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Abstract

Aster Yellows phytoplasma (AYp; *Candidatus* Phytoplasma asteris) is associated with diseases of herbaceous plants, including ornamentals and important commercial vegetable and grain crops. The aster leafhopper (ALH; *Macrosteles quadrilineatus* Forbes) is the predominant vector of these bacteria, though other leafhopper species can acquire and transmit AYp. Potentially inoculative leafhoppers are reported to overwinter in the southern United States and migrate to northern latitudes in the spring. Examining the genetic similarities and differences in AYp associated with southern and northern populations of ALH may provide insight into the role that migrating ALH play in AYp disease development. To investigate similarities among geographically distinct populations of ALH and characterize the variation in AYp associated within these populations, we identified genetic variations in subgroup designation and the relative proportions of secreted AY-WB proteins from field-collected populations of AYp isolated from ALH from select locations in the southern (Arkansas, Kansas, Oklahoma, and Texas) and the northern United States (Wisconsin) in 2016, 2017, and 2018. Isolated phytoplasma were tested for variation of AYp genotypes, numbers of potentially inoculative (AYp-positive) ALH, and presence of specific AYp virulence (effector) genes. Geographically distinct populations of ALH collected in northern and southern regions were similar in CO1 genotype but carried different proportions of AYp genotypes. While similar AYp strains were detected in geographically distinct locations, the proportion of each genotype varied over time.

Key words: Aster leafhopper, Aster Yellows phytoplasma, hemiptera, migration, insect vector

Aster yellows (AY) is a disease of plants caused by the Aster Yellows phytoplasma (AYp), a small, cell-wall-less prokaryotic organism currently placed in the provisional genus 'Candidatus Phytoplasma' under 'Ca. Phytoplasma asteris' species name (Lee et al. 2000, IRPCM Phytoplasma/Spiroplasma working team—Phytoplasma taxonomy group 2004, Hogenhout et al. 2008) and has common ancestry with members of the order Acholeplasmataceae within the Class Mollicutes (Lee et al. 2000). More than 24 leafhopper species are known to transmit AYps (Mahr et al. 1989), but the bacterial pathogen is predominantly spread by the aster leafhopper (ALH), *Macrosteles quadrilineatus* Forbes (Drake et al. 1965) in the United States. The AYp is regarded as an unculturable obligate colonizer of plants and insects that transmits via insect vector to infect herbaceous plants, including commercially important crops encompassing processing carrot (*Daucus carota* ssp. *sativus* L.), lettuce (*Lactuca sativa* L.) and celery (*Apium graveolens* L.), as well as the grains wheat (*Triticum aestivum* L.), and oats (*Avena sativa* L.) (Lee et al. 2000). Infection by certain subgroups of AYp in carrot results in the development of AY disease symptoms, including witch's broom (proliferation of shoots), phyllody (retrograde development of flowers into leaves), virescence (flower organs remaining green), bolting, formation of shortened internodes and elongate petioles, yellowing, and the production of epicormic (e.g., hairy) roots (Hogenhout et al. 2008, Rodgers et al. 2011). The persistence and continued prevalence of AYp as a pathogen depends on the mutualistic relationship between AYp and associated leafhoppers, as well as the parasitism between AYp and herbaceous plants (Hogenhout et al. 2008, Sugio et al. 2011a). Transmission of AYp occurs primarily

horizontally, from insect vector to plants and back to insect vectors. Vertical transmission of AYp to the progeny of infected ALH, for example, via transovarial passage within egg-laying females, is unlikely to occur, and thus feeding on an infected host plant is required for acquisition and subsequent inoculation of AYp (Rodgers et al. 2011). Further, ALH overwinters in the egg life stage and would require infected plants to become inoculative.

The ALH has previously been described as a migratory insect that moves with low-level directional winds associated with major synoptic weather systems that occur in the spring (Chiykowski et al. 1965). Historically, ALH abundance data suggest that ALH initiate migration into regions of the upper Midwest in late spring and into early summer and have historically been regarded as the principle source of early season AYp inoculation to susceptible crops (Chiykowski et al. 1965). Depending upon variation in directionality of winds and warming trends, ALH migratory movement can vary significantly from year to year. However, degree day models generated by Frost et al. (2013) provide a reliable and robust model to predict ALH arrival into the upper Midwest. This model is partially based on monitoring data collected from agricultural growers in the upper Midwest (Pest Pros, Division of Allied Cooperative, Adams, WI). Based on the degree day models developed for ALH, overwintered populations in Wisconsin do not eclose as adults until early June and peak abundance of these adult leafhoppers is not observed until the beginning of July (Julian day ~190) (Frost et al. 2013). Thus, the population at the time of peak abundance in central Wisconsin represents a combination of first-generation offspring (nymphs) from southern migratory populations and recently matured adults from overwintered northern populations. This putative population of ALH is likely able to transmit and spread AYp.

