MOLECULAR AND BIOCHEMICAL GENETIC STUDIES ON SOME LEAFHOPPER TRANSMITTED PLANT PATHOGENS

DISSERTATION

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Abstract

Aster yellows is a disease that sporadically damages leafy green vegetables, especially lettuce. The disease is caused by a phytoplasma and vectored by phloem-feeding aster leafhoppers. Aster yellows induces various symptoms in lettuce including yellowing and witches’-broom of the leaves, and phyllody and virescence of the flowers, which can lead to severe economic losses. Previously, four aster yellows phytoplasma strains were identified and characterized based on their symptoms in lettuce fields in Ohio. In our study, a thorough assessment of the role that these different strains play in aster yellows epidemics in Ohio was made over time during two growing seasons (2008 and 2009). In addition, new aster yellows isolates that may contribute to the aster yellows phytoplasma strain complex were identified based on their 16SrRNA sequences. The role of herbaceous plants such as cilantro and parsley, which are commonly produced in lettuce production areas, was studied as well. Surveys of aster leafhoppers and host plants (lettuce, cilantro and parsley) were conducted to assess the distribution of these known strains (AY-WB, AY-S, AY-DB2 and AY-BW) of aster yellows phytoplasma in two major vegetable production areas (Celeryville and Hartville) in northern Ohio. Strains in two 16SrRNA group I subgroups (16SrI-A and 16SrI-B) were detected in both locations and years. Strain AY-WB belonged to subgroup 16SrI-A while strains AY-S, AY-BD2 and AY-BW belonged to 16SrI-B. Multiplex PCR assays for aster yellows phytoplasma were developed and used to screen leafhopper and plant
samples to detect known phytoplasma strains. In both locations during both years, populations of aster leafhoppers collected were higher in romaine lettuce during the month of August. There was a noticeable increase in the proportion of infected/inoculative aster leafhoppers collected early in the season compared to that observed 3-8 years previously. Aster yellows infection of surveyed leafhoppers and plants was the highest early in the growing season, declining late in the season, in both years.

All lettuce types (romaine, red leaf and green leaf), cilantro and parsley plants were infected with both 16SrI-A and 16SrI-B strains. Aster yellows phytoplasma strains were distributed evenly across hosts (red, green and romaine lettuce, cilantro and parsley) and there was no association between aster yellows phytoplasma strain and plant host. Strain AY-S (16SrI-B) appeared to be relatively rare (2.2-3.2%) in leafhoppers and plants in both years at both locations; however, there were no significant differences in strain distribution in either location. Other strains in subgroup 16SrI-B (AY-BD2 and AY-BW) were numerically more abundant in leafhoppers and plants in this study.

Phylogenetic analysis of 16S rRNA gene sequences of sixteen aster yellows isolates that were detected amongst host plants and aster leafhoppers associated with these plants indicated that these isolates belonged to aster yellows subgroup 16SrI-A and subgroup 16SrI-B.
In the last five years, cilantro and parsley production increased dramatically in intensive lettuce production areas in Ohio. While these plants remained symptomless in the field, the proportions of aster yellows-infected plants were no different than the proportions of aster yellows-infected lettuce plants. Therefore, it is very likely that parsley and cilantro play an important role in aster yellows epidemiology, providing a natural reservoir of aster yellows phytoplasmas.
Dedication

To my mom

and

My children Yassmine and Seif
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Major Field: Plant Pathology
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CHAPTER 1

Background on Phytoplasmas Diseases, Distribution, Detection and Classification

Aster Yellows Phytoplasmas Disease of Lettuce in Ohio

Introduction

Phytoplasmas cause more than 400 plant diseases worldwide, causing significant losses globally (McCoy et al. 1989; Lee et al. 2000 and Bertaccini 2007). Many of these diseases, affecting cultivated and wild plants, are economically important, including aster yellows in vegetable crops like lettuce, carrot, onion and celery (Errampalli and Fletcher 1991; Lee et al. 1993b; Zhang et al. 2004; Oshima et al. 2004; Beanland et al. 2005; Chung et al. 2007 and Duduk et al. 2006; 2008). Other diseases associated with phytoplasmas include tomato big bud (Shaw et al. 1993 and Streten and Gibb 2003), tomato and pepper little leaf (Santos-Cervantes et al. 2008), potato witches’-broom (Doi et al. 1967 and Khadhair et al. 2003), sweet potato little leaf (Streten and Gibb 2003 and Saqib et al. 2006), rice yellow dwarf (Nakashima et al. 1993), maize bushy stunt (Harrison 1996 and Bedendo et al. 2000) and maize redness (Duduk et al. 2006 and Jovic et al. 2009). Phytoplasmas also contribute to major fruit crop diseases including coconut lethal yellowing (Harrison and Richardson 1994 and Mpunami et al. 1999), peach X-disease (Lee et al. 1992; Ahrens et al. 1993 and Zhu et al. 1996); strawberry yellows
(Valiunas et al. 2006), pear decline (Davies et al. 1992), grapevine yellows (Daire et al. 1993; Chen et al. 1994 and Bertaccini and Stefani 1995), and apple proliferation (Lee et al. 1995 and Seemüller and Schneider 2007). Ornamental plants such as gladiola, hydrangea, China aster, and purple coneflower are also affected (Stanosz et al. 1997; Sawayanagi et al. 1999; Zhang et al. 2004 and Lee et al. 2008). Herbaceous plants such as parsley (Khadhair et al. 1998) and cilantro (Zhou et al. 2003 and Lebsky and Poghosyan 2007) can also be hosts of phytoplasma diseases. Furthermore, phytoplasma diseases like elm and ash yellows have destroyed historical and new plantations of forest trees (Sinclair et al. 1996; Griffith et al. 1999 and Lee et al. 2004b).

Phytoplasma diseases were originally thought to be caused by viruses because they shared similar symptoms, are insect-transmitted, and could not be cultured (Kunkel 1926, 1957). Kunkel (1926) was the first to describe a phytoplasma disease as aster yellows, which caused a yellowing of the China aster leaves. In 1967, a group of Japanese scientists discovered that the plant pathogen that caused yellowing symptoms in plants was similar to the animal pathogen ‘mycoplasmas’: both were pleiomorphic, 80×800 nm in size, and sensitive to the antibiotic tetracycline. Subsequently, the term mycoplasma-like organism (MLO) was coined to describe these plant pathogens (Doi et al. 1967). In 1980, Woese and others suggested that MLOs originated from a single lineage diverged from species of Acholeplasma, namely Bacillus/Clostridium ancestors (Gundersen et al. 1996). Moreover, Lim and Sears (1989) compared the 16SrRNA gene sequence from an MLO (Oenothera virescence phytoplasma) from the aster yellows group with those of
mycoplasmas, which indicated that MLOs are members of the class Mollicutes and are evolutionarily distinct from animal mycoplasmas. Phylogenetic analyses based on conserved genes followed the lateral comparison to confirm that MLOs represent a clearly distinct, monophyletic clade within the class Mollicutes (Lim and Sears 1991 and 1992; Kuske and Kirkpatrick 1992; Namba et al. 1993; Seemüller et al. 1994; Toth et al. 1994; Gundersen et al. 1994 and Jomantiene et al. 1998). In 1994, the 10th Congress of the International Organization of Mycoplasmology announced that the name ‘phytoplasmas’ would replace ‘MLOs’. Subsequently, with the development of new techniques for identification and characterization of phytoplasmas such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and phylogenetic analyses, the International Research Program on Comparative Mycoplasmology (IRPCM) suggested in 2004 that phytoplasmas be placed within the genus ‘Candidatus’ (IRPCM 2004). Thus far, 27 Ca. phytoplasma species have been described and for any strain to be described as a novel ‘Ca. Phytoplasma’ species, its 16SrRNA gene sequence has to have < 97.5% similarity to any previously described ‘Ca. Phytoplasma’ species (Zreik et al. 1995; Davis et al. 1997; Griffiths et al. 1999; Sawayanagi et al. 1999; Montano et al. 2001; Jung et al. 2002, 2003; Verdin et al. 2003; Lee et al. 2004a, b; Marcone et al. 2004; Hiruki and Wang 2004; IRPCM 2004; Seemüller and Schneider 2004; Schneider et al. 2005; Firrao et al. 2005; Valiunas et al. 2006; Arocha et al. 2007; Al-Saady et al. 2008; Hohenhout et al. 2008 and Zhao et al. 2009).
Phytoplasmas indeed have unique characteristics amongst microorganisms, including repeat-rich genomes, which promote rapid adaptation of these plant pathogens to new environments (Bai et al. 2006). Furthermore, phytoplasmas have many copies of transporter genes that encode proteins to facilitate importation of different types of sugars from plant and insect hosts, such as maltose from plant sieve tubes and trehalose from insect haemolymph. Phytoplasmas lack some cell metabolism and division genes, which explain the dependence of phytoplasmas on their hosts (plants and insect vectors) for survival (Oshima et al. 2004; 2007; Bai et al. 2006 and Kube et al. 2008).

In the plant host, phytoplasmas reside mainly in the phloem sieve tubes and spread from the leaves to the roots by moving through the pores of the sieve plates that divide the phloem sieve tubes (Doi et al. 1967; Whitcomb and Tully 1989 and Olivier et al. 2007). Once phytoplasmas have infected a plant it can take from 1 week up to 24 months, depending on the phytoplasma and plant species, for symptoms to develop. For example, aster yellows symptoms take 3 weeks to develop on lettuce (Lee et al. 2000). Although phytoplasmas can be spread by vegetative propagation such as grafting, rhizomes, or bulbs (Lee et al. 1992) they are not transmitted through seeds. There has been no evidence that the phloem sieve tubes that harbor phytoplasmas have a direct connection to the seed.

Insect vectors are the means of phytoplasma dispersal in nature (Bosco et al. 1997; Sforza et al. 1998; Carraro et al. 1998; Marzachi et al. 1998; Maixner and Reinert 1999;
Insect vectors are phloem-feeders, mainly from the Order Hemiptera, including leafhoppers (*Cicadellidae*) (Maramorosch and Harris 1979; Grylls 1979; McClure 1982 and Kawakita *et al.* 2000), psyllids (*Psyllidae*), (Weintraub and Beanland 2006) and planthoppers (*Fulgoromorpha, Delphacidae* and *Derbidae*) (Khan and Saxena 1984). Insect vectors acquire phytoplasmas through their stylets during feeding in the phloem of infected plants (Lherminier *et al.* 1990 and Nakashima and Hayashi 1995).

This feeding period, which allows the insect vector to acquire sufficient titer of phytoplasmas, is called acquisition access period (AAP). The AAP can range from a few minutes to a few hours (Purcell 1987). Phytoplasmas then pass through the foregut and penetrate the midgut, passing through the epithelial cells to the haemolymph where they replicate (Purcell 1987; Nault 1997; Lett *et al.* 2001 and Ammar and Hogenhout 2006).

In order for the insect vector to transmit phytoplasmas, they must pass through the three salivary gland barriers (the basal lamina, the basal plasmalemma, and the apical plasmalemma), where they accumulate in high titers and are injected as the insect feeds on new plants. This period between initial acquisition of phytoplasmas and the ability of the insect vector to transmit the phytoplasma is called the latent period (LP). The latent period ranges from 7 to 80 days, which can increase with low temperature (Nagaich *et al.* 1974; Murral *et al.* 1996 and Moya-Raygoza and Nault 1998). Once the insect vector becomes infectious, it is able to transmit phytoplasmas for life. However, if the phytoplasmas fail to penetrate any of the three insect salivary gland barriers, the insect vector becomes a dead-end host and is unable to transmit the phytoplasmas to new host.
plants (Vega et al. 1993, 1994; Lefol et al. 1993; Wayadande et al. 1997 and Fletcher et al. 1998). Some plant hosts such as cilantro and parsley may become infected with phytoplasmas by feeding insects without showing any symptoms, but still serve as reservoirs for the phytoplasmas (Khadhair et al. 1998 and Lee et al. 2003).

Generally, phytoplasmas do not affect their insect vector negatively, in fact, the insect vector can benefit from its association with phytoplasmas by showing improved overwintering ability and increased fecundity and longevity (Beanland et al. 2000 and Hogenhout et al. 2008). Aster yellows phytoplasma-infected aster leafhoppers lay nearly double the amount of eggs on host plants than non-infected leafhoppers do (Beanland et al. 2000). In 2009, Bai et al. demonstrated that phytoplasmas produce effector proteins that target the plant nuclei, changing plant gene expression to increase the attractiveness of the young shoots and leaves to leafhoppers, and making it more accessible for insect vector feeding and oviposition compared to healthy plants (Peterson 1973 and Schultz 1973).

Insects that vector phytoplasmas can be monophagous, oligophagous or polyphagous (Backus et al. 2005). For example, North American aster yellows phytoplasma strains are transmitted by the polyphagous aster leafhopper to at least 250 plant species (Swenson 1971; McCoy et al. 1989 and Weintraub and Beanland 2006). On the other hand, the American elm yellows phytoplasma is transmitted by the monophagous vector
*Scaphoideus luteolus*, and causes disease in only a few plant species in the genus Ulmus (Lee et al. 1992, 1994).

