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PHYLOGENETIC ANALYSIS OF KENTUCKY STRAINS OF XYLELLA FASTIDIOSA

J. NICOLE MUNDELL
University of Kentucky

ABSTRACT OF MASTER'S THESIS

J. NICOLE MUNDELL

The Graduate School

University of Kentucky

2005

PHYLOGENETIC ANALYSIS OF KENTUCKY STRAINS OF *XYLELLA FASTIDIOSA*

ABSTRACT OF THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master's of Science in the College of Agriculture
at the University of Kentucky

By

J. Nicole Mundell

Lexington, Kentucky

Director: Dr. Christopher L. Schardl, Professor of Plant Pathology

Lexington, Kentucky

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ABSTRACT OF THESIS

PHYLOGENETIC ANALYSIS OF KENTUCKY STRAINS OF *XYLELLA FASTIDIOSA*

Phytopathogenic bacterium, *Xylella fastidiosa*, causes a number of economically important diseases, including Pierce's disease (PD) of grape and bacterial leaf scorch (BLS) of a number of landscape trees. In Kentucky (KY), BLS affects a number of shade trees including many oak and maple species. In 2001, PD was diagnosed in grapevines in western KY. *Xylella fastidiosa* is also detected in many asymptomatic landscape plants and grasses. It was the goal of this research to identify hosts of *X. fastidiosa* around KY and use phylogenetic analysis to compare sequences of the 16S rDNA and gyrase B (*gyrB*) genes between samples. This research tests the hypothesis that sequence comparison can identify asymptomatic hosts and vectors that serve as a source of inoculum for pathogenic strains of *X. fastidiosa*.

Plant collections were done in urban areas of KY between 2002 and 2004 and samples were tested for the presence of *X. fastidiosa* by ELISA and PCR. A number of symptomatic and asymptomatic plants were found to be hosts. Primer sets specifically developed for *X. fastidiosa* were used to amplify part of the 16S rDNA and the *gyrB* gene from DNA extracted directly from plant tissue. Sequence data from these specifically amplified products were assembled using Phrap, aligned with ClustalW, then phylogenetic analysis was done with Paup 4.0b10 beta. Comparisons with strains outside of Kentucky were also done using *X. fastidiosa* sequence obtained from NCBI.

Maximum parsimony (MP) trees from the 16S rDNA showed a clade of sequence from oak and grass samples that is an outgroup to sequence from NCBI and other samples in this study. According to BLAST, sequences in this outgroup clade seem to be more closely related to the genera *Xanthomonas* or *Stenotrophomonas* than *Xylella*. However, the *gyrB* gene MP tree showed sequence from three of the samples that were part of this outgroup clade as being closely related to those *X. fastidiosa* sequences that are part of the ingroup of both 16S rDNA and *gyrB* trees. The topology difference between the 16S rDNA and *gyrB* trees suggest there may have been recombination in the genomic region containing one of these genes.

KEYWORDS: *Xylella fastidiosa*, Bacterial Leaf Scorch, Pierce's disease, 16S rDNA, *gyrB* gene

J. Nicole Mundell

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By

J. Nicole Mundell

Christopher L. Schardl

_____

Director of Dissertation

Lisa J. Vaillancourt

_____

Director of Graduate Studies

05/06/2005

_____

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Chapter 1

Introduction

Background

History of *Xylella fastidiosa* diseases

The plant pathogenic bacterium, *Xylella fastidiosa*, is the causal agent of leaf scorching diseases in a number of plant hosts. In Kentucky, it is known to cause bacterial leaf scorch (BLS) of shade trees, particularly in urban landscapes, and also Pierce's disease (PD) of grape (Davis et al. 1978). BLS affects a wide range of plant hosts, including oaks (Chang and Walker 1988), maple (Sherald et al. 1987), mulberry (*Morus rubra*)(Kostka et al. 1986) and elm (*Ulmus americana*)(Sherald 1993; Wester and Jylkka 1959). It also affects sycamore (*Platanus occidentalis*) in paper plantations (Sherald et al. 1983).

The problem of BLS in Kentucky goes back to the late 1970's with the first observations of marginal leaf necrosis and early leaf drop, typical symptoms of BLS, around Lexington, Kentucky (J.R. Hartman, personal communication). *Xylella fastidiosa* was first diagnosed in pin oaks (*Quercus palustrus*) in Lexington in 1987 using symptoms, ELISA and electron microscopic observation of bacterial cells in the xylem vessels (Hartman et al. 1991). Positive diagnosis was not possible until the bacterium was identified and described (Wells et al. 1987).

Surveys conducted between 1989 and 1990 resulted in positive diagnoses of BLS in pin oaks in 11 cities across Kentucky (Hartman et al. 1991), and northern red oaks (*Quercus rubra*) in Lexington and Owensboro. In surveys conducted over the following five years, bur oak (*Quercus macrocarpa*), shingle oak (*Quercus imbricaria*), sycamore, sweetgum (*Liquidambar styraciflua*) and sugar maple (*Acer saccharum*) were added to the list of hosts in Kentucky (Hartman et al. 1992; Hartman et al. 1995; Hartman et al. 1996).

PD was diagnosed on Mars cultivar grapevines from a Hancock County, Kentucky vineyard in September 2001. Since this is the first occurrence of PD in Kentucky, the source of the bacterium is in question. The vines originated at a nursery in Arkansas, but other stock from the same nursery was uninfected. Also in 2001, PD was diagnosed on Chancellor grapevines in an Indiana vineyard. The PD in Hancock County could either be a genuine PD strain from a southern state or it may not be a true PD strain, but rather a BLS strain infecting grapevine.

BLS causes major economic damage in cities where severely infected trees have to be removed and replaced. It is also costly in plantations of sycamore. In spite of this economic impact, not much is known about the strains of *X. fastidiosa* that cause BLS. PD and citrus variegated chlorosis (CVC) of sweet orange (Rossetti et al. 1990) are the two most economically important of the diseases caused by *X. fastidiosa*. Consequently, much of what is known about *X. fastidiosa* was discovered through research on these higher profile diseases.

PD was first described in the 1880's by Newton B. Pierce (Pierce 1882), one of a small group of American plant pathologists with formal training at that time (Hopkins and Purcell 2002). Pierce was investigating a "mysterious disease" of grape that was devastating vineyards in the Los Angeles Basin. He was unable to complete Koch's postulates and identify the causal agent, and grape production in the L.A. Basin eventually stopped altogether. PD also affected grape production in the southeastern U.S., but was not studied for the simple reason that it was so deadly and widespread that susceptible crops could not be cultivated (Hopkins and Purcell 2002).

Research on PD resumed between 1939 and 1945 when another outbreak threatened grapevines in Northern California. During this time, vectors were identified to be xylem-feeding leafhoppers and spittlebugs (Freitag and Frazier 1954). The most recent research effort began with the introduction of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, to California in 1989 (Varela

et al. 2001). GWSS is able to travel farther than the native Californian sharpshooters, and is thereby able to transmit PD over longer distances, threatening more vineyards. Also, unlike the native vectors, GWSS is able to penetrate older, lignified canes and the bark on the lower portion of the vines which makes removal of infected tissue difficult.

The history of CVC is more recent than that of PD. First noticed in orange groves of Argentina in the 1980s, CVC spread from there to groves in Brazil. The epidemic of CVC in Brazil grew from just a few trees in 1987 (Rossetti et al. 1990) to over 120 million diseased trees in 2002 (Hopkins and Purcell 2002). The severity and fast spread of CVC in Brazil, coupled with the threat CVC posed to citrus production in North America made this disease an important focus for research efforts.

Taxonomy and Biology of *Xylella fastidiosa*

Xylella fastidiosa is a gram-negative rod-shaped bacterium with thick, rippled cell walls (Wells et al. 1987), and resides in the xylem vessels of host plants (Wells et al. 1987). *Xylella* is a genus in the Xanthomonas group, which is part of the Gamma subdivision of the Proteobacteria (Wells et al. 1987). *Xylella fastidiosa* is the only species in the genus, but there is evidence that may justify the description of subspecies. Three subspecies have been proposed (Schaad et al. 2004), *X. fastidiosa* subsp. *piercei*, *X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *pauca*. DNA-DNA relatedness studies and sequence analysis of the 16S-23S intergenic spacer region are considered diagnostic for these subspecies.

Definitive host ranges have not been determined for every strain of *X. fastidiosa*, and cross-infectivity studies give confusing results. For example, PD strains do not infect oleander and oleander leaf scorch (OLS) strains do not infect grape (Purcell et al. 1999) but both strains infect, and cause disease in, almond (Hopkins and Purcell 2002). Further confounding the differentiation of strains is the likelihood that symptom development is more dependent on host response

than on distinct *X. fastidiosa* strain factors (Li et al. 2001). Therefore, an almond tree exhibits the same symptoms whether infected with isolates of the PD or the OLS pathotypes.

When the bacteria are deposited into a xylem vessel, they multiply and fill the space with their microcolonies and extracellular polysaccharides (da Silva et al. 2001; Purcell et al. 1996; Tyson et al. 1985). When the xylem vessel is full, the bacteria break through the cell walls and spread into adjacent vessels. In this manner, the bacteria colonize the xylem of the plant. The physical clogging of the xylem vessels effectively causes water stress in the plant, resulting in the drought-like symptoms that are typical of *X. fastidiosa* infection in susceptible hosts. Due to the time it takes for the bacteria to fully colonize and occlude the xylem vessels, symptom development can take 5 months or more (Varela et al. 2001).

The bacteria do not survive cold winter temperatures, so the cycle of colonization and occlusion of xylem vessels occurs each year in systemically infected plants. This explains the appearance of symptoms of diseases caused by *X. fastidiosa* in late July or early August in temperate climates. It also explains why PD is more severe in warmer climates like Florida (Hopkins and Purcell 2002). Research on grape showed that viable *X. fastidiosa* population is reduced, though not completely eliminated, in temperatures below 10°C (Feil and Purcell 2001). Diseases caused by *X. fastidiosa* rarely occur in parts of North America that experience cold winters (Hopkins and Purcell 2002).

The geographic distribution and range of symptomatic hosts of *X. fastidiosa* suggest that it is native to the Americas. Diseases caused by *X. fastidiosa* are known to occur only in North and South America with the exceptions of pear leaf scorch in Taiwan (Leu and Su 1993) and one report of PD in Kosovo (Berisha et al. 1998). Also, PD is more severe in the European grape species *Vitis vinifera* than in *Vitis* species native to the southeastern United States. The occurrence of BLS in native North American tree species is more likely to be an effect of the

stressful environment of the urban landscape than the pathogenicity of *X. fastidiosa*.

Avirulence and hypersensitive response genes have not been found after studying genomes of two different strains of *X. fastidiosa* (Simpson et al. 2000; Van Sluys et al. 2003). This lack of evidence of evolved plant-pathogen interaction, coupled with the presence of the *X. fastidiosa* in asymptomatic hosts and the fact that population level within the plant appears to be the primary factor for disease development suggest that this bacterium originated as a benign endophyte.

Symptom Development

The characteristic symptom of BLS on shade trees is marginal scorching of the leaves separated from green tissue by a faint band of chlorosis (Fig. 1.1) (Chang and Walker 1988). Early infection affects single branches, but 2-3 years after infection the entire crown will show scorching (Fig. 1.2). Additional symptoms include early leaf drop and dieback (Fig. 1.3).

Symptoms of PD on grape begin with mild interveinal chlorosis of the leaves that turns into marginal leaf necrosis coupled with a band of chlorosis between dead and green leaf tissue by late summer. As the disease progresses, infected vines develop green islands, or isolated areas of green tissue on lignified branches caused by irregular maturation of woody tissue (Varela et al. 2001). In some grape varieties, the petioles of leaves stay attached to the branches after leaves fall off. Infection also results in dieback and the production of shriveled, unmarketable grapes.

Early symptoms of CVC on sweet orange are the formation of chlorotic lesions between veins of the leaves (Rossetti et al. 1990). As infection progresses, newly produced leaves and fruit are smaller than from healthy trees. Like grapes of PD infected vines, the oranges of symptomatic trees are also unmarketable.

In addition to the many symptomatic hosts, the long list of hosts for *X. fastidiosa* includes asymptomatic hosts. In asymptomatic hosts, *X. fastidiosa* is an endophyte that can be systemic or non-systemic through the xylem of the plant (Purcell and Saunders 1999; Hill and Purcell 1995b). The bacterial titer within asymptomatic hosts does not reach levels that occlude the xylem, and these hosts do not experience water stress as do symptomatic hosts. Even hosts that are locally but not systemically infected can be sources of inoculum for the bacterium (Hill and Purcell 1997).

Transmission of *Xylella fastidiosa* and Disease Management

Xylella fastidiosa is limited to the xylem vessels of the plant; therefore, transmission requires transfer of infested xylem fluid. This is primarily done by xylophagous leafhoppers in the *Cicadellidae*, subfamily *Cicadellinae*, and spittlebugs in the *Cercopidae* (Freitag and Frazier 1954; Hopkins 1989). *Xylella fastidiosa* can also be transmitted by grafting (Wester and Jylkka 1959).

Limited studies have been done to identify vectors of BLS. *Xylella fastidiosa* was detected in the following leafhopper species collected in oak trees in Washington, D.C.: *Aulacizes irrorata*, *Graphocephala coccinea*, *G. versuta*, *Oncometopia undata*, *Erythroneura* spp. and *Typhlocybia* spp. (Pooler et al. 1997). In Kentucky, treehoppers in the genera *Enchenopa*, *Ophiderma*, *Archasia*, *Telamona*, *Glossonotus* and *Microcentrus* were collected on pin oaks and may be associated with *X. fastidiosa* transmission (Johnson and Freytag 1997). Other potential vectors of BLS in Kentucky have been identified based on their xylem-feeding ability and their relationship to known vectors of PD and CVC (R.T. Bessin, personal communication). These potential vectors include *G. coccinea*, *G. versuta*, *O. orbona*, *Orientus ishidae* and *Paraulacizes irrorata*.

Vectors of PD in Florida and California and CVC in Brazil are better-studied than those of BLS. Besides GWSS, the introduced vector, PD is also known to be vectored in California by native leafhoppers including the

bluegreen sharpshooter (BGSS), *G. atropunctata*, the red-headed sharpshooter (RHSS), *Carneocephala fulgida,* and the green sharpshooter (GSS), *Draeculacephala minerva* (Freitag and Frazier 1954). Nielsen (1968) found that *O. orbona* (Fabr.) and *G. versuta* (Say) vector PD in the southeastern U.S. Extensive leafhopper transmission studies done with a PD strain of *X. fastidiosa* demonstrate the polyphagous behavior of these insects (Freitag 1951; Freitag and Frazier 1954; Hill 1995b), and a definitive host range of these vectors has not been established.

The CVC bacterium in South America is also transmitted by sharpshooter leafhoppers. Brlansky et al. (2002) found that *O. nigricans*, a PD vector native to Florida, is able to transmit the CVC bacterium, an important discovery because *O. nigricans* is known to feed on citrus trees as well.

Following acquisition, vectors can transmit the bacterium without a latent period (Varela et al. 2001). Electron microscopy studies on BGSS revealed that the bacteria attach to the foregut and multiply within the sharpshooter (Purcell et al. 1979). In a study by Hill and Purcell (Hill and Purcell 1995a), as the bacterial population within the foregut of BGSS grew between 1 hour and 7 days post-acquisition, transmission efficiency increased from 56% at 1 hour to 92% at 7 days. Regardless of transmission efficiency, the sharpshooters were able to transmit the *X. fastidiosa* when population levels were below the detectable limit of 100 cells. Once the vector acquires *X. fastidiosa*, it is able to transmit the bacteria for the entirety of its life.

The life cycles of the vectors vary by species. BGSS has only one generation per year, GWSS has two, GSS has three and RHSS can have four generations in a year. All of these species overwinter as adults, laying eggs in the spring. Leafhopper transmissions done in the spring by the adults that have overwintered are most important for disease development. Early spring infections allow time for bacterial populations to grow within the plant to levels that cause occlusion of the xylem and the leaf scorching symptoms observed in late summer (Hopkins 1981). These early infections also allow enough time for

colonization of the root parts of the infected plant where the bacteria survive cold winter temperatures.

Currently, vector control and rogueing infected vines or trees are the recommended PD and CVC management strategies (Varela et al. 2001). It is also recommended that growers manage vegetation surrounding vineyards and orchards in order to eliminate vector breeding ground and potential sources of PD and CVC inoculum.

BLS management is more complicated because neither the plants nor the insects can be controlled in urban landscapes as they can in agricultural situations. Despite these difficulties, there are two recommended management methods. Injecting BLS-infected trees with antibiotics can reduce bacterial titer (Kostka et al. 1985), and extend the life of trees, but it does not eliminate the bacteria and is costly to the tree owners. Injections are also costly to the health of the tree because injections introduce wound sites through the bark where opportunistic pathogens can enter and infect the tree. Recommendations for BLS include regular watering, particularly during droughts, and generally maintaining the infected tree's health.

Genomic Studies of *Xylella fastidiosa*

The absence of adequate controls for CVC coupled with its spread and severity, have made this a disease of great importance to researchers in Brazil. In 2000, the genome sequence of *Xylella fastidiosa* strain 9a5c (CVC pathotype) was published by a Brazil-based consortium (Simpson et al. 2000), making *X. fastidiosa* the first non-viral plant pathogen to have its entire genome sequenced. Since then, the genomes of a PD strain, Temecula, an almond leaf scorch strain, Dixon, and an oleander leaf scorch strain, Ann1, have been completely sequenced (Bhattacharyya et al. 2002; Van Sluys et al. 2003).

Comparison of the genomes of strains 9a5c, Dixon and Ann1 revealed factors related to pathogenicity, such as fimbrial genes and afimbrial adhesins,

and protein clusters that are specific for each strain (Bhattacharyya et al. 2002). Comparative analysis of the Temecula and CVC strains (Van Sluys et al. 2003) showed genes that are likely involved in the interaction between *X. fastidiosa* and hosts, like the toxin-producing genes colicin and bacteriocin. This analysis also revealed the presence of genomic islands that are unique for each strain.

