

## ABSTRACT

Title of Document: EPIDEMIOLOGY AND POPULATION STRUCTURE OF *XYLELLA FASTIDIOSA*, THE CAUSAL AGENT OF BACTERIAL LEAF SCORCH, AMONG URBAN TREES IN THE DISTRICT OF COLUMBIA

Jordan L. Harris, Master of Science, 2014

Directed by: Dr. Yilmaz Balci, Department of Plant Sciences and Landscape Architecture

A survey of urban trees affected by bacterial leaf scorch caused by *Xylella fastidiosa* was conducted in the District of Columbia during 2012 and 2013. Disease occurred most frequently with *Quercus palustris*, *Q. rubra*, *Ulmus americana*, and *Platanus occidentalis*. Eight other symptomatic and five asymptomatic tree species were found infected. The bacterium was also detected on asymptomatic portion of seven tree species. The occurrence of crown dieback was found significantly associated with *X. fastidiosa*-infection on *Q. palustris*, *Q. rubra*, *U. americana*, and *P. occidentalis*. A multi-locus sequence typing analysis using 10 housekeeping loci for *X. fastidiosa* revealed five clonal strains among the urban trees. These strains were host specific, with only one clone being associated with members of the red oak family, American elm, American sycamore, and two clones being associated with mulberry. Long-term management strategies aimed at mitigating the occurrence of bacterial leaf scorch disease are discussed.

EPIDEMIOLOGY AND POPULATION STRUCTURE OF *XYLELLA FASTIDIOSA*,  
THE CAUSAL AGENT OF BACTERIAL LEAF SCORCH, AMONG URBAN TREES  
IN THE DISTRICT OF COLUMBIA

By

Jordan Lee Harris

Thesis submitted to the Faculty of the Graduate School of the  
University of Maryland, College Park, in partial fulfillment  
of the requirements for the degree of  
Master of Science  
2014

Advisory committee:

Dr. Yilmaz Balci, Chair

Dr. Michael Cummings

Dr. Shirley Micallef

© Copyright by

Jordan Lee Harris

2014

## Table of Contents

<b>Table of Contents</b>	<b>ii</b>
<b>List of Tables</b>	<b>iv</b>
<b>List of Figures</b>	<b>v</b>
<b>Chapter 1: Literature Review</b>	<b>1</b>
1.0 History	1
1.01 First Discovery	1
1.02 A Viral Pathogen	2
1.03 A Rickettsia-like Bacteria (RLB) / A Xylem-Limited Bacteria (XLB)	3
1.04 Demonstration of Koch's Postulates and Taxonomic Classification	4
1.05 Genome Sequence of <i>X. fastidiosa</i>	5
1.06 <i>X. fastidiosa</i> subspecies	5
1.1 Pathology	8
1.1.1 Disease and Economic Loss Caused by <i>X. fastidiosa</i>	8
1.1.2 Morphology of <i>X. fastidiosa</i>	9
1.1.3 Transmission	10
1.1.4 Host range	10
1.1.5 Geographic Distribution and Origin of <i>X. fastidiosa</i>	11
1.2 Variation Observed Among Strains of <i>Xylella fastidiosa</i>	12
1.2.1 Host Specificity	12
1.2.2 Genomic Variation	13
1.3 Overview of Relevance to the Urban Forest	14
1.3.1 Bacterial Leaf Scorch in Urban Ecosystems	14
1.3.2 Epidemiology of <i>X. fastidiosa</i> in Landscape Trees	16
1.3.3 Current Management Options for Amenity Trees	17
1.4 Research Objectives	18
1.4.1 Justification	18
1.4.2 Objective 1: Survey of <i>X. fastidiosa</i> Infection in the District of Columbia	20
1.4.3 Objective 2: Population Assessment	21
<b>Chapter 2: Bacterial Leaf Scorch in the District of Columbia: Distribution, Host Range, and Presence of <i>Xylella fastidiosa</i> Among Urban Trees</b>	<b>23</b>
2.0 Abstract	23
2.1 Introduction	24
2.2 Materials and Methods	25
2.2.1 Sample Collection	25
2.2.2 Detection of <i>X. fastidiosa</i> Using ELISA	30
2.2.3 DNA Extraction and PCR Analysis	31
2.2.4 Statistical Analyses	32
2.3 Results	32
2.3.1 Occurrence of BLS in the District	32
2.3.2 Detection of <i>X. fastidiosa</i> in the Asymptomatic Foliage of Infected Trees	35
2.3.3 Detection of <i>X. fastidiosa</i> in Asymptomatic Trees	36
2.3.4 PCR Results and Strain Characterization	36
2.3.5 Association of ELISA values with DBH (Diameter at Breast Height), Tree Height, Crown Dieback, and Leaf Scorching	38
Discussion	40

<b>Chapter 3: Population structure of the bacterial pathogen <i>Xylella fastidiosa</i> among street trees in Washington D.C</b>	<b>46</b>
3.0 Abstract	46
3.1 Introduction	47
3.2 Materials and Methods	50
3.2.1 Selection of <i>X. fastidiosa</i> Infected Trees	50
3.2.2 DNA extraction, Amplification and Sequencing	55
3.3.3 Sequence Analysis and Submission	57
3.3.4 Genetic Relatedness Analysis	58
3.4 Results	58
3.5 Discussion	70
<b>Chapter 4: Application of Knowledge and Broader Impacts</b>	<b>74</b>
4.0 Project Summary	74
4.0.1 Summary of field sampling and BLS incidence among amenity trees	74
4.0.2 Summary of Multi-locus Sequence Analysis	75
4.1 Personal Views	76
4.1.1 Outlook	76
4.1.2 Future Direction	79
4.2 Concluding Remarks for Managing BLS in an Urban Environment.	80
<b>Bibliography</b>	<b>82</b>

## List of Tables

Table 1: Number of <i>X. fastidiosa</i> -infected urban trees surveyed in the District of Columbia in 2011 and 2012.....	28
Table 2: Analysis of variance of mean ELISA values and standard deviation (Stdv) of asymptomatic and symptomatic foliage of <i>X. fastidiosa</i> -infected trees. ....	35
Table 3:Subspecies and strain genotypes of <i>Xylella fastidiosa</i> from environmental samples obtained from petioles of infected urban tree species. ....	37
Table 4: Contingency table: Crown dieback of <i>X. fastidiosa</i> -infected and non-infected trees. Infected trees were determined with ELISA. ....	38
Table 5: Location and site information of all trees used in the multi-locus sequencing analysis.....	52
Table 6: Samples collected for the MLST analysis.. ....	54
Table 7: Primers and gene regions selected for the multi-locus analysis. ....	56
Table 8: Allelic profiles of each sample in the analysis. ....	59
Table 9: Consensus of <i>Xylella fastidiosa</i> sequence types from tree petiole samples. ....	64
Table 10: All polymorphic sites found for each allele in the analysis.....	66

## List of Figures

Figure 1: A, Satellite image showing an oak tree with a discolored canopy that is selected for sampling; and B, Study sites with positive detection of <i>X. fastidiosa</i> in seven of the eight District wards. ....	27
Figure 2: Bacterial leaf scorch symptoms of amenity trees in the District of Columbia and in neighboring Maryland municipalities. ....	33
Figure 3: Logistic fit of ELISA values in correlation with scorch rating on the most commonly sampled urban trees infected with <i>X. fastidiosa</i> . ....	39
Figure 4: Location of sample collection sites in Washington D.C. ....	51
Figure 5: Distance tree of sequence types found infecting urban trees. ....	65

# Chapter 1: Literature Review

## 1.0 History

### 1.01 First Discovery

Agricultural diseases associated with the xylem-limited bacterium *Xylella fastidiosa* (127) have been recognized for over a century. In the late 1800's, Newton B. Pierce studied a disease known at the time as California vine disease (103). This disease, which resulted with the scorch and decline of grapevine, could not be isolated, cultured, nor identified, although Pierce suspected that a "minute microorganism" was involved. The disease was eventually given the name Pierce's disease of grapevine, named after Pierce himself.

While California vine disease was investigated on the west coast by Pierce, a peach tree dwarfing disease, known as phony peach disease, was first detected in Marshallville, Georgia in 1885 (64). Although geographically separated, Pierce's disease of grapevine and phony peach diseases shared a common causal agent. At the time of first detection, the scarce occurrence of stunted peach trees did not warrant concern. Although the peach tree itself possessed compact dense growth that looked healthier, the fruit size was considerably smaller, and branch dieback was evident after a period of five years. By 1915, the disease became such a problem that a prominent peach tree owner by the name of J.H. Hale requested aid from the United States department of agriculture. By then the disease had made its way into parts of Alabama and Mississippi. Research efforts at the time determined that the disease was only transmitted via root grafting and "by some means not yet understood," and a large-scale eradication effort was put forth. In addition,



the Federal Plant Quarantine No. 67 became effective June 1, 1929, prohibiting the shipment of stunted peach tree nursery stock to other parts of the nation (64).

### **1.0.2 A Viral Pathogen**

During the majority of the 1900's, an alleged viral agent was believed to be responsible for Pierce's disease of grapevine, phony peach disease, and alfalfa dwarf disease. In 1930, transmission of phony peach disease was accomplished with root grafting, leading L.M. Hutchins to believe that a viral agent was responsible for the disease causing reduced growth of peach trees (64). In 1936, the graft transmission of alfalfa dwarfing disease, a disease similar to phony peach disease, again suggested the causal agent was viral (126). Experiments conducted by W.B. Hewitt supported the belief that Pierce's disease was viral in nature due to the successful transmission of PD with infected buds and grafts. Several years thereafter, Hewitt also established the leafhopper as the primary vector of PD, and determined that both PD and alfalfa dwarf were caused by the same infectious agent (50, 51). J.H. Freitag demonstrated the wide host range of the alleged virus using vector transmission tests with indicator plants, identifying over 100 common riparian plant species that harbor the virus, although most of the hosts did not express symptoms (31). Two plant pathologists working for the U.S. National Park Service, Horace V. Wester and Edward W. Jylkka, were the first to report an association between leaf-scorched elm trees in Washington D.C. and a pathogen that develops within the xylem (129). The results demonstrated that, although the pathogen was incapable of being transmitted by bark patching, it was capable of being transmitted via chip and bud grafts.

### **1.0.3 A Rickettsia-like Bacteria (RLB) / A Xylem-Limited Bacteria (XLB)**

It wasn't until 1971 that the pathogen's bacterial etiology was revealed after soil drenches of the antibiotic tetracycline demonstrated successful suppression of Pierce's disease of grapevine (59). In 1973, electron microscopy provided the first visual evidence of xylem-limited bacteria (XLB) confined to the xylem vessels of grapevines and alfalfa plants. Two independent discoveries of the xylem-limited bacteria were made concurrently that same year. Using ultra thin sections of PD affected grapevines and alfalfa plants; observation of Rickettsiae-like bacteria (RLB) that were rod shaped (0.4  $\mu\text{m}$  in diameter and 3.2  $\mu\text{m}$  in length) and confined to the xylem elements of the host plants were reported. Although still unable to culture the bacteria due to the nutritionally fastidious nature of the pathogen, Goheen and colleagues were able to demonstrate that hot water treatment of propagating wood successfully eliminated the disease (35). On the other side of the nation, Donald L. Hopkins and Hilton H. Mollenhauer reported that an obligate parasitic bacterium resembling a rickettsia is consistently associated with PD (57). Once the etiological discoveries of PD were published, the same rickettsia-like organism was observed in association with phony peach disease (56, 58, 94), almond leaf scorch (ALS) (88), plum leaf scald (69), and periwinkle wilt (82). In 1975, the successful transmission of almond leaf scorch to almond seedlings and grape seedlings confirmed a close relationship between ALS and PD (4). Elm leaf scorch was also determined to be caused by the same bacterial agent in 1978 (112).

#### 1.0.4 Demonstration of Koch's Postulates and Taxonomic Classification

In 1978, Davis and colleagues successfully isolated PD and ALS bacterium by blotting petiole extract onto modified isolation media, and then successfully incite PD symptoms after inoculating grapevine with the isolated agent (24). Interestingly, the media was modified with bovine serum albumin and hemin at quantities that support the bacteria *Rochalimaea quintana*, the etiological agent responsible for trench foot fever that belongs to the family Rickettsiaceae. Shortly after the initial description of the culture media for PD, an isolation media for plum leaf scald and phony peach disease was formulated (128). It was at this time that many landscape trees were recognized as being deleteriously affected by XLB, including elm, oak, sycamore, mulberry, and maple (46, 73, 113, 116). In 1982, Sherald et al. was the first to fulfill Koch's postulates for a landscape tree, successfully isolating the bacteria from sycamore petioles, inciting sycamore leaf scorch after inoculation, and re-isolating the bacterial causal agent. Finally, in 1987 the XLB was classified under a single species name, *Xylella fastidiosa* (127). Serological data, fatty-acid analysis, and DNA hybridization studies strongly suggested that the 25 different strains of XLB derived from 10 different plant hosts were homogenous. Multiple 16S rRNA sequence analyses of *X. fastidiosa* and bacteria of similar morphology and pathogenicity closely relate this species to the Xanthomonads, particularly the species *Pseudomonas boreopolis* and *Xanthomonas campestris* (85). Citrus variegated chlorosis, another disease caused by *X. fastidiosa*, was first reported during an outbreak in the northwest region of the state of São Paulo that same year (14).

### **1.0.5 Genome Sequence of *X. fastidiosa***

In 2000, the 9a5c clone of *X. fastidiosa* was the first plant pathogenic bacterium to have its genome entirely sequenced (117). This particular strain was isolated from twigs of Brazilian Valencia sweet oranges affected by citrus variegated chlorosis. *X. fastidiosa* is characterized as having a 52.7% GC-rich 2,679,305-bp circular chromosome and two plasmids of 51,158 bp and 1,285 bp. Interestingly, orthologues of several genes coding for virulence were only found in bacteria of animals and humans. This is an indication that the molecular basis for bacterial pathogenicity is most likely independent of plant hosts, and that at least 83 genes had been acquired from bacteriophage-mediated horizontal gene transfer. Efficient metabolic mechanisms were found as predicted and explain the ability of this bacterium to thrive in the nutritionally poor xylem of plants (117).

### **1.0.6 *X. fastidiosa* subspecies**

Since the initial description of *X. fastidiosa* by Wells et al. in 1987 (127), five pathogenic varieties of this bacterium have been described. These include i) *X. fastidiosa* subsp. *fastidiosa* (108), ii) *X. fastidiosa* subsp. *multiplex* (108), iii) *X. fastidiosa* subsp. *pauca* (108), iv) *X. fastidiosa* subsp. *sandyi* (109), and v) *X. fastidiosa* subsp. *tashke* (104). The first three subspecies were described together based on serological and phenotypic information, DNA-DNA homology, and sequencing of the 16S-23S intergenic spacer (ITS) region (108), while the later two subspecies were described individually thereafter once discovering separate monophyletic clade formations when constructing phylogenies using multi-locus sequence data (104, 109).

A diverse array of molecular techniques have been implemented in an ongoing attempt to genetically characterize the *X. fastidiosa* population and elucidate the mechanisms associated with host specificity and virulence. These include restriction fragment length polymorphism (RFLP)(17), randomly amplified polymorphic DNA (3, 20, 47, 49, 97), multi-primer assays (48, 49, 86), microsatellite and simple sequence repeat (SSR) analysis (2, 80, 104), 16S rDNA, 16S-23 ISR region, and internal transcribed spacer (ITS) sequencing (47, 49, 63, 85, 86, 104), and more recently multilocus sequencing (93, 96, 107, 109, 132). All previously cited studies provided a general consensus of the following described subspecies.

*X. fastidiosa* subsp. *fastidiosa*

Erroneously named *X. fastidiosa* subsp. *piercei* when first described, this subspecies was renamed *X. fastidiosa* subsp. *fastidiosa* after discovering that the nomenclature was in violation of the rules set forth by the International Code of Nomenclature of Bacteria (108). This subspecies is most notable for causing Pierce's disease of grapevine (*Vitis vinifera*). However, this subspecies is also known for causing disease to almond (*Prunus dulcis*), maple (*Acer* spp.), and alfalfa (*Medicago sativa*) (108). The geographic distribution of this particular subspecies is throughout Northern and Central America and Peru (65). The sequenced genome representative of this subspecies is Temecula1 (124).

*X. fastidiosa* subsp. *multiplex*

The *multiplex* subspecies was described concurrently with subspecies *fastidiosa* and subspecies *pauca* (108). This subspecies is most notable for causing phony peach disease (*Prunus persica*). Additionally, it also causes diseases to plum (*Prunus domestica*), pigeon grape (*Vitis aestivalis*), pecan (*Carya illinoensis*), and almond (*Prunus dulcis*),

as well as numerous landscape ornamentals including elm (*Ulmus* spp.) and sycamore (*Platanus occidentalis*) (49, 86, 108). Differentiation of *X. fastidiosa* strains using RAPD analysis further delineated the *multiplex* subspecies into two distinct genotypes, *multiplex* ALSI and *multiplex* ALSII (3). Such interspecific variation within the *multiplex* subspecies was further supported with multilocus sequence analyzes (96, 107). The sequenced genome representative of *X. fastidiosa* subsp. *multiplex* is M12 (124).

*X. fastidiosa* subsp. *pauca*

*X. fastidiosa* subsp. *pauca* was originally classified to subspecies with subspecies *fastidiosa* and *multiplex* (108). This subspecies is known for causing citrus variegated chlorosis. It has also been suggested that this subspecies causes coffee leaf scorch (26, 79). Its geographic distribution includes Argentina and Brazil, and this is currently the only described subspecies that is not present in North America (132). Due to its potential threat to US agriculture, the CVC strain of *X. fastidiosa* is included in the USDA-APHIS select agent list. The reference genome for this particular subspecies is 9a5c (117).

*X. fastidiosa* subsp. *sandyi*

*X. fastidiosa* subsp. *sandyi* was classified to subspecies after differences in host specificity and phylogenetic relatedness warranted separation from *X. fastidiosa* subsp. *fastidiosa* (109). This subspecies is most notable for causing oleander leaf scorch (*Nerium oleander*). However, based on RAPD-PCR and 16S-23S rDNA ISR, additional hosts including daylily (*Hemerocallis* spp.), jacaranda (*Jacaranda mimosifolia*), and magnolia (*Magnolia grandiflora*) have also been found harboring this particular subspecies (49). This subspecies has been found in California, Texas, and Florida (109). This reference genome for this species is Dixon (8).

### *X. fastidiosa* subsp. *tashke*

*X. fastidiosa* subsp. *tashke* was discovered infecting chitalpa (*Chitalpa tashkentensis*) in southern California (104). No other associated hosts or incidences have been documented for this particular subspecies. A reference genome does not exist for this subspecies.

### *X. fastidiosa* subsp. *morus*

*X. fastidiosa* subsp. *morus* is specifically pathogenic to members of the mulberry genus (*Morus* spp.). This subspecies is an intersubspecific homologous recombinant of *X. fastidiosa* subsp. *multplex* and *X. fastidiosa* subsp. *fastidiosa* (68). This chimeric strain type has the reference genome MUL0034 (109).

## **1.1 Pathology**

### **1.1.1 Disease and Economic Loss Caused by *X. fastidiosa***

*X. fastidiosa* is an established and problematic bacterial pathogen that is responsible for numerous diseases of agricultural crops throughout the Americas, as well as the urban forest of many urban environments throughout the eastern-Atlantic region (9, 42-44, 84, 115). The most significant diseases caused by *Xylella fastidiosa* (127) include the Pierce's disease of grapevine (24), citrus variegated chlorosis (14), almond leaf scald (18), phony peach disease (64), plum leaf scald (69), alfalfa dwarf (81), and leaf scorch of landscape trees (110). In several Maryland and New Jersey municipalities, the cost for maintaining and removing bacterial leaf scorch affected oak trees has been estimated to exceed one million dollars over a period of 5 to 10 years, an economic burden that well exceeds most normal tree budget allocations (36).

The symptoms expressed by *X. fastidiosa*-infected hosts can vary dramatically. *X. fastidiosa* is responsible for dwarfing diseases such as phony peach disease (64), alfalfa dwarf (81), and ragweed stunt (11), as well as leaf scorching diseases such as almond leaf scorch (75), plum leaf scald (69), and the leaf scorch diseases of numerous landscape woody ornamentals (110). In many instances, *X. fastidiosa* is capable of living as a harmless endophyte without causing any noticeable symptoms (31, 62, 84). The relationship between genetic diversity, nutritional preference, symptom development, and host specificity is still far from being understood.

### **1.1.2 Morphology of *X. fastidiosa***

*Xylella fastidiosa* is a gram-negative bacterium. The bacterium is non-motile, non-flagellate, oxidase negative, catalase positive, strictly aerobic, non-fermentative, and non-pigmented. All bacteria within the *X. fastidiosa* species share similar morphological features, and possess furrowed or rippled cell walls resembling those of the family *Rickettsiaceae* and *Legionellaceae* families (127). Their morphology is predominately single, straight rod (0.25 to 0.35 by 0.9 to 3.5  $\mu\text{m}$ ) with long filamentous strains possible under some cultural conditions (127). The optimum temperature for bacterial growth in woody perennials is between 26-28°C (30). Nutritional preferences differ among strains of *X. fastidiosa*, and all are incapable of being grown on conventional media (23, 25, 34, 82, 128). Multiple 16S rRNA sequence analyses of *X. fastidiosa* and bacteria of similar morphology and pathogenicity closely relate this species to the Xanthomonads, particularly the species *Psuedomonas boreopolis* and *Xanthomonas campestris* (85).



### 1.1.3 Transmission

Transmission of *X. fastidiosa* to new hosts occurs during xylem sap feeding by insect vectors, including treehoppers (Membracidae), sharpshooter leafhoppers (Cicadellidae) and spittlebugs (Aphrophoridae and Clastopteridae) (100, 105, 133). Bacterial cells inoculated into host plants by insect vectors develop into dense xylem-plugging colonies and extensive non-plugging bacterial communities that restrict sap flow and cause drought like symptoms and stunting of growth (33, 89). Although transmission experiments have never been performed for landscape trees, leafhopper species within the subfamily Cicadellinae were the most abundant xylem-feeding insects among elm trees in a survey of potential BLS vectors in the District (6). Additionally, the treehopper *Ophiderma definita* is suggested to be the most dominant implied vector in New Jersey oak canopies (133). Although never proven for landscape trees, root transmissibility has been demonstrated in citrus (45).

### 1.1.4 Host range

*X. fastidiosa* has a wide host range, affecting over 100 plant species in at least 30 monocotyledonous and dicotyledonous plant families (110). Diseases associated with this pathogen are damaging to a plethora of agricultural crops, including grapevine (*Vitis vinifera*), peach (*Prunus persica*), almond (*Prunus dulcis*), pecan (*Carya illinoensis*), citrus (*Citrus* spp.), and coffee (*Coffea arabica*) to name a few (65). In an urban forest, *X. fastidiosa* can cause decline of mature oak (*Quercus* spp.), elm (*Ulmus* spp.), sycamore (*Platanus* spp.), maple (*Acer* spp.), and red mulberry (*Morus rubra*) (46, 73, 116).

Although *X. fastidiosa* has a broad host range, in urban ecosystems the impact is most noticeable pin and red oaks (77).