Comparative genetic analysis has revealed 46 'Candidatus Phytoplasma' species, including AYp subclade ('Candidatus Phytoplasma asteris' also known as 16SrI phytoplasma group). Further analysis of the 16S ribosomal RNA region has established that the AYp subclade is comprised of multiple subgroups (Lee et al. 2004, Hogenhout et al. 2008, Yang et al. 2017). The classification of these subgroups is strictly based on polymorphic differences in the highly conserved 16S rRNA region. The distribution of these AYp subgroups has also been reported to vary considerably among different geographical regions. Lee et al. (2004) further reports that AYp subgroups 16SrI-A, 16SrI-B, 16SrI-E, 16SrI-K, 16SrI-O, 16SrI-P, and 16SrI-R have all been isolated in the contiguous United States. Members of these subclades were assigned to 'Ca. Phytoplasma asteris' (Lee et al. 2004).

Symptoms of AYp associated diseases are highly variable and some strains do not induce obvious symptoms at all (Lee et al. 2004). Aster Yellows phytoplasma and other phytoplasmas induce disease symptoms via the secretion of specific virulence factors, called effectors, which are released into the plant and interact with plant transcription factors involved in regulation of developmental processes, including excessive shoot formation and the development of leaf-like flowers (Hogenhout et al. 2008, Bai et al. 2009, Sugio et al. 2011a). For the 16SrI-A Aster Yellows phytoplasma strain Witches Broom (AY-WB), it was demonstrated that the effector secreted AY-WB Protein (SAP) 11, which destabilizes teosinte-branched cycloidea PCF (TCP) transcription factors, leads to stem proliferations and changes in leaf development (Sugio et al. 2011b, Sugio et al. 2014). Alternatively, the effector SAP54 degrades MADS-box transcription factors (MTFs) via the 26S proteasome shuttle factor RAD23, leading to phyllody and virescence symptoms (MacLean et al. 2011, MacLean et al. 2014). Both SAP11 and SAP54 homologs have also been identified in other phytoplasmas (Sugio et al. 2012, Maclean

et al. 2014, Orlovskis et al. 2016), probably due to horizontal transfer of potential mobile units (PMUs), which harbor the majority of phytoplasma effector genes (Bai et al. 2006, Toruno et al. 2010, Chung et al. 2013, Ku et al. 2013). Interestingly, SAP11 and SAP54 also promote colonization of ALH on AY-WB infected plant; secreted AY-WB protein 11 promoted egg laying of ALH in no-choice tests and SAP54 attracts ALH to AY-WB-infected plants in choice tests (Sugio et al. 2011b, MacLean et al. 2014, Orlovskis et al. 2016). Therefore, the SAPs are likely to contribute to the development of AYp epidemics via increasing insect vector populations and the numbers of potentially inoculative insects in these populations.

In the current study, our primary goal was to investigate the similarities among geographically distinct populations of ALH and characterize the variation in AYp associated within these populations. Examining the distribution of AYp genotype, prevalence of AYp in ALH and effector distribution may lead to a better understanding of AYp population dynamics, including AYp epidemics in northern United States. Based on previous investigations of AYp infectivity in dispersing ALH populations (Chiykowski et al. 1965, Frost et al. 2013), we hypothesize that rates of AYp infection in leafhoppers will be higher in field collections from the northern US when compared with those from the southern United States, and that populations of ALH collected from different geographic areas (e.g., northern versus southern United States) are genetically similar resulting from annual mixing with southern migrants.

Materials and Methods

Ethics Statement

No specific permits were required for field collection or experimental treatment of *M. quadrilineatus* for the study described.

Aster Leafhopper Collections

Adult ALHs were collected from eight wheat fields in the southern U.S. states of Arkansas, Kansas, and Oklahoma on 14 and 15 April 2016. In 2017, adult ALHs were collected between 4 and 19 April from five wheat fields in these same states and Texas. Again, in 2018 ALH collections were conducted from 4 wheat fields in Kansas and Oklahoma. Adults were obtained from each sample site using a standard, 15″ sweep net with a total of 100 pendulum sweeps of the plant canopy. Immediately after sweeps, insects were placed into 3.8 l sealable plastic bags and placed on ice for shipment to the University of Wisconsin–Madison for processing. In 2016, adult ALHs were collected from nine unique northern carrot fields in central Wisconsin using sweep collections in August and September. In 2017, collections were conducted in central Wisconsin in three wheat fields in May and four additional carrot fields in July and August. Adult ALH collections were again conducted in Wisconsin in 2018 from one wheat field in May and one carrot field from June to August. The geographical locations of all fields sampled in the study are presented in Fig. 1, and the specific geographical coordinates of each field site can be found in Supp Table 1 [online only]. The number of adult ALH obtained at each field location is presented in Supp Tables 2–4 [online only]. A taxonomic key to the family Cicadellidae was used to confirm the identity of all adult ALH (Dietrich 2005).