In addition to valuable information about pathogenicity of *X. fastidiosa* and the discovery of genetic features that could be useful for differentiation of strains, the comparative analyses of these genomes showed evidence for horizontal gene transfer (HGT). Bhattacharyya et al. (2002) looked for HGT by comparing the sequence of open reading frames (ORFs) and determining which, if any, shared stronger similarity to ORFs from outside *X. fastidiosa*. They found a number of putative candidates for HGT, including genes in the type IV secretion pathway and an ADP/ATP carrier protein. Van Sluys et al. (2003) found evidence for three genomic reorganizations facilitated by phage.

Research Questions

There are a number of interesting research questions raised in regards to the plant pathogen *Xylella fastidiosa*. The wide host range and lack of control for diseases caused by *X. fastidiosa* highlight the need to further characterize and differentiate the species. In a study in which subspecies were proposed (Schaad et al. 2004) only 26 strains of *X. fastidiosa* were included. Notably absent from the study are strains of *X. fastidiosa* associated with BLS of oaks and mulberry. The effort to differentiate strains of *X. fastidiosa* requires further study, including surveys to identify and isolate from asymptomatic hosts. The difficulty of isolating *X. fastidiosa* from all hosts means that molecular methods for differentiation may have to be adapted for use with DNA extracted directly from plant tissue.

The ability to differentiate strains of *X. fastidiosa* may yield information about sources of inoculum for pathogenic strains. This avenue of research could

be useful in controlling diseases caused by *X. fastidiosa*. Studies to identify potential hosts for BLS strains of *X. fastidiosa* have not been done, but a number of studies have identified potential hosts of the PD bacterium around California vineyards (Purcell and Saunders 1999; Freitag 1951). However, these greenhouse studies have failed to establish that hosts capable of supporting PD strains of *X. fastidiosa* actually host these pathogenic strains in nature, and the means of making such an assertion are still questionable, even if a gene chosen for comparison lacks sequence differences between the strains.

Phylogenetic analyses of the 16S rDNA and 16S-23S intergenic spacer region are commonly used to compare strains of *X. fastidiosa* (Chen et al. 2000; Chen et al. 2000; Mehta and Rosato 2001; Rodrigues et al. 2003). In addition to these, the gyrase B gene has also been used for phylogenetic analysis. It is ubiquitous and conserved but gives greater variation than the 16S rDNA (Rodrigues et al. 2003).

These studies may not give an accurate picture of the relationships since they neither address nor account for the impact of HGT and recombination in the genomes. HGT has been well-documented in bacteria (Ochman et al. 2000), and comparative genomics suggests the occurrence of HGT in *X. fastidiosa* in particular (Bhattacharyya et al. 2002; Van Sluys et al. 2003).

It was the goal of this research to collect from potential plant and insect hosts of *X. fastidiosa* around the state of Kentucky and test them for the presence of the bacteria; then, using phylogenetic analysis to compare sequence from the different sources of *X. fastidiosa*, to identify potential sources of inoculum and determine relationships between bacteria found in the different plant and insect hosts. Phylogenetic analysis might also answer the question of whether the Kentucky PD strain is more likely to be a true PD strain or rather a BLS strain infecting grapevine. The hypothesis driving this research was that sequence comparison can identify asymptomatic hosts and vectors that serve as a source of inoculum for pathogenic strains of *X. fastidiosa*.

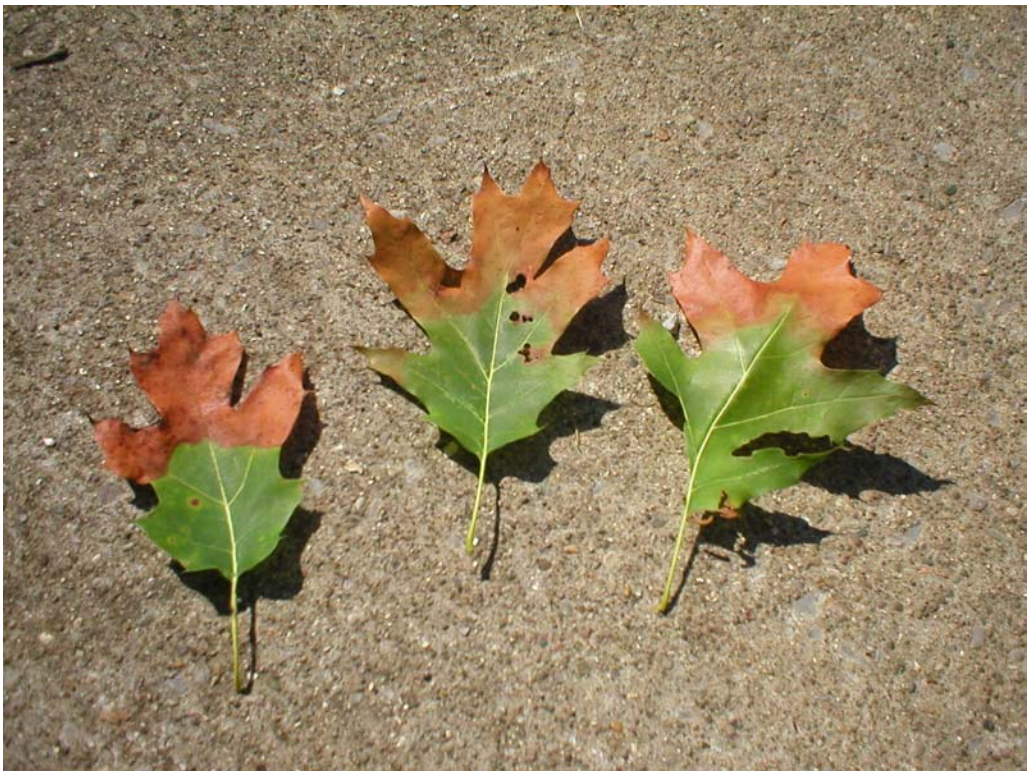

Figure 1.1: BLS symptoms on northern red oak leaves.

Figure 1.2: BLS leaf scorching symptoms on northern red oak.

Figure 1.3: BLS dieback and leaf scorching symptoms on pin oak.

Chapter 2

Isolation and Detection of *Xylella fastidiosa*

Introduction

Annual surveys for hosts of *X. fastidiosa* in Kentucky have been done since ELISA detection has been available (Hartman et al. 1992; Hartman et al. 1995; Hartman et al. 1991; Hartman et al. 1996). The list of identified host species in Kentucky, including the host type and detection method, is given in Table 2.1. This list of known Kentucky hosts is varied and includes both asymptomatic and symptomatic hosts, as indicated (Table 2.1). Surveys have also been done to determine potential vectors of BLS in Lexington, but these have not yet successfully yielded confirmed leafhopper vectors of BLS (R.T. Bessin, personal communication).

The ELISA kit used for detection of *X. fastidiosa* is the PathoScreen-Xf kit (Agdia, Inc., Elkhart, IN). This ELISA test as a detection method can give false positives because it is based on a polyclonal antibody which can allow binding with multiple proteins. It also requires populations of 10^6-10^5 CFU/ml for detection (Wang et al. 1999), and it does not differentiate pathotypes of *X. fastidiosa*. Isolation is definitive confirmation of *X. fastidiosa*, but the fastidious nature of this pathogen makes it difficult, if not impossible, to culture from all suspected hosts using currently available techniques and media. The problems associated with these detection methods have made necessary the development and use of molecular detection methods. For the purpose of this study, the word strain is used to indicate isolates of *X. fastidiosa* and sample refers to *X. fastidiosa* DNA extracted directly from plant material. Pathotype is used for groups of strains that are associated with disease in a particular host, without necessarily implying a particular degree of relationship between strains in a pathotype. For example the PD pathotype is the set of strains that cause disease in grapevines.

Polymerase chain reaction (PCR) has emerged as the technique of choice for relatively quick confirmation of the presence of *X. fastidiosa*. In 1995, specific primers for detection of *X. fastidiosa* were developed by using RAPD analysis (Pooler and Hartung 1995). Schaad et al. (2002) published a protocol for real-time PCR detection of *X. fastidiosa* by using primers designed for the 16S rDNA and the 16S-23S intergenic spacer region. These primer sets, though specific, do not yield enough meaningful sequence for phylogenetic analysis. Rodrigues et al. (2003) developed primer sets for specific amplifications of the 16S rDNA and gyrase B gene of *X. fastidiosa*.

This study continues the annual surveys to identify new symptomatic and asymptomatic hosts of *X. fastidiosa* in Kentucky. It also aims to use PCR detection to definitively associate *X. fastidiosa* with potential leafhopper vectors identified based on their relationship to known vectors in California and the southeastern United States. ELISA, multiple isolation techniques and multiple PCR techniques were used for detection.

Materials and Methods

Collections

Plant collections were begun in late July and ended in October of 2002, 2003 and 2004. Table 2.2 lists the plants collected in 2002 and 2003 with their sample identification and the collection location. In 2002, all plants collected had tested positive for the presence of *X. fastidiosa* by ELISA previous to this study. In 2003, plants were collected on the basis of symptoms of marginal necrosis coupled with a band of chlorosis as is typical of *X. fastidiosa* infection.

Whereas the collections from 2002 and 2003 were done primarily in Lexington, Kentucky, the 2004 collections were made from Owensboro, Paducah and Louisville in Kentucky, as shown in Table 2.3. These urban areas were chosen because of previous diagnosis of BLS of shade trees (Hartman et al. 1991)

and because these cities are in different geographic areas of Kentucky. Samples collected from trees in 2004 were selected based on the typical BLS symptoms. Grass samples were collected in close proximity to symptomatic trees and harborage of suspected vectors. Grass plants were sampled based on preliminary observations that grass samples collected under canopies of BLS-infected oak trees sometimes also tested positive for *X. fastidiosa* by ELISA (J.R. Hartman, personal communication). Other plant material included in the 2004 collection data came from Minnesota.

In addition to the plant collections done in 2002, 2003 and 2004, leafhoppers collected in the spring and summer of 2002 were included in subsequent analyses. Dr. Ric Bessin, Extension Entomologist at the University of Kentucky, collected leafhoppers by sweepnet or on yellow sticky cards. Leafhopper collection data are in Table 2.4.

Isolation and culture maintenance

Isolations were attempted from all plant material collected in 2002. Five of the methods tried were followed as described in publications (Chang and Donaldson 1993; Fry et al. 1990; Huang et al. 2004; McElrone et al. 1999; Schaad et al. 2001). Besides these published protocols, the squeeze method was tried (J. Hartung, personal communication). This method required cutting leaf petioles into 1 cm sections and surface disinfesting them by a 3 min soak in 1% sodium hypochlorite followed by 3 rinses in sterile water. Individual sections were then picked up with sterile tweezers and squeezed until liquid was visible on the ends. The ends of the section with the exudate were then lightly pressed onto an agar plate.

Five different culture medium recipes were initially tried with all the above isolation methods. CS-20, PD2, PW, and Supplemental PW medium were prepared according to recipes published in the *Laboratory Guide for Identification of*

Plant Pathogenic Bacteria (Shaad and Chun 2001). Xf-26 medium was prepared according to the published recipe (Chang and Donaldson 1993).

In 2003, isolations were attempted from all plant material collected, using only the Huang and Sherald (2004) method with Supplemental PW medium plates. Isolations were also attempted from the leafhoppers in Table 2.4 using the method published by Hill and Purcell (1995a) and Supplemental PW medium.

The 2004 isolations were attempted only from samples RO1, MP2, PO-Don, HB1 and SO2. These isolations were done using the Huang and Sherald (2004) and Chang (1993) methods as described, on New PW medium plates. The recipe for New PW agar (C.J. Chang, personal communication), is 4.0 g phytone peptone, 1.0 g tryptone, 1.2 g K_2HPO_4, 1.0 g KH_2PO_4, 0.4 g $MgSO_4$-$7H_2O$, 4.0 g L-glutamine, 2.0 g soluble potato starch, 15 g BactoAgar, 10 ml of 0.1% hemin chloride, and 10 ml of 0.2% Phenol Red dissolved in 1110 ml of distilled water. Prior to autoclaving, New PW medium was brought to a pH range of 6.9-7.2 with 5M NaOH.

Cultures were incubated in the dark at 29°C. Lacking a definitive source for subculture intervals, subculturing from plates was first done monthly. This was changed to every 7-10 days, as suggested by C.J. Chang. Supplemental PW medium was used for subculturing until December 2003, then New PW medium was used exclusively.

Freezer cultures were made of two isolates, MB1 and KYPD1, by putting 0.75 ml of sterile glycerol and 0.75 ml of Supplemental PW broth culture in cryotubes. The tubes were placed in Nalgene's Cryo 1°C Freezing container, put in a -80°C freezer and lowered to the freezer temperature in 1°C increments over four hours. After the tubes reached freezer temperature, they were transferred from the freezing container to a freezer box for long-term storage.

DNA Extractions

In 2002 and 2003, DNA extraction was done directly from plant material that tested positive by ELISA or PCR or by both methods. DNA extractions in 2002 were done on samples KYPD1, RO1, PO1 and HB1 as published in Green et al. (1999).

The 2003 DNA extractions from plant material were done using the woody plant extraction method from Green et al. (1999) and the wasp DNA extraction method, developed in Jim Whitfield's lab by combining other published protocols (Hillis et al. 1990; Sambrook et al. 1989) was used for extracting DNA from leafhoppers in Table 2.4.

DNA extraction was done from all plant material collected in the 2004 season using the Rapid Homogenization Plant Leaf DNA Amplification kit (Cartagen Molecular, Inc., Seattle, WA), according to the manufacturer's instructions. Another round of DNA extraction was done on most of the plant material using the QIAamp DNA Stool mini kit (QIAgen Inc., Valencia, CA), according to the manufacturer's instructions. DNA extraction from pure culture was done using the protocol from Green et al. (1999). For each plant sample, the DNA extraction method used is indicated in Table 2.3.

Detection

ELISA detection of *Xylella fastidiosa* from plant material was done using the PathoScreen-Xf kit (Agdia, Inc.), according to the manufacturer's instructions. The kit was previously tested for efficacy of detecting BLS in sycamore and elm, by Sherald and Lei (1991), and it was used in combination with electron microscopy during early Kentucky surveys for *X. fastidiosa* (Hartman et al. 1992; Hartman et al. 1995; Hartman et al. 1991; Hartman et al. 1996). A number of grass samples were collected in this study, and the PathoScreen-Xf kit (Agdia, Inc.) has not been tested previously for potential cross-reactions with plant sap from grasses. Therefore, tall fescue and perennial ryegrass were germinated

from seed in a closed container, and the seedlings were tested with the PathoScreen-Xf kit (Agdia, Inc.) and by PCR using primer set 272-int (Table 2.6). The protocol for this primer set is described below.

In 2002, PCR detection of *X. fastidiosa* was done using the primer set 27f and 143r from Lane (1991), using a PCR recipe and protocol optimized for use on the SmartCycler (Cepheid, Sunnyvale, CA). Reaction volume was 25 μl reaction containing 4 μM concentrations of each primer, 1 mM $MgCl_2$, 12.5 μl of 2X Brilliant SYBR Green QPCR Mastermix (Stratagene, La Jolla, CA), 5.0 μl of 5X SAR and 1 μl DNA [1-4ng/μl]. The protocol was as follows: 94°C for 30 sec followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec.

From 2003-2004, PCR detection of *X. fastidiosa* was done using the primers 272-1-int and 272-2-int from Pooler and Hartung, (1995); the primer sequences are shown in Table 2.5. Amplification was performed in a SmartCycler in a 25 μl reaction containing 4 μM concentrations of each primer, 1 mM $MgCl_2$, 12.5 μl of 2X Brilliant SYBR Green QPCR Mastermix (Stratagene, La Jolla, CA), 5.0 μl of 5X SAR and 1 μl DNA [1-4ng/μl]. The protocol used was optimized from the Brilliant SYBR Green QPCR Mastermix (Stratagene) suggested protocol for products longer than 400 bp. The protocol began with a 10 min incubation at 95°C, followed by 40 cycles of 30 sec at 95°C, 60 sec at 62°C and 90 sec at 72°C. Cycling was followed by a final extension at 72°C for 4 min and melt curve analysis. With this primer set, DNA was considered positive for the presence of *X. fastidiosa* if the PCR product had a melt temperature (Tm) between 79.4°C-80.9°C.

Protocols for all of the primer sets used in this study differed only by annealing temperature, listed in Table 2.6. Annealing temperatures were optimized for use on the SmartCycler. The expected melt temperatures (Tm) for each primer set were determined empirically by comparing melt temperatures of runs with known-positive DNA with gel images to check for the expected band size. Figure 2.1 shows two graphs generated from the optimization of primer set

272-int. Figure 2.1A is the SYBR green fluorescence curve, and Figure 2.1B shows the melt derivative graph. On the melt derivative graph, the peaks indicate product Tm. Figure 2.2 shows the gel confirmation of the PCR products. The Tm ranges for all primer sets are listed in Table 2.6 along with expected product sizes.

The first PCR done on DNA extracted from each plant sample by each DNA extraction method included controls for PCR inhibition. For each sample, a reaction was done with just the extracted DNA and another reaction was done with both extracted DNA and positive control *X. fastidiosa* DNA (ATCC 35881D). If this control did not amplify, inhibition was suspected and that extracted DNA was diluted 1:10 or 1:100 until the control amplified. Also, for each set of reactions run on the SmartCycler, a reaction with positive control DNA (ATCC 35881D) and another reaction as a negative control containing no DNA were included to test for contamination in the reagents.

PCR detection for DNA extracted from each leafhopper sample (Table 2.4) was done initially with primer set 272-int and later with the *gyrB* primer set. Genomic DNA was electrophoresed in a 1.5% Seakem agarose gel to confirm the presence of DNA. The presence of leafhopper DNA was confirmed by testing sample LH5/8 with primers developed on Vector NTI 7.0, by using sequence of the 18S rDNA and 28S rDNA from leafhoppers *Paraulicizes irrorata* and *Graphocephala hieroglyphica* (data obtained from NCBI). These primer sequences are listed in Table 2.5 as LH18SRNAf with LH18SRNAr and LH28SRNAf with LH28SRNAr. The PCR recipe and protocol were the same as that described above for the 272-int primer set with an annealing temperature of 62°C instead of 60°C. The length of expected product for the LH18SRNA primer set is 1156bp, and approximately 800bp for the LH28SRNA primer set.

