Detection and Variability of Aster Yellows Phytoplasma Titer in Its Insect Vector, *Macrosteles quadrilineatus* (Hemiptera: Cicadellidae)

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ABSTRACT The aster yellows phytoplasma (AYp) is transmitted by the aster leafhopper, *Macrosteles quadrilineatus* Forbes, in a persistent and propagative manner. To study AYp replication and examine the variability of AYp titer in individual aster leafhoppers, we developed a quantitative real-time polymerase chain reaction assay to measure AYp concentration in insect DNA extracts. Absolute quantification of AYp DNA was achieved by comparing the amplification of unknown amounts of an AYp target gene sequence, elongation factor TU (*tuf*), from whole insect DNA extractions, to the amplification of a dilution series containing known quantities of the *tuf* gene sequence cloned into a plasmid. The capabilities and limitations of this method were assessed by conducting time course experiments that varied the incubation time of AYp in the aster leafhopper from 0 to 9 d after a 48 h acquisition access period on an AYp-infected plant. Average AYp titer was measured in 107 aster leafhoppers and, expressed as Log_{10} (copies/insect), ranged from 3.53 ($\pm$0.07) to 6.26 ($\pm$0.11) occurring at one and 7 d after the acquisition access period. AYp titers per insect and relative to an aster leafhopper chromosomal reference gene, *cp6* wingless (*cp6*), increased $\approx$100-fold in insects that acquired the AYp. High quantification cycle values obtained for aster leafhoppers not exposed to an AYp-infected plant were interpreted as background and used to define a limit of detection for the quantitative real-time polymerase chain reaction assay. This method will improve our ability to study biological factors governing AYp replication in the aster leafhopper and determine if AYp titer is associated with frequency of transmission.

KEY WORDS aster leafhopper, aster yellows, aster yellows phytoplasma, *Macrosteles quadrilineatus*, qPCR

Aster yellows (AY) is a widespread disease of plants caused by the aster yellows phytoplasma (AYp), a small, wall-less prokaryotic organism that is currently placed in the provisional genus *Candidatus* (Lee et al. 2000, IRPCM Phytoplasma/Spiroplasma working team–Phytoplasma taxonomy group 2004). The AYp has an extensive and diverse host range infecting over 350 plant species including many common vegetable, ornamental, and agronomically important field crops, and several noncrop plant species (Kunkel 1926; Chiykowski 1965, 1967; Chiykowski and Chapman 1965; Westdal and Richardson 1969; Peterson 1973; Lee et al. 1998, 2000, 2003; Hollingsworth et al. 2008). The most common disease phenotypes are vein clearing, chlorosis, stunting, twisting and proliferation of plant stems, and the development of adventitious roots (Kunkle 1926, Bloomquist 2002). In vegetable crops, these symptoms can lead to yield and quality losses. For root vegetables, processing problems can result from an inability to obtain clean raw product because of adventitious root growth and associated field soil contamination.

Although, more than 24 leafhopper species are known to acquire and transmit AYp organisms (Mahr 1989, Christensen et al. 2005), the aster leafhopper, *Macrosteles quadrilineatus* Forbes, is considered to be the primary vector of the AYp because of its prevalence in Midwestern susceptible crops (Drake and Chapman 1965, Hoy et al. 1992). The aster leafhopper is a polyphagous insect species that uses over 300 different plant species for food, oviposition, and shelter and many of these are susceptible to AYp infection, (Wallis 1962, Peterson 1973). Aster leafhopper host plant species can be classified into two primary groups based on utilization patterns to include: 1) feeding hosts or 2) feeding and reproductive hosts. Other factors such as plant community composition (Lee and Robinson 1958, Wallis 1962, Schultz 1979), plant physiological state (Peterson 1973) and seasonal or geographic location (Lee and Robinson 1958, Wallis 1962, Peterson 1973) can also affect host preferences of aster leafhopper in the field. In Wisconsin, cultivated grains are hosts for overwintering eggs and also serve as early feeding and reproductive hosts for the

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aster leafhopper (Drake and Chapman 1965). In addition to grain crops, the aster leafhopper feeds and is moderately abundant in mixed broadleaf weeds and grasses that border crop fields (Shultz 1979).

AYp has been reported to be circulative and propagative in the aster leafhopper (Maramorosch 1952, Sinha and Chiykowski 1967, Lee et al. 2000); vector competence involves acquisition, pathogen replication, and circulation to result in successful transmission to a susceptible host (Matthews 1991). Using indirect methods (see below) and histological studies of other fastidious prokaryotic organisms, a model has been developed for acquisition and transmission of AYp. Briefly, the aster leafhopper acquires AYp by feeding on vascular tissues of infected plants for extended periods of time (hours to days) (Murral et al. 1996). The phytoplasma moves from the mouthparts through the alimentary canal to the lumen of the midgut (Sinha and Chiykowski 1967, Fletcher et al. 1998). Once in the midgut lumen, the organism moves through the epithelium (Fletcher et al. 1998, Kwon et al. 1999), circulates in the insect body and replicates in the hemolymph and other insect tissues (Maramorosch 1952a, Sinha and Chiykowski 1967, Fletcher et al. 1998). Ultimately, AYp infects the accessory salivary glands where it replicates, moves into the salivary ducts and is introduced into plant hosts with insect saliva during the egestion phase of feeding (Fletcher et al. 1998, Kwon et al. 1999). These events occur during a two to three week latency period or the period after acquisition during which the insect cannot transmit the pathogen. Once infectious, the leafhopper may transmit AYp to healthy plants during relatively short inoculation feeding periods and remains infectious for the remainder of its adult life (Maramorosch 1953b). Although transovarial transmission of AYp is not thought to occur, there is mounting evidence that transovarial transmission of phytoplasmas does occur for some insect-pathogen combinations (Alma et al. 1997, Kawakita et al. 2000).

Because AYp organisms have an obligatory association with their plant and insect hosts and have not been successfully cultured in the laboratory, much of what is known about phytoplasma replication in the insect has been derived using indirect methods of measurement. For example, replication of AYp organisms in their insect host was studied using dilution series experiments where insect extracts were microinjected into populations of uninfected insects (Black 1941; Maramorosch 1952a,b, 1955). The infectivity of the resulting microinjected populations was measured and the limiting dilution (the dilution at which leafhoppers lost the ability to transmit) was determined after serial transfers of the pathogen. These early experiments measured 100-fold, or higher, increases of AYp in its insect vector. More recently, DNA hybridization (Rahardja et al. 1992, Bloomquist and Kirkpatrick 2002), competitive polymerase chain reaction (PCR) (Liu et al. 1994), and quantitative real-time PCR (qPCR) (Marzachi and Bosco 2005; Bosco et al. 2007a,b) have been used in an attempt to directly quantify the titer of phytoplasma organisms in their insect host.

Recent research in pathogen-vector interactions suggests that the titer of circulative, propagative pathogens in their insect vectors may influence the likelihood of successful transmission events to a susceptible host plant. For example, Rotenberg et al. (2009) demonstrated that single thrips (*Frankliniella occidentalis*) containing higher titers of Tomato spotted wilt virus (TSWV) transmitted the virus more frequently to susceptible plants. Only a few studies have reported phytoplasma titer of an insect and its relationship to transmission success (Bosco et al. 2007a, Galetto et al. 2009). Additionally, the relationship between AYp titer and frequency of transmission has not been examined for individual aster leafhopper. To better understand AYp replication in the aster leafhopper, our objectives were 1) to develop a quantitative assay to measure AYp titer in individual leafhoppers, 2) to examine the variability of AYp titer in aster leafhoppers, and 3) to use the assay to characterize the temporal dynamics of AYp titer among a population of AYp-infected insects.

Materials and Methods

Aster Leafhopper Colony. An aster leafhopper colony was established from adult aster leafhopper populations collected in wheat (*Triticum* spp.) fields in central Missouri (April 2009) and southern Wisconsin (May–June 2009). Adult leafhoppers were initially maintained on oat (*Avena sativa* L.) seedlings in a controlled environment with a 16:8 (L:D) photoperiod (24°C light; 19°C dark). To ensure a phytoplasma-free colony of leafhoppers, the field caught adult female leafhoppers were initially placed on oat seedlings and allowed a 36 h oviposition period after which time all adult leafhoppers were removed. First instar nymphs resulting from those eggs were subsequently moved onto new oat seedlings not previously visited by aster leafhoppers. Additionally, leafhoppers were periodically checked for phytoplasma or AYp infection by PCR, using primers P1 and 16sSr (Lee et al. 2006) or F4 and R1 (Davis and Lee, 1993; reaction conditions described below). All oat plants were grown in a glasshouse and plants were established by sowing oat seed into 10 cm square pots containing Metro Mix 300 (Sun Gro Horticulture, Canada CM Ltd.) with each pot receiving ≈1 g of Osmocote time-release fertilizer (The Scotts Company LLC, Marysville, OH).

