 2004). Scanning electron microscopy of BGSS that fed on *X. fastidiosa*-inoculated grapevine was used by Almeida and Purcell (2006) to corroborate studies by Purcell and Finlay (1979) and Almeida and Purcell (2003b) that indicated *X. fastidiosa* must be present in the sharpshooter foregut, specifically the precibarium, for transmission to plants to occur. Almeida and Purcell (2006) further demonstrated that detachment of *X. fastidiosa* cells from the precibarium during insect feeding is important for transmission of the bacterium. Nevertheless, questions concerning vector efficiency remain unanswered. Almeida and Purcell (2006) found that five out of 12, or 41.7%, of BGSS with *X. fastidiosa* cells attached to their precibariums did not transmit the bacterium to plants. The researchers in that and another study (Almeida and Purcell 2003b) also found that the GWSS is a less-efficient vector than the BGSS in transmitting *X. fastidiosa* to grapevine. In the 2006 study, Almeida and Purcell examined 30 GWSS and found *X. fastidiosa* cells in the

precibarium of only one, and that insect failed to transmit the bacterium to grapevine. The researchers surmised that differences in feeding behavior among sharpshooters may explain variability in efficiency of transmission of *X. fastidiosa*, as the pathogen has low to no vector specificity (Frazier 1965, Purcell 1989, Daugherty and Almeida 2009), and the ability of bacterial cells to attach to the foregut of different species should be similar.

Sources of variability in transmission rate of insect vector-borne plant pathogens include vector species (Palermo et al. 2001, Daugherty and Almeida 2009), vector abundance (Ng and Perry 2004, Daugherty and Almeida 2009), host species (Wistrom and Purcell 2005, Lopes et al. 2009), pathogen strains (Lucio-Zavaleta et al. 2001, Lopes et al. 2009) and local climate (Sylvester 1964, Shih et al. 1995, Murral et al. 1996, Lucio-Zavaleta et al. 2001, Dohm et al. 2002, Anhalt and Almeida 2008, Daugherty et al. 2009). Statistical models based on binomial and Poisson distributions of empirical data were proposed by Purcell (1981) and used by Daugherty and Almeida (2009) to predict the probability of transmission of *X. fastidiosa* by sharpshooters as a function of vector abundance, pathogen acquisition period and inoculation access period. As predicted by Purcell (1981), Costa et al. (2000) and Daugherty and Almeida (2009) found that sharpshooter numbers are positively related to *X. fastidiosa* transmission rate. Hill and Purcell (1995) determined that acquisition efficiency of *X. fastidiosa* by sharpshooters is dependent on pathogen populations in host plants, which in turn is associated with disease symptoms (Krivanek et al. 2005). Purcell and Finlay (1979) and Almeida and Purcell (2003b) also determined that acquisition efficiency of *X. fastidiosa* by sharpshooters is dependent on length of the acquisition period. Low bacterial populations in host plants and short insect feeding periods reduce the probability of sharpshooters acquiring *X. fastidiosa* (Daugherty and Almeida 2009). Inoculation efficiency of *X. fastidiosa* by sharpshooters also is positively related to the

length of inoculation period through insect feeding (Purcell and Finlay 1979, Hill and Purcell 1995, Almeida and Purcell 2003b, Daugherty and Almeida 2009). As determined by Daugherty and Almeida (2009) with the Poisson statistical model and with greenhouse studies, transmission efficiency of PD in grapevine by the GWSS is substantially lower than by the BGSS. Of vector number, acquisition period and inoculation period, only vector number was positively associated with GWSS transmission efficiency of PD in grapevine in the statistical model. Neither acquisition nor inoculation period increased transmission efficiency of PD in grapevine by the GWSS. Conversely, vector number had a weaker effect than acquisition and inoculation period in transmission efficiency of PD in grapevine by the BGSS. Both acquisition and inoculation period contributed substantially to transmission efficiency of PD in grapevine by the BGSS. Daugherty and Almeida (2009) concluded that high vector numbers are not related to greater *X. fastidiosa* populations in plants, but may drive disease dynamics by decreasing the time for vectors to reacquire the pathogen and thus increasing the likelihood of secondary spread. They further concluded that if transmission efficiency is a function of number of inoculation events more so than total inocula supplied, then insect feeding period would be expected to be less significant than insect numbers, especially for an inefficient vector such as the GWSS.

Genetic diversity of *X. fastidiosa*. All strains of *X. fastidiosa* are currently classified as a single species (Wells et al. 1987). The 25 strains of *X. fastidiosa* isolated from 10 plant hosts that Wells et al. (1987) grouped into a single species showed similar phenotypic and genetic characteristics, with 51-53% GC content and at least 85% DNA sequence homology. Prior to the 1990s, pathogenic relationships among strains of the bacterium were not well characterized and appeared complex (Hopkins 1989). Reciprocal transmission experiments indicated that PD, almond leaf scorch (ALS) and alfalfa dwarf are caused by the same *X. fastidiosa* strains

(Hopkins 1989), while PPD and plum leaf scald are caused by a different group of *X. fastidiosa* strains (Wells et al. 1981b, Hopkins 1989). Inoculation experiments indicated periwinkle wilt and PD strains of *X. fastidiosa* were pathogenic to periwinkle; however, the PD strains caused only mild chlorosis of older leaves, and the periwinkle strains multiplied in grapevine but did not cause disease (Davis et al. 1983, Hopkins 1989). Thus, strains of these two pathogens were pathologically distinct (Hopkins 1989). Mechanical inoculation indicated ragweed stunt strains of *X. fastidiosa* infected plum and periwinkle without producing symptoms and did not infect grapevine, peach or citrus (Timmer et al. 1983, Hopkins 1989). Conversely, PPD strains infected a low percentage of ragweed plants. Although pathologically distinct, strains causing ragweed stunt were more closely related to PPD or plum leaf scald than to PD strains (Timmer et al. 1983, Hopkins 1989). Field surveys and greenhouse inoculation studies also have shown that PD strains do not infect oleander and OLS strains do not infect grapevine (Purcell et al. 1999).

Studies beginning in the mid-1980s revealed there are indeed genetic differences among strains, with a number of studies indicating the existence of host-specific subgroups of *X. fastidiosa*. DNA sequence homology was used by Kamper et al. (1985) to differentiate PD strains from strains causing PPD, plum leaf scald and periwinkle wilt. Restriction fragment length polymorphisms (RFLP) were used by Chen et al. (1992) to confirm that strains associated with PD, alfalfa dwarf and ALS form a closely related taxonomic group. Randomly amplified polymorphic DNA (RAPD) was used by Chen et al. (1995) to demonstrate that strains isolated from oak are closely related to strains isolated from grapevine and are distantly related to strains isolated from periwinkle and plum. Pooler and Hartung (1995a) also used RAPD to demonstrate that PD strains are more similar to a ragweed strain than to an almond strain and are less similar

to CVC strains. Thus, there appears to be a grapevine-alfalfa-almond-ragweed group, an oak group, a plum-elm group, a mulberry group and a citrus group (Doddapaneni et al. 2006).

Hendson et al. (2001) and Almeida and Purcell (2003b) reported that ALS could be caused by two genotypes: one that would also cause PD in grapevine and one that would not. Single nucleotide polymorphisms in conserved 16S rDNA sequences from *X. fastidiosa* strains Temecula and Dixon were used by Chen et al. (2005b) to design two primer pairs for multiplex polymerase chain reaction (PCR) assays to detect and distinguish between the two genotypes in almond. ALS strains that also cause PD in grapevine were designated as G-type strains of the bacterium, while ALS strains that do not cause PD in grapevine were designated as A-type.

Analysis of the 16S-23S rDNA intergenic spacer (ITS) region has successfully determined taxonomic relationships between and within many groups of bacteria (Schaad et al. 2004, Martinati et al. 2007). Schaad et al. (2004) conducted DNA-DNA hybridization and ITS sequence comparison analyses to assign *X. fastidiosa* strains to three subspecies. Strains from grapevine, alfalfa and maple, and some strains from almond were assigned to *X. fastidiosa* subsp. *fastidiosa*. Strains from peach, plum, almond, sycamore, elm and pigeon grape were assigned to *X. fastidiosa* subsp. *multiplex*. Strains from citrus were assigned to *X. fastidiosa* subsp. *pauca*. Schuenzel et al. (2005) used multilocus sequence typing data from 10 genes common to *X. fastidiosa* strains Temecula, Dixon, Ann-1 and 9a5c to construct a maximum likelihood tree for 26 *X. fastidiosa* isolates, which resulted in oleander leaf scorch (OLS) strains of the bacterium being assigned, based on phylogeny, to a fourth subspecies that they proposed be called *sandyi*. To differentiate and assign PD, ALS and OLS strains to *X. fastidiosa* subsp. *fastidiosa*, *multiplex* or *sandyi*, Hernandez-Martinez et al. (2006b) designed three primer pairs for use in multiplex

PCR assays. They demonstrated that multiplex PCR also allowed further distinction of two genotypes, ALSI and ALSII, within *X. fastidiosa* subsp. *multiplex*.

Detection of *X. fastidiosa*. Prior to culturing and the use of Koch's postulates by Davis et al. (1978) to identify the causal agent of PD in grapevine, Freitag (1951) used insects to demonstrate that PD could be transmitted to 75 of 100 host plant species. During the past three decades, a number of other methods have been used to detect and also differentiate *X. fastidiosa* strains, including light, scanning electron, phase contrast and fluorescence microscopy; biochemical and serological tests such as enzyme-linked immunosorbent assay (ELISA), nutritional requirement and whole-cell protein profiles, multigene coding loci analysis, DNA homology and DNA pulse field gel electrophoresis. Beginning in the 1990s, detection and differentiation of *X. fastidiosa* strains shifted to a more rapid and sensitive molecular approach, with various types of PCR that use primers designed to target genes and regions in *X. fastidiosa* genomes and DNA templates extracted from pure cultures (Chen et al. 1992, Minsavage et al. 1994, Chen et al. 1995, Pooler and Hartung, 1995a, Pooler and Hartung, 1995b, Pooler et al. 1997, Albibi et al. 1998, Banks et al. 1999, Chen et al. 1999, McElrone et al. 1999, Chen et al. 2000, da Costa et al. 2000, Hendson et al. 2001, Mehta et al. 2001, Mehta and Rosato 2001, Chen et al. 2002, Schaad et al. 2002, Almeida and Purcell 2003a, Rodrigues et al. 2003, Ciapina et al. 2004, Schaad et al. 2004, Bextine et al. 2005, Chen et al. 2005a, Chen et al. 2005b, Chen and Civerolo 2005, Lin et al. 2005, Scally et al. 2005, Doddapaneni et al. 2006, Francis et al. 2006, Hernandez-Martinez et al. 2006b, Huang et al. 2006, Bextine and Child 2007, Doddapaneni et al. 2007, Martinati et al. 2007, Bextine et al. 2009, Yuan et al. 2010, Nissen *unpublished data*). Types of PCR used include conventional, multiplex, nested, immunocapture, repetitive extragenic palindromic (REP), reverse transcription (RT), and real-time and

quantitative real-time with TaqMan®, SYBR® Green, EvaGreen® and Takara SYBR® Green probes. Molecular methods used in conjunction with PCR include RFLP, RAPD, amplified fragment length polymorphisms (AFLP), single nucleotide polymorphisms (SNPs), multilocus sequence typing (MLST), simple sequence repeat (SSR) and variable numbers of tandem repeat (VNTR) markers, and single locus markers (16S rRNA, 16S-23S rDNA ITS and *gyr*B). Both the 16S rRNA and 16S-23S rDNA ITS loci have successfully determined taxonomic relationships between and within groups of bacteria, including among strains of *X. fastidiosa*; however, the latter offers more diversity in length and sequence (Barry et al. 1991, Jeng et al. 2001, Schaad et al. 2004, Martinati et al. 2007).

During the past decade, other methods to detect and differentiate *X. fastidiosa* strains have included the use of commercial kits that extract total genomic DNA from plant and insect tissue for use as template (Bextine et al. 2004a, Bextine et al. 2004b, Costa et al. 2006, Huang et al. 2006, Myers et al. 2007, Nissen *unpublished data*), which increases efficiency by eliminating the need to isolate *X. fastidiosa* and purify genomic DNA prior to PCR. However, the presence of PCR inhibitors in both types of extracts has been an inherent problem in detection and differentiation of *X. fastidiosa* strains that few protocols address (Doddapaneni et al. 2007). Pooler et al. (1997) used immunomagnetic separation and Ciapina et al. (2004) used Chelex 100, both along with nested PCR, to avoid inhibitors in plant and insect extracts and permit the detection of *X. fastidiosa*. A BIO-PCR protocol was developed by Fatmi et al. (2005) to avoid inhibitors and permit detection of *X. fastidiosa* in sap from grapevine and citrus. Chen et al. (2008) developed a PCR protocol to detect *X. fastidiosa* using pulverized freeze-dried tissue from almond instead of extracted genomic DNA.

Due to its simplicity in sample preparation and ease of use, ELISA is the most common method to detect *X. fastidiosa* in field surveys and epidemiological studies involving large numbers of plant and insect samples (Minsavage et al. 1994, Smith et al. 1992, Huang et al. 2006). However, the method is limited in its sensitivity, prohibiting detection of low numbers of bacterial cells, and cannot be used to differentiate strains beyond the species level. ELISA can be used to detect *X. fastidiosa* from cultures and extracts of buds, leaf veins, petioles and other woody ornamental plant tissues (Sherald and Lei 1991), but only at a sensitivity greater than 10^6 colony forming units per ml (Sherald and Kostka 1992). ELISA is 10-100-fold less sensitive than culturing (Sherald and Lei 1991, Smith et al. 1992) and at least 100-fold less sensitive than conventional PCR (Minsavage et al. 1994) for detection of *X. fastidiosa*. While ELISA was used by Chang et al. (2009) to detect BLSB strains in SHB, molecular methods to detect and characterize the pathogen in plants and insect vectors have not been studied.

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CHAPTER 3

CHARACTERIZATION OF *XYLELLA FASTIDIOSA* STRAINS THAT CAUSE BACTERIAL LEAF SCORCH OF SOUTHERN HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM* INTERSPECIFIC HYBRIDS) IN SOUTH GEORGIA

INTRODUCTION

Xylella fastidiosa is a fastidious, gram-negative, xylem-limited plant pathogenic bacterium that is vectored primarily by xylem-feeding leafhoppers (Mizell et al. 2003, Costa et al. 2006). Although not fully known, the host range of *X. fastidiosa* is diverse and vast, encompassing more than 30 families and 153 species of monocotyledonous and dicotyledonous plants (Freitag 1951, Hopkins 1989, Mizell et al. 2003, Costa et al. 2006). Pathotypes of *X. fastidiosa* are the causal agents of Pierce's disease of grapevine (PD) (Davis et al. 1978), citrus variegated chlorosis (CVC) (Chang et al. 1993, Lee et al. 1993), phony peach disease (Hopkins et al. 1973, Hopkins and Mollenhauer 1973, Davis et al. 1981a, Davis et al. 1981b, Wells et al. 1981, Wells et al. 1983), plum leaf scald (Kitajima et al. 1975, Davis et al. 1981a, Davis et al. 1981b, Wells et al. 1981, Raju et al. 1982), almond leaf scorch (ALS) (Mircetich et al. 1976, Davis et al. 1980) and oleander leaf scorch (OLS) (Purcell et al. 1999).

Chang et al. (2009) completed Koch's postulates in 2006 to demonstrate that *X. fastidiosa* is the causal agent of a new disease, bacterial leaf scorch of blueberry (BLSB), observed in varieties of southern highbush (SHB) (*Vaccinium corymbosum* interspecific hybrids) in Georgia. Blueberries surpassed peaches in farm gate value in 2004 to become the most economically important fruit crop in the state (Boatright and McKissick 2010). Nearly 6,615

hectares in the state are planted in blueberry, which represents a 192% increase since the year 2000. Blueberry production in Georgia has continued to increase in value and currently stands at more than $102.4 million. However, BLSB is a threat to the industry (Brannen et al. 2010).

Symptoms of *X. fastidiosa* vary depending on host, but generally involve a loss of plant vigor, branch dieback and eventual death of the plant (Hopkins 1989, Mizell et al. 2003). Specific symptoms may include delayed leafing in the spring, mottling and marginal scorching of leaves, interveinal chlorosis and necrosis, shoot dwarfing, wilting and premature coloring of fruit, and root death. Initial symptoms of BLSB include chlorosis and subsequent necrosis of older leaves that begins at the margin and progresses throughout the entire leaf, resulting in a scorched appearance similar to that seen with extreme drought or fertilizer salt burn (Chang et al. 2009). New shoots often are abnormally thin, produce fewer flower buds and develop a yellow "skeleton-like" appearance once affected leaves drop. Death of the plant follows leaf drop, typically within two years after the onset of symptoms.

All strains of *X. fastidiosa* are currently classified as a single species (Wells et al. 1987). However, various studies have found phenotypic and genetic differences among strains, suggesting genetic diversity within the species. Hendson et al. (2001) and Almeida and Purcell (2003) reported that ALS could be caused by two genotypes: one that would also cause PD in grapevine and one that would not. Single nucleotide polymorphisms in conserved 16S rDNA sequences from *X. fastidiosa* strains Temecula and Dixon were used by Chen et al. (2005) to design two primer pairs for multiplex polymerase chain reaction (PCR) assays to detect and distinguish between the two genotypes in almond. ALS strains that also caused PD in grapevine were designated as G-type strains of the bacterium, while ALS strains that did not cause PD in grapevine were designated as A-type. In that same study, researchers observed distinct colony

morphologies on PWG medium (Hill and Purcell 1995) that also distinguished between the two genotypes. The researchers reported that colonies of G-type strains typically were circular in form, convex and had entire margins, while colonies of A-type strains appeared "pit"-like, were circular to spindle shaped in form, convex, pulvinate or umbonate, and had rough margins. To determine taxonomic relatedness among *X. fastidiosa* strains, Schaad et al. (2004) conducted DNA-DNA hybridization and 16S-23S intergenic spacer (ITS) sequence comparison analyses to assign strains to three subspecies. Strains from grapevine, alfalfa and maple, and some strains from almond were assigned to *X. fastidiosa* subsp. *fastidiosa*. Strains from peach, plum, almond, sycamore, elm and pigeon grape were assigned to *X. fastidiosa* subsp. *multiplex*. Strains from citrus were assigned to *X. fastidiosa* subsp. *pauca*. Schuenzel et al. (2005) used multilocus sequence typing data from 10 genes common to *X. fastidiosa* strains Temecula, Dixon, Ann-1 and 9a5c to construct a maximum likelihood tree for 26 *X. fastidiosa* isolates, which resulted in OLS strains of the bacterium being assigned to a fourth subspecies that they proposed be called *sandyi*. Temecula is a PD strain, Dixon is an ALS strain, Ann-1 is an OLS strain and 9a5c is a CVC strain of *X. fastidiosa*. To differentiate and assign PD, ALS and OLS strains to *X. fastidiosa* subsp. *fastidiosa*, *multiplex* or *sandyi*, Hernandez-Martinez et al. (2006) designed three primer pairs for use in multiplex PCR assays. They demonstrated that the multiplex PCR also allowed further distinction of two genotypes, ALSI and ALSII, within *X. fastidiosa* subsp. *multiplex*.

The objective of this research was to characterize BLSB strains by determining their similarity to G- or A-type strains of *X. fastidiosa*. Species-specific and strain-specific primer pairs were used in PCR assays to compare *X. fastidiosa* strains isolated from blueberry with strains isolated from grapevine, alfalfa, almond, plum, oak, sycamore and oleander. The 16S-23S

rDNA ITS sequences of two BLSB strains also were determined and used to compare DNA homology of the BLSB strains with similar sequences from known *X. fastidiosa* genomes. Based on those results, a single primer pair was designed that eliminated the need for multiplex PCR assays to detect and distinguish A- and G-type strains of *X. fastidiosa*.

MATERIALS AND METHODS

Strains used in this study. Pure cultures of 51 strains of *X. fastidiosa* were obtained from Dr. Chung-Jan Chang, Department of Plant Pathology, The University of Georgia, Griffin, GA (Table 3.1). Lyophilized genomic DNA of 20 strains of *X. fastidiosa* was obtained from Dr. Jianchi Chen, U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS), Parlier, CA (Table 3.2). A strain of *X. fastidiosa* (TH949) was isolated during this study (method described in Chapter 4) from an introduction of SHB ('Camellia' × 'Reveille') located at a horticulture research plot at the Griffin campus of The University of Georgia. The strain was identified as *X. fastidiosa* using the RST32-RST33, 2594F-2594R and XF1968F3-XF1968R3 primer pairs (Table 3.3). BLSB strains BT2V and HT5S used in this study were isolated (as described in Chapter 4) in October 2009 from leaf tissue (petiole and midvein) and stem sap, respectively, from the 'Star' variety of symptomatic SHB at fields in Berrien and Brantley counties in southeast Georgia.

Preparation of genomic DNA. Bacterial cell suspensions were made from pure cultures by harvesting 7-10-d old *X. fastidiosa* colonies from plates with a sterile inoculation loop and suspending them into 3 ml of sterile TE buffer (10mM Tris-HCL, 1 mM EDTA, pH 7.4) or sterile deionized water. Cell suspensions of approximately 10^9 colony forming units (CFU) per ml were estimated by optical density at 600 nm with a spectrophotometer. Cells in 1-ml aliquots were pelleted by centrifuging 10 min at 20,000 × g, and then all but approximately 50 µl of each

supernatant was removed. Cell pellets were stored at -20°C until genomic DNA was purified using the MasterPure™ DNA Purification Kit (EPICENTRE® Biotechnologies, Madison, WI). DNA then was stored at -20°C. Lyophilized genomic DNA samples received from Dr. Chen were stored at 4°C. Sterile TE buffer (50 µl) was added to each sample to dissolve the DNA. After incubating for 24 h at 4°C, samples were stored at -20°C.

PCR primers and protocols. Sequences and sizes of expected amplicons for primers used in this study are listed in Table 3.3. PCR samples were prepared using the MasterAmp™ *Tfl* DNA Polymerase kit (EPICENTRE Biotechnologies, Madison, WI) and contained 3X PCR Enhancer with betaine, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 1X PCR Buffer, 25 pmol of each primer, 10-100 ng genomic DNA, 0.5 U MasterAmp™ *Tfl* DNA Polymerase and sterile deionized water, for a total reaction volume of 25 µl. Amplification was conducted in thin-wall PCR tubes using a GeneAmp® PCR System 9600 thermocycler (Perkin-Elmer Corporation, Norwalk, CT). The thermocycler program for all but the ALMF2-ALMR3 and XF1968F3-XF1968R3 primer pairs was as follows: initial denaturation of 4 min at 94°C; 40 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C; and a final extension of 10 min at 72°C. The thermocycler program for the ALMF2-ALMR3 primer pair was as follows: initial denaturation of 4 min at 94°C; 40 cycles of 30 sec at 94°C and 1 min at 70°C; and a final extension of 10 min at 72°C. The thermocycler program for the XF1968F3-XF1968R3 primer pair was as follows: initial denaturation of 4 min at 94°C; 40 cycles of 30 sec at 94°C, 1 min at 65°C, and 30 sec at 72°C; and a final extension of 10 min at 72°C. Amplicons were resolved by electrophoresis in a 1% agarose gel containing 50 µg/ml of ethidium bromide in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. PCR products were visualized using an ultraviolet light transilluminator and documented using either an Eagle Eye II Video Imaging System (Stratagene Corporation, La

Jolla, CA) or BioSpectrum® 300 Imaging System (UVP LLC, Upland, CA). The size of amplicons was determined by comparison to either a 100-base pair (bp) or 1-kb DNA ladder (New England BioLabs Inc., Ipswich, MA).