DNA Isolation From Aster Leafhoppers

Insect samples were processed as individuals or aggregates of two to five insects, depending on ALH abundance at each field location. Insect samples were placed in corresponding 1.5-ml sterile microcentrifuge
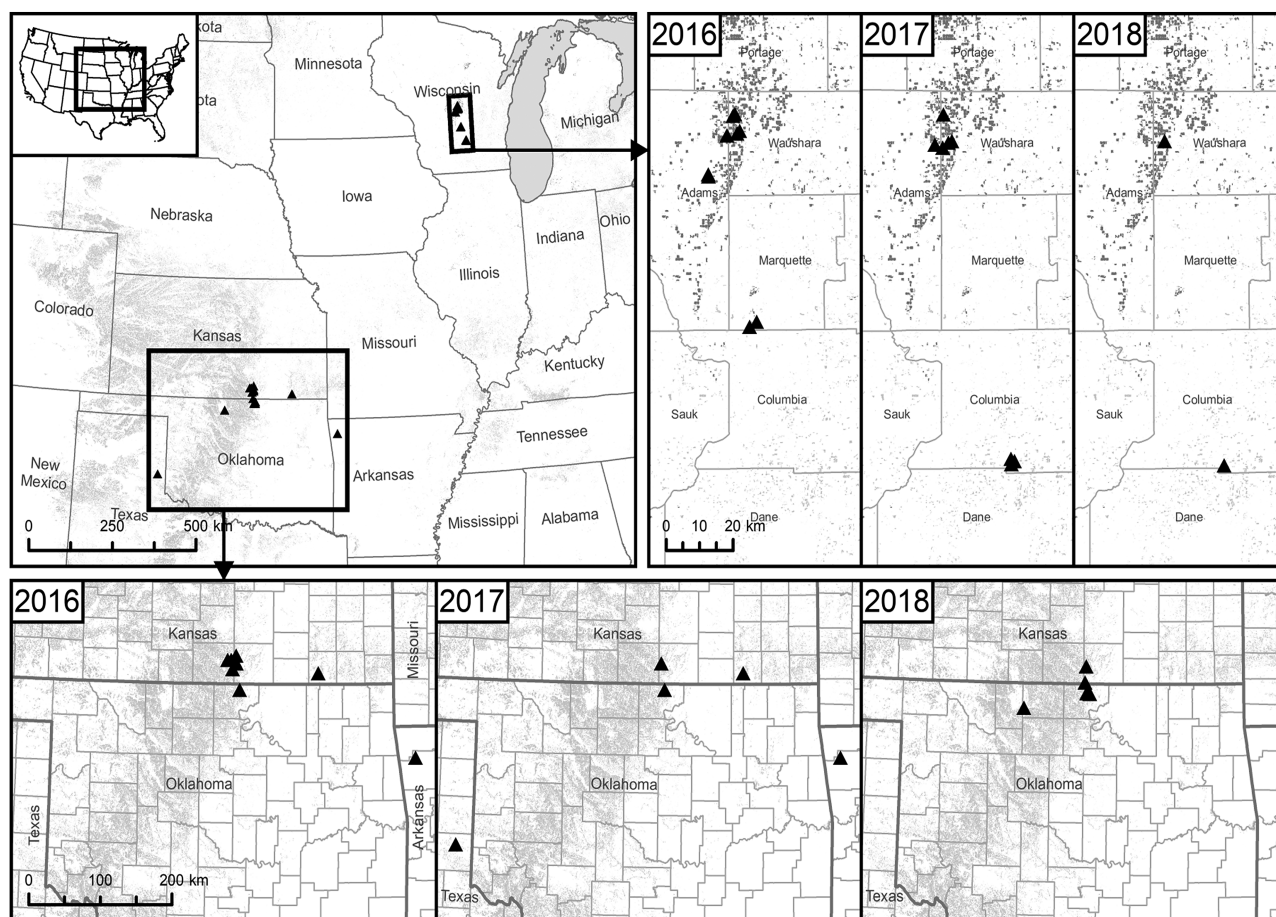
Fig. 1. Map of the central United States with the geographic locations of northern and southern *Macrosteles quadrilineatus* collection sites. Triangles represent field locations sampled.

tubes and homogenized with a sterile plastic pestle in 500-µl 2% CTAB (cetyl-trimethyl-ammonium-bromide; bioWORLD, Dublin, OH) buffer with 1 µl of 0.2-ng/µl RNase A (Thermo Fisher Scientific, Waltham, MA). Homogenates were incubated at 60°C for 30 min, centrifuged at 12,000*g* for 5 min, and the supernatant was transferred to a fresh tube. A single volume of chloroform was added and the samples were gently mixed for 10 min. Samples were then centrifuged for 10 min at 12,000*g* and the supernatant was again transferred to a fresh tube. DNA was precipitated by adding 1 volume of cold isopropanol and mixed by inversion for 10 min. Samples were centrifuged to pelletize DNA and washed with 75% ethanol. After washing, ethanol was discarded and samples were allowed to completely air dry; methodology was adapted from Marzachi et al. (1998). DNA was suspended in DNase/RNase-free H_2O, and subsequently quantified using a NanoDrop, microvolume spectrophotometer (Thermo Fisher Scientific), and brought to a final volume of between 20 and100 µl depending on the measured concentration. Samples were then frozen at –20°C pending further analysis.