Phytoplasma Isolate Collection. AYp isolates were initially obtained by placing three groups of 20–30 field-caught adult aster leafhoppers per group onto three 96-well flats containing carrot seedlings for a 96 h acquisition access period (AAP). Carrot plants that subsequently expressed symptoms typical of aster yellows were individually transplanted into 15 cm pots and assayed for the presence of phytoplasma by PCR using the universal phytoplasma nested primer set P1 and 16sSr followed by a second round of amplification with R16F2n and R16R2 (Lee et al. 2006; reaction

Table 1. Designed qPCR primer set names, sequences, sources, efficiencies, and product sizes

Primer name	5' Sequence 3'	Target organism	Efficiency[a]	Product size
cp6F	GGGCAAGAAGGGCAAGTA	Aster leafhopper[b]	2.01	91
cp6R	AGGCTCCAGATACACTAGGTC			
tufF	CCAGGTTCTGTTAAGCCTCATTC	Aster yellows phytoplasma[c]	1.97	198
tufR	AACTACTAATTCAGCGTTGTCACC			
lysSF	CTTGAGAATTGCCACCGAATTG	Aster yellows phytoplasma[c]	1.97	158
lysSR	GCATATCAGCATAAGCCAAGTAAG			

[a] Primer efficiencies were calculated as $E = 10^{(-1/\text{slope})}$ and slopes were established from dilution curves of target DNA fragments.
[b] Primers were designed from GenBank FJ001411.
[c] Primers were designed from GenBank AJ271323.

conditions described below). AYp infection was confirmed using AYp-specific16s rDNA primers F4 and R1. Plants testing PCR-positive for the presence of the AYp were placed into insect-proof cages and 10 fourth- or fifth-instar aster leafhopper nymphs were isolated on each infected plant for a 48 h AAP. Nymphs were then aspirated and transferred to rye (*Secale cereal* L.) seedlings where they were maintained for 14 d to complete adult eclosion. As adults, they were again transferred (≈5 leafhoppers for each plant species) and isolated onto either 6-wk old aster (*Callistephus chinensis*) 'Aster-Tiger Paws' or periwinkle (*Catharanthus roseus*) plants. Adult aster leafhopper placed onto periwinkle were allowed an indefinite inoculation access period (IAP) to recover the AYp in planta for long-term storage. Before use in isolation, Chinese aster (*Callistephus chinensis*) seed was sown into 96 well seedling trays containing Metro Mix 300 and amended with Osmocote time-release fertilizer at a rate 2.5 kg per M^3 of soil. When the aster plants had reached the 2–3 true leaf stage, they were transplanted to 10 cm square pots. Aster plants were in the 5–7 leaf stage when phytoplasma isolation was initiated. Plants were maintained in insect-proof cages in the greenhouse at ≈25–29°C under natural light supplemented with a 16:8 L:D photoperiod.

DNA Extraction From Leafhoppers. A cetyltrimethylammonium bromide (CTAB) method (modified from Doyle and Dickson, 1987) was used to extract DNA from individual insects. Briefly, insects were placed in 1.5 ml microfuge tubes and washed with 400 μl of CTAB buffer (2% CTAB, 1.2 M NaCl, 100 mM TRIS-HCl, 20 mM EDTA, and 0.2% β-mercapto ethanol) that was later discarded. Twenty microliters of CTAB buffer was added back to each tube and individual insects homogenized with sterile blue Kontes pestles (Kimble Chase Life Science Research, Vineland, NJ). The buffer volume in each tube was brought to 600 μl with CTAB buffer and tubes were incubated for 30 min in a 60°C water bath. Six hundred μl of chloroform:isosamyl alcohol (24:1) was added to each tube and tubes were inverted 20 times. After centrifugation at 12,000 × *g* for 15 min, the aqueous phase was transferred to a clean 1.5 ml microfuge tube and 600 μl of cold isopropanol was added to each tube. Samples were incubated overnight at −20°C and then centrifuged at 16,100 × *g* for 15 min. The supernatants were discarded and each pellet was washed with 1000 μl of 70% ethanol. Samples were again centrifuged at 16,100 × *g* for 15 min, and again supernatants were discarded and pellets dried in a SpeedVac model SS1 (Savant Instruments, Inc., Farmingdale, NY). DNA extracts were resuspended in 50 μl of sterile distilled water and the quantity and quality of the extracted DNA was assessed by scanning 1.5 μl of each sample in a NanoDrop spectrophotometer (Thermo Fisher, Inc., Waltham, MA). Extracted DNAs were stored at −80°C until used in PCR. Before quantitative qPCR, all leafhopper DNA extracts were diluted to15 ng/μl.

Design of qPCR Primers and Calibration Curves. Two AYp gene sequences, elongation factor TU (*tuf:* GenBank AJ271323) and lysyl-tRNA synthetase (*lysS:* GenBank AJ271323), were used as AYp targets for amplification. Both gene targets were selected based on their performance in an evaluation of 10 candidate AYp gene targets (data not shown). The two genes consistently generated similar results and only primed the specified AYp targets of interest. The remaining eight candidate primers were eliminated because of nonspecific amplification of unknown targets in uninfected insects (i.e., possibly uncharacterized endosymbionts) that produced inconsistent results among primer sets. One aster leafhopper gene sequence, Wingless (*cp6:* GenBank FJ001411), was selected as a target for amplification of the leafhopper chromosomal DNA and served as a reference gene for relative quantification among samples with unknown quantities of AYp target sequence and also confirmed that the DNA extraction method being used produced consistent results among samples. All primers (Table 1) were designed using Beacon Designer (PREMIER Biosoft International) and target sequence locations with significant structure were avoided.

To develop calibration curves of our AYp and leafhopper gene targets, PCR products of each target were generated with each of our designed primer pairs. Two microliters of DNA extracts from AYp infected periwinkle or aster leafhoppers in varying concentrations were used as template for the reactions. Amplified PCR products were separated by gel electrophoresis, purified using activated silica beads (modified from Vogelstein and Gillespie 1979), and sequenced at the University of Wisconsin-Madison Biotechnology Center DNA sequencing facility (http://www.biotech.wisc.edu/). Using BLAST, PCR products were compared with the NCBI database and the original gene sequence used to design the primers. Products with the appropriate sequences were cloned into a

pGEM-T Easy plasmid (Promega, Madison, WI) according to the manufacturer's instructions. *Escherichia coli* DH5α were transformed with the plasmid constructs containing target sequences. Transformants were selected using blue-white selection and assayed for the presence of the cloned target using PCR. Plasmids were purified from overnight cultures of *E. coli* transformants using a QIAprep Spin Miniprep Kit (Qiagen) and standard plasmids were termed pCP6–5, pTUF-2, and pLysS-4. Purified plasmid preparations were quantified using a NanoDrop spectrophotometer and DNA concentrations (expressed as plasmid copy number/μl) were calculated as follows assuming the average weight of a nucleotide base pair was 660 Daltons:

$$\text{DNA (copies/}\mu\text{l)} = \text{DNA (ng/}\mu\text{l)}/((\text{DNA (bp)} * 1 \times 10^9 \text{ (ng/g)} * 660 \text{ (Da/bp))}/6.022 \times 10^{23} \text{ (copies/mol))}$$

Two independent 10-fold dilution series for each gene target based on plasmid copy number/μl were prepared in concentrations ranging from 10^8 to 10 copies/μl. All standards were diluted in 0.1 $\times$ TE buffer (pH = 8.0). Initially, the analytical sensitivity of our primers was evaluated using the full dilution series and primer efficiency (E), based on standards ranging from 10^6 to 10 copies/μl, was calculated as:

$$E = 10^{-1/\text{slope of dilution curve}}$$

To evaluate the variability associated with calibration curve preparation, four total reactions of each standard (i.e., two technical replicates per concentration for each independently prepared calibration curve) ranging from 10^6 to10 copies/μl were run on a single plate (data analysis described below).

All experimental 96-well plates with samples containing unknown amounts of AYp target included a 10-fold dilution series of standards that ranged from 10^6 to 10 copies/μl for quantification purposes. Primer efficiencies were calculated as described above and plate-to-plate variability of primer efficiency was characterized (see Data Analysis).

PCR and qPCR Conditions. The presence of phytoplasma in plant and insect tissue extracts was detected using PCR or nested PCR. Nested PCR reactions were performed as described by Lee et al. (2006) with minor modifications. The first-round of amplification used universal phytoplasma primers P1 and 16sSr. Reactions were carried out in 25 μl of 1$\times$ GoTaq Green Master mix (Promega) containing 1 μM of each forward and reverse primer, 2 μl of DNA extract in varying concentrations as template and water. For a nested amplification, 1 μl of the diluted (1:30) PCR product from the first round of amplification was used as template and the universal phytoplasma primers R16F2n and R16R2 were used in the PCR mixture. Briefly, the reactions were denatured at 94°C for 10 min followed by 38 cycles of 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. The last cycle was followed by a final extension of 10 min at 72°C, and held at 4°C. Reaction conditions for the AYp specific primer set F4 and R1 (Davis and Lee 1993), were the same as above with slight changes to the thermo cycler program. Reactions were denatured at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The last cycle was followed by a final extension of 10 min at 72°C and held at 4°C. Positive and negative controls were run with each set of reactions and all reactions were conducted in a MyiQ Cycler thermo cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Ten microliters of each PCR product was subjected to electrophoresis in a 1.2% agarose gel, stained with ethidium bromide and visualized under UV light. Positive detections resulted in the production of a 1,600 bp amplicon (P1 and 16sSR), a 1,200 bp amplicon for the nested reaction (R16F2n and R16R2) or a 660 bp amplicon for primers F4 and R1.