16S-23S rDNA ITS region sequencing. Amplicons produced by primers 16S-23SF and 16S-23SR were extracted from an agarose gel using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA) and then directly sequenced, using the same primers, at the Georgia Genomics Facility (The University of Georgia, Athens, GA). Sequences were aligned with similar sequences from known *X. fastidiosa* genomes using the Basic Local Alignment Search Tool (BLAST) through the network service of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Limits of detection. Bacterial cell suspensions (2×10^8 CFU/ml) of *X. fastidiosa* strain TH949 were generated in sterile deionized water, sterile TE buffer and phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4), as previously described. Suspensions were serially diluted 10-fold, and the 10^{-5}, 10^{-6} and 10^{-7} dilutions were spotted (20 µl) in triplicate on PD2 and CS20 agar media. Plates were incubated at 28°C, and spots were observed for colony growth after 6 d. Aliquots (1 ml) of the remaining suspensions were incubated 10 min at 100°C and then cooled to room temperature. DNA from lysed cells was analyzed by PCR, as previously described, using 10 µl of boiled cells as template and primer pairs RST32-RST33, 2594F-2594R and XF1968F3-XF1968R3. Genomic DNA from BLSB strain BB9 was serially diluted 1/4 starting at 1 ng/µl, and dilutions were then used as template in PCR using the same primers.

RESULTS AND DISCUSSION

PCR analyses. All strains used in this study were confirmed as *X. fastidiosa*, as species-specific primer pairs RST31-RST33 and RST32-RST33 amplified DNA fragments of approximately 733 bp and 600 bp, respectively (results not shown). To improve specificity for PD strains of *X. fastidiosa*, new forward and reverse primers (PDconsvdF and PDspec685) were designed from published primers XF176f and XF686r. Banks et al. (1999) designed XF176f and XF686r, based on random amplified polymorphic DNA (RAPD) sequence data, to amplify PD strains. The new primers were designed based on analysis of a multiple sequence alignment using the ClustalW software available through the network service of the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/). For the analysis, sequences for Temecula1, Ann-1, Dixon and 9a5c strains of *X. fastidiosa* were aligned with the sequence of a PD-specific RAPD fragment (GenBank accession number AF130454) that contains a serine tRNA gene.

To correct what appeared to be an error in the sequence of XF176f (Appendix A), a single nucleotide (G) was deleted to make the new forward primer PDconsvdF. To make the new reverse primer PDspec685, the sequence of XF686r was shifted, creating a GC clamp at that primer's 3′ end with genomic DNA from PD strains and a terminal one-base mismatch with genomic DNA from ALS, OLS and CVC strains. As shown in Figure 3.1, the new primer pair produced the expected 514-bp amplicon with DNA from four PD strains from grapevine, but no prominent amplicon was produced with DNA from six BLSB strains. The weak band of approximately 500 bp produced from BLSB strains was considered to be nonspecific amplification, as the bands appeared smaller than the amplicons produced from the PD strains. Thus, the BLSB strains tested appeared to be different from PD strains of *X. fastidiosa*.

Since BLSB strains appeared to be different than PD strains, a study was initiated to determine what group BLSB strains of *X. fastidiosa* belonged to based on a comparison of sequences from the conserved 16S-23S rDNA ITS region. This region was used instead of the 16S rRNA locus because of its greater diversity and longer length. Analysis using the ITS region has successfully resolved taxonomic relationships between and within groups of bacteria, including among strains of *X. fastidiosa* (Barry et al. 1991, Jeng et al. 2001, Schaad et al. 2004, Martinati et al. 2007). The ITS regions of BLSB strains BB8 and BB20 were amplified using primer pair 16S-23SF/16S-23SR, and the approximately 650-bp target amplicons were gel purified and directly sequenced in both directions. A BLAST analysis revealed that 520 bases from the ITS regions of the BLSB strains were identical (results not shown) and that they were 99.81% identical to the ITS regions of M12 and Dixon, two A-type strains of *X. fastidiosa* isolated in California from almond with ALS, and to Ann-1 that was isolated in California from oleander (Figure 3.2). ITS regions of the BLSB strains were also 98.65% identical to the ITS regions of Temecula1 and M23, and 99.04% identical to the ITS region of 9a5c. Temecula1 and M23 are *X. fastidiosa* strains that were isolated in California from grapevine and almond, respectively, and are G-type strains, grouped into *X. fastidiosa* subsp. *fastidiosa*. Ann-1 is an OLS strain, grouped into *X. fastidiosa* subsp. *sandyi*, which is considered neither A-type nor G-type (Chen et al. 2010, Chen et al. 2005, Hernandez-Martinez et al. 2006). 9a5c, a strain of CVC isolated in Brazil, is grouped into *X. fastidiosa* subsp. *pauca*, which also is neither A or G type. As indicated in a multiple sequence alignment of ITS regions using ClustalW (Figure 3.2), BB8 differed from Dixon, M12 and Ann-1 by one nucleotide, with the difference being in the BLSB strain. BB8 differed by seven nucleotides from Temecula1 and M23, with the differences being in the PD strains. BB8 differed by five nucleotides from 9a5c, with four of the differences being

in the CVC strain. Analysis of the ITS regions thus confirmed that BLSB strains differed from PD strains and determined that BLSB strains are most closely related to ALS and OLS strains of *X. fastidiosa*.

Discovering the BLSB strains are most closely related to Ann-1, Dixon and M12, the M12 strain was targeted for the design of a primer pair to detect BLSB strains in plants and insect vectors, as it is the only one of the three strains whose genome is completely sequenced (Chen et al. 2010). To begin the process, 54 genes unique to Dixon and M12 were accessed from a *X. fastidiosa* database (http://fresno.ars.usda.gov/citrusdisease/CVC_index.htm) and visually evaluated based on function and length of nucleotide sequence. The database is maintained by Doddapaneni et al. (2006), who identified genes unique to *X. fastidiosa* strains, based on DNA variation in coding and non-coding regions, single nucleotide polymorphisms and insertions-deletions of one to several hundred base pairs. Locus FX2594, which has a sequence of 234 nucleotides and is predicted to encode a phage DNA packaging protein GP2 (terminase large subunit), was selected for the development of a primer pair. OLIGO Primer Analysis Software (Molecular Biology Insights, Inc., Cascade, CO) was used to facilitate the design of 2594F and 2594R, which flank FX2594 in the M12 genome, as shown in Appendix B. Designed to amplify a 1323-bp DNA fragment from A-type strains of *X. fastidiosa*, the primers generated the expected amplicon from genomic DNA of known A-type strains provided by J. Chen (Figure 3.3). They also generated the same amplicon from all 22 BLSB strains tested (Figure 3.4), indicating that they are A-type strains.

However, contrary to expectations, the 2594F-2594R primer pair also generated multiple amplicons approximately 700 bp and larger from G-type strains (Figure 3.3). Generation by the 2594F-2594R primer pair of different banding patterns for A- and G-type strains was similar in

behavior to primers used in the multiplex PCR assay developed by Chen et al. (2005), based on single nucleotide differences in the 16S rDNA loci, to distinguish between the two strain genotypes. Thus, in this study, the 2594F-2594R primer pair was more efficient and reliable than the multiplex primers (results not shown) and can replace the need for multiplex PCR to differentiate A- and G-type strains of *X. fastidiosa*.

Since the 2594F-2594R primer pair was not specific to A-type strains, published primer pairs ALM1-ALM2 and XF1968-L/XF1968-R, which were demonstrated by Hernandez-Martinez et al. (2006) to be specific to OLS and/or ALS strains of *X. fastidiosa*, were evaluated for use in this study as subspecies-specific primers. Primers ALM1 and ALM2 were designed to amplify a 521-bp fragment from an intergenic region in ALS strains (Appendix C), while primers XF1968-L and XF1968-R were designed to amplify a 638-bp fragment from the XF1968 gene in ALS and OLS strains of *X. fastidiosa* (Appendix D). However, we could not reproduce the specificity reported by these researchers for either primer pair. Thus, specificity of the primers was further evaluated, based on a multiple sequence alignment using ClustalW, with *X. fastidiosa* strains M12, Dixon, Ann-1, Temecula1, M23 and 9a5c, and as a result, one primer was modified and three new primers were designed.

ALMF2 was modified from forward primer ALM1 by lengthening the sequence with the addition of "G" nucleotides at both ends, which enhanced a mismatched alignment with non-ALS strains at the 3′ end (Appendix C). ALMR3 is a new reverse primer that was designed such that six bases at the 3′ end and four other internal bases would uniquely match a target in ALS strains. The ALMF2-ALMR3 primer pair was designed to amplify from ALS strains, such as Dixon and M12, and from CVC strain 9a5c (although the primers were not used to test 9a5c because genomic DNA from the strain was not available). As expected, the primers amplified an

approximately 541-bp DNA fragment from all BLSB and other A-type strains of *X. fastidiosa* tested (results not shown). However, the primer pair was not robust, as BLSB and other A-type strains sometimes failed to amplify, while amplification was unexpectedly observed with some PD strains. Thus, the decision was made to not use the primers for distinguishing A-type strains.

Primers XF1968F3 and XF1968R3, which were designed to replace XF1968-L and XF1968-R, were expected to amplify a 371-bp DNA fragment from within the XF1968 gene locus in strain 9a5c. A multiple sequence alignment using ClustalW predicted that the primers would amplify DNA from Ann-1 and ALS strain Dixon, but not from ALS strain M12 or PD strains (Appendix D). As previously stated, the new primers were not used to test 9a5c. The new primers performed as expected by amplifying the target fragment from Dixon, two OLS strains from California and three BLSB strains (Figure 3.5). As expected, the 371-bp fragment was not amplified from M12. However, a weak DNA fragment of approximately 700 bp also was amplified from the Dixon, two OLS, three BLSB and M12 strains. A weak DNA fragment of approximately 1200 bp was also amplified from two PD strains. Interestingly, only the 371-bp fragment was amplified from A-type *X. fastidiosa* strains isolated from oak in Florida and plum in Georgia. Thus, as expected, the XF1968F3-XF1968R3 primer pair differentiated A-type strains Dixon and M12, and BLSB strains were determined to be like Dixon. These primers also might be used to differentiate BLSB from some A-type strains isolated from shade trees in Georgia and elsewhere in the Southeast, but more strains need to be tested to confirm this hypothesis.

Limits of detection. In screening plants and insects for the presence of *X. fastidiosa*, it is important to know the minimum number of cells or DNA concentration necessary for successful amplification of target DNA using the previously described primer pairs. Calculated based on the

average number of colonies produced through drop dilution plating, approximately 80 CFU was the minimum CFU needed to detect *X. fastidiosa* when using the RST32-RST33 primer pair and boiled cells as template in a 25-µl PCR reaction (Figure 3.6). This was approximately 100-fold and 1000-fold more sensitive in detecting *X. fastidiosa* than the XF1968F3-XF1968R3 and the 2594F-2594R primer pairs, respectively (Figure 3.6).

The PCR detection threshold for the same three primer pairs was also determined by serially diluting genomic DNA from BLSB strain BB9 for use as template. Primer pairs RST32-RST33 and XF1968F3-XF1968R3 were comparable in sensitivity, producing visible amplicons with 0.04 pg of genomic DNA in a 25-µl reaction (Figure 3.7). Primer pair 2594F-2594R was approximately four-fold less sensitive than the RST32-RST33 and XF1968F3-XF1968R3 primer pairs, producing a visible amplicon at 0.16 pg in a 25-µl reaction (Figure 3.7). Comparing the limits of detection of the two species-specific primer pairs, RST31-RST33 and RST32-RST33 were comparable in sensitivity, producing visible amplicons with 0.04 pg of genomic DNA in a 25-ul reaction (Figure 3.7). However, intensity of the amplicon was greater with the RST32-RST33 primer pair. The detection threshold of *X. fastidiosa* observed in PCR assays was less variable when genomic DNA rather than boiled cells was used as template.

It was observed in drop dilution assays that growth of BLSB colonies was greater on both PD2 and CS20 when water was used to generate a cell suspension. Colony growth was the least on both media when PBS was used to generate a cell suspension. But as also observed in isolation assays using plant tissue, more but smaller BLSB colonies grew on CS20 than on PD2. Colonies on CS20 also tended to develop 1-4 d later than on PD2. Morphology of colonies on the two media also was distinctive. Colonies on CS20 were opalescent, creamy in color, circular in form and had entire margins. Colonies on PD2 were shiny, opalescent, golden in color, circular

in form, convex and exhibited twitching motility. Colonies of BLSB strains on PD2 resembled the "pit"-like colonies of A-type strains grown on PWG agar media described by Chen et al. (2005).

Cultivation *in vitro* is the most definitive and direct method for detection and identification of bacterial plant pathogens, due to observation of whole cells and their biochemical and physiological properties. However, PCR-based methods are more specific and sensitive by allowing targeted detection of genetic variation in bacterial genomes using specific primers (Henson and French 1993, Chen et al. 2005, Chen et al. 2008). Restriction fragment length polymorphisms (RFLP) of RST31-RST33 primer pair products may be used to type BLSB strains, but only if the concentration of amplified DNA is sufficient. RFLP analysis described by Minsavage et al. (1994) uses the *Rsa*I endonuclease to separate amplicons from non-PD strains into two smaller fragments, while there is no digestion of amplicons generated using the RST32-RST33 primer pair.

Based on this study, BLSB strains are not unique at this point in analysis. BLSB strains differ from PD, or G-type, strains and are most similar to Dixon among A-type strains of *X. fastidiosa*. In regard to template used in PCR, boiled cells may have provided a lower concentration of DNA, or cellular debris may have interfered with PCR amplification by the primers used, resulting in less sensitive detection of *X. fastidiosa* than by using purified genomic DNA. Primer pair RST32-RST33 is not commonly used by researchers for general detection of *X. fastidiosa*. From the literature, the RST31-RST33 primer pair appears to be the standard for general detection of the bacterium, as PCR products also can be used in RFLP analysis to type strains. However, it was demonstrated in this study that the RST32-RST33 primer pair was as sensitive as the RST31-RST33 primer pair, and in fact, generated more intense amplicons. Thus,

it is suitable for general detection of *X. fastidiosa*. Although four-fold less sensitive than the RST32-RST33 primer pair, the 2594F-2594R primer pair is suitable for both detection and typing of *X. fastidiosa* strains within the context of a single PCR assay. This eliminates the need for multiplex PCR. Primer pair XF1968F3-XF1968R3 may be used to differentiate BLSB from some A-type strains besides M12, such as oak and plum. The possibility warrants further study. However, as the epidemiology of BLSB strains still is not known, other conserved loci in the *X. fastidiosa* genome also need to be examined to further characterize BLSB strains endemic to Georgia.

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Table 3.1. Cultures from *Xylella fastidiosa* strains included in this study that were provided by Dr. C.J. Chang, Department of Plant Pathology, The University of Georgia, Griffin, GA.

Original strain	Assigned name	Host	Host location	Date sampled
705	BB1	Blueberry	Alma, GA	12/2007
1105	BB2	Blueberry	Alma GA	12/2007
1310	BB3	Blueberry	Alma, GA	12/2007
1109	BB4	Blueberry	Alma, GA	12/2007
905-PW	BB5	Blueberry	Alma, GA	12/2007
905-CS20	BB6	Blueberry	Alma, GA	12/2007
311	BB7	Blueberry	Alma, GA	12/2007
BB Root 1	BB8	Blueberry	Brantley Co., GA	3/2006
BB Root 2	BB9	Blueberry	Brantley Co., GA	3/2006
BB Root 2 few lg	BB10	Blueberry	Brantley Co., GA	3/2006
BB Stem 1	BB11	Blueberry	Brantley Co., GA	3/2006
BB Stem 2	BB12	Blueberry	Brantley Co., GA	3/2006
BB Stem 3	BB13	Blueberry	Brantley Co., GA	3/2006
GHS CK (6/9/06)[a]	BB14	Blueberry	Brantley Co., GA	3/2006
5RBB (95)	BB15	Blueberry	Brantley Co., GA	3/2006
V1S1	BB16	Blueberry	Greenhouse	7/2006
V1S2	BB17	Blueberry	Greenhouse	7/2006
V1S4	BB18	Blueberry	Greenhouse	7/2006
V1R1	BB19	Blueberry	Greenhouse	7/2006
V1R2	BB20	Blueberry	Greenhouse	7/2006
V1R3	BB21	Blueberry	Greenhouse	7/2006
V1R4	BB22	Blueberry	Greenhouse	7/2006
Crabapple 1[b]	G1	Grape	Alpharetta, GA	8/2006
Crabapple 2[b]	G2	Grape	Alpharetta, GA	8/2006
Crabapple 3[b]	G3	Grape	Alpharetta, GA	8/2006
Crabapple 4[b]	G4	Grape	Alpharetta, GA	8/2006

Crabapple 6[b]	G5	Grape	Alpharetta, GA	8/2006
Wolf Mt.[c]	G6	Grape	Dahlonega, GA	8/2006
3SV-G[c]	G7	Grape	Dahlonega, GA	8/2006
Stonepile[c]	G8	Grape	Habersham Co.,	10/2006
GHS-D[d]	G9	Grape	Griffin, GA	N/A
GHS-E[d]	G10	Grape	Griffin, GA	N/A
Alpha 1[e]	G11	Grape	Alpharetta, GA	2006
Alpha 2[e]	G12	Grape	Alpharetta, GA	2006
Alpha 3[e]	G13	Grape	Alpharetta, GA	2006
Alpha 4[e]	G14	Grape	Alpharetta, GA	2006
18A3[f]	G15	Grape	Aiken, SC	7/2006
18B-70A3[f]	G16	Grape	Aiken, SC	7/2006
15A75[f]	G17	Grape	Aiken, SC	7/2006
15A95[f]	G18	Grape	Aiken, SC	7/2006
16A1[f]	G19	Grape	Aiken, SC	7/2006
17A17[f]	G20	Grape	Aiken, SC	7/2006
17A26[f]	G21	Grape	Aiken, SC	7/2006
17A27[f]	G22	Grape	Aiken, SC	7/2006
E(5/10)[g]	S1	Sycamore	Gainestown, AL	~2001
4rd+1[h]	S2	Sycamore	Athens, GA	9/2005
4rd+2[h]	S3	Sycamore	Athens, GA	9/2005
2rd+2[h]	S4	Sycamore	Athens, GA	9/2005
2rd+3[h]	S5	Sycamore	Athens, GA	9/2005
OL1	OL1	Oleander	St. Simons	4/1999
OL2	OL2	Oleander	St. Simons	4/1999

[a] Entire plant sent to Department of Plant Pathology, The University of Georgia, Griffin, GA, where maintained in greenhouse and used for isolations.

[b] Plant samples collected at Crabapple Vineyard.

[c] Plant samples collected at Wolf Mountain, Three Sisters or Stonepile vineyards, respectively.

[d] Strains from muscadine grape plants maintained many years in greenhouse at Griffin campus of The University of Georgia.

[e] Plant samples likely collected at Crabapple Vineyard.

[f] Plant samples collected at Montmorenci Vineyards.

[g] Plant samples collected from injected trees.

[h] Plant samples obtained from the Athens campus of The University of Georgia, near the U.S. Department of Agriculture Forest Service building.

Table 3.2. Genomic DNA from *Xylella fastidiosa* strains included in this study that was provided by Dr. Jianchi Chen, U.S. Department of Agriculture-Agricultural Research Service, Parlier, CA.

Strain name	Strain type	Host	Host location	Year isolated
GA G#1	G	Grape	Georgia	2003
C13R1P3-2	G	Grape	California	2003
Teme	G	Grape	California	2003
SN1	G	Alfalfa	California	2005
T. Oak 35	A	Turkey Oak	Florida	2005
Red Oak	A	Oak	Georgia	2005
GA Plum 19a	A	Plum	Georgia	2005
GA Plum 18a	A	Plum	Georgia	2005
FSUR5T20	G	Almond	California	2004
K3	G	Almond	California	2003
M12α	A	Almond	California	2003
M22	A	Almond	California	2003
M23	G	Almond	California	2003
M24	G	Almond	California	2003
M25	A	Almond	California	2003
M26	A	Almond	California	2003
P23	A	Almond	California	2005
P29	A	Almond	California	2005
P45	G	Almond	California	2005
Dixon[a]	A	Almond	California	2003
T1B[a]	A[b]	Oleander	California	2003
T5C[a]	A[b]	Oleander	California	2003

[a]Strains received by Dr. Chen from Sandy Purcell, retired professor of entomology, University of California at Berkeley.

[b]Determined in this study using the XF1968F3-XF1968R3 primer pair.

Table 3.3. Oligonucleotide primers used for PCR detection of *Xylella fastidiosa* in this study.

Primer pair	Sequence (5′ to 3′)	Amplicon (bp)	Specificity[a]	Citation
RST31 RST33	GCGTTAATTTTCGAAGTGATTCGATTGC CACCATTCGTATCCCGGTG	733	Species	Minsavage et al. 1994
RST31 RST32	GCGTTAATTTTCGAAGTGATTCGATTGC CGAACAAAGGCAATGCCGC	600	Species	Minsavage et al. 1994
XF176f XF686r	AAACAATCACAGGGGACTGC ATATTCATAGATTTCCGTCGA	511	PD	Banks et al. 1999
PDconsvdF Pdspec685	AAACAATCACAGGGACTGC GACGGAATCTATGAATATCGCC	514	PD	This study
16S-23SF 16S-23SR	GATGACTGGGGTGAAGTCGT GACACTTTTCGCAGGCTACC	650	16S-23S intergenic spacer region	Martinati et al. 2007
XF1968-L XF1968-R	GGAGGTTTACCGAAGACAGAT ATCCACAGTAAAACCACATGC	638	ALS OLS	Hernandez-Martinez et al. 2006
XF1968F3 XF1968R3	CGCACCGTTACCGCCATTC CCTGATATTGGGCGCATCG	371	ALS (like Dixon) OLS CVC	This study
ALM1 ALM2	CTGCAGAAATTGGAAACTTCAG GCCACACGTGATCTATGAA	521	ALS	Hernandez-Martinez et. al. 2006

ALMF2 ALMR3	GCTGCAGAAATTGGAAACTTCAGG CATGCCCACACCACCCAAT	541	ALS CVC	This study
2594F 2594R	GAAACTGGCACGGACCGCT AGCAGCTTGCCGACCCTCGATA	1323	ALS OLS	This study

[a] PD = Pierce's disease strains; ALS = almond leaf scorch strains; OLS = oleander leaf scorch strains; and CVC = citrus variegated chlorosis strains.

Figure 3.1. Primer pair PDconsvdF-Pdspec685, which is specific to Pierce's disease (PD) strains of *Xylella fastidiosa*, produced the expected 514-base-pair amplicon from genomic DNA of four PD strains isolated from grape (lanes 8-11) but not from six strains of *X. fastidiosa* that cause bacterial leaf scorch of blueberry (lanes 2-7). The negative control (lane 12) consisted of master mix and water instead of a DNA template. The size of amplicons was determined by comparison to a 1-kb DNA ladder (lane 1).