Confirmation of Aster Yellows Phytoplasma Presence and Genetic Determination of Phylogenetic Subgroup

To confirm the presence of AYp from DNA isolations of adult ALH, a P1/P7 polymerase chain reaction (PCR) amplification was conducted, followed by a R16F2n/R16R2 nested PCR on the P1/P7 PCR product to amplify the 16S rRNA gene sequence. DNA primers

were obtained from Smart et al. 1996 (P1/P7) and Davis et al. 2003 (R16F2n/R16R2) and are presented in Supp Table 5 [online only]. Specifically, 25-µl PCR were conducted with GoTaq Green Master Mix (Promega Corporation, Madison, WI). Reaction conditions included 240 s at 94°C as the initial denaturing step, followed by 30 cycles of 30 s at 94°C for denaturation, 60 s at 64°C (P1/P7) or 60°C (R16F2n/R16R2) for annealing, 90 s at 72°C for extension and a final extension of 300 s at 72°C. PCR amplification products were separated on a 1.5% agarose gel to confirm presence of corresponding DNA fragments. The corresponding DNA fragment observed from the P1/P7 amplification was 1.8 kbp, while the nested product was 1.2 kbp. As an additional diagnostic confirmation for the presence of AYp, the R16F2n/R16R2 PCR products were submitted and sequenced by Macrogen USA (Rockville, MD). 16S rDNA sequences were searched against the nonredundant (nr) nucleotide NCBI database using blastn. Adult ALHs were considered AYp-infected if the presence of the phytoplasma was confirmed by the sequencing result. The proportion of AYP-infected ALH was calculated using a maximum likelihood estimator for composite samples (Lund et al. 1985). An analysis of variance (ANOVA) was conducted in KaleidaGraph to test the correlation between the response variable (prevalence of AYp in ALH) and independent variables (region and year) using an α = 0.05.

To further confirm the phytoplasma ribosomal subgroup within the *Candidatus* (*Ca.*) Phytoplasma genus, the nucleotide sequence of the 16s rRNA gene amplified by R16F2n/R16R2 was analyzed. Sequencing results were cleaned and aligned using Clustal X (Version

2.0, http://www.cluster-x.org/) and the generated matrices were cleaned and analyzed using BioEdit (Version 7.0.5.3). The identity and position of six unique, single nucleic acid polymorphisms were compared between sequences to identify AYp subgroup. Furthermore, a restriction fragment length polymorphism (RFLP) assay, with the restriction endonuclease Hha1 (Promega Corporation), was conducted at 37°C for 90 min and run on 1.5% agarose gel. The RFLP assay was conducted on extracted DNA from each AYp-positive leafhopper sample. RFLP was used to confirm the subgroup designation of the sequencing data (Lee et al. 2004). An ANOVA was conducted in KaleidaGraph to test the correlation between the response variable (subgroup) and independent variables (region and year) again using an α = 0.05.

Prevalence of SAP Effector Genes in Aster Yellows Phytoplasma-Positive Leafhopper Vectors

We chose to examine the prevalence of several important secreted AY-WB protein genes, including SAP11 and SAP54, which are known to influence vector–host interactions (Sugio et al. 2011a, Maclean et al. 2014), and SAP68 and SAP67, which lie on the same potential mobile unit as SAP11 (Bai et al. 2009) and have been hypothesized to also have significant host–vector interactions. DNA primers were designed to AYWB genome CP000061.1 (Bai et al. 2009) to detect the presence of SAP11, SAP54, SAP67, and SAP68 sequences within AYp-positive ALH. A nested PCR was performed to detect the presence of SAP54. Briefly, 25-µl PCR were conducted with GoTaq Green Master Mix (Promega Corporation). Reaction conditions were 240 s at 94°C as the initial denaturing step, followed by 30 cycles of 30 s at 94°C for denaturation, 60 s at 55°C (SAP11), 62°C (SAP54), or 62°C (SAP67, SAP68, SAP54 nested) for annealing, 90 s at 72°C for extension and a final extension of 300 s at 72°C. PCR product was run on a 1.5% agarose gel to confirm presence of specific effectors. In addition, the PCR product was sequenced by Macrogen USA as a supplemental, confirmatory assay to identify the presence of specific effectors. Primers designed for amplification of specific effectors can be found in Supp Table 5 [online only]. An ANOVA was conducted in KaleidaGraph to test the correlation between the response variable (effectors) and the independent variables (region and year) using an α = 0.05.