Real-time qPCR reactions were performed in iQ 96-well PCR plates sealed with Micro-Seal 'B' film (Bio-Rad Laboratories). Each reaction contained 1$\times$ iQ SsoFast EvaGreen Supermix (Bio-Rad Laboratories), 0.2 μM of each forward and reverse primer (Table 1), 8 μl of each leafhopper extract (at 15 ng DNA/μl) or 8 μl plasmid standard (at varying copies/μl), and sterile distilled water for a final reaction volumes of 20 μl. DNA extracts containing unknown quantities of AYp or leafhopper DNA were assayed in triplicate (three technical replicates) and plasmid DNA standards at concentrations ranging from 10^6 to 10 copies/μl (or 8.0×10^6 to 80 copies per reaction) were assayed in a total of four reactions (four technical replicates, two from each duplicate dilution series). The reactions were denatured at 94°C for 10 min followed by 40 cycles of 94°C for 30 s and annealing at 55°C for 30 s. The last cycle was followed by a final extension of 10 min at 72°C, and a melt curve was created by increasing the temperature from 65 to 95°C by 0.5 degree increments per 10 s. Real-time PCR reactions were conducted in a MyiQ instrument fitted with a One-Color Real-Time PCR Detection System (Bio-Rad Laboratories).

Aster Leafhopper Weights. The average leafhopper weight was estimated to better understand if differences between AYp or aster leafhopper target concentrations of the aster leafhopper sexes was because of DNA yield per male and female insect or, in the case of AYp, differential growth of the phytoplasma in male versus female aster leafhopper. A random selection of 50 adult male and 50 adult female leafhoppers was collected from the leafhopper colony. Carbon dioxide was used to immobilize the leafhoppers and insects were weighed in batches of five individuals on an analytical balance (Mettler-Toledo International Inc., Switzerland). The average weight per insect for each batch was calculated and the average weight for aster leafhoppers was reported as the mean of the 10 batches for both male and female leafhopper groups.

Time Course Experiment. To assess the potential utility of our qPCR method, we produced a population of leafhoppers with varying AYp titer. The leafhopper population was created by providing a group of 200

aster leafhopper a 48-h AAP on an AYp-infected aster after which time the insects were removed from the acquisition host and placed on rye seedlings to allow for the propagation of the phytoplasma within the leafhoppers. The rye plants, on which incubation occurred, were grown in 10 cm circular pots and covered with a mesh-top, cylindrical plastic tube and held in an environmental cabinet under similar light and temperature as the aster leafhopper culture (see above). Following the AAP, the first group of 15 aster leafhoppers was sampled immediately (0 d post-AAP) and subsequent sampling occurred on 1, 2, 3, 5, 7, and 9 d post-AAP. An additional group of 15 leafhoppers not exposed to an AYp-infected aster served as our control group. At sampling, individual insects were placed into 1.5 ml microfuge tubes, sex was determined, and leafhopper specimens were stored at −80°C until DNA extraction (described above). The presence of phytoplasma in aster leafhoppers was confirmed using a single round of amplification with the primer sets P1/16sSr, followed by a second round of amplification with R16F2n/R16R2.

Data Analysis. *Data Export and Organization.* Quantification cycles (Cq) were calculated automatically by the MyiQ Optical System Software (Version 1.0, Bio-Rad Laboratories) and reports with relevant data (i.e., Cq values, starting quantities, starting quantities in unknown samples, etc.) were exported as text (*.txt) files. Exported data were concatenated in a single spreadsheet, combined with the biological data recorded for each sample, and imported into R version 2.11.1 (R Development Core Team 2009) for statistical analysis. Unless specified, all functions used in the analysis can be found in the base distribution of R and are italicized in the text.

Standard Curve Repeatability. A linear regression approach (i.e., analysis of covariance [ANCOVA]) was used to examine the variability associated with standard curve preparation and test the hypothesis that slopes and intercepts of different standard curve preparations were equivalent (Burns et al. 2005). The intent of this analysis was to determine the repeatability of standard curve preparation. Linear models were fit in R using the *lm* function and test statistics were extracted using the *anova* function.

A linear mixed effects model was used to examine the plate-to-plate variability of the standard curves run on the plates containing samples for quantification. This model corresponded to the simple linear regression of the estimated Cq value (Y_{ij}) in the j^{th} reaction within the i^{th} plate on the known starting concentration of the reaction (x_{ij}) and can be formulated as:

$$Y_{ij} = (B_0 + b_{0i}) + (B_1 + b_{1i})\, x_{ij} + \varepsilon_{ij}$$

$$b_{0i} \approx N(0, \sigma_i^2);\ b_{1i} \approx N(0, \sigma_s^2);\ \varepsilon_{ij} \approx N(0, \sigma_r^2).$$

In this model, B_0 and B_1 are the fixed effects parameters corresponding to the intercept and slope. b_{0i} and b_{1i} are the random effects vectors assumed to be independent and normally distributed for different 96-well plates and ε_{ij} are the within-plate errors assumed to be independent of the random effects. The intent of this analysis was to examine the overall, or average, slope and intercept values of the population of five independent 96-well plates (for each primer target) and to explicitly examine plate-to-plate variability associated with our standard curves. The homogeneity of variance of our standard curves throughout the target concentration range was evaluated by examining the residual plots of the mixed models (not shown). We also examined the within-plate Cq value variances averaged for the five plates and the standard deviation of the average variation (among plates) for each reaction containing different known starting quantities of standard. Linear mixed models were fit in R using the *lme* function (Package nlme: Pinheriro and Bates 2000) and parameter estimates and test statistics were extracted using the *summary* and *anova* functions.

Insect Samples. Calibration curves were run on each 96 well plate and the same standard curve preparation (for each primer set) was used for all experimental (time course) insect samples. Thus, estimates of starting quantities of the AYp targets in the experimental samples were adjusted for plate-to-plate variability and estimated starting quantities could be compared among all 96-well reaction plates. The estimated copy number for each of the three technical replicates were aggregated for each leafhopper and average copy number for each insect was used for further calculations and statistical analysis. An AYp positive detection was defined as having a copy number 3 s or greater than the mean copy number of the control group of aster leafhoppers for each qPCR primer set.

A simple logistic regression model was used to evaluate the effect of AYp concentration (X_i) on the ability to detect AYp using conventional and nested PCR (Kutner et al. 2004). The outcomes of one hundred conventional and nested PCR reactions of aster leafhopper DNA extracts with varying starting copy numbers of AYp were used for this analysis. The results of PCR and nested PCR were coded in binary fashion (Y = 1 if the PCR resulted in a visually detectable band under UV light in an agarose gel and Y = 0 if there was no visible band) and initial AYp copy number was estimated using our qPCR results. The standard logistic regression model has the form:

$$Y_i = \exp(Z_i)/(1 + \exp(Z_i))$$

where $Z_i = B_0 + B_1 X_i$

This equation can be rearranged and the slopes (B_1) and intercepts (B_0) of the regression models are more easily interpreted in the context of the following regression equation:

$$\text{Ln}\ (Y_i/1 - Y_i) = B_0 + B_1 X_i$$

where the odds of a positive detection is $Y_i/1\text{-}Y_i$ and X_i is the starting copy number (Log_{10}) in the PCR reaction. For each regression model, the number of copies necessary to have the probability of a positive detection (Y) be 0.5 (similar to ED_{50}) is estimated by

substituting 0.5 for Y and solving for X, the number of AYp copies (log-transformed). The logistic regression models were fit in R using the *glm* function with a logit link function (family binomial) (package MASS: Venables and Ripley 2002). Parameter estimates, (chi-squared) test statistics, model predictions, and ED_{50} estimates (±SE) were extracted using the *summary*, *anova*, *predict*, and *dose.p* functions.

The titer of AYp present in individual aster leafhopper extracts was estimated assuming one AYp organism possessed a single copy of the target DNA and time was considered a categorical covariate. A linear model was then used to test the null hypothesis that there were no differences of AYp titer between sexes or among aster leafhopper that underwent different incubation times. Linear models were fit in R using the *lm* function and differences among the mean length of incubation time were determined using Tukey's honest significant difference method (function Tukey's HSD). Parameter estimates and test statistics were extracted using the *summary* and *anova* functions. When data were unbalanced, the *drop1* function was used to extract the marginal sum of squares for model factors or the effect of a factor conditional on all other terms entering the model first.

