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RESEARCH ARTICLE

Detection, characterisation and transmission by *Macrosteles* leafhoppers of watercress yellows phytoplasma in Hawaii

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Abstract

A new yellows disease of watercress (*Nasturtium officinale*) in Hawaii has symptoms of reduced leaf size, leaf yellowing and crinkling, and occasionally witches' brooms. This disease is found on all watercress farms on Oahu but has not yet been found on other Hawaiian islands. Watercress plants were tested for phytoplasma infection by polymerase chain reaction assays using phytoplasma-specific primers. Amplicons of the expected sizes were produced from all symptomatic plants but not from healthy plants raised from seed. Phylogenetic analysis of the 16S rRNA gene indicated that watercress yellows was caused by a phytoplasma in the aster yellows group, with sequence similarity to onion yellows from Japan. Six weed species collected from the vicinity of affected watercress farms, *Amaranth* sp., *Eclipta prostrata*, *Emilia sonchifolia*, *Plantago major*, *Myriophyllum aquaticum* and *Sonchus oleraceus*, were also determined to be hosts of this phytoplasma. Leafhoppers, identified as *Macrosteles* sp. (Hemiptera, Cicadellidae), collected from symptomatic watercress transmitted this phytoplasma to watercress, plantain and lettuce (*Lactuca sativa*) in greenhouse experiments.

Introduction

Phytoplasmas are noncultivable, phloem-limited bacterial pathogens that cause many serious diseases of woody and herbaceous plants worldwide (McCoy *et al.*, 1989). The classification of phytoplasmas has been based on restriction fragment length polymorphism (RFLP) and sequence analysis of several phytoplasma genes (Lee *et al.*, 1992, 1998; Seemüller *et al.*, 1998). Lee *et al.* (1998) differentiated 34 representative phytoplasma strains into 14 major groups or clades and 41 subgroups based on RFLP analysis of the 16S rRNA gene. Smart *et al.* (1996), Kirkpatrick *et al.* (1994) and Schneider *et al.* (1995) analysed sequence data from the spacer region that separates the 16S and 23S ribosomal genes of phytoplasmas and developed a series of PCR primer pairs that are also useful in differentiating phytoplasmas from one another. PCR primers that amplify ribosomal protein genes *rps19*, *rpl22* and *rps3* from phytoplasmas have also been useful in classifying certain phytoplasma groups (Lim & Sears, 1992). Schneider *et al.* (1997) developed detection and classification schemes based upon analysis of the elongation factor Tu (*Tuf*), a region of the phytoplasma genome that is more variable than either the 16S rDNA or spacer regions. These and other molecular-based typing approaches have been used as benchmarks for phytoplasma detection and classification, primarily because of the absence of other typing methods. Phytoplasmas are currently placed within the genus '*Candidatus* Phytoplasma' in the class *Mollicutes*, based on phylogenetic analyses of these conserved genes. Within this genus, 13 subtaxa are currently described whose 16S rRNA gene sequences differ by more than 97.5% from one another (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004).

Watercress (*Nasturtium officinale* L.) is grown in Hawaii on small farms of 10 acres or less that are irrigated by naturally occurring fresh water springs. The crop is

vegetatively propagated from cuttings, and growers can harvest up to eight crops per year from established farms. There are few diseases associated with this crop in Hawaii; the primary limitation on production has been the diamondback moth (*Plutella xylostella*, Lepidoptera), whose larvae can consume large amounts of the leafy canopy if not controlled. Recently, watercress in Hawaii has been afflicted with a yellows disease with symptoms of leaf yellowing and crinkling, reduced leaf size and occasionally witches' brooms (Borth *et al.*, 2002). This disease is present in all the watercress farms on the island of Oahu but has not yet been found on any of the other Hawaiian Islands. Nutritional deficiencies or toxicities, water salinity, and insect or mite feeding damage were investigated but were not implicated in the aetiology of this disease (W.B. Borth, unpublished data).

However, we have found that leafhoppers commonly collected from affected watercress farms are a species newly introduced to Hawaii. These leafhoppers have been tentatively identified as *Macrosteles* sp. nr. *severini* (*sensu* A. Hamilton, herein *Macrosteles*). The location of origin of this leafhopper is not known. The association of this invasive leafhopper with the disease (both spatially and temporally), as well as the symptoms displayed on the watercress, suggested that phytoplasmas might be involved in the aetiology of this disease. We used molecular approaches to test if phytoplasmas were associated with this watercress disease and also conducted transmission experiments to determine if this leafhopper found in large numbers on afflicted farms could be a vector of this possible aetiological agent. A preliminary report has been previously published (Borth *et al.*, 2002).