Species Confirmation and Genetic Variation Between Southern and Northern Populations of Aster Leafhoppers

To confirm species identity and determine the degree of genetic relatedness of adult ALH between northern and southern ALH populations in the southern and northern United States, an analysis of the cytochrome *c* oxidase 1 (*CO1*) gene was conducted on a representative subsample of both AYp positive and negative ALHs. Using primers developed by Folmer et al. (1994), a 701-bp segment of the *CO1* gene was amplified from representatives of both southern and northern populations. Again, 25-µl PCR were conducted with GoTaq Green Master Mix (Promega Corporation), and conditions were 240 s at 94°C as an initial denaturing step, followed by 35 cycles of 30 s at 94°C for denaturation, 60 s at 50°C for annealing, 90 s at 72°C for extension and a final extension of 420 s at 72°C. PCR product was run on a 1.5% agarose gel to confirm presence of CO1. Further PCR product was sequenced by Macrogen USA. Sequencing results were cleaned and aligned using Clustal X (Version 2.0, http://www.cluster-x.org/), and the generated matrices were cleaned and analyzed using BioEdit (Version 7.0.5.3) to characterize and evaluate differences in the *CO1* gene.

Results

Determination of the prevalence of Aster Yellows phytoplasma in aster leafhoppers in southern and northern regions of the United States

Aster Yellows phytoplasma was detected in ALH using primers for amplification of the conserved 16S rRNA region of the AYp genome. Overall, the proportion of AYp-infected ALH ranged from 0 to 12.8% among the locations evaluated (2016, 2017, and 2018 data). Cross comparison of AYp prevalence from northern and southern sample locations revealed similar rates of AYp infection, ranging from 0 to 11.2% (northern) and 0–12.8% (southern) in 2016, whereas in 2017, AYp prevalence ranged from 0 to 10.2% (northern) and 0 to 6.6% (southern) and 2018 AYp prevalence ranged from 1.2 to 8.3% (northern) and 1.1 to 2.6% (southern). Estimated proportions of AYp-infected ALH are illustrated in Table 1. In 2016, a mean estimated AYp prevalence of 3.6 ± 4.4% was observed in populations of ALHs collected in southern locations in April, 3.2 ± 4.3% in the northern locations in August, and 0.6 ± 0.9% in September. In 2017, a mean estimated AYp prevalence of 1.4 ± 2.8% was observed in populations of ALHs collected among southern populations in April, and prevalence in the north of 6.3 ± 3.7% in May, 4.2 ± 4.8% in July, and 2.1 ± 1.8% in August. In 2018, a mean estimated AYp prevalence of 1.8 ± 0.7% was observed in populations of ALHs collected among southern populations in April, whereas prevalence of 8.3, 1.2, 3.3, 5.6, and 6.4% were observed in May, June, July, August, and September, respectively. No significant correlation was found between prevalence and regions (north and south), and prevalence and year (2016, 2017, and 2018). Statistical analysis can be found in Supp Table 6 [online only].

Aster Yellows Phytoplasma Subgroup Determination

Aster Yellows phytoplasma subgroups were characterized among populations collected from northern and southern locations. From the analysis of the 16S rRNA gene, it was determined that all of the AYp collected in this study belonged to either the AYp subgroup 16SrI-A or 16SrI-B. Based on these evaluations, it appeared that some ALHs were co-infected by both subgroups (16SrI-A + 16SrI-B). The overall percentage of subgroups for all 3 yr was 16SrI-A (45.9%), 16SrI-B (47.1%), and 16SrI-A + 16SrI-B (6.8%) (Fig. 2A). In 2016, the most common subgroup observed in the northern population was the 16SrI-A (57.1%), whereas the most common subgroup in southern populations was the 16SrI-B (66.6%). In 2017, the predominant subgroup observed in northern populations was the 16SrI-B (53.8%), whereas the predominant subgroup detected in southern populations was the 16SrI-A (66.6%). In 2018, the predominant subgroup observed in northern populations was the 16SrI-A (60%), whereas the predominant subgroup detected in southern populations was also 16SrI-A (58.3%) (Fig. 2B and C). When we examined the overall data set encompassing all AYp-positive insects over 3 yr, we found a significant correlation between year and individual subgroup proportion of co-infection (16SrI-A + 16SrI-B) (*P* = 0.05). A nonsignificant but notable trend between year and the proportion of 16SrI-A (*P* = 0.13) was also observed. Region was not a significant factor in the proportion of subgroups with 16SrI-A (*P* = 0.34) and 16SrI-B (*P* = 0.28). Statistical analysis can be found in Supp Table 6 [online only].

Prevalence of Aster Yellows Phytoplasma-Specific Effector Genes

Aster Yellows phytoplasma effectors manipulate molecular and biological functions of pathogen–host and pathogen–insect interactions.