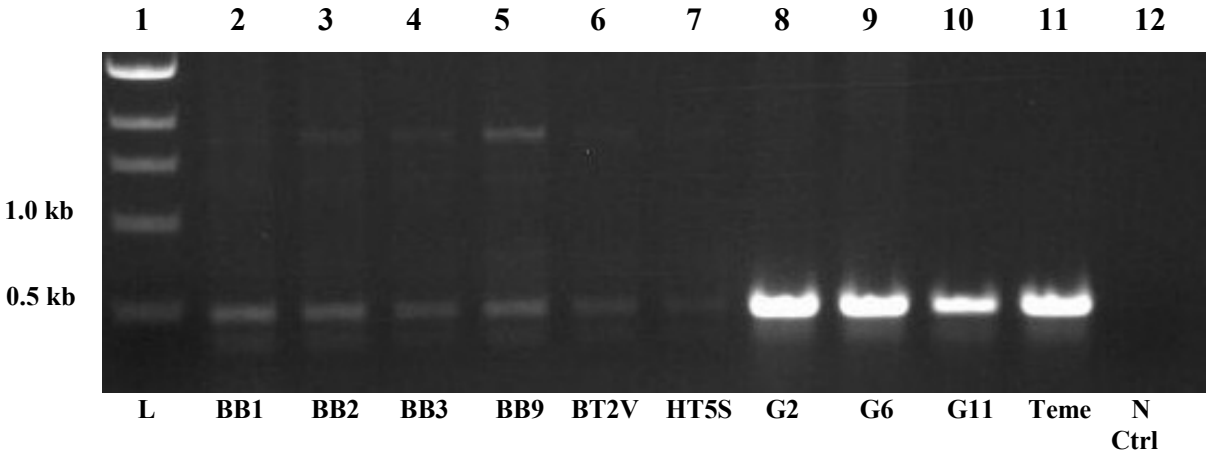
1 2 3 4 5 6 7 8 9 10 11 12
1.0 kb
0.5 kb
L BB1 BB2 BB3 BB9 BT2V HT5S G2 G6 G11 Teme N Ctrl

Figure 3.2. Multiple sequence alignment of the 16S-23S rDNA intergenic spacer regions from selected *Xylella fastidiosa* strains. Bacterial leaf scorch of blueberry strain BB8 was compared to Dixon, M12, Ann-1, Temecula1, M23 and 9a5c. Dixon and M12 are A-type strains that cause almond leaf scorch. Temecula1 and M23 are G-type strain that cause Pierce's disease and almond leaf scorch, respectively. Ann-1 causes oleander leaf scorch, and 9a5c causes citrus variegated chlorosis. Blue highlighting denotes differences in alignment.

```
BB8             GTGCGGCTGGATCACCTCCTTTTGAGTATGGTGAATATAATTGTCTTATCAGGCGTCCTC
Dixon           GTGCGGCTGGATCACCTCCTTTTGAGTATGGTGAATATAATTGTCTTATCAGGCGTCCTC
M12             GTGCGGCTGGATCACCTCCTTTTGAGTATGGTGAATATAATTGTCTTATCAGGCGTCCTC
Ann-1           GTGCGGCTGGATCACCTCCTTTTGAGTATGGTGAATATAATTGTCTTATCAGGCGTCCTC
Temecula1       GTGCGGCTGGATCACCTCCTTTTGAGTATGGTGAATATAATTGTCTTACCAGGCGTCCTC
M23             GTGCGGCTGGATCACCTCCTTTTGAGTATGGTGAATATAATTGTCTTACCAGGCGTCCTC
9a5c            GTGCGGCTGGATCACCTCCTTTTGAGTATGGTGAATATAATTGTCTTATCAGGCGTCCTC
                ********************************************** ***********

BB8             ACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATGTTGGCTATTT
Dixon           ACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATGTTGGCGATTT
M12             ACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATGTTGGCGATTT
Ann-1           ACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATGTTGGCGATTT
Temecula1       ACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATGTTGGCGATTT
M23             ACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATGTTGGCGATTT
9a5c            ACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATGTTGGCGATTT
                ************************************************** ****

BB8             TTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAGGGGGTCGTCG
Dixon           TTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAGGGGGTCGTCG
M12             TTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAGGGGGTCGTCG
Ann-1           TTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAGGGGGTCGTCG
Temecula1       TTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAGGGGGTCGTCG
M23             TTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAGGGGGTCGTCG
9a5c            TTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAGGGGGTCGTCG
                ************************************************************

BB8             GTTCGATCCCGACAGGCTCCACCATGAAAGTATTTATGGGTCTGTAGCTCAGGTGGTTAG
Dixon           GTTCGATCCCGACAGGCTCCACCATGAAAGTATTTATGGGTCTGTAGCTCAGGTGGTTAG
M12             GTTCGATCCCGACAGGCTCCACCATGAAAGTATTTATGGGTCTGTAGCTCAGGTGGTTAG
Ann-1           GTTCGATCCCGACAGGCTCCACCATGAAAGTATTTATGGGTCTGTAGCTCAGGTGGTTAG
Temecula1       GTTCGATCCCGACAGGCTCCACCATGAAAGTATTTATGGGTCTGTAGCTCAGGTGGTTAG
M23             GTTCGATCCCGACAGGCTCCACCATGAAAGTATTTATGGGTCTGTAGCTCAGGTGGTTAG
9a5c            GTTCGATCCCGACAGGCTCCACCATGAAAGTATTTATGGGTCTGTAGCTCAGGTGGTTAG
                ************************************************************

BB8             AGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACCCACCAATGTTA
Dixon           AGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACCCACCAATGTTA
M12             AGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACCCACCAATGTTA
Ann-1           AGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACCCACCAATGTTA
Temecula1       AGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACCCACCAATGTTA
M23             AGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACCCACCAATGTTA
9a5c            AGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACCCACCAATGTTA
                ************************************************************

BB8             TATCAATTATTCTGAATGTGGTTTGCGCATTTTTTATGCTTATCAGCCTTGGAGCTGTGA
Dixon           TATCAATTATTCTGAATGTGGTTTGCGCATTTTTTATGCTTATCAGCCTTGGAGCTGTGA
M12             TATCAATTATTCTGAATGTGGTTTGCGCATTTTTTATGCTTATCAGCCTTGGAGCTGTGA
Ann-1           TATCAATTATTCTGAATGTGGTTTGCGCATTTTTTATGCTTATCAGCCTTGGAGCTGTGA
Temecula1       TATCAATTATTCTGAATGTAGTTTGCGCATTTTT-ATGCTTATCAGCCTTGGAGCTGTGA
M23             TATCAATTATTCTGAATGTAGTTTGCGCATTTTT-ATGCTTATCAGCCTTGGAGCTGTGA
9a5c            TATCAATTATTCTGAATGTGGTTTGCGCATTTTTTATGCTTATCAGCCTTGGAGCTGTGA
                ****************** ************** ************************
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BB8             AGCGTTCTTTTATAATTTGATGATGTAGCAAGCGTTTGAACTTTTTATTAATAATTTCTC
Dixon           AGCGTTCTTTTATAATTTGATGATGTAGCAAGCGTTTGAACTTTTTATTAATAATTTCTC
M12             AGCGTTCTTTTATAATTTGATGATGTAGCAAGCGTTTGAACTTTTTATTAATAATTTCTC
Ann-1           AGCGTTCTTTTATAATTTGATGATGTAGCAAGCGTTTGAACTTTTTATTAATAATTTCTC
Temecula1       AGCGTTCTTTTATAATTTGATGATGTAGCAAGCGTTTGAAATTTTTATTAAAAATTTCTC
M23             AGCGTTCTTTTATAATTTGATGATGTAGCAAGCGTTTGAAATTTTTATTAAAAATTTCTC
9a5c            AGCGTTCTTTTATAATTTGATGATGTAGCAAGCGTTTGAACTTTTTATTAATAATTTCTC
                **************************************** ********** ********

BB8             ATTGGAAGCCTTAAGTGACAATGTTTATCCATTGTCTTGTAGATTTTGAGGCGACTTTGG
Dixon           ATTGGAAGCCTTAAGTGACAATGTTTATCCATTGTCTTGTAGATTTTGAGGCGACTTTGG
M12             ATTGGAAGCCTTAAGTGACAATGTTTATCCATTGTCTTGTAGATTTTGAGGCGACTTTGG
Ann-1           ATTGGAAGCCTTAAGTGACAATGTTTATCCATTGTCTTGTAGATTTTGAGGCGACTTTGG
Temecula1       ATTTGAAGCCTTAAGTGACAATGTTTATCCATTGTCTTGTAGATTTTGAGGCGACTTGGG
M23             ATTTGAAGCCTTAAGTGACAATGTTTATCCATTGTCTTGTAGATTTTGAGGCGACTTGGG
9a5c            ATTGCAAGCCTTAAGTGGCAATGTTTATCCATTGTCTTATAGATTTTGAGGCAACTTTGG
                ***  ************ ******************** ************* **** **

BB8             GTTATATGGTCAAGCGAATAAGCGCACATGGTGGATGCCT
Dixon           GTTATATGGTCAAGCGAATAAGCGCACATGGTGGATGCCT
M12             GTTATATGGTCAAGCGAATAAGCGCACATGGTGGATGCCT
Ann-1           GTTATATGGTCAAGCGAATAAGCGCACATGGTGGATGCCT
Temecula1       GTTATATGGTCAAGCGAATAAGCGCACATGGTGGATGCCT
M23             GTTATATGGTCAAGCGAATAAGCGCACATGGTGGATGCCT
9a5c            GTTATATGGTCAAGCGAATAAGCGCACATGGTGGATGCCT
                ****************************************
```

Figure 3.3. Primer pair 2594F-2594R distinguished between A- and G-types of *Xylella fastidiosa*. The primers were designed to amplify a 1323-base pair DNA fragment from A-type strains of *X. fastidiosa*. However, the primers also generated amplicons approximately ≥ 700 base pairs from G-type strains. G, G-type strains; A, A-type strains; L, 1-kb DNA ladder.

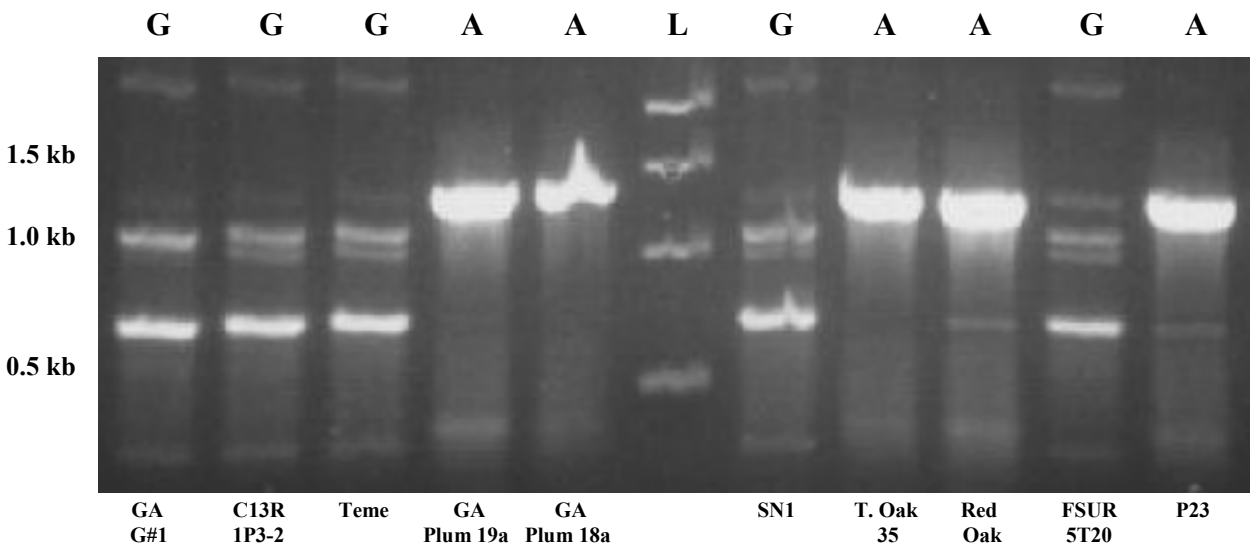
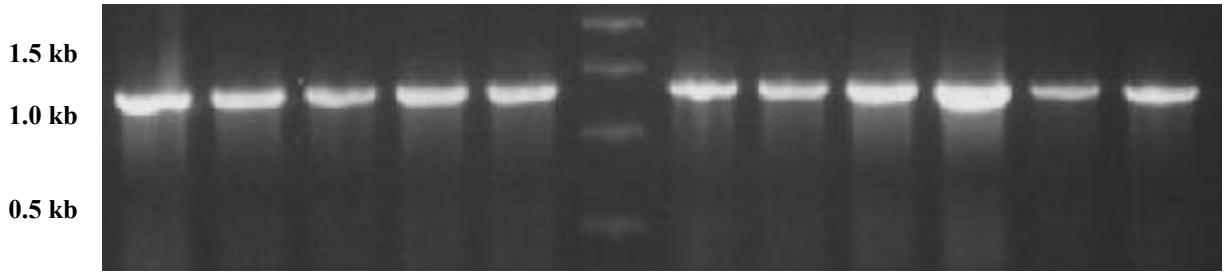
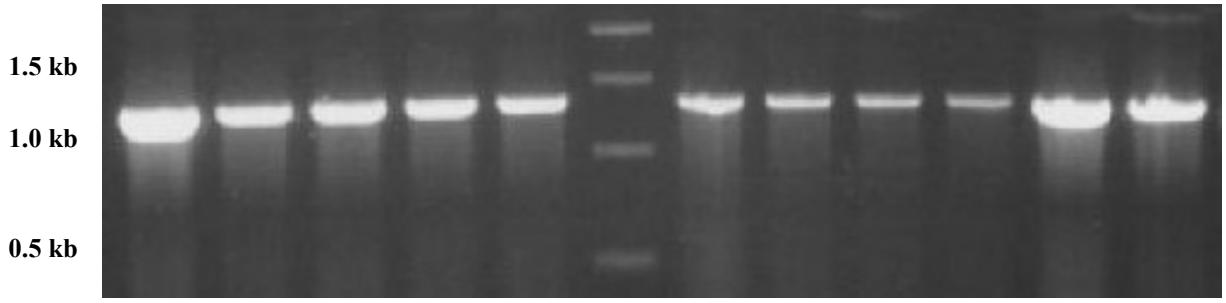

G G G A A L G A A G A
1.5 kb
1.0 kb
0.5 kb
GA G#1
C13R 1P3-2
Teme
GA Plum 19a
GA Plum 18a
SN1
T. Oak 35
Red Oak
FSUR 5T20
P23

Figure 3.4. Primer pair 2594F-2594R amplified a target DNA fragment of approximately 1323 base pairs in 22 strains of *Xylella fastidiosa* isolated from southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) in Alabama, Georgia and South Carolina, indicating that they are A-type strains of the bacterium. L, 1-kb DNA ladder.



BB1 BB2 BB3 BB4 BB5 L BB6 BB7 BB8 BB9 BB10 BB11



BB12 BB13 BB14 BB15 BB16 L BB17 BB18 BB19 BB20 BB21 BB22

Figure 3.5. Primer pair XF1968F3-XF1968R3 amplified a target DNA fragment of approximately 371 base pairs from *Xylella fastidiosa* strains isolated from blueberry, oak, plum, one of two strains from almond and two of three strains from oleander. As expected, the amplicon was not produced from the M12 strain isolated from almond, due to deletions of base pairs in the XF1968F3 primer site in this genome. The negative control consisted of master mix and water instead of a DNA template. L, 1-kb DNA ladder.

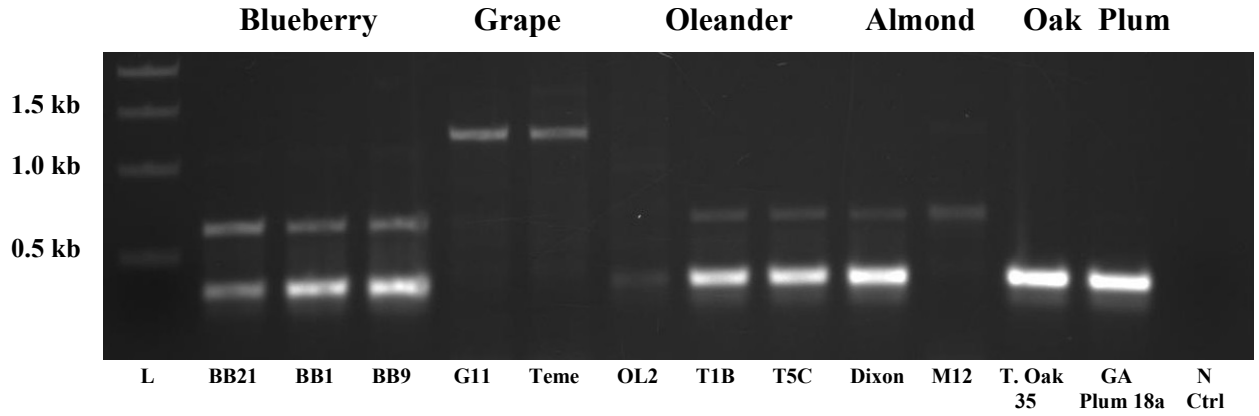
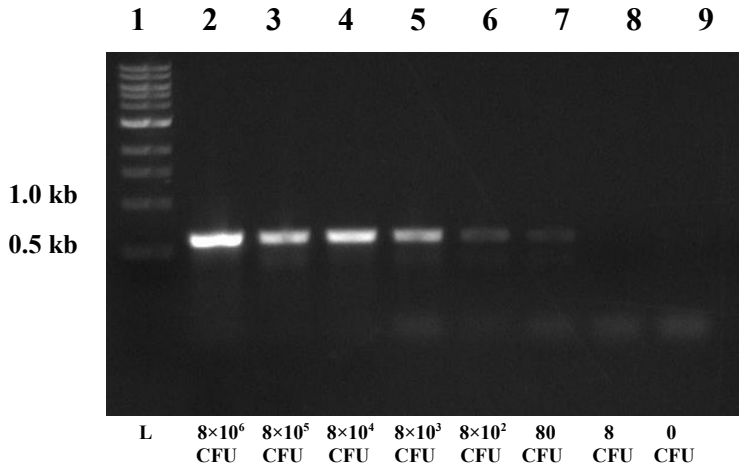
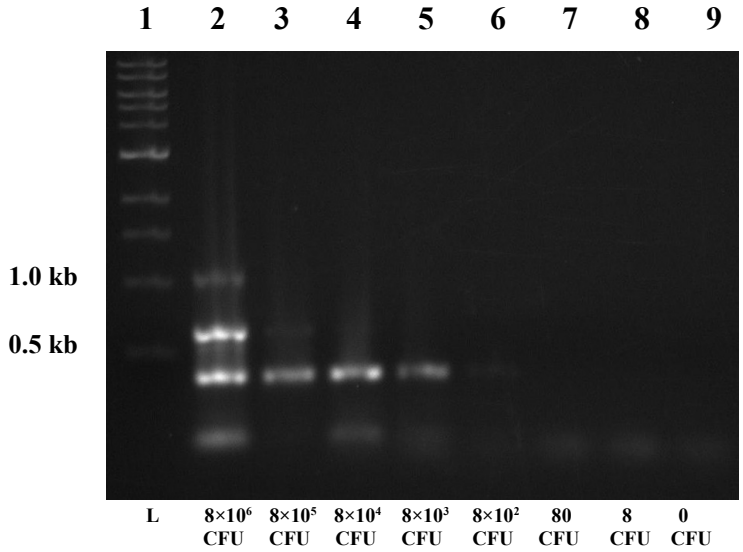

Blueberry
Grape
Oleander
Almond
Oak
Plum
1.5 kb
1.0 kb
0.5 kb
L
BB21
BB1
BB9
G11
Teme
OL2
T1B
T5C
Dixon
M12
T. Oak 35
GA Plum 18a
N Ctrl

Figure 3.6. Primer pair RST32-RST33 produced the best amplification in PCR from low concentrations of *Xylella fastidiosa* cells from a suspension of strain TH949. A) PCR using the RST32-RST33 primer pair produced a detectable product with as few as 80 colony forming units (CFU) (lane 7) when boiled cells were used as template in a 25-µl PCR reaction. B) The XF1968F3-XF1968R3 primer pair was approximately 100-fold less sensitive than the RST32-RST33 primer pair, producing a barely detectable PCR product with 8×10^2 CFU in a 25-µl reaction (lane 6). C) The 2594F-2594R primer pair was approximately 1000-fold less sensitive than the RST32-RST33 primer pair, producing a PCR product with 8×10^4 CFU in a 25-µl reaction (lane 4). L, 1-kb DNA ladder.



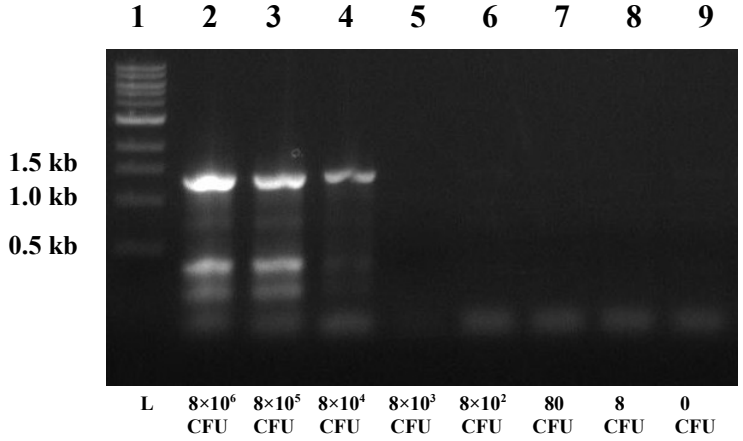
A)

RST32-RST33



B)

XF1968F3-XF1968R3

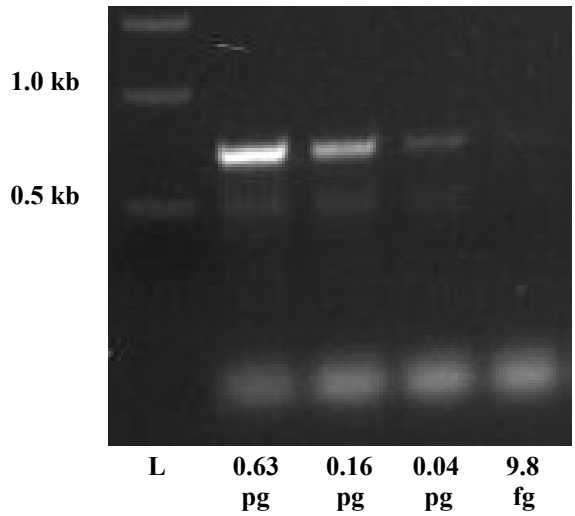


C)

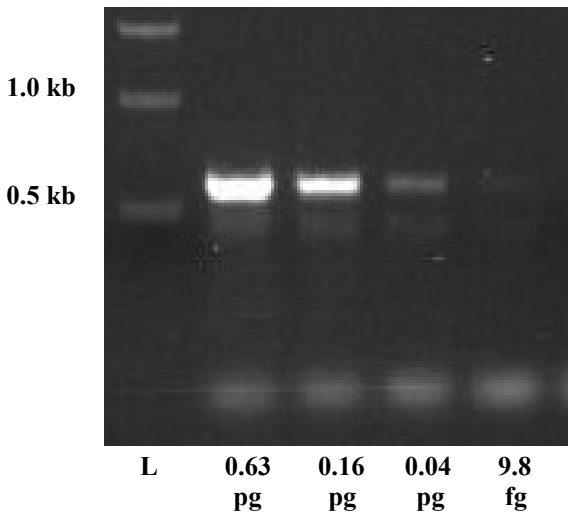
2594F-2594R

Figure 3.7. The threshold of detection for *Xylella fastidiosa* was determined using serially diluted genomic DNA from strain BB9 as template in PCR reactions using primer pairs RST31-RST33, RST32-RST33, 2594F-2594R and XF1968F3-XF1968R3. The amount of genomic DNA in each 25-µl PCR reaction is shown. L, 1-kb DNA ladder.

