Taxa of Leafhoppers Carrying Phytoplasmas at Sites of Ash Yellows Occurrence in New York State

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ABSTRACT

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Leafhopper (Homoptera: Cicadellidae) populations were sampled and leafhopper carriers of ash yellows (AshY) phytoplasmas were identified as first steps toward vector identification. Nearly 5,000 leafhoppers were collected in malaise traps at two sites of high AshY incidence in New York state in 1996 and 1997. These insects comprised 33 taxa, including representatives of 13 genera known to contain phytoplasma vectors. The most abundant genus was *Scaphoideus*, with numbers about six times greater than any other genus. A total of 1,632 insects were assayed individually for phytoplasmas by polymerase chain reaction (PCR) amplification of phytoplasmal 16S rDNA and restriction fragment length polymorphism analyses of PCR products using restriction enzymes *TaqI* and *RsaI* separately. Phytoplasmas were detected in 35 insects, all but one in the subfamily Deltocephalinae. AshY phytoplasmas were detected in 19 of 812 individuals of *Scaphoideus* spp. and 1 of 87 of *Colladonus clitellarius*. Phytoplasmas of the *Prunus* X-disease group were detected in 1 *Scaphoideus* sp., 4 individuals of *C. clitellarius*, and 4 of 83 *Scaphytopius acutus* individuals. Phytoplasmas of the aster yellows group were detected in 1 of 68 individuals of *Gyponana* spp. and 5 of *S. acutus*. AshY phytoplasma carriers merit testing for possible vector ability.

Most natural transmission of phytoplasmas occurs via phloem-feeding, homopteran insects, primarily leafhoppers (Cicadellidae). A few species of planthoppers (Cixiidae), psyllids (Psyllidae), and a froghopper (Cercopidae) are other known homopteran vectors (5,18,22,34,37). Three stinkbugs in the genus *Halyomorpha* (Hemiptera: Pentatomidae) are the only reported phytoplasma vectors not in the Homoptera (8,13). Phytoplasmas circulate and multiply within the bodies of their insect vectors. They can be transmitted into plants only after passing from the gut into the hemocoel and thence into salivary glands. Therefore, days to weeks elapse between phytoplasma acquisition and transmission to plants via the insect saliva (16,26,34).

Ash yellows (AshY), a disease putatively caused by phytoplasmas, induces slow growth and premature decline of *Fraxinus* spp., notably *F. americana* L. (white ash). The symptoms, impact, and pathogen of AshY have been characterized (11,32), but the insect vectors are not known. Although the leafhopper *Para-*

phlepsius irroratus (Say) and the froghopper *Philaenus spumarius* (L.) were reported to transmit naturally acquired phytoplasmas into ash test plants (20), the phytoplasmas were not identified, and laboratory colonies of these insect species failed to transmit AshY phytoplasmas (30).

Searches for vectors of phytoplasmas have been facilitated by molecular techniques, such as polymerase chain reaction (PCR), that have enabled the sensitive detection of phytoplasmal DNA in insects (4,5,12,18,19,37). Although detection of phytoplasmas in an insect does not prove a vector relationship (35), the technique is useful for narrowing the search for potential vectors among many taxa. Knowledge of the vectors of AshY would enable a more complete understanding of the spread and distribution of the pathogen. Vector host and habitat preference, movement, and life cycle all have profound influence on the epidemiology of phytoplasmal diseases (5,9). Objectives of this study were to examine the phenology of leafhopper populations on sites of AshY occurrence and to identify carriers of AshY phytoplasmas as first steps in vector identification.

MATERIALS AND METHODS

Insect collection. Insects were trapped on two sites of high AshY incidence near Ithaca, New York, in 1996 and 1997. Site 1 (elevation 305 m) was a plantation of *F. pennsylvanica* Marsh. (green ash) in which trees were 7 years old in 1996. Over 50% of the 720 trees present were known to

have AshY (33). Ground cover was composed primarily of *Asclepias syriaca* L., *Convolvulus arvensis* L., *Solidago rugosa* Mill., and grasses. Site 2 (elevation 427 m) was a small clearing in a dense stand of white ash saplings in the understory of a failed experimental plantation of *Prunus serotina* Ehrh. and *Pinus strobus* L. Other plants present included a *Fragaria* sp., *Prunus virginiana* L., *Rhamnus cathartica* L., *Rubus allegheniensis* Porter, *S. rugosa*, *Viburnum dentatum* L., and *V. lentago* L. AshY incidence on this site increased an average of 8% per year in 1991 to 1994 (31), and was estimated to be near 75% in 1996. The landscape surrounding both sites was a mixture of agricultural fields and forested plots. The distance between sites 1 and 2 was approximately 1.5 km.