### **1.1.5 Geographic Distribution and Origin of *X. fastidiosa***

Only several rare occurrences of *X. fastidiosa* infection have been reported outside the Americas. In Taiwan, it was suggested that *X. fastidiosa* is the causal agent of pear leaf scorch (78), although there is evidence that the pear strains were not related to this group of bacteria based on DNA hybridization and phylogenetic analysis using 16S-23S region (85). More recently, comparison of the 16S rRNA and 16S-23S rRNA regions of strains isolated from pear were genetically distinct from 20 other strains isolated from numerous other hosts, and the inconclusive results suggest that the strain responsible for pear leaf scorch belongs in a separate subspecies of *X. fastidiosa* that has yet to be described (120). In 2013, *X. fastidiosa* subsp. *fastidiosa* was reported infecting grapevine in Taiwan, and was the first ever report of Pierce's disease on the Asian continent (119). The strain isolated from grapevine was genetically distinct from the strain isolated from pear using 16S rRNA and 16S-23S rRNA ITS, suggesting that the two strains possibly evolved independently (119). In Europe, only a single case of *X. fastidiosa* infection of grapevine was reported on an imported grapevine from the U.S (7). There was no outbreak or spread of *X. fastidiosa* observed thereafter (93). Recently in 2013, *X. fastidiosa* was discovered infecting orchards of olive in southern Italy. Amplification and sequencing of the RNA polymerase sigma 70 factor gene confirmed the existence of a molecularly distinct strain of *X. fastidiosa* infecting these olive orchards (29).

Aside from the formally mentioned, *X. fastidiosa* is restricted to the Americas (93). *X. fastidiosa* subsp. *fastidiosa* lacks any significant amount of genetic diversity regardless of locality within the U.S., suggesting that a single introduced genotype is responsible for Pierce's disease of grapevine in North America (93). Based on multi-locus sequence typing (MLST) of 7 housekeeping genes of *X. fastidiosa*, the greater amount of genetic diversity of *X. fastidiosa* subsp. *fastidiosa* found in Costa Rica lead Nunney et al. to believe that this subspecies is native to Central America. Based on the high level of nucleotide polymorphisms observed for *X. fastidiosa* subsp. *multiplex* (five time greater than that of *X. fastidiosa* subsp. *fastidiosa*) (132), and based on the slower evolutionary rate of *X. fastidiosa* subsp. *multiplex* due to temperate-region (U.S.) climate, it has been suggested that *X. fastidiosa* subsp. *multiplex* is native to the U.S. (93). In South America, the subspecies infecting citrus, subsp. *pauca*, is highly differentiated from the other subspecies based on MLST, suggesting long-term isolation in that region (132). No incident reports of *X. fastidiosa* subspecies *tashke* or *X. fastidiosa* subspecies *sandyi* have been reported outside the United States.

## **1.2 Variation Observed Among Strains of *Xylella fastidiosa***

### **1.2.1 Host Specificity**

Host specificity has been demonstrated after cross-inoculation of isolated bacteria from different hosts has failed to demonstrate reciprocated symptom development in several instances (32, 99, 111). Sub-specific pathotypes of *X. fastidiosa* are capable of infecting the same host species. For example, almond plants are susceptible to isolates from grape plants but not vice versa (3). Although subspecies *pauca* is the implied causal agent of

coffee leaf scorch, it has been shown that subspecies *fastidiosa* can also infect coffee plants (93). Both the *fastidiosa* and *multiplex* subspecies have been found to infect almond trees, as well as co-inhabit the same host plant (18). Conversely, host specificity is also observed at the sub-specific level. There is evidence that cross-inoculation of *X. fastidiosa* subsp. *multiplex* from different host species does not result in reciprocated symptom development (32, 111). Similarly, isolates of bacteria from coffee could not colonize citrus when cross-inoculation was performed, although both can be colonized by *X. fastidiosa* subsp. *pauca* (99).

### **1.2.2 Genomic Variation**

Since the initial sequencing of the genome of strain 9a5c, four additional genomes have been completely sequenced, including *Temecula1*, a strain isolated from California grapevine (124); the strain *GB514* isolated from Texas grapevine (no corresponding publication, performed by the University of Houston-Downtown); and the *M12* and *M23* strains isolated from infected almond trees (21). Nine additional draft genomes are available, including almond isolate (*Ann1*) and oleander (*Dixon*) (8), a bio-control strain isolated from elderberry *EB92-1* (134), as well as several strains from coffee (*X. fastidiosa* 32 and 6c) (no corresponding publication, performed by Universidade de Mogi das Cruzes). Sequenced genomes of strains isolated from landscape trees include mulberry (*Mul-MD*) (125) and red oak (*Griffin1*) (19). Little information is provided for the two draft genomes ATCC 35871 (Doe Joint Institute) and PLS229 (USDA) on the NCBI database.

When performing a genome wide analysis of the strains 9a5c, Dixon, Ann1, and Temecula1, all four strains possessed 1,579 genes and 194 non-coding homologous sequences, which account for 76.2% and 3.9% respectively, of the sequenced genomes. The number of genes unique to each strain was 241 (9a5c), 96 (Dixon), 145 (Ann1) and 10 (Temecula1). Of these genes, 60 (9a5c), 54 (Dixon), 83 (Ann1) and 9 (Temecula1) did not share homology with any other known organism with available sequence data. INDELs (insertions or deletions of bases in a genome) were the main source of genetic variation among the four genomes. Multiple alignments of the four genomes identified 12,754 SNPs (single nucleotide polymorphisms) and 14,449 INDELs in the 1,528 commonly shared genes. In the shared 194 non-coding regions, 20,779 SNPs and 10,075 INDELs were identified. The average SNP frequency for the four genomes was  $1.08 \times 10^2$  per base pair of DNA, while the average INDEL frequency was  $2.06 \times 10^{-2}$ . The average rates of synonymous and non-synonymous substitutions were 60.33% and 39.67%, respectively. When analyzing the strain specific genes, significant differences in terms of codon usage and GC composition suggest that the genes are of xenologous origin (transferred horizontally) (28).

### **1.3 Overview of Relevance to the Urban Forest**

#### **1.3.1 Bacterial Leaf Scorch in Urban Ecosystems**

*Xylella fastidiosa* (127) causes a chronic leaf-scorching disease of landscape woody ornamentals (110). Leaf scorch symptoms first develop on an isolated branch or region within the crown of a tree and an annual progression of leaf scorch ensues until the entire crown is affected. *X. fastidiosa* can cause decline of mature oak (*Quercus* spp.), elm

(*Ulmus* spp.), sycamore (*Platanus* spp.), maple (*Acer* spp.), and red mulberry (*Morus rubra*) (46, 73, 116). Although *X. fastidiosa* has a broad host range, it has been suggested that BLS primarily affects pin oaks and red oaks in New Jersey (77). In New Jersey, incidences as high as 30% were noted for northern red oak, pin oak, and scarlet oak (77). Further south, an ELISA-based survey of trees found BLS occurring on symptomatic pin oak, red oak, shingle oak (*Q. imbricaria*) and white oak (*Q. alba*) in 16 cities and towns in Kentucky (40). The study also detected BLS in scarlet oak, post oak (*Q. stellata*), water oak (*Q. nigra*), swamp oak (*Q. bicolor*), chestnut oak (*Q. prinus*), and willow oak in most major metropolitan areas in Tennessee. Within the Carolinas, positive ELISA-detection of *X. fastidiosa* was reported in pin oak and southern red oak (*Q. falcata*) (43). The association of BLS with oak decline in urban settings extends as far south as Florida and includes turkey oak (*Q. laevis*) and southern red oak (5).

Previously conducted surveys of BLS provide compelling evidence of a well-established occurrence of BLS among urban trees in the District (46, 84, 113, 115, 129). The 2010 Forest Action Plan for the District developed in accordance with the National Association of State Foresters and the U.S. Forest Service included BLS as an urban forest disease of concern. The economic implications associated with the maintenance and removal of BLS-affected trees within the District have forced regional researchers and arborists to devise management strategies in response to this chronic disease. As this pathogen continues to perpetuate uncontrolled in urban environments, there is a pressing need to identify long-term management strategies that abate disease.

### 1.3.2 Epidemiology of *X. fastidiosa* in Landscape Trees

There is a lack of knowledge regarding the persistence of *X. fastidiosa* within an infected landscape tree, asymptomatic neighboring trees, and non-susceptible trees that may still harbor the bacterium. Many wild plants latently harbor the bacterium, including grasses, sedges and trees (31, 49). In an investigation of the natural occurrence of *X. fastidiosa* in a Maryland nursery, crape myrtle (*Lagerstroemia indica*) within the nursery, as well as sassafras (*Sassafras albidum*) and mimosa (*Albizia julibrissin*) trees bordering the nursery were found to harbor *X. fastidiosa* without expressing leaf scorch symptoms (62). A survey of trees within the District of Columbia discovered *X. fastidiosa* in asymptomatic boxelder (*Acer negundo*) and buckeye (*Aesculus x hybrid*) (84). Symptomless hosts with latent infections of *X. fastidiosa* can serve as a reservoir of inoculum for vector dispersal (60).

The bacterium is capable of systemic movement in plants such as citrus, grapevine (*Vitis* spp.), and symptomless blackberry (*Rubus procerus*), but does not move systemically in most symptomless hosts (45, 46, 55, 60). Although never proven for landscape trees, root transmissibility has been demonstrated in citrus (45). Using electron microscopy, *X. fastidiosa* has been observed colonizing the asymptomatic portion of *X. fastidiosa*-infected elm trees (46). The uncertainties regarding the persistence of *X. fastidiosa* in an urban landscape present a difficult challenge when prescribing management options that seek to remove infested plant material. If inoculum were abundantly available to vectors beyond the visibly symptomatic plant material, such a management option would likely be inefficient, particularly since only a very small number of bacterial cells are required

for successful transmission from a leafhopper vector (52). The extent to which population size influences systemic spread within a landscape tree has yet to be determined, though it is suggested that bacterial multiplication to high concentrations ( $10^7$  to  $> 10^9$  CFU/g) is needed for systemic movement in grapevine (52)

Many urban environments are embodied in an urban heat island created by the excess heat from urban surfaces. During the summer in the District of Columbia, it has been found that by mid-morning, diurnal temperature differences can be as great as  $10^{\circ}\text{C}$  between the urban environment and nearby woodlands (67). This may have implications regarding disease incidence in urban environments. It has been suggested that regions with warm day and night summer temperatures should expect less interruption to exponential phase growth of *X. fastidiosa in planta* compared with regions with similarly warm days and cool nights (30). If urban environments are moderating cooler temperatures during evening hours, a lag phase of growth in *X. fastidiosa* could become nonexistent, and permit unrestricted bacterial growth throughout the summer. Since the rate of systemic movement in plants has been found to require bacterial multiplication to high concentrations (52), urban environments may be more conducive for disease severity and incidence. This might be one explanation why *X. fastidiosa*-associated tree mortality is observed in cities but not in neighboring forest areas.

### **1.3.3 Current Management Options for Amenity Trees**

A curative management option for bacterial leaf scorch is nonexistent. Maintaining plant vigor can help alleviate the symptoms of disease (83). Root-flare injections of the antibiotic oxytetracycline into the xylem of infected trees and soil drenches of the plant



growth regulator paclobutrazol are the management prescriptions currently recommended by arborists (27, 39, 72). However, these management options only help alleviate the deleterious symptoms of this disease. Use of the insecticide imidacloprid and thiamethoxam as well as the use of kaolin to disrupt feeding behavior have also been used to help suppress vector populations with some success in an agricultural setting (13, 65, 122). However, despite the efforts of any resource manager, trees begin a slow and irreversible decline once infected.

## **1.4 Research Objectives**

### **1.4.1 Justification**

Leaf scorch is a symptom of *X. fastidiosa*-infection among susceptible landscape trees. Aside from xylem occlusion by *X. fastidiosa*, leaf scorch can be caused by numerous other abiotic stresses including drought, high temperatures with hot and dry winds, fertilizer burn, root damage from construction, and impeded root development due to anthropogenic infrastructure. The incidence of *X. fastidiosa*-infection in relation to leaf-scorched trees in the District has not been extensively evaluated and the scale of pathogen-associated leaf scorch remains unclear.

Once a tree becomes infected, repeated vector probing and systemic movement of bacteria are methods in which the bacterium is capable of moving within and between tree canopies (1, 52). It has been suggested that pruning may help reduce the incidence of *X. fastidiosa*-infection within a tree (110). Knowledge of the extent of latent infection throughout a tree's canopy can provide baseline information regarding the efficacy of pruning symptomatic branches to manage disease. Knowledge of whether asymptomatic

neighboring trees possess latent infections of *X. fastidiosa* can help with determining whether removal of infected trees is a feasible management option after visual recognition of an infected tree.

Understanding the genetic diversity and population structure of this pathogen within the District is an important factor to consider when evaluating any host management program. Population structures of pathogens can become altered when populations isolated by geography or host preference are allowed to interbreed after a migration event or introduction to a commonly shared host species, respectively. Genetic mutation, horizontal gene transfer, and host selection pressure can also influence the population structure within an environment. By analyzing sequence data of multiple loci from this pathogen in various tree species, it may be possible to identify candidate host species that facilitate genetic reassortment of genetically distinct *X. fastidiosa* strains. Inferring migration rates between populations of *X. fastidiosa* may provide evidence of exclusivity or genetic recombination between two populations specific to different hosts. Consequently, knowledge of the genetic variation within the *X. fastidiosa* population can assist with understanding disease epidemiology.

The extent to which the infected portion of a tree contributes to bacterial acquisition by vectors is unknown. In a recent New Jersey study, vectors (Cicadomorpha) of *X. fastidiosa* were found less frequently in symptomatic red oak trees compared to asymptomatic trees (133). It has been suggested that the inoculum source for vectors could derive from porcelain berry, wild grape, and wild mulberry, and that removal of

extrinsic wild hosts that harbor the bacterium could be an effective means of controlling diseases caused by *X. fastidiosa* (63). However, the potential for an infected ornamental tree to be the sole source of inoculum for all ornamental trees within a given site has not yet been evaluated. Whether the vectors (Cicadomorpha) of *X. fastidiosa* acquire the inoculum from weedy host reservoirs or infected landscape trees is unclear. Additionally, it is unknown whether systemic movement of the bacteria or repeated inoculation by vectors is the primary mechanism of *X. fastidiosa* dissemination within the crown of a tree. Determining the genetic diversity of bacteria from different portions of an individual tree could provide information regarding the origins of the bacteria, more particularly whether the bacterial population within a site is homogeneous and from a single origin, or derived from multiple introductions. Population differences within a single host or site can illustrate dissemination patterns and ranges, and potentially elucidate the inoculum sources causing infection.

#### **1.4.2 Objective 1: Survey of *X. fastidiosa* Infection in the District of Columbia**

In an attempt to clarify the extent of latent infection and bacterial colonization in urban landscape trees, the first objective of this investigation is to determine the occurrence and host range in the District of Columbia as well as the presence of *X. fastidiosa* in asymptomatic trees and within the canopy of an infected tree. This objective attempts to answer;

- 1) What is the incidence of *X. fastidiosa*-infection in relation to trees expressing leaf-scorch in the District?
- 2) Which trees are most frequently found associated with *X. fastidiosa* and which infected trees are most vulnerable to decline?

- 3) Is there latent infection in asymptomatic trees in close proximity (>25m) to infected trees?
- 4) Can the bacterium be readily detected within the asymptomatic portion of infected trees?

Evaluating the prevalence of *X. fastidiosa* among leaf-scorched trees will help clarify the role this pathogen has in causing leaf-scorch disease in the District. This survey will illustrate the extent of latent infection both within an infected tree and within neighboring asymptomatic trees. The survey will also evaluate decline rates in relation to infection severity among urban trees. Establishing a list of host species most susceptible to disease and species most detrimentally affected by disease can help urban foresters make sound management decisions when selecting trees to replant at a site.

#### **1.4.3 Objective 2: Population Assessment**

An extensive population assessment will elucidate strain diversity and host specificity behaviors of *X. fastidiosa*. Information about the geographic distribution of distinct *X. fastidiosa* strains may reveal bacterial dissemination patterns when taking into account the location, host species, and distance between microsites, and could potentially provide useful information regarding the dissemination dynamics of *X. fastidiosa*. Within a single infected tree, knowledge regarding the genetic diversity within two opposing areas of the canopy can provide valuable information regarding the etiology of *X. fastidiosa*. The presence of multiple strains or a single strain throughout the canopy of a tree can determine whether multiple introductions of bacteria occur, whether bacteria from one side of the canopy is more similar to the bacteria in a neighboring infected tree than

bacteria within its own canopy, or whether the bacteria within a host is entirely homogeneous. A comparison of populations within and between tree canopies among multiple infection sites within a region will clarify epidemiological patterns associated with bacterial leaf scorch in the District.

## **Chapter 2: Bacterial Leaf Scorch in the District of Columbia: Distribution, Host Range, and Presence of *Xylella fastidiosa* Among Urban Trees**

### **2.0 Abstract**

A survey of urban trees affected by bacterial leaf scorch (BLS) caused by *Xylella fastidiosa* was conducted in the District during 2011 and 2012. Over 20 species of urban trees were evaluated at 95 sites. Symptomatic and asymptomatic foliage from trees with BLS symptoms and foliage from neighboring asymptomatic trees were sampled. *X. fastidiosa*-specific ELISA and a PCR assay was used to detect and identify the strains from environmental samples. Symptomatic trees testing ELISA-positive for *X. fastidiosa* occurred most frequently with *Quercus palustris*, *Q. rubra*, *Ulmus americana*, and *Platanus occidentalis*. The bacterium was also less frequently identified on eight other symptomatic and on five asymptomatic tree species. On infected trees, the bacterium was also detected on the asymptomatic portion of seven tree species. All strains were identified as the *X. fastidiosa* subsp. *multiplex* genotype ALSII except on *Morus alba* where the genotype ALSI and the subsp. *sandyi* was detected. The occurrence of crown dieback was found significantly associated with *X. fastidiosa*-infection on *Q. palustris*, *Q. rubra*, *U. americana*, and *P. occidentalis*. As this pathogen continues to perpetuate uncontrolled in urban environments, there is a pressing need to identify long-term management strategies that abate disease.

## 2.1 Introduction

*Xylella fastidiosa* (127) is the causal agent of bacterial leaf scorch (BLS), a chronic leaf-scorching disease of landscape woody ornamentals (110). Leaf scorch symptoms first develop on an isolated branch within the crown of a tree and an annual progression of leaf scorch ensues until the entire crown is affected (110). *X. fastidiosa* can cause decline of mature oak (*Quercus* spp.), elm (*Ulmus* spp.), sycamore (*Platanus* spp.), maple (*Acer* spp.), and red mulberry (*Morus rubra*) (46, 73, 116). Numerous surveys have established the incidence of BLS within multiple jurisdictions throughout the eastern-Atlantic region (42, 43, 84, 115). In several Maryland and New Jersey municipalities, the cost for maintaining and removing BLS-affected oak trees has been estimated to exceed one million dollars over a period of 5 to 10 years, an economic burden that well exceeds most normal tree budget allocations (36).

Previous surveys of BLS provide evidence of a well-established occurrence of BLS among urban trees in the District (46, 84, 115, 129). The economic implications associated with the maintenance and removal of BLS-infected trees within the District have forced regional researchers and arborists to devise management strategies in response to this chronic disease. Palliative responses such as oxytetracycline injections and plant growth regulators such as paclobutrazol are not curative and only help alleviate the deleterious symptoms of this disease (27, 39, 72). Pruning to remove infected branches of a tree has not yet been proven as an effective method for mitigating the occurrence or severity of BLS, and little is known about the prevalence of this pathogen beyond the visibly symptomatic portions of host trees.

Identification of various *X. fastidiosa* subspecies among urban trees can assist with understanding disease epidemiology and control. Currently, there are five subspecies of *X. fastidiosa* that have been described, including subspecies *fastidiosa*, known for causing Pierce's disease of grapevine (*Vitis* spp.); subspecies *multiplex*, known for causing leaf scorching diseases of numerous landscape ornamentals; subspecies *pauca*; known for causing citrus variegated chlorosis on citrus; subspecies *sandyi*, known for causing leaf scorch of oleander (*Nerium oleander*); and a newly described subspecies *tashke*, known for leaf scorch of chitalpa (*Chitalpa tashkentensis*) (104, 108, 109).

In order to determine the distribution, host range, and subspecies identity of *X. fastidiosa* in the District, a survey of symptomatic and asymptomatic trees was conducted. Our specific objectives were to outline latent infection among urban trees and identify genetic variability of *X. fastidiosa* within infected plant tissue of different tree species. The host range of BLS among various urban trees in the District was determined with ELISA. A PCR assay was used to determine the subspecies of *X. fastidiosa* infecting multiple species of urban trees in the District. Results from this investigation will provide baseline information for developing management options in response to BLS in urban municipalities.

## **2.2 Materials and Methods**

### **2.2.1 Sample Collection**

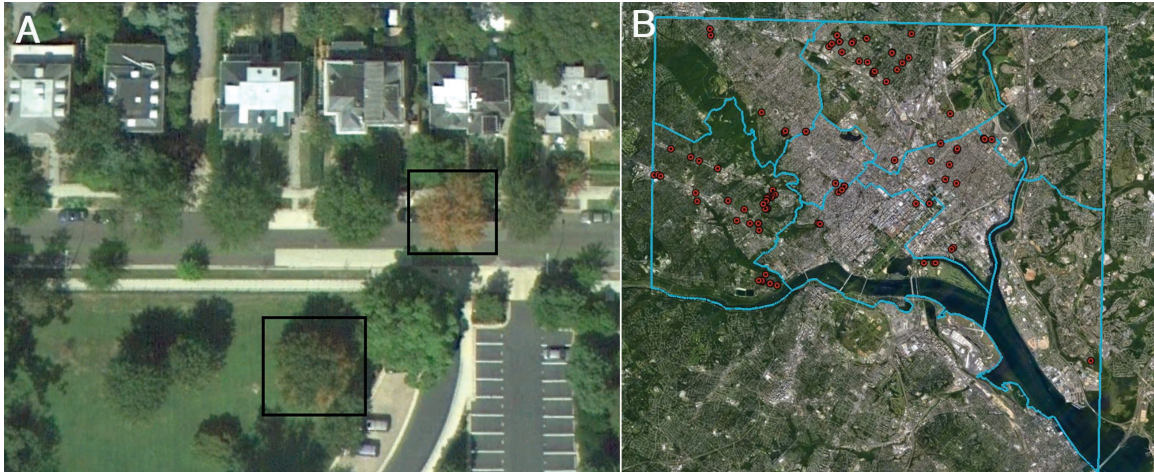
During the first year of the study (August 30 to October 26, 2011), 169 leaf-scorched trees within the District were identified using Google Earth (Google, Mountain View,



CA; accessed in August of 2011) (Fig. 1A). Trees that appeared discolored on satellite images were visited and sampled if symptoms resembled BLS. For the 2011 survey year, samples were collected from 20 different trees species and the location of each tree was recorded (Table 1).

A total of 64 sites with trees that tested ELISA-positive for *X. fastidiosa* in 2011 were revisited for more extensive sampling in 2012 (August 15 to October 12). In addition to the 64 previously sampled sites, 31 new sites with trees displaying symptoms of leaf scorch were sampled. Additional sites were selected when symptomatic trees were observed during field collection. Sampling in 2012 included; 1) symptomatic and asymptomatic foliage of trees determined to be infected in 2011, 2) symptomatic and asymptomatic foliage of neighboring symptomatic trees, and 3) foliage from asymptomatic neighboring trees. Neighboring trees were selected within a 25 m radius of an infected tree, and were generally located within 5 to 10 m. A total of 95 sampling sites comprised of over 340 urban trees were sampled throughout the District during the 2012 survey.

**Figure 1:** A, Satellite image showing an oak tree with a discolored canopy that is selected for sampling; and B, Study sites with positive detection of *X. fastidiosa* in seven of the eight District wards.