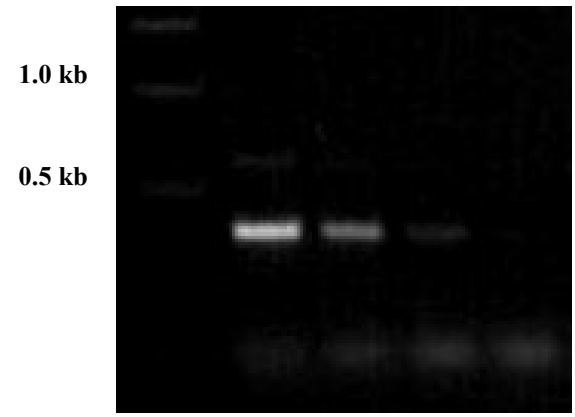
RST31-RST33



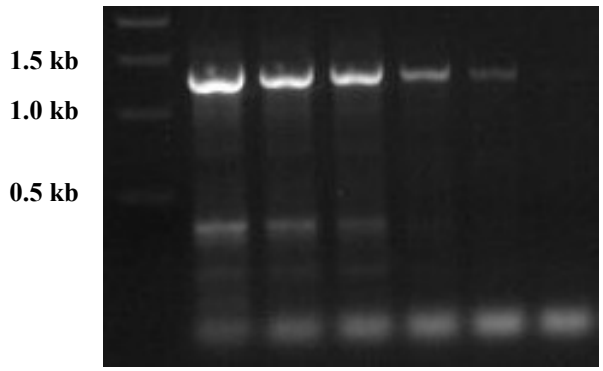
RST32-RST33



XF1968F3-XF1968R3



2594F-2594R



CHAPTER 4

DETECTION OF *XYLELLA FASTIDIOSA* STRAINS IN SOUTHERN HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM* INTERSPECIFIC HYBRIDS) IN SOUTH GEORGIA

INTRODUCTION

A disorder described as "yellow twig" or "yellow stem" was observed beginning in 2004 in the 'FL 86-19' (also known as 'V1') variety of cultivated southern highbush blueberry (SHB) (*Vaccinium corymbosum* interspecific hybrids) in the Flatwoods of southeastern and south-central Georgia. Two years later, Chang et al. (2009) completed Koch's postulates, demonstrating that the disease, bacterial leaf scorch of blueberry (BLSB), was caused by *Xylella fastidiosa*. Since its appearance, BLSB has become a major disease in SHB, affecting other varieties such, as 'O'Neal' and 'Star,' while 'V1' remains the most susceptible (Brannen et al. 2010). Rabbiteye (*Vaccinium virgatum* Aiton) blueberry varieties, which are native to southern Georgia, northern Florida and southeastern Alabama, are thought to be largely resistant to BLSB (Scherm and Krewer 2003, Brannen et al. 2010). Although the pathogen will colonize rabbiteye (Chang et al. 2009), it is rarely found, and only a few varieties exhibit symptoms under field conditions (P. Brannen, personal communication). Although no specific yield loss data are available, survey data collected in 2008 indicate a near 100% loss for 'V1' and a 30% loss for 'Star' are projected to occur within 10 years of the onset of symptoms (P. Brannen, personal communication). Thus, BLSB is considered a threat to the blueberry industry in Georgia (Brannen et al. 2010).

Blueberry production has grown considerably in Georgia since its inception in the mid-1950s, and has been particularly rapid the past 10 years (Scherm and Krewer 2003, Boatright and McKissick 2010). Blueberries passed peaches in farm gate value in 2004 to become the most economically important fruit crop in the state. The value of blueberry production continues to increase and currently stands at more than $102.4 million.

X. fastidiosa is a fastidious, gram-negative, xylem-limited plant pathogenic bacterium that is vectored primarily by xylem-feeding leafhoppers (Mizell et al. 2003, Costa et al. 2006). Symptoms of *X. fastidiosa* vary by host. Initial symptoms of BLSB include chlorosis and subsequent necrosis of older leaves that begins at the margin and progresses throughout the entire leaf, resulting in a scorched appearance similar to that seen with extreme drought or fertilizer salt burn (Chang et al. 2009). New shoots often are abnormally thin, produce fewer flower buds and develop a yellow "skeleton-like" appearance once affected leaves drop. Death of the plant follows leaf drop, typically within two years after the onset of symptoms.

All strains of *X. fastidiosa* are classified as a single species (Wells et al. 1987). However, various studies beginning in the 1990s found phenotypic and genetic differences among strains, suggesting genetic diversity within the species (Schaad et al. 2004). Based on single nucleotide polymorphisms in conserved 16S rDNA sequences from *X. fastidiosa* strains Temecula and Dixon, Chen et al. (2005) distinguished between two genotypes of *X. fastidiosa* in almond. Almond leaf scorch (ALS) strains that also cause Pierce's disease (PD) in grapevine were designated as G-type, while ALS strains that do not cause PD in grapevine were designated as A-type.

X. fastidiosa has been well studied in a number of host plant systems, including almond, citrus, grapevine, oleander and peach. Little is known about BLSB epidemiology, thus methods

are needed to facilitate detection of strains in plants. The objective of this research was to detect BLSB strains by polymerase chain reaction (PCR) analyses of total genomic DNA extracted from plant tissue. Leaves and stems for assays were selected from 'Star' and 'V1' varieties of SHB at production fields in Berrien, Brantley and Ware counties in Georgia and at an experimental field at Alma in Bacon County, GA. A method also was developed to reduce PCR inhibitors co-extracted from plant tissue.

MATERIALS AND METHODS

Collection of plant samples. *October 2009*. Stems and leaves of the 'Star' variety of SHB were sampled from a total of 35 plants distributed among three fields, one each in Berrien, Brantley and Ware counties (Figure 4.1). Of these plants, five were asymptomatic and 30 were symptomatic for BLSB. Severity of disease varied among the 30 symptomatic plants and was based on the presence of yellow and necrotic leaves, yellow twiggy stems, desiccation and/or plant death. Samples were placed in labeled, 1-gallon plastic bags and transported on ice to the Department of Plant Pathology at the Griffin campus of The University of Georgia, where they were stored at 4°C. Samples were used within 2 d for colony isolation of *X. fastidiosa* and within 4 d for genomic DNA extraction.

July 2010. Stems and leaves of the 'Star' and 'V1' varieties of SHB were sampled from a total of 60 plants distributed among 3 fields, including the same field in Berrien County sampled in October 2009, an experimental field at the airport in Alma in Bacon County, GA (Figure 4.1), and a different field in Brantley County. Of these plants, 30 were asymptomatic and 30 were symptomatic for BLSB. Samples were handled as previously described and transported to the Department of Plant Pathology at the Athens campus of The University of Georgia, where they

were stored at 4°C. Samples were used within 2 d for colony isolation of *X. fastidiosa* and within 4 d for genomic DNA extraction.

Isolation of *X. fastidiosa* from blueberry plant samples. *October 2009: Maceration of plant tissue*. The petiole and midvein from one leaf from each of the 35 plants sampled were surface sterilized with a 0.8% sodium hypochlorite solution for 3 min and then triple rinsed (5 min each rinse) in sterile deionized water, as described by Chang et al. (2009). Each petiole-midvein was macerated separately in CS20 broth (Chang and Walker 1988) at a 1:3 (wt/vol) ratio of tissue to broth in a disposable Petri dish using a flame-sterilized razor blade. One loopful (~ 10 µl) of each macerate was streaked onto both PD2 (Davis et al. 1980) and CS20 agar media. The plates were incubated at 28°C and periodically examined with a dissecting microscope for up to 14 d. Cultures positive for *X. fastidiosa*, based on colony morphology and growth characteristics, were maintained by subculturing on PD2 and CS20 agar media every 7 d.

October 2009: Squeezed stem sap. The same stems from which the 35 petiole-midveins were obtained were used for colony isolation. Stems were cut with hand pruners into approximately 2-inch-long segments and then surface sterilized as previously described. Stem segments were placed in a 3-inch bench vise and the ends freshly cut with flame-sterilized hand pruners. Segments from the same stem were squeezed with the vise, and sap that exuded from cut ends was collected with a micropipettor and pooled in a microcentrifuge tube. Sap was then spotted (10 µl) onto PD2 and CS20 agar media and streaked with a sterile inoculation loop. Incubation and examination of the plates and maintenance of positive cultures was as previously described.

July 2010: Maceration of plant tissue. Thirty samples, each consisting of five petioles from different leaves on different stems of the same plant, were used for colony isolation of *X.*

fastidiosa, as previously described, on PD2 and CS20 agar media. Of the 30 samples, 15 were from asymptomatic and 15 were from symptomatic plants.

July 2010: Squeezed stem sap. Thirty samples, each consisting of sap pooled from two to four different stems on the same plant, were used for colony isolation of *X. fastidiosa* on PD2 and CS20 agar media, as previously described. Stems were different from those used to obtain petioles for colony isolation. Of the 30 samples, 15 were from asymptomatic and 15 were from symptomatic plants.

Preparation of genomic DNA. Genomic DNA was prepared from pure cultures of *X. fastidiosa*, either as previously described in Chapter 3, or by using the DNeasy® Plant Mini Kit (QIAGEN Inc., Valencia, CA) according to the supplier's instructions. In 2009, total genomic DNA was extracted from plant tissue as described by Costa et al. (2006), incorporating the use of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4), mesh-lined maceration bags (Agdia Inc., Elkhart, IN) and the DNeasy® Plant Mini Kit. Tissue was macerated within a few days after being collected, but was then stored at -20°C for several months before extracting DNA. In 2010, DNA was prepared from frozen macerate and fresh plant samples using the DNeasy® Plant Mini Kit with modifications as described in Appendix E. Extracted DNA was stored at -20°C.

PCR primers and protocols. Sequences and sizes of expected amplicons for primers used in this study are listed in Table 3.3. Primer pairs used in this study were RST31-RST33, RST32-RST33 and 2594F-2594R. Thermocycler conditions and PCR reactions using the three primer pairs were as previously described in Chapter 3, except 5 µl of DNA extract from plant tissue also was used as template. Samples (10 µl) of PCR reactions were loaded on a 1% agarose gel containing 50 µg/ml of ethidium bromide, and after electrophoresis, amplicons were

visualized and imaged with the BioSpectrum® 300 Imaging System (UVP LLC, Upland, CA). The size of amplicons was determined by comparison to a 1-kb DNA ladder (New England BioLabs Inc., Ipswich, MA).

Restriction digestion. Aliquots (9 µl) of PCR products of the RST31-RST33 primer pair were digested in a 10.5-µl reaction containing 1X NEBuffer #4, 5 U of *Rsa*I restriction endonuclease (New England BioLabs Inc., Ipswitch, MA) and 0.5 µl sterile deionized water. Reactions were incubated at 37°C for 3 h and then terminated by adding 2 µl of 6X blue gel loading dye (New England BioLabs Inc., Ipswitch, MA). Restriction fragment length polymorphisms (RFLP) were identified by electrophoresis in a 1% agarose gel, with all of digested samples being loaded into wells. Visualization and determination of size of separated DNA fragments was as previously described.

RESULTS AND DISCUSSION

Characterization of *X. fastidiosa* cultures isolated from blueberry. *October 2009*. Ten percent (14) of the total 140 isolations from five asymptomatic and 30 symptomatic plants were positive for *X. fastidiosa* based on colony growth and morphology. Positive isolations were from one symptomatic plant at the Berrien County field and four symptomatic plants at the Brantley County field. Of the positive isolations, 57% were generated from both leaf tissue and sap from the same stem, 29% were generated from only sap and 14% were generated from only leaf tissue. Contamination was generally less on PD2 plates streaked with sap. Genomic DNA obtained from pure cultures of all 14 strains tested positive as A-type strains of *X. fastidiosa* by PCR using the RST32-RST33 and 2594F-2594R primer pairs (results not shown).

July 2010. Six percent (7) of the total 120 isolations from 30 asymptomatic and 30 symptomatic plants were positive for *X. fastidiosa* based on colony growth and morphology.

Positive isolations were from one symptomatic plant at the Berrien County field and three symptomatic plants at the Alma field. Of the positive isolations, 86% were generated from leaf tissue, while 14% were generated from sap. Contamination increased, regardless of the isolation method and type of agar media, according to the degree of BLSB symptoms. Genomic DNA obtained from pure cultures of all seven strains tested positive as A-type strains of *X. fastidiosa* by PCR using the RST32-RST33 and 2594F-2594R primer pairs (results not shown).

The earliest that *X. fastidiosa* colonies were observed on PD2 agar media was 6 d post-isolation. Colonies on CS20 developed 1 to 4 d later. Colonies on PD2 were fewer and larger than colonies on CS20. Morphology of colonies on the two media also was distinctive (Figure 4.2). Colonies on CS20 were opalescent, creamy in color, circular in form and had entire margins. Colonies on PD2 were opalescent, bronze colored, circular in form, convex and exhibited twitching motility. Colonies of BLSB strains on PD2 resembled the "pit"-like colonies of A-type strains grown on PWG agar media described by Chen et al. (2005).

Extraction and analysis of DNA directly from blueberry plant tissue. *October 2009*. Total genomic DNA was extracted from 70 petiole-midveins (two petiole-midveins from different stems from each of the 35 plants sampled). Initial PCR assays to detect *X. fastidiosa* in 20 of the DNA extracts using the species-specific RST32-RST33 primer pair were negative, despite the fact that extracts were from symptomatic plants. It is common for DNA extracts from plants to contain PCR inhibitors, so it seemed likely these results were false negatives. This suspicion was confirmed when PCR assays remained negative despite the addition (spiking) of pure *X. fastidiosa* genomic DNA to reaction tubes. Inhibition also was demonstrated when serial dilution of extracts with water prior to PCR analysis resulted in the expected amplicon. The degree of inhibition varied between individual extracts, but was more prevalent in the first eluate

from the DNeasy spin column than in subsequent eluates (Figure 4.3). PCR inhibitors apparently eluted more readily from the column than did the *X. fastidiosa* DNA. Diluting the samples 1/15 with water allowed the detection of *X. fastidiosa* in eight of the 20 previously negative extracts, using the RST32-RST33 primer pair in PCR assays (results not shown). Genomic DNA extraction of the remaining 50 samples was postponed until a method was developed to reduce inhibitors in extracts. The 50 samples later tested negative for *X. fastidiosa* by PCR using the RST32-RST33 primer pair. However, some results could have been false due to possible degradation of any bacterial DNA in extracts due to long-term storage of leaf macerate in PBS at -20°C. In an attempt to type the strains, the eight extracts positive for *X. fastidiosa* were further analyzed by PCR using the 2594F-2594R primer pair. Dilution of extracts 1/30 produced a weak target DNA fragment of approximately 1323 base pairs (bp) in three samples, indicating DNA was from A-type strains (results not shown).

July 2010. Total genomic DNA was extracted from 60 samples consisting of three petioles pooled from different stems on the same plant. Of the 60 samples, 30 were from asymptomatic plants and 30 were from symptomatic plants. None of the extracts from plants at the Brantley County field were positive for *X. fastidiosa* using the RST32-RST33 primer pair and undiluted DNA extract in PCR (results not shown). Four of 10 DNA extracts from symptomatic plants at the Alma field and one of 10 DNA extracts from symptomatic plants at the Berrien County field tested positive for *X. fastidiosa* using the RST32-RST33 primer pair and undiluted DNA extract in PCR (results not shown). PCR-negative DNA extracts were not subsequently tested for inhibitors, either through dilution or spiking of genomic DNA because assays during development of the modified extraction method indicated it was effective in reducing PCR inhibitors, with minimal loss of genomic DNA. All five PCR-positive DNA

extracts also produced a weak amplicon of the expected size on an agarose gel after PCR using the 2594F-2594R primer pair, indicating DNA was from A-type strains of *X. fastidiosa* (results not shown).

RFLP analysis of PCR products. In a second attempt to type DNA in the eight extracts from October 2009 that tested positive for *X. fastidiosa*, the extracts were subjected to RFLP analysis using *Rsa*I (Minsavage et al. 1994). RFLP analysis using *Rsa*I separates PCR products from the RST31-RST33 primer pair into 572-bp and 149-bp DNA fragments, but only for A-type strains of *X. fastidiosa* (Minsavage 1994, Chen et al. 2005). PCR products from the RST31-RST33 primer pair do not separate into smaller DNA fragments if the strain of *X. fastidiosa* is G-type. Although digested products appeared faint on a 1% agarose gel, this method allowed DNA in three additional extracts to be designated as from A-type strains of *X. fastidiosa* (results not shown). Thus, bacteria in only two of the eight DNA extracts could not be typed either by RFLP analysis or PCR using the 2594F-2594R primer pair, presumably because the DNA concentration was below the detection threshold for these methods.

Optimization of *X. fastidiosa* detection methods. PCR inhibition observed in DNA extracts from SHB processed with the method described by Costa et al. (2006) prompted the development of a new DNA extraction method (Appendix E). Optimization assays showed that PCR inhibition was reduced when plant tissue (but not bacteria) was allowed to settle in the maceration buffer and only the supernatant was used for DNA extraction. The concentration of inhibitors also was reduced in DNA extracts when TE buffer instead of PBS was used to macerate plant tissue. No major difference in PCR inhibition was observed in DNA extracts prepared from plant tissue macerated in a mesh-lined bag (Agdia Inc., Elkhart, IN) or microcentrifuge tube. However, a microcentrifuge tube was used to contain and reduce transfer

of macerate, thus reducing the potential for loss of *X. fastidiosa* cells. Although effective in reducing PCR inhibitors co-extracted from SHB, the new extraction method was inefficient in terms of labor and time, particularly when handling a large number of samples. Nevertheless, this method was substantially faster than colony isolation for detecting *X. fastidiosa* and detected the presence of the pathogen at approximately the same frequency. Pure cultures of isolated bacteria provided sufficient genomic DNA for detection and typing assays that was free of inhibitors. However, viable bacteria are needed to generate a culture. The time required to culture *X. fastidiosa* also allowed growth of contaminating non-target bacteria that hindered *X. fastidiosa* growth.

While colony growth and morphology are used to identify *X. fastidiosa*, PCR-based methods are more specific and sensitive (Henson and French 1993). RFLP analysis of RST31-RST33 products could be used instead of PCR analysis using the 2594F-2594R primer pair to detect and type DNA of *X. fastidiosa* from SHB, but only if the DNA concentration is at least 4 pg/µl, based on detection threshold assays described in Chapter 3.

Although similar results were obtained in October 2009 and July 2010 for both colony isolation and DNA extraction from plant tissue, detection of *X. fastidiosa* in SHB was low relative to the number of samples from symptomatic plants. In the literature, the extent of blocked xylem vessels by *X. fastidiosa* is commonly attributed to the development and severity of disease (Hopkins 1989, Fry and Millholland 1990, Newman et al. 2003, Krivanek and Walker 2005). Gambetta et al. (2007) developed a quantitative PCR assay to determine the relationship between *X. fastidiosa* populations and the development of leaf scorch symptoms in artificially inoculated, greenhouse-grown and naturally inoculated, field-grown grapevines susceptible to PD. The researchers examined *X. fastidiosa* populations across the lamina of leaves with varying

severity of symptoms, and then related these populations to symptom development. The distribution of *X. fastidiosa* across leaves was found to be patchy, and some leaves exhibited severe leaf scorch symptoms in the absence of detectable *X. fastidiosa*. The researchers subjected their data to statistical analysis and concluded there is little or no correlation between bacterial populations and symptom development in individual leaves.

Since the severity of disease symptoms may not be an indicator of the detectability of *X. fastidiosa*, a more comprehensive and systematic method for sampling must be developed to increase detection of BLSB in symptomatic plants. A study is needed to determine frequency of sampling as well as what, how much and where tissue needs to be sampled on the plant. Other methods to extract DNA from plant tissue need to be developed and evaluated for efficiency in terms of labor and time, effectiveness in reducing or eliminating PCR inhibitors and efficiency in recovering *X. fastidiosa* DNA. Alternative PCR protocols also need to be explored to increase sensitivity of detection of low concentrations of *X. fastidiosa* DNA in plant extracts.

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Figure 4.1. Map of the state of Georgia, indicating the counties (shown with a red dot) from where plant and insect samples were collected. 'Star' and 'FL 86-19' (also known as 'V1') varieties of southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) were sampled from fields in Bacon, Berrien, Brantley and Ware counties. Glassy-winged sharpshooters [*Homalodisca vitripennis* (Germar)] (Hemiptera: Cicadellidae) were collected using yellow sticky traps from fields in Berrien, Brantley and Ware counties. The field in Bacon County is experimental rather than a production field like the others.