To better define the population dynamics of AYp in aster leafhopper, iteratively reweighted nonlinear regression analysis was used to fit a three parameter logistic growth model to absolute AYp titer expressed as Log_{10}-transformed copies per insect (N) and relative AYp titer expressed as Log_{10}-transformed AYp gene copies per *cp6* gene copies (R) averaged by incubation time. The logistic growth equation was:

$$Y_i = \Phi / (1 + \exp((((4 * \mu)/\Phi) * (\lambda - X_i)) + 2)$$

where Y_i is AYp titer relative to AYp titer at time 0 (i.e., $N_i - N_0$ or $R_i - R_0$), X_i was incubation time and i indexes incubation time. Parameters Φ, μ, and λ represent the asymptote, the maximum growth rate, and the lag time associated with bacterial population growth (Zwietering et al. 1990). Nonlinear models were fit using the *nls* function with start values estimated from visual examination of the plotted data points. Parameter estimates, standard errors, and test statistics were extracted using the *summary* function and pseudo-R^2 values (pseudo-R^2 = 1 – (variance of residuals/total variance))) were used as a measures of goodness-of-fit (Schabenberger and Pierce 2002).

Finally, Pearson's correlation coefficient and simple linear regression was used to compare estimates of AYp copy number per microliter (Log_{10}) of *tuf* and *lysS* in each aster leafhopper DNA extract. The *lm*, *cor.test*, *anova*, and *summary* functions were used to complete this analysis.

Results

Calibration Curve Preparation for Absolute Quantification of AYp. Two independent standard curves were prepared for each of the three primer sets shown in Table 1 using serial dilutions of pCP6–5, pTUF-2, and pLysS-4 plasmid. For each standard curve, regressions of the Cq value on the standard starting quantity showed that a significant proportion of the variability in quantification cycle could be predicted by the starting quantity (Fig. 1). All regression slopes were significantly different than zero with coefficients of determination for all regressions >0.99 (Table 2; Fig. 1). Additionally, there was no significant effect of dilution series preparation on the slope (Table 2: B_1 x Standard Preparation) or intercepts (Table 2: Standard Preparation) of the best fit lines for each primer set. All plasmid standards examined together, the standard deviation of the mean Cq value increased with increasing dilution of the plasmid targets ($t = -3.8$; df = 16; $P < 0.005$; $R^2 = 0.48$). However, when 80 or more copies of plasmid template was used per reaction, the precision of the technical replicates remained high with the standard deviation among Cq values for technical replicates averaging 0.40 (±0.17), 0.25 (±0.10), and 0.42 (±0.17) for *cp6*, *lysS* and *tuf* gene targets, respectively.

Intra- and Inter-Assay Variability. A linear mixed effects model was used to examine the plate-to-plate variability of the standard curves run on each of the experimental plates containing AYp DNA extracted from leafhopper samples. The typical, or average, slope (B_1) and intercept (B_0) values for each primer target run on five independent 96-well plates did not vary (i.e., standard errors of the fixed effects were all <0.05) and average efficiencies for our primer sets were 1.99, 1.86, and 1.92 for *cp6*, *lysS*, and *tuf* genes, respectively (Table 3). Plate-to-plate variability of the slopes (σ_s) was low when compared with the magnitude of the slope estimates at 1, 0, and 3% of the magnitude of the typical slope (B_1) of the *cp6*, *lysS*, and *tuf* gene primer sets, respectively. Similarly, the plate-to-plate variability of the intercepts (σ_i) was 2.7, 2.5, and 4.6% of the magnitude of the typical intercept estimates (B_0). The residual variability (σ_r) of 0.22, 0.27, and 0.32 for pCP6–5, pLysS-4, and pTUF-2 represents the within plate error and other experimental error. These values can be interpreted as a measure of variation because of technical replication and can be used as an additional measure of calibration curve repeatability similar to the average variance of Cq values of pCP6–5, pLysS-4, and pTUF-2 standards (Table 3). In general, the precision of the technical replicates remained high with an average variance of the Cq values of 0.18 (±0.05) when 80 copies of plasmid template was used per reaction (all primer sets together). Although the variance of the mean Cq value increased with increasing dilution of the plasmid targets ($t = 4.53$; df = 88; $P < 0.001$).

Aster Leafhopper Samples. Of the 200 aster leafhoppers used in this experiment, 15 were used as a control group and 185 were allowed access to an AYp-infected plant. Because of mortality, only 93 live aster leafhoppers that had access to the AYp source plant were recovered resulting in a total of 108 aster leafhopper DNA extracts, of which, one was not quantified because of poor DNA quality. In general, female aster leafhopper weighed more than male aster leaf-

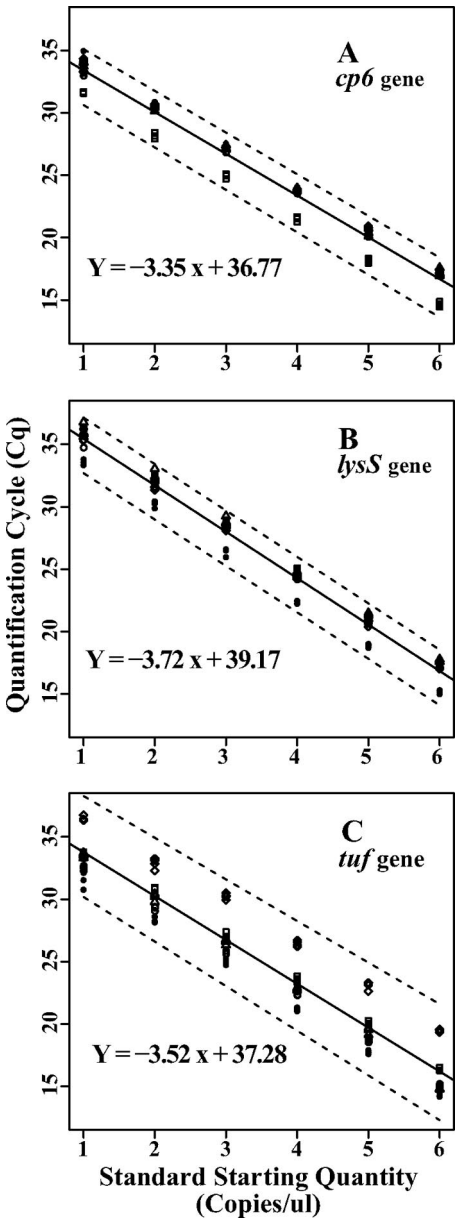

Fig. 1. Calibration curves used to quantify the copy number of aster leafhopper sequence target (*cp6*–Panel A) or AYp sequence target (*lysS*–Panel B and *tuf* Panel C) in individual aster leafhopper DNA extracts. Points for each starting quantity represent four total reactions of each standard (i.e., four technical replicates per starting concentration) ranging from 10^6 to 10 copies per microliter run on five different 96-well plates. The four technical replicates resulted in a total of replicates for each starting quantity grouped by 96-well plate as 1 (□), 2 (Δ), 3 (●), 4(◇), and 5(▲). Unbroken lines represent the typical slope and intercept estimated using a linear mixed effects model and dashed lines describe a 95% prediction interval for each gene sequence calibration curve.

hopper and weights averaged 1.32 and 0.90 mg, respectively. Similarly, more total DNA was extracted from individual female insects (9,278 ± 74 ng) than male insects (2,956 ± 138 ng). The CTAB DNA extraction method yielded high quality DNA with 260/280 ratios averaging 2.11 (±0.004) and 260/230 ratios averaging 1.30 (±0.03) for all aster leafhopper extracts. The average yields for our CTAB DNA extractions from aster leafhopper were higher than Chen et al. (2010), who reported yields of 2200 ng/mg tissue from CTAB extractions of western corn rootworm beetles (*Diabrotica virgifera vergifera* LeConte).

Of the 92 leafhoppers exposed to an AYp-infected plant, AYp was detected in 37 and 95% of the insects using the primer set P1/16sSr and R16F2n/R16R2 (nested in P1/16sSr), respectively (Table 4). AYp was detected in 73 and 82% of the aster leafhopper samples using the *lysS*, and *tuf* gene primer sets, respectively. For *lysS* and *tuf* gene sequence targets, the limits of detection calculated were 58 and 22 copies per reaction for *lysS* and *tuf* genes, respectively, and below the minimum range of our standard curve (80 copies/reaction). For the average sized female (9,280 ng) these detection limits correspond to 4,485 and 1,700 copies per insect for the *lysS* and *tuf* gene targets, respectively. For the average male (2,960 ng) aster leafhopper these detection limits are reduced to 1,430 and 543 copies per insect for the *lysS* and *tuf* gene targets, respectively.