Materials and methods

Plant material

Watercress plants with and without yellows symptoms were collected from 12 watercress farms on Oahu. Total DNA was extracted from fresh tissues or from tissues that had been stored at −20°C for up to 2 months. DNA was extracted from 100–200 mg of leaf, stem or root tissues using DNeasy® kits (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. We used the same procedure to extract DNA from 42 weed species found either within or in the immediate vicinity of watercress farms that had high disease incidences and large *Macrosteles* populations. We tested 3–10 individuals of each weed species. Plantain (*Plantago major* L.) was grown from seed collected from various locations on the island of Oahu. Barley (*Hordeum vulgare* L.) and lettuce (*Lactuca sativa* L.) cultivars and healthy watercress were grown from commercially available seeds.

Leafhopper rearing

Macrosteles leafhopper colonies were established on barley with insects collected from watercress farms on the island of Oahu. Adults collected from the field were allowed to oviposit on barley seedlings in cages, and hatching nymphs were transferred weekly onto fresh barley seedlings in cylindrical cages. This was repeated until the insects matured. Adult insects from the colonies were allowed to oviposit on barley seedlings in the same cages for 1 week, after which adult leafhoppers were removed from the cages. Barley plants with eggs were kept in cages for 2 weeks to allow nymphs to hatch. Nymphs were then transferred to fresh barley seedlings at weekly intervals until the insects had matured into adults. Barley plants always tested negative for phytoplasma, and most phytoplasmas are not vertically transmitted (Lee *et al.*, 2000). Insects randomly collected from these colonies always tested negative in PCR analyses.

Leafhopper DNA isolation

DNA was isolated from leafhoppers by grinding individuals with plastic pestles in 250 μL extraction buffer containing 2% (w/v) hexadecyl-trimethylammonium bromide, 1.4 M NaCl, 100 mM Tris and 20 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0. Extracts were incubated at 65°C for 30 min, cooled to room temperature and extracted with an equal volume of chloroform : isoamyl alcohol (24:1). Nucleic acids precipitated from the recovered aqueous phase by adding 2.5 volumes ethanol were collected by centrifugation, washed in 70% ethanol, dried and resuspended in 50–100 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was used immediately or stored at −20°C before use in PCR.

Phytoplasma detection and characterisation assays

Phytoplasma-specific PCR primer pairs P1/P7 (Schneider *et al.*, 1995), R16mF2/R16mR1 (Lee *et al.*, 1993) and fU5/rU3 (Lorenz *et al.*, 1995), which prime the amplification of 16S rDNA from all phytoplasmas, were used in PCR assays to determine the presence or absence of phytoplasma DNA in symptomatic and nonsymptomatic watercress and other plants growing in the vicinity of watercress farms. Other primer pairs tested include P1/AYint that primes the amplification of the spacer region between the 16S and 23S rDNA of aster-yellows-type phytoplasmas (Smart *et al.*, 1996), fTufAY/rTufAY (Schneider *et al.*, 1997) that primes aster-yellows-specific sequences from the *Tuf* gene and rpF1/rpR1 that primes

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sequences from the ribosomal protein gene operon of phytoplasmas (Lim & Sears, 1992). Primer pairs P1/P7 and fTufAY/rTufAY were also used to amplify phytoplasma DNA from leafhoppers. All reactions were performed in volumes of 30–50 μL using Amplitaq Gold® Taq polymerase (Applied Biosystems, Inc., Foster City, CA, USA), 20–100 ng DNA and reaction conditions as specified for each primer pair (see Lim & Sears, 1992; Lee *et al.*, 1993; Lorenz *et al.*, 1995; Smart *et al.*, 1996; Schneider *et al.*, 1995, 1997). Reaction products were separated on 1% (w/v) agarose gels in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) and visualised under ultraviolet light after staining with ethidium bromide.