Phytoplasmas have been difficult to detect due to their low and often variable titre in hosts, their variable distribution in the sieve tubes of infected plants (Doi et al. 1967; Martinez et al. 2000 and Firrao et al. 2007), and the inability to culture them. Prior to the development of molecular techniques, the characterization of phytoplasmas relied on biological properties such as symptoms, host range, and the relationship between the phytoplasma and its leafhopper vector (Davis et al. 1990; Chen et al. 1992; Daire et al. 1992; Davis and Lee 1993 and Davies et al. 1995). Transmission (Jones et al. 1974; Parthasarathy 1974; Schneider 1977; Braun and Sinclair 1979; Bertaccini and Marani 1982; Cousin et al. 1986; Singh 1991; Ahrens and Seemüller 1994 and Tanaka et al. 2000) and scanning (Haggis and Sinha 1978; Marcone and Ragozzino 1996 and Poghosyan et al. 2004) electron microscopy are the principal diagnostic techniques that allow *in situ* observation of phytoplasmas in plants and leafhoppers. A method to stain the DNA with the fluorescent dye 4', 6-diamidino-2-phenylindole (DAPI) was developed for light microscopy (Schaper and Converse 1985 and Hiruki and Rocha 1986) and used with success for the diagnosis of phytoplasmas even in woody hosts such as coconut palm (Schuiling and Forstel 1992), ash (Sinclair et al. 1989 and 1992) and pear (Malinowski et al. 1996). However microscopic methods are time-consuming, not widely accessible and limited in capacity for phytoplasma identification beyond the genus (*Candidatus Phytoplasma*) level.
Polyclonal and monoclonal antibodies were amongst the earliest tools of biotechnology used for detection of phytoplasmas affecting host plants (Davis et al. 1988; Clark et al. 1989; Chen et al. 1992a, b; Lee and Davis 1992; Chang et al. 1995; Srinivasulu and Narayanasamy 1995; Lin and Chen 1996; Seddas et al. 1996; Gomez et al. 1996; Guo et al. 1998; Thomas and Balasundaran 2001; Loi et al. 2002; Bertaccini 2007 and Firrao et al. 2007). Polyclonal antibodies against phytoplasmas belonging to different taxonomic groups were developed for use in immunosorbent electron microscopy (IEM) (Sinha et al. 1983), fluorescence microscopy (Lin and Chen 1986; Clark et al. 1989 and Chen et al. 1993), western blots (Clark et al. 1989 and Seemüller and Schneider 2004) and ELISA (Hobbs et al. 1987; Davies and Clark 1992; Sarindu and Clark 1993 and Canik and Ertunc 2007). However, some of the polyclonal antibodies have a high level of cross-reactivity with other phytoplasmas, and therefore are not useful for differentiation between phytoplasma groups (Sinha et al. 1983; Clark et al. 1989 and Schneider et al. 1997). The development of monoclonal antibodies against some phytoplasmas such as aster yellows (Lin and Chen 1985; 1996), apple proliferation (Loi et al. 2002), maize bushy stunt (Chen and Jiang 1988) and grapevine yellows (Chen et al. 1993) permitted some differentiation between different groups of phytoplasmas. Monoclonal antibodies have been used successfully to detect different phytoplasmas in potential vectors by immunofluorescence (Boudon-Padieu et al. 1986; Boudon-Padieu et al. 1989; Lherminier et al. 1989; Scheltinga et al. 1989 and Liefting and Kirkpatrick 2003), immunosorbent electron microscopy (Sinha and Chiykowski 1986; Lherminier et

More recently, another approach for phytoplasma detection and classification based on nucleic acid-based techniques was developed, which mainly depends on high quality phytoplasma DNA. Different protocols for phytoplasma DNA isolation have been reported for use in detection of phytoplasmas in their hosts in the laboratory and in the field (Ahrens and Seemüller 1992; Gibb and Padovan 1994; Levy et al. 1994; Daire et al. 1997; Zhang et al. 1998; Green et al. 1999; Prince et al. 1999; Guo et al. 2003; Goto et al. 2009; and Tomlinsona et al. 2010). The development of nucleic acid-based techniques has facilitated routine detection and identification of phytoplasmas and indication of their distribution in plants and leafhopper vectors (Kirkpatrick et al. 1987; Lee et al. 1993; Webb et al. 1999; Christensen et al. 2004 Wei et al. 2004; Bertolini et al. 2007; Hren et al. 2007 and Martini et al. 2007). Of all the nucleic acid-based techniques, PCR is used most extensively for this purpose since it is rapid, specific and highly sensitive for the detection of phytoplasmas both from symptomatic and asymptomatic plants and from insect vectors. Universal primers prime the amplification of common sequences and can be used to detect phytoplasmas 7 days before symptoms appear in the plants (Lee et al. 1991; 1992; 1994; Deng and Hiruki 1991; Firrao et al. 1993 and Smart et al. 1996). Specific primers that distinguish between phytoplasma groups (Griffiths et al. 1994 and Weintraub and Beanland 2006) have been developed from different positions on the ribosomal RNA operon, such as the 16SrRNA gene and the variable
16S-23S intergenic region (Deng and Hiruki 1991; Davis and Lee 1993; Namba et al. 1993; Rhode et al. 1993; Schneider et al. 1993; Ahrens et al. 1994; Kirkpatrick et al. 1994; Lorenz et al. 1995; Maixner et al. 1995; Gundersen et al. 1996; Malisano et al. 1996; Smart et al. 1996; Schneider et al. 1997; White et al. 1998; Zhang et al. 1998; Griffiths et al. 1999; Guo et al. 2000; Montano et al. 2001; Wang et al. 2003; Lee et al. 2005; 2006; Wei et al. 2007 and Firrao et al. 2007). Phytoplasmas may be detected by direct PCR assays but in some cases can be present in low titers, so nested PCR assays are commonly used. In nested PCR, two sets of primers are used in two consecutive runs of PCR; the second set of the primers amplify DNA within the first PCR product, which increases the assay detection sensitivity. Furthermore, for the complete identification of detected phytoplasmas to the strain level it may be necessary to perform multiplex PCR and/or RFLP analysis or sequencing of 16SrRNA amplicons (Lee et al. 1992; Daire et al. 1997 and Zhang et al. 2004). In a multiplex PCR assay, multiple phytoplasma strain-specific primer sets are used within a single PCR mixture to produce amplicons of varying sizes, which results in ‘fingerprints’ unique to specific phytoplasma strains. Assays utilizing strain-specific primer sets in a multiplex mixture can distinguish between different phytoplasma strains within specific phytoplasma groups precisely with less time and less cost than multiple independent assays (Oliveira et al. 2007 and Gomes et al. 2004).

In addition to the PCR assays, RFLP analysis has been used to identify and classify different groups and subgroups of phytoplasmas from different plant and leafhopper
sources based on the size of the DNA fragments produced after using restriction enzymes (Lee et al. 1993; Schneider et al. 1993; Langer et al. 2003 and Zhang et al. 2004 and Bertaccini 2007). In 1993, Lee et al. proposed a classification scheme that included ten major phytoplasma groups and 15 subgroups based on their RFLP profiles. This scheme was later expanded to 28 groups and 57 subgroups, and new groups are continually being added (Lee et al. 1993 and 1994; Gundersen et al. 1994; Seemüller et al. 1998; Lee et al. 1998 and 2000; Marcone et al. 2000; Lee et al. 2004a, b; Al-Saady et al. 2008).

DNA sequencing is considered the most appropriate nucleic-acid based technique for phytoplasma classification because it provides the most information and helps define the evolutionary relationships between different groups and subgroups. Also, this technique may be used to compare between different phytoplasma strains within the same subgroup/group based on the sequence of specific genes such as the 16SrRNA gene (Bertaccini 2007). Based on these distinctive sequences in the 16SrRNA gene, several phytoplasma-specific PCR assays have been developed and more than 350 different, nearly full length sequences of phytoplasma 16SrRNA genes have been obtained to date (Ahrens and Seemüller 1992; Firrao et al. 1993; Lee et al. 1993; Namba et al. 1993; Gibb et al. 1995; Lorenz et al. 1995; Padovan et al. 1995; Smart et al. 1996; Seemüller et al., 1998b; Lee et al., 2000; IRPCM 2004 and Zhao et al. 2009).

One of the first phytoplasma groups to be classified was the aster yellows group (Group I), which is the main focus in this study. In Ohio, aster yellows significantly damages leafy
green vegetables, especially lettuce, although the incidence of the disease varies from year to year (Murral et al. 1996). The disease is caused by ‘Ca. Phytoplasma asteris’ which induces various symptoms in lettuce including yellowing of the leaves, witches’-broom (excessive proliferation of shoots), phyllody (the development of leafy structures instead of floral parts), and virescence (the development of chloroplasts in flower parts) (Lee and Davis 1992; Lee et al. 2000; Firrao et al. 2007 and Hogenhout et al. 2008). Aster yellows disease is transmitted by the aster leafhopper (Macrostelea quadrilineatus) (Kunkel 1957), which feeds and reproduces on over 400 plant species. Preferred hosts include small grain crops (wheat, oats, barley, and rye), vegetable crops (lettuce, carrot, onion, tomato, and pepper), and various herbs (parsley, cilantro, and dill) (Kunkel 1957; Lee et al. 2003, 2004a and Hollingsworth et al. 2008). The movement of these leafhoppers can be influenced by the dispersion of their host plants over the growing season and can have a significant impact on aster yellows epidemics. During spring, phytoplasma-infected aster leafhopper populations migrate from grain growing areas in Texas, Louisiana, Oklahoma and Arkansas, acquire the phytoplasma before or during migration, start feeding on winter wheat and oats, and then emerge in the vegetable growing areas of midwestern states of the United States and the Great Lakes area of Canada in winds originating from the Gulf Coast region and the northern Plains (Wallis 1962; Meade and Peterson 1964; Drake and Chapman 1965; Chiykowski and Chapman 1965; Nichiporick 1965 and Hoy et al. 1992). Infected migratory aster leafhoppers are considered to be the main source of aster yellows in many vegetable crops in the midwestern United States. However, there were some observations that aster leafhoppers overwinter in the egg stage in Minnesota (Meade and
Peterson 1964) and Wisconsin (Chiykowski and Chapman 1965). No evidence of transovarial transmission has been reported for aster yellows phytoplasma in *M. quadrilineatus* (Ammar and Hogenhout 2006).

Once aster yellows-infected aster leafhoppers migrate into lettuce fields, they begin feeding on and infecting early plantings of lettuce. As the growing season progresses, leafhoppers originating from earlier plantings or from other migrant infected leafhoppers begin feeding on the later plantings of lettuce, which explains the clustered pattern of aster yellows-infected lettuce plants caused by different strains observed in Oklahoma and Ohio (Errampalli et al. 1991; Madden et al. 1995; Murral et al. 1996 and Beanland et al. 2005). Aster yellows phytoplasma strains distribute differently within and between host plants in the field (Madden et al. 1995). The diversity of aster yellows symptoms in lettuce fields depends on the type of aster yellows phytoplasma strains present (Errampalli and Fletcher 1991 and Murral et al. 1996). For example, Beanland et al. (2000) observed that aster leafhoppers infected with aster yellows phytoplasma strain AY-WB laid more eggs than those infected with AY-S, which suggests that the AY-WB strain could become more common in the lettuce fields than the AY-S strain.

In Ohio, five different strains of aster yellows phytoplasma were identified previously in lettuce fields: aster yellows witches’-Broom (AY-WB) (16SrI-A), aster yellows severe (AY-S) (16SrI-B) (D’Arcy and Nault 1982; Hoy et al. 1992 and Murral et al. 1994), aster yellows bolt white (AY-BW) (16SrI-B), aster yellows bolt distortion no. 2 (AY-BD2)
and aster yellows bolt distortion no. 3 (AY-BD3) (16SrI-B) (Zhang et al. 2004). Initially, the five aster yellows phytoplasma strains were identified and characterized based on their symptoms in both lettuce and China aster plants (Zhang et al. 2004). Distinguishing symptoms included chlorosis, wilting and witches’-broom (AY-WB); stunting, chlorosis, phyllody and virescence (AY-S) (Murral et al. 1996); chlorosis of newly emerging leaves (AY-BW) and leaf and stem distortion (AY-BD2/AY-BD3) (Zhang et al. 2004). Zhang et al. (2004) used the universal primer sets P1/P7 (Deng and Hiruki 1991 and Smart et al. 1996) and F2n/R2 (Lee et al. 1992) to identify these aster yellows phytoplasma strains by direct and nested PCR. They also developed strain-specific primers to distinguish between these strains; i.e. AY-WB can be distinguished from the other strains by using the primer pair BF/BR. AY-WB and AY-S can be distinguished from the other strains by using the primer pair S1/S2. AY-WB, AY-S, AY-BD2, AY-BD3 and AY-BW have been detected and characterized successfully using multiple single reaction PCR assays but not using multiplex PCR assays.

Epidemics of aster yellows disease in Ohio depend on several factors, including 1) host plant susceptibility to phytoplasma infection; 2) phytoplasma strains, which are distributed between and within the host plants and 3) the infected insect vector feeding behavior and its preference for particular host plants throughout the growing season (Hoy et al. 1992 and Zhou et al. 2003). While leafhopper feeding behavior and phytoplasma distribution between and within lettuce plants have been described extensively in the past two decades (Hoy et al. 1992; Madden et al. 1995; Murral et al. 1996; Beanland et al. 1996).
2000 and Zhang et al. 2004), a thorough assessment of the role of different aster yellows strains and other herbaceous crops in lettuce aster yellows epidemiology has not been done. Therefore the aims of this study were: 1) to document the distribution of known strains of aster yellows phytoplasma in lettuce intensive production areas, 2) to determine if the composition of the aster yellows strain complex varies over time in different vegetable crops, 3) to determine the role of the herbaceous plants such as cilantro and parsley as reservoirs for the aster yellows phytoplasma within the lettuce-intensive production areas and 4) to identify any new aster yellows isolates that may contribute to the aster yellows phytoplasma strain complex.
CHAPTER 2
Development of a Multiplex PCR Assay for the Detection and Identification of Aster Yellows Phytoplasma Strains

Introduction

Effective prevention techniques for aster yellows disease in lettuce and other crops rely mainly on early detection and accurate diagnosis of phytoplasmas in their plant and leafhopper hosts. Since phytoplasmas cannot be cultured in vitro and have low titers and/or variable distribution in their host plants, identification and differentiation of phytoplasmas has been traditionally based on the symptoms they produce in plants and microscopic observation of their cells (Lee and Davis 1992). Diagnosis based on symptoms can be unreliable since symptoms such as leaf distortion, chlorosis and vein clearing are similar to those caused by viruses or nutritional deficiencies. Microscopy, while useful in confirming the actual presence of the phytoplasma, is time-consuming and can be limited by the distribution or titer of the phytoplasma within the host (plant and/or insect vector). In addition, neither microscopy nor plant symptoms can distinguish between phytoplasma groups sub-groups or strains causing the disease. The development of molecular techniques such as PCR and DNA sequencing has increased the specificity
and sensitivity of phytoplasma detection from symptomatic and asymptomatic plants and insect vectors, as well as the detection time (Kirkpatrick et al. 1987; Lee et al. 1993; Webb et al. 1999; Christensen et al. 2004; Bertolini et al. 2007; Hren et al. 2007 and Martini et al. 2007). Phytoplasma-specific PCR assays generally utilize primers specific to conserved regions of the 16SrRNA gene and to the variable 16S-23S intergenic region (Deng and Hiruki 1991; Ahrens et al. 1992 and 1993; Schneider et al. 1993; Rhode et al. 1993; Davis and Lee 1993; Kirkpatrick et al. 1994; Lee et al. 1994 and 1995; Lorenz et al. 1995; Maixner et al. 1995; Gundersen et al. 1996; Smart et al. 1996; White et al. 1998; Zhang et al. 1998; Griffiths et al. 1999; Montano et al. 2001; Wang et al. 2003 and Firrao et al. 2007). Both universal (P1/P6, P1/P7 and F2n/R2; Lee et al. 1991; 1992; Deng and Hiruki 1991; Firrao et al. 1993 and Smart et al. 1996) and group-specific (F4/R1 for aster yellows group; fPD/rPDS for pear decline; fB1/rULWS1 for elm yellows group; fB1/rASHYS for ash yellows and P1/PYLRint for apple proliferation group; Griffiths et al. 1994; Smart et al. 1996 and Weintraub and Beanland 2006) primers have been developed and are used routinely to detect and identify phytoplasma infections in host plants and insect vectors. In addition, the use of PCR assays with universal or group-specific primers has been especially practical for the early detection of aster yellows-infected leafhoppers in leafy green vegetables such as lettuce. Early detection before the symptoms develop is critical to prevent the disease from spreading, minimize inoculum within lettuce fields and avoid major loss of yield by developing preventative insecticide spray programs to manage disease onset (Errampalli and Fletcher 1991 and Zhang et al. 2004).
Although PCR assays including strain-specific PCR (Lorenz et al. 1995; Smart et al. 1996; Lee et al. 1998 and Zhang et al. 2004), nested multiplex PCR (Daire et al. 1997), real-time multiplex PCR (Christensen et al. 2004) and RFLP analysis (Lee et al. 1993; and Schneider et al. 1993; Langer et al. 2003; Zhang et al. 2004 and Bertaccini 2007) have been developed over the last ten years, they still require multiple steps and have not always been reproducible. However, these techniques have been used as tools for the detection and identification of many plant pathogens including viruses (Ali et al. 2009), bacteria (Soustrade et al. 2010), and fungi (Lievens and Thomma 2005). Even though there are few publications on multiplex PCR assays, they are favored over RFLP assays (Chamberlain et al. 1994; Edwards et al. 1994 and Henegariu et al. 1997) because they are less time-consuming and require fewer steps to completion. Although developing multiplex PCR assays to identify phytoplasma group and subgroup can be tedious, once all conditions are optimized, they require very little sample material, are sensitive and can be used to quickly identify multiple strains of aster yellows.