Table 1. Estimated percentage of Aster Yellows phytoplasma-infected aster leafhoppers detected from northern and southern collection sites in 2016, 2017, and 2018

| Year | FieldID | North | | | | | South | |
		May (%)	Jun. (%)	Jul. (%)	Aug. (%)	Sep. (%)	FieldID	Apr. (%)
2016	16-01	–	–	–	0	1	16-10	4.7
	16-02	–	–	–	0	0	16-11	0
	16-03	–	–	–	0	2.4	16-12	5.5
	16-04	–	–	–	0	0	16-13	0.6
	16-05	–	–	–	6.5	0	16-14	0
	16-06	–	–	–	7.1	–	16-15	0
	16-07	–	–	–	11.2	0	16-16	5.2
	16-08	–	–	–	-	1	16–17	12.8
	16-09	–	–	–	1.2	–		
	Mean	–	–	–	3.2	0.6	–	3.6
2017	17-01	–	–	0	3.2	–	17-08	0
	17-02	–	–	8.5	0	–	17-09	0
	17-03	–	–	8.3	3.3	–	17-10	6.6
	17-04	–	–	0	–	–	17-11	0
	17-05	6.2	–	–	–	–	17-12	0.7
	17-06	2.7	–	–	–	–		
	17-07	10.2	–	–	–	–		
	Mean	6.3	–	4.2	2.1	–	*Mean*	1.4
2018	18-01	8.3	–	–	–	–	18-03	2.4
	18-02	–	1.2	3.3	5.6	6.4	18-04	2.6
							18-05	1.2
							18-06	1.1
	Mean	8.3	1.2	3.3	5.6	6.4		1.8

– indicates samples were not collected during this time period.

The prevalence of AYp effector genes SAP11, SAP54, SAP67, and SAP68 were examined among geographically distinct populations of ALH (presence of the effectors was only examined in samples that had a distinct subgroup classification and not in co-infected individuals). There was a higher prevalence of all four effector genes in AYp detected in northern populations of ALH in 2016. In contrast, only a single AYp-positive ALH sample from among all eight southern locations possessed any of the effector genes. However, this specific positive sample had amplification products of all four SAP examined (Table 2). In 2017, the distribution of the effector sequences in northern and southern populations was more evenly distributed between these geographical locations, but regional differences persisted with regard to AYp subgroups. Similar trends were observed in 2018, with effector genes being detected in similar proportions between geographic regions. When we examined the overall data set, encompassing all AYp-positive insects over the 3 yr of the investigation, there is a trend between year and detection of SAP54 ($P = 0.08$), and there was a significant correlation between year and the detection of SAP67 ($P = 0.04$), SAP68 ($P = 0.05$), and SAP11 ($P = 0.05$). There were significant correlations between regions and detection of SAP68 ($P = 0.02$) and a trend between regions and detection of SAP11 ($P = 0.06$). Statistical analysis can be found in Supp Table 6 [online only].

Genetic Variation Between Aster Leafhoppers Collected From Northern and Southern Populations

Genetic variation in the *CO1* gene of ALH was examined from individuals collected from both southern and northern field locations in 2016, 2017, and 2018. A 701-bp region of the *CO1* gene was amplified using PCR and the resulting product was sequenced to compare genetic differences among insects. Following sequence analysis and comparison of CO1 sequences, no genetic dissimilarities were observed among any of the samples, suggesting that the selected ALH used in this evaluation were genetically similar and are likely to consist of the same species bio-geographically. Sequence alignments of all adult insects evaluated in the current study also confirmed the leafhopper specie to be *M. quadrilineatus*.