Sampling on symptomatic trees included, 1) 8 to 12 leaves from the most severely scorched portion of the canopy and, 2) 8 to 12 asymptomatic leaves from branches located as far as possible from the symptomatic portion of the canopy. If trees were entirely scorched and there was an absence of asymptomatic foliage, trees were sampled from the most scorched portion of the crown. On asymptomatic trees, 2 to 3 leaf samples were obtained from four opposing directions, resulting with a combined total of 8 to 12 leaves. This procedure was performed as scrupulously as the branch architecture permitted; however, inability to access upper canopy foliage confined the leaf collection range to the lower 7 m of the canopy. For sample collection from high branches, a 6.4 m silky Hayauchi extension pole-saw (UM Kogyo, Inc., Ono, Japan) was used. Branches that could be reached without the assistance of a pole-saw were sampled with felco F-2 hand-pruners (Pygar USA Inc., Kirkland, WA). After collecting leaves, the petioles and midribs, where *X. fastidiosa* bacteria are found in highest concentrations (46, 54), were

harvested by cutting away excessive leaf tissue. All collection utensils; the pole-saw, the hand-pruners, and the scissors, were consistently surface disinfested with 70% ethanol between sample collections. The petioles and midribs of an individual sample were stored in a sterile 15 ml test tube and transferred in a cooler to the lab the same day and frozen at -20°C (74).

In 2012, samples were collected from 19 different species of urban trees (Table 1). Information regarding the site location and physical characteristics of each tree, including tree species, height, and stem diameter at breast height (DBH) was recorded. Each tree was assigned a rating based on the amount of crown dieback: 0 = no tip dieback, minimal deadwood < 2.5 cm in diameter; 1 = < 25% dieback, dieback observed at tips of branches, deadwood generally < 5 cm in diameter; and, 2 =  $\geq$  25% dieback, reduction in crown size, deadwood > 5 cm in diameter, failure of entire limbs and growth of epicormic sprouts. In addition, every tree was assigned a visually estimated leaf scorch percentage (0 to 100%). This approximation represented the percentage of the tree crown that displayed leaf scorch.

**Table 1:** Number of *X. fastidiosa*-infected urban trees surveyed in the District of Columbia in 2011 and 2012. *X. fastidiosa*-specific double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) was used to detect the pathogen in leaves with symptoms resembling those of bacterial leaf scorch. In 2012, asymptomatic trees neighboring a symptomatic tree and, if present, asymptomatic foliage of symptomatic trees was also tested for *X. fastidiosa*. Values indicate the number of positive detection /

total number of samples and (percent of detection). n.s. indicates that no samples were collected/present for this category.

Tree species	2011 Survey		2012 Survey	
	symptomatic trees	asymptomatic trees	symptomatic trees	
	symptomatic foliage	asymptomatic foliage	asymptomatic foliage	symptomatic foliage
<i>Acer negundo</i>	ns	ns	0/1 (0)	0/1 (0)
<i>Acer platanoides</i>	1/3 (33)	0/3 (0)	0/6 (0)	0/7 (0)
<i>Acer rubrum</i>	0/6 (0)	0/7 (0)	0/5 (0)	0/9 (0)
<i>Aesculus hippocastaneum</i>	0/4 (0)	ns	ns	ns
<i>Catalpa speciosa</i>	ns	ns	0/1 (0)	0/1 (0)
<i>Cladrastis kentukea</i>	ns	ns	ns	0/1 (0)
<i>Cornus florida</i>	ns	0/1 (0)	ns	ns
<i>Fraxinus pennsylvanica</i>	0/4 (0)	ns	ns	ns
<i>Ginkgo biloba</i>	3/9 (33)	0/9 (0)	0/7 (0)	1/13 (8)
<i>Liquidambar styraciflua</i>	1/1 (100)	ns	ns	ns
<i>Liriodendron tulipifera</i>	1/2 (50)	ns	1/3 (33)	1/5 (20)
<i>Magnolia</i> sp.	0/1 (0)	ns	ns	ns
<i>Morus alba</i>	1/1 (100)	1/2 (50)	3/4 (75)	3/4 (75)
<i>Platanus occidentalis</i>	12/18 (67)	0/12 (0)	4/14 (29)	10/15 (67)
<i>Platanus x acerifolia</i>	ns	0/2 (0)	0/1 (0)	0/1 (0)
<i>Quercus alba</i>	ns	0/1 (0)	ns	ns
<i>Quercus bicolor</i>	0/1 (0)	ns	ns	ns
<i>Quercus coccinea</i>	0/2 (0)	1/1 (100)	0/1 (0)	2/2 (100)
<i>Quercus macrocarpa</i>	3/5 (60)	0/2 (0)	0/2 (0)	1/2 (50)
<i>Quercus palustris</i>	18/19 (95)	7/37 (19)	18/29 (62)	38/40 (95)
<i>Quercus phellos</i>	1/5 (20)	0/1 (0)	1/1 (100)	1/1 (100)
<i>Quercus prinus</i>	0/2 (0)	ns	ns	ns
<i>Quercus rubra</i>	36/48 (75)	7/60 (12)	29/52 (56)	57/61 (93)
<i>Tilia americana</i>	0/1 (0)	ns	0/3 (0)	0/3 (0)
<i>Ulmus americana</i>	19/35 (54)	6/21 (29)	12/18 (67)	16/20 (80)
<i>Ulmus parvifolia</i>	0/2 (0)	ns	ns	ns
<b>Sum</b>	<b>96/169 (57)</b>	<b>22/159 (14)</b>	<b>68/148 (46)</b>	<b>130/186 (70)</b>

### 2.2.2 Detection of *X. fastidiosa* Using ELISA

The *X. fastidiosa*-specific, double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) (Agdia Inc. Elkhart, IN) was used in accordance with the manufacturer's protocol in 2011. In 2012, instead of using the mesh bags provided by the manufacturer, the MP FastPrep-24 instrument was used for maceration to provide greater consistency when processing samples (MP Biomedicals, Solon, OH). This step involved dissecting 8 to 12 leaf petioles into approx. 0.5 mm pieces using a sterile razor blade and then placing 0.1 g of tissue into a 2 ml lysing matrix A test tube (MP Biomedicals, Solon, OH), with 1 ml of general extraction buffer. Sample tubes were then processed twice for 40 s at a speed of 4.5 m/s using the MP FastPrep-24 instrument. Each sample was assigned three test wells to ensure consistent ELISA readings, along with positive and negative controls for each plate.

In 2011, absorbance values were not measured and positive detection was determined based on color changes according to the manufacturer's protocol. In 2012, the optical absorbance values of all ELISA reactions were read at 650 nm ( $A_{650}$ ) using a BIO-RAD Benchmark microplate reader (BIO-RAD, Hercules, CA). A reaction was determined positive if the sample well had an absorbance value (absorbance unit) greater than the mean absorbance value of all negative control wells plus four times the standard deviation (121). In this study, a sample was deemed positive for *X. fastidiosa* when the ELISA absorbance values were  $\geq 0.30$  ( $n = 62$ , negative control =  $0.18 \pm 0.03$ ; positive control =  $1.76 \pm 0.30$ ).

### 2.2.3 DNA Extraction and PCR Analysis

Using samples collected in 2012, subsets consisting of no more than ten samples for each ELISA-positive tree species were selected for analysis using a multiprimer PCR assay (48). Total DNA was extracted from ELISA-positive petiole samples using DNeasy kit (Qiagen Inc, Valencia, CA) according to the manufacturer's protocol, with the following modification. The maceration step in the protocol was carried out in 2 ml lysing matrix tubes consisting of lysing matrix A, and samples were processed twice in a MP FastPrep-24 instrument for 40 s at a speed of 4.5 m/s.

A multiprimer PCR assay designed by Hernandez-Martinez et al. (48) was performed with the following modifications. For all reactions, 2 µl of total DNA extract was added to 12.5 µl of 2X GoTaq Green Master Mix (Promega Corporation, Madison, WI), 0.25 µl (100 µM solution) of the following primers: XF2542-R (5'-CAGTACAGCCTGCTGGAGTTA-3'), XF2542-L (5'-TTGATCGAGCTGATGATCG-3'), XF1968-R (5'-ATCCACAGTAAAACCACATGC-3'), XF1968-L (5'-GGAGGTTTACCGAAGACAGAT-3'), ALM1 (5'-CTGCAGAAATTGGAAACTTCAG-3'), ALM2 (5'-GCCACACGTGATCTATGAA-3') (Invitrogen, Grand Island, NY), and 9 µl of molecular grade water for a total volume of 25 µl per reaction (48). These three primer sets allow the differentiation of strains *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *sandyi*, and *X. fastidiosa* subsp. *multiplex*, with distinction between the ALSI and ALSII genotypes within the *multiplex* subspecies. All amplifications were performed in a BIO-RAD S1000 Thermal Cycler (BIO-RAD, Hercules, CA), and carried out with the following cycle program: 5 min at

94°C, followed by 39 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension step of 10 min at 72°C. PCR products were run in a 1.5% agarose gel (Fisher Scientific, Pittsburgh, PA) in 1X sodium boric acid conductive medium (12), and post-stained with GelRed (Biotium, Hayward, CA) at a 1:3,300 ratio of stock reagent to distilled water.

#### **2.2.4 Statistical Analyses**

The association between crown dieback ratings and presence or absence of *X. fastidiosa* on four common amenity trees was evaluated using Chi-square contingency tables and the significance of the difference identified using the Likelihood ratio and Pearson test. A simple linear regression analysis was used to test ELISA values (independent variable) in relation to scorch percentage (dependent variable) of trees. ELISA values of asymptomatic and symptomatic foliage of infected trees among the four most frequently sampled tree species were compared and differences in variances of means were separated using one-way analysis of variance (ANOVA) at the significance level identified using an *F*-test. Transformation of ELISA values for ANOVA was not necessary because data were normally distributed. The statistical software JMP 10.0.2 (2012, SAS Inst. Inc, Carry, NC) was used in all statistical analyses.

### **2.3 Results**

#### **2.3.1 Occurrence of BLS in the District**

BLS was found in seven of the eight District wards delineated by the District Department of Transportation Urban Forestry Administration (Fig. 1B). Of the 95 testing sites, 88

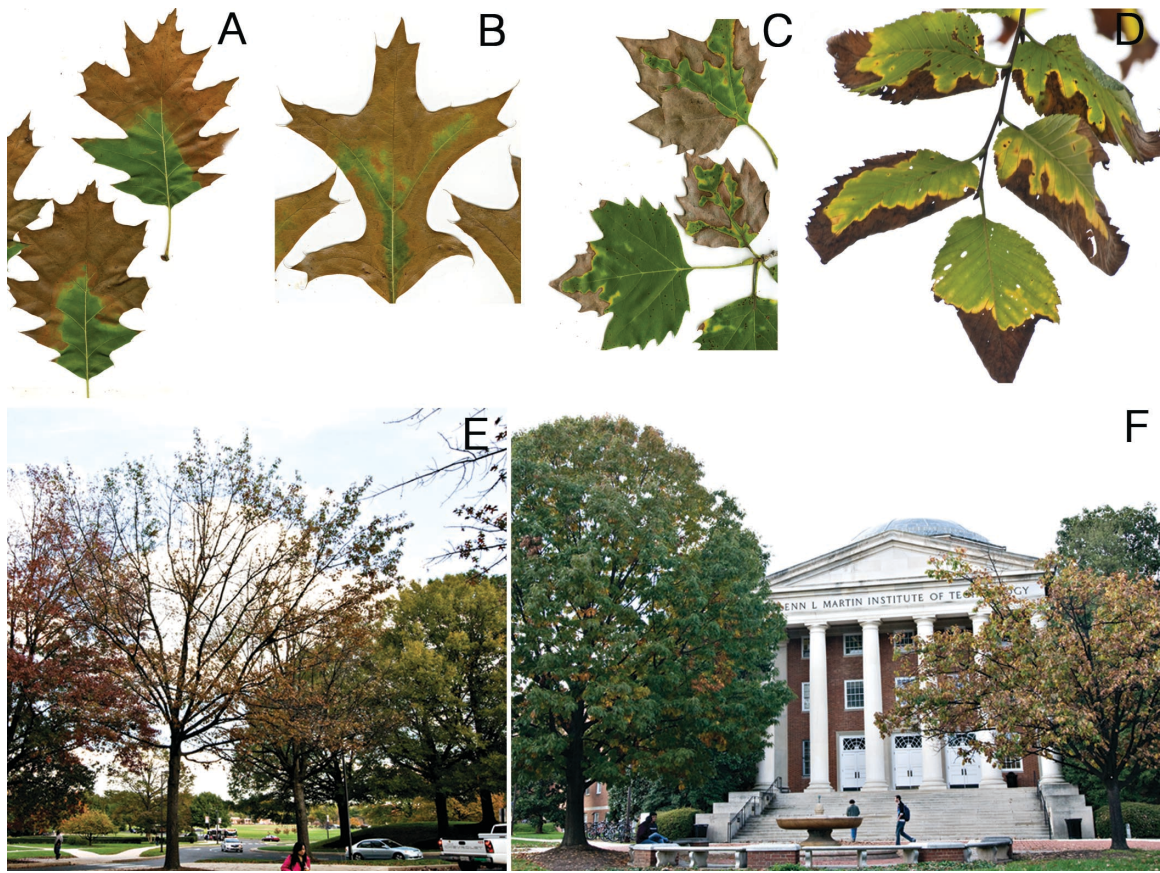
possessed a leaf-scorched tree positive for *X. fastidiosa*, and 47 contained more than one *X. fastidiosa*-infected tree. Red oak, pin oak, elm, and sycamore trees were the most common leaf-scorched trees found in the District based on satellite imagery and roadside observation (Table 1).

Leaf scorch symptoms developed late in August during both the 2011 and 2012 survey years. Symptoms displayed on infected trees were consistent with previously reported descriptions of BLS symptoms (46, 110) (Fig. 2A-D). Early leaf senescence was commonly noticed on BLS-affected pin oaks, with symptoms appearing much earlier than in other tree species (Fig. 2E). *X. fastidiosa*-infected red oaks were observed with severe dieback and stunting (Fig. 2F).

In 2011, 169 symptomatic trees belonging to 20 tree species were sampled and tested for *X. fastidiosa* (Table 1). Overall, 57% (96/169) of trees with symptoms of BLS tested positive for *X. fastidiosa* using ELISA. The percentage of infected trees varied based on tree species and was highest for pin oak (*Quercus palustris*), followed by northern red oak (*Q. rubra*), American elm (*Ulmus americana*), and sycamore (*Platanus occidentalis*), all of which were the most frequently sampled tree species (Table 1).

**Figure 2:** Bacterial leaf scorch symptoms of amenity trees in the District of Columbia and in neighboring Maryland municipalities: Leaf scorch symptoms on; A, red oak; B, pin oak; C, sycamore (*Platanus occidentalis*); and D, elm (*Ulmus americana*). Severe symptoms common with oaks; E, Early leaf drop on pin oak (*Q. palustris*); F, Stunting and chronic scorching on red oak (*Q. rubra*); tree on the right is infected.





The majority of revisited trees with a positive detection of *X. fastidiosa* in 2011 had a positive detection of *X. fastidiosa* in 2012 (Table 1). The exceptions occurred with one Norway maple (*A. platanooides*), two ginkgos (*Ginkgo biloba*) within a single site, and one bur oak (*Q. macrocarpa*). These trees tested positive for *X. fastidiosa* in 2011 and displayed BLS symptoms, but failed to provide a positive ELISA result in 2012. A total of 27 additional sites (27/31) were found with at least one tree ELISA-positive for *X. fastidiosa*. Of the 186 leaf-scorched trees examined in 2012, 70% (130/186) were ELISA-positive for *X. fastidiosa* (Table 1). Tree species within the red oak family and elm, white mulberry (*Morus alba*), and sycamore with characteristic BLS symptoms generally tested positive for the detection of *X. fastidiosa* in ELISA. The greatest mean ELISA value was recorded with pin oak (Table 2).

**Table 2:** Analysis of variance of mean ELISA values and standard deviation (Stdv) of asymptomatic and symptomatic foliage of *X. fastidiosa*-infected trees.

Tree species	asymptomatic		symptomatic		<i>F</i>	<i>P</i> <sup>a</sup>
	<i>n</i>	foliage ± Stdv	<i>n</i>	foliage ± Stdv		
<i>Quercus palustris</i>	25	1.30 ± 0.6	38	1.75 ± 0.3	15.75	0.0002*
<i>Quercus rubra</i>	36	1.10 ± 0.5	57	1.43 ± 0.4	15.05	0.0002*
<i>Platanus occidentalis</i>	4	0.89 ± 0.5	10	1.39 ± 0.3	4.92	0.0460*
<i>Ulmus americana</i>	18	0.56 ± 0.3	16	1.03 ± 0.3	21.22	<0.0001*

An asterisk (\*) indicates significant differences at  $P < 0.05$ .

Tree species that displayed BLS symptoms but were consistently ELISA-negative for *X. fastidiosa* in both 2011 and 2012 included red maple (*Acer rubrum*) and American linden (*Tilia americana*). An ELISA-positive detection of *X. fastidiosa* was possible with single samples of ginkgo and Norway maple, however all other symptomatic trees from these two tree species were ELISA-negative. Other less extensively evaluated symptomatic species that were ELISA-negative included horse chestnut (*Aesculus hippocastanum*), yellowwood (*Cladrastis kentukea*), green ash (*Fraxinus pennsylvanica*), London plane (*P. x acerfolia*), flowering dogwood (*Cornus florida*), Magnolia (*Magnolia* sp.), white oak (*Q. alba*), swamp white oak (*Q. bicolor*) chestnut oak (*Q. prinus*), Chinese elm (*Ulmus parvifolia*), boxelder (*A. negundo*), and catalpa (*Catalpa speciosa*) (Table 1).

### 2.3.2 Detection of *X. fastidiosa* in the Asymptomatic Foliage of Infected Trees

When asymptomatic leaves from *X. fastidiosa*-infected trees were tested for latent infections of *X. fastidiosa*, trees in the red oak family including pin oak, red oak, scarlet oak, and willow oak (*Quercus phellos*) had a high incidence of ELISA-detection with

over half of the samples yielding positive detection (Table 1). Similarly, 67% (12/18) of asymptomatic samples derived from infected elm tested ELISA-positive. Asymptomatic leaves derived from an infected mulberry were generally ELISA-positive (75% 3/4). When mean ELISA values of symptomatic and asymptomatic foliage from infected trees were compared for the four most frequently sampled tree species, symptomatic foliage always had a significantly greater mean ELISA value than the asymptomatic foliage (Table 2).

### **2.3.3 Detection of *X. fastidiosa* in Asymptomatic Trees**

Fourteen asymptomatic tree species in close proximity to a *X. fastidiosa*-infected tree were examined for *X. fastidiosa*. Out of 159 entirely asymptomatic trees, 22 (14%) trees were positive for *X. fastidiosa* in ELISA (Table 1). An ELISA-positive detection for *X. fastidiosa* was possible for pin oak, red oak, scarlet oak (*Q. coccinea*), mulberry, and elm, despite the absence of BLS symptoms (Table 1). In several instances asymptomatic pin oak and elm trees tested ELISA-positive for *X. fastidiosa*, and although characteristic leaf scorch symptoms were absent, the trees displayed stunting of foliage, chlorosis, and epicormic sprouting on tree limbs.

### **2.3.4 PCR Results and Strain Characterization**

All ELISA-positive samples tested with PCR were positive, except for single samples of ginkgo and tulip poplar (data not shown). The resulting banding pattern for *X. fastidiosa*-positive elm, sycamore, red oak, and pin oak samples were consistent with the banding pattern for *X. fastidiosa* subsp. *multiplex*/ALSII (Table 3). However, positive samples of

mulberry resulted with banding patterns different than these tree species. Due to the low number of mulberry trees sampled, all ELISA-positive mulberry samples were tested, which included the asymptomatic samples from infected trees. Three mulberry trees displayed a single band indicating subsp. *sandyi* whereas two mulberry trees exhibited the banding pattern for subsp. *multiplex*/ALSI, and the subsp. *sandyi* (Table 3). On these two trees, there was no relation between symptom expression and subspecies present. On one mulberry tree, the symptomatic portion of the crown possessed the banding pattern for subspecies *sandyi* while the asymptomatic portion of the crown possessed the banding pattern for *multiplex* ALS1. On the second tree, this pattern was reciprocated; the symptomatic portion of the crown demonstrated the banding pattern for *multiplex* ALSI and the asymptomatic portion of the crown possessed the banding pattern for subspecies *sandyi*.

**Table 3:**Subspecies and strain genotypes of *Xylella fastidiosa* from environmental samples obtained from petioles of infected urban tree species.

Host Species	n	PCR assay fragments <sup>a</sup>			Strain subspecies /genotype
		XF1968	ALM	XF2542	
<i>Morus alba</i>	5	+	-	-	<i>sandyi</i>
<i>M. alba</i>	2	+	+	-	<i>multiplex</i> /ALSI
<i>Platanus occidentalis</i>	10	+	+	+	<i>multiplex</i> /ALSII
<i>Quercus coccinea</i>	3	+	+	+	<i>multiplex</i> /ALSII
<i>Q. macrocarpa</i>	1	+	+	+	<i>multiplex</i> /ALSII
<i>Q. palustris</i>	10	+	+	+	<i>multiplex</i> /ALSII
<i>Q. phellos</i>	1	+	+	+	<i>multiplex</i> /ALSII
<i>Q. rubra</i>	10	+	+	+	<i>multiplex</i> /ALSII
<i>Ulmus americana</i>	10	+	+	+	<i>multiplex</i> /ALSII

<sup>a</sup>PCR products were amplified using three primer sets (see methods). (+) DNA product was obtained and visually observed after gel electrophoresis. (-) DNA product was not obtained.

### 2.3.5 Association of ELISA values with DBH (Diameter at Breast Height), Tree Height, Crown Dieback, and Leaf Scorching

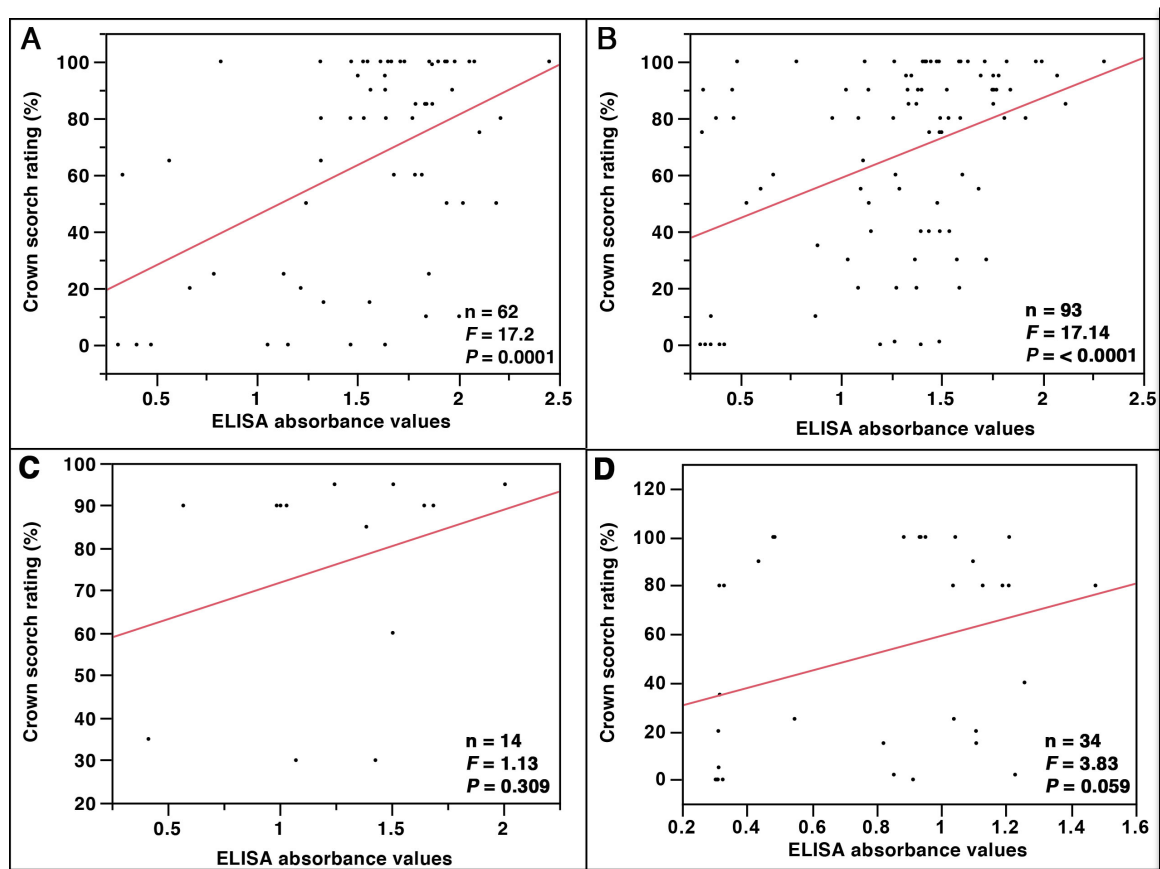
There was a significant association between crown dieback and incidence of *X. fastidiosa* (Table 4). However, no association existed between the presence of *X. fastidiosa* and tree DBH or height for any species tested (data not shown). Scorch percentage was positively correlated for red and pin oaks (*F* was significant at  $P = < .001$  for both species), however, this was not true for sycamore and elm trees (*F* was non significant at  $P = 0.309$  and  $P = 0.059$ , respectfully) (Fig. 3A-D).