In this study, two multiplex PCR assays were developed to facilitate the differentiation of four strains of phytoplasmas (AY-WB, AY-S, AY-BD2 and AY-BD3) within the 16SrI group of phytoplasmas (aster yellows phytoplasma group) using primers previously described by Zhang et al. (2004).
Materials and Methods

Rearing of aster leafhoppers.

Laboratory colonies of aster leafhoppers were the progeny of leafhoppers originally collected in 1992 from vegetable crops grown in Celeryville, OH (41.00°N, 82.45°W) (Murral et al. 1996). Leafhoppers were reared by placing ~200 nymphs on four-six 7-day-old oat seedlings (Avena sativa L. var. Armor) contained within rearing cages (20 cm x 40 cm x 40 cm) covered with Dacron organdy netting. The rearing cages were constructed using an aluminum frame with a solid floor and two hinged plexiglass doors (Figure 1; D’Arcy and Nault 1982). Leafhoppers were maintained in an insectary under fluorescent lights (16/8 hrs) at 27°C. Every 3 months, 50 leafhoppers were randomly selected and tested using universal primers P1/P7 (Deng and Hiruki 1991) and F2n/R2 (Smart et al. 1996 and Lee et al. 1992) to confirm the colonies remained phytoplasma-free.

Maintenance of leafhoppers infected with aster yellows phytoplasma reference strains.

Leafhoppers collected from plants in commercial, symptomatic lettuce fields in Celeryville, OH previously were purified into four colonies, each infected with a different strain of aster yellows phytoplasma (Zhang et al. 2004; Table 1). Colonies were maintained on China aster (Callistephus chinensis) cv. Matsumoto Red (Stokes Seeds Ltd., Buffalo, NY) (D’Arcy and Nault, 1982; Hoy et al. 1992; Murral et al. 1996 and Zhang et al. 2004) as described below. Pure colonies of each strain were generated by
placing non-infected aster leafhoppers nymphs (2nd and 3rd instars) into leafhopper rearing cages containing symptomatic lettuce and/or China aster (one symptom type per cage) for an acquisition access period (AAP) of 21 days. The leafhoppers were maintained in the insectary as described for the non-infected colonies. After the AAP, the infected leafhoppers were transferred to new cages containing 3-wk-old healthy, leafhopper-free China aster seedlings for a variable inoculation access period (IAP). After the IAP, the aster plants were removed from the rearing cages, sprayed with 1100 Pyrethrum aerosol insecticide (0.5% pyrethroid, 2.0% piperonyl butoxide; Whitmire Micro-Gen Research Laboratories Inc. St. Louis, MO) and left in a greenhouse for a day to assure no live nymphs or leafhoppers were still harbored by the plants. Plants were subsequently transferred to a greenhouse containing 400 W high-pressure sodium lights. The plants were maintained at a daytime temperature of 25±2°C and a nighttime temperature of 20±2°C. Plants were monitored daily for any hatched eggs or feeding nymphs and for symptom development. Once the symptoms were observed, within 20-45 days after the initiation of IAP, the infected plants were placed into clean rearing cages containing non-infected nymphs and maintained in the insectary for the AAP described above. The transmission cycle of phytoplasmas from infected China aster plants to the non-infected ones by the leafhoppers was repeated for each aster yellows phytoplasma strain.
Evaluation of aster yellows phytoplasma symptom types.

The aster yellows phytoplasma-infected China aster plants were maintained in a special greenhouse, after the successful transmission of different aster yellows phytoplasma strains (four plants for each cage contain one aster yellows phytoplasma strain type), at a daytime temperature of 25±2°C and a nighttime temperature of 20±2°C. China aster plants were monitored daily for strain-specific symptom development within 3-5 wks (after the initiation of IAP). Once the symptoms developed, another serial transmission cycle was initiated.

Total genomic DNA extraction from aster yellows infected and non-infected leafhoppers and plant tissues.

Total genomic DNA from ten individual laboratory-reared leafhoppers infected with each aster yellows phytoplasma strains (AY-WB, AY-S, AY-BD2 and AY-BD3), a total of 40 leafhoppers, was extracted using the Wizard SV96 DNA extraction kit (Promega Corp., Madison, WI). Total genomic DNA from ten individual laboratory-reared non-infected leafhoppers was extracted to serve as negative control. For the plant samples, total genomic DNA from 0.5 g leaf tissue of five individual China aster plants infected switch one of the aster yellows phytoplasma strains (AY-WB, AY-S, AY-BD2 and AY-BD3), a total of 20 plants, was extracted using DNeasy plant mini kit (Qiagen, Valencia, CA). In addition, total genomic DNA from 0.5 g leaf tissue from five non-infected China aster plants was extracted to serve as negative control. For both extraction protocols the manufacturer’s instructions were followed. DNA from leafhoppers and plant tissues was
diluted in TE buffer (TE; pH 7.4, 10mM Tris-HCl pH 7.4, 1mM EDTA) to 50 ng/µl and used as template for the uniplex and multiplex PCR assays.

**Development of aster yellows phytoplasma-strain specific multiplex PCR assays.**

Aster yellows-strain specific multiplex PCR assays were developed using DNA from the aster yellows phytoplasma reference strains AY-WB, AY-S, AY-BD2 and AY-BD3 isolated from leafhoppers and China aster plants, and with strain specific primer sets BF/BR, S1/S2, 15F/15R and 21F/21R (Table 2; Zhang *et al.* 2004). An optimal annealing temperature was established for each strain-specific primer pair using a temperature gradient-PCR protocol. A PCR master mix (1.25 µl of each primer (10 µM), 12.5 µl of GoTaq Green Master Mix (Go-Taq DNA polymerase at 100 units/ml, 400 µM dNTP and 3 mM MgCl₂ per reaction; Promega Corporation, Madison, WI), and 10 µl of sterile DNAase-free water) was prepared for each reference strain and each mixture was identical except for the primer pair used. Twenty-four microliters of the master mix was combined with 1 µl of the corresponding reference strain DNA (50 ng/µl) and the following program was used to amplify the specific gene sequence: 2 min at 95°C, 30 cycles of 30 sec at 94°C, 30 sec at a temperature range of 47-57°C (0.83°C intervals), 1 min at 72°C, and a 10 min final extension at 72°C. PCR products (10 µl) were separated by horizontal gel electrophoresis in 1.5% agarose in 1X TTE buffer (Tris-Triton-EDTA; 1M Tris-HCl (pH 8.0), 0.25M EDTA, Triton X-100) for 20 min at 130 V. Gels were stained in dilute GelRed™ (6X in water), and DNA was visualized under UV light and photographed using the Kodak Electrophoresis Documentation and Analysis System.
PCR assays were carried out in a PTC-200 thermocycler with a gradient alpha block (MJ Research Inc., Waltham, MA). The intensity of the amplicons for each temperature and PCR assay was observed and the optimal annealing temperature most common for all assays (55.2°C) was selected and used for the uniplex and multiplex PCR assays. Aster yellows phytoplasma strain-specific primer sets BF/BR, S1/S2, 15F/15R and 21F/21R were used in independent uniplex PCR reactions with aster yellows phytoplasma strain AY-WB-, AY-S-, AY-BD2- and AY-BD3-infected leafhoppers and aster plants (Table 2). A PCR master mix (as described above) was prepared for each leafhoppers and aster sample infected with each reference strain and each mixture was identical except for the primer pair used. Twenty-four microliters of the master mix was combined with 1 μl of the corresponding DNA samples from each leafhopper and aster infected with aster yellows strains (50 ng/μl) and the following program was used: 2 min at 95°C, 30 cycles of 30 sec at 94°C, 30 sec at a temperature range of 55.2°C, 1 min at 72°C, and a 10 min final extension at 72°C. PCR products (10 μl) were separated as described above and DNA was visualized and photographed. Aster yellows phytoplasma strain-specific primer sets were used in three combinations of primer sets: CPA1 (BF/BR and S1/S2); CPA2 (BF/BR and 15F/15R) and CPA3 (BF/BR, S1/S2, 15F/15R, 21F/21R); Table 3) for testing with aster yellows phytoplasma-infected leafhoppers and aster plants to ensure no multiplex assay produced two amplicons of the same size. Each PCR mixture contained each primer at 10μM and GoTaq Green Master Mix as described above. The volume of water for each assay was adjusted so that the final volume of reaction mixture was 25 μl. The following
amplification program was used for each assay: 2 min at 95°C, 30 cycles of 30 sec at 94°C, 30 sec at 55.2°C, 1 min at 72°C, and a 10 min final extension at 72°C in a PTC-100 thermocycler (MJ Research Inc., Waltham, MA) to find the minimal number of reactions that could distinguish all four strains of aster yellow phytoplasma. PCR products (10 μl) were separated by horizontal gel electrophoresis, stained and DNA visualized as described above. All the above experiments were repeated once.

**Results**

**Symptoms of aster yellows phytoplasma on China aster plants infected with strains of aster yellow phytoplasma.**

**AY-WB.** China aster plants began to show symptoms 2-3 weeks after inoculation with AY-WB-infected aster leafhoppers. Newly emerged leaves showed vein clearing, chlorosis (Figure 1), stunting, wilting and necrosis. At the late stage of infection, leaves start showing witches-broom symptoms (Figure 2) and the flowers became virescent.

**AY-S.** China aster plants began to show symptoms 7 days post-inoculation with AY-S-infected aster leafhoppers and were stunted during all growth stages. Newly emerged leaves were chlorotic and smaller than healthy leaves, and within 14 days leaves turned dark green (Figure 3). In the late stage of infection, the flowers were green and had a leaf-like morphology (phyllody) (Figure 4).

**AY-BD2.** China aster plants began to show symptoms 7-14 days post-inoculation with AY-BD2-infected aster leafhoppers and developed symptoms similar to plants infected
with AY-WB. Newly emerged leaves and stems were distorted and showed vein clearing and chlorosis. After 21 days of infection, the leaves were clustered and chlorotic (Figure 5).

**AY-BD3.** AY-BD3 symptoms were difficult to distinguish from AY-BD2 symptoms. Twenty-one days post-inoculation with AY-BD3, newly emerged leaves showed vein clearing and chlorosis (Figure 6) and in the late stages of infection, the aster plants were stunted.

**Development of aster yellows phytoplasma-strain specific multiplex PCR assay.**

Temperature gradient-PCR using aster yellows phytoplasma-strain specific primers BF/BR, S1/S2, 15F/15R and 21F/21R (Table 2) and DNA from the corresponding strains (AY-WB, AY-S, AY-BD2 and AY-BD3) produced the expected size amplicon for each annealing temperature in the assay (Figure 7B and 8A, B), with the exception of the assay using BF/BR primers at the 47.9°C annealing temperature (Figure 7A). However, a PCR product was amplified at all the other temperatures above and below 47.9°C. Although bands were visible at every annealing temperature up to the 57°C threshold, sensitivity decreased once the temperature exceeded 55.5°C for PCR assays with primer pairs BF/BR (Figure 7B) and 21F/21R (Figure 8B). Therefore, an annealing temperature of 55.5°C was selected and subsequently used in the multiplex PCR assays.

In the uniplex PCR reaction (Table 4) with primer set BF/BR, only one 900-bp amplicon was generated with strain AY-WB, and none with the other aster yellows
strains. Primer set S1/S2 generated one 490-bp amplicon with AY-WB and AY-S strains and no amplicons were generated from AY-BD2 and AY-BD3 (Figure 9A, 9B). PCR with primer set 15F/15R generated one 390-bp amplicon, whereas with 21F/21R one 700-bp amplicon was amplified when tested with AY-BD2 (Figure 8) and AY-BD3 (data not shown). No amplicons were generated from AY-WB or AY-S using these primers (data not shown).

The multiplex PCR reaction (Table 4) CPA1 (BF/BR and S1/S2) generated 490-bp and 900-bp amplicons from AY-WB and one 490-bp amplicon from AY-S, but none from AY-BD2 or AY-BD3 (Figures 9 and 10). CPA2 (BF/Br and 15F/15R) generated 390-bp and 900-bp amplicons from AY-WB and one 390-bp amplicon from AY-BD2 and AY-BD3 (Table 5). No amplicons were generated from AY-S.