The PCR products amplified from watercress and other test plants by Platinum High-Fidelity® Taq polymerase (Invitrogen, Inc., Carlsbad, CA, USA) and primer pairs P1/P7, fTufAY/rTufAY or rpF1/rpR1 were used for cloning and sequencing. Amplicons were ligated into plasmid pGEM-T Easy® (Promega, Inc., Madison, WI, USA) and used to transform *Escherichia coli* DH5α competent cells. Plasmid DNA was isolated from 3 to 5 mL of liquid cultures of these clones grown overnight at 37°C using QIAprep Spin® miniprep kits (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Isolated plasmid DNAs were sequenced on both strands using appropriate primers with the Applied Biosystems 377XL DNA sequencers at the Greenwood Molecular Biology Facility at the University of Hawaii. The 16S rDNA sequences of nine phytoplasmas and *Acholeplasma* were obtained from GenBank for phylogenetic analysis. The accession numbers and acronyms are AYWB, aster yellows witches'-broom (CP000061); BB, big bud (L33760); CPh, clover phyllody (L33762); MBS, maize bushy stunt (AY265208); OY, onion yellows (AP006628); PWB, peanut witches'-broom (L33765); SAY, severe aster yellows (M86340); STOL, stolbur (X76427); WAY, watercress aster yellows (AY665676) and *Acholeplasma laidlawii* (M23932). Partial 16S rDNA sequences (1423 bp) of these mollicutes were aligned with ClustalX v.1.82 (Chenna *et al.*, 2003) and the alignment manually checked. Phylogenetic analyses were conducted using PAUP* 4.0b10 (Swofford, 2002); maximum parsimony and distance (Neighbour-Joining) searches were performed using all defaults with 1000 bootstraps.

Insect transmission of phytoplasma

Two approaches were used to determine if *Macrosteles* could vector the agent causing watercress yellows. First, we collected adult insects from farms with symptomatic watercress plants. These insects were then transferred to healthy plantains for either 2-, 4-, or 12-day inoculation access periods. The plants were then sprayed to kill adult insects and hatching nymphs and were kept at 25–28°C for symptom development. The number of insects caged on plantain was either 5 or 10 adults per plant, and the number of test plants varied from 6 to 20, depending on the number of insects collected. This experiment was replicated four times. In another experiment, leafhoppers collected as described previously were placed on healthy lettuce plants for a 5-day inoculation access period and then removed as described above, and the plants were kept at 25–28°C. In this experiment, five insects were caged on each of 18 lettuce plants. Specific parameters of these inoculation experiments are summarised in Table 1.

The second approach was to collect symptomatic plants from watercress farms and allow laboratory-reared leafhopper nymphs (third or fourth stage instars) to acquire the pathogen under controlled conditions. Two replicates were performed. Symptomatic plants that had tested positive for phytoplasma in PCR assays were collected from the field and maintained in shallow trays filled with coarse silica sand flooded with water to simulate conditions in watercress fields. Twenty nymphs were kept on each of three or eight symptomatic watercress plants for a 3-day acquisition access period at 25–28°C. We used relatively short acquisition access periods because leafhopper survival was low under these conditions, probably because of the loss of vigour of symptomatic transplanted watercress plants. Surviving leafhoppers from each symptomatic plant were transferred to barley seedlings for 14 or 18 days at 25–28°C. Leafhoppers were

Table 1 Transmission of watercress phytoplasma by *Macrosteles* sp. nr. *severini* collected from two watercress farms with high (Farm A) and low (Farm B) incidence of watercress yellows

Experiment No.	Date	Farm	Test Plants	No. of Test Plants	No. of Insects Per Plant	Inoculation Period (Days)	No. of PCR (+) Plants
1	29 May 2003	A	Lettuce	18	5	5	8
2	12 November 2003	B	Plantain	6	10	2	1
3	10 December 2003	B	Plantain	9	5	4	0
4	03 March 2004	B	Plantain	20	10	2	0
5	24 March 2004	B	Plantain	20	10	12	2

transferred to fresh barley seedlings each week. Our goal was to maintain the leafhoppers on a host plant that allowed insect survival during the latent period. Surviving leafhoppers were transferred in groups of five individuals to healthy watercress plants in the greenhouse. Details of these transmission experiments are summarised in Table 2. All plants were kept at 25–28°C, monitored for symptom development and analysed by PCR to confirm phytoplasma infections.