Discussion

The ALH is regarded as an annually occurring and potentially damaging pest species of several ornamental, grain and vegetable crops in the upper Midwest region due to its competence as a vector of AYp. Alone, the direct feeding damage imposed by ALH would be regarded as only very minor, or even insignificant, but because the insect is considered the principal vector of AYp, it is regarded as a key pest species in susceptible crops. The AYp, which is introduced into plants via secretions from the accessory salivary glands of AYp-carrier insects, can lead to major crop damage through multiple negative phenotypes which result from AYp infection. While AY disease can be managed through the use of tolerant germplasm and broad-spectrum insecticides, the prevalence of AYp remains a major concern for growers. Whereas long-range movements and migration patterns of the ALH vectors have been well studied (Chiykowski et al. 1965, Frost et al. 2013), far fewer investigations focused on the presence of AYp prevalence within ALH and genotypes associated with AY disease throughout the contiguous United States. The current investigation showed that there are notable differences between rates of AYp subgroup designation and effector gene presence, both within and between two distinct geographical regions and consecutive years in the United States. The results provide producers and the scientific community with an improved understanding of the
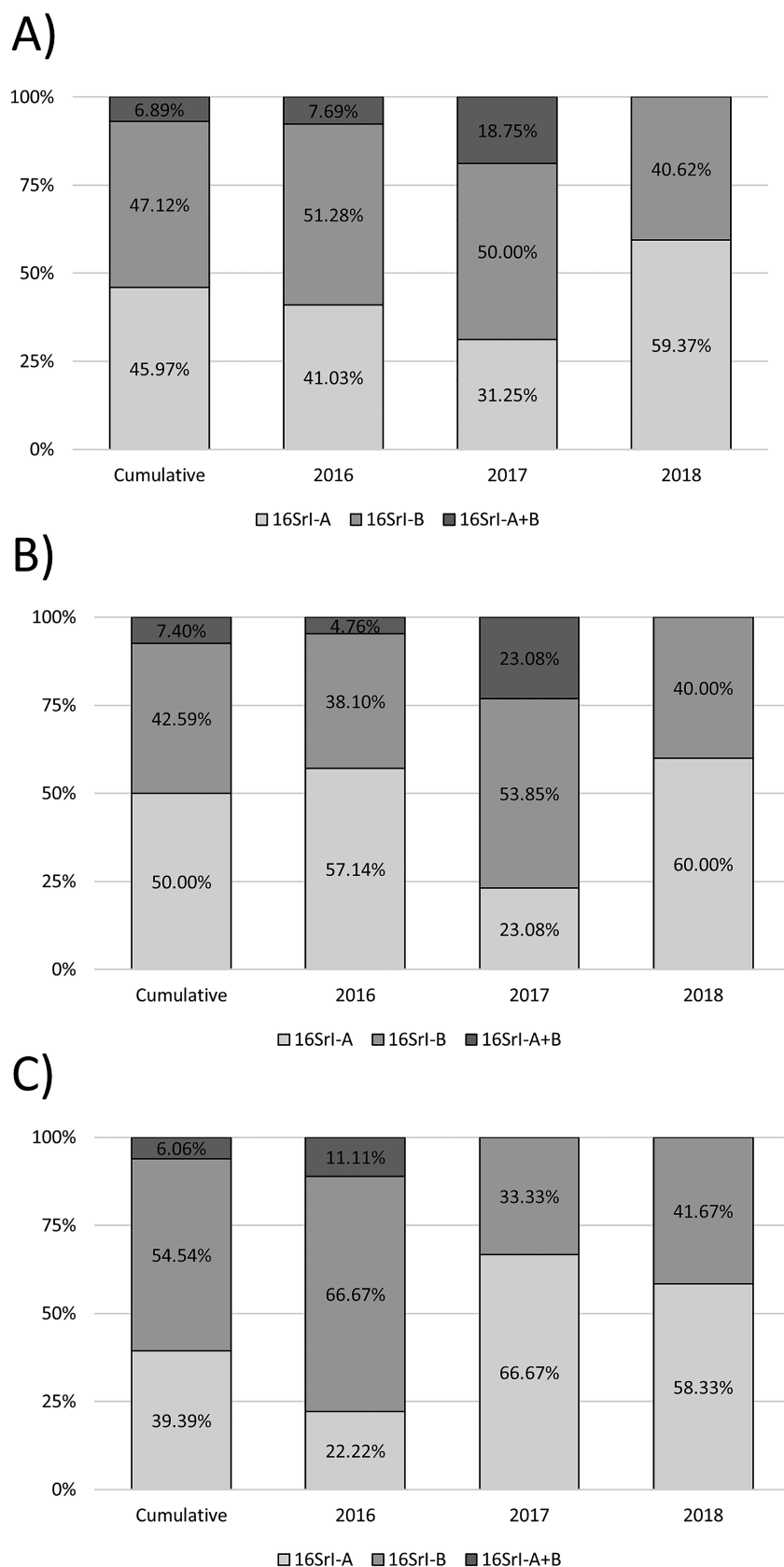
Fig. 2. Estimated mean proportion of Aster Yellows phytoplasma subgroups in A) both regions, B) the northern region, and C) the southern region.

Table 2. Estimated proportions of specific effector genes detected from among Aster Yellows phytoplasma-positive samples detected through PCR from northern and southern populations of aster leafhopper

	Specific effector genes			
	SAP11 (%)	SAP54 (%)	SAP67 (%)	SAP68 (%)
North 16SrI-A (2016)	58	25	41	75
South 16SrI-A (2016)	25	25	25	25
North 16SrI-B (2016)	0	0	0	0
South 16SrI-B (2016)	0	0	0	0
North 16SrI-A (2017)	100	0	100	100
South 16SrI-A (2017)	100	0	50	100
North 16SrI-B (2017)	0	0	0	14
South 16SrI-B (2017)	0	0	0	0
North 16SrI-A (2018)	66	25	66	75
South 16SrI-A (2018)	57	57	57	57
North 16SrI-B (2018)	12	0	0	12
South 16SrI-B (2018)	0	0	20	20

potential routes for the spread of AYp and the prevalence of AYp infection in ALH host insects.