Logistic regression was used to examine the relationship between the starting copy number in PCR reactions and the outcome of the conventional PCR assay (Table 5). Using a cutoff of 0.5, these models predicted the observed conventional PCR outcome 90 and 88% of the time when copy number was calculated using AYp concentration estimated from *lysS* and *tuf* gene primer sets, respectively. The copy number necessary to have a 0.5 probability of detecting AYp by conventional PCR was estimated to be (Log_{10}) 3.86 (±0.16) and 3.72 (±0.16), respectively for both the *lysS* and *tuf* gene primer sets. For nested PCR, the logistic regression models predicted the observed outcomes 87 and 88% using copy number estimates obtained from *lysS* and *tuf* gene primer sets, respectively. For nested PCR, the copy number necessary to have a 0.5 probability of detecting AYp was estimated to be (Log_{10}) 1.78 (±0.17) and 1.50 (±0.16) corresponding to 60 and 32 copies per reaction.

AYp Titer as a Function of Sex and Time. No AYp was detected in the control group of aster leafhoppers (those not having access to an AYp-infected plant) and the highest AYp titers were among individual insects that underwent longer incubation times (Table 6). Averaging over sex, AYp titer measured using the *lysS* gene primer set and expressed as copies per insect (Log_{10}), ranged between 3.94 (±0.18) to 6.34 (±0.13) occurring at one and 7 d postacquisition. However, the variability of AYp titer in individual leafhoppers was greater ranging from 3.68 to 7.02 total copies. Similar results were obtained using the *tuf* gene primer set with average titer ranging between 3.50 (±0.07) to 6.27 (±0.12) copies at one and 7 d after

Table 2. ANOVA tables for regression analysis evaluating the effect of independent preparation of dilution series standards

Standard	Ho: slopes do not vary between calibration curve preparations			Ho: intercepts do not vary between calibration curve preparations		
	Parameter	*F*-value	*P* value[a]	Parameter	*F*-value	*P* value[b]
pCP6–5	B_1	2537	<0.001	B_1	2640	<0.001
	Standard preparation	0.06	0.81	Standard preparation	0.06	0.80
	B_1 × standard preparation	0.18	0.68			
pTuf-2	B_1	3493	<0.001	B_1	3549	<0.001
	Standard preparation	0.54	0.47	Standard preparation	0.55	0.47
	B_1 × standard preparation	0.66	0.42			
pLysS-4	B_1	5247	<0.001	B_1	5336	<0.001
	Standard preparation	0.37	0.47	Standard preparation	0.37	0.54
	B_1 × standard preparation	0.65	0.42			

[a] *P* values calculated using df = 1, 20.
[b] *P* values calculated using df = 1, 21.

acquisition and variation among individual insects ranging from 3.19 to 6.93 copies.

In the subset of AYp-infected aster leafhoppers, when AYp concentration was expressed as copies per ng DNA (Log_{10}) there was no significant interaction of sex and incubation time (Time × Sex effect: *tuf:* $F = 1.00$; df = 5, 62; $P = 0.42$; *lysS:* $F = 1.06$; df = 4, 55 ; $P = 0.39$). Linear models were refit without the interaction term and AYp concentration differences were detected among incubation times (Time effect; *tuf:* $F = 45.6$; df = 6, 67; $P < 0.001$; *lysS:* $F = 28.1$; df = 6, 59; $P < 0.001$) and between male and female insects (Sex effect; *tuf:* $F = 17.66$; df = 1, 67; $P < 0.001$; *lysS:* $F = 9.14$; df = 1, 59 ; $P < 0.005$). AYp titer was approximately threefold higher in male aster leafhopper than in female aster leafhopper.

When AYp titer was expressed as copies per insect, which accounts for approximate insect size, there was no significant interaction of sex and incubation time (Time × Sex effect: *tuf:* $F = 1.17$; df = 5, 62; $P = 0.33$; *lysS:* $F = 0.86$; df = 4, 55; $P = 0.50$). Again, linear models were refit without the interaction term. However, no differences of AYp concentration were detected between male and female insects (Sex effect; *tuf:* $F = 0.56$; df = 1, 67; $P = 0.46$; *lysS:* $F = 0.05$; df = 1, 59; $P = 0.83$) and titer differed among incubation times (Time effect; *tuf:* $F = 47.2$; df = 6, 67; $P < 0.0001$; *lysS:* $F = 29.5$; df = 6, 59; $P < 0.0001$). Using Tukey's HSD method, infected insects could be grouped into approximately three categories based on their AYp titer per insect (Table 6).

To better define the population dynamics of AYp in potentially infective aster leafhoppers, iteratively reweighted nonlinear regression analysis was used to fit a three-parameter logistic growth model to AYp titer (absolute copy number per insect and relative to a

Table 3. Regression analysis of standard curves and standard deviations of Cq values for different concentrations of target DNA sequences on circular plasmids calculated from five 96-well plates per target

Target	Parameters ± SE[a]	Plate-to-plate variability[b]	Target conc. (cps/reaction)[c]	Average Cq variance[d] (SD)
pCP6–5	B_1[e] = −3.35 (0.02)*	$\sigma_s = 0.04$	8,000,000	0.04 (0.03)
	B_0 = 36.8 (0.45)*	$\sigma_i = 1.00$	800,000	0.05 (0.03)
		$\sigma_r = 0.22$	80,000	0.02 (0.01)
			8,000	0.03 (0.02)
			800	0.03 (0.03)
			80	0.15 (0.20)
pLysS-4	B_1 = −3.72 (0.01)*	$\sigma_s = 0.00$	8,000,000	0.01 (0.01)
	B_0 = 39.2 (0.44)*	$\sigma_i = 0.98$	800,000	0.03 (0.01)
		$\sigma_r = 0.27$	80,000	0.02 (0.01)
			8,000	0.07 (0.03)
			800	0.08 (0.05)
			80	0.24 (0.19)
pTUF-2	B_1 = −3.52 (0.05)*	$\sigma_s = 0.11$	8,000,000	0.01 (0.01)
	B_0 = 37.3 (0.77)*	$\sigma_i = 1.73$	800,000	0.06 (0.04)
		$\sigma_r = 0.32$	80,000	0.03 (0.02)
			8,000	0.04 (0.02)
			800	0.09 (0.06)
			80	0.16 (0.26)

[a] Slopes (B_1) and intercepts (B_0), obtained from the linear mixed model can be interpreted as the result of a "typical" standard curve observed in our experiments. Fixed effects followed by * are significant at $P < 0.0001$ by an *F*-test df = 1, 112.
[b] Standard deviation of the random effects (i.e., on the same scale as the fixed effects) and can be interpreted as a measure of the variability of the slope (σ_s) and intercept (σ_i) among plates and the residual, within-plate, variability (σ_r).
[c] Target concentrations were calculated as described above and copy numbers were based on 8 μl of standard per reaction.
[d] Average Cq variance can be interpreted as a measure of within plate variability of 4 PCR reactions on run on five 96-well plates while its SD represents a measure of the among plate variability.
[e] Average primer efficiency can be calculated as $E = 10^{(-1/B1)}$.

Table 4. Number of positive AYp detections (%) for aster leafhoppers undergoing different incubation times after a 48 h acquisition access period in experiment 2

Incubation (days)	No. aster leafhoppers	Primer sets used for AYp detection			
		P1/16sSr	R16F2n/R16R2n[a]	lysSF/lysSR[b]	tufF/tufR[b]
C	15	0 (0)	0 (0)	0 (0)	0 (0)
0	15	0 (0)	14 (93)	6 (40)	7 (60)
1	15	1 (7)	14 (93)	8 (53)	11 (73)
2	15	1 (7)	14 (93)	9 (60)	13 (87)
3	15	8 (53)	13 (87)	14 (93)	14 (93)
5	14	9 (64)	14 (100)	13 (93)	13 (93)
7	15	14 (93)	15 (100)	14 (93)	14 (93)
9	3	1 (33)	3 (100)	3 (100)	3 (100)

[a] Second round of amplification nested in primer set P1/16sSr.
[b] A positive detection had a value of 3 s or greater than the mean of the control.

aster leafhopper chromosomal reference gene) as a function of time (Fig. 2A). The nonlinear models described the average AYp titer in aster leafhoppers over time with overall model fits having pseudo-R^2 values of 0.97 and 0.98, for *lysS* and *tuf* genes, respectively. Estimated AYp lag times were 2.06 and 1.51 d and maximum growth rates were (Log_{10}) 1.22 and 0.81 d^{-1} for *lysS* and *tuf* genes corresponding to doubling times of ≈570 and 850 min. AYp titer increased ≈100-fold and became asymptotic 6 d after completion of the AAP. Similar results were obtained when AYp titer was expressed relative to the number of aster leafhopper *cp6* gene copies present in the sample (Fig. 2B).