**Table 4:** Contingency table: Crown dieback of *X. fastidiosa*-infected and non-infected trees. Infected trees were determined with ELISA.

Tree species	Status of <i>X. fastidiosa</i>	Crown dieback <sup>a</sup>			Test	$\chi^2$	$P > \chi^2$
		0	1	2			
<i>Quercus palustris</i>	absent	24	12	6	Likelihood ratio	36.41	< 0.0001
	present	6	16	40	Pearson	33.91	< 0.0001
<i>Quercus rubra</i>	absent	48	20	12	Likelihood ratio	38.48	< 0.0001
	present	15	39	39	Pearson	36.93	< 0.0001
<i>Platanus occidentalis</i>	absent	16	5	6	Likelihood ratio	14.587	0.0007
	present	1	2	11	Pearson	13.196	0.0014
<i>Ulmus americana</i>	absent	12	10	3	Likelihood ratio	13.026	0.0015
	present	3	19	12	Pearson	12.511	0.0019

<sup>a</sup> Crown dieback rating: 0 = No tip dieback (minimal deadwood < 2.5 cm in diameter), 1 = < 25% dieback (dieback observed at tips of branches, deadwood generally < 5 cm in diameter), 2 =  $\geq$  25% dieback (reduction in crown size, deadwood > 5 cm in diameter as well as failure of entire limbs, epicormic sprouting).

**Figure 3:** Logistic fit of ELISA values in correlation with scorch rating on the most commonly sampled urban trees infected with *X. fastidiosa*. A, *Quercus palustris*; B, *Q. rubra*; C, *Platanus occidentalis*; and D, *Ulmus americana*.



## Discussion

*X. fastidiosa* was detected in 12 urban tree species in the District. All these species have previously been reported as hosts, except for Norway maple and tulip poplar. While we have not fulfilled Koch's postulates for the latter species, an ELISA-positive detection of *X. fastidiosa* suggests an association of *X. fastidiosa* with these tree species. Over the past 30 years, more than 40 species of landscape trees have been found to harbor *X. fastidiosa* (62, 110). Trees that are detrimentally affected by BLS include many species of oak (5, 40, 46, 55, 61, 70), elm (71, 111, 129), sycamore (9, 44, 55, 113), and maple (9, 41, 116). The impact of BLS in the District is prevalent on oak species, particularly pin and red oak, evident by the large amount of crown dieback observed in these two species. Other urban trees such as elm and sycamore were found with BLS but did not exhibit the crown dieback witnessed on red and pin oaks. Various other oak species are adversely impacted by BLS in other metropolitan areas along the east coast. In New Jersey, incidences as high as 30% were noted for northern red oak, pin oak, and scarlet oak (77). Further south, an ELISA-based survey of trees found BLS occurring on symptomatic pin oak, red oak, shingle oak (*Q. imbricaria*) and white oak (*Q. alba*) in 16 cities and towns in Kentucky (40). The study also detected BLS in scarlet oak, post oak (*Q. stellata*), water oak (*Q. nigra*), swamp oak (*Q. bicolor*), chestnut oak (*Q. prinus*), and willow oak in most major metropolitan areas in Tennessee. Within the Carolinas, positive ELISA-detection of *X. fastidiosa* was reported in pin oak and southern red oak (*Q. falcata*) (43). The association of BLS with oak decline in urban settings extends as far south as Florida and includes turkey oak (*Q. laevis*) and southern red oak (5).

In our study, the majority of asymptomatic trees neighboring *X. fastidiosa*-infected trees were free of the bacterium (137 out of 159 trees). However, *X. fastidiosa* was still detected in several samples of red and pin oak, elm, and mulberry. Other studies have found similar results when symptomless oak and elm trees were tested in areas where *X. fastidiosa* was established (5, 15, 46, 114). Tree species including boxelder (*Acer negundo*) and buckeye (*Aesculus x hybrid*) were previously reported with asymptomatic infection in the District (84). Similarly, in an investigation of the natural occurrence of *X. fastidiosa* in a Maryland nursery, crape myrtle (*Lagerstroemia indica*) within the nursery, as well as sassafras (*Sassafras albidum*) and mimosa (*Albizia julibrissin*) trees bordering the nursery were found to harbor *X. fastidiosa* without expressing leaf scorch symptoms (62). The reason for inconsistent development of symptoms among various tree species after *X. fastidiosa*-infection remains unresolved.

Approximately half of our study sites possessed more than one infected tree. It is not clear why disease incidence is limited to individual trees at certain sites, or the rate in which *X. fastidiosa* is disseminated among susceptible neighboring trees. The spread of BLS is primarily attributed to xylem-feeding insect vectors including a wide range of treehoppers (Membracidae) and leafhoppers (Cicadellidae), which are found abundantly in urban environments (6, 133). The amount of vector feeding required before a tree becomes infected is unknown, and the inoculation pressure sufficient to cause infection in some plants may differ from other plants (17). If each tree species has a different vector inoculation pressure threshold required for infection, the amount of inoculum available to insect vectors within the area may play a significant role in determining which trees become infected. Consequently, understanding which tree species are source plants for



vector acquisition of *X. fastidiosa* is an important consideration when assessing management options for a particular infection site (131).

For the majority of samples, ELISA results corresponded with PCR results when testing for *X. fastidiosa*. However, discrepancies in detection of *X. fastidiosa* occurred for ginkgo and tulip poplar. Each species tested positive for *X. fastidiosa* with ELISA, but not in PCR. Failure to detect *X. fastidiosa* with PCR may be due to the inability to remove plant-originating inhibitory compounds during the DNA extraction process. For example, with tulip poplar, the lysing product after the maceration step was extremely viscid and the resulting supernatant required additional extraction buffer in order to permeate the DNA binding membrane during DNA extraction; the success of DNA extraction with this anomaly is therefore questionable. In other studies, inhibitory compounds originating from plant or insect tissue were suspected not to be entirely removed during DNA extraction resulting in an inability to amplify DNA using nested PCR (84, 98). This dilemma was partially solved using immunomagnetic separation to isolate bacteria from insect vectors; however, the use of this technique to separate DNA from plant hosts proved unsuccessful (84, 98).

The subspecies *multiplex* was associated with BLS on elm, pin oak, red oak, scarlet oak, bur oak, and sycamore in the District. This subspecies was previously reported to be responsible for disease of woody perennial hosts including the ones found in this study (86, 92, 108). However, subspecies *multiplex* genotype ALSI and subspecies *sandyi* were found associated with mulberry leaf scorch. The subspecies responsible for disease of mulberry has not yet been described (49), and although our PCR assay was instrumental

in determining the genetic variability of the strains found among urban trees, additional molecular analysis is required before assigning the strains found in mulberry to a definitive subspecies. If the strains identified in mulberry are not responsible for disease of amenity trees, then an eradication effort aimed at reducing uncultivated mulberry would not be effective at reducing *X. fastidiosa* infection among amenity trees. Another limitation of the *in vivo* PCR assay used in our investigation is the inability of accounting for multiple subspecies within the same plant sample. Similar to two mulberry trees in this study, cohabitation of genetically variable strains of *X. fastidiosa* has been observed in almond trees (18). If such a cohabitation is occurring in landscape trees, alternative methods must be employed to further characterize the *X. fastidiosa* strains within the District. A probe-based real-time PCR method for multilocus melt typing of *X. fastidiosa* strains in plant samples was suggested to resolve any genotypic diversity without the need for culturing (10).

This study did not attempt to quantify the population of *X. fastidiosa* within each host; however, significant differences in ELISA values were noted among various tree species, as well as among symptomatic and asymptomatic plant tissue. In a previous study, larger ELISA optical absorbance values were found indicative of larger *X. fastidiosa* concentrations within grapevine (*Vitis* spp.) (74). If greater ELISA values are indicative of a larger bacterial population, our data suggests that a larger bacterial population is correlated with severe leaf scorch and crown dieback for red and pin oak. If accurate, this suggests that oak may be a good candidate host plant for *X. fastidiosa* acquisition from insect vectors. Real-time PCR (95) could validate the association between bacterial

concentration, symptom severity, and tree species and ELISA may prove to be a very useful method for quantifying pathogen population.

ELISA values of asymptomatic foliage of infected trees provided new insight regarding the etiology of *X. fastidiosa* within the canopy. The bacterium was commonly detected within asymptomatic foliage of infected red oak, pin oak, and elm, with over half of the samples testing positive in ELISA. Similarly, the bacterium was found in about 50% of tracheary elements taken from symptomless branches of diseased elm trees using electron microscopy (46). The significantly lower ELISA values of asymptomatic foliage compared to the symptomatic foliage suggest that larger bacterial titer is required before symptoms become apparent. On this note, we lack considerably on knowledge of 1) whether systemic movement or repeated inoculation by vectors is the primary mechanism of *X. fastidiosa* dissemination within the crown of a tree, and 2) the duration of latent infection prior to the onset of symptoms.

An economical management option for BLS such as pruning infected branches to eliminate or reduce the spread of *X. fastidiosa* has not been experimentally demonstrated. Although the presence of *X. fastidiosa* in asymptomatic branches is indicative that pruning branches will not always eliminate *X. fastidiosa* from a tree, it could reduce the amount of inoculum available to vectors. Effective acquisition of *X. fastidiosa* by a vector was shown to require a large bacterial population within host tissue (53), and thus removal of infected tree branches that possess a larger bacterial population may reduce the rate in which vectors acquire the bacterium. In this regard, pruning might still be a useful management option for reducing the spread of the bacterium in urban ecosystems.

The 2010 Forest Action Plan for the District developed in accordance with the National Association of State Foresters and the U.S. Forest Service included BLS as an urban forest disease of concern. This study illustrates the regional extent of BLS in an urban environment. The four most commonly infected trees in this study were red oak, pin oak, elm, and sycamore. These four tree species represent 4.9% of urban trees and 19.7% of the total canopy coverage in the District (90). When infected with *X. fastidiosa*, these trees were found with a significantly greater incidence of crown dieback. Although BLS has been known to affect landscape ornamentals in the District for over 50 years (129), less is known about how climate change may influence the epidemiology of this disease in the coming years. Abiotic conditions such as water stress was shown to exacerbate BLS disease severity (83), and the chronic nature of *X. fastidiosa* infection may become increasingly acute if environmental conditions become optimal for vector activity (118) and bacterial propagation in host woody ornamentals (30).

## **Chapter 3: Population structure of the bacterial pathogen *Xylella fastidiosa* among street trees in Washington D.C**

### **3.0 Abstract**

Bacterial leaf scorch, caused by the bacterial pathogen *Xylella fastidiosa*, is a widely established and problematic disease of landscape ornamentals in Washington D.C. A multi-locus sequence typing analysis was performed using 10 housekeeping loci for *X. fastidiosa* strains in order to better understand the epidemiology of leaf scorch disease in this municipal environment. Samples were collected from 7 different tree species located throughout the District of Columbia, consisting of 101 samples of symptomatic and asymptomatic foliage from 84 different trees. Five clonal strains of the bacteria were discovered. These strains were host specific, with only one particular clone being associated with members of the red oak family, American elm, American sycamore, and two clones being associated with mulberry. Strains found for asymptomatic foliage were the same as strains from the symptomatic foliage on individual trees. Cross transmission of the clonal strains was not observed at sites with multiple species of infected trees within an approx. 25 m radius of one another. *X. fastidiosa* strain specificity observed for each genus of tree suggests a highly specialized host-pathogen relationship.

### 3.1 Introduction

*Xylella fastidiosa* (127) is a bacterial pathogen and causal agent of numerous diseases of agricultural crops throughout the Americas. Several of the most significant diseases caused by *X. fastidiosa* (127) include the Pierce's disease of grapevine (24), citrus variegated chlorosis (14), almond leaf scald (18), and phony peach disease (64). In addition to leaf-scorching diseases inflicted on many woody perennial cash crops, urban forests of several eastern-Atlantic municipalities share a similar fate (9, 42-44, 84, 115). In urban environments, *X. fastidiosa* (127) causes a chronic leaf-scorching disease often referred to as bacterial leaf scorch (BLS) (110). Leaf scorch symptoms first develop on an isolated tree branch and an annual progression of leaf scorch ensues, eventually leading to outright tree mortality.

*X. fastidiosa* is responsible for the decline of mature oak (*Quercus* spp.), elm (*Ulmus* spp.), sycamore (*Platanus* spp.), maple (*Acer* spp.), and red mulberry (*Morus rubra*) (46, 73, 116). In New Jersey, disease incidence as high as 30% was noted for northern red oak (*Q. rubra*), pin oak (*Q. palustris*), and scarlet oak (*Q. coccinea*) (77). This disease is also prevalent in Washington D.C. (the District), where a significant association was found between crown dieback and BLS infection on several common street trees including red oak, pin oak, American sycamore (*P. occidentalis*) and American elm (*U. americana*) (38). Despite the efforts of municipal resource managers, trees begin a slow and irreversible decline once infected by *X. fastidiosa*. Consequently, BLS was declared an urban forest disease of concern the 2010 Forest Action Plan for the District (<http://www.forestactionplans.org/states/district-columbia>).

Since the initial description of *X. fastidiosa* (127), six subspecies have been described. These include i) *X. fastidiosa* subsp. *fastidiosa* (108), ii) *X. fastidiosa* subsp. *multiplex* (108), iii) *X. fastidiosa* subsp. *pauca* (108), iv) *X. fastidiosa* subsp. *sandyi* (109), v) *X. fastidiosa* subsp. *tashke* (104), and iv) *X. fastidiosa* subsp. *morus* (91). The first three subspecies were described together based on serological and phenotypic information, DNA-DNA homology, and sequencing of the 16S-23S intergenic spacer (ITS) region (108), while the later three subspecies were described individually thereafter once discovering separate monophyletic clade formations when constructing phylogenies using multi-locus sequence data (104, 109). Differences in host range are evident not only among these six subspecies (49, 91, 108, 109), but also within particular subgroups, as different genotypes within a subspecies also demonstrate host specificity (92).

In several agricultural systems, the dynamics of host specificity for particular subspecific clonal complexes was demonstrated with multi-locus sequence typing (MLST), a method widely used to detect recombination and sequence typing of *X. fastidiosa* [25]. For example, *X. fastidiosa* subsp. *fastidiosa* is known for causing Pierce's disease (PD) of grapevine and subsp. *multiplex* is known for causing almond leaf scorch. Both subspecies are capable of infecting almond trees (18), although subsp. *multiplex* cannot infect grapevine (3, 18). This anomaly of non-reciprocal symptom development after cross-inoculation was also evident in a MLST based phylogeny. While the almond leaf scorch strains were nested within both the subsp. *fastidiosa* clade and the subsp. *multiplex* clade, all PD strains were confined to the subsp. *fastidiosa* clade (107). Using isolates derived from four regions of Brazil, *X. fastidiosa* strains from citrus plants were not capable of sustainable colonization of coffee plants and strains isolated from coffee plants failed to

develop symptoms in citrus (2). The strains isolated from each host species exhibited phylogenetically distinct subgroups (2). Similarly, clade differentiation was evident among subspecies *fastidiosa* and subspecies *sandyi* (109), two subspecies that were proven to be incapable of infecting each other's major host plant, grapevine and oleander, respectively (101).

Our previous survey identified several subspecies of *X. fastidiosa* responsible for bacterial leaf scorch in the District of Columbia (38). *X. fastidiosa* subsp. *multiplex* genotype ALSII was found in association with decline of oak, sycamore, and elm. Moreover, white mulberry was found associated with subsp. *sandyi* and subsp. *multiplex* genotype ALSI. Some of this information agrees with previous findings for oak and sycamore (92, 108). However, it has been proposed that white mulberry is infected with the newly described *X. fastidiosa* subsp. *morus* (92), and that subsp. *multiplex* (108), or at least an intermediate form of subsp. *multiplex* (91), is responsible for elm leaf scorch. It is clear that in urban environments, there exists differentiation of *X. fastidiosa* at the subspecies level (49). Furthermore, whether these subspecies remain confined to a single host in a system where numerous different strains of the bacteria are endemic has not yet been demonstrated.

In order to better understand the epidemiology of leaf scorch disease caused by *X. fastidiosa* in urban environments, a MLST scheme for *X. fastidiosa* was used to genetically characterize *X. fastidiosa* among urban trees in the District. Our objective was to determine the strain diversity of *X. fastidiosa* infecting various tree species and elucidate any major clonal complexes that demonstrate host specificity.

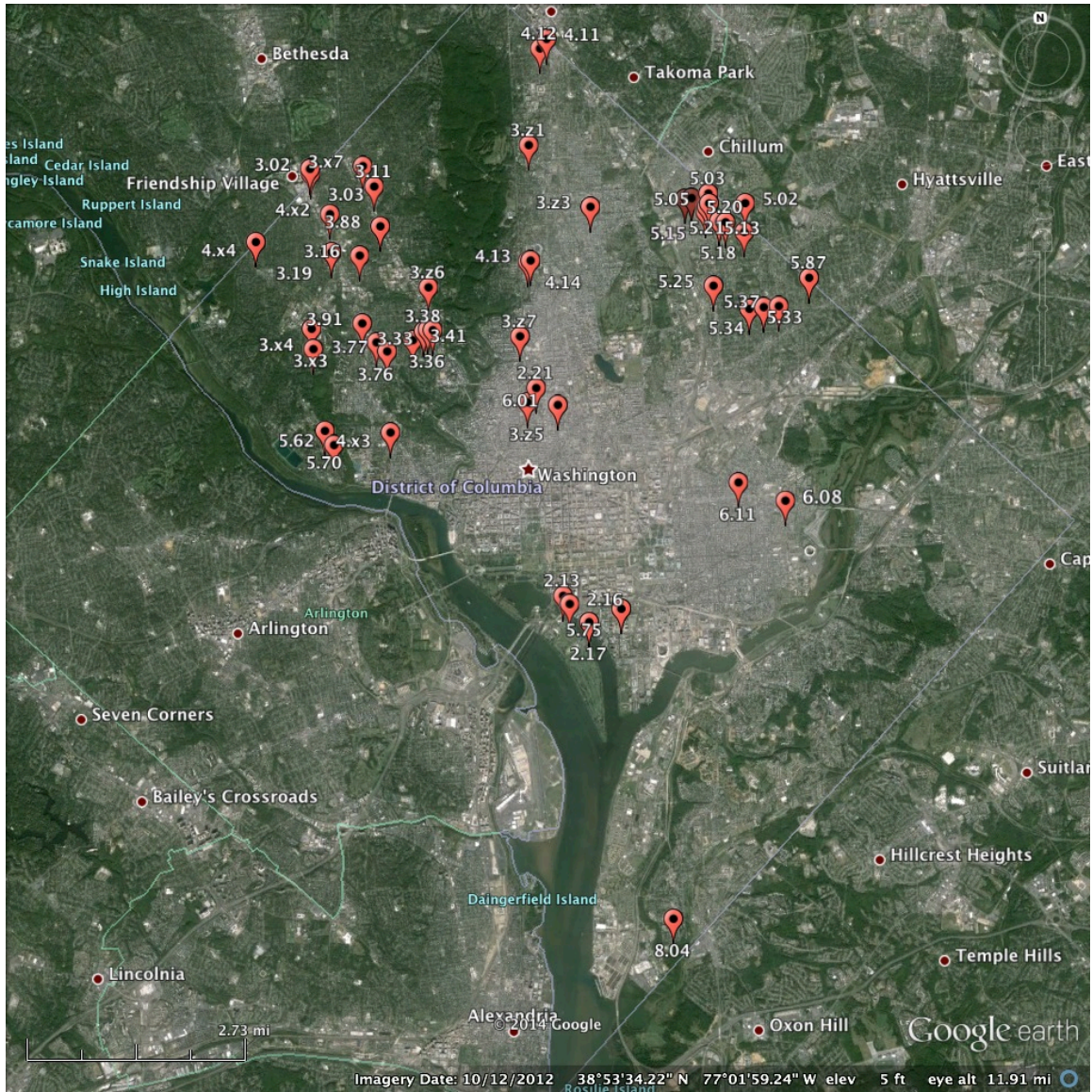


## 3.2 Materials and Methods

### 3.2.1 Selection of *X. fastidiosa* Infected Trees

Sites that included trees infected with *X. fastidiosa* were selected throughout the District (Fig. 4). A site is defined as all infected trees within an approx. 25 m radius of each other. Trees were previously determined to be infected with *X. fastidiosa* based on the results of a survey that used an enzyme-linked immunosorbent assay (ELISA) and PCR (38). Samples chosen from the 2012 survey and analyzed in this investigation consisted of symptomatic and asymptomatic foliage of infected trees and symptomatic and asymptomatic foliage of neighboring infected trees. A total of 101 samples from 84 urban trees at 56 different sites were used for the MLST analysis (Table 5). The sampled tree population consisted of 7 different species, including 20 red oaks (*Quercus rubra*), 29 pin oaks (*Quercus palustris*), 2 scarlet oaks (*Quercus coccinea*), 1 willow oak (*Quercus phellos*), 17 American elms (*Ulmus americana*), 11 American sycamores (*Platanus occidentalis*), and 4 white mulberries (*Morus alba*) (Table 6). Of the 84 sampled trees, 17 were sampled from both the symptomatic and asymptomatic portion of the crown, and 14 trees were entirely asymptomatic.

Figure 4: Location of collection sites in Washington D.C.