Results

Phytoplasma detection

The PCR assays with phytoplasma- and aster-yellows-group-specific primers produced amplicons of the expected sizes from all symptomatic plants, whereas no products were amplified from healthy plants raised from seed (Fig. 1). All samples from symptomatic watercress collected from various farms on Oahu produced amplicons of identical sizes using these primer pairs. Weed plants *Eclipta prostrata, Emilia sonchifolia, Sonchus oleraceus, Myriophyllum aquaticum, P. major* and *Amaranth* sp. were also identified as hosts of the phytoplasma in the field using this same approach. Sixty percent of *Eclipta prostrata*, 50% of *Emilia sonchifolia,* 30% of *S. oleraceus,* 40% of *M. aquaticum,* 30% of *P. major* and 30% of *Amaranth* sp. were found to be infected based on PCR analyses. Leafhoppers collected from farms with watercress yellows also tested positive by PCR for the WAY phytoplasma (data not shown). The sequences of the amplicons produced from weeds and leafhoppers were identical (100% similarity) to those produced from symptomatic watercress collected from the field and from plants used in transmission tests (data not shown).

Watercress yellows phytoplasma characterisation

Sequence analyses confirmed the association of watercress yellows with a phytoplasma (WAY) similar to OY, an aster yellows strain from onions in Japan (Namba *et al.*, 1993), and SAY, a severe strain of western North American aster yellows phytoplasma (Kuske & Kirkpatrick, 1992). The OY and SAY phytoplasmas belong to the 16SrI-B phytoplasma group (Lee *et al.*, 1998; Seemüller *et al.*, 1998). The WAY sequence amplified using primer pair P1/P7 is 100% similar to OY and differs from the SAY sequence at two positions. Other WAY amplicons sequenced, including the spacer region between the 16S and 23S rDNA genes, and the 5′ portion of the 23S rDNA, are 100% similar to that of OY and SAY. Phylogenetic trees generated using the 16S rDNA sequence of WAY and other phytoplasmas and a mollicute confirmed that it belongs within the aster yellows clade (Fig. 2). Sequence and phylogenetic analyses of the *Tuf* gene from WAY provided further evidence that the phytoplasma from watercress in Hawaii is most closely related to OY from Japan and more distantly related to SAY (data not shown). The WAY *Tuf* sequence differs from the OY at one position and from SAY at seven positions. Sequence analysis of the cloned rpF1/rpR1 amplicon also indicated that WAY is closely related to OY, but no sequence information is available for the *rp* genes of SAY. The sequences generated with primer pairs fTufAY/rTufAY and rpF1/rpR1 have been deposited in GenBank with accession numbers DQ402361 (*Tuf*) and DQ406588 (*rp*). Combined, these results suggest that WAY may have been introduced into Hawaii from Japan.

Insect transmission

The infectivity levels of leafhoppers collected from watercress farms afflicted by WAY were variable when assayed with transmission experiments. About 50% of lettuce test plants became infected with phytoplasma when insects collected from symptomatic watercress with high leafhopper populations were used in transmission tests (Farm A, Table 1). However, leafhoppers collected from a watercress farm with a low incidence of WAY transmitted the phytoplasma at rates of only ~10% to test plants when groups of 5 or 10 insects were caged on individual test plants (Farm B, Table 1). Leafhoppers that were raised on barley for multiple generations and then allowed acquisition access of 3 days on infected watercress only transmitted the phytoplasma to healthy watercress in a single case (Table 2). The short acquisition access periods used in these experiments with laboratory-reared leafhoppers may have reduced the efficiency of acquisition from infected watercress and led to lowered transmission

Table 2 Transmission of watercress phytoplasma by laboratory-reared *Macrosteles* sp. nr. *severini* using watercress as host plants. All insects were given 3-day acquisition access periods on field-collected, WAY-infected watercress plants

Experiment No.	No. of Infected Plants	No. of Insects per Plant	Latent Period (Days)	No. of Test Plants	No. of Insects per Plant	Inoculation Period (Days)	No. of PCR (+) Plants
1	3	20	14	7	5	28	1
2	8	20	18	5	5	14	0