Comparison of *lysS* Primer Set to *tuf* Primer Set. The *tuf* and *lysS* gene primers sets were both designed to target gene sequences of AYp. As expected, the two primer sets produced similar estimates of target sequence concentration that, when compared, were significantly correlated (Pearson's: $R = 0.97$; $t = 37.5$; df = 94; $P < 0.001$). Regression analyses showed that a significant proportion of the variability in titer as measured by the *tuf* gene primer set could be predicted by estimates of AYp titer obtained using the *lysS* gene primer set (Fig. 3: Slope: $B_1 = 1.02$; $t = 37.5$; df = 94; $P < 0.0001$; Intercept: $B_0 = 0.22$; $t = -0.22$; df = 94; $P < 0.0001$). The slope of the regression line was not significantly different from one ($t = 0.68$; df = 94; $P = 0.25$) and the y-intercept was significantly different from zero suggesting a constant bias may be present among AYp titers measured by the different primer sets.

Table 5. Simple logistic regression analysis of 100 detection outcomes of PCR and nested PCR reactions used to detect varying amounts of AYp in aster leafhopper DNA extracts

	Predictor[a]	Estimate	SE	χ^2	df	Pr ($>\chi^2$)	Odds ratio
PCR	Intercept - B_0	−8.87	1.66				
	LysS - B_1	2.30	0.43	59.9	1	<0.0001	9.97
	Intercept - B_0	−7.94	1.49				
	Tuf - B_1	2.14	0.40	56.9	1	<0.0001	8.50
NestedPCR	Intercept- B_0	−4.09	1.49				
	LysS - B_1	2.29	0.69	64.1	1	<0.0001	9.87
	Intercept- B_0	−3.66	1.27				
	Tuf - B_1	2.44	0.68	55.3	1	<0.0001	11.47

[a] "Logistic" regression model fit in R with *glm* function and arguments were set to binomial family with a logit link function.

Concentrations of *cp6* (AsterLeafhopper) in Insect DNA Extracts. To ensure the accuracy of our DNA extraction methodology and ability to measure and dilute insect samples for AYp detection, the aster leafhopper *cp6* gene target was amplified to be used as a reference chromosomal marker. When *cp6* concentration was expressed as copies per ng DNA (Log_{10}), the set of values was normally distributed (Shapiro-Wilk: $W = 0.99$; $P = 0.36$; $n = 107$) with a mean of 2.70 (±0.02). Differences were detected among insects undergoing different incubation times (Time effect; $F = 2.69$; df = 7, 92; $P = 0.01$) and between leafhopper sex (Sex effect; $F = 8.75$; df = 1, 92 ; $P < 0.005$) with DNA extracts from male insects having about twofold more copies of CP6 than females insects per ng DNA (M: 2.99 ± 0.03; F: 2.61 ± 0.02). There was no interaction of sex and incubation time on *cp6* gene sequence concentration (copies/ng DNA) (Time × Sex effect: $F = 0.93$; df = 6, 92; $P = 0.48$).

With *cp6* gene sequence concentration expressed as copies per insect, the number of *cp6* copies in male aster leafhoppers was not different from female aster leafhoppers (M: 3,115,136 ± 270,081; F: 4,110,462 ± 171,458; Sex effect; $F = 0.67$, df = 1, 92, $P = 0.41$) but the number of *cp6*gene copies did differ among incubation times (Table 6: Time effect; $F = 2.15$, df = 7, 92, $P < 0.05$). Again, there was no interaction of sex and incubation time on *cp6* gene concentration (copies/ng DNA) (Time × Sex effect: $F = 0.58$; df = 6, 92; $P = 0.75$).

Discussion

Several studies have reported the development of qPCR assays for the identification and quantification of mollicutes in their insect and plant hosts (Marzachi and Bosco 2005; Lee et al. 2006; Inoue et al. 2009; Lopes et al. 2009; Wenbin et al. 2008, 2009a,b; Yvon et al. 2009). To our knowledge, however, the current study is the first to report a method for directly quantifying AYp titer in the aster leafhopper (*M. quadrilineatus*) and contributes to the growing body of research of phytoplasma replication in their insect host by describing the AYp growth pattern and titer variation among individual aster leafhopper. One of the primary contributions of this study was

Table 6. Mean copy no. (±SE) of AYp and insect genome sequence targets, expressed as Log_{10} copies per insect (cp/in), for aster leafhoppers undergoing different incubation times after a 48 h acquisition access period

Incubation	Gene target[a]					
	No. aster leafhoppers[b]	*lysS*	No. aster leafhoppers[b]	*tuf*	No. aster leafhoppers	*cp6*
(Days)		log(cp/in)[c]		log(cp/in)[c]		log(cp/in)
C	15	ND[d]	15	ND	15	6.65 (0.05)AB
0	6	3.94 (0.18)A	9	3.63 (0.16)A	15	6.53 (0.04)AB
1	8	3.92 (0.07)A	10	3.50 (0.07)A	15	6.58 (0.05)A
2	9	4.16 (0.12)A	13	4.19 (0.12)B	15	6.58 (0.06)AB
3	14	5.10 (0.17)BC	14	4.79 (0.16)B	15	6.54 (0.05)AB
5	13	5.73 (0.21)BC	13	5.64 (0.19)C	14	6.51 (0.05)AB
7	14	6.34 (0.13)C	14	6.27 (0.12)C	15	6.42 (0.07)B
9	3	5.96 (0.26)C	3	5.73 (0.32)C	3	6.58 (0.18)AB

[a] Titers for a specific gene target followed by the same letter are not different (Tukey's HSD; $P = 0.05$).
[b] Number of aster leafhoppers testing PCR positive and used in the mean calculation. A positive detection had a value of 3 s or greater than the mean of the control (C).
[c] Only aster leafhopper extracts testing positive for AYp were used in the mean comparisons of the titer estimates obtained from *lysS* and *tuf* targets of experimental treatments (i.e., samples defined as different from the control).
[d] ND = not detected.

to demonstrate qPCR as a reliable and accurate method for measuring AYp titer in aster leafhoppers and detecting differences in AYp titers among insect individuals. Because absolute quantification of AYp DNA was achieved by comparing the amplification of unknown amounts of an AYp target gene sequence to the amplification of a dilution series containing known starting quantities of the targets sequences cloned into a plasmid, the factors leading to variation of the calibration curves within and among experiments was evaluated to examine the capabilities and limitations of the method.

A critical factor that relates directly to the reproducibility of any qPCR assay (for absolute quantification) is the accurate measurement of the initial plasmid standard DNA concentrations, which we estimated using a spectrophotometer, and preparation of calibration curves (Rutledge and Cote 2003, Burns et al. 2005, Bustin et al. 2009, Montes-Borrego et al. 2011). Because we could not reliably use spectrophotometry to estimate the low DNA concentrations that exist in our calibration curves (they were below our spectrophotometer's limit of detection) we examined the variation that may occur among calibration curve preparations made using two independently prepared and measured plasmid standards. Several studies have reported differences among slopes (Atallah et al. 2009) and slopes and intercepts (McNeil et al. 2004, Montes-Borrego et al. 2011) of different standard curve preparations and/or runs. We used an ANCOVA approach to compare the slopes and intercepts of two independently prepared standard curves, which demonstrated that the variation because of standard curve preparation was less than the variation because of the technical replication (or within plate variation) of the standard curves (Burns et al. 2005). Our result is similar to Montes-Borrego et al. (2011) in that the reproducibility of our standard curve was not affected by the origin of the plasmid standard or our ability to measure the plasmid standard and prepare a dilution series.

Intra- and inter-assay variation of standard curves has been examined using multiple methodologies. Specifically, Rutledge and Cote (2003) and Burns et al. (2005) have discussed the use of calculating standard deviations among (and within) standard curve replicates and ANCOVA for the characterization of calibration curve variability, respectively. In our study, we used a linear mixed model to simultaneously examine both intra- (within plate) and inter-assay (plate-to-plate) variation. Because this model associates common random effects to observations sharing the same level of a classification factor, it most accurately represents the covariance structure induced by the inherent 96-well plate groupings (Pinheiro and Bates 2000). This approach allowed us to examine the typical slope (B_1) and intercept (B_0) values for standard curves run on a "population" of five independent 96-well plates (for each primer target). It also allowed us to characterize plate-to-plate variability associated with the slope (σ_s) and intercept (σ_i) of our standard curve as well as the residual within-plate variability (σ_r). From these values (Table 3) we can approximate 95% confidence intervals for the typical slope and intercepts. For example, plate-to-plate variation in the slope of *cp6* gene calibration curve corresponds to a standard deviation of 0.04 Cq-values per Log_{10} (Copy Number), and as a result, slope values as low as $(-3.35–2*0.04) = -3.43$ or as high as -3.27 Cq-values per Log_{10} (Copy Number) among calibration curves run on different 96-well plates would be expected. For the *cp6* gene primer set, this corresponds to the calculated efficiency of $98.9 \pm 1.7\%$ that is consistent with the range slopes that would be estimated by fitting individual linear regressions for each run (Murtaugh 2007) and consistent with the findings of Rutledge and Cote (2003). Few studies have reported the intra- and inter-assay variation of slope (i.e., efficiency) and intercept in this way and no guidelines exist to determine acceptable levels of slope variation for a qPCR assay. Formal tests to deter-