**Table 5:** Location and site information of all trees used in the multi-locus sequencing analysis.

\* denotes that at least two trees of the same species were sampled at the site

\*\* denotes that an infected tree of a different genus was within an a 25 m radius

<u>Isolate Name</u>	<u>Coordinates (N)</u>	<u>Coordinates (W)</u>	<u>Host Scientific Name</u>
2.13 AS/S1 *	38° 52'44.8"	77° 00' 49.2"	<i>Quercus palustris</i>
2.13 S1 *	38° 52'44.8"	77° 00' 49.2"	<i>Quercus palustris</i>
2.13 S4 *	38° 52'44.8"	77° 00' 49.2"	<i>Quercus palustris</i>
2.16 S1	38° 52'42.1"	77° 00' 45.3"	<i>Platanus occidentalis</i>
2.17 AS/S4 **	38° 52' 27.2"	77° 01' 29.3"	<i>Morus alba</i>
2.17 S1 **	38° 52' 27.2"	77° 01' 29.3"	<i>Platanus occidentalis</i>
2.17 S4 **	38° 52' 27.2"	77° 01' 29.3"	<i>Morus alba</i>
2.21 S1	38° 55' 02.8"	77° 02' 04.0"	<i>Quercus rubra</i>
3.02 AS3	38° 57' 33.9"	77° 05' 05.6"	<i>Quercus palustris</i>
3.11 AS3	38° 57' 20.3"	77° 04' 12.2"	<i>Quercus rubra</i>
3.16 S7 *	38° 56' 35.32"	77° 04' 27.65"	<i>Quercus rubra</i>
3.19 S1	38° 56' 38.6"	77° 04' 50.9"	<i>Ulmus americana</i>
3.33 S1	38° 55' 44.2"	77° 03' 28.9"	<i>Quercus palustris</i>
3.36 AS3 *	38° 55' 44.7"	77° 03' 33.1"	<i>Quercus palustris</i>
3.36 S1 *	38° 55' 44.7"	77° 03' 33.1"	<i>Quercus palustris</i>
3.38 AS3	38° 55' 43.8"	77° 03' 36.6"	<i>Quercus palustris</i>
3.41 S1	38° 55' 38.3"	77° 03' 46.1"	<i>Quercus rubra</i>
3.76 AS3	38° 55' 37.8"	77° 04' 16.8"	<i>Quercus palustris</i>
3.77 AS4 *	38° 55'31.7"	77° 04' 8.2"	<i>Quercus palustris</i>
3.77 S3 *	38° 55'31.7"	77° 04' 8.2"	<i>Quercus palustris</i>
3.88 AS3 *	38° 55'54.0"	77° 04' 08.7"	<i>Quercus palustris</i>
3.88 S1 *	38° 55'54.0"	77° 04' 08.7"	<i>Quercus palustris</i>
3.88 S2 *	38° 55'54.0"	77° 04' 08.7"	<i>Quercus palustris</i>
3.91 AS/S1	38° 55' 51.2"	77° 04' 28.2"	<i>Quercus palustris</i>
3.91 S1	38° 55' 51.2"	77° 04' 28.2"	<i>Quercus palustris</i>
3.x3 S1	38° 55' 48.83"	77° 5' 11.11"	<i>Quercus palustris</i>
3.x4 S2	38° 55' 36.48"	77° 5' 9.996"	<i>Platanus occidentalis</i>
3.x7 S1	38° 57' 33.91"	77° 04' 21.00"	<i>Quercus rubra</i>
3.z1 AS/S5 *	38° 57' 42.56"	77° 1' 59.96"	<i>Quercus palustris</i>
3.z1 S1 *	38° 57' 42.56"	77° 1' 59.96"	<i>Quercus palustris</i>
3.z1 S2 *	38° 57' 42.56"	77° 1' 59.96"	<i>Quercus palustris</i>
3.z1 S3 *	38° 57' 42.56"	77° 1' 59.96"	<i>Quercus palustris</i>
3.z1 S5 *	38° 57' 42.56"	77° 1' 59.96"	<i>Quercus palustris</i>
3.z1 S8 *	38° 57' 42.56"	77° 1' 59.96"	<i>Quercus palustris</i>

3.z3 S1	38° 57' 00.33"	77° 01' 09.90"	<i>Quercus rubra</i>
3.z5 S1	38° 54' 53.71"	77° 2' 11.90"	<i>Ulmus americana</i>
3.z7 S1 *	38° 55' 37.35"	77° 2' 15.50"	<i>Quercus palustris</i>
3.z7 S4 *	38° 55' 37.35"	77° 2' 15.50"	<i>Quercus palustris</i>
4.11 AS/S1	38° 58' 51.9"	77° 01' 40.1"	<i>Quercus palustris</i>
4.11 S1	38° 58' 51.9"	77° 01' 40.1"	<i>Quercus palustris</i>
4.12 S1	38° 58' 46.1"	77° 01' 46.3"	<i>Quercus coccinea</i>
4.13 S2	38° 56' 26.7"	77° 02' 03.32"	<i>Quercus palustris</i>
4.14 S1 **	38° 56' 25.7"	77° 02' 05.2"	<i>Platanus occidentalis</i>
4.14 S2 **	38° 56' 25.7"	77° 02' 05.2"	<i>Ulmus americana</i>
4.98 S1	-	-	<i>Ulmus americana</i>
4.x2 S1	38° 57' 3.56"	77° 4' 50.73"	<i>Quercus rubra</i>
4.x3 AS/S1	38° 54' 38.28"	77°04' 9.30"	<i>Platanus occidentalis</i>
4.x3 S1	38° 54' 38.28"	77°04' 9.30"	<i>Platanus occidentalis</i>
4.x4 AS4 **	38° 56' 47.84"	77° 05' 54.59"	<i>Quercus coccinea</i>
4.x4 S1 **	38° 56' 47.84"	77° 05' 54.59"	<i>Quercus rubra</i>
5.02 AS2 **	38° 56' 57.6"	76° 58' 58.8"	<i>Morus alba</i>
5.02 AS3 **	38° 56' 57.6"	76° 58' 58.8"	<i>Quercus rubra</i>
5.02 S1 **	38° 56' 57.6"	76° 58' 58.8"	<i>Quercus rubra</i>
5.03 S1 *	38° 57' 05.1"	76° 59' 30.0"	<i>Platanus occidentalis</i>
5.03 S2 *	38° 57' 05.1"	76° 59' 30.0"	<i>Platanus occidentalis</i>
5.05 S1	38° 56' 58.8"	76° 59' 30.5"	<i>Platanus occidentalis</i>
5.13 S1	38° 56' 56.5"	76° 59' 32.0"	<i>Quercus palustris</i>
5.15 AS/S1	38° 56'52.6"	76° 59' 33.0"	<i>Platanus occidentalis</i>
5.15 S1	38° 56'52.6"	76° 59' 33.0"	<i>Platanus occidentalis</i>
5.18 AS2	38° 56' 46.8"	76° 59' 22.3"	<i>Quercus rubra</i>
5.2 S1 **	38° 56' 45.9"	76° 59' 16.5"	<i>Quercus palustris</i>
5.2 S3 **	38° 56' 45.9"	76° 59' 16.5"	<i>Platanus occidentalis</i>
5.21 AS3 *	38° 56' 38.8"	76° 59' 01.3"	<i>Quercus rubra</i>
5.21 S1 *	38° 56' 38.8"	76° 59' 01.3"	<i>Quercus rubra</i>
5.25 S1	38° 56' 04.3"	76° 59' 29.6"	<i>Quercus rubra</i>
5.33 S1	38° 55' 48.1"	76° 59' 00.6"	<i>Ulmus americana</i>
5.34 S1	38° 55' 48.5"	76° 58' 48.3"	<i>Ulmus americana</i>
5.37 AS/S2 *	38° 55' 49.0"	76° 58' 35.3"	<i>Ulmus americana</i>
5.37 S1 *	38° 55' 49.0"	76° 58' 35.3"	<i>Ulmus americana</i>
5.37 S2 *	38° 55' 49.0"	76° 58' 35.3"	<i>Ulmus americana</i>
5.62 AS/S1 **	38° 54' 32.2"	77° 04' 57.2"	<i>Quercus rubra</i>
5.62 S1 **	38° 54' 32.2"	77° 04' 57.2"	<i>Quercus rubra</i>
5.62 S3 **	38° 54' 32.2"	77° 04' 57.2"	<i>Ulmus americana</i>
5.7 S2	38° 54' 41.5"	77° 05' 04.2"	<i>Ulmus americana</i>
5.75 AS/S2 *	38° 52' 35.1"	77° 01' 01.2"	<i>Quercus rubra</i>
5.75 S1 *	38° 52' 35.1"	77° 01' 01.2"	<i>Quercus rubra</i>
5.75 S2 *	38° 52' 35.1"	77° 01' 01.2"	<i>Quercus rubra</i>

5.82 AS/S1	38° 57' 02.1"	76° 59' 49.4"	<i>Ulmus americana</i>
5.82 S1	38° 57' 02.1"	76° 59' 49.4"	<i>Ulmus americana</i>
5.83 AS/S1	38° 57' 02.3"	76° 59' 44.5"	<i>Ulmus americana</i>
5.83 S1	38° 57' 02.3"	76° 59' 44.5"	<i>Ulmus americana</i>
5.87 AS/S1 **	38° 56' 06.6"	76° 58' 08.3"	<i>Quercus palustris</i>
5.87 AS/S2 **	38° 56' 06.6"	76° 58' 08.3"	<i>Quercus palustris</i>
5.87 AS/S4 **	38° 56' 06.6"	76° 58' 08.3"	<i>Morus alba</i>
5.87 S1 **	38° 56' 06.6"	76° 58' 08.3"	<i>Quercus palustris</i>
5.87 S2 **	38° 56' 06.6"	76° 58' 08.3"	<i>Quercus palustris</i>
5.87 S4 **	38° 56' 06.6"	76° 58' 08.3"	<i>Morus alba</i>
5.92 AS/S1	38° 57' 05.0"	76° 59' 29.86"	<i>Quercus phellos</i>
5.92 S1	38° 57' 05.0"	76° 59' 29.86"	<i>Quercus phellos</i>
6.01 S1 **	38° 54' 51.18"	77° 54' 46.42"	<i>Platanus occidentalis</i>
6.01 S2 **	38° 54' 51.18"	77° 54' 46.42"	<i>Quercus palustris</i>
6.08 AS/S5 **	38° 53' 40.4"	76° 58' 38.2"	<i>Morus alba</i>
6.08 S2 **	38° 53' 40.4"	76° 58' 38.2"	<i>Ulmus americana</i>
6.08 S5 **	38° 53' 40.4"	76° 58' 38.2"	<i>Morus alba</i>
6.11 S1	38° 53' 53.7"	76° 59' 18.0"	<i>Quercus rubra</i>
8.04 AS3 *	38° 49' 9.66"	77° 0' 31.36"	<i>Ulmus americana</i>
8.04 S1 *	38° 49' 9.66"	77° 0' 31.36"	<i>Ulmus americana</i>

**Table 6:** Samples collected for the MLST analysis. Symptomatic and asymptomatic trees infected with *X. fastidiosa* were sampled. Some trees were sampled from both the asymptomatic and symptomatic portion of the canopy.

Tree Species	Total # of Trees	Total # of Samples	Asymptomatic foliage	Symptomatic foliage
Elm	17	20	4	16
Mulberry	4	7	4	3
Pin Oak	29	35	12	23
Red Oak	20	22	6	16
Scarlet Oak	2	2	1	1
Sycamore	11	13	3	10
Willow				
Oak	1	2	1	1
<b>Total</b>	<b>84</b>	<b>101</b>	<b>31</b>	<b>70</b>

### 3.2.2 DNA extraction, Amplification and Sequencing

Total DNA was extracted from petiole samples collected from infected trees using DNeasy kit (Qiagen Inc, Valencia, CA) according to the manufacturer's protocol, with the following modification. The maceration step in the protocol was carried out in 2 ml lysing matrix tubes consisting of lysing matrix A, and samples were processed twice in a MP FastPrep-24 instrument for 40 s at a speed of 4.5 m/s. Ten housekeeping loci were selected to form the basis of MLST typing of *X. fastidiosa* in the District (Table 7). These pre-established gene regions were chosen for this organism due to the consistent level of sequence type diversity among at least 5 of the loci, variety in biochemical functions, and possession of  $K_A/K_S$  values typical of moderately constrained genes ( $< 1$ ),  $K_A$  representing synonymous mutations and  $K_S$  representing non-synonymous mutations (107, 109, 132).

For all reactions, 2  $\mu$ l of total DNA extract was added to 12.5  $\mu$ l of 2X GoTaq Green Master Mix (Promega Corporation, Madison, WI), 1.5  $\mu$ l (10  $\mu$ M solution) of each primer set designed for ten housekeeping loci of *X. fastidiosa* (Table 7), and 7.5  $\mu$ l of molecular grade water for a total volume of 25  $\mu$ l per reaction. Dilutions of DNA extracts were performed at a 1:100 ratio with nuclease free water (Promega Corporation, Madison, WI) for optimal amplification. All amplifications were performed in a BIO-RAD S1000 Thermal Cycler (BIO-RAD, Hercules, CA), and carried out with the following cycle program: 5 min at 94°C, followed by 39 cycles of for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension step of 10 min at 72°C. The extension time was lengthened from 1 min to 90 seconds for genes greater than 1,000 base pairs. PCR products were run in a 1.5% agarose gel pre-stained with GelRed (Biotium, Hayward,

CA) at a 1:10,000 ratio of stock reagent to molten agarose (Fisher Scientific, Pittsburgh, PA) and 1X sodium boric acid conductive medium (12).

**Table 7:** Primers and gene regions selected for the multi-locus analysis.

\* denotes a newly designed primer for this analysis

Temecula Annotation	bp	Gene	Primer sequences	Primer References
PD0104	379	<i>holC</i>	5'-ATGGCACGCGCCGACTTCT-3' 5'-ATGTCGTGTTTGTTCATGTGCAGG-3'	Yuan et al. 2010
PD0210	429	<i>rfbD</i>	5'-TTTGGTGATTGAGCCGAGGGT-3' 5'-CCATAAACGGCCGCTTTC-3'	Scally et al. 2005
PD0259	557	<i>nuoL</i>	5'-TAGCGACTTACGGTTACTGGGC-3' 5'-ACCACCGATCCACAACGCAT-3'	Yuan et al. 2010
PD0261	1311	<i>nuoN</i>	5'-GGGTAAACATTGCCGATCT-3' 5'-CGGGTTCCAAAGGATTCCTAA-3'	Scally et al. 2005
PD1516	951	<i>gltT</i>	5'-TTGGGTGTGGGTACGTTGCTG-3' 5'-CGCTGCCTCGTAAACCGTTGT-3'	Scally et al. 2005
PD1840	1170	<i>cysG</i>	5'-GGCGGCGGTAAGGTTG-3' 5'-GCGTATGTCTGTGCGGTGTGC-3'	Scally et al. 2005
PD1775	531	<i>petC</i>	5'-CTGCCATTCGTTGAAGTACCT-3' 5'-CGTCCCTCCCAATAAGCCT-3'	Scally et al. 2005
PD0148	873	<i>pilU</i>	5'-CAATGAAGATTCACGGCAATA-3' 5'-ATAGTTAATGGCTCCGCTATG-3'	Scally et al. 2005
PD1047	1218	<i>leuA</i>	5'-GGGCGTAGACATTATCGAGAC-3' 5'-GTATCGTTGTGGCGTACACTG-3'	Scally et al. 2005
PD1465	642	<i>lacF</i>	5'-TTGCTGGTCCTGCGGTGTTG-3' 5'-CCTCGGGTCATCACATAAGGC-3'	Scally et al. 2005
PD1516*	504	<i>gltT</i>	5'-TTTTTCAGGGGTGTCGCGC-3' * 5'-TTCCAACGTTACTGGACGCT-3' *	This study
PD1840*	800	<i>cysG</i>	5'-CCAAACATAGAAGCACGCCG-3' * 5'-CGTATGTCTGTGCGGTGTG-3' *	This study
PD1047*	564	<i>leuA</i>	5'-GGCCAGTGCTGTGTTTTGTT-3' * 5'-GGGCTACTTGCTGGAGGAAG-3' *	This study
PD1465*	829	<i>lacF</i>	5'-TTCTTTGGTGGGTGGGTGT-3' * 5'-CACACAGCATCAACGTCGTC-3' *	This study

Once there was visual confirmation of the target length gene product, the PCR products were cleaned using the EXOSAP-IT PCR purification kit (Affymetrix, Inc., Santa Clara, CA). When necessary, gel extractions of target length gene product were performed using the gel extraction kit Nucleospin (Macherey-Nagel Inc., Bethlehem, PA). The cleaned PCR products were sequenced with an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA) by MC Lab (San Francisco, CA).

### **3.3.3 Sequence Analysis and Submission**

All sequence data was processed using the software Geneious v6.1.6 (66). The forward and reverse read of each locus were quality-trimmed, *de novo* assembled, and ambiguities were resolved with visual analysis of the chromatograph and re-sequencing when necessary. The consensus sequence was extracted from each assembly and sequences were aligned using the Geneious alignment option (66). In order to compare the STs in our study to those published in [www.pubmlst.org](http://www.pubmlst.org), an additional set of primers were required in order to meet the sequence requirements necessary for website entry. The new primers were designed to span the gene region at each locus not formerly covered by the original primers used in the analysis. The newly designed primers were created using the primer3 v0.4.0 plug-in for Geneious (123). A subset of 3 samples for each ST at each of the seven MLST loci were further sequenced and included in the sample assemblies before an allelic profile of each host species was submitted to [pubmlst.org](http://pubmlst.org). In addition, all *X. fastidiosa* alleles found for each host species was uploaded to GenBank, accession numbers KM487213-KM487276 and KM590452-KM590457.



### 3.3.4 Genetic Relatedness Analysis

All ten loci were concatenated for each bacterial sequence type (ST) and a distance tree was created using the Geneious tree building option in Geneious (66). A phylogenetic tree would be inappropriate given the historical intersubspecific homologous recombination (IHR) events previously determined for strains in our analysis (91). The program was run using the Tamura-Nei genetic distance model with a neighbor-joining tree-building algorithm. Summary of the topology posteriors was visualized in a majority rule consensus tree. A bootstrap re-sampling technique was used for approximating sampling distributions. All STs were run with 5 reference sequences in order to determine relatedness of strains from the District with strains representative of well-defined subspecies. The reference strains included in the phylogenetic analysis were; Temecula 1 (124), EB92.1 (134), M23(21), represented subsp. *fastidiosa*; M12 (21), represented subsp. *multiplex*; and 9a5c (117), represented the subspecies *pauca* which was used as an outgroup.

### 3.4 Results

The *X. fastidiosa* allelic profiles from 101 samples derived from 84 infected trees yielded 5 unique STs (Table 8). The STs in our analysis corresponded to previously established STs for *X. fastidiosa* published at pubmlst.org (Table 9). Each tree genus was generally associated with a single unique *X. fastidiosa* ST; ST-1 (ST-9 in pubmlst.org), associated with members of the red oak family; red oak, pin oak, scarlet oak, and willow oak; ST-2 (ST-8) associated with American sycamore, ST-3 (ST-41) associated with American elm, and ST-4 and ST-5 (ST-29) associated with mulberry (Table 9). The allelic profiles of the

three loci, *gltT*, *holC*, and *cysG*, were capable of distinguishing the sequence types found within the each genus of host.

**Table 8:** Allelic profiles of each sample in the analysis.

\* denotes an outlying allelic profile from the consensus host specific sequence types

( ) Parenthesis indicate the allele numbers found on pubmlst.org after resequencing with additional primers

Tree species	Tree Code	<i>holC</i>	<i>nuoL</i>	<i>gltT</i>	<i>cysG</i>	<i>petC</i>	<i>leuA</i>	<i>lacF</i>	<i>rfbD</i>	<i>nuoN</i>	<i>pilU</i>
Elm	3.19 S1	2 (9)	-	-	-	-	-	-	-	-	-
Elm	4.14 S2	2 (9)	-	2	2	-	-	-	-	-	-
Elm	4.98 S1	2 (9)	-	2	-	-	-	-	-	-	-
Elm	5.33 S1	2 (9)	-	2	-	-	-	-	-	-	-
Elm	5.34 S1	2 (9)	1(3)	2	2	1 (3)	1	1 (5)	-	1	1
Elm	5.37 S1	2 (9)	1 (3)	2 (3)	2 (18)	1 (3)	1 (3)	1 (5)	1	1	1
Elm	5.37 AS/S2	2 (9)	1 (3)	2	-	1 (3)	-	1 (5)	-	1	1
Elm	5.37 S2	2 (9)	1 (3)	2 (3)	2	1 (3)	1	1 (5)	-	1	1
Elm	5.62 S3	2	1 (3)	2	-	1	1	1	-	1	1
Elm	5.7 S2	2 (9)	-	-	-	-	-	-	-	-	-
Elm	5.82 AS/S1	2	-	-	-	-	-	1	-	-	-
Elm	5.82 S1	2 (9)	1 (3)	2	2	1 (3)	1	1 (5)	-	1	1
Elm	5.83 AS/S1	2 (9)	-	-	-	-	1 (3)	-	-	-	-
Elm	5.83 S1	2 (9)	1 (3)	2	2 (18)	1 (3)	1	1 (5)	1	1	1

Elm	6.08 S1	2 (9)	-	2	-	-	-	-	-	-	-
Elm	6.08 S2	2 (9)	1 (3)	2 (3)	2 (18)	1 (3)	1	1 (5)	-	1	1
Elm	8.04 S1	2 (9)	-	-	-	-	-	-	-	-	-
Elm	8.04 AS3	2 (9)	-	2 (3)	-	-	-	-	-	-	-
Elm	3.z5 S1	2 (9)	1 (3)	2	2 (18)	1	1	1 (5)	1	1	1
<b>Elm</b>	<b>3.z6* S1</b>	<b>1 (4)</b>	-	<b>1 (4)</b>	<b>1 (5)</b>	-	-	-	-	-	-
Mulberry	2.17 AS/S4	3 (5)	2 (4)	2	2 (18)	1 (3)	2	2	1	3	2
Mulberry	2.17 S4	3 (5)	2 (4)	2 (3)	2 (18)	1 (3)	2	2	1	2	2
Mulberry	5.02 AS2	3 (5)	2 (4)	2 (3)	2 (18)	1 (3)	2	2	1	3	2
Mulberry	5.87 AS/S4	3 (5)	2 (4)	2	2 (18)	1 (3)	2 (4)	2 (6)	1	3	2
Mulberry	5.87 S4	3 (5)	2 (4)	2	2	1 (3)	2 (4)	2 (6)	1	3	2
Mulberry	6.08 AS/S5	3 (5)	2 (4)	2	2 (18)	1	2 (4)	2 (6)	1	2	2
Mulberry	6.08 S5	3 (5)	2 (4)	2	2 (18)	1 (3)	2	2	1	2	2
Pin Oak	2.13 AS/S1	1 (4)	1 (3)	1 (4)	1	1 (3)	1	1	1	1	1
Pin Oak	2.13 S1	1 (4)	1 (3)	1 (4)	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	2.13 S4	1 (4)	1 (3)	1 (4)	1	1 (3)	1	1	1	1	1
Pin Oak	3.02 AS3	1 (4)	-	1	-	-	-	-	-	-	-
Pin Oak	3.33 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	3.36 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	3.36 AS3	1 (4)	-	1	1	-	-	-	-	-	-
Pin Oak	3.38 AS3	1 (4)	-	1	1 (5)	-	-	-	-	-	-

Pin Oak	3.76 AS3	1 (4)	-	1	1 (5)	-	-	-	-	-	-
Pin Oak	3.77 S3	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	3.77 AS4	1 (4)	-	1	1 (5)	-	-	-	-	-	-
Pin Oak	3.88 S1	-	-	1	-	-	-	-	-	-	-
Pin Oak	3.88 S2	1 (4)	-	1	-	-	-	-	-	-	-
Pin Oak	3.91 AS/S1	1 (4)	1 (3)	1 (4)	1	1 (3)	1	1	1	1	1
Pin Oak	3.91 S1	1 (4)	1 (3)	1 (4)	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	4.11 AS/S1	-	-	-	-	1 (3)	-	-	-	-	1
Pin Oak	4.11 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	4.13 S2	1 (4)	1 (3)	1	1 (5)	1 (3)	1 (3)	1 (5)	1	1	1
Pin Oak	5.13 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	5.2 S1	1 (4)	-	1	1 (5)	-	-	-	-	-	-
Pin Oak	5.87 AS/S1	1 (4)	1 (3)	1	1	1 (3)	1	1	1	1	1
Pin Oak	5.87 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	5.87 AS/S2	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	5.87 S2	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	5.87 S3	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	6.01 S2	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	3.x3 S1	1 (4)	1 (3)	1	1	1 (3)	1	1	1	1	1
Pin Oak	3.z1 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	3.z1 S2	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1