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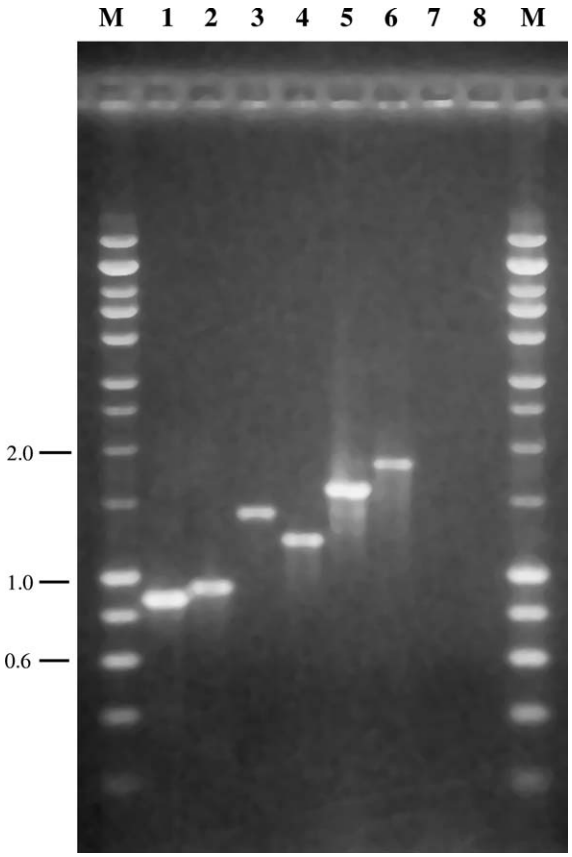

Figure 1 Agarose gel of PCR products amplified from symptomatic watercress using phytoplasma-specific primer pairs. Lanes: M, molecular weight marker (sizes in kilobases); lane 1, primer pair fU5/rU3; lane 2, primer pair fTufAY/rTufAY; lane 3, primer pair R16mF2/R16R1; lane 4, primer pair rpF1/R1; lane 5, primer pair P1/AYint and lanes 6, 7 and 8, primer pair P1/P7. Lanes 1, 2, 3, 4, 5 and 6 contained DNA amplified from symptomatic watercress. Lane 7 contained DNA from healthy watercress. Lane 8 is a water control.

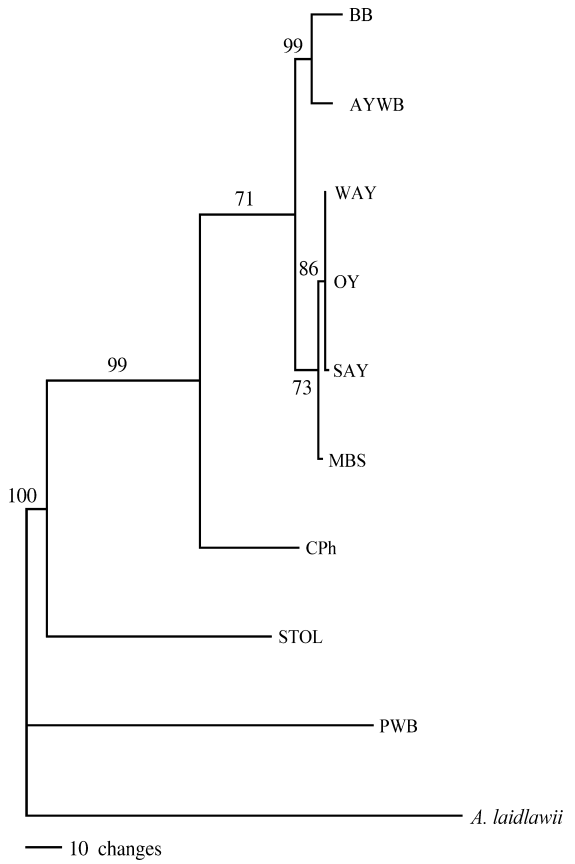

Figure 2 Phylogenetic placement of watercress aster yellows (WAY) using 16S rDNA sequences. One maximum parsimony tree is shown; other parsimony trees and the neighbour-joining tree were similar. The sequences used in the analysis and their GenBank accession numbers are: AYWB, aster yellows witches'-broom (CP000061); BB, big bud (L33760); CPh, clover phyllody (L33762); MBS, maize bushy stunt (AY265208); OY, onion yellows (AP006628); PWB, peanut witches'-broom (L33765); SAY, severe aster yellows (M86340); STOL, stolbur (X76427); WAY, watercress aster yellows (AY665676) and *Acholeplasma laidlawii* (M23932). Values on nodes represent percentage of 1000 bootstraps using maximum parsimony. Bootstrap values below 70% are not shown.

rates of WAY to watercress plants. These transmission experiments demonstrate that this *Macrosteles* species is capable of vectoring WAY to watercress and other unrelated plants.