DNA extracted from the 2004 collections was also tested for the presence of *X. fastidiosa* DNA by using the forward primer S-S-X.fas-0838-a-S-21 (Rodrigues et al. 2003) with the reverse primer 1525r (Lane 1991); the sequences

of these primers are listed in Table 2.5. This primer set is designated 16S 2nd in Table 2.6. Amplification with this primer set was performed in the SmartCycler in a 100 μl reaction containing 0.3 μM each primer, 1 mM $MgCl_2$, 50 μl 2X Brilliant SYBR Green QPCR Mastermix (Stratagene), 20 μl of 5X SAR and 4 μl DNA [1-4ng/μl].

In addition to PCR for detection, PCR was used to generate products for sequencing. All of the primer sets used were designed by Rodrigues et al. (2003) to be specific for *X. fastidiosa*. These putatively specific primers were used because DNA was extracted directly from plant material. To confirm specificity, they were tested against cell suspensions of *Xanthomonas vesicatoria* strain XV56 and *Xanthomonas axonopodis* pv vesicatoria strain 75.3, both strains provided by the University of Kentucky Plant Pathology Extension Lab. Primer set 16S Set B consists of primers S-S-X.fas-0067-a-S-19 and S-S-X.fas-0838-a-S-21 (Rodrigues et al. 2003), and was used in PCR to amplify the partial first half of the 16S rDNA. 16S Set C consists of primers S-S-X.fas-0838-a-A-21 and S-S-X.fas-1439-a-A-19 (Rodrigues et al. 2003) and was used in PCR to amplify the partial second half of the 16S rDNA. PCR with primer set 16S 2nd also was used to amplify the second half of the 16S rDNA, and was preferred over use of 16S Set C because it generates a larger product. Primer set *gyrB* includes primers FXYgyr499 and RXYgyr907, and was used in PCR to amplify 429 bp of the gyrase B gene. The PCR recipe for all these primer sets was the same as that described above for 16S 2nd.

Generation of PCR product for the 16S-23S intergenic spacer region, from the end of the 16S rDNA to the 23S rDNA, was attempted with multiple primer sets. Forward primer 1439-a-S-19 is the reverse complement of primer S-S-X.fas-1439-a-A-19 (Rodrigues et al. 2003) and was used because of its putative specificity for *X. fastidiosa*. This forward primer was coupled with each of the reverse primers 23S uni 322, G1, 23S-, 23S-1998 and 23r; the sequences and references for these are listed in Table 2.5. Forward primer, Xf 16S 1430F was

developed using the genome sequence of PD strain Temecula on Vector NTI 7.0 and NCBI BLAST to find a primer specific for *X. fastidiosa*. This forward primer was tried with reverse primer 23S uni 322. Optimization of each 16S-23S sequencing primer set was done using the same recipe as described for set 16S 2nd and consisted of trying a range of annealing temperatures with the protocol described for the 272-int and 16S rDNA sequencing primer sets.

RESULTS

Isolations

From the isolations during 2002, pure cultures of strains KYPD1, MB1 and MP1 were obtained on PW-Supplemental medium by using the Huang and Sherald method (2004). These cultures were maintained by monthly subculturing that proved to be insufficient for maintaining viable cells because all three isolates died. The KYPD1 strain was reisolated from a PD-positive grapevine that was collected from the Hancock County vineyard and maintained in a greenhouse. This isolate was subcultured every 7-10 days (C.J. Chang, personal communication). Freezer cultures of the MB1 strain and KYPD1 strain did not grow when streaked onto fresh New PW agar plates.

DNA Extractions

DNA was obtained with all extractions done in 2002 using the Green et al. (1999) method. These DNA extractions tested positive for *X. fastidiosa* DNA by PCR using the 27f-143r primer set. Table 2.7 shows the DNA yield and Tm of PCR products obtained from the samples collected in 2002. The DNA yield was fairly low, ranging from 1-7 ng/μl, but was sufficient for PCR detection.

Results from the DNA extractions done on material collected in 2003 are listed in Table 2.8. For each plant, either the Green et al. (1999) method or the Wasp DNA extraction method or both were used. DNA yield was generally higher when the Wasp DNA extraction method was used, as shown in Table 2.8.

DNA yield from the leafhopper DNA extractions is listed in Table 2.4. Fig. 2.3 shows a 2% Seakem agarose gel electropherogram showing the leafhopper genomic DNA. The smears for the DNA extracted from leafhoppers indicate that, for most of the samples, large amounts of DNA of varying size were obtained.

Figure 2.4 shows a 1.5% Seakem agarose gel electropherogram of some of the DNA extracted using the QIAamp DNA Stool mini kit (QIAgen). The gel shows that adequate quantities of DNA were obtained for all the samples for DNA extraction. PCR results for both the Rapid Homogenization and QIAamp DNA Stool mini extraction kits are listed in Table 2.3. These kits had different efficiencies for obtaining amplifiable *X. fastidiosa* DNA from samples, as shown by the difference in PCR positives. Extractions done using the Rapid Homogenization Plant Leaf DNA Amplification kit (Cartagen) required a 1:100 dilution to overcome inhibitors, and yielded only 23 PCR positives out of 123 attempted (18.7%). The QIAamp DNA Stool mini kit (QIAgen) gave the best results of any of the previously tried DNA extraction techniques, yielding PCR positives for 46 out of 109 samples (42.2%).

Detection

Results for ELISA and PCR detection method are listed in Tables 2.2 and 2.3. Table 2.9 gives a summary that compares ELISA with PCR results for all material collected from 2002-2004. The two largest groups of plant hosts tested by both methods were the grasses and oaks. ELISA gave a positive result for 100% of the grasses and 95.4% of the oaks versus 38.5% and 48.5% positive, respectively, by PCR. As described in the methods, the PathoScreen-Xf kit (Agdia, Inc.) was tested against negative controls consisting of, tall fescue and perennial ryegrass seedlings that had no prior exposure to *X. fastidiosa* vectors. This testing gave a positive result for germinated seedlings.

PCR results for these negative control grass samples, conducted using the 272 internal primers, were negative. This result suggested that the consistent ELISA reactions to grass samples were due to cross-reaction with constituents of grass sap, rather than specific detection of *X. fastidiosa*.

PCR tests for specificity of the primer sets 16S Set B, 16S 2nd and *gyrB* demonstrated negative results when *Xa. vesicatoria* and *Xa. axonopodis* pv vesicatoria DNA was used as template, confirming the specificity of these primer sets under the PCR conditions used in this study.

PCR was also used to confirm the presence of intact leafhopper genomic DNA in extracts from collected samples, and sample LH5/8 was positive for both the LH18SRNA and LH28SRNA products. This result supports the validity of the Wasp DNA extraction technique.

Attempts to generate 16S-23S ITS product were unsuccessful, at the optimization of annealing temperature step, for all primer sets tried.

Discussion

The goal of this study was to find different hosts of *Xylella fastidiosa* in different geographic regions of Kentucky. In addition to the plant material collected in Kentucky, BLS was confirmed both by ELISA and PCR test to be present in pin oak from Illinois and pin oak from Minnesota. Collection efforts were centered on urban areas where BLS was previously found to occur and collection was done with the intent of finding possible sources of inoculum for the BLS and PD strains of *X. fastidiosa*.

Collections done in 2002 and 2003 were done to find and test the efficacy of protocols for isolation in culture and detection. With the isolation of strains KYPD1, MB1 and MP1 in culture, the isolation technique adopted for general use was the Huang and Sherald (2004) method. Isolation proved to be a difficult, time-consuming task that yielded only these 3 isolates, 2 of which died after less than a year.

DNA extraction became the primary means of processing collected plant material because it does not require live bacterial cultures, so plant tissue could be collected and stored for months before being analyzed. DNA extraction has its drawbacks, especially with woody plant tissue. PCR inhibition from DNA extract was a major problem. In 2004, two kits were compared for their ease and efficiency of DNA extraction. The QIAgen kit is equipped with inhibitex tablets that bind PCR inhibitors, making PCR amplification possible from most of the DNA extracts without further treatment or dilution.

The comparison of ELISA and PCR may be more dependent on the sample extraction method than the inherent value of either method alone, but that was not evaluated in this study. ELISA gave an overwhelmingly higher number of positives, but there is no way to know how many of those are false positives. The results of ELISA and PCR analysis of uninfected grass samples (negative controls) strongly suggested cross-reaction of tall fescue and perennial ryegrass sap with the *X. fastidiosa* antibodies. All of the grass samples collected in 2004 tested positive by ELISA, but fewer than half of them were positive by PCR. This result strongly suggests that PCR is a more reliable test for *X. fastidiosa* than is ELISA.

The leafhopper study yielded only one positive association of *X. fastidiosa* with a sample of 2-3 *G. versuta* leafhoppers collected in April of 2002 at the University of Kentucky South Research Farm in Lexington, KY. This result could be accurate, in the sense that there may actually have been only one positive sample out of the 38 leafhopper samples collected. This result could also reflect other problems with the collection and detection of leafhopper vectors. First, the earliest collections were done in April and may have thereby missed overwintering leafhoppers that may have been carrying *X. fastidiosa*. Second, vector suppression in the Hancock County, KY vineyard prevented the collection of great numbers of leafhoppers and may have resulted in collection only of leafhoppers not infested with *X. fastidiosa* that flew in from surrounding areas.

Third, the DNA extraction technique used on each sample of 2-3 leafhoppers was developed for use with wasps and may not have been effective at extracting the bacterial DNA from the foregut of these insects. Immunomagnetic capture has been used in other studies to obtain *X. fastidiosa* DNA from vector insects (Pooler et al. 1997) and, though laborious, may well be superior to the techniques tried here. All of these potential problems might be resolved for future vector collection and detection studies by collecting earlier in the Spring and trying immunomagnetic capture, and perhaps different DNA extraction techniques. The issue of vector suppression in the vineyard that was PD-positive in 2001 and 2002 cannot be directly resolved, but collections could be done from trees in urban areas affected by BLS.

The conclusion of these experiments in isolation and detection of *X. fastidiosa* in Kentucky plants is that PCR is the most reliable method available; however, it is not necessarily the easiest due to the necessity of extracting DNA. DNA extraction is particularly necessary when the plant material is older and dry. One drawback to PCR is the potential for false positives due to contamination, since this method is far more sensitive than ELISA. Another drawback is that PCR does not differentiate between live and dead bacterial cells, so it truly is a method for associating *X. fastidiosa* with symptomatic tissue and cannot be used as the sole means of diagnosing the cause of disease (ELISA may suffer the same drawback). A significant benefit of PCR with the Rodrigues et al. (2003) 16S rDNA and *gyrB* gene primer sets, is that sequencing of these products allows validation and comparison of bacterial genotypes in the plant samples.

Table 2.1: Current list of known hosts of *Xylella fastidiosa* in Kentucky.

Scientific Name	Common Name	Year of Confirmation	Method of Detection	Host Type	Reference
Quercus palustrus	pin oak	1987	ELISA, EM	symptomatic	Hartman et al. 1991
Quercus rubra	Northern red oak	1989	ELISA	symptomatic	Hartman et al. 1991
Quercus macrocarpum	bur oak	1994	ELISA	symptomatic	Hartman et al. 1995
Quercus imbricaria	Shingle oak	1994	ELISA	symptomatic	Hartman et al. 1995
Platanus occidentalis	sycamore	1994	ELISA	symptomatic	Hartman et al. 1995
Acer saccharum	sugar maple	1995	ELISA, EM	symptomatic	Hartman et al. 1996
Liquidambar styraciflua	sweetgum	1995	ELISA, EM	symptomatic	Hartman et al. 1996
Acer rubrum	red maple	2001	ELISA	symptomatic	Hartman 2001
Acer saccharinum	silver maple	2001	ELISA	symptomatic	Hartman 2001
Lolium perenne	perennial ryegrass	2001	ELISA	asymptomatic	Hartman et al. 2001
Poa pratensis	Kentucky bluegrass	2001	ELISA	asymptomatic	Hartman et al. 2001
Muhlenbergia shreberi	nimblewill	2001	ELISA	asymptomatic	Hartman et al. 2001
Lolium pratense	tall fescue	2001	ELISA	asymptomatic	Hartman et al. 2001
Polygonum aviculare	prostrate knotweed	2001	ELISA	asymptomatic	Hartman et al. 2001
Osmanthus americanus	osmanthus	2001	ELISA	asymptomatic	Hartman et al. 2001
Lonicera japonica	honeysuckle	2001	ELISA	asymptomatic	Hartman et al. 2001
Vinca minor	dwarf periwinkle	2001	ELISA	asymptomatic	Hartman et al. 2001
Pieris floribunda	andromeda	2001	ELISA	asymptomatic	Hartman et al. 2001
Quercus alba	white oak	2002	ELISA	symptomatic	Hartman 2002
Quercus phellos	Willow oak	2002	ELISA	symptomatic	Hartman 2002
Platanus acerifolia	London plane	2002	ELISA	symptomatic	Hartman 2002
Ulmus americana	Elm	2002	ELISA	symptomatic	Hartman 2002
Celtis occidentalis	hackberry	2002	ELISA, PCR	symptomatic	Hartman et al. 2002
Quercus coccinea	Scarlet oak	2002	ELISA, PCR	symptomatic	Hartman et al. 2002
Morus rubra	mulberry	2002	ELISA, PCR	symptomatic	Hartman et al. 2002
Quercus bicolor	swamp white oak	2003	ELISA, PCR	symptomatic	This study
Hedera helix	english ivy	2004	PCR	asymptomatic	This study
Quercus stellata	post oak	2004	ELISA, PCR	symptomatic	This study
Vitis vinifera	Mars grapevine	2001	ELISA, PCR	symptomatic	This study

Table 2.2: 2002 and 2003 collection and detection data. For each detection method, a (+) indicates a positive result, and a (–) indicates a negative result. PCR detection was from template DNA isolated by the Green et al. or Wasp DNA extraction methods and with primer set 272-int (Table 2.6).

Plant Species	ID	Year	Location	ELISA	PCR	Isolation
red oak	RO1	2002	Lexington, KY	+	+	-
Mars grapevine	KYPD1	2002	Hancock Co., KY	+	+	+
pin oak	PO1	2002	Lexington, KY	+	+	-
mulberry	MB1	2002	Lexington, KY	+	+	+
sugar maple	MP1	2002	Lexington, KY	+	+	+
hackberry	HB1	2002	Lexington, KY	+	+	-
hackberry	HB2	2002	Lexington, KY	+	+	-
scarlet oak	SO1	2002	Lexington, KY	+	-	-
grapevine	IN-PD1	2002	Indiana	+	-	-
swamp white oak	SWO1	2003	Henderson Co., KY	+	+	-
red oak	RO-Shaw	2003	Lexington, KY	+	+	-
pin oak	POTr	2003	Lexington, KY	+	+	-
pin oak	POTl	2003	Lexington, KY	+	+	-
pin oak	PODon	2003	Lexington, KY	+	+	-
pin oak	POLime	2003	Lexington, KY	+	+	-
pin oak	POCp1	2003	Lexington, KY	+	+	-
red oak	RO2	2003	Lexington, KY	+	+	-
red oak	RO3	2003	Lexington, KY	+	+	-
scarlet oak	SO2	2003	Lexington, KY	+	+	-
sugar maple	MP2	2003	Lexington, KY	+	+	-
pin oak	ILPO1	2003	Illinois	+	+	-
gingko	GK-1	2003	Lexington, KY	-	-	-
ash	AH1	2003	Lexington, KY	-	-	-
sugar maple	MP-E	2003	Lexington, KY	-	-	-

Table 2.3: 2004 collection and detection data. For each detection method, a (+) indicates a positive result, a (-) indicates a negative result and nt indicates the detection method was not tried.