Pin Oak	3.z1 S3	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	3.z1 AS/S5	1 (4)	1 (3)	1	1	1 (3)	1	1	1	1	1
Pin Oak	3.z1 S5	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Pin Oak	3.z1 S8	1 (4)	1 (3)	1	1	1 (3)	1	1	1	1	1
Pin Oak	3.z7 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1 (3)	1 (5)	1	1	1
Pin Oak	3.z7 S4	1 (4)	-	1	1 (5)	-	-	-	-	-	-
Pin Oak	2.21 S1	1 (4)	1	1	-	1 (3)	1	1	-	1	1
Red Oak	3.03 S1	1 (4)	-	1	1 (5)	-	-	-	-	-	-
Red Oak	3.11 AS3	1 (4)	-	-	-	-	-	-	-	-	-
Red Oak	3.16 S7	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Red Oak	3.41 S1	1 (4)	-	1	1 (5)	-	-	-	-	-	-
Red Oak	5.02 S1	1 (4)	-	1	1 (5)	-	-	-	-	-	-
Red Oak	5.02 AS3	1 (4)	-	1	1 (5)	-	-	-	-	-	-
Red Oak	5.18 AS2	1 (4)	-	-	-	-	-	-	-	-	-
Red Oak	5.21 S1	1 (4)	1 (3)	1	1	1 (3)	1	1	1	1	1
Red Oak	5.21 AS3	1 (4)	-	1	-	-	-	-	-	-	-
Red Oak	5.25 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Red Oak	5.62 AS/S1	1 (4)	-	1	1 (5)	-	1	1 (5)	-	-	-
Red Oak	5.62 S1	1 (4)	-	1	1 (5)	-	-	-	-	-	-
Red Oak	5.75 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1 (3)	1	1	1	1
Red Oak	5.75 AS/S2	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1

Red Oak	5.75 S2	1 (4)	1 (3)	1 (4)	1	1 (3)	1	1	1	1	1
Red Oak	6.11 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1 (3)	1 (5)	1	1	1
Red Oak	3.x7 S1	1 (4)	-	1	1 (5)	-	-	-	-	-	-
Red Oak	3.z3 S1	1 (4)	-	1	1	-	-	-	-	-	-
Red Oak	4.x2 S1	1 (4)	1 (3)	1 (4)	1 (5)	1 (3)	1	1	1	1	1
Red Oak	4.x4 S1	1 (4)	1 (3)	1 (4)	1 (5)	1 (3)	1	1	1	1	1
Red Oak	4.x4 S3	1 (4)	1 (3)	1 (4)	1 (5)	1 (3)	1	1	1	1	1
Scarlet Oak	4.12 S1	1 (4)	1 (3)	1	1 (5)	1 (3)	1	1	1	1	1
Scarlet Oak	4.x4 AS4	1 (4)	-	-	-	-	-	-	-	-	-
Sycamore	2.16 S1	1 (4)	1 (3)	3	1 (5)	1 (3)	1 (3)	1 (5)	1	1	1
Sycamore	2.17 S1	1 (4)	1 (3)	3	1 (5)	1 (3)	1	1	1	1	1
Sycamore	4.14 S1	1 (4)	1 (3)	3	1 (5)	1 (3)	1	1 (5)	1	1	1
Sycamore	5.03 S1	1 (4)	-	3	1 (5)	-	-	-	-	-	-
Sycamore	5.03 S2	1 (4)	-	3 (7)	1 (5)	-	-	1 (5)	-	-	-
Sycamore	5.05 S1	1 (4)	1 (3)	3	1 (5)	1 (3)	1	1	1	1	1
Sycamore	5.15 AS/S1	1 (4)	-	3	1 (5)	-	1	1 (5)	-	-	-
Sycamore	5.15 S1	1 (4)	1 (3)	3	1 (5)	1 (3)	1	1	1	1	1
Sycamore	5.2 S3	1 (4)	-	3 (7)	1 (5)	-	1 (3)	-	-	-	-
Sycamore	6.01 S1	1 (4)	1 (3)	3	1 (5)	1 (3)	1	1	1	1	1
Sycamore	3.x4 AS/S1	1 (4)	-	-	-	-	-	-	-	-	-
Sycamore	4.x3 AS/S1	1 (4)	-	3 (7)	1 (5)	-	1	1 (5)	-	1	1

Sycamore	4.x3 S1	1 (4)	1 (3)	3 (7)	1 (5)	1 (3)	1	1	1	1	1
Willow Oak	5.92 AS/S1	1 (4)	1 (3)	1 (4)	1 (5)	1 (3)	1	1 (5)	-	1	1
Willow Oak	5.92 S1	1 (4)	1 (3)	1 (4)	1 (5)	1 (3)	1 (3)	-	-	1	1

**Table 9:** Consensus of *Xylella fastidiosa* sequence types from tree petiole samples.

( ) Values in parenthesis correspond to alleles and sequence types on PubMLST.org

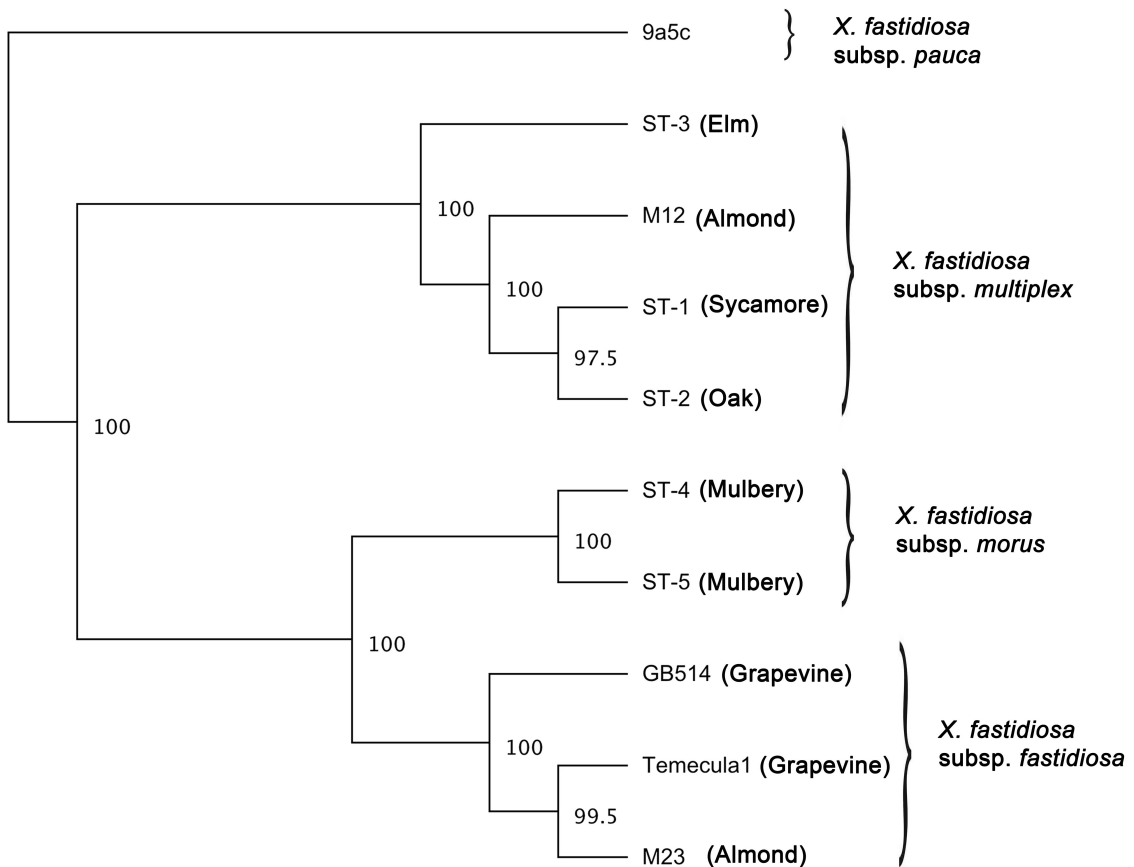
\* asterisk denotes additional loci examined for sequence typing in this analysis

Sequence Type	Allelic Profiles of STs in the District of Columbia									
	<i>holC</i>	<i>nuoL</i>	<i>gltT</i>	<i>cysG</i>	<i>petC</i>	<i>leuA</i>	<i>malF</i>	<i>rfbD</i> *	<i>nuoN</i> *	<i>pilU</i> *
ST-1 (ST-9)	1 (4)	1 (3)	1 (4)	1 (5)	1 (3)	1 (3)	1 (5)	1	1	1
ST-2 (ST-8)	1 (4)	1 (3)	3 (7)	1 (5)	1 (3)	1 (3)	1 (5)	1	1	1
ST-3 (ST-41)	2 (9)	1 (3)	2 (3)	2 (18)	1 (3)	1 (3)	1 (5)	1	1	1
ST-4 (ST-29)	3 (5)	2 (4)	2 (3)	2 (18)	1 (3)	2 (4)	2 (6)	1	2	2
ST-5 (ST-29)	3 (5)	2 (4)	2 (3)	2 (18)	1 (3)	2 (4)	2 (6)	1	3	2

Once the sequence types were identified, a distance tree was created using concatenated sequences of all ten loci totaling 7,416 bp for each ST. Two distinct clades were found in the distance tree: the mulberry strains nested close with the *X. fastidiosa* subsp. *fastidiosa* reference strains Temecula1, GB92.1, and M23, while the amenity tree strains were nested closely with the subsp. *multiplex* strain M12 (Fig. 5). The two clonal complexes in our analysis, defined as members within a complex sharing at least seven of ten loci, were from the mulberry strains and the amenity tree strains. The two strains from mulberry that comprise one of these complexes differed from each other by a single SNP at the *nuoN* locus (Table 10). Within the amenity tree complex, the sycamore and oak

strains differed from each other by a single SNP at the *gltT* locus. Elm was the outlier in this complex, only sharing 7 loci with oak and sycamore. The elm alleles for *gltT* and *cysG* were identical to the alleles for the mulberry strains and different from the oak and sycamore strains.

**Figure 5:** Distance tree of sequence types found infecting urban trees. A distance tree was constructed with 7,416 bp of concatenated sequence data for each *X. fastidiosa* sequence type. The two mulberry strains form a clade that represents the newly described subspecies *morus*, while amenity tree strains nest closely within the subsp. *multiplex* clade. Percentages represent bootstrap support from the re-sampling distribution.





**Table 10:** All polymorphic sites found for each allele in our analysis. Reference strains M12 (subsp. *multiplex*) and Temecula1 (subsp. *fastidiosa*) were also included for comparison.

<b>PD0261 / <i>nuoN</i> gene</b>	<b>Temecula Coordinates of DNA Polymorphisms</b>						
1,216 bp (post-trim)	331,327	331,401	331,524	331,630	331,639	331,654	331,774
Allele 1	G	G	G	T	C	C	C
Allele 2	A	A	C	C	G	T	A
Allele 3	A	A	C	C	G	T	A
subsp. <i>fastidiosa</i>	A	G	C	C	C	T	C
subsp. <i>multiplex</i>	G	G	G	T	C	C	C
	331,870	331,888	332,129	332,131	332,132	332,242	332,266
Allele 1 (Continued)	G	G	C	C	T	C	T
Allele 2	T	C	A	T	C	T	G
Allele 3	G	C	A	T	C	T	G
subsp. <i>fastidiosa</i>	G	C	A	T	C	T	G
subsp. <i>multiplex</i>	G	G	C	C	T	C	T
	332,287	332,389					
Allele 1 (Continued)	C	T					
Allele 2	T	T					
Allele 3	T	T					
subsp. <i>fastidiosa</i>	T	C					
subsp. <i>multiplex</i>	C	T					

<b>PD0104 / <i>holC</i> gene</b>	<b>Temecula Coordinates of DNA Polymorphisms</b>						
360 bp (post-trim)	134,004	134,053	134,059	134,089	134,194	134,266	134,287
Allele 1	T	G	A	G	G	T	C
Allele 2	C	A	A	G	G	T	C
Allele 3	C	A	A	G	A	C	C
subsp. <i>fastidiosa</i>	C	A	A	A	A	C	T
subsp. <i>multiplex</i>	T	G	G	G	G	T	C
	134,289	134,290	134,293	134,298	134,299	134,320	134,367
Allele 1 (Continued)	C	G	C	G	G	T	A
Allele 2	C	G	C	G	G	T	A
Allele 3	T	A	T	T	A	C	A
subsp. <i>fastidiosa</i>	T	G	C	G	G	T	C
subsp. <i>multiplex</i>	C	G	C	G	G	T	A

<b>PD1465 / <i>lacF</i> gene</b>	Temecula Coordinates of DNA Polymorphisms						
632 bp (post-trim)	1,707,664	1,707,676	1,707,751	1,707,756	1,707,813	1,707,921	1,707,931
Allele 1	C	T	T	A	A	A	A
Allele 2	C	T	G	G	G	G	T
subsp. <i>fastidiosa</i>	A	C	G	G	G	G	T
subsp. <i>multiplex</i>	C	T	T	A	A	A	A
	1,708,024	1,708,031	1,708,138	1,708,181	1,708,193	1,708,199	1,708,234
Allele 1 (Continued)	C	G	G	C	A	G	C
Allele 2	A	A	A	G	G	G	C
subsp. <i>fastidiosa</i>	A	A	A	G	G	G	A
subsp. <i>multiplex</i>	C	G	G	C	A	C	C

<b>PD0148 / <i>pilU</i> gene</b>	Temecula Coordinates of DNA Polymorphisms						
854 bp (post-trim)	1,345,667	1,345,673	1,345,789	1,345,823	1,345,892	1,345,928	1,345,940
Allele 1	G	C	G	C	G	A	T
Allele 2	T	T	A	T	A	G	C
subsp. <i>fastidiosa</i>	T	T	A	T	A	G	C
subsp. <i>multiplex</i>	G	C	G	C	G	A	T
	1,345,947	1,346,042	1,346,054	1,346,094	1,346,143	1,346,171	1,346,183
Allele 1 (Continued)	T	A	A	C	C	A	A
Allele 2	C	G	G	G	G	C	G
subsp. <i>fastidiosa</i>	C	G	G	G	G	C	G
subsp. <i>multiplex</i>	T	A	A	C	C	A	A
	1,346,206	1,346,210	1,346,240	1,346,241	1,346,243		
Allele 1 (Continued)	C	C	C	T	G		
Allele 2	A	T	T	C	A		
subsp. <i>fastidiosa</i>	A	T	T	C	A		
subsp. <i>multiplex</i>	C	C	C	T	G		

<b>PD0210 / <i>rfbD</i> gene</b>	Temecula Coordinates of DNA Polymorphisms						
413 bp (post-trim)	265,222	265,231	265,236	265,243	265,260	265,291	265,309
Allele 1	C	C	C	C	G	C	G
subsp. <i>fastidiosa</i>	T	T	T	T	A	T	T
subsp. <i>multiplex</i>	C	C	C	C	G	C	G
	265,413	265,567	265,576	265,601	265,625		
Allele 1 (Continued)	T	G	T	A	G		
subsp. <i>fastidiosa</i>	G	G	T	A	G		
subsp. <i>multiplex</i>	T	A	C	C	A		

<b>PD1840 / <i>cysG</i> gene</b>	Temecula Coordinates of DNA Polymorphisms						
905 bp (post-trim)	2,156,101	2,156,122	2,156,417	2,156,474	2,156,484	2,156,509	2,156,614
Allele 1	A	T	C	G	T	A	G
Allele 2	A	T	C	G	T	A	G
subsp. <i>fastidiosa</i>	G	C	T	A	C	G	G
subsp. <i>multiplex</i>	A	T	C	G	T	A	A
	2,156,721	2,156,728	2,156,869	2,156,896	2,156,944	2,156,951	2,156,958
Allele 1 (Continued)	A	C	C	T	C	G	C
Allele 2	G	T	C	C	T	A	T
subsp. <i>fastidiosa</i>	G	T	G	C	T	A	C
subsp. <i>multiplex</i>	A	C	C	T	C	G	C
	2,156,977	2,156,987					
Allele 1 (Continued)	C	A					
Allele 2	T	G					
subsp. <i>fastidiosa</i>	T	G					
subsp. <i>multiplex</i>	C	A					

<b>PD1047 / <i>leuA</i> gene</b>	Temecula Coordinates of DNA Polymorphisms						
1,119 bp (post-trim)	1,249,732	1,249,875	1,250,004	1,250,127	1,250,412	1,250,637	1,250,639
Allele 1	G	T	C	C	C	T	A
Allele 2	G	T	C	C	C	C	G
subsp. <i>fastidiosa</i>	A	A	G	T	T	C	G
subsp. <i>multiplex</i>	G	T	C	C	C	T	A
	1,250,665	1,250,667	1,250,685	1,250,688	1,250,748		
Allele 1 (Continued)	A	T	G	T	C		
Allele 2	G	C	T	C	T		
subsp. <i>fastidiosa</i>	G	T	T	C	T		
subsp. <i>multiplex</i>	A	T	G	T	C		

<b>PD1775 / <i>petC</i> gene</b>	Temecula Coordinates of DNA Polymorphisms					
533 bp (post-trim)	2,066,328	2,066,372	2,066,448	2,066,499	2,066,638	2,066,701
Allele 1	G	T	T	T	C	A
subsp. <i>fastidiosa</i>	A	C	C	C	G	G
subsp. <i>multiplex</i>	G	T	T	T	C	A

<b>PD1516 / <i>gltT</i> gene</b>	Temecula Coordinates of DNA Polymorphisms						
842 bp (post-trim)	1,762,901	1,763,045	1,763,162	1,763,195	1,763,201	1,763,265	1,763,270
Allele 1	G	G	G	A	T	A	G
Allele 2	G	G	G	A	T	G	G
Allele 3	G	G	G	A	T	A	G
subsp. <i>fastidiosa</i>	A	A	A	G	G	G	A
subsp. <i>multiplex</i>	G	G	G	A	T	G	G
	1,763,369	1,763,556	1,763,615	1,763,630	1,763,681	1,763,702	1,763,729
Allele 1 (Continued)	C	A	A	A	G	C	A
Allele 2	C	A	G	A	G	C	A
Allele 3	C	A	G	A	G	C	A
subsp. <i>fastidiosa</i>	G	G	G	G	A	T	T
subsp. <i>multiplex</i>	C	A	G	A	G	C	A

<b>PD0259 / <i>nuoL</i> gene</b>	Temecula Coordinates of DNA Polymorphisms						
540 bp (post-trim)	328,737	328,863	328,989	329,033	329,077	329,112	329,120
Allele 1	G	C	T	C	G	T	T
Allele 2	G	C	T	C	A	T	T
subsp. <i>fastidiosa</i>	A	A	A	T	A	C	C
subsp. <i>multiplex</i>	G	C	T	C	G	T	T
	329,205	329,227					
Allele 1 (Continued)	A	A					
Allele 2	G	C					
subsp. <i>fastidiosa</i>	A	C					
subsp. <i>multiplex</i>	A	A					

Strain STs found within the asymptomatic portions of infected trees and entirely asymptomatic trees were consistently associated with the STs found in the symptomatic canopies of the respective tree genus. The only exception occurred for a single mulberry tree that possessed both ST-4 on one side of the canopy and ST-5 on the other side of the canopy. Of the 56 sites selected for this study, 9 of them possessed two different tree species that were infected with *X. fastidiosa* and were within 25 m of each other. Cross transmission of the *X. fastidiosa* STs between different species of tree was not observed

at any of these 9 sites. The only instance where a ST was found infecting more than one genus of tree was with the oak ST-1 strain found infecting one elm tree. The symptomatic elm tree was in an area that did not have a neighboring infected oak tree within 25 m.

### **3.5 Discussion**

For the past century, *X. fastidiosa* has continuously eluded our understanding of the mechanisms in which it selects hosts and causes disease. Former literature demonstrates our misconceptions of the etiological agent responsible of Pierce's disease of grapevine and phony peach disease throughout the better part of the 20<sup>th</sup> century (58). More recently, with the progression of sequencing technologies and bioinformatics, we have been provided increasingly advanced molecular tools that enable greater insight into the biochemical composition of this organism. However, there still exists a great deal of scientific uncertainty regarding mechanisms responsible for host selectivity and methods aimed at reducing its effects in an agricultural or municipal setting.

Sequence typing assignments in this study are concurrent with previous ST classifications for each host specific ST (91, 92). Peak discrepancies were not observed at any of the polymorphic sites used for typing. Although this would suggest an absence of co-infection of multiple STs in a single sample for most of the trees in the analysis, a multi-locus melt analysis could further validate this finding (10). In addition, caution should be taken when collecting sequence data from DNA that is directly extracted from infected plant tissue without the isolation of the query organism. Non-specific amplification was an issue for several American elm DNA extracts and gel extraction of the target gene product was necessary. Furthermore, when using a Sanger based approach for sequencing

(such as the one used for this study); the dominant ST may mask multiple STs in an individual sample during base calling. It has been shown that samples with DNA from PD (subsp. *fastidiosa*) and ALS (subsp. *multiplex*) are mixed together, the strain with the higher concentration is identified during a quantitative PCR melt analysis, and that dual peaks do not occur (87).

The evidence of host-pathogen incompatibility between each genus of tree and each subspecific ST is perhaps the most important observation in this study. This suggests that either i) genes conferring pathogenicity are marginally altered orthologs which are the product of a long evolutionary selection that allowed each pathovar to either outcompete or thrive in a given host xylem, or ii) vector-pathogen dynamics are dictating the occurrence of infection. The latter is a less likely scenario, as vectors have occasionally been found to harbor multiple subspecies (22, 87). Additionally, cross-inoculations of *X. fastidiosa* subsp. *multiplex* from different hosts have failed to demonstrate reciprocated symptom development in several instances (108, 111). Such specificity was demonstrated when two strains of *X. fastidiosa* isolated from elm and sycamore were only pathogenic to the seedlings of their respective host plant, and cross inoculation of the isolated strains did not cause symptoms, nor could be recovered by culturing, when introduced to the reciprocate plant host (111). A single elm tree infected with ST-1 was the only discrepancy in host specificity observed for each ST. This would suggest that ST-1 is capable of infecting both oaks and elms.

The only occurrence of intraspecific (same strain) diversity of *X. fastidiosa* within a single host was observed with the 2 STs responsible for mulberry disease in this study.

Mulberry is the only tree species in this analysis that is propagated from pollinated seed. If amenity trees are clonally propagated from a nursery and disseminated throughout a city, a single virulent haplotype of *X. fastidiosa* could be responsible for devastating an entire population of a tree species in the absence of host selective pressure on the pathogen, as is evident in our study. Although the presence of interspecific (different strain) specificity suggests a specialized host-pathogen relationship, the lack of intraspecific strain diversity among the amenity trees may suggest a strong selective pressure brought on by the genetically uniform host. Similarly, in a former study that investigated the genetic diversity of *X. fastidiosa* strains within coffee and citrus hosts, no haplotypes were shared between the two host species, and a greater diversity of strains was found among the coffee plants compared to the citrus plants (102, 130). It was suggested that the greater genetic diversity of the cross-pollinated coffee hosts and the longer period of coffee production in Brazil corresponded with greater genetic diversity of the pathogen population compared to the pathogen population infecting the recently introduced and clonally propagated citrus plants (130).