When CPA1 and CPA2 were run in conjunction, three of the four strains were distinguished (AY-WB, AY-S and AY-BD2) (Figure 11). AY-BD2 and AY-BD3 could not be distinguished from one another by any PCR assay in this study. CPA3 multiplex PCR, which utilized four primer sets, did not amplify any PCR products under the conditions used in this study (data not shown). No amplicons were generated in any of these assays when DNA from non-infected leafhoppers and non-infected China aster plants was used as template.
Discussion

The four strains of aster yellows phytoplasma (AY-WB, AY-S, AY-BD2, and AY-BD3) used in this study were isolated from lettuce fields in Celeryville, Ohio. Since these aster yellows phytoplasma strains are closely related, they produce similar symptoms. Using these symptoms as tools to differentiate between different aster yellows phytoplasma strains requires comparing the infected plants at the same stage of infection and then comparing several stages of infection in each plant to monitor the development of aster yellows disease. Further, mixed infections of two or more phytoplasma strains have been reported in different vegetables, fruits and trees (Errampalli et al. 1986; Errampalli and Fletcher 1991; Lee et al. 1993, 1994 and 1995), which make it very difficult to differentiate between strains based on host symptoms. PCR assays are now the method of choice for phytoplasma detection and diagnosis, but using PCR assays with universal primers can only confirm the presence of the phytoplasma, not classify it within any group or subgroup. Thus, Zhang et al. (2004) developed strain-specific primers including the primer set BF/BR, which was developed from an aster yellows phytoplasma strain AY-WB genomic DNA clone. Primer sets S1/S2, 15F/15R and 21F/21R were developed based on the sequence variation between PCR products of aster yellows phytoplasma strains amplified by primer pair AY19p and AY19m (Schaff et al. 1992). These strain-specific primer sets were used in the development of the multiplex PCR assays in this study. Multiplex PCR assays developed in this study made it possible to detect and differentiate three of the four aster yellows
phytoplasma strains previously observed in Ohio lettuce fields, without the need to wait for symptom development or to run multiple uniplex PCR assays.

Initially, the individual primer sets were amplified in uniplex reactions by gradient PCR to optimize their annealing temperature in order to have strong amplification of PCR amplicon(s) and reduce any non-specific amplification. Although DNA of aster yellows phytoplasma strains was amplified using strain-specific primers at 57°C in a previous study (Zhang et al. 2004), our experiment showed that lowering the annealing temperature to 55.5°C was critical for these strains to be re-amplified in multiplex mixtures and to produce highly reliable amplification products.

Developing reliable multiplex PCR assays that target sequences of more than one aster yellows phytoplasma reference strain can be challenging, but we utilized previously developed primers (Zhang et al. 2004) that distinguish between those strains in multiple uniplex reactions. When BF/BR (detects AY-WB) and S1/S2 (detects AY-WB and AY-S) primer sets were pooled in the multiplex PCR assay CPA1, they generated two amplicons from AY-WB (490 and 900-bp), one amplicon from AY-S (490-bp) and none for AY-BD2 and AY-BD3. When 15F/15R (detects AY-BD2 and AY-BD3) was combined with BF/BR in the multiplex PCR assay CPA2, two amplicons from AY-WB (390 and 900-bp) and one amplicon from AY-BD2 and AY-BD3 (390-bp) were generated. The CPA2 multiplex assay detected AY-BD2 and AY-BD3 but did not distinguish them from one another. Strains AY-BD2 and AY-BD3 are very similar,
causing nearly identical symptoms in lettuce and China aster, and could not be
distinguished with the primers used in this study. However, Zhang et al. (2004)
differentiated these two strains by PCR with primers (S10F/S10R) developed from AY-S
genomic DNA with high sequence identity to an AL1-like protein gene. However, this
primer set reacted with AY-S and other strains in this study and therefore was not
selected for use in multiplex PCR development. Quadriplex PCR assays, which utilized
S1/S2, BF/BR, 15F/15R and 21F/21R primers, failed to generate any amplicons of the
band sizes observed in the uniplex reactions for the same DNA extracts from aster
yellows phytoplasma strains. Failure to generate visible amplicons from the quadriplex
PCR assays could be the result of the limited size range of PCR amplicons (Chen et al.
2005) or due to several compatible primers (primer-primer interactions) (Clair et al. 2003
and Lopez et al. 2009).

These multiplex PCR assays can be convenient in the diagnostic lab to distinguish
between different aster yellows phytoplasma strains during routine diagnosis of leafy
green samples. Both CPA1 and CPA2 assays were used to test for the presence of aster
yellows phytoplasma strains in individual aster leafhoppers, captured flying in Ohio
fields of lettuce, cilantro and parsley and from leaves and roots of these crops (Chapter
3).
<table>
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<th>Strain and Subgroup affiliation</th>
<th>Symptoms on Lettuce</th>
<th>Symptoms on Aster</th>
<th>Year of collection</th>
</tr>
</thead>
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<tr>
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<td>Vein clearing</td>
<td>Vein clearing</td>
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<tr>
<td>Aster yellows severe (AY-S)(^1) 16SrI-B</td>
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<td>1996</td>
</tr>
<tr>
<td>Aster yellows bolt distortion No.2 (AY-BD2)(^2) 16SrI-B</td>
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<td>2003</td>
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<tr>
<td>Aster yellows bolt distortion No.3 (AY-BD3)(^2) 16SrI-B</td>
<td>Bolting</td>
<td>Vein clearing</td>
<td>2003</td>
</tr>
</tbody>
</table>

\(^{1,2}\) Aster yellows phytoplasma strains were described previously by \(^1\)Murral \textit{et al.} 1996 and \(^2\)Zhang \textit{et al.} 2003.

**Table 1.** Aster yellows phytoplasma strains collected from lettuce in Ohio and maintained in China aster plants.
Table 2. Sequences of the oligonucleotide primer sets developed from rRNA operon of aster yellows phytoplasma reference strains by Zhang *et al.* (2004) for detection of aster yellows phytoplasma (AY) strains in uniplex PCR assays. AY-WB = aster yellows witches’-broom; AY-S = Aster yellows severe; AY-BD2 and AY-BD3 = aster yellows bolt-distortion; AY-BW = aster yellows bolt white.

<table>
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<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Tm (°C)</th>
<th>G+C %</th>
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<th>Strain specificity</th>
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<tr>
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</tr>
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<td>26.9</td>
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<tr>
<td>21R</td>
<td>TGATGGTGGTTCTATGTAGC</td>
<td>48.5</td>
<td>38.1</td>
<td>700</td>
<td>AY-BD2, AY-BD3</td>
</tr>
<tr>
<td>Primer combination</td>
<td>Strains to be identified</td>
<td>Amplicon size</td>
<td>Multiplex PCR assay</td>
<td></td>
<td></td>
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<tr>
<td>--------------------</td>
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<td>---------------</td>
<td>---------------------</td>
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<td></td>
</tr>
<tr>
<td>BF/BR+S1/S2</td>
<td>AY-WB</td>
<td>490 and 900 bp</td>
<td>CPA1</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>AY-S</td>
<td>490 bp</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>AY-BD2, AY-BD3</td>
<td>No amplicon</td>
<td></td>
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<tr>
<td>BF/BR+15F/15R</td>
<td>AY-WB</td>
<td>390 and 900 bp</td>
<td>CPA2</td>
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<tr>
<td></td>
<td>AY-S</td>
<td>No amplicon</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>AY-BD2, AY-BD3</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>BF/BR+S1/S2+15F/15R</td>
<td>AY-WB</td>
<td>390, 490 and 900 bp</td>
<td>CPA3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AY-S</td>
<td>490 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AY-BD2, AY-BD3</td>
<td>390 and 519 bp</td>
<td></td>
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**Table 3.** Specificity of aster yellows strain-specific primer sets (Zhang *et al.* 2004); expected amplicons sizes and primer combinations used in multiplex PCR for detection and differentiation of aster yellows strains.
Table 4. Differentiation of aster yellows phytoplasma strains by uniplex and multiplex PCR. Template DNA was from leafhoppers infected with one of four phytoplasma strains. DNA isolated from non-infected leafhoppers was used as a negative control. AY-WB = aster yellows witches’-broom; AY-S = Aster yellows severe; AY-BD2 and AY-BD3 = aster yellows bolt-distortion; AY-BW = aster yellows bolt white.
Table 5. Primers, primer sequences and number and size of amplicons produced in two multiplex PCR assays to differentiate strains of aster yellows phytoplasma.

<table>
<thead>
<tr>
<th>Multiplex PCR assay</th>
<th>Primer Identification</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Number of amplicons produced</th>
<th>PCR amplicon sizes (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA1</td>
<td>BF</td>
<td>AGG ATG GAA CCC TTC AAT GTC</td>
<td>2</td>
<td>490 and 900</td>
</tr>
<tr>
<td></td>
<td>BR</td>
<td>GGA AGT CGC CTA CAA AAA TCC</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>S1</td>
<td>CGC TAA CAA ATG TAA AGG CAA G</td>
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</tr>
<tr>
<td></td>
<td>S2</td>
<td>CTT TAA TAG GAC TAT GAG GG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPA2</td>
<td>BF</td>
<td>AGG ATG GAA CCC TTC AAT GTC</td>
<td>2</td>
<td>390 and 900</td>
</tr>
<tr>
<td></td>
<td>BR</td>
<td>GGA AGT CGC CTA CAA AAA TCC</td>
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</tr>
<tr>
<td></td>
<td>15F</td>
<td>CCC TCA AAC CCA CGA AGT T</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15R</td>
<td>TAC TGT GTT CCC TTA CTC C</td>
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</tbody>
</table>

1 Primers previously described by Zhang et al. (2004).
Figure 1. China aster plant showing vein clearing and yellowing symptoms caused by aster yellows phytoplasma strain AY-WB.

Figure 2. China aster plant showing witches'-broom symptom caused by aster yellows phytoplasma strain AY-WB.
Figure 3. China aster plant showing chlorotic (A) turning to dark green leaves (B) caused by aster yellows phytoplasma strain AY-S.

Figure 4. China aster plant showing the flower phyllody symptom caused by aster yellows phytoplasma strain AY-S.
Figure 5. Infected China aster plant showing vein clearing (A) and chlorosis (B) symptoms caused by aster yellows phytoplasma strain AY-BD2.
Figure 6. China aster showing vein clearing and yellowing symptoms caused by aster yellows phytoplasma strain AY-BD3.
Figure 7. Gradient polymerase chain reaction (PCR) amplification of aster yellows phytoplasma strain AY-WB using primer pairs: (A) BF/BR; and (B) S1/S2. Lane M, 1-Kb plus ladder; Lane 1, 47°C; 2, 47.3°C; 3, 47.9°C; 4, 48.7°C; 5, 49.8°C; 6, 51.3°C; 7, 53°C; 8, 54.4°C; 9, 55.5°C; 10, 56.3°C; 11, 56.8°C; 12, 57°C.
Figure 8. Gradient polymerase chain reaction (PCR) amplification of aster yellows phytoplasma strain AY-BD2 using primer pairs: (A) 15F/15R; and (B) 21F/21R. Lane M, 1Kb plus ladder; Lane 1, 47°C; 2, 47.3°C; 3, 47.9°C; 4, 48.7°C; 5, 49.8°C; 6, 51.3°C; 7, 53°C; 8
Figure 9. Polymerase chain reaction (PCR) amplification of aster yellows phytoplasma strains (AY-WB, AY-S, AY-BD2 and AY-BD3) with (A): BF/BR primer pair; (B): S1/S2 primer pair; and (C): multiplex PCR using primer pairs BF/BR and S1/S2 (CPA1). Lane M, 1-Kb plus ladder (Invitrogen Crop., Carlsbad, CA); lane 1, AY-S; lane 2, AY-WB; lane 3, AY-BD2; lane 4, AY-BD3; lane 5, AY-S + AY-WB; and lane 6, non-infected leafhopper as a negative control.
Figure 10. Multiplex PCR amplification of aster yellows phytoplasma strains using primer pairs: (A): BF/BR, S1/S2 (CPA1); and (B): BF/BR, 15F/15R (CPA2). Lane M, 1-Kb plus ladder (Invitrogen Corp., Carlsbad, CA); lane 1, AY-S; lane 2, AY-WB; lane 3, AY-BD2; lane 4, AY-BD3; and lane 5, non-infected leafhopper.
Figure 11. Flow chart of development of two multiplex PCR assays, A: CPA1 (differentiates AY-S and AY-WB); and B: CPA2 (differentiates AY-BD2/BD3 and AY-WB).
CHAPTER 3

Diversity of Aster Yellows Phytoplasma Strains in Intensive Lettuce Production Areas in Ohio

Introduction

Lettuce fields in Ohio can be significantly affected by aster yellows disease, but disease incidence varies from year to year. In some years, disease incidence can reach 100% in individual fields and cause major economic losses (Hoy et al. 1992). The cause of aster yellows disease is a phytoplasma (Ca. Phytoplasma asteris) that affects more than 400 host plant species. Aster yellows phytoplasmas induce symptoms in their plant hosts, including yellowing of leaves, witches’-broom (excessive proliferation of shoots), stunting, and phyllody, among others (Lee and Davis 1992; Lee et al. 2000; Zhang et al. 2004; and Hogenhout et al. 2008). They are dependent on phloem-feeding aster leafhoppers (Macrosteles quadrilineatus, Forbes) as the main vector for disease transmission. Once the leafhoppers transmit phytoplasmas to a host plant, aster yellows symptoms may appear within 2-3 weeks. Some host plants may become infected with phytoplasmas and remain asymptomatic, and serve as reservoirs for the phytoplasma (Blomquist and Kirkpatrick 2002; Gatineau et al. 2001; Marzachi et al. 1998; Bertaccini 2007; Maramorosch and Harris 1979; McClure 1982 and Kawakita et al. 2000).
The distribution of aster yellows phytoplasma in Ohio depends on many factors, including host plant range, which is determined in large part by aster leafhopper feeding behavior. The aster leafhopper is a polyphagous vector, which can transmit aster yellows phytoplasma to different vegetable crops including but not limited to lettuce, carrot, onion, celery, tomato, potato, spinach, cauliflower, broccoli, cabbage and dill (Swenson 1971; McCoy et al. 1989 and Weintraub and Beanland 2006). In a recent study, Bai et al. (2009) showed that aster yellows phytoplasma produced effector proteins in infected host plants that changed the plant’s gene expression to increase the attractiveness of young shoots and leaves to the aster leafhopper compared to the non-infected plants, contributing to increased leafhopper feeding and oviposition (Peterson 1973 and Schultz 1973). Furthermore, aster yellows phytoplasma-infected leafhoppers showed improvement in overwintering ability and an increase in fecundity and longevity. Beanland et al. (2000) observed that aster yellows phytoplasma-infected leafhoppers laid twice the amount of eggs on host plants as non-infected leafhoppers.