Designation	Plant Host	Location	ELISA	PCR	
				Cartagen Kit	Qiagen Stool Kit
3H	maple	Lexington, KY	nt	-	nt
4H	nimblewill	Lexington, KY	nt	-	nt
12H	tall fescue	Lexington, KY	nt	-	+
2H	pin oak	Lexington, KY	nt	-	nt
13H	Kentucky bluegrass	Lexington, KY	nt	-	+
7H	pin oak	Lexington, KY	nt	+	+
11H	pin oak	Lexington, KY	nt	+	+
14H	perennial ryegrass	Lexington, KY	nt	-	+
10H	pin oak	Lexington, KY	nt	-	nt
8H	pin oak	Lexington, KY	nt	+	+
1H	pin oak	Lexington, KY	nt	+	+
RO-Shaw	northern red oak	Lexington, KY	+	+	+
6H	pin oak	Lexington, KY	nt	-	nt
5H	pin oak	Lexington, KY	nt	-	nt
16H	vinca minor	Lexington, KY	+	-	-
17H	Plantain	Lexington, KY	nt	-	-
18H	tall fescue	Lexington, KY	nt	-	-
9H	English ivy	Lexington, KY	nt	+	-
19H	Kentucky bluegrass	Lexington, KY	nt	-	+
20H	perennial ryegrass	Lexington, KY	nt	-	-
21H	Kentucky bluegrass	Lexington, KY	nt	-	-
22H	white oak	Minneapolis, MN	-	-	nt
23H	white oak	Minneapolis, MN	-	-	nt
24H	white oak	Minneapolis, MN	-	-	nt
25H	white oak	Minneapolis, MN	-	-	nt
26H	oak	Owensboro, KY	+	-	+
27H	oak	Owensboro, KY	+	-	+
28H	grass	Owensboro, KY	+	-	+
29H	oak	Owensboro, KY	+	-	-
30H	grass	Owensboro, KY	+	-	+
31H	oak	Owensboro, KY	+	-	-
32H	grass	Owensboro, KY	+	-	+
33H	maple	Owensboro, KY	+	-	-
34H	oak	Owensboro, KY	+	-	+
35H	oak	Owensboro, KY	+	-	+
36H	grass	Owensboro, KY	+	-	-
37H	pin oak	Owensboro, KY	+	-	+
38H	pin oak	Owensboro, KY	+	-	+
39H	pin oak	Owensboro, KY	+	+	+
40H	pin oak	Owensboro, KY	nt	+	+

Table 2.3: continued

Designation	Plant Host	Location	ELISA	PCR	
				Cartagen Kit	Qiagen Stool Kit
42H	magnolia	Lexington, KY	+	-	-
43H	red maple	Lexington, KY	+	-	+
44H	red maple	Lexington, KY	+	-	+
50H	post oak	Paducah, KY	+	-	-
51H	grass	Paducah, KY	+	-	-
52H	post oak	Paducah, KY	+	-	-
53H	grass	Paducah, KY	+	-	+
54H	post oak	Paducah, KY	+	-	-
55H	grass	Paducah, KY	+	-	-
56H	post oak	Paducah, KY	+	-	-
57H	Kentucky bluegrass	Paducah, KY	+	-	+
58H	post oak	Paducah, KY	+	-	-
59H	post oak	Paducah, KY	+	-	-
60H	grass	Paducah, KY	+	-	-
61H	post oak	Paducah, KY	+	-	-
62H	grass	Paducah, KY	+	-	-
63H	post oak	Paducah, KY	+	-	-
64H	grass	Paducah, KY	+	-	-
65H	post oak	Paducah, KY	+	-	-
66H	post oak	Paducah, KY	+	-	-
67H	grass	Paducah, KY	+	-	-
68H	post oak	Paducah, KY	+	-	-
69H	post oak	Paducah, KY	+	-	-
70H	post oak	Paducah, KY	+	-	-
41H	post oak	Paducah, KY	+	-	-
71H	grass	Paducah, KY	+	-	-
72H	grass	Paducah, KY	+	-	-
73H	grass	Paducah, KY	+	-	-
45H	post oak	Paducah, KY	+	-	-
46H	grass	Paducah, KY	+	-	+
74H	post oak	Paducah, KY	+	-	-
75H	grass	Paducah, KY	+	-	-
49H	mulberry	Louisville, KY	+	-	+
47H	oak	Louisville, KY	+	-	-
48H	grass	Louisville, KY	nt	-	+
76H	northern red oak	Louisville, KY	nt	-	+
77H	grass	Louisville, KY	nt	-	+
78H	pin oak	Louisville, KY	+	-	-
79H	grass	Louisville, KY	+	-	-
82H	oak	Lexington, KY	+	-	-
83H	oak	Lexington, KY	+	-	+
84H	grass	Lexington, KY	+	-	-

Table 2.3: continued

Designation	Plant Host	Location	ELISA	PCR	
				Cartagen Kit	Qiagen Stool Kit
85H	pin oak	Lexington, KY	+	+	+
86H	pin oak	Lexington, KY	+	+	+
87H	pin oak	Lexington, KY	nt	+	+
88H	pin oak	Lexington, KY	+	-	-
89H	pin oak	Lexington, KY	+	+	+
90H	oak	Lexington, KY	+	-	-
91H	grass	Lexington, KY	+	-	nt
92H	oak	Lexington, KY	+	-	-
93H	oak	Lexington, KY	+	-	-
94H	grass	Lexington, KY	+	-	nt
PO-Tl	pin oak	Lexington, KY	+	+	+
PO-Tr	pin oak	Lexington, KY	+	+	+
95H	oak	Lexington, KY	+	-	-
96H	oak	Lexington, KY	+	-	-
97H	grass	Lexington, KY	+	-	-
98H	oak	Lexington, KY	+	-	-
99H	grass	Lexington, KY	+	-	-
100H	southern red oak	Lexington, KY	+	-	-
101H	grass	Lexington, KY	+	-	-
PO-Don	pin oak	Lexington, KY	+	+	+
SO2	scarlet oak	Lexington, KY	+	+	+
HB1	hackberry	Lexington, KY	+	-	+
HB2	hackberry	Lexington, KY	+	-	+
106H	shrub	Lexington, KY	+	-	-
107H	pin oak	Lexington, KY	+	-	-
108H	sugar maple	Lexington, KY	+	-	+
109H	pin oak	Lexington, KY	+	-	+
110H	pin oak	Lexington, KY	+	+	+
111H	pin oak	Minneapolis, MN	+	-	+
RO1	northern red oak	Lexington, KY	+	+	+
RO3	northern red oak	Lexington, KY	+	+	+
PO-Lac1	pin oak	Lexington, KY	nt	+	+
MP1	sugar maple	Lexington, KY	+	-	nt
MP2	sugar maple	Lexington, KY	+	-	+
117H	grass	Lexington, KY	nt	-	nt
118H	pin oak	Lexington, KY	+	+	+
119H	northern red oak	Lexington, KY	+	-	-
PO-Cp1	pin oak	Lexington, KY	nt	-	+
121H	pin oak	Lexington, KY	+	-	-
122H	pin oak	Lexington, KY	+	-	-
123H	pin oak	Lexington, KY	+	-	-
124H	northern red oak	Lexington, KY	+	-	-
125H	pin oak	Lexington, KY	+	-	-
126H	grass	Lexington, KY	+	-	+

Table 2.3: continued

Designation	Plant Host	Location	ELISA	PCR	
				Cartagen Kit	Qiagen Stool Kit
127H	pin oak	Lexington, KY	+	-	+
128H	grass	Lexington, KY	+	-	+
129H	pin oak	Lexington, KY	+	-	-
130H	grass	Lexington, KY	+	-	+
131H	pin oak	Lexington, KY	+	-	-
132H	grass	Lexington, KY	+	-	+
133H	pin oak	Lexington, KY	+	-	-
134H	pin oak	Lexington, KY	+	+	+
135H	oak	Lexington, KY	+	-	-
136H	oak	Lexington, KY	+	-	-
137H	pin oak	Lexington, KY	+	-	-
138H	pin oak	Lexington, KY	+	+	+
139H	pin oak	Lexington, KY	+	-	-
140H	pin oak	Lexington, KY	nt	-	+
141H	pin oak	Lexington, KY	+	-	+
142H	grass	Lexington, KY	+	-	+

Table 2.4: 2002 leafhopper collection data. NA indicates that DNA was not obtained for this leafhopper, + indicates a positive result and – indicates a negative result. nt indicates that DNA concentration was not checked by fluorometer.

Sample	Leafhopper Species	Date	Location Collected	PCR	[DNA] (ng/μl)
LH1	*Graphocephala versuta*	16-May-2002	Hancock Co., Hawesville	-	94
LH2	*G. versuta*	16-Jul-2002	Hancock Co., near vineyard	-	86
LH3	*G. versuta*	4-Sep-2002	Vineyard	-	98
LH4	*G. versuta*	15-Jul-2002	Hancock Co.	-	184
LH5 & 8	*G. versuta*	1-Oct-2002	Hancock Co.	-	117
LH6	*G. versuta*	22-Jul-2002	Hancock Co.	-	73
LH7	*G. versuta*	29-Jul-2002	Hancock Co.	-	99
LH9	*G. versuta*	28-May-2002	Vineyard, Hancock Co.	-	81
LH10	*G. versuta*	21-Jul-2002	Hancock Co.	-	88
LH11	*G. versuta*	28-Jul-2002	Hancock Co.	-	nt
LH12	*G. versuta*	14-Jul-2002	Hancock Co.	-	nt
LH13	*G. versuta*	21-Aug-2002	Hancock Co., near vineyard	-	231
LH14	*G. versuta*	1-Jul-2002	Hancock Co.	-	nt
LH15	*G. versuta*	8-Jul-2002	Hancock Co.	-	nt
LH16	*G. versuta*	1-Jul-2002	Hancock Co.	-	nt
LH17	*G. versuta*	19-Jun-2002	Lexington, KY	-	nt
LH18	*G. versuta*	15-Apr-2002	Fayette Co.	+	nt
LH19	*G. versuta*	17-Jul-2002	Fayette Co., UKSF grapes	-	nt
LH20	*G. versuta*	19-Jun-2002	Lexington, KY	-	nt
LH21	*G. versuta*	17-Jul-2002	Fayette Co., UKSF grapes	-	nt
LH22	*G. coccinea*	22-Jul-2002	Hancock Co., vineyard	NA	nt
LH23	*G. coccinea*	19-Jun-2002	Lexington, KY	-	nt
LH24	*Paraulacizes irrorata*	8-Jul-2002	Hancock Co.	-	nt
LH25	*P. irrorata*	4-Jun-2002	Hancock Co., grapes	-	nt
LH26	*P. irrorata*	21-Aug-2002	Hancock Co., near vineyard	-	443
LH27	*P. irrorata*	19-Jun-2002	Lexington, KY	-	nt
LH28	*Draculacephala antica*	21-Jul-2002	Hancock Co.	-	nt
LH29	*D. antica*	21-Aug-2002	Hancock Co., near vineyard	-	176
LH30	*D. antica*	17-Jul-2002	Fayette Co., UKSF grapes	-	nt
LH31	*Orientus ishidae*	29-Jul-2002	Hancock Co.	-	nt
LH32	*O. ishidae*	10-Jun-2002	Hancock Co., grapes	-	nt
LH33	*O. ishidae*	4-Jun-2002	Hancock Co., grapes	-	nt
LH34	*O. ishidae*	28-May-2002	Hancock Co., grapes	-	nt
LH35	*O. ishidae*	26-Jun-2002	Hancock Co.	-	nt
LH36	*O. ishidae*	28-Oct-2002	Hancock Co., vineyard	-	122
LH37	*O. ishidae*	17-Jul-2002	Fayette Co., UKSF grapes	-	nt
LH38	*O. ishidae*	17-Jun-2002	Lexington, KY	-	nt

Table 2.5: Table of primers used in this study.

Primer Name	Sequence	Reference	Application
272-1-INT	CTGCACTTACCCAATGCATCG	Pooler and Hartung (1995)	Detection
272-2-INT	GCCGCTTCGGAGAGCATTCCT	Pooler and Hartung (1995)	Detection
27f	AGAGTTTGATCMTGGCTCAG	Lane (1991)	Detection
143r	GTCCCCCACGATAAGGTAGA	Lane (1991)	Detection
S-S-X.fas-0067-a-S-19	CGGCAGCACATTGGTAGTA	Rodrigues et al. (2003)	16S Sequencing
S-S-X.fas-0838-a-S-21	GCAAATTGGCACTCAGTATCG	Rodrigues et al. (2003)	16S Sequencing
S-S-X.fas-0838-a-A-21	CGATACTGAGTGCCAATTTGC	Rodrigues et al. (2003)	16S Sequencing
S-S-X.fas-1439-a-A-19	CTCCTCGCGGTTAAGCTAC	Rodrigues et al. (2003)	16S Sequencing
1525r	AAGGAGGTGWTCCARCC	Lane (1991)	16S Sequencing
1439-a-S-19	GTAGCTTAACCGCGAGGAG	Modified from Rodrigues et al. (2003)	16S-23S Sequencing
Xf 16S 1430F	AGCAGGTAGCTTAACCGCGA	Mundell, this study	16S-23S Sequencing
23S uni 322	GGTTCTTTTCGCCTTTCCCTC	Honeycutt et al. (1995)	16S-23S Sequencing
G1	GAAGTCGTAACAAGG	Jensen et al. (1993)	16S-23S Sequencing
23S-	TAC GGC CCT TTC GGA TAC AG	Mundell, this study	16S-23S Sequencing
23S-A-1998	ACCAGGTTCGCCTCCTTGAG	Mundell, this study	16S-23S Sequencing
23r	GTGCCAAGGCATCCACC	Li and de Boer (1995)	16S-23S Sequencing
FXYgyr499	CAGTTAGGGGTGTCAGCG	Rodrigues et al. (2003)	*gyrB* Sequencing
RXYgyr907	CTCAATGTAATTACCCAAGGT	Rodrigues et al. (2003)	*gyrB* Sequencing
LH18SRNAf	CGCGGTAATTCCAGCTCC	Mundell, this study	LH18SRNA
LH18SRNAr	CGGTGTGTACAAAGGGCAGG	Mundell, this study	LH18SRNA
LH28SRNAf	GAGAGTTCAAGAGTACGTGA	Mundell, this study	LH28SRNA
LH28SRNAr	CAGCTCTGACGATCGATTG	Mundell, this study	LH28SRNA

Table 2.6: Table of primer sets used in this study.

Primer Set	Forward Primer	Reverse Primer	Application	Product Length (bp)	Positive Tm Range (°C)	Annealing Temperature (°C)
27f-143r	27f	143r	Detection	116	83.01-84.3	60
272-int	272-1-int	272-2-int	Detection	472	79.4-80.9	62
16S Set B	S-S-X.fas-0067-a-S-19	S-S-X.fas-0838-a-S-21	16S Sequencing	771	82.05-84.28	56
16S Set C	S-S-X.fas-0838-a-A-21	S-S-X.fas-1439-a-A-19	16S Sequencing	619	83.63-84.48	56
16S 2nd	S-S-X.fas-0838-a-A-21	1525r	16S Sequencing	707	83.45-83.74	56
*gyr*B	FXYgyr499	RXYgyr907	*gyr*B Sequencing	429	79.72-80.27	54
LH18SRNA	LH18SRNAf	LH18SRNAr	Leafhopper 18S rDNA	1156	82.76-83.63	62
LH28SRNA	LH28SRNAf	LH28SRNAr	Leafhopper 28S rDNA	800	89.6-89.95	62

Table 2.7: 2002 DNA extraction results from the Green method (1999). DNA was quantified on the Hoefer DyNAquant 200 fluorometer (Amersham Pharmacia Biotech, San Francisco, CA). Melt temperatures (Tm) came from the SmartCycler.

Strain	[DNA] (ng/μl)	Tm (°C)
KYPD1	2	83.91
RO1	7	83.37
PO1	1	83.75
HB1	2	83.52

Table 2.8: 2003 DNA extraction results from the Green method (1999) and the Wasp DNA extraction method. nt indicates that the DNA extraction method was not tried or the DNA quantification was not done. DNA was quantified on the Hoefer DyNAquant 200 fluorometer (Amersham Pharmacia Biotech, San Francisco, CA). PCR detection was done with primer set 272-int.

	Green et al. 1999		Wasp DNA Extraction	
Designation	**[DNA] (ng/μl)**	**PCR**	**[DNA] (ng/μl)**	**PCR**
KYPD1	1	+	2	+
RO1	1	+	nt	nt
HB1	2	-	nt	nt
MB1	1	+	nt	nt
MP1	2	+	nt	nt
IL-PO1	3	+	5	+
SO2	nt	nt	5	+
RO2	nt	nt	0	+
RO3	nt	nt	0	+
PO-Lime	nt	nt	0	+
PO-Don	nt	nt	0	+
HB2	0	-	nt	+
PO-Tr	nt	nt	1	+
PO-Tl	nt	nt	8.5	+
MP2	nt	nt	nt	+

Table 2.9: Summary table of 2002-2004 ELISA and PCR detection results by plant host group. This is a summary based on Tables 2.2 and 2.3. Grasses and oaks were the largest host groups sampled. nt = not tested.

Host Group	Total # Collected	# Tested by ELISA	# Positive by ELISA	% Positive by ELISA	# Tested by PCR	# Positive by PCR	% Positive by PCR
magnolia	1	1	1	100	1	0	0
English ivy	1	0	0	0	1	1	100
grasses	39	29	29	100	39	15	38
oaks	101	87	83	95	101	49	49
hackberry	2	2	2	100	2	2	100
maple	9	8	7	88	9	6	67
mulberry	2	2	2	100	2	2	100
nimblewill	1	0	0	nt	1	0	0
vinca minor	1	1	1	100	1	0	0
plantain	1	0	0	nt	1	0	0
shrub	1	1	1	0	1	0	0
grapevine	2	2	2	100	2	1	50
gingko	1	1	0	0	1	0	0
ash	1	1	0	0	1	0	0

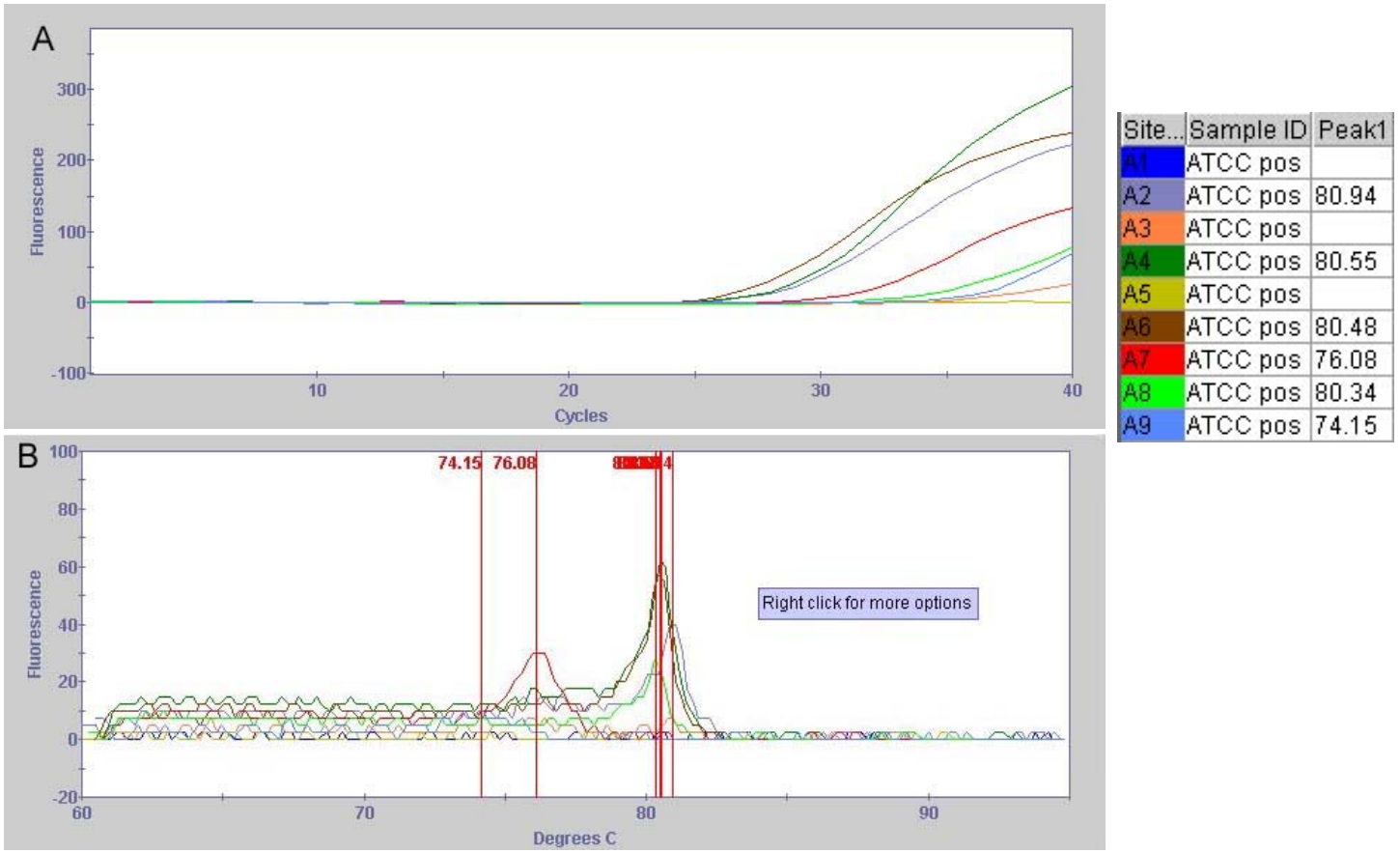

Figure 2.1: SmartCycler graphs from optimization of primer set 272-int. Graph A is the SYBR Green fluorescence curve, Graph B is the derivative of the melt curve analysis, showing peaks at the melt temperature (Tm) of the product. To the right of the graphs is the key for the lines and the Tm of each product.