One 6-m malaise trap (3) with wet collection heads (John W. Hock Company, Gainesville, FL) was set up at each site. An A-frame support system (36) was used at site 1 due to lack of large trees to which the trap could be anchored. Trapped insects fell into mason jars containing 80% ethanol. Insects were harvested and clean ethanol-loaded jars installed every 2 to 5 days. Collections were sorted in ethanol, and leafhopper, planthopper, and froghopper taxa were saved. Ethanol-preserved and pinned voucher specimens of each taxon were retained. Specimens in 80% ethanol were stored at -20°C. Species were identified or their identities verified by C. H. Dietrich of the Illinois Natural History Survey, Champaign.

Assays for phytoplasmal DNA. Total nucleic acid was extracted from preserved insects using a protocol slightly modified from one devised by J. Zhang and S. A. Miller (Ohio Agricultural Research and Development Center, Wooster; *personal communication*). Ethanol was blotted from insects before the extraction procedure. Individual insects <6 mm long were crushed and coarsely ground using a glass microcapillary tube in a 600- μ l Eppendorf tube containing 30 μ l of NaOH (0.5 N) for approximately 30 s. Then, 36 μ l of ice-cold Tris buffer (1.0 M, pH 8.0) was added. The suspension was centrifuged at $2,500 \times g$ for 8 min. The supernatant was transferred to a new tube, and 60 μ l of 70% ethanol (-20°C) was added. After 1 h at -20°C, the suspension was centrifuged at $3,500 \times g$ for 10 min. The ethanol was decanted and the pellet dried at 65°C. The pellet was resuspended in 60 μ l of Tris-EDTA (TE) buffer and stored at 4°C. Reagent volumes

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were multiplied by 1.5 to accommodate insects >6 mm long.

DNA was also extracted by the method of Dellaporta et al. (6,11) from *Catharanthus roseus* (L.) G. Don plants singly infected with phytoplasma strains AshY1 (ash yellows group), CX (X-disease group), and NJAY (aster yellows group). The latter two strains, obtained from L. N. Chiykowski and T. A. Chen, respectively, were used as standards for comparison with field-detected phytoplasmas in PCR-restriction fragment length polymorphism (RFLP) analyses.

Nucleic acid samples extracted from individual insects were diluted 1:30 with sterile deionized water, and 1 µl of dilute solution was used as template in each PCR mixture. Procedures and conditions for PCR were as described previously (11). Nucleic acids extracted from laboratory-reared *Macrosteles quadrilineatus* (Forbes) which had fed or not fed on aster yellows (AY)-phytoplasma-infected *Callistephus chinensis* (L.) Nees were used for positive and negative controls, respectively, in PCR. The phytoplasma-universal primer

pairs P1/P7 (7,29) and R16F2/R2 (15) were used to prime the amplification of fragments of ribosomal DNA. Primer pair P1/P7 mediates the amplification of a 1.8-kilobase (kb) fragment containing nearly the entire 16S rRNA gene, the interspacer region, and a portion of the 5' end of the 23S rRNA gene. Primer pair R16F2/R2 mediates amplification of a 1.24-kb fragment of the 16S rRNA gene internal to the P1/P7 priming sites. A 5-µl sample of each reaction mixture obtained with primer set P1/P7 was electrophoresed in a 0.7% agarose gel, and the amplimers were stained with ethidium bromide and visualized by UV transillumination. If an amplimer was observed, 2 µl of the primary reaction mixture was diluted 1:1,000 with sterile deionized water, and 1 µl of the diluted product was used as template in a second PCR using primer pair R16F2/R2. The second amplification was performed for two reasons. First, the primary amplification did not consistently yield enough DNA for phytoplasma differentiation by RFLP analysis. Second, it was desirable to use R16F2/R2 for identification purposes

because published RFLP profiles of nine distinct phytoplasma groups (14,15) were derived by analyses of R16F2/R2 amplimers. DNA from infected plant samples was diluted 1:10 with sterile deionized water, and phytoplasmal DNA was amplified using R16F2/R2. Amplimers were visualized as described above.