Infected leafhoppers migrating from southern states are considered to be the main source of aster yellows in vegetables in the Midwestern United States (Hoy et al. 1992). However, there were some reports that aster leafhoppers overwinter in the egg stage in Minnesota (Meade and Peterson 1964) and Wisconsin (Chiykowski and Chapman 1965). However, there was no evidence of aster leafhoppers overwintering as adults or eggs in Ohio (Teraguchi 1986).
Once they arrive in lettuce fields, leafhopper movement depends on the distribution of lettuce and other host crops over the growing season. For example, infected leafhoppers move to a lettuce field, and feed on and inoculate the lettuce plants. They eventually fly to another nearby lettuce field and continue the cycle of inoculation (Wallis 1962; Meade and Peterson 1964; Drake and Chapman 1965; Chiykowski and Chapman 1965; Nichiporick 1965 and Hoy et al. 1992). A clustered pattern of aster yellows-infected lettuce plants caused by different aster yellows phytoplasma strains transmitted by aster leafhoppers has been documented (Errampalli et al. 1991; Madden et al. 1995; Mural et al. 1996 and Beanland et al. 2005). Aster yellows phytoplasma strains carried by infected leafhoppers distribute differently within and between lettuce plants in the field, which affects their transmission (Madden et al. 1995) and the diversity of the symptoms they express (Mural et al. 1996 and Errampalli and Fletcher 1991). For example, Beanland et al. (2000) showed that leafhoppers infected with aster yellows phytoplasma strain AY-WB produced significantly more nymphs and had more fitness than those infected with strain AY-S. Leafhoppers infected with AY-WB were better adapted to the aster yellows pathogen as documented by higher fecundity and longevity, suggesting that the strain AY-WB could become more common in the lettuce fields than the AY-S strain.

In the past, aster yellows phytoplasma detection relied on biological properties such as symptoms, host range, and the relationship between the phytoplasma and its leafhopper vector (Davis et al. 1990; Chen et al. 1992; Daire et al. 1992; Davies et al. 1996).
1995 and Davis and Lee 1993). Currently, aster yellows phytoplasma detection and classification rely on molecular techniques. Direct and nested PCR, using universal primers P1/P7 and F2n/R2, are used extensively for the detection of the aster yellows phytoplasmas both from symptomatic and asymptomatic plants and from aster leafhoppers. Also, specific primers that distinguish among aster yellows phytoplasma strains have been developed from different positions on the ribosomal RNA operon, such as the 16SrRNA gene and the variable 16S-23S intergenic region (Smart et al. 1996; Schneider et al. 1993; Gundersen et al. 1996; Lorenz et al. 1995; Rhode et al. 1993; Davis and Lee 1993; Deng and Hiruki 1991; Ahrens et al. 1994; Kirkpatrick et al. 1994; Lee et al. 1994; 1995; 2005; 2006; Griffiths et al. 1999; Zhang et al. 1998; Schneider et al. 1997 and Zhang et al. 2004). Sequences of the aster yellows phytoplasma 16SrRNA gene provide sufficient information for aster yellows phytoplasma classification to the strain level. There are at least 14 subgroups with different strains distributed worldwide in the aster yellows phytoplasma group. Although these different strains have high similarity in 16SrRNA sequences between them, they represent the genetic diversity of the aster yellows group in different ecological niches (Gundersen et al. 1994; Seemüller et al. 1994, 1998b; Vibio et al. 1996; Lee et al. 1998, 2000, 2002; Marcone et al. 2000 and Jomantiene et al. 2002).

In Ohio, five different strains of aster yellows phytoplasma were identified previously in lettuce fields, based on their symptoms and RFLP patterns: aster yellows Witches’-Broom (AY-WB) (16SrI-A) and aster yellows Severe (AY-S) (16SrI-B) (D’Arcy and
Nault 1982; Hoy et al. 1992 and Murral et al. 1994), aster yellows Bolt White (AY-BW) (16SrI-B), aster yellows Bolt Distortion no. 2 (AY-BD2) (16SrI-B), and aster yellows Bolt Distortion no. 3 (AY-BD3) (16SrI-B) (Zhang et al. 2004). Initially, the five aster yellows phytoplasma strains were identified and characterized based on their symptoms in both lettuce and China aster plants (Zhang et al. 2004). Direct and nested PCR using universal primer sets P1/P7 and F2n/R2 were used to identify these isolates as phytoplasmas. Next, strain-specific primers were developed to distinguish among these strains; i.e. AY-WB can be distinguished from the other strains by using the primer pair BF/BR. AY-WB and AY-S can be distinguished from the other strains by using the primer pair S1/S2. AY-WB, AY-S, AY-BD2, AY-BD3 and AY-BW were detected successfully using multiple single reaction PCR assays. In Chapter 2 of this study, multiplex PCR assays were developed to distinguish between the aster yellows phytoplasma reference strains using previously developed primers. These rapid assays allowed us to screen a large number of leafhopper and plant samples at different times from multiple locations and identify aster yellows strains quickly and economically.

Other factors that may affect the distribution of aster yellows phytoplasma strains in Ohio vegetable fields throughout the growing season may include host plant preference by the leafhoppers, time of sampling and variation in transmission rate of different aster yellows phytoplasma strains. Herbaceous crops including cilantro and parsley, which are widely grown in the lettuce production areas included in this study, do not commonly exhibit aster yellows disease symptoms, but may serve as alternative host plants
(Khadhair et al. 1998; Zhou et al. 2003 and Lebsky and Poghosyan 2007). However, their role in the distribution of aster yellows strains in lettuce and other economically important vegetable crops is unknown. While several aster yellows phytoplasma strains have been identified in lettuce, it is likely that others are present in leafhoppers and vegetable crops. The aims of this study were to document the geographic and temporal distribution of known strains of aster yellows phytoplasma in lettuce, cilantro and parsley, and identify new isolates that may contribute to the aster yellows phytoplasma strain complex.

**Materials and Methods**

**Rearing of healthy aster leafhoppers.**

The rearing of healthy aster leafhoppers was as described in Chapter 2. Fifty leafhoppers were randomly selected from each rearing cage and tested in groups of five by nested PCR using aster yellows phytoplasma universal primers P1/P7 (Deng and Hiruki 1991 and Smart et al. 1996) followed by F2n/R2 (Lee et al. 1992) every 3 months to insure they were not contaminated with phytoplasmas (Table 6).

**Maintenance of aster yellows reference strains.**

The maintenance of the aster yellows phytoplasma strains was as previously described in Chapter 2.
Leafhopper and plant sample collection.

From June to September 2008 and 2009, aster leafhoppers were collected in lettuce production areas near Celeryville and Hartville, OH from commercial fields planted to red leaf lettuce, green leaf lettuce, romaine lettuce, cilantro or parsley. At least 50 leafhoppers were sampled from the fields within each crop (depending on availability) following a zigzag pattern with a vacuum leafhopper net comprised of a model BG 72 leaf blower with a fine-mesh leafhopper net bag taped to the end of the intake nozzle to capture the leafhoppers (Stihl Inc., Virginia Beach, VA) (Zhou et al. 2003). Geographic coordinates of each field were recorded (Table 7) using a hand-held global positioning system (GPS). Leafhoppers trapped in the nets from each sampling unit were transferred to individual 3.78L double locked plastic bag (Ziploc® Brand freezer bags with smart zip™ seal, S.C. Johnson and Son, Inc., Racine, WI) and stored on ice up to 7 hrs prior to processing. Sampled leafhoppers were frozen and aster leafhoppers were identified and separated from other leafhopper species and packed individually (1 leafhopper per tube) into 8-well tube strips (Fisher Scientific, Pittsburgh, PA) labeled with the location, date of collection, and the host plant. Individual leafhoppers were stored at -20°C prior to DNA extraction.

Samples of lettuce, cilantro and parsley plants were collected in 2009. Plant samples were from the same commercial fields where leafhopper samples were collected, with a total of eight collections beginning in June and continuing through September 2009. Five plants were selected from each field along a zigzag pattern and removed from the soil by
hand. Plants with aster yellows symptoms were preferentially selected when present. If no symptomatic plants were observed, samples were selected at random. Samples were placed in individual 3.78L double locked plastic bags and stored on ice during the collection. The collection date, host plant type, and transect point GPS coordinates were recorded. Roots and leaves from each sample were selected, rinsed with water to remove residual soil, placed into clean plastic bags, and stored at -20°C prior to DNA extraction.

**Total genomic DNA extraction from leafhopper and plant tissue.**

Total genomic DNA from individual field-collected and laboratory-reared leafhoppers (infected and non-infected) was extracted using the Wizard SV96 DNA extraction kit (Promega Corp., Madison, WI). For the plant samples, five upper leaves (8-10 cm in length) and five lateral roots (~2-3 cm in length) from lettuce and five upper leaves (4-6 cm in length) and five lateral roots (~2-3 cm in length) from cilantro and parsley were frozen in liquid nitrogen and ground in a sterilized mortar and pestle. DNA was extracted from a 200 mg sample of this tissue using DNeasy plant mini kit (Qiagen, Valencia, CA). For both extraction protocols the manufacturer’s instructions were followed. DNA from leafhopper and plant tissue was diluted in Tris-EDTA (TE; pH 7.4, 10 mM) to 50 ng/µl and used as template for PCR.

**Optimization of the sample size required for initial screening of leafhopper and plant samples.**

DNA extracts from individual AY-WB-infected leafhoppers were previously pooled in groups of five and used as template for PCR (Zhou et al. 2003). The feasibility of
increasing the leafhopper pool to eight individuals was tested to accommodate the design of the Wizard SV96DNA and DNeasy plant mini extraction kits. DNA from eight individual AY-WB-infected leafhoppers and eight individual non-infected leafhoppers reared in the laboratory was extracted. One µl of DNA from each AY-WB-infected leafhopper extract was pooled to be used as positive control (8AY) for the experiment (a total of 8 µl) and 1 µl of DNA from each non-infected leafhoppers were pooled to be used as negative control (8N) for the experiment (a total of 8 µl). Also, 1 µl of DNA from seven AY-WB-infected leafhoppers were pooled (7AY) with 1 µl of DNA from a non-infected leafhopper (1N). Different ratios of DNA from AY-WB-infected leafhoppers and non-infected leafhoppers were pooled (6AY:2N; 5AY:3N; 4AY:4N; 3AY:5N; 2AY:6N; 1AY:7N) to be tested for the optimum sample size. One µl aliquot of DNA extract from each pooled sample was used in PCR for each combination tested (Figure 12).

For plant samples, DNA from 200 mg root tissue from eight individual AY-WB-infected aster plants and eight individual non-infected aster plants was extracted. One µl of DNA from each AY-WB-infected aster plant was pooled to be used as positive control (8AY) for the experiment (a total of 8 µl) and 1 µl of DNA from each non-infected aster plants was pooled to be used as negative control (8N) for the experiment (a total of 8 µl). Also, 1 µl of DNA from seven AY-WB-infected aster plants were pooled together (7AY) with 1 µl of DNA from non-infected aster plants (1N). Different ratios of DNA from AY-WB-infected aster plants and non-infected aster plants were pooled together
(6AY:2N; 5AY:3N; 4AY:4N; 3AY:5N; 2AY:6N; 1AY:7N) to be tested for the optimum sample size. A 1μl aliquot of DNA extract from each pooled sample was used in PCR for each combination tested. DNA samples were tested by nested PCR assay; the PCR mixture and program are described below. This experiment was repeated one time.

For both leafhoppers (Figure 12) and plant extracts (data not shown), the PCR assay was sufficiently sensitive to detect one aster yellows phytoplasma-infected leafhopper or plant sample extract in a total of eight pooled extracts. Therefore pools of eight DNA extracts from individual leafhoppers or plants were used to identify positive samples. The individual samples that comprised the phytoplasma-positive pooled samples were then tested individually as described below.

Detection of phytoplasmas in leafhopper and plant samples.

Phytoplasma direct PCR.

PCR was performed using the universal phytoplasma primer pair P1/P7 (Deng and Hiruki 1991 and Smart et al. 1996) (Table 6). Each PCR reaction mixture contained 1 μl of the pooled DNA diluted to 50 ng/μl, and 24 μl of master mix (1.25 μl of each 10 μM primer, 12.5 μl of GoTaq Green Master Mix (Go-Taq DNA polymerase at 100 units/ml, 400 μM dNTP, and 3 mM MgCl2; Promega Corporation, Madison, WI) and 10 μl sterile distilled water. The following program was used to amplify the DNA: 1 min at 94°C, 30 cycles of 2 min at 94°C, 1 min at 55°C, 2 min at 72°C, and a 10-min final extension at 72°C. All PCR assays described in this study were carried out in a PTC-100
thermocycler (MJ Research Inc., Waltham, MA). The PCR product was stored at -20°C and used as template for the nested PCR.

**Phytoplasma nested PCR.**

Nested PCR was performed on the PCR products obtained above using the universal PCR primer pair F2n/R2 (Lee *et al.* 1992) (Table 6). PCR reactions were prepared as described above except 1 μl of the PCR product from the direct PCR was used as a template, the annealing temperature was 50°C for 2 min and the extension time was 3 min. PCR products (10 μl) were separated by horizontal gel electrophoresis in 1.5% agarose in 1X Tris-Triton-EDTA (TTE, 1M Tris-HCl (pH 8.0), 0.25 M EDTA, Triton X-100) buffer for 20 min at 130 V. Gels were stained in dilute GelRed™ (3X in H₂O), and PCR products were visualized under UV light and photographed using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Eastman Kodak Company, New Haven, CT). From each pooled sample that tested positive with phytoplasma universal primer sets, the eight individual samples that comprised each pooled sample were recovered from storage and tested by nested PCR using the phytoplasma universal primer pairs as described above.

**Detection and identification of aster yellows phytoplasma strains using multiplex PCR.**

Individual leafhopper and plant samples that tested positive for aster yellows were further evaluated for strain identity using multiplex PCR with CPA1 and CPA2 primer
complexes (Chapter 2). Leafhopper and plant samples that tested negative with CPA1 and CPA2 were re-tested using AY-BW specific PCR primers BWF/BWR (Zhang et al. 2004). The reaction mixture was as described above for the aster yellows phytoplasma-specific PCR and the program was as follows: 2 min at 94°C, 30 cycles of 30 s at 94°C, 40 s at 55°C, and 1 min at 72°C, and then a 10-min final extension at 72°C. PCR products were separated and visualized as described above.

**Statistical analyses.**

Statistical analyses were performed using linear mixed models in SAS software (SAS Institute, Cary, NC) to determine the effect of year, location, host and sampling time on the distribution of the aster yellows phytoplasma strains found in vegetable production areas in Ohio. Because the number of infected samples (from host plants and leafhoppers) was not always the same, the proportion of the infected samples was calculated as follows: number of the infected samples for each strain/total number of the infected samples tested. All factors including host plant (red leaf, green leaf, romaine lettuce, cilantro and parsley) and sampling time (June, July, August and September) were considered fixed effects in the analysis. Preliminary analysis showed that there were no significant differences between the two locations and the two years, so year and location were combined and treated as a blocking factor (Loc_Year) and considered random effects in the analysis. For the host plant and the sampling time, mean comparisons were performed using Proc mixed and mean separation by letters was performed using the macro PDMIX800.sas (Saxton 1998).
Phylogenetic analysis of unidentified phytoplasma isolates.