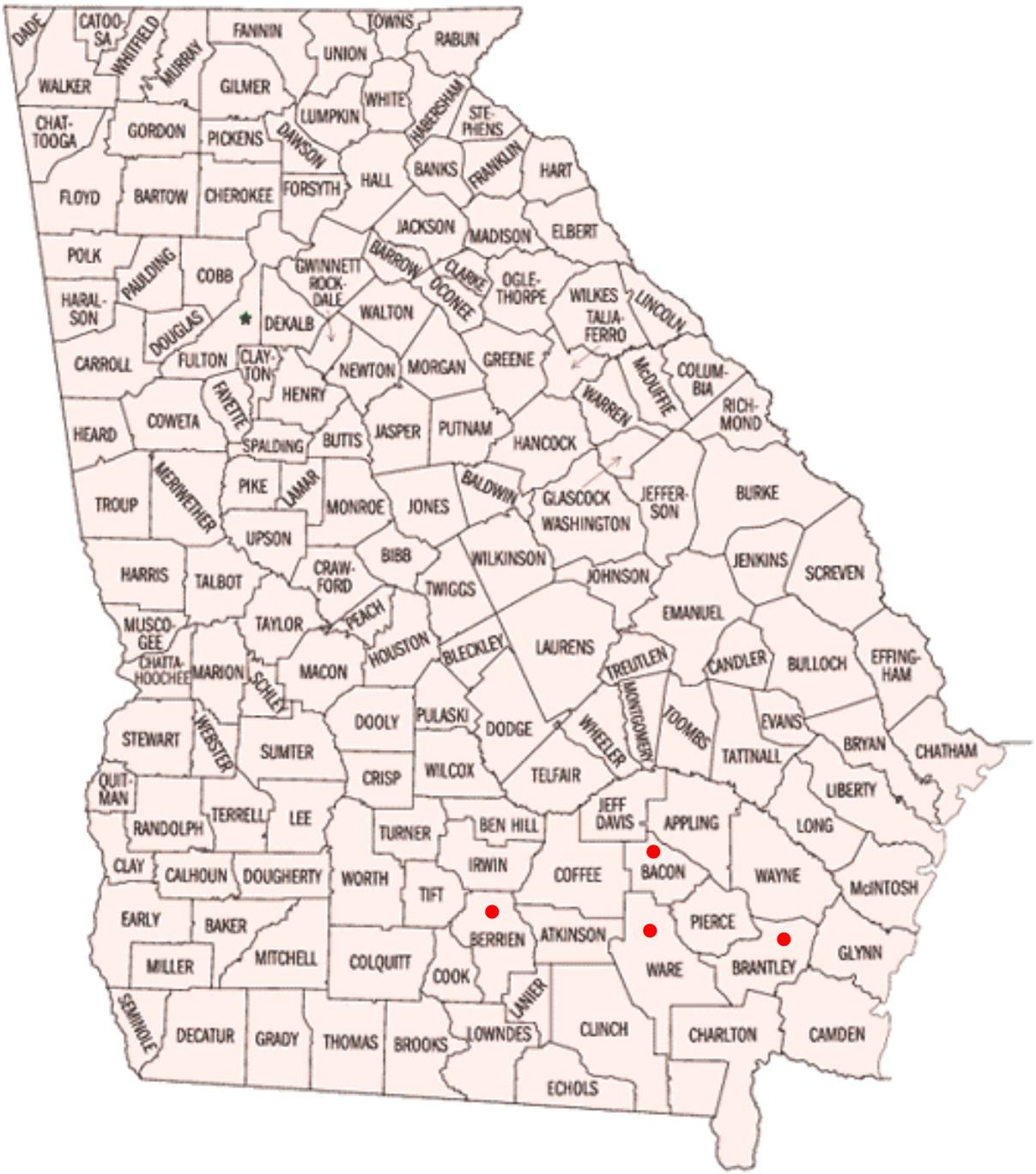
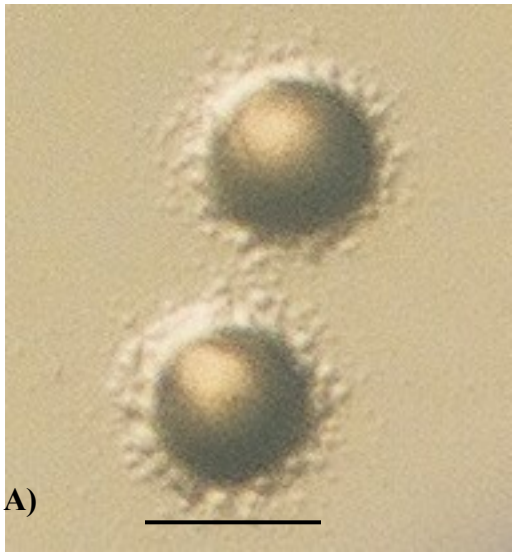
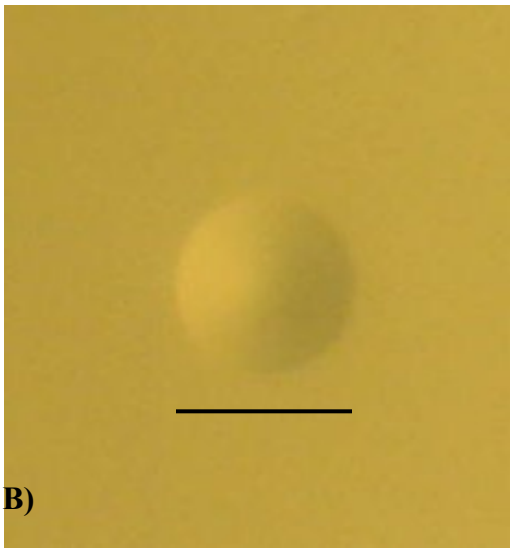

DADE
CATOOSA
WHITFIELD
MURRAY
FANNIN
TOWNS
RABUN
UNION
WALKER
GILMER
WHITE
HABERSHAM
STEPHENS
LUMPKIN
CHATTOOGA
GORDON
PICKENS
DAWSON
FRANKLIN
HART
BANKS
HALL
FORSYTH
FLOYD
BARTOW
CHEROKEE
JACKSON
MADISON
ELBERT
POLK
PAULDING
COBB
GWINNETT
BARROW
CLARKE
OGLETHORPE
WILKES
LINCOLN
OCONEE
TALIAFERRO
HARALSON
DOUGLAS
DEKALB
WALTON
CARROLL
FULTON
CLAYTON
NEWTON
MORGAN
GREENE
COLUMBIA
McDUFFIE
WARREN
RICHMOND
FAYETTE
HENRY
HEARD
COWETA
SPALDING
BUTTS
JASPER
PUTNAM
HANCOCK
TROUP
MERIWETHER
PIKE
LAMAR
MONROE
JONES
BALDWIN
GLASCOCK
JEFFERSON
BURKE
UPSON
WASHINGTON
HARRIS
TALBOT
CRAWFORD
BIBB
WILKINSON
JOHNSON
JENKINS
SCREVEN
TWIGGS
EMANUEL
MUSCOGEE
TAYLOR
PEACH
CHATTAHOOCHEE
MARION
SCHLEY
MACON
HOUSTON
BLECKLEY
LAURENS
TREUTLEN
CANDLER
BULLOCH
EFFINGHAM
STEWART
WEBSTER
SUMTER
DOOLY
PULASKI
DODGE
WHEELER
MONTGOMERY
TOOMBS
EVANS
BRYAN
CHATHAM
QUITMAN
CRISP
WILCOX
TELFAIR
TATTNALL
LIBERTY
RANDOLPH
TERRELL
LEE
TURNER
BEN HILL
JEFF DAVIS
APPLING
LONG
CLAY
CALHOUN
DOUGHERTY
WORTH
IRWIN
COFFEE
BACON
WAYNE
McINTOSH
TIFT
EARLY
BAKER
BERRIEN
ATKINSON
PIERCE
MILLER
MITCHELL
COLQUITT
COOK
WARE
BRANTLEY
GLYNN
SEMINOLE
DECATUR
GRADY
THOMAS
BROOKS
LOWNDES
LANIER
CLINCH
CHARLTON
CAMDEN
ECHOLS

Figure 4.2. *Xylella fastidiosa* colonies on A) PD2 and B) CS20 agar media. Bacteria were isolated from the 'Star' variety of southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) in South Georgia. Size bars are 1 mm.

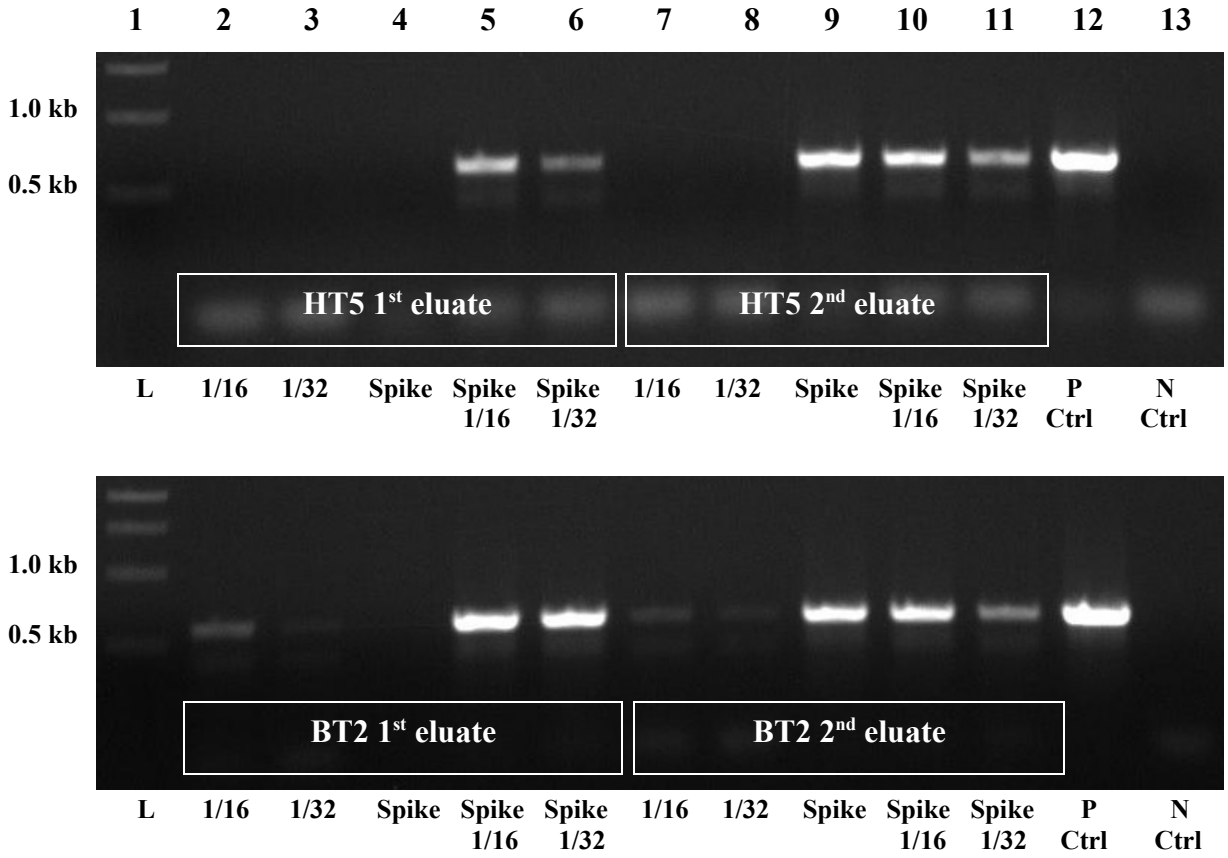


A)



B)

Figure 4.3. PCR inhibition in DNA extracts of southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) varied and was more prevalent in first eluates. The degree of PCR inhibition by DNA extracts was assessed by serial dilution of the extract and/or addition (spiking) of *Xylella fastidiosa* DNA. Amplification was successful from extract BT2 but not from extract HT5 after 1/16 and 1/32 dilutions of first and second eluates (lanes 2, 3, 7, 8). There was no amplification from first eluates despite spiking with genomic DNA (lane 4), whereas there was amplification from both undiluted second eluates spiked with genomic DNA (lane 9). Extracts that were diluted and then spiked with genomic DNA produced an amplicon (lanes 5, 6, 10, 11). Positive controls (lane 12) contained genomic DNA of *X. fastidiosa* strain BB1. Negative controls (lane 13) consisted of master mix and water instead of a DNA template.


1 2 3 4 5 6 7 8 9 10 11 12 13
1.0 kb
0.5 kb
HT5 1st eluate
HT5 2nd eluate
L 1/16 1/32 Spike Spike 1/16 Spike 1/32 1/16 1/32 Spike Spike 1/16 Spike 1/32 P Ctrl N Ctrl
1.0 kb
0.5 kb
BT2 1st eluate
BT2 2nd eluate
L 1/16 1/32 Spike Spike 1/16 Spike 1/32 1/16 1/32 Spike Spike 1/16 Spike 1/32 P Ctrl N Ctrl

CHAPTER 5

DETECTION OF *XYLELLA FASTIDIOSA* STRAINS IN GLASSY-WINGED SHARPSHOOTERS [*HOMALODISCA VITRIPENNIS* (GERMAR)] (HEMIPTERA: CICADELLIDAE) FROM FIELDS OF SOUTHERN HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM* INTERSPECIFIC HYBRIDS) IN SOUTH GEORGIA

INTRODUCTION

Native to the southeastern United States, the glassy-winged sharpshooter (GWSS) [*Homalodisca vitripennis* (Germar)] (Hemiptera: Cicadellidae) feeds on the xylem of more than 100 plant species. In Georgia, the GWSS is the primary insect vector of *Xylella fastidiosa*, the fastidious, gram-negative, xylem-limited bacterium that causes economically important plant diseases such as Pierce's disease (PD) in grapevine and phony peach disease (PPD) (Turner and Pollard 1949, Turner and Pollard 1959, Goheen et al. 1973, Hopkins et al. 1973, Hopkins and Mollenhauer 1973, Davis et al. 1978, Davis et al. 1981a, Davis et al. 1981b, Wells et al. 1981, Wells et al. 1983, Hopkins 1989, Purcell and Saunders 1999, Conklin and Mizell 2002, Almeida and Purcell 2003b, Costa et al. 2006). The GWSS is the largest leafhopper in Georgia, measuring between 1.5 and 2 cm in length at maturity (Conklin and Mizell 2002). The insect can be an efficient vector of *X. fastidiosa*, as the bacterium colonizes the foregut and is persistent throughout the life of the insect when acquired by an adult (Severin 1949, Purcell and Finlay 1979, Brlansky et al. 1983, Hopkins 1989, Hill and Purcell 1995, Conklin and Mizell 2002, Mizell et al. 2003, Almeida and Purcell 2006, Costa et al. 2006). Nymphs are not efficient vectors because the acquired pathogen is lost due to shedding of the lining of the foregut during

molting (Purcell and Finlay 1979, Purcell et al. 1979, Hopkins 1989). *X. fastidiosa* must be present in the sharpshooter foregut, specifically the precibarium, and must detach from the precibarium during feeding for transmission of the pathogen to plants (Almeida and Purcell 2006).

Beginning in 2004, a disorder described as "yellow twig" or "yellow stem" was observed in the 'FL 86-19' (also known as 'V1') variety of southern highbush blueberry (SHB) (*Vaccinium corymbosum* interspecific hybrids) cultivated in the Flatwoods of southeastern and south-central Georgia. Completed in 2006 by Chang et al. (2009), Koch's postulates demonstrated that the disease was caused by *X. fastidiosa*, and it was subsequently named bacterial leaf scorch of blueberry (BLSB). Since its appearance, BLSB has become a major disease in SHB, affecting other varieties, such as 'O'Neal' and 'Star,' while 'V1' remains the most susceptible (Brannen et al. 2010). Based on a field disease survey and serological tests of sampled plants conducted in 2008, varieties of SHB less susceptible to BLSB include 'Emerald,' 'Jewel,' 'V5,' 'Millenia,' 'Southern Belle' and 'Windsor.'

Blueberry production has grown considerably in Georgia since its inception in the mid-1950s and has been particularly rapid the past 10 years (Scherm and Krewer 2003, Boatright and McKissick 2010). Blueberries passed peaches in farm gate value in 2004 to become the most economically important fruit crop in the state. The value of blueberry production in Georgia continues to increase and currently stands at more than $102.4 million. However, BLSB is considered a threat to the industry (Brannen et al. 2010).

Detection of BLSB strains in insect vectors is important to blueberry producers in terms of management of the disease. It may be possible to slow or break the disease cycle by managing insect vectors during peak periods of host feeding activity that may correspond to periods of

pathogen infectivity, beginning in the spring and continuing into the fall. Management of leafhoppers is primarily with insecticides, particularly systemic neonicotinoids (imidacloprid), pyrethroids and organophosphates, but may also include biological and cultural controls (Triapitsyn and Phillips 2000, Bethke et al. 2001, Conklin and Mizell 2002, Brannen et al. 2008, Brannen et al. 2010).

The GWSS is considered the most probable insect vector of BLSB in Georgia, as the leafhopper constituted 97.22% of insects sampled in 2009 from plants at two SHB fields in Berrien and Hoboken counties infected with *X. fastidiosa* (Brannen et al. 2010, Tertuliano et al. 2010, Nissen *unpublished data*). Other leafhoppers sampled at the two fields were *Draeculacephala* spp. (1%), *Graphocephala versuta* (0.51%), *Homalodisca insolita* (0.25%), *Oncometopia nigricans* (0.25%) and *Paraulacizes irrorata* (0.77%) (Brannen et al. 2010, Tertuliano et al. 2010). Leafhoppers were first observed in May and were most abundant from June through early September (Tertuliano et al. 2010). Choice of plant host by the GWSS observed in caged greenhouse studies conducted by Tertuliano et al. (2010) was similar to that observed in the field, with 'Emerald' (55%) preferred to 'Star' (27%) and 'V1' (17%) varieties of SHB.

X. fastidiosa and its insect vectors have been well studied in a number of host plant systems, including grape and peach, but have not been well studied in blueberry. Since little is known about BLSB epidemiology, methods are needed to facilitate detection of the pathogen in potential insect vectors. The objective of this research was to assess whether *X. fastidiosa* could be detected by conventional PCR from total genomic DNA extracted from GWSS collected in fields of SHB. A method also was developed to reduce PCR inhibitors co-extracted from insect tissue.

MATERIALS AND METHODS

Collection of insects. Yellow sticky traps containing GWSS were obtained from a faunal study conducted by Dr. Dan Horton, Department of Entomology, The University of Georgia, Athens, GA, at three SHB fields located in Berrien, Brantley and Ware counties in southeast Georgia (Figure 4.1) varying in incidence of BLSB (discussed in Chapter 4). Traps were from 21 collection periods, starting in September 2008 and ending in August 2010 (Table 5.1).

***X. fastidiosa* strain used in this study.** BLSB strain BES2 used in this study as a positive control in PCR was isolated (method described in Chapter 4) in July 2010 from pooled petioles from the 'Star' variety of symptomatic SHB at a field in Berrien County in southeast Georgia. BES2 was identified as an A-type strain of *X. fastidiosa* using the RST32-RST33 and 2594F-2594R primer pairs (Table 3.3).

Extraction of genomic DNA. Total genomic DNA was extracted from the heads of 427 GWSS (Table 5.1) using the DNeasy® Blood & Tissue Kit (QIAGEN Inc., Valencia, CA) with modifications (Appendix F). Genomic DNA was extracted from a pure culture of BLSB strain BES2 using the DNeasy® Plant Mini Kit (QIAGEN Inc., Valencia, CA) according to the supplier's instructions. Ten microliters of a cell suspension prepared with sterile deionized water (method described in Chapter 3) was added to the lysis buffer supplied in the kit.

PCR primers and protocols. Sequences and sizes of expected amplicons for primers used in this study are listed in Table 3.3. Primer pairs used in this study were RST31-RST33, RST32-RST33 and 2594F-2594R. Thermocycler conditions and PCR reactions using the three primer pairs were as previously described in Chapter 3, except 5 µl of DNA extract from insect tissue was used as template. Samples (10 µl) of PCR reactions were loaded on a 1% agarose gel containing 50 µg/ml of ethidium bromide, and after electrophoresis, amplicons were visualized

and imaged with the BioSpectrum® 300 Imaging System (UVP LLC, Upland, CA). The size of amplicons was determined by comparison to a 1-kb DNA ladder (New England BioLabs Inc., Ipswich, MA).

RESULTS AND DISCUSSION

Extraction of genomic DNA. Preliminary assays indicated there were PCR inhibitors in DNA extracts of insect tissue prepared as described by Costa et al. (2006). Inhibition was determined through serial dilution and addition (spiking) of *X. fastidiosa* DNA. It also was observed that the membranes of spin columns supplied in the QIAGEN DNeasy® Blood & Tissue Kit were sometimes stained brown or lavender prior to DNA elution, which was correlated with PCR inhibition. Also, inhibition was greater in the first eluate than in subsequent eluates. Experiments to reduce the concentration of inhibitors that co-extracted with DNA determined that only one insect head could be used as a sample. Inhibition was reduced when insect tissue was allowed to settle in the maceration buffer and only the supernatant was used for DNA extraction. Inhibition also was reduced in extracts when TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) instead of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4) was used to not only macerate and soak insect tissue but also to wash cells/residual debris pelleted from the supernatant prior to DNA extraction. No major difference in PCR inhibition was observed in DNA extracts prepared from insect tissue macerated in a mesh-lined bag (Agdia Inc., Elkhart, IN) or microcentrifuge tube. However, a microcentrifuge tube was used to contain and reduce transfer of macerate, thus reducing the potential for loss of *X. fastidiosa* cells. Only heads of insects were used in DNA extraction, as they contain the foregut where *X. fastidiosa* is known to reside (Severin 1949, Purcell and Finlay 1979, Brlansky et al. 1983, Hopkins 1989, Brlansky et

al. 1991, Hill and Purcell 1995, Conklin and Mizell 2002, Mizell et al. 2003, Almeida and Purcell 2006, Costa et al. 2006). However, a downside to using only one insect head in DNA extraction was the reduced chance of detecting *X. fastidiosa* due to low titers of the bacterium in insect vectors (Nome et al. 1980).

Bextine et al. (2004b) evaluated the effectiveness of three standard methods (immunomagnetic separation, phenol extraction, phenol/chloroform extraction) and 11 commercial kits in extracting *X. fastidiosa* DNA from the heads of field-collected adult GWSS (negative controls), dilutions containing 10^5 *X. fastidiosa* cells, heads of field-collected adult GWSS with added 10^5 *X. fastidiosa* cells, and heads of field-collected adult GWSS that fed on *X. fastidiosa*-infected grapevines in mesh bag cages. The researchers prepared samples for DNA extraction by macerating GWSS heads in PBS in an Agdia bag or in liquid nitrogen. Using conventional PCR with primer pair RST31-RST33, Bextine et al. (2004b) determined that all 14 methods were effective in extracting *X. fastidiosa* DNA from dilutions of *X. fastidiosa* cells. *X. fastidiosa* was not detected by PCR in negative controls processed by any method. All but one method, the FTA™ Gene Card (Whatman Inc., Florham Park, N.J.), extracted *X. fastidiosa* DNA from the heads of field-collected adult GWSS with added bacterial cells. Of the 14 methods, only the QIAGEN DNeasy® Blood & Tissue Kit and Genomic DNA Purification Kit (Fermentas Inc., Glen Burnie, Md.) extracted *X. fastidiosa* DNA from the heads of field-collected adult GWSS that fed on infected grapevine. Although intensity of amplicons was faint on agarose gels, 500 and 50 *X. fastidiosa* cells were detected by PCR from extracts processed with and without insect tissue, respectively, by the QIAGEN DNeasy® Blood & Tissue Kit. Using the Fermentas Genomic DNA Purification Kit, 1,600 *X. fastidiosa* cells were detected by PCR from extracts processed without insect tissue, and the pathogen was not detected in extracts processed with

insect tissue. Similarly, using immunomagnetic separation, 1,600 and 50 *X. fastidiosa* cells were detected by PCR from extracts processed with and without insect tissue, respectively.

Studies by Costa et al. (2006) and Myers et al. (2007) also successfully used the QIAGEN DNeasy® Blood & Tissue Kit to extract *X. fastidiosa* DNA from sharpshooter heads. Costa et al. (2006) slightly modified the protocol developed by Bextine et al. (2004b), and Myers et al. (2007) slightly modified the protocol developed by Bextine et al. (2004a) to prepare sharpshooters for total genomic DNA extraction. Costa et al. (2006) used a single insect head as a sample, prepared tissue for DNA extraction through maceration in PBS in an Agdia bag, and used conventional and multiplex PCR to detect and differentiate *X. fastidiosa* strains. Myers et al. (2007) used a single insect head as a sample, prepared supernatants for DNA extraction using vacuum infiltration of insect tissue with PBS and used nested PCR to detect *X. fastidiosa* DNA. Thus, the QIAGEN DNeasy® Blood & Tissue Kit should have been the best choice for DNA extraction from sharpshooter heads in this study.