Discussion

Diseases caused by phytoplasmas were unknown in Hawaii until recently, when an indigenous shrub, *Dodonaea viscosa* (L.) Jacq., was demonstrated to be infected with a phytoplasma related to the X-disease phytoplasma (16SrIII group) (Borth *et al.,* 1995). This disease affects *D. viscosa* on all the major Hawaiian islands, but the phytoplasma associated with it has not been found to infect any other host in the state (Borth *et al.,* 1999), and the insect vectors of this disease are not known. The work presented here shows that another phytoplasma has now become established in Hawaii. Using molecular tools, we have confirmed that diseased watercress from 12 farms on Oahu were infected with a phytoplasma with high sequence similarity to OY from Japan (Namba *et al.,* 1993), which is classified as a 16SrI-B group member (Lee *et al.,* 1998; Seemüller *et al.,* 1998). No phytoplasmas from this group have previously been confirmed from Hawaii.

The incidence of phytoplasma infection in weed host plants sampled from areas near watercress farms with high levels of yellows disease and large populations of *Macrosteles* was greatest in plants of the family

Asteraceae, as might be expected for aster-yellows-type phytoplasmas. In addition, several host plants, including *Eclipta prostrata*, *Emilia sonchifolia* and *M. aquaticum*, that have not been previously reported as hosts of aster yellows (McCoy *et al.*, 1989) were found to be infected with WAY. The role of weeds as hosts of WAY in Hawaii is not understood, but they may serve as long-term reservoirs of this phytoplasma. In addition, the maintenance of WAY in weedy hosts may allow for its undetected spread in Hawaii and result in the emergence of new epidemics in other susceptible vegetable crops.

Macrosteles sp. nr. *severini* is related to *Macrosteles fascifrons* (Stål), which is an efficient vector of western North American aster yellows phytoplasma (Freitag, 1967). *Macrosteles* species have not been recorded in Hawaii prior to the outbreak of this disease in watercress; however, *Macrosteles* species have been reported to colonise watercress in California (Freitag, 1959). As both the pathogen and its insect vector were unknown in Hawaii before 2001, we hypothesise that WAY may have been introduced into Hawaii from Japan or Asia through an infective *Macrosteles* individual or group of individuals. We provide evidence that WAY is more closely related to OY than to other aster yellows phytoplasmas but have no data on the origin of the *Macrosteles* species in Hawaii. It is also possible that the introduction of WAY and the *Macrosteles* leafhoppers were independent events and that WAY was already present in Hawaii, being maintained in weed hosts by other leafhopper vectors, prior to the introduction of *Macrosteles*.

We have demonstrated that *Macrosteles* can vector WAY; however, our experimental design did not focus on the determination of transmission parameters, such as how acquisition and inoculation efficiencies vary over time. Further research is warranted to determine the basic transmission parameters of WAY by *Macrosteles* in watercress. In addition, it will also be important to identify any other leafhopper species present in Hawaii that can vector WAY. Such other leafhoppers may be able to spread WAY to weed hosts and other crops that are not hosts of *Macrosteles*.

The ecology of aster yellows phytoplasma transmission by *Macrosteles* in Hawaii is likely to be complex. Watercress cropping practices in Hawaii may be an important factor in the maintenance of this disease. This crop is routinely vegetatively propagated and is harvested by cutting the leafy canopy and allowing the remaining stems to regrow. Replanting with vegetative cuttings is only occasionally performed. Each harvest cycle is about 6–8 weeks in the Hawaiian climate. Watercress plants that are infected with WAY become severely stunted and are rapidly over-grown by surrounding uninfected watercress. The extent of disease is only apparent when the canopy is removed and the stunted, symptomatic plants are exposed (W.B. Borth and R.P.P. Almeida, unpublished data). The severe stunting of diseased plants combined with the cropping system used may influence the ability of this leafhopper to access phytoplasma-infected watercress plants. Nonsymptomatic plants may be preferred for insect feeding and limit the access of leafhoppers to infected source plants that are relatively inaccessible beneath the canopy.

We have documented the occurrence of an aster yellows group phytoplasma (WAY) in Hawaii that can infect watercress and other weedy hosts. Phylogenetic analyses revealed high similarity between WAY and OY from Japan. We have also demonstrated that a newly introduced leafhopper, *Macrosteles*, is a vector of this pathogen. Together, these introductions present a potential threat to the production of vegetable and floral crops in Hawaii. Furthermore, the confirmation that WAY is present in plants near watercress farms raises the possibility that WAY can be maintained in the environment in alternative host plants. Those hosts may function as reservoirs from which WAY might be disseminated in future epidemics by *Macrosteles* or other leafhopper vectors.

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