DNA extracts from individual plant and leafhopper samples that were phytoplasma-positive by nested PCR but negative with the aster yellows phytoplasma strain-specific primers were amplified by PCR using the universal primers P1/P7. P1/P7 PCR products were purified using the Wizard SV gel and PCR purification system (Promega, Madison, WI). The purified PCR products (40 ng/μl) and the corresponding primers P1/P7 (2 pmol/μl) were sent to the Plant-Microbe Genomic Facility (The Ohio State University, Columbus, OH) for sequencing using an automated 3730 DNA Analyzer (Applied Biosystems, Inc.). Each amplicon was sequenced from both directions. The raw sequence chromatograms were trimmed and edited, and consensus sequences were generated using Sequencher (Sequencher 3.0; Gene Codes Corporation, Ann Arbor, Michigan). The trim criteria for both ends of the sequences were set to remove no more than 25% until the last 15 bases contained less than three bases with a confidence level less than 20. Based on the trim criteria, the unreliable sequences were excluded from the analyses. Partial sequences of 16SrRNA (1136 bp) from aster yellows phytoplasma isolates detected in plants and aster leafhoppers were aligned using ClustalW algorithm (Higgins et al. 1994) in Mac Vector (Mac vector 6.1; Oxford Molecular Ltd., Beaverton, OR). The aligned sequences were compared with representative aster yellows phytoplasma strains selected from the Genbank database. Phylogenetic analyses were conducted by MEGA version 4 (Tamura et al. 2007) using the neighbor-joining method with 1000 replicates for bootstrap analysis. Reference aster yellows phytoplasma strains
from subgroup 16SrI-A: 99UW11 (Lee et al. 2002), ca2006/1 (Duduk et al. 2009), AY-WB (Zhang et al. 2004) and ParsD3 (Lee et al. 2003) and from subgroup 16SrI-B: AY-S, AY-BW, AY-BD2 (Zhang et al. 2004), SAY (Kuske and Kirkpatrick 1992), and ParsD1 (Lee et al. 2003) were included in the phylogenetic analysis (Table 16). The corresponding sequence of tomato stolbur phytoplasma (Martini et al. 2007) was used to root the phylogenetic tree.

Results

Leafhopper populations during the 2008-2009 growing seasons.

During the summer of 2008, 1326 and 1174 aster leafhoppers were collected from red leaf, green leaf and romaine lettuce, cilantro and parsley in Celeryville and Hartville, OH respectively. In 2009, 1688 and 1638 aster leafhoppers were collected from the same crops at the two locations. In 2008, at both locations, more than 75% of the leafhoppers obtained were collected during the months of July and August. Similar results were observed in 2009, with more than 80% of the leafhoppers collected during the months of July and August at both locations (Figure 13 and Tables 8 and 9).

There were significant differences in both locations during 2008 and 2009 in the total number of leafhoppers collected among plant hosts (p = 0.0006) and between the sampling dates (p < 0.0001). In addition, there was a significant effect of plant host and the sampling time (p = 0.0057) on the number of leafhoppers collected. The highest
number of the leafhoppers was found in romaine lettuce during the month of August (Table 10).

**Symptoms of aster yellows in plants.**

In 2009, 818 and 948 plants (lettuce, cilantro and parsley) samples were collected from fields in Celeryville and Hartville, respectively. Cilantro and parsley plant samples were asymptomatic for aster yellows. Early symptoms of aster yellows disease on lettuce included small, yellow, narrow, distorted leaves. By the end of the growing period, all the leaves of infected lettuce plants were yellow and the plants were wilted and stunted (Figure 14 and 15).

**Aster yellows phytoplasma infection in aster leafhoppers.**

In 2008, 735 and 525 leafhoppers from Celeryville and Hartville, respectively, were tested for phytoplasma detection by nested PCR. In 2009, 1688 and 1638 leafhoppers from Celeryville and Hartville respectively, were tested. In total, 65.9% and 75.2% of the leafhoppers from Celeryville and Hartville, respectively, were infected with phytoplasma in 2008, and 63.9% and 41.3% were positive from Celeryville and Hartville, respectively, in 2009. There were no significant differences in the proportion of phytoplasma-infected leafhoppers collected among the crops tested (p ≥ 0.59). However, the proportion was significantly higher in June and July than in August and September (p = 0.0006) (Tables 10 and 11).
Aster yellows phytoplasma infection in lettuce, parsley and cilantro plants.

In 2009, 818 and 948 plants, from Celeryville and Hartville respectively, were tested for phytoplasma infection by nested PCR. Twenty one percent of the tested plants from Celeryville were infected with phytoplasma and the number was twice that in plants from Hartville. Although there were no significant differences in the proportion of infected plants between the three lettuce types, cilantro or parsley (p ≥ 0.76), infected plants were more abundant in June, July and August than in September (p ≤ 0.01) (Tables 10 and 12).

Aster yellows phytoplasma strain distribution in leafhoppers and plants.

In 2008, the percentage of the leafhoppers infected with aster yellows phytoplasma strain AY-WB (16SrI-A) was similar to the percentage infected with strain AY-BD2 (16SrI-B) at both locations (approx. 22%; Table 13). Very few leafhoppers infected with strain AY-S (16SrI-B) were found in Celeryville, and none were detected in Hartville (Table 13). Strain AY-BW (16SrI-B) was regularly found in leafhoppers at both locations (39.7-46.6%), as was strain AY-BD2 (16SrI-B) (23.8-25.3%) (Table 13). In 2009, strain AY-WB was abundant in Celeryville (47.1%) but not in Hartville (17.6%), while the percentage of leafhoppers infected with strain AY-BW (14.4-32.4%) was lower than observed in 2008. The percentage of leafhoppers infected with aster yellows phytoplasma strain AY-S was numerically low at both locations (2.9-3.2%), as in 2008. Strains AY-BD2 and AY-BW were present in similar percentages at both locations. The percentage of plants infected with strain AY-WB was similar to the percentage of plants infected with strain AY-BW at both locations, while the percentage of plants infected
with strain AY-BD2 in Celeryville was twice the percentage infected at Hartville. The percentages of plants infected with strain AY-S were low (2.2-2.6%) and similar at both locations (Table 13).

In 2009, at both locations, the percentage of leafhoppers infected with strain AY-WB (64.7%) was twice the percentage of plants infected with strain AY-WB (39.1%), while the percentage of leafhoppers infected with strain AY-S (6.1%) was similar to that of plants infected with the same strain (4.8%). However, the percentage of plants infected with strain AY-BD2 (63.1%) was higher than that of leafhoppers infected with this strain (47.4%). The percentage of plants infected with strain AY-BW (49.7%) was similar to the percentage to the leafhoppers infected with strain AY-BW (46.8%) (Table 13).

The percentage of leafhoppers infected with subgroup 16SrI-A strain AY-WB differed significantly over time during the growing season (p = 0.0187) but did not differ among the crops (p = 0.51; Table 10). The percentage of AY-WB-infected leafhoppers collected was significantly higher in July than in September (p = 0.01) (Table 11). The proportion of leafhoppers infected with subgroup 16SrI-B strains AY-S, AY-BD2 and AY-BW did not differ by crop from which they were collected, but the proportion of leafhoppers infected with AY-BD2 differed over time (Table 10). The percentage of leafhoppers infected with AY-BD2 was significantly lower in September than in June, July or August (Table 11). There was a significant interaction between crops tested and time of sampling for the percentage of leafhoppers infected with AY-BD2 (p = 0.0012)
The percentage of leafhoppers infected with AY-BD2 was higher in romaine lettuce ($p \leq 0.001$) throughout the growing season except September.

There were no significant differences in the percentage of any of the aster yellows phytoplasma strains detected among the host plants tested (lettuce, cilantro, and parsley). On average, the percentage of aster yellows strains belong to subgroup 16SrI-B detected in the host plants tested differed over time, with the lowest proportion in September (Table 12). However, there were no significant differences in the percentages of individual 16SrI-B strains detected in these crops over time. There was no significant interaction between host plants and time of sampling for the proportion of strains detected.

**Unidentified phytoplasma isolates in infected leafhoppers and plants.**

Samples that tested positive with P1/P7 and F2n/R2 primers in the nested PCR analysis indicate phytoplasma presence in their DNA but when tested with strain-specific primers in the multiplex PCR analysis, they tested negative, which defined them as unidentified phytoplasma isolates. Of the phytoplasma-infected leafhoppers collected from Celeryville and Hartville, 23.9% (35/146) and 10.4% (11/105), respectively, were infected with previously unidentified phytoplasma isolates in 2008. In 2009, 14.2% (77/540) and 19.4% (66/339) of the phytoplasma-infected leafhoppers from Celeryville and Hartville, respectively, were infected with unidentified phytoplasma isolates (Table 14). Amongst the phytoplasma-infected plant samples collected in 2009, 14.7% (13/88) from Celeryville were infected with unidentified phytoplasma isolates. In Hartville,
28.1% (54/192) were infected with previously unidentified aster yellows phytoplasma isolates (Table 14).

**Nucleotide sequence analysis of unidentified phytoplasmas isolates.**

The 16SrRNA sequences of unidentified isolates from ten leafhoppers and five plants collected from Celeryville, and nine leafhoppers and five plants from Hartville were determined. After the pairwise distance was calculated between the different isolates, the 29 isolates from both locations were divided into 16 sequence types (Table 15). Eight sequence types originated in Celeryville. Sequence type (3) contained eight identical isolates (SE9, SE12, SE39, SE20, SE7 SE33, SE16, and SE37). Sequence type 5, 6, 7, 8, 9, 10, 11 each included one unique isolate (SE11, SE21, SE29, SE10, SE38, SE18 and SE27, respectively). Eight sequence types were also identified amongst leafhoppers and plants collected in Hartville. Sequence type (1) contained two identical isolates (SE5 and SE26), sequence type (2) consisted of four identical isolates (SE13, SE14, SE22 and SE36), sequence type (4) consisted of three identical isolates (SE4, SE31 and SE35), and sequence types 12, 13, 14, 15, 16 each contained one unique isolate (SE6, SE19, SE30, SE3 and SE8, respectively). One isolate from each sequence type (SE26, SE36, SE20, SE4, SE11, SE21, SE29, SE10, SE38, SE18, SE27, SE6, SE19, SE30, SE3 and SE8) was selected to represent its type in the phylogenetic analysis.
Phylogenetic analysis of unidentified phytoplasmas isolates.

A BLAST search using partial sequences from the 16SrRNA gene of these sixteen isolates showed they shared 99.3% sequence identity with phytoplasma reference strains from aster yellows phytoplasmas group 16Sr-I, and closely aligned to phytoplasma strains from subgroups 16SrI-A and 16SrI-B. Nucleotide sequences from positions 206 to 1342 (1136 bp) within the 16SrRNA gene of the sixteen isolates were aligned with the GenBank sequences of 10 representative aster yellows phytoplasma strains from various crops (Table 16). Six isolates (SE3, SE6, SE8 from Hartville; SE21, SE27, SE38 from Celeryville) belonged to subgroup 16SrI-A and ten isolates (SE4, SE19, SE26, SE30, SE36 from Hartville; SE10, SE11, SE18, SE20, SE29 from Celeryville) belonged to subgroup 16SrI-B. Some of the subgroup 16SrI-B isolates were found in both leafhoppers and plants, but this was not observed for subgroup 16SrI-A isolates (Table 15).

The sequence identity between the isolates belonging to subgroups 16SrI-A and the isolates belonging to subgroup 16SrI-B was 99.7% and 99.4%, respectively. The sequence identity between the six 16SrI-A isolates was 99.1%, while between the ten 16SrI-B isolates it was 99.3%. In a pairwise comparison between the six 16SrI-A isolates (SE3, SE6, SE8, SE21, SE27 and SE38) and the 16Sr-I group reference strains, these six isolates shared 99.0-99.8% nucleotide sequence identity with the reference strains that included carrot phytoplasma ca2006/1 (EU215424), aster yellows phytoplasma 99UW111 (AF268408), aster yellows phytoplasma ParsD3 (AY180940) and aster
yellows witch’s-broom (AY389828). In a pairwise comparison between the ten 16SrI-B isolates (SE4, SE10, SE11, SE18, SE19, SE20, SE26, SE29, SE30 and SE36) and the 16Sr-I group reference strains, these ten isolates shared 99.1-99.6% nucleotide sequence identity with reference strains that included severe aster yellows SAY (AF222063), aster yellows phytoplasma bolt white (AY389820), aster yellows severe (AY389824), aster yellows bolt distortion no. 2 (AY389826) and aster yellows phytoplasma ParsD1 (AY180954). Phylogenetic analysis of these sixteen aster yellows phytoplasma isolates generated one neighbor-joining tree based on their 16SrDNA gene sequence. The phylogenetic tree resolved two distinct subclades, which corresponding to subgroup 16SrI-A and 16SrI-B (Figure 16). The phylogenetic tree showed that six isolates (SE3, SE6, SE8, SE21, SE27 and SE38) were clustered with reference strains from 16SrI-A subgroup while ten isolates (SE4, SE10, SE11, SE18, SE19, SE20, SE26, SE29, SE30 and SE36) were clustered with reference strains from the 16SrI-B subgroup (Figure 16).

**Discussion**

Aster yellows phytoplasma disease causes problems sporadically in vegetable production areas in Ohio. In some years, major damage to vegetable crops, primarily lettuce, occurs and losses can reach 100% in individual fields. In other years, infection rates may be as low as 2-3% (Hoy *et al*. 1992 and Madden *et al*. 1995). Vegetable growers control this disease differently, in that some spray insecticide once they notice the aster leafhoppers in their fields, while others spray insecticide on a regular basis regardless of leafhopper presence (Hoy *et al*. 1992 and Beanland *et al*. 2000).
Alternative approaches to disease management include controlling the leafhopper population only when they are known to be infected with aster yellows (Hoy et al. 1992; Mahr et al. 1993 and Murral 1994) and cultural practices such as timing and arrangement of lettuce crop plantings throughout the season to reduce the spread of the disease from one planting to the next (Hoy et al. 1999; Zhou et al. 2003).