Amplimers obtained using R16F2/R2 were analyzed by restriction endonuclease digestion. Samples (5 µl) of each PCR product were digested separately with *TaqI* and *RsaI* (GIBCO BRL, Grand Island, NY) according to the manufacturer's instructions. Digestion of 16S rDNA of AshY phytoplasmas by *TaqI* yields an RFLP pattern unique to the AshY phytoplasma group (Group 16SrVII of Lee et al.; 14,15). Digestion by *RsaI* in conjunction with *TaqI* yields patterns that enable differentiation of phytoplasmas associated with AshY, AY (Group 16SrI), and *Prunus* X-disease (Group 16SrIII). Restriction fragments were separated by electrophoresis in a 2.5% agarose gel and were visualized as described above.

RFLP patterns obtained from R16F2/R2 amplimers were later verified via analyses

Table 1. Taxa and numbers of leafhoppers (Cicadellidae) collected at two sites of ash yellows incidence near Ithaca, New York, in 1996 and 1997 and detection of phytoplasmas in them

Leafhopper taxa	Site 1		Site 2		Proportion ^a	Identity, incidence ^b
	1996	1997	1996	1997		
Aphrodinae						
<i>Aphrodes bicincta</i> (Schrank)	7	11	9	4	0/12	
Coelidiinae						
<i>Jikradia olitoria</i> (Say)	35	25	28	18	0/46	
Agalliinae						
<i>Agallia</i> spp.	26	14	9	12	...	
<i>Agalliopsis</i> spp.	46	17	21	13	...	
Gyponinae						
<i>Gypona</i> sp.	3	2	4	0	...	
<i>Gyponana scrupulosa</i> (Spångberg)	7	4	0	0	0/6	
<i>Gyponana</i> spp.	45	38	23	16	1/68	AY, 1
Cicadellinae						
<i>Draeculacephala</i> spp.	77	53	45	49	0/24	
<i>Graphocephala</i> spp.	104	68	49	25	0/24	
<i>Tylozygus bifidis</i> (Say)	27	14	16	19	0/24	
Deltocephalinae						
<i>Allygus atomarius</i> (Fabricius)	33	28	0	4	0/36	
<i>Amblysellus curtisii</i> (Fitch)	26	31	19	6	...	
<i>Anoscopus</i> sp.	13	19	16	22	...	
<i>Athysanus argentarius</i> (Metcalf)	6	3	2	0	0/6	
<i>Chlorotettix tergatus</i> (Fitch)	17	6	9	11	0/12	
<i>Chlorotettix</i> sp.	15	15	11	7	0/18	
<i>Colladonus clitellarius</i> (Say)	55	61	8	3	5/87	AshY, 1; X-disease, 4
<i>Endria inimica</i> (Say)	42	33	14	18	0/24	
<i>Fieberiella florii</i> (Stål)	6	10	14	4	0/20	
<i>Idiodonus kennicotti</i> (Uhler)	24	14	15	7	0/24	
<i>Macrosteles</i> spp.	120	99	78	52	0/60	
<i>Orientus ishidae</i> (Matsumura)	8	17	6	14	0/24	
<i>Osbornellus</i> spp. ^c	86	104	67	62	0/153	
<i>Paraphlepsius irroratus</i> (Say)	13	16	9	14	0/24	
<i>Paraphlepsius</i> sp.	5	4	6	3	...	
<i>Prescottia lobata</i> (Van Duzee)	47	22	54	36	0/60	
<i>Scaphoideus intricatus</i> Uhler	14	7	11	9	1/34	AshY, 1
<i>Scaphoideus</i> spp. ^d	699	546	491	359	19/778	AshY, 18; X-disease, 1
<i>Scaphytopius acutus</i> (Say)	37	18	22	9	9/83	AY, 5; X-disease, 4
Other species	54	25	61	35	...	

^a Proportion carrying phytoplasmas = number in which phytoplasmas were detected/total tested in 1996 and 1997.

^b Phytoplasma identity and incidence of detection. AshY = ash yellows; AY = aster yellows; X-disease = *Prunus* X-disease.

^c Includes *O. auronitens* Provancher + other species.

^d Includes at least *S. densus* Delong and Beery, *S. elongatus* Delong and Beery, *S. minor* Osborn, *S. obtusus* Osborn, and *S. titanus* Ball.

of new PCR products obtained with primer pair P1/P7 from the original insect nucleic acid samples. This verification was made possible through the use of an optimized P1/P7 protocol that became available after the bulk of the phytoplasmal DNA assays had been completed.