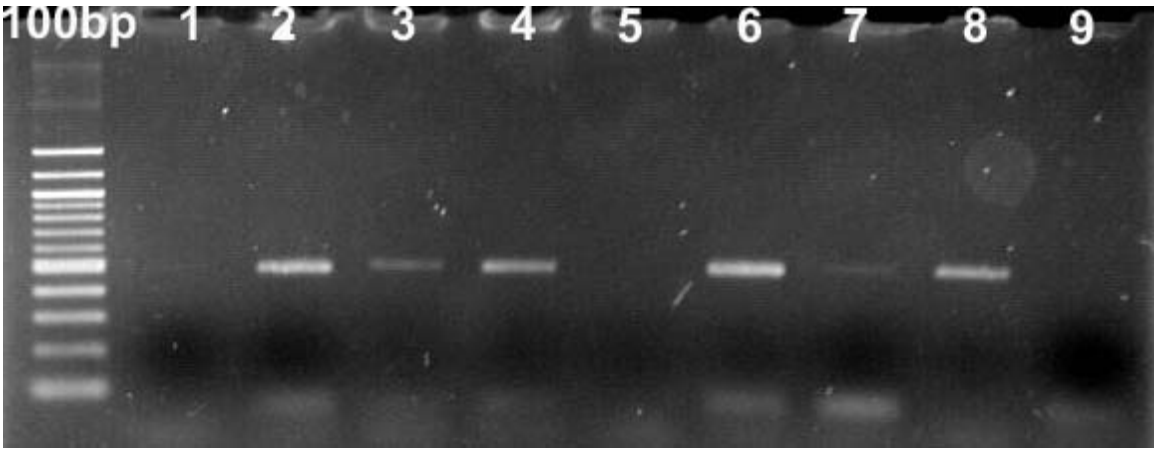

Figure 2.2: Gel image from the optimization of primer set 272-int. The expected product size is 472 bp. The first lane, labeled 100 bp, shows the 100 bp DNA ladder. Other lanes are labeled by reaction number as shown in Figure 2.1.

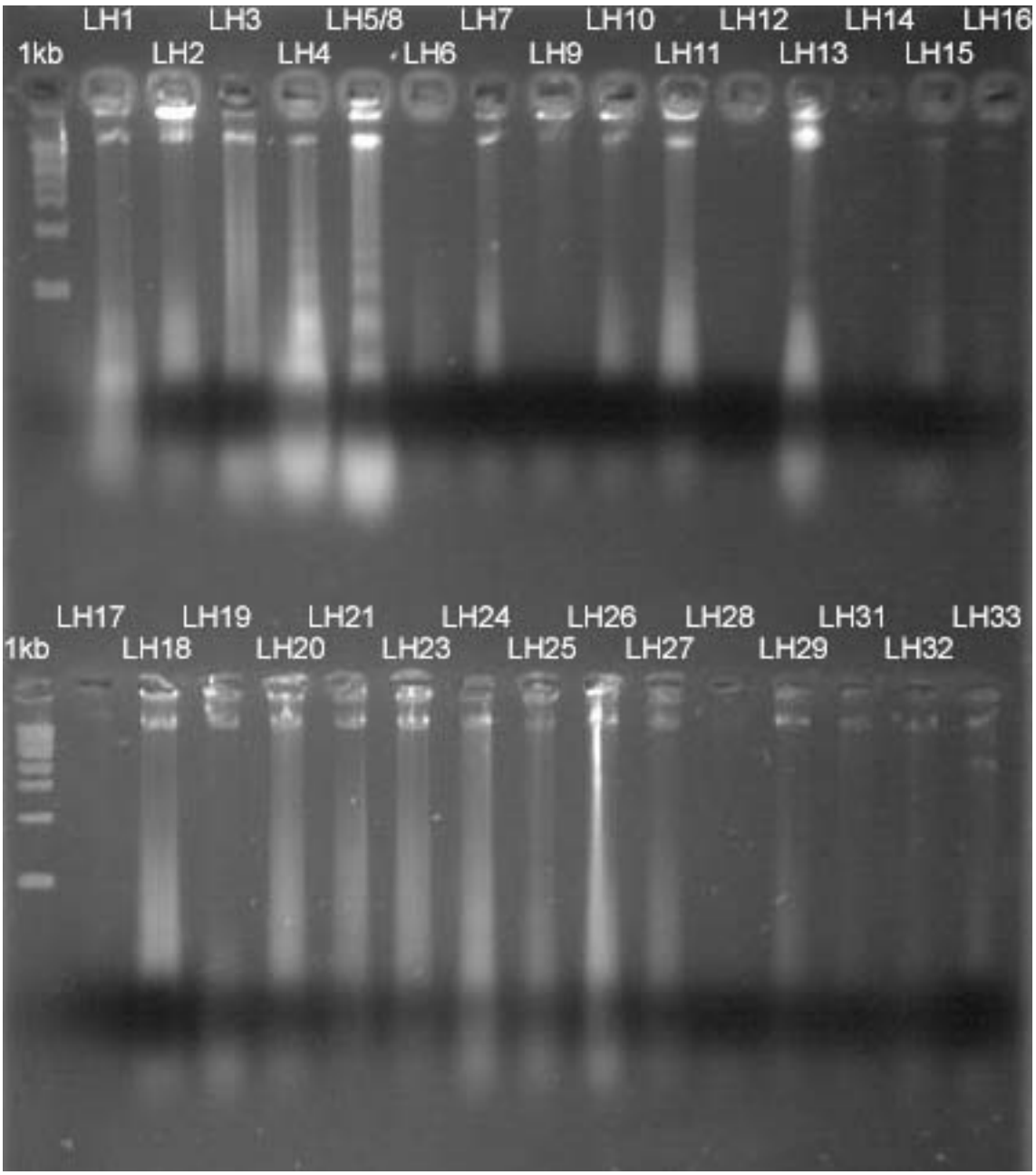

Figure 2.3: Gel image of leafhopper genomic DNA. The first lane, labeled 1 kb, shows the 1 kb DNA ladder. Other lanes are labeled by sample, according to Table 2.4.

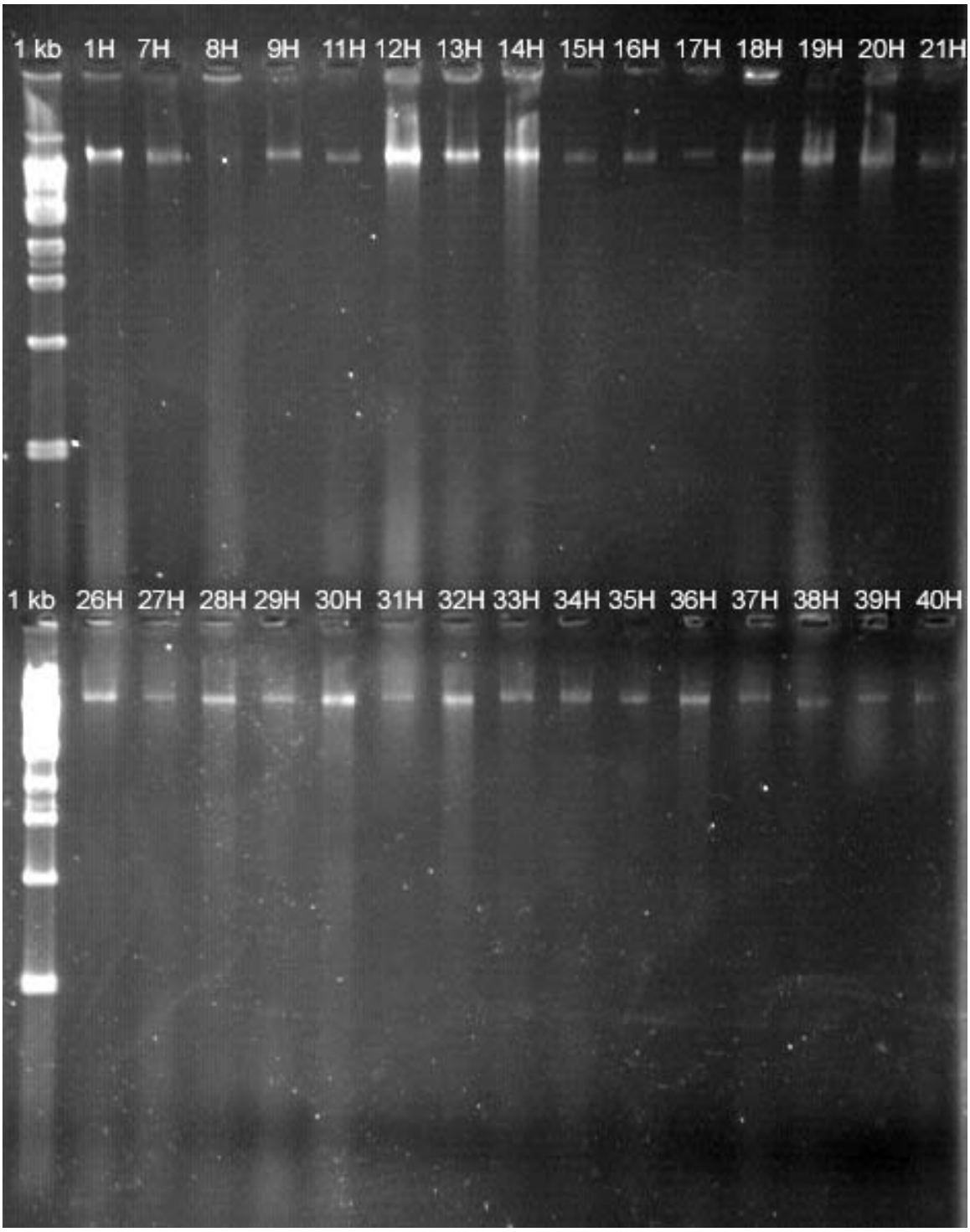

Figure 2.4: Plant genomic DNA extracted with QIAgen Stool DNA Extraction Kit in 2004. Lanes labeled 1 kb were loaded with a 1 kb genomic ladder. Other lanes are labeled with designations from Table 2.3.

Chapter 3

Phylogenetic Analysis of Strains of *Xylella fastidiosa*

Introduction

Subspecies of *Xylella fastidiosa* were proposed by Schaad et al. (2004) based on genetic data from DNA-DNA relatedness studies done by the S_1 nuclease method and also from sequence analysis of the 16S-23S intergenic spacer region. These genetic data were supported by phenotypic observations of the strains, namely, their growth on PD medium and their susceptibility or resistance to penicillin and carbenicillin. *Xylella fastidiosa* subsp. *piercei* includes the PD bacterium from *Vitis vinifera*, along with strains causing disease in alfalfa (*Medicago sativa*), almond (*Prunus amygdalus*) and maple. *Xylella fastidiosa* subsp. *multiplex*, named for its large number of strains, includes those that cause disease in peach, elm, plum, grape (*Vitis aestivalis*), sycamore and almond. The third proposed subspecies, *X. fastidiosa* subsp. *pauca*, consists only of CVC-causing strains. These proposed subspecies have only limited application within *X. fastidiosa* due to the small number of different pathotypes and small sample size from each host group.

Many genetic studies of different *Xylella fastidiosa* strains have attempted to differentiate this species into subspecies. Two common types of studies done are RAPD analyses (Albibi et al. 1998; Banks et al. 1999; Lacava et al. 2001; Pooler and Hartung1995) and phylogenetic analyses of the 16S rDNA and 16S-23S intergenic spacer region (Chen et al. 2000; Chen et al. 2000; Mehta and Rosato 2001; Rodrigues et al. 2003). Resolution of strain relationships obtained with these methods independently has not been sufficient justification for subdividing *X. fastidiosa*, but they do consistently yield certain groupings of different strains. Phylogenetic trees based on the 16S rDNA obtained by Mehta and Rosato (2001) and Rodrigues et al. (2003) place the strains of the CVC and coffee leaf scorch

pathotypes in a distinct clade from those of PD and mulberry leaf scorch pathotypes. The strain PE.PLS causes pear leaf scorch in Taiwan and was consistently separated from other strains of *X. fastidiosa*. Sequence data from the 16S-23S intergenic region shows more variability than the 16S rDNA, yielding the same general groupings but with better statistical support.

The newly proposed subspecies of *X. fastidiosa* are based on studies that included only 26 strains, and any attempt to classify other known and new strains of *X. fastidiosa* into these subspecies, or possibly into other species or subspecies, will largely depend on further genetic studies. These classification efforts may be hampered by the difficulty of culturing the bacteria. Rodrigues et al. (2003) developed primers specific for *X. fastidiosa* that amplify the 16S rDNA and the *gyrB* gene. The *gyrB* gene is ubiquitous and conserved, but yields more variable sequence than the 16S rDNA alone. These specific primer sets allow study of *X. fastidiosa* from DNA extracted directly from plant material.

The goal of this study was to determine phylogenetic relationships between samples of *X. fastidiosa* found in Kentucky, using the primer sets developed by Rodrigues et al. (2003) to specifically amplify the 16S rDNA and *gyrB* gene.

Materials and Methods

Sequencing

PCR products for sequencing were obtained with primer sets 16S Set B, 16S 2nd and *gyrB* listed in Table 2.6. PCR recipes and protocols were as described in Chapter 2. PCR products were purified using the QIAgen PCR Purification kit (QIAgen, Valencia, CA). Sequencing was done by the Advanced Genetics Technology Center (AGTC) at the University of Kentucky. Sequencing from PCR products was done with primers listed in Table 3.1.

Sequence Analysis

Sequences obtained were assembled with phredPhrap (Green 1994-1999) and edited in Consed v13.0. In addition to sequences obtained in this study, available 16S rDNA and *gyrB* sequences from *X. fastidiosa* were obtained from the GenBank database (Bilofsky and Burks 1988). Kentucky sample designations, hosts of origin and NCBI accession numbers are listed in Table 3.2. Sequence information for 16S rDNA and *gyrB* sequences obtained from NCBI are listed in Table 3.3. All sequence assemblies were aligned with ClustalW (Thompson et al. 1994) on the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw). Alignments were edited using MacClade 4.04 OS X (Maddison and Maddison 2001) and PCR primer sequence was removed.

Phylogenetic Analysis

Phylogenetic analyses of the 16S rDNA, 16S 2nd and *gyrB* alignments was done with PAUP* 4.0b10 beta version (Swofford 2002). Maximum parsimony (MP) trees were generated with the heuristic search option. Branch-swapping was turned off and step-wise addition was done for 1000 replications with random taxon additions. Bootstrap values were obtained using stepwise addition for 1000 replications of heuristic parsimony with random sequence additions.

Partition homogeneity test was done to evaluate tree topologies using concatenated 16S 2nd and *gyrB* sequence. Trees were generated using parsimony. The test was done with PAUP* 4.0b10 beta version (Swofford 2002) using heuristic search of 1000 replications.

In addition to the partition homogeneity test, the Kishino-Hasegawa (KH) and Shimodaira-Hasegawa tests for comparing tree topologies were implemented using PAUP* 4.0b10 beta version (Swofford 2002). The trees for comparison with KH and SH were generated using the maximum likelihood method. The KH test was done with normal approximation and using a two-

tailed test. The SH test was done using the RELL method and bootstrap of 1000 replicates.

Results

Sequence Analysis

For some samples, only the partial second half sequence (primer set 16S 2nd) of the 16S rDNA could be obtained. For others, the near-complete 16S rDNA or *gyrB* gene or both were sequenced. Sequence from sample HB1 was excluded from further analysis because the sequence traces showed evidence of multiple genotypes. For all sequences from other samples, traces were high-quality or had minor cases of dye-blobs or low-signal.

Phylogenetic Analysis

Figure 3.1 shows the MP tree based on the near-complete 16S rDNA (products 16S SetB and 16S 2nd combined) sequences for all samples in this study and those obtained from GenBank. A bootstrap tree for this dataset is shown in Figure 3.2. With the exceptions of 26H, 27H and OSL 92-3, all the near-complete 16S rDNA sequence from oak samples gave identical sequences. The exceptions formed a clade, labeled G, with good bootstrap support (72%). Some, but not all, of the sequences of strains from citrus and coffee also form a well-supported clade (88% bootstrap), labeled III. The strain from pear (PE.PLS) and a strain from plum (PL.788) had distinct sequences separated from the clades representing other samples and strains (99% bootstrap).

The MP tree based on 16S 2nd sequence of selected samples from this study is shown in Figure 3.3. The bootstrap cladogram for this sequence set is shown in Figure 3.4. These sequence data gave a distinct clade, labeled G, made up primarily of sequences from grass samples but also including sequences from a few oak samples (26H, 27H, 38H, 127H and OSL 92-3). This clade apparently corresponded to clade G in Figures 3.1 and 3.2. It was clearly an outgroup to the

other *X. fastidiosa* sequences. The sequences in this clade were from samples collected in different locations in Kentucky. BLAST analysis of some sequences from this clade showed closest similarity to either *Stenotrophomonas* sp. (subclade Gi) or *Xa. axonopodis* pv vesicatoria (subclade Gii) sequences. BLAST of 16S 2nd sequence from sample 26H (grouping in clade Gii) yielded closest similarity to *Xylella fastidiosa* strain OSL 92-3.