Different symptoms of aster yellows disease have been observed in lettuce fields, depending mainly on the aster yellows phytoplasma strains present (Errampalli and Fletcher 1991 and Murral et al. 1996). To date, four different strains of aster yellows phytoplasma have been detected, isolated and identified in Ohio based on different symptoms they cause in lettuce: aster yellows Witches’-Broom (AY-WB) (16SrI-A), which causes chlorosis, wilting and witches’-broom, and aster yellows severe (AY-S) (16SrI-B), which causes stunting, chlorosis, phyllody and virescence (D’Arcy and Nault 1982; Hoy et al. 1992 and Murral et al. 1994). Moreover, aster yellows bolt white (AY-BW) (16SrI-B) induces chlorosis of newly emerging leaves, and aster yellows bolt distortion no. 2 (AY-BD2) (16SrI-B) causes leaf and stem distortion (Zhang et al. 2004). Molecular markers for these four aster yellows phytoplasma strains have been developed (Zhang et al. 2004).

In order to better describe aster yellows phytoplasma strain distribution in lettuce production areas, we developed a multiplex assay optimized from previously existing strain-specific primers to identify aster yellows phytoplasma strains in samples collected.
from leafhopper and lettuce plants. Initially, we assessed strain distribution temporally, geographically and by host (from which leafhoppers were collected) only in aster leafhoppers. In the second year, in addition to the aster leafhoppers, three lettuce types (red leaf, green leaf and romaine) highly susceptible to aster yellows, cilantro and parsley were sampled randomly and tested for the presence of different strains of aster yellows phytoplasma. In the last 5 years, cilantro and parsley plantings in Ohio lettuce production areas have increased. We proposed that these herbaceous crops might serve as an additional host for the aster yellows phytoplasma and as sources of inoculum (reservoir) for the disease, which has not been considered in the model of aster yellows epidemiology (Zhou et al. 2003).

While all types of lettuce plants showed typical aster yellows phytoplasma disease symptoms, cilantro and parsley were asymptomatic. There has been only one report of wild cilantro exhibiting witches’-broom symptoms (Lebsky and Poghosyan 2007) and another that showed that parsley exhibits virescense and phyllody in the flowering stage (Lee et al. 2003). In both studies, the cilantro and parsley were not intended for human consumption (treated as weeds), and allowed to flower, at which point aster yellows symptoms appeared. Cultivated cilantro and parsley are not allowed to flower in our sampled area, which can explain the lack of observation of disease symptoms. However, while these crops were asymptomatic, the proportion of plants infected with aster yellows phytoplasma was not significantly different from that observed in the lettuce. Further, the total number of leafhoppers detected in cilantro and parsley was not significantly
different from the number detected in red leaf and green leaf lettuce. Therefore, it is very likely that parsley and cilantro play an important and previously unrecognized role as inoculum reservoirs for aster yellows phytoplasmas within the lettuce fields in this region. Weeds that surround vegetable production areas and survive inside irrigation ditches may also serve as reservoirs for phytoplasmas (Iranoff et al. 1944, Lee et al. 1998, Lee et al. 2003, Weintraub and Beanland 2006). Langer et al. (2003) stated that the weed composition around lettuce fields not only affects the levels of aster leafhopper infection with phytoplasmas but also affects the incidence and spread of the disease.

Over both years and in both locations, there was a significant increase in the total number of aster leafhoppers collected as the growing season progressed, especially in the month of August. Further, leafhopper populations were higher in romaine lettuce than in the other types of lettuce, cilantro and parsley. During the growing season of 1989 and 1990, Hoy et al. (1992) surveyed both locations (Celeryville and Hartville, Ohio), planted with vegetable crops lettuce, carrot and celery, for the presence of immigrant aster leafhoppers. Hoy documented that populations of leafhoppers were higher in July than June and varied in the month of August. The proportion of phytoplasma-infected individuals was low within the immigrant leafhopper population early in the season, although some were also inoculative upon arrival in Ohio. During the growing season of 2008 and 2009, the proportion of leafhoppers infected with aster yellows phytoplasma was high in June and July and declined by the end (September) of the growing season. This agrees with a previous survey conducted during 2001 and 2002, in which the
percentage of leafhoppers migrating from the southern United States to southern Manitoba, Canada and captured in carrot fields was high in June and July and declined by the end of the growing season (Wally et al. 2004). Further, the proportion of immigrant aster yellows-infected leafhoppers detected was higher early in the season than at the end. These results suggest that host plant infection with aster yellows phytoplasma from inoculative leafhoppers peaked early in the growing season, rather than middle or late season, because of the high proportion of phytoplasma-infected immigrant leafhoppers. Therefore, management may require spraying insecticides to significantly reduce leafhopper populations in the early part of the growing season (May, June). However, this approach should be based on early testing of immigrant leafhoppers to determine their phytoplasma infection levels.

It is possible that changes in environmental conditions such as earlier onset of warming temperatures in the spring observed recently (Baldocchi et al. 2005 and Kelaine et al. 2007) have increased the ability of leafhoppers to acquire the phytoplasma from host plants along the route of migration. In Ohio, Murral et al. (1996) found that more than 60% of aster leafhoppers acquired different strains of aster yellows phytoplasmas when the temperature was between 25-32°C, decreasing to less than 30% when the temperature was under 20°C. These results suggest a positive correlation between local temperature and the efficiency of acquisition of the aster yellows phytoplasma. The increase of acquisition efficiency by leafhoppers enhances its ability to transmit the phytoplasma at higher rates, which increases the spread of the disease. Bale et al. (2002)
stated that the main effect of climate change on insects is the increase in temperature, which directly affects their feeding behavior, their fecundity and longevity. In Europe, Flavescence dorée disease of grapevines, caused by phytoplasmas from elm yellows group and vectored by leafhoppers (Scaphoideus titanus Ball), was reported in epidemic proportions in France, Spain and Portugal (Bertin et al. 2007). There was a noticeable increase of its vector activity and population density when winters were mild in these regions. Further, the leafhopper S. titanus populations increased within its geographical distribution and successfully established new habitats out of its range. The authors theorized that the increase in temperature enhanced migration activity of the vector (Parmesan, 2006). Bois noir disease of grapevines, which is caused by phytoplasmas from the stolbur group (Daire et al. 1993), is transmitted solely from wild herbaceous plants (bindweed) to grapevine by planthoppers (Hyalesthes obsoletus Signoret) (Maixner et al. 1995). In German wine-growing areas, H. obsoletus acquire phytoplasmas exclusively from bindweed (Convolvulus arvensis) and transmit them to grapevines (Langer and Maixner 2004). But with a mild winter in 2003, the population of planthoppers increased in the following summer and started to harbor stinging nettle (Urtica dioica) as a new host plant with a prolonged feeding period (Boudon-Padieu et al. 2007). Usually, the stinging nettle population is delayed 2-3 weeks compared to bindweed population, but with the increase of the yearly mean temperature (Lüers 2003) the nettle population had a prolonged growth period, which explains the recent exploitation of it as an additional host plant for Bois noir disease (Menzel et al. 2003 and Boudon-Padieu et al. 2007).
There were no significant differences in the distribution of aster yellows phytoplasma strains from subgroup 16SrI-A or 16SrI-B in aster leafhoppers in either location. A previous study showed that leafhoppers infected with aster yellows strain AY-WB (subgroup 16SrI-A) produced more nymphs than those infected with strain AY-S (subgroup 16SrI-B) (Beanland et al. 2000). We found that strain AY-S was rare in leafhoppers and plants in both years and both locations, supporting the previous work. However, other strains in subgroup 16SrI-B were abundant in leafhoppers and plants in this study and other studies have shown a predominance of strains in subgroup 16SrI-B (Marcone et al. 2000 and Lee et al. 2000, 2003). Therefore, it is possible that the differences observed by Beanland et al. (2000) were unrelated to current taxonomic grouping.

Unidentified phytoplasma isolates (a total of 207) were collected from plants and aster leafhoppers associated with these plants and were sequenced based on their 16SrRNA gene. Sixteen strain types from a total of 29 isolates with 4 nt differences were identified and typed to subgroup 16SrI-A and 16SrI-B. Half of these isolates were collected from cilantro and parsley plants and leafhoppers from fields that were infected with subgroup 16SrI-A and 16SrI-B strains as well. These previously unreported isolates of aster yellows might play an important role in the aster yellows epidemic in lettuce crops in this region.
In addition, some of these isolates belonging to subgroup 16SrI-B were detected in both infected leafhoppers and plants, but none of the isolates belonging to subgroup 16SrI-A was detected in both. These results suggest that these newly detected phytoplasma isolates belonging to subgroup 16SrI-B might also be more widespread in the lettuce fields than subgroup 16SrI-A isolates. While Marcone et al. (2000) observed that subgroup 16SrI-B was the most dispersed and widespread subgroup of aster yellows phytoplasmas in America, Europe and Asia, Wally et al. (2008) found that subgroup 16SrI-A was more common than subgroup 16SrI-B in Canada. Additional studies are needed to clarify the biological relevance of 16SrI subgroup classification for aster yellows phytoplasma strains.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
<th>Amplicon Size (bp)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>AAGAGTTTGATCCTGGCTCAGGATT</td>
<td>Deng and Hiruki (1991)</td>
<td>1784</td>
<td>Phytoplasma</td>
</tr>
<tr>
<td>P7</td>
<td>CGTCCTTCATCGGCTCTTT</td>
<td>Smart et al. 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2n</td>
<td>GAAACGACTGCTAAGACTGG</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>R2</td>
<td>TGACGGGCGGTGTGTAACCAACCCCG</td>
<td>Lee et al. 1992</td>
<td>1239</td>
<td>Phytoplasma</td>
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</tbody>
</table>

*Table 6.* Universal primer sets used to identify phytoplasma in aster leafhoppers, lettuce, cilantro and parsley.
<table>
<thead>
<tr>
<th>Location</th>
<th>GPS Coordinates</th>
<th>Sampling Date</th>
<th>Crop Type¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celeryville, OH</td>
<td>41°00’N, 82°45’W</td>
<td>June 24, 2008; August 20, 2008; July 17, 2009</td>
<td>RL, GL, R, C, P</td>
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<td>July 18, 2008; August 8, 2008; July 9, 2009</td>
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<td>June 24, 2008; August 24, 2009</td>
<td>RL, R, C</td>
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<td>41°01’N, 82°44’W</td>
<td>August 20, 2008; July 7, 2009</td>
<td>RL, GL, R, C, P</td>
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<td>41°00’N, 82°44’W</td>
<td>September 25, 2008; July 20, 2009</td>
<td>RL, GL, R, C, P</td>
</tr>
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<td>41°01’N, 82°43’W</td>
<td>August 20, 2008; June 18, 2009</td>
<td>RL, GL, R, C, P</td>
</tr>
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<td></td>
<td>41°01’N, 82°45’W</td>
<td>June 24, 2008; July 7 and 14, 2009</td>
<td>RL, GL, C, P</td>
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<td>41°01’N, 82°45’W</td>
<td>June 24, 2008; July 25, 2009</td>
<td>RL, GL, R, C, P</td>
</tr>
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<td>41°01’N, 82°42’W</td>
<td>July 18, 2008; August 10, 2009</td>
<td>RL, GL, C, P</td>
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<td>41°00’N, 82°41’W</td>
<td>July 18, 2008; August 27, 2009; September 17, 2009</td>
<td>RL, R, C, P</td>
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<td></td>
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<td>June 24, 2008; September 10, 2009</td>
<td>GL, R, C, P</td>
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<tr>
<td>Hartville, OH</td>
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<td>June 26, 2008; July 9, 2009</td>
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<td>RL, GL, R, C, P</td>
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<td>40°57’N, 81°16’W</td>
<td>August 1, 2008; August 24, 2009</td>
<td>RL, GL, R, C, P</td>
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<td>40°58’N, 81°16’W</td>
<td>August 15, 2008; July 9, 2009</td>
<td>RL, GL, C</td>
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<td>40°59’N, 81°17’W</td>
<td>July 10, 2008; September 3, 2008; August 17, 2009</td>
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<td>August 1, 2008; June 25, 2009</td>
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<td>40°57’N, 81°15’W</td>
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<td>41°59’N, 81°16’W</td>
<td>July 10, 2008; August 12, 2009</td>
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<td>July 10, 2008; August 24, 2009</td>
<td>RL, GL, R, P</td>
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<td>41°57’N, 81°18’W</td>
<td>August 1, 2008; September 3, 2009</td>
<td>GL, R, C, P</td>
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<td></td>
<td>40°58’N, 81°17’W</td>
<td>July 10, 2008; September 18, 2009</td>
<td>RL, C, P</td>
</tr>
</tbody>
</table>

¹ RL: Red leaf lettuce; GL: Green leaf lettuce; R: Romaine; C: Cilantro; P: Parsley.

Table 7. Sampling date and GPS coordinates of fields in OH from which leafhopper and plant samples were collected in 2008 and 2009.
Figure 12. Sensitivity of PCR detection of aster yellows phytoplasma strain AY-WB-infected leafhoppers in pooled samples. Samples were assayed by nested PCR using the universal phytoplasma primers P1/P7 followed by universal primers F2n/R2. Lane M, 1-Kb plus ladder; Lane 1, AY-WB (positive control); Lanes 2-10, samples with different ratios of DNA extracts from AY-WB-infected (AY) and non-infected (N) leafhoppers: 2, 8N; 3, 1AY:7N; 4, 2AY:6N; 5, 3AY:5N; 6, 4AY:4N; 7, 5AY:3N; 8, 6AY:2N; 9, 7AY:1N; 10, 8AY; 11, non-infected leafhoppers (negative control).
Figure 13. Numbers of leafhoppers collected from lettuce, cilantro and parsley fields in Celeryville and Hartville, Ohio during summer 2008 and 2009.
Aster yellows phytoplasma subgroups 16SrI-A and subgroup 16SrI-B detected by multiplex PCR assays; total numbers of samples that tested negative with aster yellows phytoplasma strain-specific primers in the multiplex PCR assay.