RESULTS

Insect collection. Approximately 5,000 leafhoppers were collected at the two sites. This collection comprised 33 taxa and included 13 genera in the subfamilies Aphrodinae, Gyponinae, and Deltocephalinae that contain known vectors of phytoplasmas (22): species of *Aphrodes*, *Chlorotettix*, *Colladonus*, *Endria*, *Fieberiella*, *Gyponana*, *Idiodonus*, *Macrosteles*, *Orientus*, *Osbornellus*, *Paraphlepsius*, *Scaphoideus*, and *Scaphytopius* (Table 1). More insects were trapped in 1996 than in 1997 at both sites, and more were trapped at site 1 than at site 2. *Scaphoideus* spp. were collected most frequently. Due to identification difficulties, however, only *S. intricatus* Uhler was identified prior to assay for phytoplasmal DNA. Six other species of *Scaphoideus* (*S. densus* Delong and Beery, *S. elongatus* Delong and Beery, *S. minor* Osborn, *S. obtusus* Osborn, and *S. titanus* Ball) were ultimately identified among preserved specimens. The most numerous leafhoppers after *Scaphoideus* spp. were *Macrosteles* spp. and *Osbornellus* spp., which were collected at less than 20% of the frequency of *Scaphoideus*. The peak of the *Scaphoideus* population was variable between site and year, occurring between 31 July and 19 September (Fig. 1). Collections of *Gyponana* spp., *Osbornellus* spp., and *Prescottia lobata* (Van Duzee) had apparent frequency distributions similar to that of *Scaphoideus* spp. One leafhopper, *Jikradia olitoria*, did not appear in the traps until early August, and its numbers peaked in early October. Numbers of *Colladonus clitellarius* (Say) peaked earlier (mid-July) than those of other taxa. This species was approximately 10 times more common at site 1 than site 2. *Macrosteles* spp. exhibited two peaks in population, one in late July and one in early September. Other leafhopper taxa had too few individuals to represent seasonal trends, or time-of-collection data were inadequate to suggest one. *Gyponana scrupulosa* (Spångberg) was the only leafhopper not collected at both sites.

Detection and identification of phytoplasmas. A total of 1,632 insects were assayed individually for phytoplasmas. Phytoplasmal DNA was detected in 35 leafhoppers, all but 1 in the subfamily Deltocephalinae. PCR products were obtained from >95% of reaction mixtures using nucleic acid samples from *M. quadrilineatus* that had fed on AY-infected *Callistephus chinensis* and from all reaction mixtures using template DNA extracted from *Catharanthus roseus* in-

fectured with reference strains AshY1, CX, and NJAY. No phytoplasmal DNA was detected in *M. quadrilineatus* reared on healthy plants. Phytoplasmas of the AshY, AY, and *Prunus* X-disease groups were identified based on comparison of R16F2/R2 RFLP profiles (Fig. 2) to those of Lee et al. (14,15) and to those of reference strains AshY1, CX, and NJAY.

The percentage of insects carrying phytoplasmas was less than 11% in each of the five insect taxa in which phytoplasmas were detected (Table 1). AshY phytoplasmas were detected in *Scaphoideus intricatus*, other *Scaphoideus* spp., and *Colladonus clitellarius*. Phytoplasmas of the *Prunus* X-disease group were detected in *Scaphoideus* sp., *C. clitellarius*, and *Scaphytopius acutus*. Phytoplasmas of the aster yellows group were detected in *Gyponana* sp. and *S. acutus*. Phytoplasmas were not detected in 58 specimens of *Philaenus spumarius*, a froghopper species previously implicated as a potential vector of AshY (20).

Phytoplasmas were detected in *Scaphoideus* spp. and other taxa throughout the collection period at both sites. There were no clear relationships among the year, time of collection, numbers collected, and the proportion of insects in which phytoplasmas were detected.

DISCUSSION

Leafhoppers containing phytoplasmas could be vectors, dead-end hosts, or non-host carriers (27,35). Overall, the proportion of insects in which phytoplasmas were detected was low, approximately 2% of those assayed. This proportion is consistent

Fig. 1. Numbers of *Scaphoideus* spp. collected at two sites of ash yellows occurrence near Ithaca, New York, in 1996 and 1997. Dates are final days of respective collection periods.

with findings from some other phytoplasmal systems. Mpunami et al. (21), in their search for vectors of coconut lethal disease in Tanzania, were able to amplify phytoplasmal rDNA from only 7 insects among more than 5,000 individuals tested. Poggi-Pollini et al. (24) detected phytoplasmas in only a small proportion of leafhoppers in their search for possible vectors of European stone fruit yellows. Davies and Eyre (5) found the proportion of the pear psyllid, *Cacopsylla pyricola* (Foerster), carrying pear decline phytoplasmas ranged from <1% to near 40%, depending on site characteristics and time of year.