In other studies, Bextine et al. (2004a, 2005) tested the speed and efficiency of DNA extraction from the heads of infectious GWSS after using vacuum infiltration of PBS to liberate *X. fastidiosa* cells from the foregut of insects. The researchers continued to use the QIAGEN DNeasy® Blood & Tissue Kit to extract total genomic DNA from the supernatant. Then instead of conventional PCR, the researchers used SYBR® Green-based quantitative real time (QRT) PCR in an attempt to improve sensitivity of detection of *X. fastidiosa* from extracts. Bextine et al. (2004a, 2005) found using vacuum infiltration versus tissue maceration in an Agdia bag to prepare samples for DNA extraction was not statistically different in terms of the number of samples testing positive for *X. fastidiosa* or the number of detectable *X. fastidiosa* cells. However, in five of six trials, mean positives and mean relative fluorescence were greater in

macerated samples. Bextine et al. (2004a, 2005) noted that the mean number of GWSS testing positive for *X. fastidiosa* varied between trials and experiments, and was likely due to natural variation in the ability of the GWSS to harbor the pathogen. However, these researchers noted that vacuum infiltration improved the speed and efficiency of the overall DNA extraction process. They surmised that since no insect tissue was macerated, fewer inhibitors were likely released and less non-template DNA was likely co-extracted. These two factors often hinder PCR detection of the pathogen when DNA concentration is low. These researchers also noted that using QRT-PCR, which allows detection of low concentrations of bacteria from environmental samples (Lockey et al. 1998), improved sensitivity of *X. fastidiosa* detection in the heads of infectious adult GWSS 10-100 fold, from 50-500 *X. fastidiosa* cells with conventional PCR (Bextine et al. 2004b) to 5-50 *X. fastidiosa* cells per sample with QRT-PCR (Bextine et al. 2004a, 2005).

PCR analysis. Primer pairs RST31-RST33 and RST32-RST33 used in this study were expected to yield DNA fragments of 733 base pairs (bp) and 600 bp, respectively (Table 3.3). Conventional PCR using the *X. fastidiosa* species-specific RST32-RST33 primer pair did not detect the pathogen in any of the 427 DNA extracts processed from single GWSS heads (results not shown). Primer pair RST32-RST33 amplified single or multiple non-target DNA fragments in at least 130 extracts (results not shown), most of which were smaller in size than the target amplicon. Amplification by the RST32-RST33 primer pair of one non-target DNA fragment of approximately 500 bp (Figure 5.1) was particularly intense in nearly 40 DNA extracts from GWSS collected from Berrien and Brantley counties in September 2009 and in July and August 2010 (results not shown). Six of these DNA extracts from September 2009 were reanalyzed using the RST32-RST33 primer pair, producing similar results (Figure 5.1). The six extracts

were also analyzed using the RST31-RST33 and 2594F-2594R primer pairs. The RST31-RST33 primer pair produced a weak amplicon of approximately the expected size from one of the six DNA extracts (Figure 5.1). The 2594F-2594R primer pair was designed to yield a DNA fragment of 1323 bp for A-type strains of *X. fastidiosa*. As with the RST32-RST33 primer pair, the 2594F-2594R primer pair amplified multiple non-target DNA fragments in all six extracts, but not to the intensity of the RST32-RST33 primer pair (Figure 5.1).

It is unlikely that the approximately 500-bp DNA fragment amplified by the RST32-RST33 primer pair is from *X. fastidiosa*. First, it is clearly smaller than the 600-bp amplicon expected when using these primers (Figure 5.1). Second, if this 500-bp amplicon was from *X. fastidiosa*, then there would have been sufficient pathogen DNA in the extracts for the other two primer pairs to produce expected amplicons. As discussed in Chapter 3, the RST31-RST33 and 2594F-2594R primer pairs are comparable to the RST32-RST33 primer pair in their thresholds of detection of *X. fastidiosa*. However, the RST31-RST33 primer pair generated no strong amplicon of the expected size, and the 2594F-2594R primer pair produced only many nonspecific amplicons. Therefore, although suitable for analyzing DNA extracts from SHB (Chapter 4), the RST32-RST33 primer pair is not suitable for analyzing DNA extracts from GWSS due to amplification of non-target DNA.

It is unclear why *X. fastidiosa* was not detected in any of the DNA extracts from GWSS. Factors affecting detection of *X. fastidiosa* in DNA extracts from GWSS may include collection, handling and storage of insects; inefficient lysis of bacteria and recovery of pathogen DNA, inadequate removal of PCR inhibitors, co-extraction of non-target DNA, an undetectable concentration of pathogen DNA due to sample size, and inefficient pathogen acquisition and transmission by the GWSS in SHB. Sources of variability in transmission rate of insect vector-

borne plant pathogens include vector species (Palermo et al. 2001, Daugherty and Almeida 2009), vector abundance (Ng and Perry 2004, Daugherty and Almeida 2009), host species (Wistrom and Purcell 2005, Lopes et al. 2009), pathogen strains (Lucio-Zavaleta et al. 2001, Lopes et al. 2009) and local climate (Sylvester 1964, Shih et al. 1995, Murral et al. 1996, Lucio-Zavaleta et al. 2001, Dohm et al. 2002, Anhalt and Almeida 2008, Daugherty et al. 2009). The GWSS was found by Almeida and Purcell (2003b, 2006) to be a less-efficient vector than the blue-green sharpshooter (BGSS) [*Graphocephala atropunctata* (Signoret)] (Hemiptera: Cicadellidae) in transmitting *X. fastidiosa* to grapevine. In the 2006 study, Almeida and Purcell examined 30 GWSS and found *X. fastidiosa* cells in the precibarium of only one, and that insect failed to transmit the bacterium to grapevine. The researchers surmised that differences in feeding behavior among sharpshooters may explain variability in efficiency of transmission of *X. fastidiosa*, as the pathogen has low to no vector specificity (Frazier 1965, Purcell 1989, Daugherty and Almeida 2009), and the ability of bacterial cells to attach to the foregut of different species should be similar.

Studies using methods similar to what was used in this research have successfully detected *X. fastidiosa* in multiple sharpshooter species in vineyards with grapevines exhibiting symptoms of PD. Costa et al. (2006) conducted a greenhouse study to compare the relative proportion of adult GWSS that retained PD or oleander leaf scorch (OLS) strains of *X. fastidiosa* after feeding on hosts and nonhosts of each strain. They determined that PCR detection of *X. fastidiosa* is a useful predictor of transmission of the pathogen and that insects testing positive for a particular *X. fastidiosa* strain are more likely to transmit that strain. Of 46 adult GWSS that survived throughout the experiment and were exposed to both PD and OLS strains of *X. fastidiosa*, 39% transmitted one strain of the pathogen, which is within the range of transmission

rates observed for individual GWSS exposed to a single strain of *X. fastidiosa* (Almeida and Purcell 2003a,b).

Myers et al. (2007) focused on better understanding the epidemiology of PD in vineyards in the eastern Piedmont and northeastern Coastal Plain regions of North Carolina. They found that *Oncometopia orbona*, *Graphocephala versuta*, *Paraphlepsius irroratus* and *Agalliota constricta* were the most abundant sharpshooters and that of three species tested, 27% of *O. orbona*, 28% of *G. versuta* and 33% of *P. irroratus* were positive for *X. fastidiosa*. DNA analysis of *X. fastidiosa* strains from field-surveyed insects indicated strains were genetically similar to each other and to PD strains of *X. fastidiosa* endemic to California. A greenhouse study demonstrated that *O. orbona* and *G. versuta* are able to transmit *X. fastidiosa* to grapevine, with *O. orbona* being more efficient at transmission than *G. versuta*.

In studies being conducted in SHB fields in southeast Georgia to determine sharpshooter species, abundance and host preferences, Tertuliano et al. 2010 observed that the GWSS is by far the most abundant sharpshooter. These SHB fields, which were the source of GWSS used for DNA extraction in this study, are known to have plants infected with *X. fastidiosa* (Nissen *unpublished data*). Therefore, based on knowledge from studies of PD in grapevine, it was thought very probable that a detectable percentage of GWSS would carry *X. fastidiosa* in SHB. For example, as predicted by Purcell (1981), Costa et al. (2000) and Daugherty and Almeida (2009) found that sharpshooter abundance is positively related to *X. fastidiosa* transmission rate. Also, Daugherty and Almeida (2009) concluded that even though high vector numbers are not related to greater *X. fastidiosa* populations in plants, they may drive disease dynamics by decreasing the time for vectors to reacquire the pathogen and thus increasing the likelihood of secondary spread. However, both acquisition and inoculation efficiency of *X. fastidiosa* by

sharpshooters is positively related to the length of insect feeding (Purcell and Finlay 1979, Hill and Purcell 1995, Almeida and Purcell 2003b, Daugherty and Almeida 2009) and to the pathogen population in the host plant (Hill and Purcell 1995). Thus, results in this study may be explained by low bacterial populations in SHB and/or short insect feeding periods that would reduce the probability of GWSS acquiring *X. fastidiosa* that cause BLSB.

Since *X. fastidiosa* was not detected in field-collected GWSS, a controlled study is needed in which caged insects are allowed to feed exclusively on infected SHB to ensure that they acquire the pathogen. These insects can then be used to re-evalute the methods used in this study to extract and analyze *X. fastidiosa* DNA from sharpshooters. Other methods, such as vacuum infiltration described by Bextine et al. (2004a, 2005) to extract *X. fastidiosa* cells from insect heads, should be explored. Methods that increase sensitivity in detecting low concentrations of pathogen DNA, such as nested PCR and QRT-PCR, also should be explored as a means to maximize amplification of *X. fastidiosa* DNA in insect extracts. The 2594F-2594R primer pair developed in this study (Chapter 3) may be a candidate for the development of a nested PCR protocol due to the size of its target amplicon (1323 bp) and the fact that it differentiates A-type (non-PD-like) from G-type (PD-like) strains of *X. fastidiosa*.

Once the best methods to extract bacteria from sharpshooters and detect *X. fastidiosa* DNA are determined, field survey and testing of insects can resume. However, sampling needs to be comprehensive and systematic, with all observed species of sharpshooters collected weekly or bimonthly year-round from within SHB fields and surrounding vegetation. For example, Myers et al. (2007) noted that in sharpshooters collected from four vineyards over two seasons, overwintering populations of *O. orbona* and *G. versuta* had the highest proportion of potentially infective individuals. The researchers surmised that a decline in the number of sharpshooters

positive for *X. fastidiosa* later in the season most likely reflected the mortality of overwintering adults or repeated application of broad-spectrum insecticides during the growing season in some vineyards. Other studies have found that a high percentage of sharpshooters are capable of transmitting *X. fastidiosa* in early spring, followed by a decline in individuals testing positive for the pathogen during periods of nymphal development (Freitag and Frazier 1954, Purcell 1975, Myers et al. 2007). Myers et al. (2007) also noted that a higher population of *O. orbona* was observed in vineyards than was collected on yellow sticky traps, suggesting trapped insects indicate the extent of immigrating insects and primary spread of *X. fastidiosa* but do not indicate insect species present in vineyards that are less attracted to traps.

By direct observation and sampling in the field, sharpshooter species and numbers; primary, secondary and overwintering hosts; and strains of *X. fastidiosa* carried by sharpshooters can be identified. Sampling needs to occur in a variety of locations and over a several year period to account for differences in cultural conditions, topography, vegetation and climate. In regard to sampling method, a sweep net or vacuum may be preferable to yellow sticky traps. Storing insects on traps takes up tremendous space in freezers. Adhesive also makes handling of traps and removal of insects more difficult.

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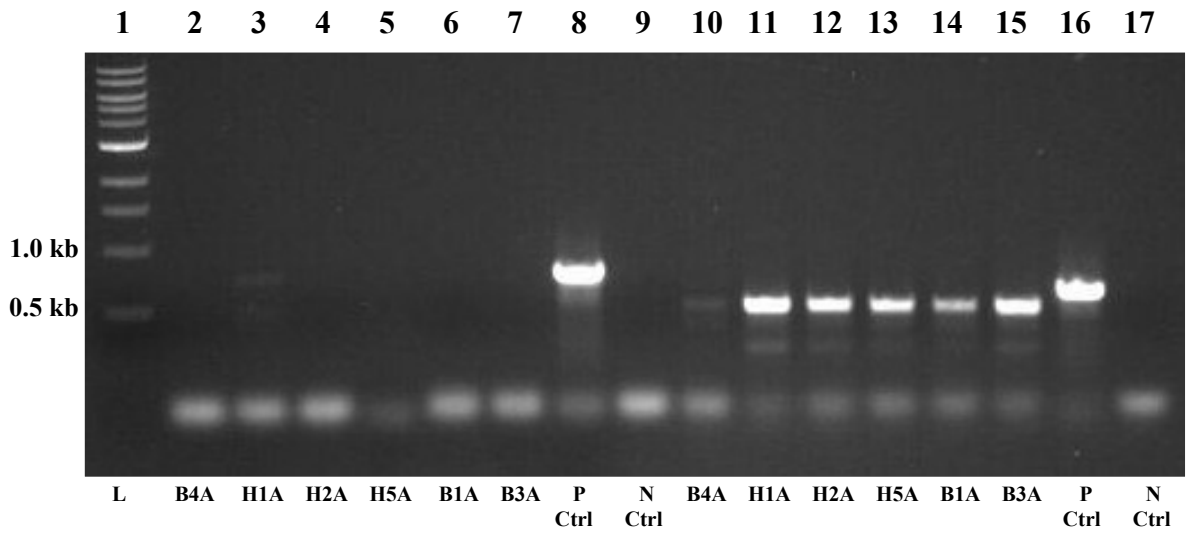
Table 5.1. Total genomic DNA was extracted from the heads of 427 glassy-winged sharpshooters [*Homalodisca vitripennis* (Germar)] (Hemiptera: Cicadellidae) and then analyzed by PCR for the presence of *Xylella fastidiosa*. Shown is the number of insects sampled per collection period per field.

Collection period[a]	Berrien County	Ware County	Brantley County	Totals
September 9, 2008	5	5	5	15
September 23, 2008	5	1	5	11
October 2008	5	5	5	15
May 5, 2009	10	1	10	21
May 19, 2009	0	0	8	8
June 2, 2009	10	0	10	20
June 30, 2009	10	10	10	30
July 14, 2009	10	0	10	20
July 28, 2009	10	0	10	20
August 11, 2009	10	5	10	25
August 25, 2009	10	3	10	23
September 8, 2009	5	0	5	10
September 22, 2009	5	1	5	11
October 6, 2009	5	0	5	10
October 20, 2009	2	1	5	8
June 15, 2010	10	10	10	30
June 29, 2010	10	10	10	30
July 13, 2010	10	10	10	30
July 27, 2010	10	10	10	30
August 10, 2010	10	10	10	30
August 24, 2010	10	10	10	30
Totals	162	92	173	427

[a] Insects were obtained from a faunal study conducted by Dr. Dan Horton, Department of Entomology, The University of Georgia, Athens, GA, at three southern highbush blueberry fields located in Berrien, Brantley and Ware counties in southeast Georgia. Insects were collected using yellow sticky traps.

Figure 5.1. Representative results of PCR using primer pairs RST31-RST33 (lanes 1-9), RST32-RST33 (lanes 10-17) and 2594F-2594R (lanes 19-26), and DNA extracted from the heads of glassy-winged sharpshooters [*Homalodisca vitripennis* (Germar)] (Hemiptera: Cicadellidae) as template. Extracts are from insects collected in September 2009 from southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) fields in Berrien (lanes 2, 6, 7, 10, 14, 15, 19, 23, 24) and Brantley (lanes 3, 4, 5, 11, 12, 13, 20, 21, 22) counties in southeast Georgia. Positive controls (lanes 8, 16, 24) contained genomic DNA of *X. fastidiosa* strain BES2. Negative controls (lanes 9, 17, 26) consisted of master mix and water instead of a DNA template.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
1.0 kb
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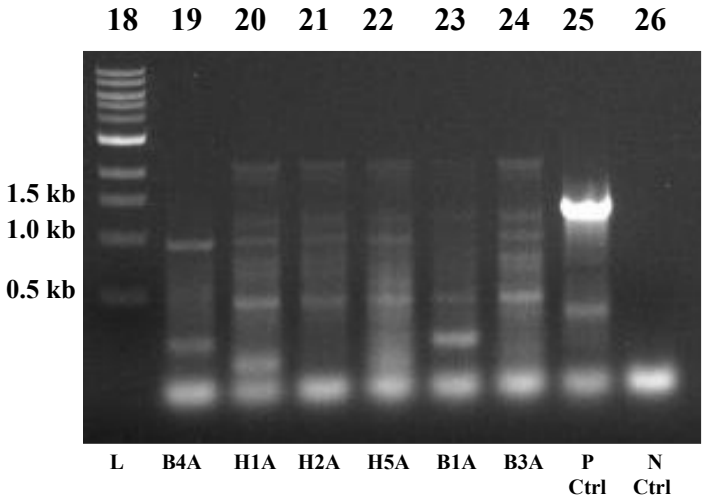
L B4A H1A H2A H5A B1A B3A P Ctrl N Ctrl B4A H1A H2A H5A B1A B3A P Ctrl N Ctrl


18 19 20 21 22 23 24 25 26
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CHAPTER 6

CONCLUSIONS

Demonstrated through Koch's postulates by Chang et al. (2009) to be caused by *Xylella fastidiosa*, bacterial leaf scorch of blueberry (BLSB) is a threat to the viability of a growing, multimillion-dollar blueberry production industry in Georgia (Brannen et al. 2010). *X. fastidiosa* and its insect vectors have been well studied in a number of host plant systems, including grape and peach, but not in blueberry. Thus, objectives of this research were to characterize BLSB strains of *X. fastidiosa* and develop assays to detect them in plants, particularly southern highbush blueberry (SHB) (*Vaccinium corymbosum* interspecific hybrids), and in the most probable insect vector, the glassy-winged sharpshooter (GWSS) [*Homalodisca vitripennis* (Germar)] (Hemiptera: Cicadellidae).

No evidence was found in this study that BLSB strains are unique. Based on PCR analyses in this study, BLSB strains differ from *X. fastidiosa* strains that cause Pierce's disease (PD) in grapevine, but are genetically similar to the *X. fastidiosa* Dixon strain that causes leaf scorch in almond. The 16S-23S rDNA intergenic spacer region was used instead of the 16S rRNA locus to characterize BLSB strains based on DNA homology with other *X. fastidiosa* strains. Both loci have successfully determined taxonomic relationships between and within groups of bacteria, including among strains of *X. fastidiosa*; however, the 16S-23S rDNA intergenic spacer region offers more diversity in length and sequence (Barry et al. 1991, Jeng et al. 2001, Schaad et al. 2004, Martinati et al. 2007). However, as the epidemiology of BLSB

strains still is not known, other semi-conserved loci in the *X. fastidiosa* genome need to be examined to further characterize BLSB strains endemic to Georgia.

Although seemingly effective in reducing PCR inhibitors, the genomic DNA extraction methods for plant and insect tissue developed in this study were inefficient in terms of labor and time, particularly when handling a large number of samples. However, the DNA extraction method for plant tissue was more efficient than colony isolation in terms of time to recover genomic DNA. While colony growth and morphology can be used to identify *X. fastidiosa*, PCR-based methods are more sensitive and specific (Henson and French 1993), as they can be used to differentiate strains of the pathogen. *X. fastidiosa* in plant extracts was detected by conventional PCR, but only when the concentration of pathogen DNA was sufficient for amplification by primers used in this study. A disadvantage of the genomic DNA extraction method for plant tissue developed in this study is that *X. fastidiosa* DNA often was not detected from symptomatic plants, which hindered detection and typing of pathogen strains, and may be problematic for future epidemiological studies. Colony isolation provided sufficient genomic DNA for detection and typing of *X. fastidiosa* strains by conventional PCR and primers used in this study. However, viable bacteria are needed to generate a culture. The time required to culture *X. fastidiosa* also often allows other bacterial and fungal contamination to overtake media and hinder *X. fastidiosa* growth.

Similar results were obtained in October 2009 and July 2010 for both colony isolation and DNA extraction from plant tissue. However, detection of *X. fastidiosa* in SHB was low relative to the number of samples from symptomatic plants. Both methods are used by researchers to detect *X. fastidiosa* in plants. However, since severity of disease symptoms may not be an indicator of the detectability of *X. fastidiosa* (Gambetta et al. 2007), a more

comprehensive and systematic method for sampling must be developed to increase detection of BLSB in symptomatic plants. A study is needed to determine frequency of sampling as well as what, how much and where tissue needs to be sampled on the plant.

X. fastidiosa was not detected in any genomic DNA extractions from insect tissue by conventional PCR and primers used in this study. Factors affecting detection of *X. fastidiosa* in DNA extracts from GWSS collected in SHB fields may include collection, handling and storage of insects; inefficient lysis and recovery of pathogen DNA, inadequate removal of PCR inhibitors, co-extraction of non-target DNA, an undetectable concentration of pathogen DNA due to sample size, and inefficient pathogen acquisition and transmission by insects in blueberry. Six species of sharpshooters were observed in SHB fields in southeast Georgia by Tertuliano et al. (2010), yet only the most abundant species, the GWSS, was tested for the presence of *X. fastidiosa* in this study. It is possible that the GWSS is not the primary insect vector of BLSB. Since *X. fastidiosa* was not detected in this sharpshooter, all observed species need to be assayed to ensure that a potential vector is not overlooked. Since *X. fastidiosa* was not detected in field-collected GWSS, a controlled study is needed in which caged insects are allowed to feed exclusively on infected SHB to ensure that they acquire the pathogen. Methods used in this study to extract and analyze *X. fastidiosa* DNA from sharpshooters then can be re-evaluated.

Other methods to extract *X. fastidiosa* DNA from plant and insect tissue need to be developed and evaluated for efficiency in terms of labor and time, effectiveness in reducing or eliminating PCR inhibitors and non-template DNA, and efficiency in recovering pathogen DNA. Alternative PCR protocols also need to be explored to increase sensitivity of detection of low concentrations of *X. fastidiosa* DNA in plant and insect extracts. RFLP analysis using *Rsa*I described by Minsavage et al. (1994) may be used to detect and type *X. fastidiosa* strains.

However, the method is limited to the detection of non-PD strains and by the amount of pathogen DNA amplified by the RST31-RST33 primer pair. The RST32-RST33 primer pair is suitable for detecting *X. fastidiosa* in plant extracts but is not suitable for detecting *X. fastidiosa* in insect extracts due to amplification of non-target DNA. The 2594F-2594R primer pair developed in this study is efficient and robust in detecting and typing strains of *X. fastidiosa*, eliminating the need for multiplex PCR described by Chen et al. (2005) and Hernandez-Martinez et al. (2006). Although further study is needed, the primer pair XF1968F3-XF1968R3 developed in this study might be useful to differentiate BLSB strains from *X. fastidiosa* strains isolated from plum and oak in the Southeast.