Temporal variation in AYp infectivity in ALH likely influences the risk of AY disease development in susceptible crops. Higher rates of infected ALH will likely lead to higher disease potential in a susceptible crop. To date, it has been understood that AYp has principally been spread by migratory adults from southern populations in early spring (Chiykowski et al. 1965). Coincidentally, when peak movement and migration of ALH has historically been documented (mid-May; Frost et al. 2013), many susceptible crops have only just been planted or are emerging from the soil. In our investigations, we observed no statistical difference in the variation in ALH infectivity between regions (southern and northern) and year (2016, 2017, and 2018). A similar prevalence between region and year would suggest that AYp is relatively stable and consistently abundant at low levels throughout its geographical range over time, and between the discrete migratory and over-wintering populations in southern and northern regions, respectively. The relatively stable prevalence of AYp in ALH further suggest that the threat of AYp infection in host–plants may extend well into the growing season and beyond the initial arrival of the spring migrants.

Isolated AYp from potentially migratory (southern) and established (northern) adult ALH were determined to belong to either 16SrI-A or 16SrI-B subgroups (or both). Both 16SrI-A and 16SrI-B are subgroup designations for genotypes that are known agents which can result in the AY disease conditions in susceptible carrots (Lee et al. 2004). Examining across sample locations in both regions, the subgroups 16SrI-A and 16SrI-B were both found in adult ALH collected from southern and northern regions. In fact, some samples were co-infected with both 16SrI-A and 16SrI-B subgroups. The composition of the AYp subgroups examined differed over the 3 yr examined but did not different between geographic regions. The differences in subgroup proportions suggest that there is high interannual variation in the composition of AYp. This would suggest that the AYp infection within leafhopper populations is constantly changing and since leafhoppers acquire the AYp from infected host plants, the infection in host plants may also differ interannually. Supplementary investigations of the AYp in both crop and potential noncrop host plants in locations similar to those where adult ALH were collected, may be appropriate in future investigations to confirm this interannual hypothesis.

The second set of genetic markers examined in the current investigation was the presence of genes for secreted AY-WB proteins commonly associated with virulence of AYp. AYp effectors induce disease symptoms and promote ALH fecundity (Sugio et al. 2011b, Maclean et al. 2014, Orlovskis et al. 2016). Whereas all bacterial effectors examined were found in both geographical locations, the distribution and prevalence varied between year and region. The effector genes were more likely amplified from samples infected with 16SrI-A phytoplasma than with those infected with 16SrI-B, in agreement with PCR primers being designed to specifically amplify effector sequences from AY-WB, which is a 16SrI-A phytoplasma. However, AY-WB effector genes were detected in some samples supposedly infected with only 16SrI-B phytoplasma. The most likely explanation of this result is that these samples were also infected with the 16SrI-A phytoplasma, though in lower concentrations than the 16SrI-B phytoplasma, leading to the preferred amplification of the 16SrI-B 16S rDNA gene. Therefore, effector genes, rather than conserved 16S rDNA sequences, may work better for the detection of mixed phytoplasma infections.

All effector detection rates had a trend toward being different between the years examined and were significantly different for SAP11, 67 and 68, in agreement with these effector genes being located adjacently to one-another in the genome of AY-WB (Sugio et al. 2012). Effector sequence proportion similarly shows the same findings observed with temporal variation of subgroup proportions. It was further determined that the proportion of SAP68 significantly varied by geographical region. The difference in the proportion of SAP68 by region could indicate that the two populations of phytoplasma (southern and northern) are geographically different. However, it is more likely that the northern AYp populations represent phytoplasma brought from southern locations and AYp acquired from northern host plants that represent a similar, but slightly different, composition of AYp. Multiple factors could explain the differences observed, including the host plants that ALHs were collected from (e.g., wheat versus carrot), and the time of year in which collections were made (e.g., spring vs summer), which may effectively bottleneck specific genetic factors of the AYp. A limitation of this study could have resulted from our inability to isolate and solely quantify DNA concentrations of AYp within samples. Specifically, we quantified the DNA concentration of both the ALH and all associated microorganisms within our insect sampling unit. If AYp was in low abundance within a sample, we might have underestimated the occurrence or prevalence of the phytoplasma, as well as some SAP effectors with our current approach. Another consideration is that effector genes often locate on genomic regions that are mobile elements, which may be lost in some AYp lineages.

In this investigation, we observed a similar infection throughout all geographical locations sampled disproving out hypothesize that rates of AYp infection in leafhoppers would be higher in field collections from the northern United States when compared with those from the southern United States. Further, the data presented here represent differences in the genetic structure of AYp in the geographically distinct populations of ALH over time and region. We demonstrate that there is interannual variation in the composition of AYp. There is also evidence that the genetic composition of AYp may differ over region. Future investigations are needed to determine the pathogen–vector–host relationship between year and region, as additional sources of variation may be influencing AYp variation on a landscape scale. This report demonstrated the genetic variability within the AYp of Northern and Southern populations of ALH.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

Acknowledgments

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