The low frequency of phytoplasma carriers detected in this study may also be due to sensitivity of the detection technique used. Both the DNA extraction procedure and the use of only direct PCR for detection of phytoplasmal DNA presumably resulted in lower frequencies of detection than could have been obtained using more rigorous DNA extraction or nested PCR. The latter procedure has enabled extremely sensitive detection of phytoplasmas in both plants and insects (4,12). We chose to pay attention only to those insects for which direct PCR products were observed, thus

Fig. 2. Representative restriction fragment length polymorphism profiles, in 2.5% agarose, of polymerase chain reaction products amplified with primer pair R16F2/R2 from individual leafhoppers and phytoplasma reference strains AshY1, CX, and NJAY. (A) Products digested by *TaqI*. (B) Products digested by *RsaI*. S1 and S2 are 1-kilobase and 100-base pair ladders, respectively (GIBCO BRL, Grand Island, NY). AshY = ash yellows; AY = aster yellows; PX = *Prunus* X-disease.

arbitrarily disregarding insects that may have harbored low or nascent populations of phytoplasmas detectable with nested PCR. The rationale for this approach was that it is biased toward detection of phytoplasmal populations that have increased to high titer within their carriers.

Seasonal population trends could be discerned for several of the most numerous leafhopper groups in the collections. These groups exhibited single population peaks between late July and early September, perhaps indicating a single generation per year. Their numbers diminished greatly before the first frosts in autumn. The delay in population peaks of *Scaphoideus* spp. and other taxa at site 2 was likely due to more shade and lower temperature, because the site was at higher elevation and was wooded. Only *Macrostes* spp. exhibited a seasonal population trend that indicated multiple generations. The seasonal trends observed may have been verified or extended if we had begun collecting in late spring. Our July starting dates reflected the desire to collect insects during the period in which phytoplasma acquisition from leaves would likely be most frequent.

The insect collection method facilitated the trapping of a large number of airborne adult leafhoppers. Insects and their DNA were preserved immediately upon falling into the ethanol (25). This method also allowed collection of taxa potentially moving between ash trees or from ground-cover plants to and from ash. Leafhopper taxa obtained with a sweep net directly from ash (*data not shown*) or collected previously from sticky traps exposed at sites of AshY incidence near Ithaca (W. A. Sinclair, *unpublished*) were among those found in the malaise-trap collections.

Scaphoideus spp., in addition to being the largest group of leafhoppers collected, also yielded the most samples testing positive for phytoplasmal DNA, almost entirely that of AshY phytoplasmas. Barnett (2) indicated that *Scaphoideus* spp. are not common and that large collections are not usually made. Therefore, little research has been completed on the host or plant relationships of this genus. Identification of the *Scaphoideus* genus is problematic. Its taxonomy is based primarily on morphology of male genitalia, making identification of females and nymphs difficult using available keys such as that by Barnett (2). In our study, females were collected most frequently, often with no associated males that appeared to be of the same species. As a further complication, intraspecific variability in genital characters of males may cause uncertainty in identification (2).

The genus *Scaphoideus* contains vectors of several other phytoplasmal pathogens of woody plants. *S. titanus* transmits the causal agent of flavescence dorée, a serious disease of grapevines in Europe. In New York, *S. titanus* was implicated as a poten-

tial vector of grapevine yellows (17). *S. luteolus* Van Duzee is a vector of elm yellows (1). *S. carinatus* Osborn and *S. diutius* DeLong and Mohr were shown to transmit the phytoplasma associated with X-disease of stone-fruit trees (23,28). Additional vector relationships of *Scaphoideus* spp. and tree-infecting phytoplasmas will likely be elucidated if the difficulty and uncertainty of identifying these insects is reduced. Only one *Scaphoideus* species, *S. obtusus*, has been reported as collected from *Fraxinus* spp. (2). It is probable that *S. intricatus* and other *Scaphoideus* spp. in which AshY phytoplasmas were detected had fed on ash, because it was the dominant plant at each site and because AshY phytoplasmas have not been detected in searches of other plant species on sites of high AshY incidence (10).

One individual of *Colladonus clitelarius* carrying AshY phytoplasmas was identified. This species is a known vector of eastern X-disease of peach (22). Perhaps it may harbor or transmit more than one type of phytoplasma. Precedents for one species serving as a host for more than one phytoplasma exist. For example, *C. montanus* (Van Duzee) and *Fieberiella florii* both transmit western X-disease and aster yellows phytoplasmas (22). Detection of AshY phytoplasmas in *C. clitelarius*, *S. intricatus*, and other *Scaphoideus* spp. indicates that insects in these taxa should be tested for AshY vector ability.

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