Table 8. Aster yellows phytoplasma detected in leafhoppers collected from lettuce, cilantro, and parsley fields (2008-2009) and in lettuce, cilantro and parsley plants (2009) in Celeryville, Ohio, by nested and multiplex PCR assays using universal and strain-specific primer sets.
<table>
<thead>
<tr>
<th>Location</th>
<th>Year</th>
<th>Vector / Plant Host</th>
<th>Samples Collected</th>
<th>Nested PCR</th>
<th>Multiplex PCR</th>
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<td></td>
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<td>Tested</td>
<td>+Phytoplasma</td>
<td>-Phytoplasma</td>
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<td>Hartville</td>
<td>2008</td>
<td>ALH RL1</td>
<td>265</td>
<td>106</td>
<td>70</td>
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<td></td>
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<td>ALH GL</td>
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<td></td>
<td></td>
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<td></td>
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<td>ALH C</td>
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<td>ALH P</td>
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<td>2009</td>
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<td>ALH R</td>
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<td>2009</td>
<td>RL</td>
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<td></td>
<td>GL</td>
<td>232</td>
<td>232</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>179</td>
<td>179</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>166</td>
<td>166</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>152</td>
<td>152</td>
<td>60</td>
</tr>
</tbody>
</table>

¹ALH: Aster leafhopper; RL: Red leaf lettuce; GL: Green leaf lettuce; R: Romaine; C: Cilantro; P: Parsley; ²Aster yellows phytoplasma subgroups 16SrI-A and subgroup 16SrI-B detected by multiplex PCR assays; ³total numbers of samples that tested negative with aster yellows phytoplasma strain-specific primers in the multiplex PCR assay.

**Table 9.** Aster yellows phytoplasma detected in leafhoppers collected from lettuce, cilantro, and parsley fields (2008-2009) and in lettuce, cilantro and parsley plants (2009) in Hartville, Ohio, by nested and multiplex PCR assays using universal and strain-specific primer sets.
Figure 14. Healthy (left) and aster yellows-infected (right) romaine lettuce in Hartville, Ohio.
Figure 15. Healthy (left, right) and aster yellows-infected (center) green leaf lettuce, Celeryville, Ohio.
<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>Total ALH(^1) collected</th>
<th>Proportion of aster yellows phytoplasma-infected</th>
<th>Proportion of 16SrI-A (AY-WB)(^2)</th>
<th>Proportion of 16SrI-B</th>
<th>Proportion of AY-S(^2)</th>
<th>Proportion of AY-BD2(^2)</th>
<th>Proportion of AY-BW(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALH</td>
<td>Plants</td>
<td>ALH</td>
<td>Plants</td>
<td>ALH</td>
<td>Plants</td>
<td>ALH</td>
</tr>
<tr>
<td>Crop</td>
<td>0.006</td>
<td>0.5929</td>
<td>0.3509</td>
<td>0.5119</td>
<td>0.6764</td>
<td>0.0353</td>
<td>0.7688</td>
</tr>
<tr>
<td>Time</td>
<td>0.0001</td>
<td>0.0006</td>
<td>0.0103</td>
<td>0.0187</td>
<td>0.5155</td>
<td>0.0001</td>
<td>0.0112</td>
</tr>
<tr>
<td>Crop*Time</td>
<td>0.0057</td>
<td>0.6656</td>
<td>0.9782</td>
<td>0.9300</td>
<td>0.8298</td>
<td>0.0956</td>
<td>0.8258</td>
</tr>
</tbody>
</table>

\(^1\)ALH: Aster leafhopper; \(^2\)AY-WB: aster yellows witches’-broom; AY-S: aster yellows severe; AY-BD2: aster yellows bolt distortion no. 2 and AY-BW: aster yellows bolt white.

Table 10. P-value of the effect of host and sampling time over the growing seasons on the distribution of aster yellows phytoplasma strains in infected aster leafhoppers and infected lettuce, cilantro and parsley plants.
Table 11. Mean estimates of the effect of sampling time over the growing seasons on the percentage of aster yellows phytoplasma-infected leafhoppers collected in Ohio lettuce, cilantro and parsley fields and on the percentage of phytoplasmas belonging to 16SrI subgroup A and B, and identified as strain AY-BD2 (also 16SrI-B). Values in each column followed by the same letter are not significantly different at p = 0.05.

<table>
<thead>
<tr>
<th>Sampling Time</th>
<th>Percentage of aster yellows phytoplasma-infected leafhoppers</th>
<th>Percentage of 16SrI-A (AY-WB)</th>
<th>Percentage of 16SrI-B</th>
<th>Percentage of AY-BD2 (16SrI-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>June</td>
<td>69.5 a</td>
<td>9.7 ab</td>
<td>6.8 bc</td>
<td>24.2 a</td>
</tr>
<tr>
<td>July</td>
<td>68.0 a</td>
<td>16.1 a</td>
<td>14.1 b</td>
<td>34.9 a</td>
</tr>
<tr>
<td>August</td>
<td>44.9 b</td>
<td>9.8 ab</td>
<td>27.8 a</td>
<td>39.4 a</td>
</tr>
<tr>
<td>September</td>
<td>36.5 b</td>
<td>3.5 b</td>
<td>3.6 c</td>
<td>8.0 b</td>
</tr>
</tbody>
</table>

Table 12. Mean estimates of the effect of sampling time over the growing season on the percentage of phytoplasma-infected lettuce, cilantro and parsley crops collected in Ohio lettuce, cilantro and parsley fields and on the percentage of aster yellows phytoplasma strains belonging to phytoplasma 16SrI-B subgroup. Values in each column followed by the same letter are not significantly different at p = 0.05.

<table>
<thead>
<tr>
<th>Sampling Time</th>
<th>Percentage of aster yellows phytoplasma-infected plants</th>
<th>Percentage of 16SrI-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>June</td>
<td>30.2 a</td>
<td>36.2 a</td>
</tr>
<tr>
<td>July</td>
<td>39.1 a</td>
<td>26.2 a</td>
</tr>
<tr>
<td>August</td>
<td>42.5 a</td>
<td>27.1 a</td>
</tr>
<tr>
<td>September</td>
<td>6.5 b</td>
<td>5.5 b</td>
</tr>
<tr>
<td>Year</td>
<td>2008</td>
<td>2009</td>
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<tr>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Host</td>
<td>Leafhopper</td>
<td>Leafhopper</td>
</tr>
<tr>
<td>Aster yellows subgroup</td>
<td>16SrI-A</td>
<td>16SrI-B</td>
</tr>
<tr>
<td>Strain</td>
<td>AY-WB</td>
<td>AY-S</td>
</tr>
<tr>
<td>Celeryville</td>
<td>21.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Hartville</td>
<td>21.0</td>
<td>0</td>
</tr>
</tbody>
</table>

1AY-WB: aster yellows witches’-broom; AY-S: aster yellows severe; AY-BD2: aster yellows bolt distortion no. 2 and AY-BW: aster yellows bolt white.

**Table 13.** Percentages of the aster yellows phytoplasma strains detected in leafhoppers and lettuce, cilantro and parsley crops in Ohio.
Table 14. Number of unidentified phytoplasma isolates collected from leafhoppers and plants samples in Celeryville and Hartville over the growing season in 2008 and 2009.

<table>
<thead>
<tr>
<th>Location</th>
<th>Number of unidentified isolates</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Celeryville</td>
<td>Total</td>
<td>Hartville</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Host</td>
<td>2008</td>
<td>2009</td>
<td>2008</td>
<td>2009</td>
<td></td>
</tr>
<tr>
<td>Leafhopper</td>
<td>35</td>
<td>77</td>
<td>112</td>
<td>11</td>
<td>66</td>
</tr>
<tr>
<td>Plant</td>
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<td>13</td>
<td>13</td>
<td>-</td>
<td>54</td>
</tr>
<tr>
<td>Aster Yellows</td>
<td>Location</td>
<td>Place of Isolation</td>
<td>Natural Host</td>
<td>Sequence Type</td>
<td>16Sr1-Subgroup</td>
</tr>
<tr>
<td>--------------</td>
<td>----------</td>
<td>-------------------</td>
<td>--------------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td>SE5</td>
<td>Plant</td>
<td>Hartville</td>
<td>Cilantro</td>
<td>Sequence type 1</td>
<td>16SrI-B</td>
</tr>
<tr>
<td>SE26</td>
<td>ALH</td>
<td>Hartville</td>
<td>Cilantro</td>
<td>Sequence type 1</td>
<td>16SrI-B</td>
</tr>
<tr>
<td>SE13</td>
<td>ALH</td>
<td>Hartville</td>
<td>Romaine lettuce</td>
<td>Sequence type 2</td>
<td>16SrI-B</td>
</tr>
<tr>
<td>SE14</td>
<td>ALH</td>
<td>Hartville</td>
<td>Romaine lettuce</td>
<td>Sequence type 2</td>
<td>16SrI-B</td>
</tr>
<tr>
<td>SE22</td>
<td>Plant</td>
<td>Celeryville</td>
<td>Red leaf lettuce</td>
<td>Sequence type 3</td>
<td>16SrI-B</td>
</tr>
<tr>
<td>SE36</td>
<td>Plant</td>
<td>Celeryville</td>
<td>Red leaf lettuce</td>
<td>Sequence type 3</td>
<td>16SrI-B</td>
</tr>
<tr>
<td>SE9</td>
<td>ALH</td>
<td>Hartville</td>
<td>Parsley</td>
<td>Sequence type 4</td>
<td>16SrI-B</td>
</tr>
<tr>
<td>SE12</td>
<td>ALH</td>
<td>Hartville</td>
<td>Parsley</td>
<td>Sequence type 4</td>
<td>16SrI-B</td>
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<td>SE7</td>
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<td>Parsley</td>
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<td>16SrI-B</td>
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<td>SE33</td>
<td>Plant</td>
<td>Celeryville</td>
<td>Parsley</td>
<td>Sequence type 4</td>
<td>16SrI-B</td>
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<td>ALH</td>
<td>Celeryville</td>
<td>Cilantro</td>
<td>Sequence type 5</td>
<td>16SrI-B</td>
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<td>Celeryville</td>
<td>Green leaf lettuce</td>
<td>Sequence type 6</td>
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<td>SE29</td>
<td>ALH</td>
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<td>Red leaf lettuce</td>
<td>Sequence type 7</td>
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<td>SE10</td>
<td>ALH</td>
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<td>Parsley</td>
<td>Sequence type 8</td>
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<td>SE38</td>
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<td>Cilantro</td>
<td>Sequence type 9</td>
<td>16SrI-A</td>
</tr>
<tr>
<td>SE18</td>
<td>Plant</td>
<td>Celeryville</td>
<td>Romaine lettuce</td>
<td>Sequence type 10</td>
<td>16SrI-B</td>
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<tr>
<td>SE27</td>
<td>Plant</td>
<td>Celeryville</td>
<td>Parsley</td>
<td>Sequence type 11</td>
<td>16SrI-A</td>
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<tr>
<td>SE6</td>
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<td>Hartville</td>
<td>Cilantro</td>
<td>Sequence type 12</td>
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<tr>
<td>SE19</td>
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<td>Romaine lettuce</td>
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<td>16SrI-B</td>
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<td>Green leaf lettuce</td>
<td>Sequence type 14</td>
<td>16SrI-B</td>
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<td>Parsley</td>
<td>Sequence type 15</td>
<td>16SrI-A</td>
</tr>
<tr>
<td>SE8</td>
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<td>Hartville</td>
<td>Red leaf lettuce</td>
<td>Sequence type 16</td>
<td>16SrI-A</td>
</tr>
</tbody>
</table>

**Table 15.** Classification of the unidentified phytoplasma isolates present in lettuce, cilantro and parsley plants and aster leafhoppers collected from Celeryville and Hartville, Ohio.
<table>
<thead>
<tr>
<th>Phytoplasma Strains</th>
<th>Place of Isolation</th>
<th>Natural Host</th>
<th>16SrI-Subgroup</th>
<th>Accession Number</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY-WB</td>
<td>Ohio, USA</td>
<td>Lettuce</td>
<td>16SrI-A</td>
<td>AY389828</td>
<td>1-1534</td>
<td>Zhang et al. 2004</td>
</tr>
<tr>
<td>AY-BW</td>
<td>Ohio, USA</td>
<td>Lettuce</td>
<td>16SrI-B</td>
<td>AY389820</td>
<td>1-1830</td>
<td>Zhang et al. 2004</td>
</tr>
<tr>
<td>AY-S</td>
<td>Ohio, USA</td>
<td>Lettuce</td>
<td>16SrI-B</td>
<td>AY389824</td>
<td>1-1830</td>
<td>Zhang et al. 2004</td>
</tr>
<tr>
<td>AY-BD2</td>
<td>Ohio, USA</td>
<td>Lettuce</td>
<td>16SrI-B</td>
<td>AY389826</td>
<td>1-1830</td>
<td>Zhang et al. 2004</td>
</tr>
<tr>
<td>Severe aster yellows (SAY)</td>
<td>California, USA</td>
<td>Celery</td>
<td>16SrI-B</td>
<td>AF222063</td>
<td>1-1830</td>
<td>Kuske and Kirkpatrick 1992</td>
</tr>
<tr>
<td>Carrot Phytoplasma (ca2006/1)</td>
<td>Serbia</td>
<td>Carrot</td>
<td>16SrI-A</td>
<td>EU215424</td>
<td>1-1720</td>
<td>Duduk et al. 2009</td>
</tr>
<tr>
<td>Tomato stolbur phytoplasma (PTV strain)</td>
<td>Italy</td>
<td>Tomato</td>
<td>16SrXII-A</td>
<td>EF193364</td>
<td>1-1222</td>
<td>Martini et al. 2007</td>
</tr>
<tr>
<td>Aster yellows (99UW111)</td>
<td>Wisconsin, USA</td>
<td>Carrot</td>
<td>16SrI-A</td>
<td>AF268408</td>
<td>1-1824</td>
<td>Lee et al. 2002</td>
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<tr>
<td>Aster yellow phytoplasma (ParsD1)</td>
<td>Texas, USA</td>
<td>Parsley</td>
<td>16SrI-B</td>
<td>AY180954</td>
<td>1-1528</td>
<td>Lee et al. 2003</td>
</tr>
<tr>
<td>Aster yellow phytoplasma (ParsD3)</td>
<td>Texas, USA</td>
<td>Parsley</td>
<td>16SrI-A</td>
<td>AY180940</td>
<td>1-1528</td>
<td>Lee et al. 2003</td>
</tr>
</tbody>
</table>

Table 16. Phytoplasma reference strains used in the phylogenetic analysis based on their 16SrRNA sequence retrieved from the GenBank database.
Figure 16. Phylogenetic tree of aster yellows unidentified isolates constructed by neighbor-joining analysis of partial length of 16SrRNA sequences collected from vegetable crops: lettuce (vars. red, green and romaine), cilantro and parsley; aster leafhoppers collected from lettuce, cilantro and parsley plants from Celeryville and Hartville in 2008 and 2009; and reference strains: AY-S (16SrI-B), SAY (16SrI-B), AY-BW (16SrI-B), AY-BD2 (16SrI-B), ParsD1 (16SrI-B), 99UW111 (16SrI-A), ca2006/1 (16SrI-A), AY-WB (16SrI-A), ParsD3 (16SrI-A); employing tomato stolbur phytoplasma as the outgroup.
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