The phylogenetic tree based on sequences from GenBank and this study for the partial *gyrB* gene is shown in Figures 3.5 and 3.6, with bootstrap values shown on the nodes. This tree shows three distinct clades. Sequences from the KYPD1 strain and other strains from grape hosts comprise the clade labeled I. The majority of the sequences fit into the clade labeled II. Sequences from strains isolated from citrus and coffee comprise the clade labeled III. This clade consists of sequence from those samples that grouped in clade III of 16S sequences (see Figures 3.1-3.4). Support for clades I and III (Fig. 3.5) was very good at 90% and 94% bootstrap values respectively. Support for clade II was meager, at 62% bootstrap.

Figure 3.6 focuses on the ingroup of the *gyrB* phylogram. This figure clearly shows the division of clades I, II and III. These clades seem to fit well in the scheme of subspecies of *X. fastidiosa*, with clade I representing subspecies *piercei* and clade III representing subspecies *pauca*.

The partition homogeneity test yielded trees very close in length with insignificant difference between them (p-value = 0.85). This result does not support the hypothesis of recombination for the 16S 2nd and *gyrB* sequence datasets. In contrast, both the KH and SH tests showed significant difference between the trees (p-values of 0.000 for both).

For most of the grass samples in clade G in Figures 3.3 and 3.4, the first half of the 16SrDNA (primer set 16S Set B) and the partial *gyrB* gene could not be amplified. There are also a number of grass samples shown in Figures 3.5 and

3.6 for which the partial *gyrB* gene was amplified but none of the 16S rDNA could be amplified. All the sequences obtained are listed in Table 3.2.

Discussion

The goal of phylogenetic analysis in this study was to determine potential lines of transmission for *Xylella fastidiosa*. The 16S rDNA has been used extensively to compare strains of *X. fastidiosa* (Chen et al. 2000; Chen et al. 2000; Mehta and Rosato 2001), but does not show sufficient variability to make conclusions about relatedness. The gyrase B gene is more variable than the 16S rDNA. Comparison of phylograms based on each of these genes was expected to give resolution of relationships between the strains.

As expected, the *gyrB* gene MP trees (Figures 3.5 and 3.6) indicated greater variability between sequences than the 16S rDNA trees. Figure 3.5 shows a clade (labeled I) with good bootstrap support at 90%, consisting of sequence from strains isolated from grape and mulberry as well as sequence from DNA extracted from grass samples. Aside from the sequences for samples from grass hosts, the samples in this clade fit into the *X. fastidiosa* subsp. *piercei*. Clade III on Figure 3.6 also has good bootstrap support, 96%, and consists of strains from citrus and coffee hosts. This clade would likely fit in the *X. fastidiosa* subsp. *pauca*. The remaining clade (labeled II) has comparably low bootstrap support at 62% on Figure 3.5. This clade, consisting of *X. fastidiosa* sequences from numerous and varied hosts, could fit in the *X. fastidiosa* subsp. *multiplex*. The trees generated for the partial 16S rDNA (Figures 3.1-3.4) did not show the distinct clades as shown in the *gyrB* trees (Figures 3.5-3.6). It is interesting that these results from the partial *gyrB* gene seem to correspond well with the subspecies designations that were made based on the 16S-23S intergenic spacer region and DNA-DNA relatedness. This may indicate that the *gyrB* is superior to the 16S rDNA for studying the relatedness of strains.

By comparing the trees based on 16S rDNA partial sequence (Figures 3.1-3.4) and partial *gyrB* gene sequence (Figures 3.5-3.6), it is possible to make some assertions about samples of particular interest from this study. On all the phylogenetic trees, these samples are shown in bold text. First, according to Figures 3.5 and 3.6, the strain KYPD1 is most likely a true PD strain and not a BLS strain that jumped hosts to infect grapevine, because it fits into clade I with the other strains isolated from grape. Also, the sequences of *X. fastidiosa* extracted from the insect *G. versuta* (LH18) fits well with BLS strains on all the phylograms. On the *gyrB* trees (Figures 3.5 and 3.6), the LH18 sequence is distinct from the sequence from grape hosts. This may mean that this particular sample of leafhoppers could be BLS vectors.

The other two samples of particular interest are 111H from pin oak and ILPO1 from pin oak. These were collected in Minnesota and northern Illinois, respectively. The positive diagnosis of BLS in these samples is surprising because of the known cold-sensitivity of *X. fastidiosa*, and raises the question of how *X. fastidiosa* survives where the top layer of soil around the roots of trees typically freeze during winter. It also raises the question of where the bacteria originated. According to the phylograms presented in this study, 16S rDNA and *gyrB* gene sequences from these samples are identical to those of other BLS samples collected around Kentucky. This could mean that the samples from Minnesota and Illinois originated in the south and migrated north, perhaps with infested leafhopper vectors.

According to the *gyrB* gene trees (Figures 3.5-3.6), host of origin has a greater effect on the relationship between sequences than geography. For example, clade I from Figure 3.5 consists of strains from grape strains originating in California, Florida, Georgia and Kentucky. Also, the sequences from oak samples group together in clade II despite the fact that they were collected in different parts of Kentucky and even different states.

The unexpected result of this study was that the 16S rDNA second half showed a well-supported, distinct clade of sequence from all grasses and some oak samples (clade G on Figures 3.1-3.4). The *gyrB* gene tree did not indicate a corresponding clade, with sequence from grass sample 77H and oak samples 26H and 27H that were in the G clade of the 16S 2nd sequence being part of the ingroup in the *gyrB* phylogram (Figures 3.5 and 3.6). The 16S rDNA partial second half and *gyrB* gene MP tree topologies were visibly different, and significantly different according to the Kishino-Hasegawa and Shimodaira-Hasegawa tests.

One possible explanation for the difference between the topologies of the 16S 2nd and *gyrB* trees could be that DNA from samples 77H, 26H and 27H contained a mixed population such that the 16S rDNA from one bacterial species was amplified while the *gyrB* of another (*X. fastidiosa*) was amplified. Using BLAST comparison, the sequences in the G clade of the 16S 2nd tree (Figure 3.4) more closely resemble either *Stenotrophomonas* sp. (subclade i) or *Xa. axonopodis* pv vesicatoria (subclade ii). The possibility of mixed populations is unlikely given that the sequencing traces from all samples included in this study showed no indication of multiple reads, and because the primers for the 16S rDNA and *gyrB* gene were developed to amplify only *X. fastidiosa* sequences. Specificity was determined by BLAST comparison and by PCR on bacterial species from different genera.

If not for the *gyrB* data, it would be tempting to dismiss the G clade samples as contaminating bacteria. An alternative explanation for the differences observed between the topologies of the 16S 2nd and *gyrB* trees is that recombination occurred between *X. fastidiosa* and a closely related bacterial species. These might be strains of *X. fastidiosa* that, through horizontal gene transfer (HGT), received and recombined sequence from the 16S rDNA from close relatives of *Stenotrophomonas* sp. and *Xanthomonas* sp. The converse explanation could be that these samples are truly *Stenotrophomonas* sp. or

Xanthomonas sp. - or related bacteria - that recombined *gyrB* gene sequence from *X. fastidiosa* into their genome resulting in a false representation of relatedness as shown in Figures 3.5 and 3.6. Either case of recombination, or the alternative that multiple bacteria were present, seem equally plausible at this point, and further investigation, including sequence analysis of other genes, would be necessary to support either explanation.

As mentioned in Results, there were some samples for which sequence of the partial first half of the 16S rDNA and *gyrB* sequence could not be obtained. There were other samples for which the *gyrB* could be amplified but none of the 16S rDNA. This inability to amplify all sequences of interest from all of the samples could be due to suboptimal PCR reaction conditions, either in the reaction recipe or in the protocol. It could also be further evidence of recombination. Perhaps for some of the samples, recombination occurred approximately in the middle of the 16S rDNA where *X. fastidiosa* DNA integrated into the genome of a close relative. This would explain the samples for which only the 16S 2nd sequence could be amplified. For those samples that only the *gyrB* could be amplified, it might be that *X. fastidiosa* DNA in the *gyrB* gene region integrated into the genome of a closely related bacterial species. The suggestion from these results is that recombination could have occurred to varying degrees, in different genomic regions and between different bacteria.

The possibility of recombination has implications for the host range and pathogenicity of *X. fastidiosa*. HGT enables a clonal organism to recombine and get the same sort of fitness benefit that sexual organisms have. Obtaining DNA from other bacterial strains, particularly pathogenic bacteria, could increase the host range of *X. fastidiosa*. This could be the result of getting genes that mask *X. fastidiosa* from plant defenses or genes that allow it to overcome plant defenses.

The presence of clade G has not been raised previously, perhaps because there is only one GenBank sequence that fits clade G, OSL92-3. Mehta and Rosato (2001) included this strain in their 16S phylogenetic analysis and it did

not group with other strains. Analysis of the 16S-23S intergenic spacer sequence of another strain from oak (88.9, not included in this study) indicated it fit in a clade with citrus and coffee strains. This discrepancy between inclusion of oak-derived strains could not be explained then, and cannot be explained in this study. Further investigation, including the attempt to complete Koch's postulates with the oak samples 27H and 26H, obtained from symptomatic oaks, is necessary to adequately address the nature of bacteria associated with clade G.

The comparison of these genes raises another concern about differentiation of the species *X. fastidiosa*. The goal of this study was to determine whether certain strains from different hosts were likely to be the same, with the hope of identifying sources of inoculum, particularly for PD. Comparing these gene sequences gives no clearer picture as to sources of inoculum or transmission. Further study is necessary to make reasonable the assertion that a strain found in a given host is the same, or originated from, another host.

Table 3.1: Sequencing primers used in this study. Colors indicate the PCR products for which the included sequencing primers were used.

PCR Product	Primer Name	Primer Sequence	Strand
16S Set B	S-S-X.fas-0067-a-S-19	CGGCAGCACATTGGTAGTA	+
16S Set B	360r	ATATTCCCCACTGCTGCCTC	-
16S Set B	572-A-20	ACTTAAATAACCACCTACGC	-
16S Set B	682-S-20	CAGTGAAATGCGTAGAGATC	+
16S Set B	S-S-X.fas-0838-a-A-21	CGATACTGAGTGCCAATTTGC	-
16S Set B	Xf 371f	GGAGGCAGCAGTGGGGAATA	+
16S Set B	Xf 629f	AATGGCAGTGGATACTGGATAGCT	+
16S Set B	Xf 612r	ATTCCCAGGTTAAGCCCCAGG	-
16S 2nd	S-S-X.fas-0838-a-S-21	GCAAATTGGCACTCAGTATCG	+
16S 2nd	1070-S-20	CGTGAGATGTTGGGTTAAGT	+
16S 2nd	1185-A-20	GGCCATGATGACTTGACGTC	-
16S 2nd	1310-S-20	TGCAACTCGACTCCATGAAG	+
16S 2nd	S-S-X.fas-1439-a-A-19	CTCCTCGCGGTTAAGCTAC	-
16S 2nd	Xf 937f	CAAGCGGTGGAGTATGTGGT	+
16S 2nd	Xf 1247f	GGGACAGAGGGCTGCAATCT	+
16S 2nd	Xf 1508r	GCACCTTCCGATACGGCTAC	-
16S 2nd	Xf1215r	ACGTGTGTAGCCCTGGTCGT	-
16S 2nd	Xf910r	CCCCGTCAATTCCTTTGAGT	-
16S 2nd	1525r	AAGGAGGTGWTCCARCC	-
gyrB	FXYgyr499	CAGTTAGGGGTGTCAGCG	+
gyrB	RXYgyr907	CTCAATGTAATTACCCAAGGT	-

Table 3.2: Information for sequences from this study. The letters ns are used to indicate sequences that were not obtained.

	NCBI Accession Number			
Sample ID	**Partial 16S**	***gyrB***	**Host of Origin**	**Geographic Origin**
1H	DQ021503	DQ022617	pin oak	Lexington, KY
7H	DQ021505	DQ022619	pin oak	Lexington, KY
8H	DQ021506	ns	pin oak	Lexington, KY
11H	DQ022848	DQ022620	pin oak	Lexington, KY
12H	ns	DQ022618	tall fescue	Lexington, KY
13H	ns	DQ022621	Kentucky bluegrass	Lexington, KY
14H	ns	DQ022623	perennial ryegrass	Lexington, KY
26H	DQ021507	DQ022624	oak	Owensboro, KY
27H	DQ021509	DQ022625	oak	Owensboro, KY
28H	DQ021508	ns	grass	Owensboro, KY
30H	DQ021511	ns	grass	Owensboro, KY
32H	DQ021510	ns	grass	Owensboro, KY
34H	ns	DQ022626	oak	Owensboro, KY
35H	DQ021504	DQ022622	oak	Owensboro, KY
37H	DQ021513	DQ022627	pin oak	Owensboro, KY
38H	DQ021514	ns	pin oak	Owensboro, KY
39H	DQ021512	DQ022629	pin oak	Owensboro, KY
40H	DQ022849	DQ022630	pin oak	Owensboro, KY
43H	DQ022850	DQ022628	red maple	Lexington, KY
44H	ns	DQ022631	red maple	Lexington, KY
46H	DQ021517	ns	grass	Paducah, KY
48H	DQ022851	ns	grass	Louisville, KY
49H	ns	DQ022633	mulberry	Louisville, KY
53H	DQ022852	ns	grass	Paducah, KY
57H	ns	DQ022634	Kentucky bluegrass	Paducah, KY
76H	ns	DQ022632	red oak	Louisville, KY
77H	DQ021516	DQ022635	grass	Louisville, KY
83H	DQ021515	DQ022636	oak	Lexington, KY
85H	DQ021519	DQ022637	pin oak	Lexington, KY
86H	DQ021518	DQ022638	pin oak	Lexington, KY
87H	DQ022853	DQ022639	pin oak	Lexington, KY
89H	DQ022854	DQ022641	pin oak	Lexington, KY
108H	DQ021520	DQ022640	sugar maple	Lexington, KY
109H	DQ022855	DQ022643	pin oak	Lexington, KY
110H	DQ021522	ns	pin oak	Lexington, KY
111H	DQ021521	DQ022644	pin oak	Minnesota

Table 3.2: continued

Sample ID	NCBI Accession Number		Host of Origin	Geographic Origin
	Partial 16S	*gyrB*		
126H	DQ021524	ns	grass	Lexington, KY
127H	DQ021523	ns	pin oak	Lexington, KY
128H	DQ021525	ns	grass	Lexington, KY
130H	DQ021526	ns	grass	Lexington, KY
132H	DQ021527	ns	grass	Lexington, KY
134H	DQ022856	DQ022642	pin oak	Lexington, KY
138H	DQ022857	DQ022645	pin oak	Lexington, KY
140H	ns	DQ022646	pin oak	Lexington, KY
141H	DQ022858	DQ022647	pin oak	Lexington, KY
142H	DQ021530	ns	grass	Lexington, KY
HB2	ns	DQ026291	hackberry	Lexington, KY
ILPO1	DQ021529	DQ026290	pin oak	Illinois
KYPD1	DQ021528	DQ022649	grapevine	Lexington, KY
LH18	DQ022859	DQ022654	leafhopper	Lexington, KY
MB1	DQ021532	DQ022648	mulberry	Lexington, KY
MP1	DQ021531	DQ022652	sugar maple	Lexington, KY
MP2	DQ022860	ns	sugar maple	Lexington, KY
PO1	DQ021535	DQ022651	pin oak	Lexington, KY
POCp1	DQ022861	ns	pin oak	Lexington, KY
PODon	DQ022862	DQ022650	pin oak	Lexington, KY
POLac1	DQ021533	DQ022653	pin oak	Lexington, KY
POLime	DQ021538	DQ026289	pin oak	Lexington, KY
POTl	DQ021536	ns	pin oak	Lexington, KY
POTr	DQ021534	DQ026288	pin oak	Lexington, KY
RO1	DQ021540	DQ026287	red oak	Lexington, KY
RO2	DQ021537	ns	red oak	Lexington, KY
RO3	DQ021539	DQ026286	red oak	Lexington, KY
ROShaw	DQ021541	DQ026285	red oak	Lexington, KY
SO2	ns	DQ022655	scarlet oak	Lexington, KY

Table 3.3: Information for sequences obtained from GenBank. The letters na indicate information or sequence that was not available.

Species	Strain	NCBI Accession Number		Host of Origin	Geographic Origin	Tree Topology Tests
		16S	*gyrB*			
Xylella fastidiosa	ALS-BC	AF536770	AF534973	almond	California, U.S.	included
X. fastidiosa	SL1	AF536766	AF534969	citrus	Minas Gerais, Brazil	included
X. fastidiosa	B14	AF536765	AF534968	citrus	São Paulo, Brazil	included
X. fastidiosa	9a5c	NC002488	NC002488	citrus	São Paulo, Brazil	included
X. fastidiosa	CVC93-2	AF159575	na	citrus	Brazil	not included
X. fastidiosa	CI.52	AF203389	na	citrus	Brazil	not included
X. fastidiosa	Plana-5	AF224736	na	citrus	Brazil	not included
X. fastidiosa	P3	AF536769	AF534972	coffee	São Paulo, Brazil	included
X. fastidiosa	CRS2	AF536768	AF534970	coffee	São Paulo, Brazil	included
X. fastidiosa	CM1	AF536767	AF534974	coffee	São Paulo, Brazil	included
X. fastidiosa	CO.01	AF203390	na	coffee	Brazil	not included
X. fastidiosa	Café 20	AF224739	na	coffee	Brazil	not included
X. fastidiosa	Found 4	AF224737	na	coffee	Brazil	not included
X. fastidiosa	ELM-1 (ATCC 35873)	AF224738	AF534966	elm	Washington, D.C.	included
X. fastidiosa	Elm	AF224734	na	elm	Washington, D.C.	not included
X. fastidiosa	Temecula	AF536760	AF534960	grapevine	California	included
X. fastidiosa	PD28-5	AF159574	na	grapevine	Florida	not included
X. fastidiosa	r116v11	AF159573	na	grapevine	Georgia	not included
X. fastidiosa	PCE-FG (ATCC 35881)	AF159572	AF534961	grapevine	Florida	not included
X. fastidiosa	PCE-FF (ATCC 35879)	AF192343	na	grapevine	Florida	not included
X. fastidiosa	MUL-3	AF536763	AF534965	mulberry	Massachusetts	included
X. fastidiosa	Mul-2	AF159576	na	mulberry	Nebraska	not included

Table 3.3: continued.