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APPENDIX A

MULTIPLE SEQUENCE ALIGNMENT SHOWING LOCATIONS OF REDESIGNED PRIMERS SPECIFIC TO *XYLELLA FASTIDIOSA* STRAINS CAUSING PIERCE'S DISEASE

As shown here in this multiple sequence alignment of the *X. fastidiosa* tRNA-Ser gene (GenBank Accession No. AF130454) with *X. fastidiosa* strains Temecula1, Ann-1, Dixon and 9a5c, forward primer PDconsvdF (5′-AAACAATCACAGGGACTGC-3′) and reverse primer PDspec685 (5′-GACGGAATCTATGAATATCGCC-3′) were designed from primer XF176f (5′-AAACAATCACAGGGGACTGC-3′) and primer XF686r (5′-TCGACGGAATCTATGAATAT-3′) to improve specificity for Pierce's disease (PD) strains (Temecula1). To correct what appeared to be an error in the sequence of XF176f, a single nucleotide "G" was deleted to make PDconsvdF. The reverse primer sequence was shifted, creating a GC clamp at that primer's 3′ end with genomic DNA from PD strains and a terminal one-base mismatch with strains that cause almond leaf scorch (Dixon), oleander leaf scorch (Ann-1) and citrus variegated chlorosis (9a5c).

```
AF130454        CAGGCCCTTCATGGTCTATTTGAAATCGGCACAGGCAGCACCATCAAGACAATGTGCCAC
Temecula1       ----CCCTTCATGGTCTATTTGAAATCGGCACAGGCAGCACCATCAAGACAATGTGCCAC
Ann-1           -----CCTTCATGGTCTATTTGAAATCGGCACAGGCAGCACCATCAAGACACTGTGCCAC
Dixon           -----CCTTCATGGTCTATTTGAAATCGGCACAGGCAGCACCATCAAGACACTGTGCCAC
9a5c            -----CCTTCATCGTCTATTTGAAATCGGCACAGGCAGCACCATCGAGACACTGTGCCAC
                     ******* ******************************** ***** ********

AF130454        TTGTATTCGGACTACTCCGGCTACCTGTAAGAACGCCACACATCCGCGTGACGCACTTAA
Temecula1       TTGTATTCGGACTACTCCGGCTACCTGTAAGAACGCCACACATCCGCGTGACGCACTTAA
Ann-1           TTGTATTCGGACTACTCCGGCTACCTGTAAGAACGTCACACATCCGCGTGACGCACTTAA
Dixon           TTGTATTCGGACTACTCCGGCTACCTGTAAGAACGTCACACATCCGCGTGACGCACTTAA
9a5c            TTGTATTCGGACTACTCCGGCTACCTGTAAGAACGTCACACATCCGCGTGACGCACTTAA
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AF130454        AACCCAATGATGACAACATGCATTGTATTTTTAAATGCAAAAGTAGGAAAAGAACAAACA
AF130454        AACCCAATGATGACAACATGCATTGTATTTTTAAATGCAAAAGTAGGAAAAGAACAAACA
Temecula1       AACCCAATGATGACAACATGCATTGTATTTTTAAATGCAAAAGTAGGAAAAGAACAAACA
Ann-1           AACCCAATGATGACAACGTGCATTGTATTTTTAAATGCAAAAGTAGGAAAAGAACAAACA
Dixon           AACCCAATGATGACAACGTGCATTGTATTTTTAAATGCAAAAGTAGGAAAAGAACAAACA
9a5c            AACCCAATGATGACAACGTGCATTGTATTTTTAAATGCAAAAGTAGGAAAAGAACAAACA
                ***************** ****************************************

AF130454        ATCACAGGGGACTGCCAAGTTATTTTTTGAAGATTTCAAATTAAAGCATAAGGCATTTCA
AF130454        ATCACAGGG-ACTGCCAAGTTATTTTTTGAAGATTTCAAATTAAAGCATAAGGCATTTCA
Temecula1       ATCACAGGG-ACTGCCAAGTTATTTTTTGAAGATTTCAAATTAAAGCATAAGGCATTTCA
Ann-1           ATCACAGGG-ACTGCCAAGTTATTTTTTGAAGATTTCAAATTAAAGCATAAGGCATTTCA
Dixon           ATCACAGGG-ACTGCCAAGTTATTTTTTGAAGATTTCAAATTAAAGCATAAGGCATTTCA
9a5c            ATCACAGGA-ACTGCCAAATTATTTTTTGAAGATTTCAAATTCAAGCATAAGGCATTTCA
                ********  ******** ********************** ******************

AF130454        AAAAATTCTTTCTGCTGGCTGTTCGTTAGCAACATAGATTAGATACAAAAAATAAACCGC
Temecula1       AAAAATTCTTTCTGCTGGCTGTTTGTTAGCAACATAGATTAGATACAAAAAATAAACCGC
Ann-1           AAAAATTCTTTCTGCTGGCTGTTTGTTAGCAACGTAGATTAGATACCAAAAATAAACCGC
Dixon           AAAAATTCTTTCTGCTGGCTGTTTGTTAGCAACGTAGATTAGATACCAAAAATAAACCGC
9a5c            AAAAATTCTTTCTGCTGGCTGTTTGTTAGCAACGTAGATTAGATACCAAAAATAAACCGC
                ********************** ********* ************ *************

AF130454        ATCAAAATAACCAACATCTCCAATGCGAATTTAAATATTCAATAAAATACCAACTCACTC
Temecula1       ATCAAAATAACCAACATCTCCAATGCGAATTTAAATATTCAATAAAATACCAACTCACTC
Ann-1           ATCAAAATAACCAACATCTCCAATGCGAATTTAAATATTCAATAAAATACCAACTCACTA
Dixon           ATCAAAATAACCAACATCTCCAATGCGAATTTAAATATTCAATAAAATACCAACTCACTA
9a5c            ATCAAAATAACCAACATCTCCAATGCGAATTTAAATATTCAATAAAATGCCGACTCACTA
                ********************************************** ** *******

AF130454        GCATATGCTTAATCATAGAGAACACGGAACCAAAGAAGCATCACGCAATACTCACGTATA
Temecula1       GCATATGCTTAATCATAGAGAACACGGAACCAAAGAAGCATCACGCAATACTCACGTATA
Ann-1           GCATATGCTTAATCATAGAGAACACGGAACCAAAGAAGCATCACGCAATACTCACGTATA
Dixon           GCATATGCTTAATCATAGAGAACACGGAACCAAAGAAGCATCACGCAATACTCACGTATA
9a5c            GCATATGCTTAATCATAGAGAACACGGAACCAAAGAAGCACCACGCAATACTCACGTATA
                ************************************* ******************

AF130454        AACGAGAAATGATGTTGGTTGCTCTATGCTCTATCTGAGTAAACCAATTCAACCACAACA
Temecula1       AACGAGAAATGATGTTGGTTGCTCTATGCTCTATCTGAGTAAACCAATTCAACCACAACA
Ann-1           AACGAGAAATGATGTTGGTTGCTCTATGCTCTATCTGAGTAAACCAATTCAACCACAACA
Dixon           AACGAGAAATGATGTTGGTTGCTCTATGCTCTATCTGAGTAAACCAATTCAACCACAACA
9a5c            AACGAGAAATGATGTTGGTTGCTCTATGCTCTATCTGAGTAAACCAATTCAACCACAACA
                ************************************************************

AF130454        CCGCTTTGAGATTCCATCAAGTCAGCCTACGAAACTGTATTTCAAGAGCGCTGATAGAAA
Temecula1       CCGCTTTGAGATTCCATCAAGTCAGCCTACGAAACTGTATTTCAAGAGCGCTGATAGAAA
Ann-1           CCGCTTTGAGATTCCATCAAGTCAGCCTACGAAACGGGATTTCAAGAGTGCTGATAGAAA
Dixon           CCGCTTTGAGATTCCATCAAGTCAGCCTACGAAACGGGATTTCAAGAGTGCTGATAGAAA
9a5c            CCGCTTTGAGATTCCATCAAGTCAGCCTACGAAACGGGATTTCAAGAGTGCTGATAGAAA
                ********************************** * ********** ***********

AF130454        CAATGCCCAATCAGGAGTCACTTCAGAAAACCTCACTGACCACAGGGTATAGAAGCAACG
Temecula1       CAATGCCCAATCAGGAGTCACTTCAGAAAACCTCACTGACCACAGGGTATAGAAGCAACG
Ann-1           CAATGCCCAATCAGGAGTCACTTCAGAAAACCTCACTGACCACAGGGTATAGAAGCAACG
Dixon           CAATGCCCAATCAGGAGTCACTTCAGAAAACCTCACTGACCACAGGGTATAGAAGCAACG
9a5c            CAATGGCCAATCAGGAGTCACTTCAGAAAACCTCACTGACCACAGGGTATAGAAGCAACG
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AF130454        ACACCATCTAAAGAACTGCTTTAGGCCACTATTGAACAGAACTCTAAAATACTAGATAGA
Temecula1       ACACCATCTAAAGAACTGCTTTAGGCCACTATTGAACAGAACTCTAAAATACTAGATAGA
Ann-1           ACACCATCTAAAGAACCACTTTAGGCCACTATTGAACAGAACTCTAAAATACTAGATAGA
Dixon           ACACCATCTAAAGAACCACTTTAGGCCACTATTGAACAGAACTCTAAAATACTAGATAGA
9a5c            ACACCATCTAAAGAACCGCTTTAGGCCGCTATTGAACAGAACTCTAAAATACTAGATAGA
                **********************  ********* ********************************

AF130454        AATGAATCGACGGAATCTATGAATATCGCCATAGCAAAGAGGATTTACAAAAGCTTCCAC
AF130454        AATGAATCGACGGAATCTATGAATATCGCCATAGCAAAGAGGATTTACAAAAGCTTCCAC
Temecula1       AATGAATCGACGGAATCTATGAATATCGCCATAGCAAAGAGGATTTACAAAAGCTTCCAC
Ann-1           AATGAATCCACGGAACCTATGAACATCGCCATAGCAAAGAGGATTTATAAAAGCTTCCAC
Dixon           AATGAATCCACGGAACCTATGAACATCGCCATAGCAAAGAGGATTTATAAAAGCTTCCAC
9a5c            AATGAATCCACGGAACCTATGAACATCGCCATAGCAAAGAGGATTTATAAAAGCTTCCAC
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APPENDIX B

LOCATION OF 2594F AND 2594R PRIMER SITES FLANKING THE FX2594 LOCUS

WITHIN THE M12 GENOME

Yellow denotes forward primer 2594F (5′-GAAACTGGCACGGACCGCT-3′)

Red denotes reverse primer 2594R (5′-TCGTCGAACGGCTGGGAGCTAT-3′)

Aqua denotes sequence for gene FX2594

```
LOCUS       NC_010513    1800 bp    DNA    linear    BCT 24-MAR-2008
DEFINITION  Xylella fastidiosa M12, complete genome
ACCESSION   NC_010513 REGION: 1823701..1825500

ORIGIN
        1 ctacatttaa cccatcgcac tcacacgttt gctgcccctt gcaacatcgc ggcgacaatg
       61 acataccagt caaacagcac ccggcacaca acgcggccag cgatggcatt acatccccgg
      121 tcacagacct gcaccatcgc ggctaacggc acgacctgca acaacggttc ccagcagtgc
      181 cagcggctta acgcactgca gttacttcct gcctgcgaca gcgttaacgt cccatatgcc
      241 gtcacgcacc acgcgatggt cttggctggt tcagtcatcc acgtgagtcc cacatgacat
      301 ccgccgcgtg actgcagtcc cccttagcat ccggcaccct ggttgcacat cgaccttact
      361 agatatgtcg ccactgaaac tggcacggac cgctcttgcg atcaccgtag aacaacgcaa
      421 ccaatgactg atcaccgcat caacacgcca acgcacaacc gccccctacc atgacaagcg
      481 ggacaatcgc gcggctgcac catctcacgc acaaccgccg caacgagtac gggaattgtt
      541 tctaacgttt ttatggccat accgcccccc ttccggctgt ttagcggtcc gacatgcaga
      601 gcggtctcgc cgtagcccac cggcttcgca tagatacgcc tgcaaccgca ggccttctaa
      661 aaacttgatg agcttaatgc cttcgtcgct aatcgtttgc atgcggatgc tccaccacgc
      721 gaaaaaccgc cagaaggcgg tatgggccaa aaaaaatccc tctcgccact acaagaacag
      781 ataagcgcat tatgggcggc gctcagctcc gttcacagcg gcgcagcttc aagccatcga
      841 ctcgctcaac atggtaggta cattagccaa cggcttggcc acactgagcc tagaaggcga
      901 tgccgccacc actggcgcct ctcgctatta cggcaccaat gcgaacagcg taaaaggctg
      961 gcacccgctt cccacacgtg taacctgcta atcctcatac gttctctcta gccagcctct
     1021 gcccgcagta acgagcgtga cgccgtaggc cgcgaaaaat ggcaagcaga taccgttcat
     1081 tgggtatggt ttgatgaaga accgcccgaa gatgtctact tcgaaggcat cacccgcacc
     1141 aatcgcacgt ttgggcccgt ctttatgacg tttacgccat taaaaggcat gtcaaccgtc
     1201 gtacggcgct ttttgacaga agacgccgca gaccgtggct atataaaaga tgccgaacac
     1261 tattccgccg aagagtgcgc acgcatcatc gcaagctatc caccacgcag tgcccaccag
     1321 cggagcgccg aattgctgag tcctcagcgt ccaagcctgc ggcgcgcttc ttgtccaatg
     1381 cgcaccaaca ccgtgcggta gtttgtgcag cgcaatgcca acccaccaca cgaggacacc
     1441 acaatgcctg cacccaagcg cgccagcacc gacaccgtag ttcaccgaga gccgttccat
     1501 gacgtgagcg aagcgttgtt catggagaac ttctcggcac acggcaagaa gcccgaagac
     1561 tcgttgctcg ccagttgcta cgacattcgc tccaacgccg tgcagcagtg catggacctg
     1621 gtcaacagcg tgcggcgcgt ctacgccaac cccacgctca actccgtgca gcaggatatc
     1681 gagggtcggc aagctgctga agccccgcat gaccgagcgc atcaacggct tcgatgcttt
     1741 gcgccagcgc gtgctggagc gaagggatga ggtggcccac gagatcgacg tcaagctgcg
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APPENDIX C

REDESIGN OF PRIMERS TO THE REGION OF THE M12 GENOME ORIGINALLY TARGETED BY THE ALM1-ALM2 PRIMER PAIR

***Xylella fastidiosa* ALM locus**

Dr. Rufina Hernandez-Martinez (ruhernan@cicese.mx) sent the following unpublished 521-bp sequence showing the region of an almond leaf scorch (ALS) strain of *X. fastidiosa* that should amplify with ALM1 and ALM2 primers (Hernandez-Martinez et al. 2006. Plant Disease 90:1382-1388), with regions homologous to the primers in highlighted capital letters:

CTGCAGAAATTGGAAACTTCAGGtgggtgttcattgaagtgagcttcatggagcaagagact
tcatctgattagcgacttcggtccgatgcaagccttgcctgttgtatcccaagtgctatcca
cttcccacagctaagccaatatccccaggttgattctgtactccgttaaggtcacttggctt
gctttactatcggcatcgtcatctttcgagttcttttcaagactttcttagtgagaagcagc
tccaatcagtgggctagatagaagtcgttccatctccccccgtttacttctcggatctctac
cttttgttgtttttcaagtgtcattgggcgccacctttctgctgttttactctgtgtatgt
gtatttatttttttattgtgaatcgtgtcacttttttatcaagttctttttgtcttcctatg
attcggatctgcttagtttttgtttaatgcttctctgttgaggatcacaatggattgtgatc
gtctcTTCATAGATCAGCGTGTGGC

This sequence is 99.81% identical (520/521 bases) to nucleotides 188931 to 189450, located near one end of a large (1361 base pairs) intergenic region in the genome of M12 (NCBI NC_010513), a strain that causes ALS. The coding sequences flanking this region are predicted to encode a hypothetical protein (Xfasm12_0153) and a glycine cleavage H-protein (Xfasm12_0154). A BLASTX search with this sequence on October 29, 2010 revealed no open reading frames within the intergenic region that have significant hits in the NCBI non-redundant protein database.

Published ALM1 and ALM2 primers

As shown here, the homologous regions do not align perfectly with the primer sequences in the Hernandez-Martinez et al. (2006) publication.

ALM1: Shown: 5′CTGCAGAAATTGGAAACTTCAGG 3′

Published: 5′CTGCAGAAATTGGAAACTTCAG 3′

ALM2: Shown: 5′GCCACACGCTGATCTATGAA 3′

Published: 5′GCCACACG–TGATCTATGAA 3′

Modification of existing ALM primers and design of new ALM primers

ALMF2 (5′GCTGCAGAAATTGGAAACTTCAGG 3′) is the same as ALM1, except for the addition of one "G" nucleotide to both ends. As shown below, this enhances the mismatch with non-ALS strains at the 3′ end. The longer primer also should be more specific.

ALMR3 (5′CATGCCCACACCACCCAAT 3′) is a new primer designed so that the six bases at the 3′ end should uniquely hybridize to a target in ALS and citrus variegated chlorosis (CVC) strains. Only a few of the remaining bases are unique to these strains. Thus, the ALMF2-ALMR3 primer pair should amplify a 541-bp fragment from ALS strains like Dixon and M12. It also should amplify from CVC but not from Pierce's disease and oleander leaf scorch strains.