In our analysis, we found only one instance that suggested a co-habitation of STs in a single host. A mulberry tree possessed both ST-4 and ST-5 on opposing sides of the tree. Otherwise, none of the amenity trees possessed more than a single ST within an individual canopy, and only a single elm possessed a ST uncommon to the majority. This may have implications regarding the opportunity for intersubspecific homologous recombination (IHR) of *X. fastidiosa* in urban environments. If more hosts such as elm are found capable of being infected with more than one host-specific ST, then there exists a possibility that IHR could occur during a co-infection of the host. In order for this event

to take place, an IHR clone must not only outcompete the currently long-evolved and established clone, but it must also multiply to a large enough quantity for a chance to become fixated in the population. Conversely, if each pathovar has evolved to occupy a particular genus of tree, IHR opportunities may be impeded by strong host selection pressure in the event of co-habitation. Instead, IHR may have a greater opportunity to occur within the leafhopper vector.

Many urban environments are embodied in an urban heat island created by the excess heat from urban surfaces. During a typical summer in the District, mid-morning diurnal temperature differences can be as great as 10°C between the urban environment and nearby woodlands (67). It was suggested that regions with warm day and night summer temperatures should expect less interruption to exponential phase growth of *X. fastidiosa in planta* compared with regions with similarly warm days and cool nights (30). If urban environments are moderating cooler temperatures during evening hours, a lag phase of growth in *X. fastidiosa* could become nonexistent, and permit unrestricted bacterial growth throughout the summer. Since the rate of bacterial acquisition by vectors has been found to require bacterial multiplication to threshold population levels within the host (53), urban environments may accelerate the availability of inoculum to vectors. The high recombinant tendencies of the bacteria (76, 106), the increased duration of vector acquisition time brought on by an urban heat island, and the presence of a diversity of plant material customary in urban landscape design may be a recipe for novel host shifts in the future.



## Chapter 4: Application of Knowledge and Broader Impacts

### 4.0 Project Summary

#### 4.0.1 Summary of field sampling and BLS incidence among amenity trees

Two important findings developed from the initial survey, 1) the incidence of latent infection of *X. fastidiosa* in asymptomatic foliage of susceptible hosts and 2) majority of asymptomatic trees neighboring an infected tree did not possess a latent infection of *X. fastidiosa*. First, asymptomatic foliage of infected trees possess a latent infection of *X. fastidiosa*. This evidence suggests that pruning off symptomatic branches will not rid a tree of the bacteria. Resource managers responsible for controlling bacterial leaf scorch in a municipal environment should understand that a management program aimed at removing the symptomatic branches from an infected tree will not cure the tree with bacterial leaf scorch. However, effective acquisition of *X. fastidiosa* by vectors was shown to require a large bacterial population within host tissue (53), and thus removal of infected branches that possess a larger bacterial population may reduce the rate in which vectors acquire the bacterium. In this regard, pruning might still be a useful management option for mitigating the spread of the disease. It is still unknown how long each species of infected tree can persist once infected, but it is likely influenced by environment (83), inoculum pressures from vector abundance and preferences (1), and susceptibility of the host genotype (34).

The second important observation was that the majority of asymptomatic trees neighboring an infected tree did not possess a latent infection of *X. fastidiosa*. This suggests that trees neighboring an infected tree can be regarded as uninfected and can be

considered sustainable in the event of removing an infected neighboring tree from a site. On this end, the progression of disease within a microsite may be governed by the presence of interspecies genetic resistance mechanisms in some but not all host known to be susceptible to *X. fastidiosa* infection (34). Evidence from the immunodetection survey and the MLST analysis demonstrated that although willow oak is infected with the same *X. fastidiosa* strain as red oak and pin oak, crown dieback symptoms and scorch severity were minimal suggesting greater resistance to pathogen. This could be due to genetically modulated resistance mechanisms present in willow oak and not red or pin oak, or simply due to the thinner and potentially less attractive leaf morphology of willow oak, which may not be as preferred by the leafhopper vectors as the broader red and pin oak leaves. A similar pattern is apparent with *Ulmus americana* and *U. alata*, where although both species are susceptible to infection, only *U. americana* demonstrates severe symptom development. Although the vigor of *Q. phellos* and *U. alata* does not seem to be as detrimentally impacted by *X. fastidiosa* infection as *Q. rubra* and *U americana*, their role as inoculum reservoirs for vector acquisition of *X. fastidiosa* is unclear.

#### **4.0.2 Summary of Multi-locus Sequence Analysis**

Since the first documented case of elm leaf scorch in Washington D.C. (129), bacterial leaf scorch (BLS) has continued to perpetuate uncontrolled within this urban setting. Current management strategies for suppressing the causal agent, *Xylella fastidiosa*, include injections of antibiotics and application of plant growth regulators (27, 72). However, these procedures cannot cure a tree once infected, and can only prolong the spiral of mortality after initial infection. The second study aimed to develop data for long-term management practices that mitigate the occurrence of disease. The results

provide information that can assist arboreal resource managers with selecting tree species that are not predisposed to contracting leaf scorch disease from pre-existing infected trees within the planting site. Since each genus of host was infected with a unique strain of *X. fastidiosa*, trees of the same genus should not be planted next to each other. Data generated from this study demonstrate heavy selection pressure for a particular pathovar of *X. fastidiosa* from each genus of host, suggesting that host genotype is directly associated with the pathogen genotype. Consequently, monocultures of BLS susceptible tree species (pin oak, red oak, sycamore, and elm), particularly of the same genetic variety, should be avoided when possible. When selecting these three tree genera, they should be derived from numerous nursery sources to prevent clonal establishment, and should never be planted in a site where pre-existing trees of the same genus are infected with *X. fastidiosa*.

## **4.1 Personal Views**

### **4.1.1 Outlook**

Urban ecosystems are the most rapidly expanding habitat type worldwide (37). These energy intensive anthropogenic systems are not the environments that selected for the resident biota. For the first time in ecological history, the process of natural selection has been displaced by anthropogenic selection where the selection of species is based solely on the perceived benefits of a single organism and not the surrounding biotic community. Our selection and removal of desired and undesired macroflora has repercussions at the microflora level as well. As is evident with the decimation of American elm monocultures by the fungal pathogen *Ophiostoma novo-ulmi* in the mid 20<sup>th</sup> century, the evidence in this thesis project demonstrated that a single clone of *X. fastidiosa* is

responsible for widespread disease in each genus of tree. This is likely due to host trees being of similar genetic composition, a common characteristic of plant material derived from nursery-based operations.

Nature has its own genetically constructed system of checks and balances. The presence of too much of a single host genotype over a large enough spatial and temporal scale increases the probability that the genotype will be encountered with a perpetually evolving pathogen that can overcome the genetic barriers that confer host resistance.

Once host resistance is overcome by a pathogen, the factors that govern the incidence of disease are directly related to the abundance of the susceptible genotype and the duration of an environment conducive for pathogen proliferation. It is through co-evolutionary associations between hosts and microorganisms native to a particular geographic region, which under natural circumstances has established over a significantly long duration of time, that well-balanced co-dependent relationships are achieved. Co-evolved host-pathogen relationships can cause catastrophic destruction if the pathogen is removed from its long evolved host population and introduced to a naïve host population of similar genetic composition. The pathogen perceives a single susceptible genotype even though the host is diverse in its own geographic ecology, and consequently the host population is decimated by a pathogen that is genetically constructed to fulfill a balanced niche only in the ecosystem from which it is far removed. The fungal pathogen responsible for chestnut blight, *Cryphonectria parasitica*, had co-evolved with the Chinese chestnut and was ecologically balanced with its surrounding biotic community in Asia. When the pathogen was introduced to an unfamiliar environment in the Americas, the surrounding ecology did not possess a functional niche for this organism, and consequently the organism

fulfilled a dysfunctional role. It is important to understand that nature does not select for the most virulent pathovar. Anthropogenic transport of ecologically specialized pathogens to regions where co-evolution did not occur is partly to blame for the occurrence of unnaturally severe incidents of phytopathology.

The other key anthropogenic practice that exacerbates phytopathology is the creation of disease-conducive monocultures. Pathogen evolution is mediated by changes in gene frequency over time. A mutant strain with the genetic information to confer greater virulence must regenerate at a great enough frequency over a large enough temporal scale in order for to become fixed into the natural pathogen population. Diseases caused by *X. fastidiosa* do not occur in natural forest ecosystems. This is because a virulent strain of *X. fastidiosa* would never reach fixation in a natural setting. It is at a selective disadvantage because the host would not live long enough to allow the virulent population to occur at a great enough frequency or for a long enough period of time to reach fixation. It has been shown that the leafhopper vectors that transmit disease do not prefer diseased trees when feeding (133). It is through limitations of susceptible host abundance, vector preference, and temporal constraints imposed by natural ecosystems that mediate the pathogenicity of *X. fastidiosa* in a natural setting.

A virulent strain that arises from mutation would have a greater probability of reaching fixation within either an agricultural or municipal environment due to the presence of a monoculture. An environment can change the evolution of a pathogen if placed under heavy selection pressure by a single host genotype. The greater the abundance of a single host genotype, the greater the vector feeding on this exclusive host, the greater the host's

exposure to a perpetually evolving pathogen, the greater the selective pressure for the most competitive *X. fastidiosa* strain that can outcompete all other strains inhabiting the specific host monoculture. *Xylella fastidiosa* should not be seen as a pathogen; rather, it is simply an endophytic microorganism that is filling an ecological niche to the best of its ability in an ecosystem not fashioned by natural selection, but by anthropogenic selection. In this regard, diseases caused by *Xylella* are simply due to the ecological imbalance the microorganism has with the unnaturally constructed environment in which it causes disease. Converse to the use of *Cryphonectria parasitica* as a strong selective agent for backcrossed Chinese-American chestnut hybrids, the repetitive use of a single species of plant can exert a strong selective pressure for the most host-fit strain of *X. fastidiosa*. The resulting strain causes disease that would not normally be observed if the surrounding forest community was operating under natural selection.

#### **4.1.2 Future Direction**

There are two directions to take when continuing to research this topic. One would be looking for ways to increase the biodiversity of our managed systems, and the other way would be looking for ways to suppress the pathogen. After determining the high level of specificity of *X. fastidiosa* subsp. *multiplex* and *moris* strains for a particular genus of host (e.g. sequence types), the next logical step in researching this pathogen is to elucidate the biological interactions that confer host susceptibility. The reasoning for the observed specificity may be due to vector dissemination dynamics, compatibility of each *X. fastidiosa* pathovar with host xylem nutritional composition, or susceptibility of the host xylem conduits to degradation by pathogen pit-membrane degrading enzymes (16). Vector dissemination dynamics would be the simplest factor to examine, as yellow sticky

cards placed in all susceptible species could easily illustrate dissemination dynamics and vector-pathogen associations after identifying the *X. fastidiosa* subspecies within collected vector specimens. Nutritional xylem chemistry could be analyzed using mass spectrometry on a concentrated sample of xylem fluid for each host species, and then the components could be replicated *in vitro* for compatibility analyses with different strains of *X. fastidiosa*. A more simple experiment would be with the use of a susceptible and resistant grapevine variety. By grafting the susceptible variety onto the resistant variety, it would be possible to determine if the altered xylem chemistry thereafter is capable of repressing *Xylella fastidiosa* concentrations in the susceptible variety scion. Finally, knock-out *X. fastidiosa* mutants without the pit-degrading enzyme coding region could be transformed with each type of degradation system, and the transgenic strains could be introduced to each host species to determine the role of the degradation system in host susceptibility. Once the factors that confer specificity are revealed, methods of plant propagation can be directed in ways that ensure pathogen compatibility is not achieved.

#### **4.2 Concluding Remarks for Managing BLS in an Urban Environment.**

The future protocol for resource managers that are attempting to prevent the incidence of BLS should understand that host genetic diversity is imperative for arboreal resource resiliency to any pest or pathogen, which includes *X. fastidiosa*. Obtaining plant material from numerous sources and ensuring that the origins of the nursery stock are not of a widely used clonal variety within their jurisdiction should be a common practice when looking to add new plant material to a landscape. Monocultures of trees that are of a common genus should be avoided when possible, particularly of the red and pin oak species (*Quercus palustris* and *Q. rubra*), sycamore (*Platanus occidentalis*), elm (*Ulmus*

*americana*), and the amenity tree red mulberry (*Morus rubra*). Data generated by this study suggests that pruning will not cure a tree of BLS. Removal of infected branches should be practiced within the guidelines set forth by American National Standards for arboriculture operations (ANSI A300). However, more studies are needed to determine whether removal of infected trees at a certain stage will have an impact (e.g. slow the spread of the pathogen) or influence the spatial dynamics of disease within a city environment. Uncertainty regarding the occurrence of inoculum reservoirs in wooded areas should also be explored before any widespread eradication effort is considered. An eradication effort would have minimal benefit if *X. fastidiosa* populations were abundant in unmanaged urban green spaces.



## Bibliography

1. Almeida, R. P. P., Blua, M. J., Lopes, J. R. S., and Purcell, A. H. 2005. Vector transmission of *Xylella fastidiosa*: Applying fundamental knowledge to generate disease management strategies. *Annals of the Entomological Society of America* 98:775-786.
2. Almeida, R. P. P., Nascimento, F. E., Chau, J., Prado, S. S., Tsai, C. W., Lopes, S. A., and Lopes, J. R. S. 2008. Genetic structure and biology of *Xylella fastidiosa* strains causing disease in citrus and coffee in Brazil. *Applied and Environmental Microbiology* 74:3690-3701.
3. Almeida, R. P. P., and Purcell, A. H. 2003. Biological traits of *Xylella fastidiosa* strains from grapes and almonds. *Applied and Environmental Microbiology* 69:7447-7452.
4. Auger, J., Mircetich, S. M., and Nyland, G. 1974. Interrelation between bacteria causing Pierce's disease of grapevines and almond leaf scorch. *Proceedings of the American Phytopathological Society* 1:(1975)-(1975).
5. Barnard, E. L., Ash, E. C., Hopkins, D. L., and McGovern, R. J. 1998. Distribution of *Xylella fastidiosa* in oaks in Florida and its association with growth decline in *Quercus laevis*. *Plant Disease* 82:569-572.
6. Bentz, J., and Sberald, J. 2001. Transmission of the xylem-limited bacterium *Xylella fastidiosa* to shade trees by insect vectors. *Shade tree wilt diseases*:203-208.

7. Berisha, B., Chen, Y. D., Zhang, G. Y., Xu, B. Y., and Chen, T. A. 1998. Isolation of Pierce's disease bacteria from grapevines in Europe. *European Journal of Plant Pathology* 104:427-433.
8. Bhattacharyya, A., Stilwagen, S., Ivanova, N., D'Souza, M., Bernal, A., Lykidis, A., Kapatal, V., Anderson, L., Larsen, N., Los, T., Reznik, G., Selkov, E., Walunas, T. L., Feil, H., Feil, W. S., Purcell, A., Lassez, J. L., Hawkins, T. L., Haselkorn, R., Overbeek, R., Predki, P. F., and Kyrpides, N. C. 2002. Whole-genome comparative analysis of three phytopathogenic *Xylella fastidiosa* strains. *Proceedings of the National Academy of Sciences of the United States of America* 99:12403-12408.
9. Blake, J. H. 1993. Distribution of *Xylella fastidiosa* in oak, maple, and sycamore in South Carolina. *Plant Disease* 77:1262-1262.
10. Brady, J. A., Faske, J. B., Ator, R. A., Castaneda-Gill, J. M., and Mitchell, F. L. 2012. Probe-based real-time PCR method for multilocus melt typing of *Xylella fastidiosa* strains. *Journal of Microbiological Methods* 89:12-17.
11. Brlansky, R. H., Damsteegt, V. D., and Hartung, J. S. 2002. Transmission of the citrus variegated chlorosis bacterium *Xylella fastidiosa* with the sharpshooter *Oncometopia nigricans*. *Plant Disease* 86:1237-1239.
12. Brody, J. R., and Kern, S. E. 2004. Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. *Biotechniques* 36:214-+.
13. Castle, S. J., Byrne, F. J., Bi, J. L., and Toscano, N. C. 2005. Spatial and temporal distribution of imidacloprid and thiamethoxam in citrus and impact on *Homalodisca coagulata* populations. *Pest Management Science* 61:75-84.

14. Chang, C. J., Garnier, M., Zreik, L., Rossetti, V., and Bove, J. M. 1993. Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. *Current Microbiology* 27:137-142.
15. Chang, C. J., and Walker, J. T. 1988. Bacterial leaf scorch of northern red oak: isolation, cultivation, and pathogenicity of a xylem-limited bacterium. *Plant Disease* 72:730-733.
16. Chatterjee, S., Newman, K. L., and Lindow, S. E. 2008. Cell-to-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. *Molecular Plant-Microbe Interactions* 21:1309-1315.
17. Chen, J., Chang, C. J., Jarret, R. L., and Gawel, N. 1992. Genetic variation among *Xylella fastidiosa* strains. *Phytopathology* 82:973-977.
18. Chen, J., Groves, R., Civerolo, E. L., Viveros, A., Freeman, A., and Zheng, Y. 2005. Two *Xylella fastidiosa* genotypes associated with almond leaf scorch disease on the same location in California. *Phytopathology* 95:708-714.
19. Chen, J., Huang, H., Chang, C. J., and Stenger, D. C. 2013. Draft genome of *Xylella fastidiosa* subsp. *multiplex* strain Griffin-1 from *Quercus rubra* in Georgia. *Genome Announc* 1:5.
20. Chen, J., Lamikanra, O., Chang, C. J., and Hopkins, D. L. 1995. Randomly amplified polymorphic DNA analysis of *Xylella fastidiosa* Pierce's-disease and oak leaf scorch pathotypes. *Applied and Environmental Microbiology* 61:1688-1690.

21. Chen, J., Xie, G., Han, S., Chertkov, O., Sims, D., and Civerolo, E. L. 2010. Whole genome sequences of two *Xylella fastidiosa* strains (M12 and M23) causing almond leaf scorch disease in California. *Journal of Bacteriology* 192:4534-4534.
22. Costa, H. S., Guzman, A., Hernandez-Martinez, R., Gispert, C., and Cooksey, D. A. 2006. Detection and differentiation of *Xylella fastidiosa* strains acquired and retained by glassy-winged sharpshooters (Hemiptera : Cicadellidae) using a mixture of strain-specific primer sets. *Journal of Economic Entomology* 99:1058-1064.
23. Davis, M. J., French, W. J., and Schaad, N. W. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. *Current Microbiology* 6:309-314.
24. Davis, M. J., Purcell, A. H., and Thomson, S. V. 1978. Pierce's disease of grapevines: isolation of causal bacterium. *Science* 199:75-77.
25. Davis, M. J., Raju, B. C., Brlansky, R. H., Lee, R. F., Timmer, L. W., Norris, R. C., and McCoy, R. E. 1983. Periwinkle wilt bacterium - axenic culture, pathogenicity, and relationships to other gram-negative, xylem-inhabiting bacteria. *Phytopathology* 73:1510-1515.
26. de Lima, J. E. O., Miranda, V. S., Hartung, J. S., Brlansky, R. H., Coutinho, A., Roberto, S. R., and Carlos, E. F. 1998. Coffee leaf scorch bacterium: Axenic culture, pathogenicity, and comparison with *Xylella fastidiosa* of citrus. *Plant Disease* 82:94-97.

27. DeStefano, D. A., Grybauskas, A. P., Sherald, J. L., Momen, B., Huang, Q., and Sullivan, J. H. 2007. Effect of the growth regulator paclobutrazol on growth of the bacterial pathogen *Xylella fastidiosa*. *Arboriculture & Urban Forestry* 33:246-252.
28. Doddapaneni, H., Yao, J. Q., Lin, H., Walker, M. A., and Civerolo, E. L. 2006. Analysis of the genome-wide variations among multiple strains of the plant pathogenic bacterium *Xylella fastidiosa*. *Bmc Genomics* 7:225.
29. Elbeaino, T., Yaseen, T., Valentini, F., Ben Moussa, I. E., Mazzoni, V., and D'Onghia, A. M. 2014. Identification of three potential insect vectors of *Xylella fastidiosa* in southern Italy. *Phytopathologia Mediterranea* 53:328-332.
30. Feil, H., and Purcell, A. H. 2001. Temperature-dependent growth and survival of *Xylella fastidiosa* in vitro and in potted grapevines. *Plant Disease* 85:1230-1234.
31. Freitag, J. H. 1951. Host range of the Pierce's diseases virus of grapes as determined by insect transmission. *Phytopathology* 41:920-934.
32. French, W. J. 1982. Reciprocal transmission of plum leaf scald and phony disease of peach. *Phytopathology* 72:452-453.
33. Fry, S. M., and Milholland, R. D. 1990. Multiplication and translocation of *Xylella fastidiosa* in petioles and stems of grapevine resistant, tolerant, and susceptible to Pierce's disease. *Phytopathology* 80:61-65.
34. Fry, S. M., and Milholland, R. D. 1990. Response of resistant, tolerant, and susceptible grapevine tissues to invasion by the Pierce's disease bacterium, *Xylella fastidiosa*. *Phytopathology* 80:66-69.

35. Goheen, A. C., Nyland, G., and Lowe, S. K. 1973. Association of a rickettsia-like organism with Pierce's disease of grapevines and alfalfa dwarf and heat therapy of disease in grapevines. *Phytopathology* 63:341-345.
36. Gould, A. B., Hamilton, G., Vodak, M., Grabosky, J., and Lashomb, J. 2004. Bacterial leaf scorch of oak in New Jersey: Incidence and economic impact. *Phytopathology* 94:S36-S36.
37. Grimm, N. B., Faeth, S. H., Golubiewski, N. E., Redman, C. L., Wu, J. G., Bai, X. M., and Briggs, J. M. 2008. Global change and the ecology of cities. *Science* 319:756-760.
38. Harris, J. L., Di Bello, P. L., Lear, M., and Balci, Y. 2014. Bacterial leaf scorch in the District of Columbia: Distribution, host range, and presence of *Xylella fastidiosa* among urban trees. *Plant Dis*:140521144958000.
39. Hartman, J., Dixon, E., and Bernick, S. 2010. Evaluation of therapeutic treatments to manage oak bacterial leaf scorch. *Arboriculture & Urban Forestry* 36:140-146.
40. Hartman, J. R., Eshenaur, B. C., and Jarlfors, U. E. 1995. Bacterial leaf scorch caused by *Xylella fastidiosa*: A Kentucky survey: a unique pathogen; and bur oak, a new host. *Journal of Arboriculture* 21:77-82.
41. Hartman, J. R., Jarlfors, U. E., Fountain, W. M., and Thomas, R. 1996. First report of bacterial leaf scorch caused by *Xylella fastidiosa* on sugar maple and sweetgum. *Plant Disease* 80:1302-1302.
42. Hartman, J. R., Kaiser, C. A., Jarlfors, U. E., Eshenaur, B. C., Bachi, P. R., and Dunwell, W. C. 1991. Occurrence of oak bacterial leaf scorch caused by *Xylella fastidiosa* in Kentucky. *Plant Disease* 75:862-862.