Species	Strain	NCBI Accession Number		Host of Origin	Geographic Origin	Tree Topology Tests
		16S	*gyrB*			
X. fastidiosa	MUL1	AF224740	AF534964	mulberry	Massachusetts	included
X. fastidiosa	OSL92-3	AF159577	na	oak	Florida	not included
X. fastidiosa	ATCC 35874	AF224735	na	oak	Washington, D.C.	not included
X. fastidiosa	PD Conn C	na	AY789017	grapevine	California	not included
X. fastidiosa	CRS1	na	AF534967	coffee	São Paulo, Brazil	not included
X. fastidiosa	ELM-OK	na	AJ863558	elm	Oklahoma	not included
X. fastidiosa	PP4-5	AF159580	na	peach	Georgia	not included
X. fastidiosa	PE.PLS	AF203392	na	pear	Taiwan	not included
X. fastidiosa	PWT-22 (ATCC 35878)	AF159578	AF534971	periwinkle	Florida	included
X. fastidiosa	PLM G83	AF536761	AF534962	plum	Georgia	included
X. fastidiosa	PLS2-9	AF159579	na	plum	Georgia	not included
X. fastidiosa	PL.788 (ATCC 35871)	AF203388	na	plum	Georgia	not included
X. fastidiosa	RGW-R	AF536762	AF534963	ragweed	Florida	included
Xanthamonas axonopodis pv vesicatoria	na	AY288081	na	na	na	not included
Stenotrophomonas sp.	na	AJ884481	na	na	na	not included
Pseudomonas syringae pv tomato	DC 3000	AE016853	AE016853	tomato	na	included
Bacillus subtilis	B53	AY808064	na	na	na	not included
Agrobacterium tumefaciens	na	AY221180	na	na	na	not included
Xa. campestris pv campestris	L2	AF159581	na	cabbage	Georgia	not included
Xa. axonopodis pv citri	306	AE 012082	AE011623	na	na	included
Stenotrophomonas maltophilia	Vun 10,012	AF068009	AY642281	na	na	included
Xa. campestris pv campestris	ATCC 33913	AE012540	AE012093	na	na	included

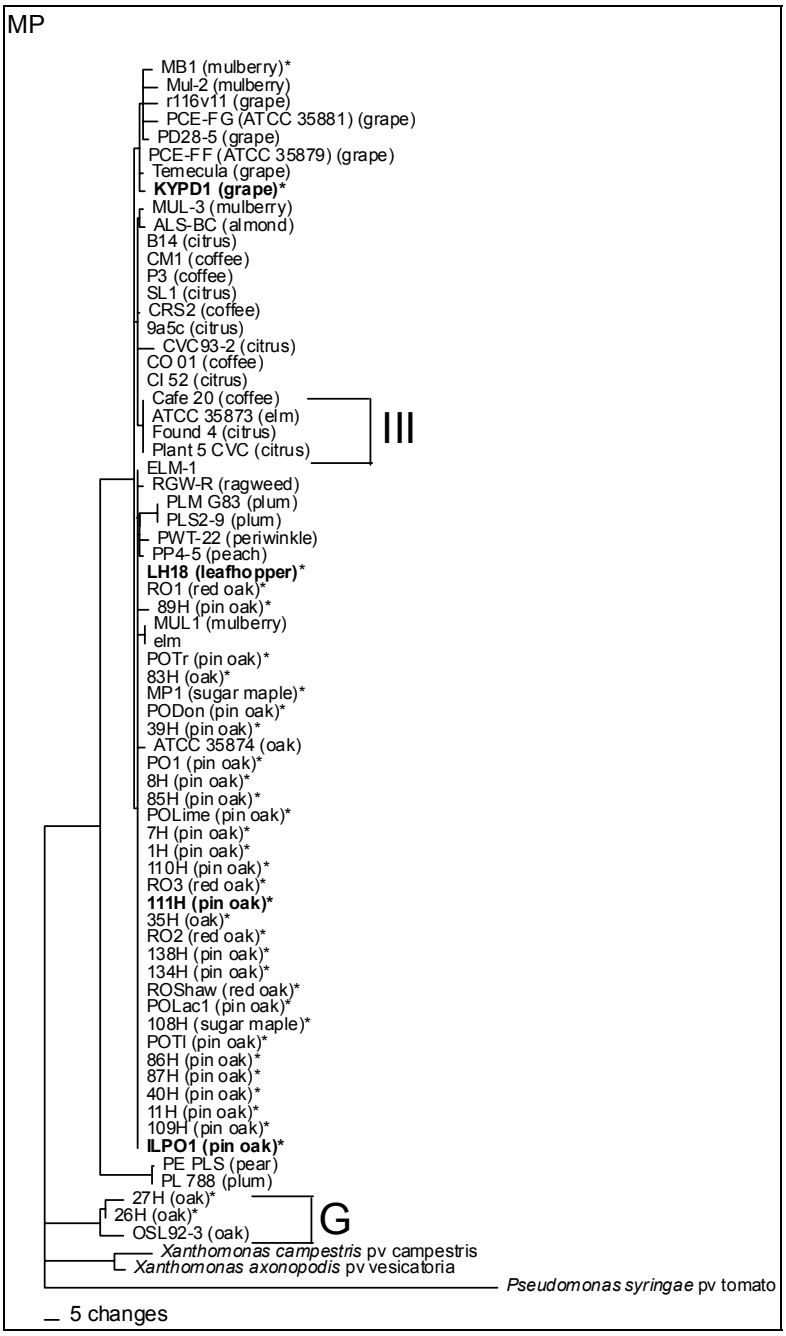

Figure 3.1: MP phylogram for near-complete 16S rDNA data. An asterisk (*) by the name indicates the sequence data from samples in this study. Unmarked names indicate sequences obtained from NCBI. Names in bold are sequences from samples of particular interest generated in this study. The G and III are used to indicate two clades for easy comparison with clades on trees in other figures.

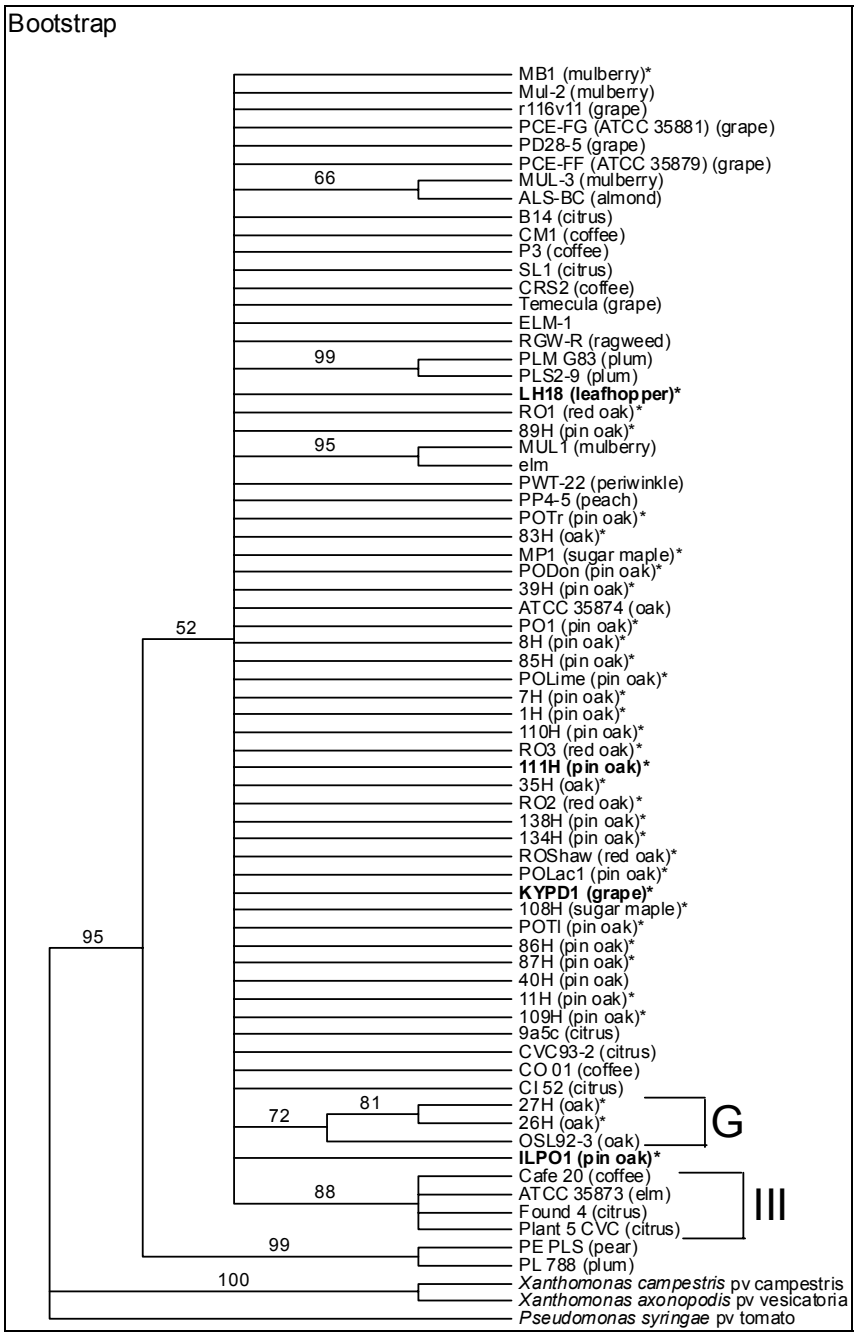

Figure 3.2: Bootstrap cladogram for near-complete 16S rDNA data. An asterisk (*) by the name indicates the sequence data from samples in this study. Unmarked names indicate sequences obtained from NCBI. Names in bold are sequences from samples of particular interest generated in this study. The G and III are used to indicate two clades for easy comparison with clades on trees in other figures.

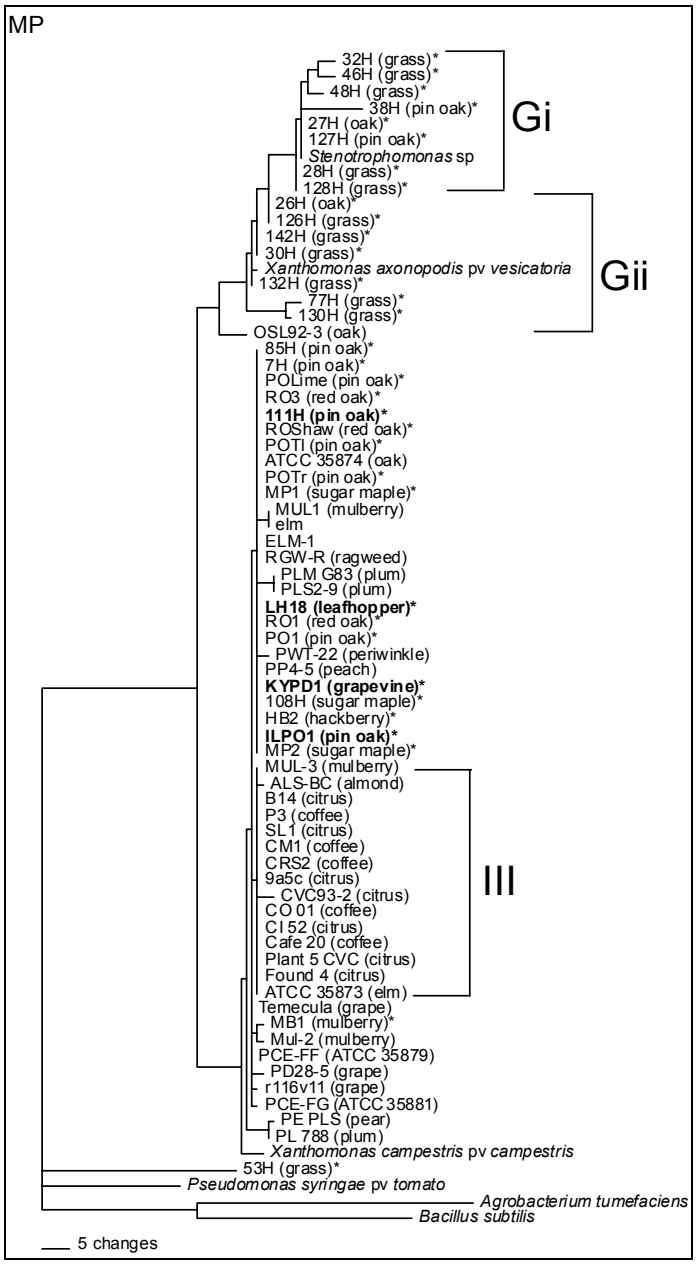

Figure 3.3: MP phylogram for 16S 2nd rDNA data. An asterisk (*) by the name indicates the sequence data from samples in this study. Unmarked names indicate sequences obtained from NCBI. Names in bold are sequences from samples of particular interest generated in this study. Clades Gi and Gii correspond to clade G on the near-complete 16S rDNA phylogram (Fig. 3.1). The clade consisting primarily of sequence from citrus and coffee strains is labeled III.

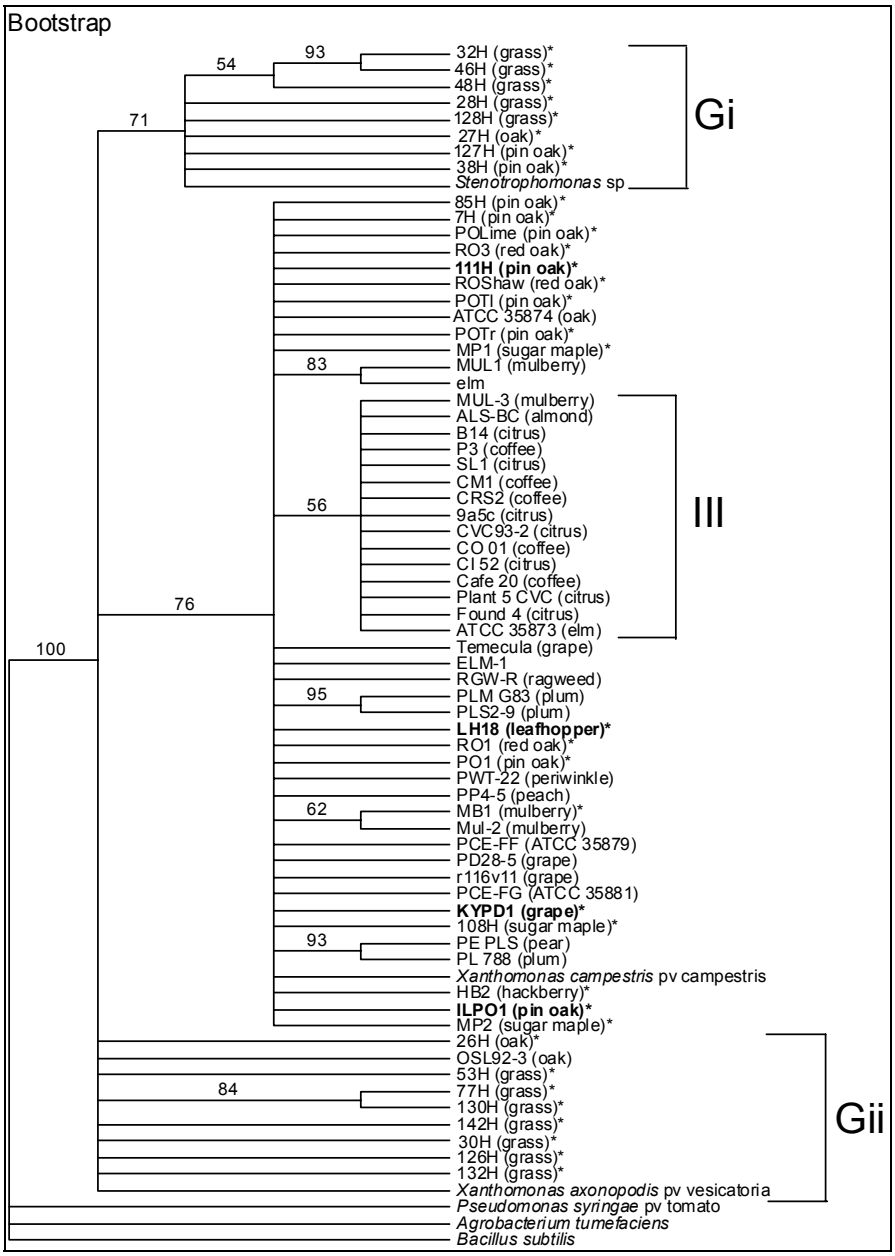

Figure 3.4: Bootstrap cladogram for 16S 2nd rDNA data. An asterisk (*) by the name indicates the sequence data from samples in this study. Unmarked names indicate sequences obtained from NCBI. Names in bold are sequences from samples of particular interest generated in this study. Clades Gi and Gii correspond to clade G on the near-complete 16S rDNA bootstrap cladogram (Fig. 3.2). The clade consisting primarily of sequence from citrus and coffee strains is labeled III.