Location of new ALM primers

This multiple sequence alignment of the ALM target region shows the sequences to which the new ALMF2 and ALMR3 primers will hybridize. Note that these primers likely will also amplify this region from CVC strains like 9a5c.

```
M23_PD      ATGGTGGTCAGTAAGGACTCTGGAAAGGTCGCGTTGCAGAAATCGGCAACTTCTCCTGTG 886
Ann-1_OLS   ATGGTGGTCAGTAAGGACTCTGGAAAGGTCGCGTTGCAGAAATCGGCAACTTCTCTTGTG 891
Teme_PD     ATGGTGGTCAGTAAGGACTCTGGAAAGGTCGCGTTGCAGAAATCGGCAACTTCTCCTGTG 854
M12_ALS     ATGGTGGTCTGTAAAGACGCTGGGAAGGTTGCGCTGCAGAAATTGGAAACTTCAGG--TG 955
Dixon_ALS   ATGGTGGTCTGTAAAGACGCTGGGAAGGTTGCGCTGCAGAAATTGGAAACTTCAGG--TG 874
9a5c_CVC    ATGGTGGTCTGTAAAGACGCTGGGAAGGTCGCGCTGCAGAAATTGGCAGCTTCTGG--TG 846
            ********* **** *** **** ***** *** ********* ** * ****    ***
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M23_PD      GGTGCTCATTGAAGTGAGCTTCAATGAGCAGTTGACTTCATCTGATTGGCGACTATGGTC 946
Ann-1_OLS   GGTGCTCATTGAAGTGAGCTTCACGGAGCAGTTGACTTCATCTGATTGGCGACTATGGTC 951
Teme_PD     GGTGCTCATTGAAGTGAGCTTCAATGAGCAGTTGACTTCATCTGATTGGCGACTATGGTC 914
M12_ALS     GGTGTTCATTGAAGTGAGCTTCATGGAGCAAGAGACTTCATCTGATTAGCGACTTCGGTC 1015
Dixon_ALS   GGTGTTCATTGAAGTGAGCTTCATGGAGCAAGAGACTTCATCTGATTAGCGACTTCGGTC 934
9a5c_CVC    GGTGTTCATTGAAGTGAGCTTCATGGAGCAAGAGACTTCATCTGATTGGCGACTCCGGTC 906
            **** ******************  *****   ************** ******  ****

M23_PD      CGATGCAAGCTTTGACTGTTGTATCGCAAGTGATATCCACTTCTCACAGCTAAGCCAATA 1006
Ann-1_OLS   CGATGCAAGCTTTGACTGTTGTATCGCAAGTGATATCCACTTCTCACAGCTAAGCCAATA 1011
Teme_PD     CGATGCAAGCTTTGACTGTTGTATCGCAAGTGATATCCACTTCTCACAGCTAAGCCAATA 974
M12_ALS     CGATGCAAGCCTTGCCTGTTGTATCCCAAGTGCTATCCACTTCCCACAGCTAAGCCAATA 1075
Dixon_ALS   CGATGCAAGCCTTGCCTGTTGTATCCCAAGTGCTATCCACTTCCCACAGCTAAGCCAATA 994
9a5c_CVC    CGATGCAAGCCTTGTCTGTTGTATCGCAAGTGCTATTCACTTCCCACAGCTAAGCCAATA 966
            ********** *** ********** ****** *** ****** ****************

M23_PD      TTCCCAGGTTAATTCTGTACTGCATTAATGTCACTTGGCTTGCTTTACTATCGGAATCGC 1066
Ann-1_OLS   TTCCCAGGTTAATTCTGTACTGCATTAATGTCACTTGGCTTGCTTTACTATCGGAATCGC 1071
Teme_PD     TTCCCAGGTTAATTCTGTACTGCATTAATGTCACTTGGCTTGCTTTACTATCGGAATCGC 1034
M12_ALS     TCCCCAGGTTGATTCTGTACTCCGTTAAGGTCACTTGGCTTGCTTTACTATCGGCATCGT 1135
Dixon_ALS   TCCCCAGGTTGATTCTGTACTCCGTTAAGGTCACTTGGCTTGCTTTACTATCGGCATCGT 1054
9a5c_CVC    TCCCCAGGTTAATTCTGTACTGCGTTAAGGTCACTTGGCTTGCTTTACTATCGGCATCAC 1026
            * ******** ********** * **** ************************ ***

M23_PD      CATCTTTCGAGTTCTTTTCAAGACTCTCTTATAGTGGGAAGCCGCTTCAATCAGCGGGTT 1126
Ann-1_OLS   CATCTTTCGAGTTCTTTTCAAGACTCTCTTATAGTGGGAAGCCGCTTCAATCAGCGGGTT 1131
Teme_PD     CATCTTTCGAGTTCTTTTCAAGACTCTCTTATAGTGGGAAGCCGCTTCAATCAGCGGGTT 1094
M12_ALS     CATCTTTCGAGTTCTTTTCAAGACTTTCTTA--GTGAGAAGCAGCTCCAATCAGTGGGCT 1193
Dixon_ALS   CATCTTTCGAGTTCTTTTCAAGACTTTCTTA--GTGAGAAGCAGCTCCAATCAGTGGGCT 1112
9a5c_CVC    CATCTTTCGAGTTCTTTTCAAGACTTTCTTA--ATGGGAAGCCGCTCCAATCAGTGGGCT 1084
            ************************** *****   ** ***** *** ******* *** *

M23_PD      AGATAGAAATCGTCCCATCTCACCCTGGATTACTTTTCGGATATCTACCTCTTGTTGTTT 1186
Ann-1_OLS   AGATAGAAATCGTTCCATCTCACCCTTGATTACTTTTCGGATATCTACCTCTTGTTGTTT 1191
Teme_PD     AGATAGAAATCGTCCCATCTCACCCTGGATTACTTTTCGGATATCTACCTCTTGTTGTTT 1154
M12_ALS     AGATAGAAGTCGTTCCATCTC-CCCCCGTTTACTTCTCGGATCTCTACCTTTTGTTGTTT 1252
Dixon_ALS   AGATAGAAGTCGTTCCATCTC-CCCCCGTTTACTTCTCGGATCTCTACCTTTTGTTGTTT 1171
9a5c_CVC    AGATAGAAGCCGTTCCATCTCACCCCCGCTTACTTCTCGGATCTCTACCTCTTGTTGTTT 1144
            ********  *** ******* ***  * ****** ****** ******* *********

M23_PD      TGCAAGTGTCATTGGGCGCCACCTTT-TCTGCTGTTTTACTCTGTGTATTTGTATTT--- 1242
Ann-1_OLS   TGCAAGTGTCATTGGGCGCCACCTTT-TCTGCTGTTTTACTCTGTGTATTTGTATTT--- 1247
Teme_PD     TGCAAGTGTCATTGGGCGCCACCTTT-TCTGCTGTTTTACTCTGTGTATTTGTATTT--- 1210
M12_ALS     TTCAAGTGTCATTGGGCGCCACCTTT-TCTGCTGTTTTACTCTGTGTATGTGTATTTAT- 1310
Dixon_ALS   TTCAAGTGTCATTGGGCGCCACCTTT-TCTGCTGTTTTACTCTGTGTATGTGTATTTAT- 1229
9a5c_CVC    TGCAAGTCTTATTGGGTGCCACCTTTATCTGCTGTTTTACTCTGTGTATGTGTATTTATA 1204
            * ***** * ****** ********* ********************** *******

M23_PD      -TCTTACCTGTGGATCGTGTCACTTTTTC-TCAGGTTCTTTTGGGCCTACCTATGATTCG 1300
Ann-1_OLS   -TCTTACCTGTGGATCGTGTCACTTTTTC-TCAGGTTCTTTTGGGCCTACCTATGATTCG 1305
Teme_PD     -TCTTACCTGTGGATCGTGTCACTTTTTC-TCAGGTTCTTTTGGGCCTACCTATGATTCG 1268
M12_ALS     TTTTT-ATTGTGAATCGTGTCACTTTTTTTATCAAGTTCTTTTTGTCTT-CCTATGATTCG 1368
Dixon_ALS   TTTTTTATTGTGAATCGTGTCACTTTTTTTATCAAGTTCTTTTTGTCTT-CCTATGATTCG 1288
9a5c_CVC    TTTTTTATTGTGAATAGCGTCACTTTTTT--CAGATTCTTTTGGGCTT-TCTATGATTCG 1261
             * **   **** ** * **********   **  ******* * * *  **********

M23_PD      GATCTGCTTA-GTTTTTGTTTAATGCTTCTCTGTTGAGGATTACGATGGATTGTGATCGT 1359
Ann-1_OLS   GATCTGCTTA-GTTTTTGTTTAATGCTTCTCTGTTGAGGATTACGATGGATTGTGATCGT 1364
Teme_PD     GATCTGCTTA-GTTTTTGTTTAATGCTTCTCTGTTGAGGATTACGATGGATTGTGATCGT 1327
M12_ALS     GATCTGCTTA-GTTTTTGTTTAATGCTTCTCTGTTGAGGATCACAATGGATTGTGATCGT 1427
Dixon_ALS   GATCTGCTTA-GTTTTTGTTTAATGCTTCTCTGTTGAGGATCACAATGGATTGTGATCGT 1347
9a5c_CVC    GATCTCCTTAAGTTTTTGTTTAATGCTTCTCTGTTGAGGATCACAATGGATTGTGATCGT 1321
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M23_PD      CTCTCCATAGATCAGTGTGTGGGTGGCTATTGGGTGGGGGATGGATGAAAG-----ATTC 1414
Ann-1_OLS   CTCTCCATAGATCAGTGTGTGGGTGGCTATTGGGTGGGGGATGGATGAAAG-----ATTC 1419
Teme_PD     CTCTCCATAGATCAGTGTGTGGGTGGCTATTGGGTGGGGGATGGATGAAAG-----ATTC 1382
M12_ALS     CTCTTCATAGATCAGCGTGTGGCTATTGGGTGGTGTGGGCATGGAGGAAAGGAAAGATTC 1487
Dixon_ALS   CTCTTCATAGATCAGCGTGTGGCTATTGGGTGGTGTGGGCATGGAGGAAAGGAAAGATTC 1407
9a5c_CVC    CTCTCCATAGATCAGCGTGTGGCTATTGGGTGGTG-GGGCATGGAGGAAAGGAAAGATTC 1380
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APPENDIX D

DESIGN OF NEW PRIMERS TO THE REGION OF THE M12 GENOME ORIGINALLY TARGETED BY THE XF1968-L/XF1968-R PRIMER PAIR

***Xylella fastidiosa* XF1968 locus**

This locus, originally defined in citrus variegated chlorosis (CVC) strain 9a5c, encodes a putative methyltransferase of the restriction methylation system (Simpson, A.J.G. et al. 2000. Nature 406:151-157). This putative gene is on the complementing strand between nucleotides 11877948 and 1879552 of the 9a5c genomic sequence (NCBI NC_002488). The 1605 nucleotides should encode a protein with 535 amino acids. The sequence of this gene is 95% identical in strain M12 that causes almond leaf scorch (ALS) and 96% identical in strain Ann-1 that causes oleander leaf scorch (OLS), but it is only 80% identical in Pierce's disease (PD) strains Temecula 1 and M23. Most of the sequence variation seen in the PD strains is in the 5′ half of the coding sequence.

Published XF1968-L and XF1968-R primers

These primers were described by Hernandez-Martinez et al. (2006) (Plant Disease 90:1382-1388.). XF1968-L hybridizes to a region of the CVC strain about 780 base pairs (bp) downstream from the start codon for the XF1968 open reading frame. Although the analogous region is very different in the genomes of PD strains Temecula 1 and M23, it is identical in the genomes of ALS strains M12 and Dixon, and the OLS strain Ann-1. XF1968-R hybridizes to a region of the 9a5c open reading frame about 1400 bp downstream from the start codon. However, this reverse primer is non-specific, because the analogous region is completely

conserved in M12, Temecula 1, M23, and Ann-1 (this region is missing from the incomplete Dixon shotgun sequence).

Design of new XF1968 primers

A multiple sequence alignment of the 5′ end of the XF1968 locus (see below) showed that it would be easy to design primers that do not hybridize to PD strains, but virtually impossible to design primers specific to ALS strains due to the high homology of this region in CVC and OLS strains. XF1968F3 (5′ CGCACCGTTACCGCCATTC 3′) is a new primer designed to hybridize to all strains except M12. This is possible because this primer is targeted to a region approximately 340 bp downstream from the start codon where there is a unique 24-bp deletion in the coding sequence from M12. XF1968R3 (5′ CCTGATATTGGGCGCATCG 3′) is a new primer that should hybridize approximately 690 bp downstream from the start codon. Almost all the bases in the 3′ half of this primer are unique to non-PD strains. Thus, the XF1968F3-XF1968R3 primer pair should amplify a 371-bp fragment from ALS strains like Dixon but not ALS strains like M12. It also should amplify from CVC and OLS but not PD strains (similar to the published primers).

Location of new XF1968 primers

This multiple sequence alignment of the relevant region of the XF1968 locus shows the sequences to which the new XF1968F3 and XF1968R3 primers will hybridize.

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19a5c_CVC         TTTGTCTACAACGACTGCTTCATTGACAAGACGCACCGTTACCGCCATTCACTGTGGCTG 360
Ann-1_OLS       TTTGTCTACAACGACTGTTTCATTGACAAGACGCACCGTTACCGCCATTCACTGTGGCTG 360
M12_ALS         TTTGTCTACAACGACTGCTTCATTGACAAG----------------------TGGCTG 336
Dixon*_ALS      TTTGTCTACAACGACTGCTTCGTCGACAAGACGCACCGTTACCGCCATTCACTGTGGCTG 360
Teme_PD         TTTGTCTACAACGACTGTTTCATTGACAAGACGCACCGTTACCGCCATTCACTGTGGCTG 360
M23_PD          TTTGTCTACAACGACTGTTTCATTGACAAGACGCACCGTTACCGCCATTCACTGTGGCTG 360
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9a5c_CVC        GAATTCATGTACCGCCGATTGCAACTCGCCAAGGAACTGCTTGCCGATGATGGCGTCATT 420
Ann-1_OLS       GAATTCATCTATCGCCGTTTGCAGCTGGCCAAGGAACTTCTTGCCGATGATGGCGTGATT 420
M12_ALS         GAATTCATCTATCGCCGTTTGCAGTTGGCCAAGGAACTTCTGCCCGATGATGGCGTCATT 396
Dixon*_ALS      GAATTCATGTACCGCCGATTGCAACTCGCCAAGGAACTGCTTGCCGATGATGGCGTTATT 420
Teme_PD         GAATTCATCTATCGCCGTTTGCAGCTGGCCAAGGAACTTCTTGCCGATGATGGCGTGATT 420
M23_PD          GAATTCATCTATCGCCGTTTGCAGCTGGCCAAGGAACTTCTTGCCGATGATGGCGTGATT 420
                ******** ** ***** *****  * ********** **  ************ ***

9a5c_CVC        TTTGTCAGCATTGATGACAACGAGTTGTTCCGCTTGGGGATGTTGATGGACCGGGTGTTT 480
Ann-1_OLS       TTTGTCAGTATTGATGACAACGAGTTGTTCCGCTTGGGGATGTTGATGGACCGGGTGTTT 480
M12_ALS         TTTGTCAGCATTGATGACAACGAGTTGTTCCGCTTGGGAATGTTGATGGATCGGGTGTTT 456
Dixon*_ALS      TCTGTCAGCATTGATGACAACGAGTTGTTCCGCTTGGGGATGTTGATGGACCGGGTGTTT 480
Teme_PD         TTTGTCAGTATTGATGACAACGAATTATTCCGCTTGGGGATGTTGATGGACCGGGTGTTT 480
M23_PD          TTTGTCAGTATTGATGACAACGAATTATTCCGCTTGGGGATGTTGATGGACCGGGTGTTT 480
                * ****** ************** ** *********** *********** *********

9a5c_CVC        GGTGAGCAGAACTTCGTTGCGAATTTTATTTGGAATCACCGGAAATCAAGCCAAAACGAT 540
Ann-1_OLS       GGTGAGCAGAACTTCGTTGCGAATTTTATTTGGAATCACCGGAAATCAAGCCAAAACGAT 540
M12_ALS         GGTGAGCAGAACTTTGTTGCGAACTTTATCTGGAATCACCGGAAATCAAGCCAAAACGAT 516
Dixon*_ALS      GGTGAGCAGAACTTCGTTGCGAATTTTATTTGGAATCACCGGAAATCAAGCCAAAACGAT 540
Teme_PD         GGACATGATCATTTCATCGCCAATGTAATTTGG---CAGAAGATTTTTTCTCCAAAGAAT 537
M23_PD          GGACATGATCATTTCATCGCCAATGTAATTTGG---CAGAAGATTTTTTCTCCAAAGAAT 537
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9a5c_CVC        ACTG----ATGTGTCATTGGCA--CACAACTACACACTGTGCTATGCCCGGACTCGTGAT 594
Ann-1_OLS       ACTG----ATGTGTCATTGGCG--CACAACTACACACTGTGCTACGCCAGGACTCGTGAT 594
M12_ALS         ACTG----ATGTGTCATTGGCA--CACAACTACACACTGTGCTATGCACGGACTCGTGAT 570
Dixon*_ALS      ACTG----ATGTGTCATTGGCG--CACAACTACACACTGTGCTACGCCCGGACTCGTGAT 594
Teme_PD         ACAGCTCAACATTTTTCTGATGATCACGAATACATCCTCGTTTTTGCAAGAAACCGTACT 597
M23_PD          ACAGCTCAACATTTTTCTGATGATCACGAATACATCCTCGTTTTTGCAAGAAACCGTACT 597
                ** *    *  * *   **     *** * ****  **    *  **  * *  ***  *

9a5c_CVC        CGCTTCTCCCTCAATCCATTGCC---TGTTGATGCC---GATAA---ATTCAATAACACT 645
Ann-1_OLS       CGCTTCTCCCTCAATCCATTGCC---TGTTGATGCC---GATAA---ATTCAATAACACT 645
M12_ALS         CGCTTCTCCCTCAATCCATTGCC---TGTTGATGCC---GATAA---ATTCAATAACACT 621
Dixon*_ALS      CGCTTCTCCCTCAATCCATTGCC---TGTTGATGCC---GATAA---ATTCAATAACACT 645
Teme_PD         TTATGGAGATCGAATCCATTACCGCGTAGCGAGACGCAGGATAAGGCATACAAAAACCCA 657
M23_PD          TTATGGAGATCGAATCCATTACCGCGTAGCGAGACGCAGGATAAGGCATACAAAAACCCA 657
                   *        ******** **   *   **  *    *****   ** *** *** *

9a5c_CVC        GATGGTGACCAGAGAGGTCCTTGGGTTGCCGATCCTTTCGATGCGCCCAATATCAGGAAG 705
Ann-1_OLS       GATGGTGACCAGAGAGGTCCTTGGGTTGCCGATCCTTTCGATGCGCCCAATATCAGGAAG 705
M12_ALS         GATGGTGACCAGAGAGGTCCTTGGGTTGCTGATCCTTTCGATGCGCCCAATATCAGGAAG 681
Dixon*_ALS      GATGGTGACCAGAGAGGTCCTTGGGTTGCTGATCCTTTCGATGCGCCCAATATCAGGAAG 705
Teme_PD         GACAACGATCCGCGTGGCCCTTGGACTTCTGGCGATTTATCGGCAAGGAATTTCTACAGT 717
M23_PD          GACAACGATCCGCGTGGCCCTTGGACTTCTGGCGATTTATCGGCAAGGAATTTCTACAGT 717
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APPENDIX E

PROTOCOL FOR TOTAL GENOMIC DNA EXTRACTION FROM SOUTHERN HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM* INTERSPECIFIC HYBRIDS)

1. Select 3 leaves symptomatic for *Xylella fastidiosa* from different stems from the same blueberry shrub (pooled sample).
2. Use a flame-sterilized razor blade and half of a sterile Petri dish to slice petioles ~ 1mm thick. Place sliced petioles into a 1.5 ml microcentrifuge tube containing 300 µl sterile TE buffer (pH 8.0). Allow sliced petioles to soak in the TE at room temperature while preparing other samples.
3. Use autoclaved wood applicator to macerate sliced petioles in TE in tube. Liquid will become pigmented from macerate.
4. Add another 1200 µl sterile TE buffer (pH 8.0) to tube.
5. Vortex mix tube 10s, making sure macerate does not collect in the tip. Place tube upright in ice 30 min to settle macerate and allow any bacterial cells of *X. fastidiosa* to float out.
6. Do not centrifuge tube. Use P1000 and P200 pipettors (the latter set at 100 µl) to transfer all of the liquid to a new 1.5 ml microcentrifuge tube. Avoid sucking up macerate. Place new tube on ice while transferring liquid of other samples.
7. Centrifuge new tube at full speed (~ 14,000 rpm) for 10 min to pellet any bacterial cells and fine plant debris.
8. Use P1000 pipettor to remove all but ~ 50 µl supernatant from tube. Discard supernatant.

a. Optional: Remove all but ~ 100 µl supernatant from tube. Resuspend cell/debris pellet by dragging tube on a tube rack. Add 1 ml sterile TE buffer (pH 8.0) to tube and vortex mix 10s. Centrifuge tube at full speed (~ 14,000 rpm) for 10 min to re-pellet any bacterial cells and fine plant debris. Use a P1000 pipettor to remove all but ~ 50 µl supernatant from tube. Discard supernatant.

9. Resuspend cell/debris pellet by dragging tube on a tube rack.

10. Proceed with total genomic DNA extraction using the Qiagen Dneasy® Plant Mini Kit, with the following modifications:

a. Beginning with Step 2 of the bench protocol, add 20 mg of PVPP (not provided in kit) to tube along with 400 µl of Buffer AP1 (provided in kit). Do not add 4 µl of RNase A (100 mg/ml; provided in kit) to tube at this point.

b. Vortex mix tube 10s and then incubate in a 37°C water bath 30 min to lyse cells and allow PVPP to bind with polyphenols.

c. Centrifuge tube at full speed (~ 14,000 rpm) for 5 min to pellet cell debris.

d. Use a P200 pipettor set at 100 µl to transfer all of the lysate to a new 1.5 ml microcentrifuge tube. First transfer ~ 300 ul of the lysate and then slide the tip of the pipettor down to the bottom of the tube to get the remaining lysate, which may be milky colored and foamy. Any residual PVPP in the lysate will be removed during a subsequent step to precipitate proteins and polysaccharides.

e. Add 4 µl of RNase A (provided in kit) to tube and vortex mix 10s. Incubate tube in a 65°C heating block 10 min. Invert mix tube 2-3X during incubation.

f. Cool tube to room temperature (~ 5 min) and then add 130 µl of Buffer AP2 (provided in kit). Flick tube or vortex to mix.

g. Place tube upright in ice 5 min to precipitate residual PVPP, proteins and polysaccharides. Centrifuge tube at full speed (~ 14,000 rpm) for 5 min. Do not return tube to ice at this point. Use a P200 pipettor set at 100 µl to transfer all the lysate to a new 1.5 ml microcentrifuge tube.

h. Place new tube upright in ice 5 min to continue precipitation. Repeat centrifugation. Transfer all the lysate to a QIAshredder Mini Spin Column in a 2 ml collection tube (provided in kit). Centrifuge column in collection tube at full speed (~ 14,000 rpm) for 4 min.

i. Transfer flow through to a new 1.5 ml microcentrifuge tube without disturbing pellet. Recovered lysate ~ 450 µl.

j. Add 1.5 volumes (~ 675 µl) of Buffer AP3/E (provided in kit) to tube and mix by pipetting 15X. Use a mini personal spinner to briefly spin down tube.

k. Transfer 650 µl of mixture to a DNeasy Mini Spin Column in a 2 ml collection tube (provided in kit). Centrifuge spin column in collection tube at 8,000 rpm for 1 min. Discard flow through. Reuse the collection tube. Repeat step with remaining mixture.

l. Place Dneasy Mini Spin Column into a new 2 ml collection tube (provided in kit). Add 500 µl of Buffer AW (provided in kit) to spin column. Centrifuge spin column in new collection tube at 8,000 rpm for 1 min. Discard flow through. Reuse the collection tube.

m. Add another 500 µl of Buffer AW to spin column. Centrifuge spin column in collection tube at full speed (~14,000 rpm) for 4 min. Discard flow through. Reuse the collection tube. Repeat centrifugation to ensure no residual ethanol is carried over during elution.

n. Transfer the Dneasy Mini Spin Column to a new 1.5 ml microcentrifuge tube. Pipette 100 µl of Buffer AE (provided in kit) directly onto spin column membrane to elute total genomic DNA. Incubate column in microcentrifuge tube at room temperature 5 min and then centrifuge column in tube at 8,000 rpm for 1 min. Repeat step with new 1.5 ml microcentrifuge tube.

o. Store elutions at -20°C.

APPENDIX F

PROTOCOL FOR TOTAL GENOMIC DNA EXTRACTION FROM GLASSY-WINGED SHARPSHOOTERS [*HOMALODISCA VITRIPENNIS* (GERMAR)]

1. Use a flame-sterilized razor blade and half of a sterile Petri dish to dissect the head (behind the frons) from the body of 1 glassy-winged sharpshooter. Place head in a 1.5 ml microcentrifuge tube containing 200 µl sterile TE buffer (pH 8.0). Allow head to soak in the TE at room temperature while preparing other samples.
2. Use autoclaved wood applicator to macerate head in TE in tube. Liquid will become pigmented from macerate.
3. Add another 1300 µl sterile TE buffer (pH 8.0) to tube.
4. Vortex mix tube 10s, making sure macerate does not collect in the tip. Place tube upright in ice 30 min to settle macerate and allow any bacterial cells of *Xylella fastidiosa* to float out.
5. Do not centrifuge tube. Use P1000 and P200 pipettors (the latter set at 100 µl) to transfer all of the liquid to a new 1.5 ml microcentrifuge tube. Avoid sucking up macerate. Place new tube on ice while transferring liquid of other samples.
6. Centrifuge new tube at full speed (~ 14,000 rpm) for 10 min to pellet any bacterial cells and fine insect debris.
7. Use a P1000 pipettor to remove all but ~ 100 µl supernatant from tube. Discard supernatant. Resuspend cell/debris pellet by dragging tube on a tube rack. Add 1 ml sterile TE buffer (pH 8.0) to tube and vortex mix 10s. Centrifuge tube at full speed (~ 14,000 rpm) for 10 min to

re-pellet any bacterial cells and fine insect debris. Use a P1000 pipettor to remove all but ~ 50 µl supernatant from tube. Discard supernatant.

8. Resuspend cell/debris pellet by dragging tube on a tube rack.
9. Proceed with total genomic DNA extraction using the Qiagen Dneasy® Blood & Tissue Kit, with the following modifications:
    a. Beginning with Step 2 of the bench protocol, add 180 µl of Buffer ATL (provided in kit) to tube along with 20 µl of proteinase K (provided in kit).
    b. Pulse vortex mix tube 10s and then incubate in a 56°C heating block 75 min to lyse cells. Pulse vortex mix tube 10s every 10 min during lysis. (Note: 1 h is sufficient for cell lysis. However, another 15 min is added to account for mixing time during lysis.)
    c. Cool tube to room temperature (~ 5 min) and then add 4 µl of RNase A (10mg/ml). Pulse vortex mix tube 10s and then incubate at room temperature 5 min to digest RNA.
    d. Pulse vortex mix tube 10s and then place upright in ice 5 min. Centrifuge tube at full speed (~ 14,000 rpm) for 5 min. Do not return tube to ice at this point. Use a P200 pipettor set at 100 µl to transfer all the lysate to a new 1.5 ml microcentrifuge tube.
    e. Repeat incubation on ice and centrifugation. Transfer all the lysate to a new 1.5 ml microcentrifuge tube. (This and the previous step are critical to removing potential PCR inhibitors from residual insect tissue that has been lysed. This must be done prior to precipitation of total genomic DNA.)
    f. To precipitate total genomic DNA, add 200 µl of Buffer AL (provided in kit) to tube. Vortex mix 15s. Add 200 µl ethanol (96-100%). Vortex mix 15s. Use a mini personal spinner to briefly spin down tube.

g. Transfer all of the mixture to a DNeasy Mini Spin Column in a 2 ml collection tube (provided in kit). Centrifuge spin column in collection tube at 8,000 rpm for 1 min. Discard flow through and collection tube.

h. Place Dneasy Mini Spin Column into a new 2 ml collection tube (provided in kit). Add 500 µl of Buffer AW1 (provided in kit) to spin column. Centrifuge spin column in new collection tube at 8,000 rpm for 1 min. Discard flow through and collection tube.

i. Place Dneasy Mini Spin Column into a new 2 ml collection tube (provided in kit). Add 500 µl of Buffer AW2 (provided in kit) to spin column. Centrifuge spin column in new collection tube at full speed (~ 14,000 rpm) for 5 min. Discard flow through. Reuse collection tube. Repeat centrifugation to ensure no residual ethanol is carried over during elution.

j. Transfer the Dneasy Mini Spin Column to a new 1.5 ml microcentrifuge tube. Pipette 150 µl of Buffer AE (provided in kit) directly onto spin column membrane to elute total genomic DNA. Incubate column in microcentrifuge tube at room temperature 5 min and then centrifuge column in tube at 8,000 rpm for 1 min. Repeat step with new 1.5 ml microcentrifuge tube.

k. Store elutions at -20°C.