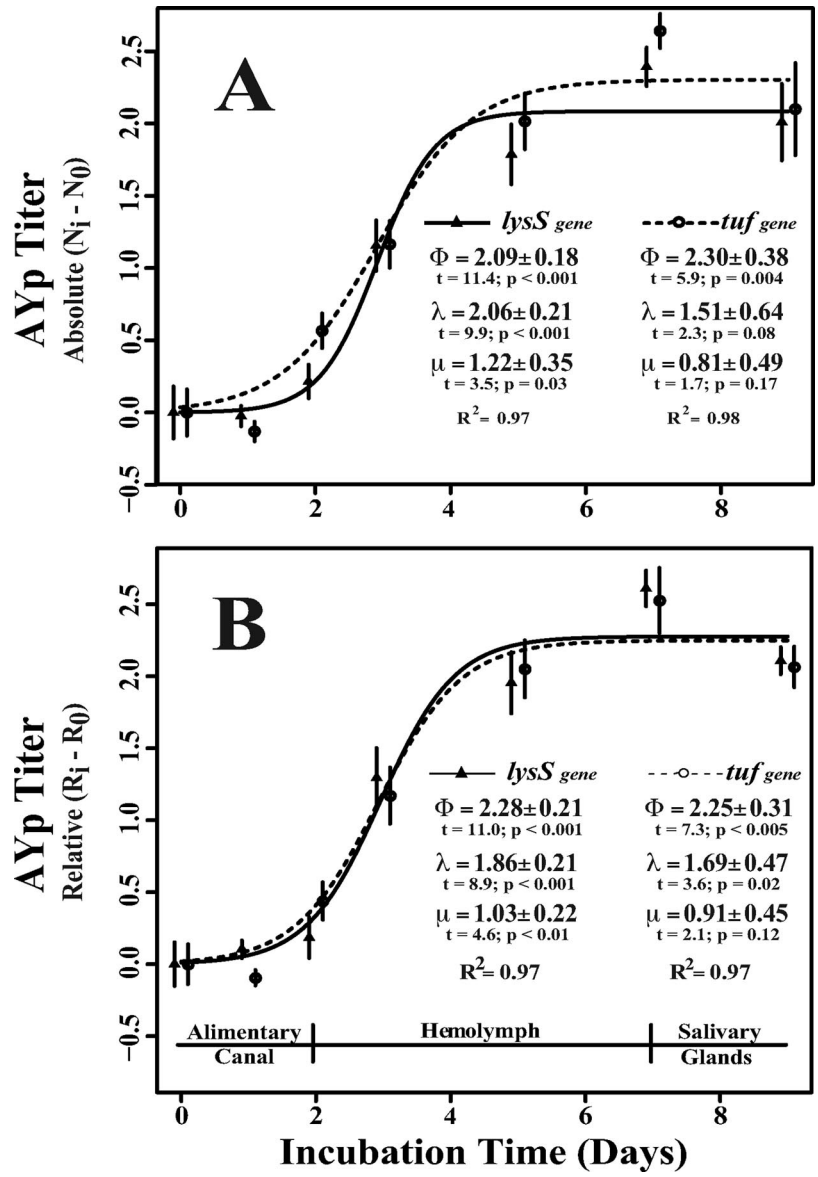

Fig. 2. (A) AYp titer is expressed as Log_{10}-transformed copies per insect (N) averaged by incubation time relative to AYp concentration at Time 0 versus incubation time. (B) AYp titer is expressed as Log_{10}-transformed AYp (*lysS* or *tuf*) gene copies per *cp6* gene copies (R) averaged by incubation relative to AYp concentration at Time 0 versus incubation time. In A and B, the solid and dashed lines represent the best fit three-parameter logistic growth curve (iteratively reweighted least squares) describing AYp titer in the aster leafhopper as a function of time. The logistic growth curve had the form: $Y_i = \Phi / (1 + \exp((((4*\mu)/\Phi)*(\lambda - X_i)) + 2)$ where Y_i is AYp titer relative to AYp titer at time 0, X_i was incubation time and *i* indexes incubation time. Parameter estimates (±SE), test statistics and *P* values for the asymptote (Φ), the maximum growth rate (μ), and the lag time (λ) associated with bacterial population growth are reported for each of the regression fits and are included in the respective panel. Time is days after a 48 h AAP. Four degrees of freedom were used for all *t*-tests and a pseudo-R^2 value (pseudo-R^2 = 1 – (SSR/SST)) was used as a measure of goodness-of-fit (Schabenberger and Pierce 2002). The bar in the lower portion of panel B is a temporal portrayal of AYp recovery from the aster leafhopper alimentary canal, hemolymph, and salivary glands as reported by Sinha and Chiykowski (1967).

mine differences among slopes of calibration curve slopes are available, but are often not reported.

Currently, the amount of sample DNA added to each of our qPCRs is measured using spectrophotometry and standardized to 120 ng. We originally thought this standardization could be avoided if a chromosomal aster leafhopper gene target whose concentration (copies/ng DNA) among insect DNA extracts remained stable could be used as a reference to standardize our assay for all insect individuals (Marzachi

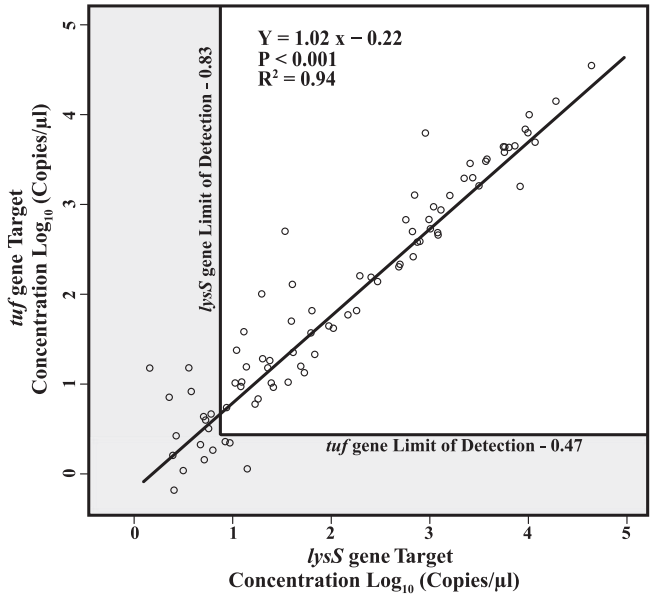

Fig. 3. AYp concentrations quantified using the *tuf* gene target sequence versus AYp concentrations quantified using the *lysS* gene target sequence. AYp titer concentrations measured using both primer sets were significantly correlated ($R = 0.95$; $P < 0.001$).

and Bosco 2005, Wong and Medrano 2005, Bustin et al. 2009). However, female aster leafhoppers had fewer copies of the *cp6* gene per ng of DNA (Log_{10}) than male aster leafhoppers. This difference might be explained by the presence of RNA, because our DNA extraction did not include a ribonuclease step, or extracrhomosomal DNA in our insect extracts. For example, female aster leafhoppers are larger than male aster leafhoppers, having approximately twice the volume and weighing 150% more than males. Therefore, female aster leafhoppers cells and bodies likely contain more nongenomic DNA and RNA (i.e., mitochondrial DNA, endosymbiont DNA, etc.) than male aster leafhopper and, to our knowledge, the relative contributions of the different DNA and RNA sources to the total nucleotide pool have not been measured for male and female aster leafhoppers. We assumed that these sources of variability, including the presence of RNA in our samples, occurred for every insect in our experiment and could be considered a consistent source of random variation. Thus our deduced copy number might be biased, but the relative differences among experimental treatments would not be expected to vary interactively with the occurrence of these errors because they were present in all samples at some average level.

Calculations of the absolute AYp titer on a per insect basis with and without an aster leafhopper reference chromosomal marker were equivalent; mathematically the units cancel to give the same result. To further evaluate the utility a aster leafhopper reference chromosomal marker, we directly compared the relative AYp titer, calculated with and without the use of the *cp6* gene target (Fig. 2), and found that using the *cp6* gene as a reference did not change the results of our statistical analysis. Parameter estimates for the nonlinear regressions were not different (test statistics not reported) although, visually, the use of the *cp6* gene did improve the consistency among parameter estimates for AYp titer measured using the *lysS* and *tuf* gene targets. This result implies that the additional qPCR step to quantify an aster leafhopper reference chromosomal marker may not be needed if DNA extraction methods are consistent among samples and yield high quality DNA. In the future, the quality and concentration of DNA extracts should always be measured and reported to ensure the accuracy (or validity) of the qPCR assay results. We will continue to use the aster leafhopper *cp6* gene target a reference to study relative AYp growth in the aster leafhopper, and to identify experimental errors associated with DNA extraction or samples in which PCR inhibitors may be present. Relative titer maybe useful for studying AYp growth in aster leafhopper or other AYp hosts because it is a ratio and can be scaled to a reference value or treatment (i.e., in our experiment, Time 0). However, absolute copy number is often needed to fully understand and interpret the biological relevance of data and for a majority of our analyses AYp titer was expressed in absolute terms as AYp copies per insect or per nanogram of DNA.