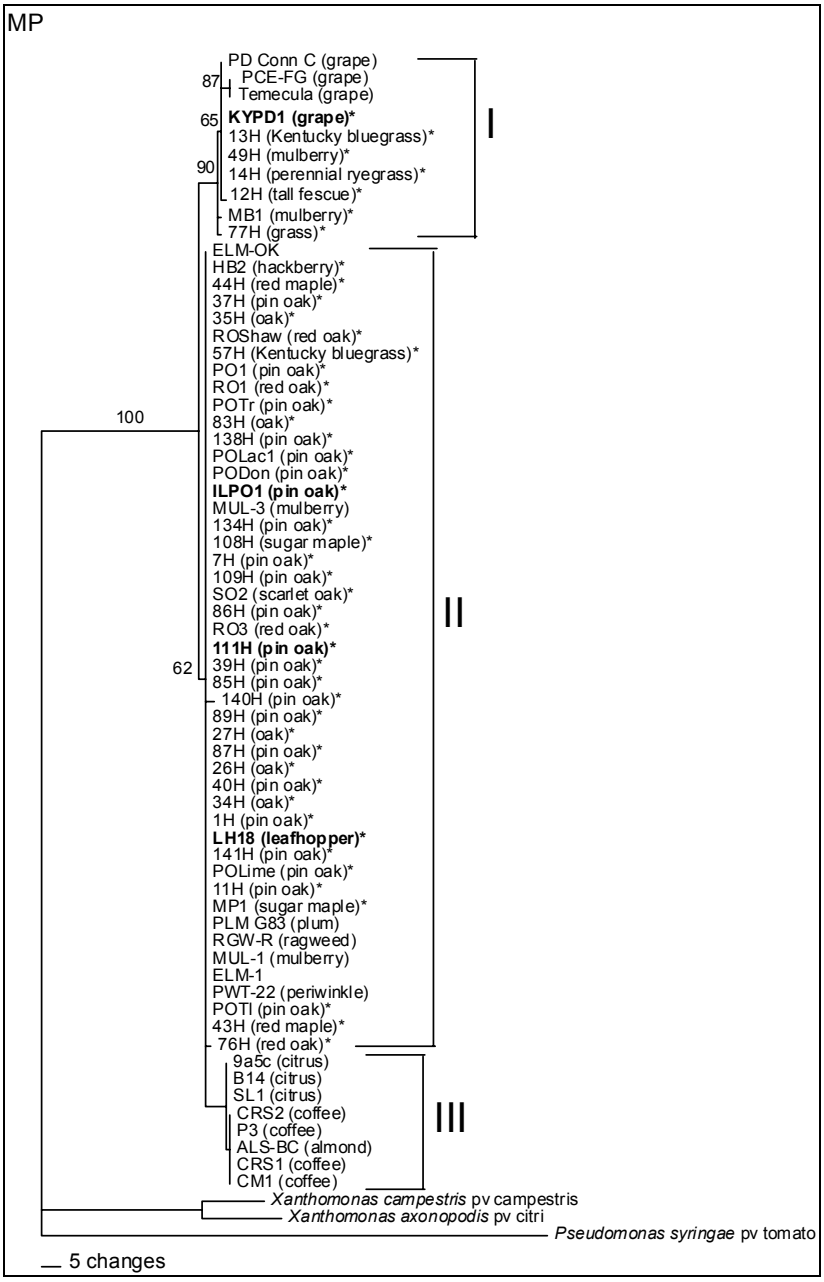

Figure 3.5: MP phylogram for *gyrB* gene data. The numbers at the nodes indicate the bootstrap value out of 1000 replications for the branch. An asterisk (*) by the name indicates the sequence data from samples in this study. Unmarked names indicate sequences obtained from NCBI. Names in bold are sequences from samples of particular interest generated in this study. Distinct clades are labeled I, II and III. Clade III corresponds to clades III from both the near-complete 16S rDNA and 16S 2nd trees (Fig. 3.1-3.4).

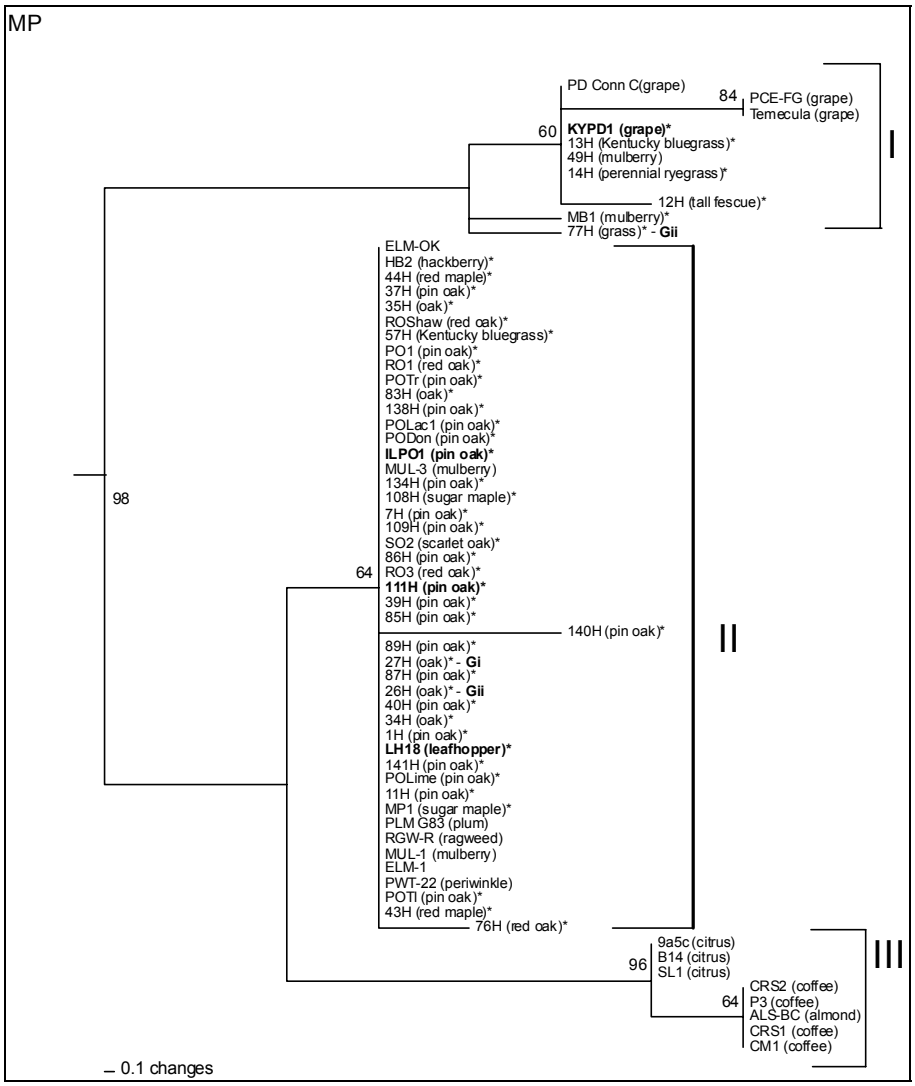

Figure 3.6: MP phylogram for *gyrB* gene data without outgroups. The outgroup root is indicated by the line at the left of the phylogram. The numbers at the nodes indicate the bootstrap value out of 1000 replications for the branch. An asterisk (*) by the name indicates the sequence data from samples in this study. Unmarked names indicate sequences obtained from NCBI. Names in bold are sequences from samples of particular interest generated in this study. Distinct clades are labeled I, II and III. Clade III corresponds to clade III from both the near-complete 16S rDNA and 16S 2nd trees (Fig. 3.1-3.4). Sequence from samples that were in the G clade are labeled with either Gi or Gii beside the name.

Chapter 4

Conclusions

This research addressed the hypothesis that sequence comparison can be used to identify potential asymptomatic hosts and vectors for pathogenic strains of *Xylella fastidiosa*. The results of the collection and detection part of this study underscore the problems associating *X. fastidiosa* with a given host, particularly those that are asymptomatic. Isolation proved to be difficult, if not impossible for *X. fastidiosa* from all hosts, and ELISA is known to cross-react with plant sap of some hosts. PCR detection methods can bypass these problems to some extent, if the primer set used is specific for *X. fastidiosa* and if the method of DNA extraction effectively eliminates PCR inhibitory compounds.

The results from phylogenetic analysis of the 16S rDNA and *gyrB* genes also had unexpected results. The MP trees from the partial sequence of these genes had significantly different topologies (Figures 3.3 and 3.5), which is an indication that recombination may have occurred between *X. fastidiosa* and another Xanthomonad. The other possible explanation for this difference in topologies is that multiple bacteria were present in the DNA extract from the plant samples.

The use of PCR for detection of *X. fastidiosa* allows the viable possibility of amplification of multiple genera of bacteria, a fact that makes it critical to use primers specifically designed to amplify *X. fastidiosa* DNA. The question of amplification from multiple genera can be addressed by using universal primers for the 16S rDNA and *gyrB* gene in PCR for all the DNA extracts used in this study to determine the bacterial populations present in each sample. Knowing the different genera of bacteria present would indicate the likelihood of amplification from multiple Xanthomonads with the specific primers developed by Rodrigues et al. (2003).

In addition to examining the potential for amplification of genes from multiple bacteria, the question of recombination should be investigated further. The first step in this direction would be to attempt culturing from the symptomatic oaks designated 26H and 27H and also from the grasses from which were obtained bacterial sequences in clade G. The importance of getting an isolate from the oaks would be to fulfill Koch's postulates and thereby confirm that the sequence comes from bacteria that are responsible for the observed symptoms. If isolates could be obtained from these samples, then additional genotypic analyses could be done to compare these strains with other strains of *X. fastidiosa*. Even if isolates could not be obtained, sequencing from genes besides the 16S rDNA and *gyrB* could be used to address the question of recombination.

Once the possibility of recombination has been resolved, and the hypothesis behind this research reassessed accordingly, the search for potential sources of pathogenic strains of *X. fastidiosa* can be continued in Kentucky. This goal can be furthered by expanding surveys to add to the list of known hosts of *X. fastidiosa* in Kentucky, particularly asymptomatic ones. This study focused on urban areas where BLS was known to occur. It would be interesting to expand the survey for hosts to natural, forested areas. Oaks are not known to show symptoms of BLS in forested settings, but asymptomatic oaks have not been tested for the presence of *X. fastidiosa*.

Besides looking for new plant hosts in Kentucky, it would be interesting to associate *X. fastidiosa* with other potential leafhopper vectors besides *G. versuta*. This could be done in urban settings from known-infected trees beginning in early March, to try and capture overwintering infested adult insects. Also, immunomagnetic capture could be tried in addition to the Wasp DNA extraction method to improve the probability of detecting *X. fastidiosa* in collected leafhoppers. Vectors represent an important but difficult to study part of the BLS

disease cycle and further research of this disease in Kentucky should include a vector component.

The ultimate conclusion from this study is that the detection and comparison of strains of *Xylella fastidiosa* is dependent on reliable, molecular methods. This requires the use of DNA extraction techniques that successfully access the DNA of *X. fastidiosa* in the xylem while minimizing the effect of PCR inhibitory compounds. It also requires the use of primers developed to specifically amplify *X. fastidiosa* DNA, and particularly to amplify evolutionarily meaningful genes or genomic regions that can be used in phylogenetic analysis.

Appendix A

Multiple Sequence Alignment for the Near-Complete 16S rDNA

```
1H        AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
7H        AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
11H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
26H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
27H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
35H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
37H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
39H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
40H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
43H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
77H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
83H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
85H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
86H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
87H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
89H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
108H      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
109H      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
111H      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
134H      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
138H      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
141H      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
ILPO1     AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
KYPD1     AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
LH18      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
MB1       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
MP1       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
PO1       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
PODon     AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
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RO3       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
ROShaw    AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
CM1       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
ALS-BC    AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
P3        AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
PWT-22    AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
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KYPD1     AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
LH18      AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
MB1       AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
MP1       AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
PO1       AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
PODon     AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
POLac1    AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
POLime    AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
POTl      AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
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RO1       AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
RO3       AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
ROShaw    AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
CM1       AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
ALS-BC    AAG-GAGACTGCCGGTGACAAATCGG-AGTAAGGTGGGGATGACGTCAAGTCATCATGGC
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PWT-22    AAG-GAGACTGCCGGTGACAAATCGG-AGGAAGGTGGGGATGACGTCAAGTCATCATGGC
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KYPD1     CCTTACGACCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCGATCTCGCG
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PCE-FG    CCTTACGACCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAATCTCGCG
Temecula  CCTTACGACCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAATCTCGCG
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KYPD1     AGGGTGAGCTAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCC
LH18      AGGGTGAGCTAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCC
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SL1       AGGGTGAGCTAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCC
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KYPD1     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTTGCACCAGAAGC-AG
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POLime    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
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ROShaw    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
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Multiple Sequence Alignment for the 16S 2nd

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ROShaw    AAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTTT
CM1       AAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTTT
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RGW-R     AAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTTT
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LH18      GCCTTGTACACACCGCCCGTCACACCNNNNNNNNNNNNN-NNNNNNNNNNN-NN
MB1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
MP1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
PO1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
PODon     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
POLac1    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
POLime    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
POTl      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
POTr      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
RO1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
RO3       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
ROShaw    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
CM1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
ALS-BC    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
P3        GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
PWT-22    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
CRS2      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
SL1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
B14       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
ELM-1     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
MUL-3     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
MUL1      GCCTTGTACACACCGCCCGTCACACCACGGGAGTTTGCT-GCACCAGAAGC-AG
RGW-R     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
PLM_G83   GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
PCE-FG    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
Temecula  GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
9a5c      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
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Multiple Sequence Alignment for the Partial *gyrB* Gene

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11H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
26H       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
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141H      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
ILPO1     AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
KYPD1     AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
LH18      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
MB1       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
MP1       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
PO1       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
PODon     AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
POLac1    AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
POLime    AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
POTl      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
POTr      NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCGGTCGCAAGACTGAAACTCAAAGGA
RO1       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
RO3       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
ROShaw    AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
CM1       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
ALS-BC    AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
P3        AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
PWT-22    AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
CRS2      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
SL1       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
B14       AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
ELM-1     AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
MUL-3     AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
MUL1      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
RGW-R     AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
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PCE-FG    AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
Temecula  AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
9a5c      AAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
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KYPD1     ATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAA
LH18      ATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAA
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ROShaw    ATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAA
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PWT-22    ATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAA
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SL1       ATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAA
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Temecula  ATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAA
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KYPD1       CCTTACCTGGTCTTGACATCTGCG-GAAC-TTTCCAGAGATGGATTGGTGCC-TTCGGGA
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RO3         CCTTACCTGGTCTTGACATCTGCG-GAAC-TTTCCAGAGATGGATTGGTGCC-TTCGGGA
ROShaw      CCTTACCTGGTCTTGACATCTGCG-GAAC-TTTCCAGAGATGGATTGGTGCC-TTCGGGA
CM1         CCTTACCTGGTCTTGACATCTGCG-GAAC-TTTCCAGAGATGGATTGGTGCC-TTCGGGA
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CRS2        CCTTACCTGGTCTTGACATCTGCG-GAAC-TTTCCAGAGATGGATTGGTGCC-TTCGGGA
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RGW-R       CCTTACCTGGTCTTGACATCTGCG-GAAC-TTTCCAGAGATGGATTGGTGCC-TTCGGGA
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Temecula    CCTTACCTGGTCTTGACATCTGCG-GAAC-TTTCCAGAGATGGATTGGTGCC-TTCGGGA
9a5c        CCTTACCTGGTCTTGACATCTGCG-GAAC-TTTCCAGAGATGGATTGGTGCC-TTCGGGA
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POLac1    GCCGTGAGA--CAGGTGCTGCA-TGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTT
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ROShaw    GCCGTGAGA--CAGGTGCTGCA-TGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTT
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ALS-BC    GCCGTGAGA--CAGGTGCTGCA-TGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTT
P3        GCCGTGAGA--CAGGTGCTGCA-TGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTT
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SL1       GCCGTGAGA--CAGGTGCTGCA-TGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTT
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Temecula  GCCGTGAGA--CAGGTGCTGCA-TGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTT
9a5c      GCCGTGAGA--CAGGTGCTGCA-TGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTT
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ELM-1     ATGAAGTCGGAATCGCTAGTAATCGCA-GATCAGCATTGCTGCGGTGAATACGTTCCCGG
MUL-3     ATGAAGTTGGAATCGCTAGTAATCGCA-GATCAGCATTGCTGCGGTGAATACGTTCCCGG
MUL1      ATGAAGTCGGAATCGCTAGTAATCGCA-GATCAGCATTGCTGCGGTGAATACGTTCCCGG
RGW-R     ATGAAGTCGGAATCGCTAGTAATCGCA-GATCAGCATTGCTGCGGTGAATACGTTCCCGG
PLM_G83   ATGAAGTCGGAATCGCTAGTAATCGCA-GATCAGCATTGCTGCGGTGAATACGTTCCCGG
PCE-FG    ATGAAGTCGGAATCGCTAGTAATCGCA-GATCAGCATTGCTGCGGTGAATACGTTCCCGG
Temecula  ATGAAGTCGGAATCGCTAGTAATCGCA-GATCAGCATTGCTGCGGTGAATACGTTCCCGG
9a5c      ATGAAGTTGGAATCGCTAGTAATCGCA-GATCAGCATTGCTGCGGTGAATACGTTCCCGG
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1H        GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
7H        GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
11H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
26H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
27H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
35H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
37H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
39H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
40H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
43H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
77H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
83H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
85H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
86H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
87H       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
89H       GCCTGGTACACACCGCCCGTCACACCATGGGAGNNNNNN-NNNNNNNNNNN-NN
108H      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
109H      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
111H      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
134H      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
138H      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
141H      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
ILPO1     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
KYPD1     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTTGCACCAGAAGC-AG
LH18      GCCTTGTACACACCGCCCGTCACACCNNNNNNNNNNNNN-NNNNNNNNNNN-NN
MB1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
MP1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
PO1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
PODon     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
POLac1    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
POLime    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
POTl      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
POTr      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
RO1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
RO3       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
ROShaw    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
CM1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
ALS-BC    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
P3        GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
PWT-22    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
CRS2      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
SL1       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
B14       GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
ELM-1     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
MUL-3     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
MUL1      GCCTTGTACACACCGCCCGTCACACCACGGGAGTTTGCT-GCACCAGAAGC-AG
RGW-R     GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
PLM_G83   GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
PCE-FG    GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
Temecula  GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
9a5c      GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTT-GCACCAGAAGC-AG
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Vita

Name: Jaclyn N. Mundell
Date of Birth: March 27, 1980
Place of Birth: Monterey, California

Education:

B.S., May 2002	University of Kentucky, Lexington, KY
H.S., June 1998	General H.H. Arnold H.S., Wiesbaden, Germany

Honors and Awards:

Research Challenge Trust Fund Fellow at University of Kentucky, 2004-2005
Graduate Student Travel Award, University of Kentucky, 2004
Kentucky Opportunities Fellow at University of Kentucky, 2003-2004
Singletary Fellow at University of Kentucky, 2002-2003
AAAA Scholarship Award, 1999
UK Merit Scholarship Recipient, 1998-2002
Member, Golden Key National Honor Society, since 2000
Member, Gamma Sigma Delta Honors Fraternity, since 2001