43. Haygood, R. A., Jones, R. K., and Huang, P. Y. 1988. Fastidious, xylem-inhabiting bacteria associated with scorch of pin oak and southern red oak trees in the Carolinas. *Phytopathology* 78:628-628.
44. Haygood, R. A., Witcher, W., and Jones, R. K. 1988. Outbreak of sycamore leaf scorch in the Carolinas. *Plant Disease* 72:644-644.
45. He, C. X., Li, W. B., Ayres, A. J., Hartung, J. S., Miranda, V. S., and Teixeira, D. C. 2000. Distribution of *Xylella fastidiosa* in citrus rootstocks and transmission of citrus variegated chlorosis between sweet orange plants through natural root grafts. *Plant Disease* 84:622-626.
46. Hearon, S. S., Sberald, J. L., and Kostka, S. J. 1980. Association of xylem-limited bacteria with elm, sycamore, and oak leaf scorch. *Canadian Journal of Botany- Revue Canadienne De Botanique* 58:1986-1993.
47. Hendson, M., Purcell, A. H., Chen, D. Q., Smart, C., Guilhabert, M., and Kirkpatrick, B. 2001. Genetic diversity of Pierce's disease strains and other pathotypes of *Xylella fastidiosa*. *Applied and Environmental Microbiology* 67:895-903.
48. Hernandez-Martinez, R., Costa, H. S., Dumenyo, C. K., and Cooksey, D. A. 2006. Differentiation of strains of *Xylella fastidiosa* infecting grape, almonds, and oleander using a multiprimer PCR assay. *Plant Disease* 90:1382-1388.
49. Hernandez-Martinez, R., de la Cerda, K. A., Costa, H. S., Cooksey, D. A., and Wong, F. P. 2007. Phylogenetic relationships of *Xylella fastidiosa* strains isolated from landscape ornamentals in southern California. *Phytopathology* 97:857-864.

50. Hewitt, W. B. 1939. A transmissible disease of grapevines. *Phytopathology* 29:10-12.
51. Hewitt, W. B., Houston, B. R., Frazier, N. W., and Freitag, J. H. 1946. Leafhopper transmission of the virus causing Pierce's disease of grape and dwarf of alfalfa. *Phytopathology* 36:117-128.
52. Hill, B. L., and Purcell, A. H. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. *Phytopathology* 85:1368-1372.
53. Hill, B. L., and Purcell, A. H. 1997. Populations of *Xylella fastidiosa* in plants required for transmission by an efficient vector. *Phytopathology* 87:1197-1201.
54. Hopkins, D. L. 1981. Seasonal concentration of the Pierce's disease bacterium in grapevine stems, petioles, and leaf veins. *Phytopathology* 71:415-418.
55. Hopkins, D. L., and Adlerz, W. C. 1988. Natural hosts of *Xylella fastidiosa* in Florida. *Plant Disease* 72:429-431.
56. Hopkins, D. L., French, W. J., and Mollenha.Hh. 1973. Association of a rickettsia-like bacterium with phony peach disease. *Phytopathology* 63:443-443.
57. Hopkins, D. L., and Mollenha.Hh. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. *Science* 179:298-300.
58. Hopkins, D. L., Mollenha.Hh, and French, W. J. 1973. Occurrence of a rickettsia-like bacterium in xylem of peach trees with phony disease. *Phytopathology* 63:1422-1423.
59. Hopkins, D. L., and Mortensen, J. A. 1971. Suppression of Pierce's disease symptoms by tetracycline antibiotics. *Plant Disease Reporter* 55:610-612.



60. Hopkins, D. L., and Purcell, A. H. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and other emergent diseases. *Plant Disease* 86:1056-1066.
61. Huang, Q. 2004. First report of *Xylella fastidiosa* associated with leaf scorch in black oak in Washington, DC. *Plant Disease* 88:224-224.
62. Huang, Q. 2007. Natural occurrence of *Xylella fastidiosa* in a commercial nursery in Maryland. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* 29:299-303.
63. Huang, Q., and Sherald, J. L. 2004. Isolation and phylogenetic analysis of *Xylella fastidiosa* from its invasive alternative host, porcelain berry. *Current Microbiology* 48:73-76.
64. Hutchins, L. M. 1930. The phony disease of the peach. *J. Econ. Entomology* 23:555-562.
65. Janse, J. D., and Obradovic, A. 2010. *Xylella fastidiosa*: Its biology, diagnosis, control and risks. *Journal of Plant Pathology* 92:S35-S48.
66. Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., and Drummond, A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647-1649.
67. Kim, H. H. 1992. Urban heat-island. *International Journal of Remote Sensing* 13:2319-2336.

68. Kishi, L. T., Wickert, E., and de Macedo Lemos, E. G. 2008. Evaluation of *Xylella fastidiosa* genetic diversity by fAFLP markers. *Revista Brasileira De Fruticultura* 30:202-208.
69. Kitajima, E. W., Bakarcic, M., and Fernandezvaliela, M. V. 1975. Association of rickettsia-like bacteria with plum leaf scald disease. *Phytopathology* 65:476-479.
70. Kostka, S. J., Sberald, J. L., and Tattar, T. A. 1984. Culture of fastidious, xylem-limited bacteria from declining oaks in the northeastern states. *Phytopathology* 74:803-803.
71. Kostka, S. J., Sberald, J. L., and Tattar, T. A. 1982. Isolation of bacteria from three elm species and mulberry exhibiting leaf scorch. *Phytopathology* 72:936-936.
72. Kostka, S. J., Tattar, T. A., and Sberald, J. L. 1985. Suppression of bacterial leaf scorch symptoms in American elm through oxytetracycline microinjection. *Arboriculture & Urban Forestry*:54-58.
73. Kostka, S. J., Tattar, T. A., Sberald, J. L., and Hurtt, S. S. 1986. Mulberry leaf scorch, new disease caused by a fastidious, xylem-inhabiting bacterium. *Plant Disease* 70:690-693.
74. Krivanek, A. F., and Walker, M. A. 2005. *Vitis* resistance to Pierce's disease is characterized by differential *Xylella fastidiosa* populations in stems and leaves. *Phytopathology* 95:44-52.
75. Krugner, R., Ledbetter, C. A., Chen, J., and Shrestha, A. 2012. Phenology of *Xylella fastidiosa* and its vector around California almond nurseries: An

- assessment of plant vulnerability to almond leaf scorch disease. *Plant Disease* 96:1488-1494.
76. Kung, S. H., and Almeida, R. P. P. 2011. Natural competence and recombination in the plant pathogen *Xylella fastidiosa*. *Applied and Environmental Microbiology* AEM 77:5278-5284.
77. Lashomb, J., Gould, A. G., Iskra, M. S., and Hamilton, G. 2002. Bacterial leaf scorch of amenity trees: A wide-spread problem of economic significance to the urban forest. Pages 1-20 in: United States Department of Agriculture Forest Service symposium for review of Bacterial Leaf Scorch USDA Forest Service, Rutgers University, Cook College.
78. Leu, L. S., and Su, C. C. 1993. Isolation, cultivation, and pathogenicity of *Xylella fastidiosa*, the causal bacterium of pear leaf scorch disease in Taiwan. *Plant Disease* 77:642-646.
79. Li, W. B., Zhou, C. H., Pridmore, W. D., Teixeira, D. C., Miranda, V. S., Pereira, E. O., Ayres, A. J., and Hartung, J. S. 2002. Citrus and coffee strains of *Xylella fastidiosa* induce Pierce's disease in grapevine. *Plant Disease* 86:1206-1210.
80. Lin, H., Civerolo, E. L., Hu, R., Barros, S., Francis, M., and Walker, M. A. 2005. Multilocus simple sequence repeat markers for differentiating strains and evaluating genetic diversity of *Xylella fastidiosa*. *Applied and Environmental Microbiology* 71:4888-4892.
81. Lopes, J. R. S., Daugherty, M. P., and Almeida, R. P. P. 2010. Strain origin drives virulence and persistence of *Xylella fastidiosa* in alfalfa. *Plant Pathology* 59:963-971.

82. McCoy, R. E., Thomas, D. L., Tsai, J. H., and French, W. J. 1978. Periwinkle wilt, a new disease associated with xylem delimited rickettsial-like bacteria transmitted by a sharpshooter. *Plant Disease Reporter* 62:1022-1026.
83. McElrone, A. J., Sberald, J. L., and Forseth, I. N. 2001. Effects of water stress on symptomatology and growth of *Parthenocissus quinquefolia* infected by *Xylella fastidiosa*. *Plant Disease* 85:1160-1164.
84. McElrone, A. J., Sberald, J. L., and Pooler, M. R. 1999. Identification of alternative hosts of *Xylella fastidiosa* in the Washington, D.C., area using nested polymerase chain reaction (PCR). *Journal of Arboriculture* 25:258-263.
85. Mehta, A., and Rosato, Y. B. 2001. Phylogenetic relationships of *Xylella fastidiosa* strains from different hosts, based on 16S rDNA and 16S-23S intergenic spacer sequences. *International Journal of Systematic and Evolutionary Microbiology* 51:311-318.
86. Melanson, R. A., Sanderlin, R. S., McTaggart, A. R., and Ham, J. H. 2012. A systematic study reveals that *Xylella fastidiosa* strains from pecan are part of *X. fastidiosa* subsp. *multiplex*. *Plant Disease* 96:1123-1134.
87. Mitchell, F. L., Brady, J., Bextine, B., and Lauziere, I. 2009. Seasonal increase of *Xylella fastidiosa* in Hemiptera collected from central Texas vineyards. *Journal of Economic Entomology* 102:1743-1749.
88. Moller, W. J., Sanborn, R. R., Mircetic, S., Williams, H. E., and Beutel, J. A. 1974. Newly recognized and serious leaf scorch disease of almond. *Plant Disease Reporter* 58:99-101.

89. Newman, K. L., Almeida, R. P. P., Purcell, A. H., and Lindow, S. E. 2003. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. *Applied and Environmental Microbiology* 69:7319-7327.
90. Nowak, D. J., Hoehn III, R. E., Crane, D. E., Stevens, J. C., and Walton, J. T. 2006. Washington, D.C.'s Urban Forest. Pages 17-20. Northern Research Station, 11 Campus Blvd Suite 200 Newton Square, PA 19073-3294.
91. Nunney, L., Schuenzel, E. L., Scally, M., Bromley, R. E., and Stouthamer, R. 2014. Large-scale intersubspecific recombination in the plant-pathogenic bacterium *Xylella fastidiosa* is associated with the host shift to mulberry. *Applied and Environmental Microbiology* 80:3025-3033.
92. Nunney, L., Vickerman, D. B., Bromley, R. E., Russell, S. A., Hartman, J. R., Morano, L. D., and Stouthamer, R. 2013. Recent evolutionary radiation and host plant specialization in the *Xylella fastidiosa* subspecies native to the United States. *Applied and Environmental Microbiology* 79:2189-2200.
93. Nunney, L., Yuan, X. L., Bromley, R., Hartung, J., Montero-Astua, M., Moreira, L., Ortiz, B., and Stouthamer, R. 2010. Population genomic analysis of a bacterial plant pathogen: novel insight into the origin of Pierce's disease of grapevine in the US. *Plos One* 5.11: e15488.
94. Nyland, G., Goheen, A. C., Lowe, S. K., and Kirkpatr.Hc. 1973. Ultrastructure of a rickettsia-like organism from a peach tree affected with phony disease. *Phytopathology* 63:1275-1278.

95. Oliveira, A. C., Vallim, M. A., Semighini, C. P., Araujo, W. L., Goldman, G. H., and Machado, M. A. 2002. Quantification of *Xylella fastidiosa* from citrus trees by real-time polymerase chain reaction assay. *Phytopathology* 92:1048-1054.
96. Parker, J. K., Havird, J. C., and De La Fuente, L. 2012. Differentiation of *Xylella fastidiosa* strains via multilocus sequence analysis of environmentally mediated genes (MLSA-E). *Applied and Environmental Microbiology* 78:1385-1396.
97. Pooler, M. R., and Hartung, J. S. 1995. Genetic relationships among strains of *Xylella fastidiosa* from rapid-pcr data. *Current Microbiology* 31:134-137.
98. Pooler, M. R., Myung, I. S., Bentz, J., Sherald, J., and Hartung, J. S. 1997. Detection of *Xylella fastidiosa* in potential insect vectors by immunomagnetic separation and nested polymerase chain reaction. *Letters in Applied Microbiology* 25:123-126.
99. Prado, S. D., Lopes, J. R. S., Demetrio, C. G. B., Borgatto, A. F., and Almeida, R. P. P. 2008. Host colonization differences between citrus and coffee isolates of *Xylella fastidiosa* in reciprocal inoculation. *Scientia Agricola* 65:251-258.
100. Purcell, A. H., and Hopkins, D. L. 1996. Fastidious xylem-limited bacterial plant pathogens. *Annual Review of Phytopathology* 34:131-151.
101. Purcell, A. H., Saunders, S. R., Hendson, M., Grebus, M. E., and Henry, M. J. 1999. Causal role of *Xylella fastidiosa* in oleander leaf scorch disease. *Phytopathology* 89:53-58.
102. Qin, X. T., Miranda, V. S., Machado, M. A., Lemos, E. G. M., and Hartung, J. S. 2001. An evaluation of the genetic diversity of *Xylella fastidiosa* isolated from diseased citrus and coffee in Sao Paulo, Brazil. *Phytopathology* 91:599-605.

103. Raju, B. C., and Wells, J. M. 1986. Diseases caused by fastidious xylem-limited bacteria and strategies for management. *Plant Disease* 70:182-186.
104. Randall, J. J., Goldberg, N. P., Kemp, J. D., Radionenko, M., French, J. M., Olsen, M. W., and Hanson, S. F. 2009. Genetic analysis of a novel *Xylella fastidiosa* subspecies found in the southwestern United States. *Applied and Environmental Microbiology* 75:5631-5638.
105. Roberto, S. R., Coutinho, A., Lima, J. E. O. D., Miranda, V. S., and Carlos, E. F. 1996. Transmission of *Xylella fastidiosa* by the sharpshooters *Dilobopterus costalimai*, *Acrogonia terminalis* and *Oncometopia facialis* in citrus. *Fitopatologia Brasileira* 21:517-518.
106. Rogers, E. E., and Stenger, D. C. 2012. A conjugative 38 kB plasmid is present in multiple subspecies of *Xylella fastidiosa*. *Plos One* 7.12: e52131.
107. Scally, M., Schuenzel, E. L., Stouthamer, R., and Nunney, L. 2005. Multilocus sequence type system for the plant pathogen *Xylella fastidiosa* and relative contributions of recombination and point mutation to clonal diversity. *Applied and Environmental Microbiology* 71:8491-8499.
108. Schaad, N. W., Postnikova, E., Lacy, G., Fatmi, M., and Chang, C. J. 2004. *Xylella fastidiosa* subspecies: *X. fastidiosa* subsp. *piercei*, subsp. nov., *X. fastidiosa* subsp. *multiplex* subsp. nov., and *X. fastidiosa* subsp. *pauca* subsp. nov. (vol 27, pg 290, 2004). *Systematic and Applied Microbiology* 27:763-763.
109. Schuenzel, E. L., Scally, M., Stouthamer, R., and Nunney, L. 2005. A multigene phylogenetic study of clonal diversity and divergence in North American strains

- of the plant pathogen *Xylella fastidiosa*. Applied and Environmental Microbiology 71:3832-3839.
110. Sherald, J. L. 2007. Bacterial leaf scorch of landscape trees: What we know and what we do not know. Arboriculture & Urban Forestry 33:376-385.
  111. Sherald, J. L. 1993. Pathogenicity of *Xylella fastidiosa* in American elm and failure of reciprocal transmission between strains from elm and sycamore. Plant Dis 77:190-193.
  112. Sherald, J. L., and Hearon, S. S. 1978. Bacteriallike organisms associated with elm leaf scorch. Phytopathology News:73.
  113. Sherald, J. L., Hearon, S. S., Kostka, S. J., and Morgan, D. L. 1983. Sycamore leaf scorch: Culture and pathogenicity of fastidious xylem-limited bacteria from scorch-affected trees. Plant Disease 67:849-852.
  114. Sherald, J. L., and Lei, J. D. 1991. Evaluation of a rapid ELISA test kit for detection of *Xylella fastidiosa* in landscape trees. Plant Disease 75:200-203.
  115. Sherald, J. L., Patton, E. N., Stidham, T. M., and Favre, C. L. 1994. Incidence and development of bacterial leaf scorch of elm on the National Mall. Journal of Arboriculture 20:18-23.
  116. Sherald, J. L., Wells, J. M., and Hurtt, S. S. 1987. Association of fastidious, xylem-inhabiting bacteria with leaf scorch in red maple. Plant Disease 71:930-933.
  117. Simpson, A. J. G., Reinach, F. C., Arruda, P., Abreu, F. A., Acencio, M., Alvarenga, R., Alves, L. M. C., Araya, J. E., Baia, G. S., Baptista, C. S., Barros, M. H., Bonaccorsi, E. D., Bordin, S., Bove, J. M., Briones, M. R. S., Bueno, M.



R. P., Camargo, A. A., Camargo, L. E. A., Carraro, D. M., Carrer, H., Colauto, N. B., Colombo, C., Costa, F. F., Costa, M. C. R., Costa-Neto, C. M., Coutinho, L. L., Cristofani, M., Dias-Neto, E., Docena, C., El-Dorry, H., Facincani, A. P., Ferreira, A. J. S., Ferreira, V. C. A., Ferro, J. A., Fraga, J. S., Franca, S. C., Franco, M. C., Frohme, M., Furlan, L. R., Garnier, M., Goldman, G. H., Goldman, M. H. S., Gomes, S. L., Gruber, A., Ho, P. L., Hoheisel, J. D., Junqueira, M. L., Kemper, E. L., Kitajima, J. P., Krieger, J. E., Kuramae, E. E., Laigret, F., Lambais, M. R., Leite, L. C. C., Lemos, E. G. M., Lemos, M. V. F., Lopes, S. A., Lopes, C. R., Machado, J. A., Machado, M. A., Madeira, A., Madeira, H. M. F., Marino, C. L., Marques, M. V., Martins, E. A. L., Martins, E. M. F., Matsukuma, A. Y., Menck, C. F. M., Miracca, E. C., Miyaki, C. Y., Monteiro-Vitorello, C. B., Moon, D. H., Nagai, M. A., Nascimento, A., Netto, L. E. S., Nhani, A., Nobrega, F. G., Nunes, L. R., Oliveira, M. A., de Oliveira, M. C., de Oliveira, R. C., Palmieri, D. A., Paris, A., Peixoto, B. R., Pereira, G. A. G., Pereira, H. A., Pesquero, J. B., Quaggio, R. B., Roberto, P. G., Rodrigues, V., Rosa, A. J. D., de Rosa, V. E., de Sa, R. G., Santelli, R. V., Sawasaki, H. E., da Silva, A. C. R., da Silva, A. M., da Silva, F. R., Silva, W. A., da Silveira, J. F., Silvestri, M. L. Z., Siqueira, W. J., de Souza, A. A., de Souza, A. P., Terenzi, M. F., Truffi, D., Tsai, S. M., Tshako, M. H., Vallada, H., Van Sluys, M. A., Verjovski-Almeida, S., Vettore, A. L., Zago, M. A., Zatz, M., Meidanis, J., Setubal, J. C., and *Xylella fastidiosa* Consortium, O. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* 406:151-157.

118. Son, Y., Backus, E. A., Groves, R. L., and Johnson, M. W. 2012. Pattern of stylet penetration activity by *Homalodisca vitripennis* (Hemiptera: Cicadellidae) adults in relation to environmental temperature and light conditions. *Environmental Entomology* 41:1215-1230.
119. Su, C. C., Chang, C. J., Chang, C. M., Shih, H. T., Tzeng, K. C., Jan, F. J., Kao, C. W., and Deng, W. L. 2013. Pierce's disease of grapevines in Taiwan: Isolation, cultivation and pathogenicity of *Xylella fastidiosa*. *Journal of Phytopathology* 161:389-396.
120. Su, C. C., Chang, C. J., Yang, W. J., Hsu, S. T., Tzeng, K. C., Jan, F. J., and Deng, W. L. 2012. Specific characters of 16S rRNA gene and 16S-23S rRNA internal transcribed spacer sequences of *Xylella fastidiosa* pear leaf scorch strains. *European Journal of Plant Pathology* 132:203-216.
121. Sutula, C. L., Gillett, J. M., Morrissey, S. M., and Ramsdell, D. C. 1986. Interpreting ELISA data and establishing the positive-negative threshold. *Plant Disease* 70:722-726.
122. Tubajika, K. M., Civerolo, E. L., Puterka, G. J., Hashim, J. M., and Luvisi, D. A. 2007. The effects of kaolin, harpin, and imidacloprid on development of Pierce's disease in grape. *Crop Protection* 26:92-99.
123. Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J. A. M. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35:W71-W74.
124. Van Sluys, M. A., de Oliveira, M. C., Monteiro-Vitorello, C. B., Miyaki, C. Y., Furlan, L. R., Camargo, L. E. A., da Silva, A. C. R., Moon, D. H., Takita, M. A.,

- Lemos, E. G. M., Machado, M. A., Ferro, M. I. T., da Silva, F. R., Goldman, M. H. S., Goldman, G. H., Lemos, M. V. F., El-Dorry, H., Tsai, S. M., Carrer, H., Carraro, D. M., de Oliveira, R. C., Nunes, L. R., Siqueira, W. J., Coutinho, L. L., Kimura, E. T., Ferro, E. S., Harakava, R., Kuramae, E. E., Marino, C. L., Giglioti, E., Abreu, I. L., Alves, L. M. C., do Amaral, A. M., Baia, G. S., Blanco, S. R., Brito, M. S., Cannavan, F. S., Celestino, A. V., da Cunha, A. F., Fenille, R. C., Ferro, J. A., Formighieri, E. F., Kishi, L. T., Leoni, S. G., Oliveira, A. R., Rosa, V. E., Sasaki, F. T., Sena, J. A. D., de Souza, A. A., Truffi, D., Tsukumo, F., Yanai, G. M., Zaros, L. G., Civerolo, E. L., Simpson, A. J. G., Almeida, N. F., Setubal, J. C., and Kitajima, J. P. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *Journal of Bacteriology* 185:1018-1026.
125. W, G., J, S., T, Z., and Q, H. 2014. Genome sequence of a *Xylella fastidiosa* strain causing mulberry leaf scorch disease in Maryland. *Genome Announc* 2:2.
126. Weimer, J. L. 1936. Alfalfa dwarf, a virus disease transmissible by grafting. *J. Agric. Res.* 55:87-104.
127. Wells, J. M., Raju, B. C., Hung, H. Y., Weisburg, W. G., Mandelcopaul, L., and Brenner, D. J. 1987. *Xylella fastidiosa* gen.nov., sp.nov.: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. *International Journal of Systematic Bacteriology* 37:136-143.
128. Wells, J. M., Raju, B. C., Nyland, G., and Lowe, S. K. 1981. Medium for isolation and growth of bacteria associated with plum leaf scald and phony peach diseases. *Applied and Environmental Microbiology* 42:357-363.

129. Wester, H. V., and Jylkka, E. W. 1959. Elm Scorch, graft transmissible virus of American elm. *Plant Disease Reporter* 43:519.
130. Wickert, E., Machado, M. A., and Lemos, E. G. M. 2007. Evaluation of the genetic diversity of *Xylella fastidiosa* strains from citrus and coffee hosts by single-nucleotide polymorphism markers. *Phytopathology* 97:1543-1549.
131. Wistrom, C., and Purcell, A. H. 2005. The fate of *Xylella fastidiosa* in vineyard weeds and other alternate hosts in California. *Plant Disease* 89:994-999.
132. Yuan, X., Morano, L., Bromley, R., Spring-Pearson, S., Stouthamer, R., and Nunney, L. 2010. Multilocus sequence typing of *Xylella fastidiosa* causing Pierce's disease and oleander leaf scorch in the United States. *Phytopathology* 100:601-611.
133. Zhang, J. X., Lashomb, J., Gould, A., and Hamilton, G. 2011. Cicadomorpha insects associated with bacterial leaf scorch infected oak in central New Jersey. *Environmental Entomology* 40:1131-1143.
134. Zhang, S., Flores-Cruz, Z., Kumar, D., Chakrabarty, P., Hopkins, D. L., and Gabriel, D. W. 2011. The *Xylella fastidiosa* biocontrol strain EB92-1 genome is very similar and syntenic to Pierce's disease strains. *Journal of Bacteriology* 193:5576-5577.