Because the AYp has not been successfully cultured in the laboratory and DNA extracts typically contain a background of host DNA, we were initially concerned that prokaryotic endosymbionts naturally colonizing the aster leafhopper might contain DNA sequences sufficiently conserved to cause false positives with a specific primer set. Therefore, multiple AYp gene targets were amplified to provide additional evidence for the analytical specificity of the assay. If

estimates of target copy number obtained using primers designed to amplify two different single-copy AYp gene sequence targets were similar, then the assay was likely targeting the same organism. Estimates of AYp target in aster leafhopper DNA extracts obtained by amplifying two different AYp sequence targets in each DNA extract were significantly correlated and the slope of the regression line between the estimates was not significantly different from one. In addition to providing evidence for the specificity of the qPCR assay, this finding further supported the conclusion that plasmid standards could be accurately quantified and the repeatability of calibration curve preparation is high. The costs of future experimentation could be significantly reduced by quantification of the AYp using a single primer set.

In the control aster leafhopper group we did not detect phytoplasma in any insect extracts using nested PCR. The average Cq values for the control group were 36.40 and 35.87 for the *lysS* and *tuf* gene targets, respectively, which were higher than the Cq values obtained for the most dilute standard of the *lysS* and *tuf* gene calibration curves at 35.37 and 33.42, respectively. We interpreted the qPCR signal associated with the control group of insects as background noise that may occur from nonspecific amplification products in the SsoFast EvaGreen reactions. Additional evidence supporting this interpretation came from an examination of the melt curves associated with the control group qPCR products. Melt peaks for this subset of reactions were poorly defined and of low amplitude. Thus, the background noise was used to define a diagnostic limit of detection (LOD) of three standard deviations above the mean starting copy number of the control group of aster leafhoppers. Individual insects were considered to be infected with AYp if the estimated copy number was greater than the LOD, which represented the clinical sensitivity of the assay. For insects undergoing shorter incubation times (0, 1, and 2 d), the percent positive detections using qPCR was higher than the percent positive detections when using conventional PCR but lower than the percent positive detections when using nested PCR. However, in insects undergoing longer incubation times (two or more days), the percent positive detections were consistent between qPCR and nested PCR.

Many studies have compared the outcomes of conventional and nested PCR methods to qPCR methods for the detection of specific pathogen and the comparison of methodologies has largely been qualitative in nature (Crosslin et al. 2006, Wen et al. 2009, Wenbin et al. 2009a, Zhang et al. 2010, Montes-Borrego et al. 2011). Because the outcome of (conventional) endpoint PCR depends on the starting copy number of the targeted sequence and a large number of unknown experimental variables (Freeman et al. 1999), a positive (or negative) detection can be thought of as having a probability distribution conditional on the initial copy number in the reaction. In this study, we used logistic regression to relate the initial copy number of AYp target present in each PCR reaction tube (estimated using qPCR) to the binary outcome of our conventional PCR and nested PCR assay outcomes (Kutner et al. 2004). We found that the starting copy number did relate significantly to the outcome of the conventional assay and ≈6,000 and 125,000 copies, respectively, were necessary to have a 0.5 and 0.95 probability of detecting AYp from an environmental sample when a single round of amplification was used (38 cycles). These values dropped to ≈40 and 850 when a second nested round of amplification was used for detection. Because, we did not control for numerous possible errors in our conventional PCR assays (i.e., conventional PCR was not replicated, reactions contained varying concentrations of DNA template, primer efficiencies not quantified, etc.), these estimates represent an approximate calculation of the copy numbers of target in a reaction necessary to give a positive detection with conventional PCR and should be refined with further research. The intent of this analysis was to point out that the comparison of molecular detection methods could be made more quantitative; it was not to evaluate the primers being used or assess our conventional PCR assays.

We also examined the variability of AYp titer in aster leafhoppers and characterized growth dynamics of AYp titer within a population of AYp-infected insects. Using indirect methods, Sinha and Chiykowski (1967) first reported the phenology of AYp recovery from *M. quadrilineatus* (formerly *M. fascifrons*) tissues after a 3 d AAP and found phytoplasmas in the ailementry canal, hemolymph, and salivary gland at 3, 6, and 12 d post-AAP, respectively. Previous studies have measured Chrysanthemum yellows phytoplasma titer in the insect vectors, *Euscelis incisus* Kirschbaum, *Euscelidius variegatus* Kirschbaum and *Macrosteles quadripunctulatus* Kirschbaum after a 7 or 10 d AAP (Bosco et al. 2007a,b). In those studies, the relatively long AAP and sampling intervals may have masked some of the finer scale phytoplasma population dynamics occurring in the insect. In our study, we found that AYp titer in aster leafhopper increased over a period of approximately 5 d post-AAP and became asymptotic. We used a logistic growth curve model to estimate parameters that are commonly used to describe bacterial growth in culture (Zwietering et al. 1990). We found that AYp had long doubling times, >500 min, which is not uncommon for some pathogenic bacteria such as *Mycobacterium tuberculosis* (James et al. 2000). We also found that AYp titer increased ≈100-fold in the insect, which is consistent with some of the original findings of Black (1941), but growth slowed after 6 d suggesting there is some upper limit to the AYp population size in the aster leafhopper. In the future, a consistent modeling approach of mollicute growth in their host would allow for easier comparison among multiple studies.

Under the assumption that an individual AYp organism possessed a single copy of target sequence, the average AYp titer per insect increased ≈100-fold to $10^{6.3}$ over 7 d. The number of AYp organisms present in an individual aster leafhopper, measured using *tuf* and *lysS* gene primer sets ranged from ≈$10^{3.2}$ (i.e., the LOD for a single insect) to $10^{7.0}$ over the course of the

experiment and varied as much as 100-fold within an incubation time group. These estimates are consistent with previous attempts to measure AYp titers in their insect vectors (Bloomquist and Kirkpatrick 2002). When expressed as copies per ng of DNA, the highest AYp titer in an individual insect was 2.4×10^3 copies per ng at 7 d post-AAP that is lower than previous reports of 3.1×10^4 copies per ng at 33 d post-AAP in the chrysanthemum yellows phytoplasma-leafhopper system (Marzachi and Bosco 2005). For our experiment, this suggests that Ayp titers might have continued to increase if the aster leafhopper were given longer incubation periods.

Male aster leafhoppers had higher AYp titers than female aster leafhoppers when phytoplasma concentration was expressed as copies per ng of DNA. This difference might be explained by the fact that female insects are larger and have more tissues that are not susceptible to AYp infection, such as the fat bodies and mycetomes (Sinha and Chiykowski 1967), but still contribute to the total DNA yield. However, when AYp titer was expressed on a per insect basis, females and males harbored approximately the same number of AYp organisms. Similar to our findings, Rotenberg et al. (2009) reported that male thrips harbor higher TSWV concentrations per unit RNA but female thrips harbor more molecules of TSWV on a per insect basis. In that study and in our study, the quantitative differences of pathogen load between sexes was likely because of the relative size differences of pathogen susceptible tissues between male and female insects. Rotenberg et al. (2009) also observed that male thrips transmit TSWV more frequently than female thrips even though males harbor fewer virus molecules than females. The authors hypothesized that the higher transmission efficiency of male thrips was likely because of feeding behavior and not virus titer. Similarly, Beanland et al. (1999) has reported differences in the ability of male and female aster leafhoppers to transmit the AYp, with females more likely to transmit than male insects. However, it is not known if the differences in transmission ability between male and female aster leafhopper were because of AYp titer, aster leafhopper feeding behavior or AYp distribution within the insect body, which is known to vary in concentrations among tissues of some insect vectors (Galetto et al. 2009).

The biotic and abiotic factors that influence the variation of AYp titer in aster leafhopper have not been well characterized. For example, temperature is known to influence the latent period of AYp-infected aster leafhopper (Maramorosch 1953, Murral et al. 1996), but the underlying mechanism for the temperature effect is not known and may simply be that AYp organisms grow more slowly in the insect at lower temperatures. It is the genetic composition of the aster leafhopper and AYp that determine vector competency and there may be a genetic basis for variation of AYp titer in aster leafhopper. Multiple AYp strains exist in the environment (Lee et al. 2003, Zhang et al. 2004) that differentially affect aster leafhopper fitness and have the potential to alter aster leafhopper population dynamics in the field (Beanland et al. 2000). Again, the mechanism for the fitness effect of AYp on the aster leafhopper is not known but may be related to phytoplasma growth in the aster leafhopper. The use of qPCR as a tool may be applied to address the biological relevance of AYp variability and growth within insect individual and within and among aster leafhopper populations. To date, AYp titer variation has not been described within or among field caught aster leafhopper populations and the relationship between AYp titer variation and aster leafhopper infectivity has not been established. However, the existence of AYp titer variability among aster leafhoppers and having the tools to manipulate and measure that variability is necessary for completing experiments to relate AYp titer to a leafhopper's ability to transmit.

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